Assessment of Environmental Impacts of Genetically Modified Plants

Implementation of the Biosafety Protocol
Development of Assessment Bases
FKZ 201 67 430/07

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Contents

1 Introduction

The German Federal Agency for Nature Conservation (Bundesamt für Naturschutz, BfN) has charged the Institute for Biodiversity - Network Science and Business (IBN) to carry out the project, FKZ ("Förderkennzeichen") 201 67 430/07, "Assessment of the environmental impacts of genetically modified organisms – Implementation of the Biosafety Protocol – Establishment of assessment bases" within the BfN´s research focus on „Biosafety research and Monitoring of GMOs".

There is still a multitude of open questions with regard to the environmental impact assessment of deliberate releases and placing on the market of genetically modified organisms (GMOs) in particular, genetically modified plants. The aim of this study was to compile the current international status of research on the environmental impacts of genetically modified plants. An assessment of actual research results with respect to their gaps and limits should be carried out. Singular, especially important scientific publications or issues should be critically assessed and further research need defined.

To approach these tasks, first of all results on the state of research, deliberate release and placing on the market of genetically modified organisms were recorded by regularly studying scientific journals, books (including symposia and workshop reports), publications and working papers of international organisations and UN institutions, of regulatory bodies (agriculture, environment, health) of some countries, especially OECD-countries and of relevant internet-based publications..

Already at the start of the assessment it became evident that simply a registering of research results of an explicit biosafety research runs too short because many biosafety-relevant research results do not result from an explicit biosafety research. The insights result from

1. an explicit biosafety research or risk research that refers to or accompanies deliberate releases of GMOs,

2. assessments and risk simulations of GMOs without the generation of primary./.field data,

3. ecological research that allows to draw analogy conclusions on the risks of deliberate releases,

4. research on molecular biological phenomena, that – without being biosafety research – lead to biosafety evidences,

5. biotechnological research and development, that implicate deliberate release and biosafety research related questions

6. molecular genetic analyses and analysis methods and

7. research on economic and agronomic aspects of GMOs.

It must be added, that in explicit biosafety research programmes (e.g. of the EU), too, pure biosafety research as well as research approaches are included that highlight risk aspects found in research projects that have been carried out for other purposes. Vice versa this affirms the chosen procedure not slavishly to orient to a biosafety research etiquette.

The risk assessment as based on the outlined state of research has been carried out according to the following scheme:

- In chapters 2 (Plant species genetically modified) and 3 (Transgenic methods in plants) plant species that have been genetically modified and methods of gene insertion are compiled. Single sections deal with transformation methods, promoters used, plastid transformation, and marker genes, and the risks connected to these methods.

- Chapter 4 (Gene spread) deals with the various ways through which transgenes can spread in the environment such as hybridization, horizontal gene transfer, and spread of transgenic plants.

- In chapters 5 (Traits of transgenic plants and their environmental impacts) and 6 (Secondary environmental impacts of transgenic plants) the – mostly new – traits of transgenic plants and their primary and secondary impacts on the environment are compiled and discussed. Single sections in chapter 5 deal with traits such as herbicide resistance, insect resistance, pathogen resistance, stress tolerance, phytoremediation, growth characteristics, new substances and vitamins, and pharmaceuticals and the environmental risks that go along with those traits. Subjects of chapter 6 are secondary environmental impacts due to the cultivation of transgenic plants, such as resistance development in weeds and pests and alterations in herbicide and insecticide use.

Part I Plant species and methods of genetic engineering

2 Plant species genetically modified

Transgenic plants, often referred to as genetically modified (GM) or genetically engineered (GE) plants, have been grown commercially since 1996 in a number of countries. According to James (2006), they have been cultivated in 2005 on 90 million hectares (ha) worldwide, a 52-fold increase from 1.7 million hectares in 1996. Main countries growing transgenic crops in 2005 were USA (55 % of global area), Argentina (19 %), Brasil (10 %), Canada (6 %), and China (4 %). Paraguay grew transgenic crops on 2 % of the global area and about 8 countries grew transgenic crops on the remaining 4 %. Herbicide resistant (HR) soybean was the most dominant transgenic crop representing about 60 % of the global transgenic crop area, followed by transgenic corn (insect resistant Bt corn and HR corn) grown on about 24 %. Transgenic cotton (insect resistant Bt cotton and HR cotton) occupied 11 % and HR oilseed rape about 5 % of global transgenic area. In 2005, HR crops, Bt crops, and crops with stacked traits such as HR/Bt cotton and HR/Bt corn represented 71 %, 18 %, and 11 %, respectively. Plant species other than these four main crops such as transgenic potato, squash, and papaya were grown on less than 1 %. As these data indicate, it is basically four crop species carrying herbicide resistance and/or insect resistance traits hat have been used commercially as transgenics in recent years. Virus resistance and other traits comprised less than 1 % of global transgenic crop area (James 2006). Detailed data on biotechnology products can be found at http://www.olis.oecd.org/bioprod.nsf and http://www.transgen.de.

However, on a global scale, there are many more transgenic plant species that have been released into the environment in past years. In the EU, applications that have been submitted under the Deliberate Release Directive (90/220, and, since 2001, 2001/18) comprise about 50 different plant species, transformed to express many different traits. In addition to the four main crop species grown commercially and tested in field trials, other transgenic crop plant species tested are cereal crops such as wheat, barley, and rice, and broad-leaved crops (potato, sugar beet, tobacco, sunflower, and alfalfa). The range of species has been extended to vegetables (e.g. tomato, cauliflower, chicorée, aubergine, carot, pea, lettuce) and fruits such as melons, strawberries, and raspberries and even to wild plants (e.g. wild radish). Applications for deliberate release of woody fruit species such as apple, cherry, plum, olive, orange, and wine or of forest trees such as poplar and eucalyptus have also been submitted. Transgenic flowers (e.g. carnation, marigold, petunia) have been released in countries of the EU too (http://biotech.jrc.it/deliberate/taxonomy.asp).

Data on GMO field trials in the US are provided at http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm. Summaries of GMO field trials in OECD member countries, as well as data from other countries that have been provided through UNIDO's BINAS, can be derived from: http://www.olis.oecd.org/biotrack.nsf and http://binas.unido.org/binas/trials.php3. Searches by organism and by country can be performed. Not all field releases of transgenic organisms taking place worldwide will be

listed, as probably not all countries provide data to these databanks. The most common plant species tested in field trials worldwide are corn, oilseed rape, potato, and soybean followed by tomato, cotton, tobacco, beet, wheat, rice, melon, and alfalfa. In addition to the transgenic plant species released in the EU, species such as cresping bentgrass, turnip rape, flax, sugarcane, walnut, papaya, brown mustard, and clover are listed. A great number of different transgene constructs has been used for transformation of these crop or ornamental species. Some of these data entries may not be complete or precise enough, according to Zoglauer et al. (2000), who collected data on field trials with forest, fruit, and ornamental woody species. Additional data on transgenic trees can be found at http://www.gaaget.org/map/index.html.

Table 1: Genetically engineered plant species tested in field trials
(EU, USA, OECD, as of 19. 03. 2003)

EU: http://biotech.jrc.it/deliberate/taxonomy.asp

USA: http://www.nbiap.vt.edu/cfdocs.biocharts2.cfm

OECD: http://www.olis.oecd.org/biotrack.nsf/by+organism?OpenView&Start=1

Species	**Common Name**
Field Crops	
Arachis hypogaea	peanut
Avena sativa	oat
Beta vulgaris	sugar beet, fodder beet
Brassica juncea	Indian/brown mustard
Brassica napus	oilseed rape
Brassica rapa	spring turnip rape
Glycine max	soybean
Gossypium hirsutum	cotton
Helianthus annuus	sunflower
Hordeum vulgare	barley
Ipomoea batatus	sweet potato
Lens culinaris	lentil
Linum usitatissimum	flax
Lupinus sp.	lupin
Manihot esculenta	cassava
Medicago sativa	alfalfa
Nicotiana tabacum	tobacco
Oryza sativa	rice
Papaver sp.	poppy
Saccharum officinarum	sugarcane
Solanum tuberosum	potato
Sorghum sp.	sorghum
Trifolium sp.	clover
Triticum aestivum	wheat
Triticum durum desf.	durum wheat
Zea mays	maize
Vegetables	
Allium cepa	onion
Ananas comosus	pineapple
Asparagus officinalis	asparagus

Brassica oleracea	broccoli, cauliflower, cabbage
Capsicum annuum	pepper
Chicorium intybus	chicory
Citrullus lanatus	watermelon
Cucumis melo	melon
Cucumis sativa	cucumber
Cucurbita pepo	squash
Daucus carota	carrot
Lactuca sativa	lettuce
Lycopersicon esculentum	tomato
Nasurtium officinale	watercress
Pisum sativum	pea
Raphanus sativus	radish
Sinapis sp.	mustard
Solanum tuberosum	potato
Solanum melongena	eggplant
Fruits, Wine	
Actinidia deliciosa	kiwi
Amelanchier leavis	juneberry
Carica papaya	papaya
Citrus x paradisi	grapefruit
Citrus sinensis	sweet orange
Coffea arabica	coffee
Coffea canephora	robusta
Cyphomandra crassicaulis	tamarillo
Fragaria fragaria x Fragaria ananassa	strawberry (adj. ananassa)
Fragaria vesca	wild strawberry
Fragaria virginia x Fragaria chiloensis	strawberry (virginiana x chiloensis)
Juglans regia	walnut
Malus domestica	apple
Malus pumila	paradise apple
Olea europea	olive
Persea americana	avocado
Prunus avium	sweet cherry
Prunus domestica	european plum
Pyrus communis	pear
Rubus idaeus	raspberry
Vaccinium sp.	cranberry
Vitis berlandieri x Vitis riparia	grape (berlandieri x riparia)
Vitis berlandieri x Vitis rupestris	grape (berlandieri x rupestris)
Vitis rupestris	sand grape
Vitis vinifera	grapevine
Vitis vinifera x Vitis berlandieri	grape (vinifera x berlandieri)
Ornamentals	
Dendranthema indicum	chrysanthemum
Dianthus caryophyllus	carnation
Gladiolus communis	gladiolus
Lilium longiflorum	lily
Limonium otolepis	limonium
Osteospermum ecklonis	marigold
Pelargonium odoratissimum	scented pelargonium
Petunia sp.	petunia

Rhododendron sp.	rhododendron
Rosa sp.	rose
Saintpaulia ionantha	african violet
Forages	
Agrostis stolonifera	creeping bentgrass
Cynodon dactylon	Bermuda grass
Festuca arundinacea	tall fescue
Lolium perenne	perennial ryegrass
Poa pratensis	Kentucky bluegrass
Trees	
Betula pendula	silver birch
Castanea sativa	chestnut
Eucalyptus globulus	tasmanian blue gum
Eucalyptus grandis	eucalyptus
Liquidambar sp.	sweet gum
Picea abies	norway spruce
Pinus sylvestris	scotch pine
Populus alba x Populus tremula	poplar (alba x tremula)
Populus deltoides	poplar
Populus tremula	european aspen
Populus tremuloides	quaking aspen
Others	
Arabidopsis thaliana	thale cress
Atropa belladonna	belladonna
Carthamus tinctorius	safflower
Mentha piperita	peppermint
Raphanus raphanistrum	wild radish

3 Transgenic methods in plants

3.1 Transformation/Transgene integration in plants

The two most widely practiced and successful plant transformation techniques are *Agrobacterium tumefaciens*-mediated transformation (mostly used for broad-leaved plants) and microprojectile bombardment or biolistic transformation (particularly for agronomically important cereal crops), mediating the transfer of foreign DNA to plant genomes in completely different ways. Transgenic plants can also be recovered after protoplast transformation using polyethylene glycol (PEG) and electroporation. In so called agrolistic transformation, elements of *Agrobacterium*-mediated as well as of biolistic transformation are combined (Hansen & Wright 1999, Smith et al. 2001). Subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium* may increase transformation frequencies (Trick & Finer 1997). Other transformation techniques such as the use of silicon carbide whiskers, microinjection of zygotes, and laser microbeam are in development. Methods for pollen-mediated transformation in tobacco have been described (van der Leede-Plegt et al. 1995). Special *Agrobacterium*-mediated transformation methods such as vacuum

infiltration of whole plants, floral dip, and floral spray have shown comparable transformation efficiencies in *Arabidopsis* species (Chung et al. 2000, Curtis & Nam 2001, Tague 2001). Transformation efficiency may be improved by using cell cycle synchronized plant cells (Imani et al. 2002) or by transient expression of the gene for isopentenyl transferase (ipt), a crucial enzyme in cytokinin synthesis, that is delivered by bombardment in addition to the genes of interest transferred by *Agrobacterium* mediation (Molinier et al. 2002).

„In planta" transformation of intact flowers, pollen, or shoot apices by injection and biolistic delivery of DNA or by *Agrobacterium* mediation could avoid tissue culture which is suspected to contribute to genomic changes, as studies done by Bao et al. (1999) with transgenic rice plants recovered from protoplasts indicate. Tissue culture involves disorganized cell growth, such as a callus phase, which can induce somaclonal variation, i.e. mutations, including chromosomal changes, changes in gene expression, or altered methylation patterns. Some of the observed genomic changes in transgenic plants seem to be connected to tissue culture and to the stress associated with selection for antibiotic or herbicide resistance, as can be derived from studies done by Carmona et al. (2005). In comparison to control sugarcane plants, consisting of original cultivars and plants regenerated without *Agrobacterium*-mediated transformation or marker-assisted selection, they observed significant DNA polymorphisms in genetically modified sugarcane. The agronomic performance of plants going through tissue culture can be altered too, the extent depending on the cultivar and perhaps also on growth regulators used during tissue culture (Dahleen et al. 2001, Bhat & Srinivasan 2002, Sharp et al. 2002).

Protocols for transformation of woody species such as grapevine and citrus have been published (Iocco et al. 2001, Cervera et al. 1998). Adaptation of transformation and regeneration protocols has led to increased transfer efficiencies and also to transformation of crop plants that once have been classified as recalcitrant (Hansen & Wright 1999). In citrus, high frequencies of chimeric shoots, consisting of transformed and nontransformed tissue and, in part, resulting from the union of different transformation events have been observed (Dominguez et al. 2004).

Generally, the constructs used for transformation of plants consist of the gene(s) of interest, mostly bacterial and plant sequences, fused to promoter/enhancer sequences of bacterial, viral, or plant origin and combined with marker genes (usually consisting of antibiotic or herbicide resistance genes including promoter sequences). Bacterial sequences are regularly adapted to plant codon usage. In general, transgenic plants approved for cultivation contain several genetic elements that are either isolated from foreign sources or represent synthetic DNA. To ease public debate about genetic engineering, Rommens (2004) proposed to create "intragenic" plants by all-native DNA transformation. Following this approach, native genes and regulatory elements would have to be used, but DNA transfer and selection protocols without bacterial sequences have not been routinely established. Addition of sequences for signal peptides may lead to targeting of the heterologous proteins to cellular organelles such as chloroplasts and vacuoles (Murray et al. 2002).

As gene integration in the genome is essentially random, variability of integration and expression patterns is often observed from one transgenic plant to another (Hansen & Wright 1999). Transgene expression can be influenced by the insertion site(s), but expression of

endogenous genes can also be influenced by the transgene construct. Of importance could be the presence or use of cryptic promoters that are inactive at their natural locations in the plant genome, but may become active if associated with transgenes (Tian et al. 2002). The transformation method can influence the frequency of genomic changes. According to Labra et al. (2001), particle bombardment and electroporation lead to fewer cases of DNA polymorphisms in transgenic rice plants than *Agrobacterium*-mediated or PEG-protoplast transformation.

To reduce the effect of the integration site on transgene expression, targeting of transgenes to specific sites in the plant genome has been tried, but with only modest success so far (Risseeuw et al. 1995, Puchta 1998, Kumar & Fladung 2001). Transgene integration seems to occur preferentially in telomeric and subtelomeric regions (Hansen & Wright 1999). This may be due to the fact that transgenes inserted in distal chromosome regions, enriched with coding DNA sequences, are more likely to be expressed and, therefore, plants carrying such insertion sites would be preferentially selected (Svitashev et al. 2000). Junctions of transgene DNA with genomic DNA have been found to contain scaffold attachment region (SAR) sequences, an observation suggesting that SARs may be hot spots for the insertion of transgenes into the genome of eukaryotic cells. As transfer (T)-DNA may integrate preferentially into open, transcriptionally active chromatin domains and since domain opening functions have been associated with scaffold/matrix attachment regions (S/MARs, DNA sequences that bind to the cell's proteinaceous nuclear matrix to form loop domains), Dietz-Pfeilstetter et al. (2003) proposed that T-DNA regions may be generally in proximity to S/MARs. Close alignment of T-DNA integration sites with an S/MAR may thus influence transgene regulation. Shimizu et al. (2001) showed that insertion of cloned junction sequences into expression cassettes of the antibiotic resistance gene npt II led to an increase in transformation frequency and transgene expression. The addition of MARs may also help to insulate transgenes from the influence of neighbouring regions. Gene silencing could thus be reduced and transgene expression enhanced (Han et al. 1997). Linkage of spacer elements to expression cassettes may stimulate amplification and expression of heterologous genes (Borisjuk et al. 2000). Translational activity of mRNA transcribed from transgenes can be affected by the insertion of introns into the coding regions of the genes of interest (Bourdon et al.2001).

3.1.1 Agrobacterium-mediated gene transfer

The soil phytopathogen *Agrobacterium tumefaciens*, known for some time to transfer DNA efficiently to plant cells (Zupan et al. 2000), has been used to transform a great number of plant species for the acquisition of new traits. Particularly in *Arabidopsis thaliana,* T-DNAs with selectable marker genes have also been used as insertional mutagens to characterize mutants and isolate the disrupted genes (Tax and Vernon 2001).

In *Agrobacterium*-mediated transformation, gene cassettes are spliced into T (transfer)-DNA derived from *A. tumefaciens*. The T-DNA region on the Ti (tumor-inducing) plasmid, bordered by left and right repeats, facilitates the transfer of DNA enclosed by these border regions (Gelvin 2003). Bin 19, a typical and widely used vector for *Agrobacterium*-mediated transformation is over 11.000 bp in length and has been used extensively for years before the

complete sequence has been published (Frisch et al. 1995). The plasmid is processed within the *Agrobacterium* cell and DNA placed between the left and right border of T-DNA (two imperfect 25 bp direct repeat sequences) is transferred to the plant cell. The essential virulence VirD2 protein attached to the right border establishes polarity and the importance of right borders relative to left borders (Gelvin 2003). The virulence proteins VirD2 and VirE2 are both required for efficient import of the T-DNA complex into plant nuclei. Ziemienowicz et al. (2001) suggested a model for nuclear import of the T-DNA complex, whereby the VirD2 protein pilots the T-DNA complex to the nuclear pore. The VirE2 protein, by binding and covering the ssDNA, creates a structure that enables translocation of large T-DNA molecules through the nuclear pore. Some *Agrobacterium* species carry more than one T-DNA on their Ti plasmids, leading to more than two T-DNA borders from which T-DNA can be processed (Tinland 1998). In the binary vector system, the virulence (vir) gene functions necessary for transformation are usually provided in trans on a second plasmid to secure that they are not transferred to the plant cell (Smith et al. 2001). If cointegrative vectors are used, that is, those with the vir genes linked to the T-DNA, whole plasmid transformation is the rule (Fu et al. 2000).

Large fragments (150 kb) can thus be transferred into plant nuclear genomes (Hansen & Wright 1999), but T-DNA insert size may have a dramatic effect on transformation efficiency, with larger constructs leading to reduced transformation efficiency (Frary and Hamilton 2001). Transformation with high molecular weight DNA can allow transfer of whole gene clusters for disease resistance or biochemical pathways and reduce the incidence of position effects and transgene silencing. Human genomic fragments of 150 kb transferred to tomato have been maintained and inherited through several meioses (Frary and Hamilton 2001). But since intergenic sequences of many plants contain numerous transposons which may lead to recombination and T-DNA rearrangements, high molecular DNA insertions derived from plant sequences may not be as stable.

Co-transformation of a single plant genome with two independent T-DNA regions has been described, permitting the genetic separation of the two sets of transgenes in the segregating progeny populations, under condition that the individual T-DNAs must be integrated at unlinked genomic sites (McCormac et al. 2001). Mixed strain methods comprise two T-DNA regions held on separate binary plasmids within different *Agrobacterium* cells, whereas in single strain methods the two T-DNAs may either be held as distinct regions on the same binary plasmid or on separate binary plasmids contained within the same *Agrobacterium* cell. Using the single strain/single plasmid system, McCormac et al. (2001) demonstrated co-transformation of tobacco to be dependent, at least in part, on the design of the plasmid vectors and the *Agrobacterium* strains used. Co-transfer of plasmid backbone sequences has been shown to occur too.

Models for T-DNA integration in *Agrobacterium*-mediated transformation have been discussed. Integration possibly involves regions of microhomology between the T-DNA borders and the plant genome and seems to occur by illegitimate recombination (Svitashev et al. 2002). Kumar & Fladung (2002) proposed that illegitimate recombination, achieved by single strand annealing and followed by ligation of the right border, seems to be the main mechanism of T-DNA integration. But there can also be integration events that show

truncations in the right border that may involve repair of genomic double-strand breaks. In contrast to a widely held view, integration of T-DNA may not follow a simple pattern, but rather involve deletions and additions in the course of repair activities during illegitimate recombination.

Following T-DNA integration and T-DNA mutagenesis, genetic anomalies and chromosomal rearrangements in transformants have been observed, such as substitutions, additions, and deletions, but also major chromosomal translocations (Kumar & Fladung 2002, Tax and Vernon 2001 and references therein). In a thorough analysis based on sequencing and mapping of two *Arabidopsis* T-DNA insertion lines, Tax & Vernon (2001) provided evidence for major internal chromosomal translocations. The transformants contained single T-DNA inserts and displayed straightforward genetics with regard to T-DNA segregation. Nevertheless, both lines were shown to contain, linked to the T-DNA, a duplicated fragment of a chromosome other than the chromosome in which the T-DNA was inserted. In one case, a sequence larger than 40 kb derived from chromosome V had been found to be linked to the left border of the T-DNA, inserted in a locus on chromosome I (the insertion resulted in an embryo-defective phenotype). In the other case, a sequence of about 1000 bp derived from chromosome IV had been linked to the left border of the T-DNA, integrated in a locus on chromosome V. Interestingly, the translocated genomic sequences must have been duplicated before their insertion in chromosomes I and V, respectively, as the respective genomic wildtype sequences were still present at their original locations on both chromosomes of the diploid genome. Linkage of duplicated/transferred sequences to the right border of the T-DNA was not observed. In analysis of 30 transgenic aspen lines, Kumar & Fladung (2002) observed different structures of integration sites, among them deletions of DNA, ranging in length from a few nucleotides to more than 500 bp, and filler DNAs of up to 235 bp which in most cases originated from neighbouring host genomic sequences or from the T-DNA. Left borders were less well preserved than right borders. Though sequence analysis of the insertion sites did not reveal any site preference for T-DNA integration, relatively high AT values have been found in the flanking genomic sequences. Therefore, AT-rich regions might act as recombination hotspots for the transgenes, perhaps because of steric bending of the chromosomal DNA structure.

The observed T-DNA-associated rearrangements may have resulted from a single T-DNA that underwent aborted insertion at one site but successful integration at another, thus incorporating left border-flanking plant DNA from the initial failed insertion (Tax and Vernon 2001). If in these cases the left border inserted before the right border and integration was aborted before the right border had integrated successfully, then any flanking sequences that were translocated would be expected to be found only on the left border. In general, chromosomal regions undergoing DNA repair may facilitate T-DNA insertion and also restoration of translocated sequences and maintenance of wildtype alleles at their original location. According to Tax and Vernon, the evidence collected for the wide diversity of chromosomal defects associated with T-DNA insertions indicates that T-DNA transformation processes can go awry in any number of ways. Since only detailed characterization will allow detection of such rearrangements, these sorts of rearrangements have likely been overlooked in prior analysis of T-DNA insertions.

Though the view is widely accepted that transfer of DNA from *Agrobacterium* to the plant genome results mostly in single copy insertions (as discussed above), there is evidence that the transformed plants very often contain more than one copy of the transgenes (from two up to ten copies) including antibiotic resistance genes integrated at a single locus or at multiple loci (Sedira et al. 2001, reviewed by Smith et al. 2001, Theuns et al. 2002, Dietz-Pfeilstetter et al. 2003). In many instances, multiple integration sites could be found, e.g. in a study aimed to compare transformation systems, maize plants transformed by *Agroacterium* had transgene copy numbers from one to nine. But, in general, particle bombardment resulted in higher copy numbers, with almost all plants having more than three copies and some with far more than 100 copies (Shou et al. 2004). Since transgene segregation in progeny often reports the number of functional transgene copies and may not reflect the actual number of transgene copies present, confirmation of low copy number T-DNA insertion must not depend solely on segregation patterns in progeny of transgenic plants (Smith et al. 2001).

Transgenic plants frequently contain functional and nonfunctional copies of the same transgene, possibly due to transgene silencing or truncation derived from incomplete T-DNA transfer. Transgenic poplars harboring a single rolC gene copy from *A. rhizogenes* contained incomplete T-DNA repeats too (Kumar & Fladung 2001). After transformation of apple rootstock, transgene copy numbers in individual shoots varied widely (from one to seven) and about half the transgenic plants had only part of the T-DNA (Sedira et al. 2001). Root forming capacity was strongly influenced by rolB copy number, with optimum performance in shoots containing two copies of intact T-DNA. However, multiple copy transgenes may be connected to unstable gene expression, as indicated by work with poplars transformed with the rolC gene (Kumar & Fladung 2001). T-DNA fingerprinting methods allow discrimination between different T-DNA inserts in stably transformed *Arabidopsis* plants, as shown by Theuns et al. (2002). By fingerprinting the junction regions between the T-DNA and the plant genome, it was possible to discern more than one transgene fragment in lines that had been classified as single copy transformants by Southern hybridzation.

Although *A. tumefaciens* is a soil phytopathogen, it may also encounter organisms belonging to other kingdoms such as insects and animals that feed on the infected plants. Recently, it has been shown that *A. tumefaciens* can attach to and transform human cells such as HeLa cells. The mechanism of transformation seems to be similar to that which it uses for transformation of plant cells, deduced from the observations that the integration event occurred at the right border of the T-DNA and HeLa cell transformation required the activity of *vir* genes necessary for plant transformation (Kunik et al. 2001).

Obviously *A. tumefaciens* can also persist within the transgenic plants despite treatment with the antibiotics cefotaxime and vancomycin or claforan and carbenicillin. In some transgenic plants, e.g. citrus, *Agrobacterium tumefaciens* could be found with high frequency one to two years after transformation in stem segments (Dominguez et al. 2004) and roots, (http://www.biosicherheit.de/features/printversion.php?context=1&id=114), but in tobacco transfer to the next generation has not been observed. Shoot tip culture of apical meristems of transgenic plants may reduce contamination by *A. tumefaciens* (Landsmann et al. 1999).

It is unknown at present, whether these observations are of relevance for the safety assessment of transgenic plants in general and of those containing T-DNA border sequences, sequences

that might recombine with *Agrobacterium* T-DNA. Dominguez et al. (2004) caution that the inadvertent introduction of genetically engineered bacteria into the environment may be a matter of concern particularly in the case of woody crops, such as citrus, that are vegetatively propagated and from which in vitro generated transgenic plantlets are directly transferred to the greenhouse.

Transfer, integration, and expression of T-DNA from the root-inducing (Ri) plasmid of the soil pathogen *Agrobacterium rhizogenes*, causative agent of hairy root disease in dicotyledonous plants, lead to development of the hairy root phenotype. Plants regenerated from hairy roots often show morphological changes including wrinkled leaves, shortened internodes, reduced apical dominance, reduced fertility, and altered flowering (Christey 2001). Four loci (rolA, B, C, D) on the T-DNA have been identified to be involved in hairy root formation. To induce altered phenotypes in transgenic plants, rol genes have also been transferred individually. The exact mechanisms, however, whereby the rol genes cause the various phenotypic changes are not fully known.

Recently, gene transfer to plants by diverse species of bacteria, other than *Agrobacterium*, has been described (Broothaerts et al. 2005). Plant-associated symbiotic bacteria were made competent for gene transfer by acquisition of both a disarmed T-plasmid and a suitable binary vector. *Rhizobium* sp. NGR234, a broad-host range *Rhizobium* species that nodulates over 100 different plants, the alfalfa symbiont *Sinorhizobium meliloti*, and *Mesorhizobium loti*, a representative of phyllobacteriaceae, transformed *Arabidopsis*, tobacco, and rice plants with overall transformation frequencies from 9 % to 36 %. All three plant species showed stable inheritance of the transgenic GUS and hygromycin resistance phenotypes with approximately Mendelian segregation. According to Broothearts et al. (2005), in contrast to the complex licensing landscape for *Agrobacterium* methodology, this alternative technology will be available to the international community in a "protected technology commons" optimized and improved as a BioForge project (http://www.bioforge.net), and accessible under a biological innovation for open society (BIOS) license (http://www.bios.net). As plant-associated bacteria such as *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, being symbionts or benign endo- and epiphytes, are expected to evoke plant responses different from the pathogen *Agrobacterium*, gene transfer to previously intractable cell types, explants, and plant species may be facilitated. There are no data yet with regard to copy number of integrated transgenes or to number and structure of integration sites and their potential effects on plant genomes and gene activity.

3.1.2 Direct gene transfer

Gene cassettes constructed for direct gene transfer (by microprojectile bombardment or protoplast transformation) are usually spliced into plasmid vectors of mostly bacterial origin. These vector constructs are applied to the plant cells in toto as supercoiled circular plasmids or as linearized plasmid DNA. The whole plasmid can be taken up by the cell and any part of this foreign DNA can become integrated into the plant genome. Up to a few hundred kb of DNA can thus be delivered to the plant genome at once. Integration of the whole plasmid at a single locus has been observed (Srivastava et al. 1999, Gahakwa et al. 2000, Smith et al.

2001) and stable inheritance of transgenes conforming to Mendelian ratios has been reported. Under artificial conditions, endophytic bacteria living in plant cells could also be transformed by plant transformation vectors, but there was no evidence for accidental transformation in plants (http://www.biosicherheit.de/features/printversion.php?context=1&id=114).

Very frequently, however, plasmids seem to fragment and recombine. The majority of reports reviewed by Smith and colleagues show transgene copy numbers ranging between one and twenty, in some cases surpassing this number. In many cases, more detailed molecular analysis revealed a complex integration pattern with integration of plasmid sequences at a single or at multiple sites of the plant genome (Pawlowski et al. 1998, Hansen & Wright 1999, Svitashev et al. 2000, 2002, reviewed by Smith et al. 2001), with different transformation events exhibiting integration patterns of different complexity (Clausen et al. 2000). Most maize transformants derived from particle bombardment had more than 10 transgene copies, with a third of them having about 100 and more copies, but most of them (~ 75 %) seemed to have insertions at a single locus (Shou et al. 2004). Multiple copies of full length, truncated, and rearranged transgenes can be found integrated at the same or tightly linked loci, in part with short sections of plant genomic DNA interspersed between them. Integration can clearly occur at different genomic loci.

Such compound loci and multiple copies can lead to gene silencing more frequently than simple loci and single copies (Pinto et al. 1999, Shou et al. 2004). Compound loci may also be prone to instability and rearrangement. Seemingly simple phenotypes can conceal inherent genetic instability as shown by RFLP analysis in over 300 progeny plants of a transgenic soybean line (Choffnes et al. 2001). These transgene integration patterns may be different depending on DNA constructs used. Constructs lacking vector backbone sequences generated predominantly low-copy-number transgenic rice plants, whereas rice plants transformed with supercoiled or linearized whole plasmids showed complex integration patterns with higher copy numbers and frequent transgene rearrangements (Fu et al. 2000).

The frequently observed rearrangements of transgenes include deletions from the ends of linearized transforming constructs, direct repeats, inverted repeats, deletion and ligation of fragments and concatemerization (Svitashev et al. 2000, Smith et al. 2001). However, in papers dealing with transformation of plants, only rarely data about Southern analysis of transgene integration events are provided. In spite of the wide use of Roundup Ready soybean since its first commercial cultivation in 1996, a detailed analysis of the insert has been published only recently (Windels et al. 2001). Anchored PCR analysis of the insert, containing a portion of the CaMV 35S promoter, the *Petunia hybrida* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) chloroplast transit peptide, the CP4 EPSPS coding sequence, and a portion of the 3‘ nontranslated region of the nopaline synthase gene terminator (NOS), revealed rearrangements at the 3‘NOS junction between insert and genomic plant DNA. Duplication of a 0.25 kb fragment of CP4 EPSPS was linked to a 0.54 kb fragment for which in a BLAST (basic local alignment search tool) search no sequence homology could be detected.

Using phenotypic and genotypic segregation, Southern blot and fluorescence in situ hybridization (FISH), Svitashev et al. (2000) found that half of the 16 transgenic oat lines studied had a single locus and half had two or three loci. Complex structures such as tightly

linked clusters of multiple transgene copies interspersed with oat DNA that could be megabases long have been found. A quarter of the loci showed rearrangement of chromosomes, some chromosomes carried two loci. By sequencing more than 160 kb of the internal structure of complex transgene loci in two oat lines, transformed by microprojectile bombardment, Svitashev et al. (2002) detected extensive scrambling of non-contiguous transgene and oat genomic fragments that were recombined via illegitimate recombination. The integration sites contained perfect and imperfect direct repeats and inverted repeats of the delivered DNA, intermingled with stretches of rearranged genomic DNA. Interestingly, most of the transgenic and genomic DNA fragments were of small size, with 50 of the 82 transgene fragments being shorter than 200 bp and several transgene and genomic fragments being even shorter than 15 bp, the latter perhaps served as filler DNA for repair of double strand breaks in the recipient genome. Analysis of the breakpoints revealed that most of them were associated with palindromes involving ten or more nucleotides, indicating that palindromes might be preferred targets for breakpoint formation in the transferred DNA. At one junction, a spurious open reading frame (ORF) was created. Such a high level of transgene locus complexity has been rarely recorded, most likely because it would not be detected using conventional methods for analysis of transgene loci, such as Southern hybridization, fluorescence in situ hybridization (FISH), or by sequencing only specific regions of transgene loci. Previously, complex transgene loci with transgenic DNA interspersed by host DNA have been reported for 13 transgenic oat lines too, leading to sizes of transgene loci of 35 to 280 kb (Pawlowski et al. 1998).

Transgene loci can also exhibit considerable degrees of instability in the progeny, as indicated by the analysis of over 300 progeny plants of a soybean line containing four copies of a bovine ß-caseine gene and a hygromycin resistance gene at a single locus (Choffnes et al. 2001). Rearrangements in approximately 16 % of both T1 and T2 generation resulted in plants that contained less transgene copies than the original transformant. The authors reasoned that recombination hotspots, perhaps situated in interspersed genomic DNA and active during mitosis and/or meiosis, might have separated the four-copy locus into loci with less transgene copies.

Complex integration patterns could be resolved by using the Cre-*lox* system. Transgenic wheat plants containing a transgene flanked by recombination sites (*lox*-sequences) were crossed with transgenic plants containing the Cre recombinase gene. Recombination between the outer *lox*-sites can lead to resolution of the integrated multiple copies into a single copy in the progeny, chimerism of such plants, however, is possible (Srivastava et al. 1999).

Particle bombardment allows the simultaneous transfer of many different constructs, either linked on the same cointegrate vector or introduced on separate plasmids. Co-transformation efficiency seems to be independent of linkage of the transgenes. Co-transferred plasmids can be found integrated at linked and unlinked sites. After co-transformation of rice with a mixture of 14 pUC-based plasmids (five marker genes and nine different coding sequences from rice viruses), 85 % of the transgenic plants regenerated carried more than two transgenes, 17 % contained ten and more, and three plants contained even 13 transgenes, but the latter plants proved to be sterile (Chen et al. 1998). All plants, except one, showed normal morphology and growth habit. Based on Southern blot analysis, gene copy numbers between

one and four were estimated. In single rice plants analysed, most cotransferred transgenes integrated at one site (deduced from co-segregation of transgenes with the selectable marker), sometimes at two separate loci. Simultaneous transformation of rice with nine different plasmids/genes, among them marker genes and genes for pharmaceutical proteins, controlled by four different promotors, has been reported to lead to 66 cell lines and 32 plant lines (Wu et al. 2002). The transformed cell lines and plants carried from three to nine genes, often in high copy numbers and expressed, in general, about half of the transgenes detected by PCR analysis. In the five transgenic lines analysed, expression seemed to be stable over three generations. According to progeny segregation analysis, the multiple transgenes integrated at the same locus of the rice genome, although more detailed analysis has not been undertaken.

Only limited data are available with regard to the factors controlling the diverse patterns of integration observed (Smith et al. 2001). Rearrangement may occur prior to integration, since integration patterns usually remain unchanged through repeated subculturing of tissues. Kohli et al. (1998) suggested a two-phase integration mechanism with DNA molecules becoming spliced together and integrated without interspersing plant genomic DNA in the first phase. In the second phase, such integration sites could act as hot spots for further integration of transforming molecules. Gelvin (1998) provided several possible explanations for the integration of different plasmids (and multiple copies of these plasmids) into the same site of the plant genome: First, the vector sequences could provide regions of homology leading to recombination before DNA integration. Second, a single integrated plasmid could undergo recombination with further plasmid DNA, either because the exogenous DNA was attracted to repair complexes forming at the integration site, or because recombination occured between homologous sequences in the integrated plasmid and the other plasmid sequences. Third, all plasmids integrate directly and simultaneously into a region of the genome that may be damaged and contain active DNA repair enzyme complexes, thus leading to transgene sequences interspersed with plant DNA. Since genomic integration of transgenes has been associated with DNA replication and break-repair processes, Pawlowski et al. (1998) proposed that integration of transgenes might preferentially occur at replication forks. Integration at a cluster of active replication forks could thus account for the interspersion of the transgenes with host DNA as observed in transgenic oat.

Svitashev et al. (2000) proposed a model for transgene integration that would account for (1) the clustered organization of transgenes in single loci, (2) the interspersion of transgenes within clusters with genome DNA fragments, and (3) the association of transgene integration loci with rearranged chromosomes. According to this model, delivery of DNA-coated microprojectiles to the nuclei of plant cells would likely cause extensive chromosomal breakage and an extrachromosomal pool of whole and sheared plasmid transgene DNA mixed with host DNA fragments of variable length would be created. Random preintegration ligation on DNA fragments in this extrachromosmal pool would produce interspersed structures containing transgene and host DNA which may then be integrated into single or multiple loci through a break-repair mechanism. A significant mutational load for the transgenic cell might result which possibly can be tolerated more easily by polyploid plants such as oat than by diploids. The extensive rearrangements of transgene loci in transgenic oat are, according to Svitashev et al. (2002), best explained by illegitimate recombination as the main mechanism

of transgene integration, mediated by a synthesis-dependent strand-annealing mechanism that resulted in transgene scrambling.

The vector backbone, when present, may also play a significant role in the integration process (Fu et al. 2000). First, backbone elements may promote high-copy-number integration events by providing extensive regions of homology. Second, the vector backbone can provide a number of recombination hotspots. Minimal cassettes provide shorter regions of homology and fewer recombination hotspots than whole plasmid DNA, therefore, integration patterns should be less complex in cases where backbone sequences have been removed.

Possible consequences of transgene scrambling might be gene silencing or the production of spurious open reading frames through juxtaposition of a transgene or plant promotor to scrambled genomic or transgene sequences. Integration of transgenes might occur close to retrotransposons (mobile genetic elements that transpose via the reverse transcription of an RNA intermediate) that are ubiquitous in plants and comprise significant amounts of nuclear DNA content. For the HR maize line GA 21, it has been reported that the 3' end of the recombinant DNA insertion disrupts the pol-polyprotein gene of a maize retrotransposon (Jank & Haslberger 2000). Because retrotransposons carry strong promoters within their long terminal repeats, insertions could give rise to altered spatial and temporal expression patterns of genes in close proximity. Even if interrupted, retrotransposons may still be able to transpose by the help of trans-acting factors, which can affect the genetic stability of DNA inserts.

In summary, these findings indicate that microprojectile transformation frequently leads to very complex integration patterns and chromosomal aberrations that can influence the traits of transgenic plants (silencing of transgenes and endogenous sequences and creation of open reading frames). Therefore, in transgenic plants, junction regions between inserts and plant genomic DNA should be characterised more properly, preferably before transgenic plants are released into the environment or before they are marketed. However, in most papers analysed for the present study, data about integration sites and their structure have not been presented.

3.1.3 Other transformation techniques

In protoplast transformation using PEG or electroporation, transgenes can be integrated in single copies but also in multiple copies linked together or at separate loci, rearrangement of transgene sequences has been observed too. The cell cycle stage of the protoplasts seems to play an important role with regard to the integration pattern, with protoplasts in M phase (mitotic phase) giving rise to transgenic plants containing more copies of the transforming plasmid, usually at separate loci. Protoplasts in S phase (DNA synthesis phase) result in high copy numbers and frequent rearrangement of plasmid sequences (Kartzke et al. 1990).

Agrolistic transformation has been developed to combine the advantages of particle bombardment and *Agrobacterium*-mediated transformation. It is based on co-bombarding virD genes along with T-DNA borders flanking the introduced transgene. The aim is to produce a high frequency of transgenic plants that lack the superfluous vector sequence and contain only a single copy of the transgene(s). However, rearrangement of plasmid sequences

and integration of multiple copies is not precluded with ceertainty (Smith et al. 2001). Co-integration of virDNA has been described as a frequent event (Srivastava et al. 1999).

Microinjection of DNA in plant cells and co-injection of fluorescent dyes to mark transformed cells may allow transfer of functional genes without selectable marker genes, with GFP genes potentially serving as reporter genes. Transformation rates may be increased by use of specific restriction enzymes, concomitant use of *A. tumefaciens* proteins involved in T-DNA integration, and activation of cellular DNA repair systems via UV treatment (http://www.biosicherheit.de/features/printversion.php?context=1&id=42).

3.1.4 Expression of multiple foreign genes

For future applications, such as increased stress resistance or altered secondary metabolism, the simultaneous and coordinated expression of genes encoding multiple steps in a pathway may be desirable. The expression of multiple foreign genes in plants can be achieved either by crossing plants transformed with different genes of interest to yield progeny carrying the desired genes, or by simultaneous transformation with more than one gene of interest. In the latter case, different transformation vectors may be co-transferred (Chen et al. 1998, Wu et al. 2002) or, alternatively, the various transcription units may be assembled in a single transformation vector (Hunt & Maiti 2001, Daniell & Dhingra 2002). Goderis et al. (2002) described a binary vector for *Agrobacterium*-mediated transformation that contained a multiple cloning site with various hexa- and octanucleotide restriction sites and homing endonuclease sites, thus allowing flexible insertion of up to six transcription units, each controlled by a different promoter.

However, there are drawbacks to most of these strategies. The unpredictable expression of transgenes due to gene silencing is thought to occur in connection with the repeated use of promoters and other foreign sequences and co-transformation of two T-DNA regions located on the same plasmid may result in the co-transfer of adjacent T-DNA and (unwanted) plasmid backbone (McCormac et al. 2001).

For these reasons, Hunt & Maiti (2001) suggested the development of methods for polycistronic or polygenic transgene expression in plants: While translation in eucaryotic cells usually involves monocistronic mRNA, virus translational transactivator proteins have been shown to promote expression of downstream reading frames in multicistronic mRNAs. In cases where roughly equivalent levels of expression of different cistrons in a polycistronic mRNA are desired, this may be feasible. Specific proteinases of the picornavirus family, on the other hand, can process polyproteins derived from monocistronic mRNA and thus liberate mature peptides from a polyprotein precursor. But partial processing seems to occur. Whether the expression of such viral genes is detrimental to plant growth and development is not clear at the moment (Hunt & Maiti 2001). The expression of a transgene encoding a cleavable chimeric polyprotein has been reported recently by François et al. (2002). In addition to nuclear transformation with different plasmids or expression of polyproteins, the coordinate expression of several transgenes could be also achieved in plastids, as plastids show characteristics of bacterial transcription and translation systems with polycistronic mRNAs.

Compared to nuclear transformation, transgene expression in plastids can also lead to far higher accumulation of transgene products (Daniell & Dhingra 2002).

3.1.5 Superfluous DNA

In *Agrobacterium*-mediated transformation, along with the sequences intended, DNA derived from the plasmid backbones not needed in plant transformation can be transferred to the nuclear genome, perhaps as a result of T-DNA "border skipping" or incorrect processing at the T-DNA borders (Fu et al. 2000, Frary and Hamilton 2001, McCormac et al. 2001, Smith et al. 2001, Theuns et al. 2002). However, because of their tendency to promote transgene rearrangements, possibly by plasmid-plasmid recombination events, integration of plasmid backbone sequences into the plant genome is undesirable. As they represent targets for nucleases and topoisomerases, particular DNA sequences such as plasmid origins of replication may promote replication-mediated recombination (Fu et al. 2000). The plasmid backbone can also provide recombinogenic AT-rich sequences that could lead to complex plasmid multimerization events. Genomic DNA segments may be captured, resulting in the integration of large complexes comprising both exogenous and genomic DNA. Large and complex transgenic loci tend to be silenced and be meiotically unstable, leading to (partial) excision of the locus and loss of transgene expression in subsequent generations. Transfer of vector backbone sequences could also result in the transfer of additional antibiotic resistance genes into the transgenic lines (Theuns et al. 2002). Finally, new replicons, containing plasmid origins of replication, transgenes, and plant genomic DNA, may escape into the environment by horizontal gene transfer (Fu et al. 2000).

In a review on superfluous DNA integration, Smith et al. (2001) challenge the view that from binary and cointegrative vectors in *Agrobacterium*-mediated transformation T-DNA alone is transferred to the plant cell, causing only minor deletions at the end of the T-DNA and within the plant genome during integration. They found evidence that plasmid sequences from either side of the T-DNA borders on T-DNA strands can be transferred to plant cells. In using T-DNA fingerprinting, Theuns et al. (2002) demonstrated the presence of vector backbone sequences in a number of transformed *Arabidopsis* lines. Similarly, 75 % of maize events resulting from *Agrobacterium*-mediated transformation carried some portion of T-DNA, varying in length from event to event with some having probably the entire backbone region (Shou et al. 2004).

Transfer of backbone sequences seems to occur regardless of their locations either to the left or to the right T-DNA border. But the right border might be more active than the left, perhaps due to the presence of overdrive enhancer sequences which exert their effects over larger distances. Of the eight transgenic *Arabidopsis* lines harboring backbone sequences, analysed by Theuns et al. (2002), one contained sequences at the left border, two sequences at the right border and five lines contained vector backbone sequences at both the left and the right border. The type of plasmid used and the genes of interest, inserted in the T-DNA region, could be important too in mediating T-DNA transfer. For example, tobacco transformants with large inserts in the transferred T-DNA showed an increased incidence of non-T-DNA vector backbone sequences integrated (Frary & Hamilton 2001). Co-transformation by

independent T-DNAs, held as distinct regions on the same plasmid, may simultaneously increase the risk of co-transfer of plasmid backbone sequences, as co-transformation experiments with tobacco indicate (McCormac et al. 2001).

Functional sequences located outside the T-DNA left and right borders, including GUS reporter gene and antibiotic gene sequences, have been found in transgenic plants (Srivastava et al. 1999, Smith et al. 2001). The complete binary vector sequence could be identified in transformed *Arabidopsis thaliana,* concatemers of the binary vector have been found to be integrated in the genome of tobacco plants derived from protoplasts transformed with *Agrobacterium*. Such sequences can be integrated into the plant genome even independently from T-DNA sequences at separate locations (Frary & Hamilton 2001). According to Frisch et al. (1995), approximately half of the Bin 19 plasmid consists of superfluous sequences and about 800 bp of superfluous sequence are present within the T-DNA region between the multiple cloning site and the right border. Sequences not needed for transformation include a broad host-range origin of replication, remnants of antibiotic resistance genes and fragments of other (bacterial) genes.

Though, in general, vectors used for direct gene transfer are significantly smaller than the ones used in *Agrobacterium*-mediated transformation, they can contain sequences not necessary for their function. Vectors for microprojectile bombardment do not undergo processing at specific sequences like the T-DNA left and right borders. Therefore, any DNA sequence within the plasmid has an equal chance of being incorporated into the plant genome. Carrier DNA that has been included in the transformation mix to increase the frequency of transformation can possibly promote transgene rearrangement and become integrated into the plant genome at the same or different loci as the gene(s) of interest (Smith et al. 2001). Since carrier DNA sequences are not normally probed in Southern blots, they will remain undetected. In agrolistic transformation, cointegration of VirDNA has been described as a frequent event (Srivastava et al. 1999).

According to Smith et al. (2001), the extent of superfluous vector sequences integrated into plant genomes as a result of microprojectile bombardment has not been routinely assessed. In most cases, molecular analysis has been restricted to the genes of interest and the selectable marker genes. However, where the selectable marker gene und gene(s) of interest are equal in size to the remaining DNA making up the complete vector, the total amount of superfluous vector sequence transferred to plant cells during transformation experiments is equal to the amount of selectable marker and gene of interest transferred. A certain proportion of cells transformed by particle bombardment are, therefore, likely to contain rearranged and/or multiple copies of remaining superfluous vector sequences. Based on knowledge about vector constructs, Smith et al. surmise that many transformed plant cells and regenerated transgenic plants contain multiple copies of antibiotic resistance genes and/or their regulatory sequences, bacterial origins of replication, or other vector sequences. These insertions may be located at the same loci as the gene(s) of interest and the marker gene or at unlinked single or multiple loci. In the majority of transformation experiments analysed, co-transformation frequencies of the selectable marker gene and the gene(s) of interest exceeded 50 %, suggesting that superfluous vector DNA may be integrated into the plant genome at similar frequencies. Southern blots could help in identfying unexpected fragments of vector sequences, however,

Smith et al. (2001) found no reports of superfluous plasmid sequence being used as a probe to assess the extent to which integration of these sequences has occurred.

Generally, selection pressure exerted on the selectable marker genes may affect the proportions of transformants that contain single or multiple copy transgenes (and superfluous DNA sequences). Under high selection pressure, transformants with multiple copies of a functional selectable marker gene may be more likely to produce enough enzyme to detoxify the antibiotic or herbicide. Selection of transformants with either single or multiple transgene copies can also be influenced by the strength and activity of the promoter driving the marker gene, as the amount of marker gene product will depend on promoter strength in the given explant tissue. If, depending on transformation and regeneration protocols, selection favours transformants with multiple transgene copies, multiple copies of other superfluous DNA sequences could be co-selected in such transformants (Smith et al. 2001).

Smith et al. (2001) concluded that, although there is vast evidence for the integration of superfluous vector DNA into the genomes of transgenic plants, this phenomenon has been occurring largely unchecked among researchers. This is because common practice to verify the nature of foreign DNA integration is to use DNA probes that are homologous only to sequences found within the T-DNA borders or to selectable marker genes and/or genes of interest. Therefore, the real frequency of backbone integration must be higher than recorded. In addition, bacterial sequences are probably transferred far more often than assumed, sequences that are unnecessary and superfluous if not risky because they can contain (parts of) antibiotic resistance genes or origins of replication. Such sequences could increase the risks linked to horizontal gene transfer.

More research is needed in order to better understand the processes by which DNA is transferred and integrated into the plant cell and to identify the transformation conditions leading to integration of multiple transgene copies and superfluous DNA and to DNA rearrangements at the integration sites. The vast quantity of transgenic plants and seed held by the numerous molecular plant laboratories should be reanalysed with respect to the integration of multiple transgene copies and superfluous DNA in relation to the transformation conditions, newly developed transformants should be analysed thoroughly (Smith et al. 2001, Theuns et al. 2002, Wilson et al. 2004). If multiple transgene copies and superfluous DNA sequences are found to be present in the plant genome, it has to be evaluated whether this might have an additonal impact on the environment the plant is intended to grow in or on human health if the transformed plant is intended for food use.

3.2 Promoters used in plant transformation

Promoters regulate the expression of transgenes by up and down regulating the rate of transcription into mRNA and possibly of translation into proteins. Promoters may act specifically during development or in certain tissues. Some are active in only one or a few species, others in a broad range of species. Many different promoters, derived from viruses, bacteria, or plants are used in plant transformation, resulting in various expression levels and tissue specificities. High expression levels have been linked to promoters of maize genes encoding C4 photosynthetic enzymes such as phosphoenolpyruvate carboxylase (PEPC),

leading in rice to a very high amount of transgenic protein up to 12 % of the total soluble leaf protein (Ku et al. 1999). High constitutive gene expression has been observed in using a GUS construct linked to promoter and intron sequences of a plant histone H3 gene, sequences that normally lead to abundant production of replacement histones in differentiated plant tissues (Kelemen et al. 2002). Constitutive promoters such as the ubiquitin1 promoter from maize may also be stress-inducible, e.g. in response to a pathogen attack, possibly qualifying this promoter for the regulation of transgenes that encode antifungal proteins (Oldach et al. 2001).

In contrast to viral promoters, promoters of highly expressed plant genes, such as promoter and intron sequences of plant histone genes, are presumed not to lead to (multi-copy) gene silencing (Kelemen et al. 2002). Cryptic promoters that are inactive at their natural location in the plant genome may also be used to drive the expression of transgenes. They are believed to be abundant and to generate diverse expression profiles ranging from cell specific to constitutive activity. The cryptic promoter tCUP from tobacco is active in a wide range of plant species when fused to cloned genes, such as selectable marker genes, and exhibits activities comparable to the 35S CaMV promoter (Tian et al. 2002). Tissue or organ specificity can be achieved by the use of promoters mainly or only active in certain tissues such as pollen, seed, or tubers. However, leakiness of organ specificity has been observed, potentially due to chromosomal position effects (Dietz-Pfeilstetter et al. 2003). Synthetic pathogen-inducible promotors containing cis-acting regulatory elements could mediate local gene expression after pathogen attack and wounding (Rushton et al. 2002).

Recently, Tang et al. (2004) and Wang et al. (2003a) reviewed various promoter systems responding to chemical inducers that might allow regulated spatial and temporal gene expression. These systems are based on bacterial repressor-operator systems induced by tetracycline or pristinamycin, on copper- or ethanol-inducible systems from fungi, or on steroid receptor-based transcription activation systems induced by glucocorticoids or estrogen. Such inducers have also been suggested for genetic use restriction technologies (GURTs). Chemical inducers must be degradable and non-toxic to the ecosystem, capable of being applied in the field, transportable within the plant, and highly specific. However, most inducers tested so far, e.g. antibiotica, copper, and steroids, are unsuitable for field applications because of their toxicity and environmental effects. Ethanol-inducible systems, despite their merit of being rapid and reversible and relying on an inexpensive and safe inducer, have the drawback of high volatility of the inducer ethanol which makes it difficult to restrict induction to the plants or plant parts wanted.

Quantitative differences in transgene expression between independent transformants have often been observed. They are generally ascribed to different integration sites of the transgene leading to position effects. However, detailed information on the distribution of transgene expression throughout a plant, within a tissue, or over prolonged periods of time in a tissue is rarely available.

A study with transgenic petunia (*Petunia hybrida*), transformed with the firefly luciferase (luc) reporter gene, revealed that the temporal and spatial activity of three different promoters, CaMV 35S, modified CaMV 35S, and the promoter of an *Arabidopsis* lipid transfer protein, varied not only among independent transformants, but also between leaves on the same plant and within a leaf (van Leeuwen et al. 2001). Protein activity was correlated with local luc

mRNA levels, indicating that the observed differences in average luciferase activity in leaves were a true reflection of differences in average transcription rate. Since different types of spatial expression patterns could be stably inherited to progeny plants, the authors concluded that the variation of expression must be connected to the integration site of the transgene, perhaps in addition to local physiological conditions. Therefore, differences in the integration sites with regard to flanking DNA sequences may result in different local modulations of the regulation of transgene activity in individual transformants.

Since environmental impacts of the release of GMOs will depend greatly on the level of products produced in the transgenic plants, knowledge about activity and species and organ specificity of promoters is needed. It is generally assumed that most promoters active in eukaryotic organisms such as plants do not cause an efficient gene expression if present in prokaryotes, due to the postulated specificity of the promoters. To analyse the potential of different plant promoters to direct expression in bacteria, Jacob et al. (2002) combined ten plant-specific promoters (derived from potato, tobacco, *A. tumefaciens, A. rhizogenes,* and 35S CaMV) with the coding region of the luciferase genes from *Vibrio harveyi* and transformed five bacterial species representing different taxonomic groups (*Escherichia coli, Yersinia enterocolitica, A. tumefaciens, Pseudomonas putida,* and *Acinetobacter* sp. BD413). Surprisingly, gene expression was observed in 50 % of the combinations analysed, with some plant promoters from potato consistently showing strong expression of the luciferase genes, and one of them, ST-LS1, directing expression in all bacteria tested. Additional analysis of the transcription start site of this promoter indicated that the sequences of the plant promoter themselves were recognized by the bacterial transcription apparatus and that the transcription initiation site in bacteria was located nine to ten bases upstream of the transcription initiation site in plants.

These results indicate that the transcription machinery may have been conserved during evolution to a greater extent than expected and that, for that reason, plant promoters may not be as specific as generally assumed. If such constructs were transferred to bacteria via horizontal gene transfer, the transgenes could also be expressed in bacteria. Therefore, promoters used in transformation of plants, in particular of food and feed plants, need to be analysed with respect to their potential activity in bacteria more closely. According to Jacob et al. (2002), promoters with up- and down-regulation could be generated by site-directed mutagenesis, leading to transgene constructs that are less readily expressed in bacteria.

The promoter most widely used in plant transformation is the 35S promoter of cauliflower mosaic virus (CaMV), a pararetrovirus of crucifer plants. Its DNA is transcribed in the nucleus using the 35S promoter to give RNA. Virion DNA is synthesized in the cytoplasm by reverse transcription of the 35S RNA transcript and encapsidated in the virus particles (Hull et al. 2000). According to a survey of peer-reviewed papers published in 1997 and 1998 dealing with plant transformation, 68 % of the transformations reported involved different derivatives of the CaMV 35S promoter, varying in size and subdomains (Brinker 1999). The 35S promoter leads to strong expression of the genes fused to it, an expression which is usually stronger than under the control of any other kind of promoter (Brinker 1999). It is active in a broad variety of organisms, in particular in plants, but also in green algae, yeasts, and *E. coli*, and possibly in frog and mammalian cells too (Ho et al. 2000b), and shows some activity in

the procaryotic gene expression system of tobacco chloroplasts (Stegemann et al. 2003). In combination with other promoters, such as the auxine-regulated mannopine synthase (mas) promoter, expression can be increased 10 fold (Chong & Langridge 2000).

In general, 35S directed expression was thought to be constitutive and mostly independent with respect to environmental stimuli, the plant tissue (with low activity in pollen, and developmental stage. Recently, however, it was shown that it is highly active in pollen of strawberry and possibly some other species (Cordero de Mesa et al. 2004). The 35S promoter can also be affected by environmental conditions (Tesfaye et al. 2001) and can be sensitive to photoperiod and temperature, leading to upregulation in short photoperiods and downregulation at low temperatures (Schnurr & Guerra 2000). In field tests with sugarbeet/chard hybrids, almost complete inactivation of a virus resistance gene under the control of the 35S promotor has been described, in particular on long and sunny days with high temperatures (http://www.biosicherheit.de/features/printversion.php?context=1&id=91).

The promoter has a modular composition consisting of a core promoter and subdomains with enhancer function, some of which may be required for expression in roots and leaves (Ho et al. 1999 and references therein). The fragment extending between positions -343 and -9 in tandem order was found to enhance the level of promoter activity up to 100 fold (Chong and Langridge 2000). Due to its complex structure with major enhancer domains and at least five subdomains acting synergistically, the promotor may also be able to interact with native gene enhancers (Cordero de Mesa et al. 2004). Specific expression of transgenes could be achieved by combination of the 35S promoter with motifs or fragments from plant promoters. Sequences of coding genes and terminators as well as posttranscriptional processes seem to be important too (Brinker 1999). Many transgenes under the control of the 35S promoter of CaMV exhibit silencing phenomena, possibly due to ist viral nature, which may be considered invasive by plants triggering the plant's protective mechanisms for genome integrity (Matzke & Matzke 1995). In cruciferous plants, expression of transgenes controlled by the 35S promoter can be silenced upon infection with the cauliflower mosaic virus, as studies performed by Al-Kaff et al. (2000) have shown.

The 35S promoter contains a recombination hotspot at the 3'end which may ease recombination of transgenic sequences with endogenous sequences, including dormant endogenous viruses and retrotransposons widely distributed in plant genomes. Viral sequences could be mobilized and lead to new infectious viruses. Sequences that increase recombination have been suspected to enhance genetic instability of transgenic lines and increase the probability of horizontal gene transfer (Ho et al. 1999, 2000a, 2000b, Cummins et al. 2000). In addition to the recombination hotspot of the 35S promoter, transgenic plants derived from *Agrobacterium tumefaciens* mediated transformation contain recombinogenic sequences of the left and right border of the transforming T-DNA. Hull et al (2000) argue that potential risks associated with the use of the 35S promoter are no greater than those encountered in conventional plant breeding, in particular, as pararetroviral sequences, including potentially active viral promoters, have been shown to be present in genomes of food plants such as banana and tomato (Matzke et al. 2000).

The discussion about the safety of the 35S promoter has gained additional momentum by the reports of Quist & Chapela (2001) that corn landraces in several regions in Mexico have been

found to be contaminated by transgenic sequences, including CaMV 35S promoter sequences. According to the authors, the foreign genes partly seemed to have become re-assorted and introduced into different genomic backgrounds. This part of the paper was, however, heavily critisized as being an artefact of the particular inverse PCR method (Metz & Fütterer 2002, Kaplinsky et al. 2002, answer Quist & Chapela 2002) causing "Nature" to declare the evidence available had not been sufficient to justify the publication of the original paper (Nature Editor 2002).

If on hybridization with landrace corn the novel genetic material in fact broke up into pieces that moved around in the genome, the implications might be profound, because promoter sequences could change the activity of other genes in the genome (Mann 2002). The wide use of 35S promoter elements in many species of transgenic plants used in open field trials and commercial production may, therefore, be a matter of concern with regard to the impacts of transgenic plants on the environment and on food safety. In addition, constitutive expression of transgenes, though quite often desired for crop plants, is not welcome from an environmental perspective, since the new proteins may be present in plants in unphysiological doses without tissue and developmental stage specificity and irrespective of environmental conditions, thus increasing the probability of adverse impacts on non-target organisms.

3.3 Transformation of plastids

As different cellular environments may be particularly suited for specific processes, products or energy consuming processes, plastids or other cellular compartments could be attractive production sites (Bogorad 2000). In addition to the nuclear-cytoplasmic system and the mitochondria, plant cells possess a third gene-containing compartment, the plastids. Different types of plastids are produced, characterized by morphological and functional differences, containing e.g. chlorophyll (chloroplasts), carotenoids (chromoplasts), starch (amyloplasts), or oil (elaioplasts). Plastids differentiate from common progenitors (proplastids) and, therefore, the plastids of a given plant share an identical genetic complement. The plastid genome (plastome) of 120-160 kb is present in high copy numbers within a plastid (60 - 100 copies of a circular chromosome), a typical plant cell contains 50 – 60, perhaps up to 100, chloroplasts which makes about 3 000 (up to 10 000) genome equivalents per cell (Bogorad 2000, Heifetz 2000). About 130 -150 genes code for plastid proteins, the majority of the plastid proteins, however, is coded for by nuclear genes targeted to plastids by chloroplast target peptides (CTP). Chloroplast genomes contain 40 to 50 % of non-coding spacer regions, introns, and regulatory sequences (Grevich & Daniell 2005). Gene silencing, a phenomenon common with nuclear genes, is not observed in plastid genomes and, therefore, expression is expected to be more stable despite accumulation of transcripts at a level far higher than in nuclear transformants (van Bel et al. 2001, Grevich & Daniell 2005). Chloroplast genes are often grouped in operons from which multicistronic RNAs are transcribed.

Reasons cited for plastid transformation comprise, among others, the targeting of genes to specific sites in the plastid genome, thereby reducing or abolishing position effects or epigenetic silencing, the strong expression of transgenes achievable after plastid transformation, the availability of a different genetic background and different cell

compartments allowing the compartmentalization of otherwise toxic products, and a reduced risk of pollen-mediated gene transfer as there are no chloroplasts in pollen of most crop plant species (Koop et al. 1996, Bogorad 2000, Heifetz 2000, Bock 2001, Heifetz & Tuttle 2001, van Bel et al. 2001, Daniell & Dhingra 2002). Expression of insecticide toxins in chloroplasts could also reduce potential toxicity of pollen to non-target species (Grevich & Daniell 2005). The presence of chaperones and enzymes in chloroplast might even allow for the correct assembly of complex multi-subunit proteins (Grevich & Daniell 2005).

Plant species for which DNA delivery to plastids has been demonstrated comprise tobacco, potato, *Arabidopsis thaliana*, red pepper, rice, carrot, marigold, oilseed rape, petunia, cotton, and soybean (Heifetz 2000, Hou et al. 2003, Grevich & Daniell 2005), the alga *Chlamydomonas reinhardtii* serving as a model species. Reliable and efficient plastid transformation and regeneration of fertile plastid transformants has been achieved so far mainly in tobacco (Bogorad 2000, Heifetz 2000, Bock 2001), monocot plastids have proven to be difficult to transform (Grevich & Daniell 2005). Tobacco seems to be particularly suitable to plastid transformation, in part due to its photosynthetically active chloroplasts during in vitro culture of regenerable cells and tissues, the natural sensitivity of its plastid genome to antibiotics that affect ribosomes, such as spectinomycin, streptomycin, and kanamycin, and the established regeneration protocols of fertile plants from leaf cells (Heifetz 2000). Stable transformation of tomato plastids has been reported too (Ruf et al. 2001). If stable transformation of plastids (including non-green plastids) of other crop plants should be possible, the species range might be broadened to economically important crops. Beside the expression of agronomic traits such as herbicide, insect, and pathogen resistanc, and drought and salt tolerance, future developments include the expression of human proteins and edible vaccines in chloroplasts of food crops, or modifications of plant metabolism. Review articles contain lists of traits achieved by chloroplast transformation and the plants used (Daniell et al. 2002, Grevich & Daniell 2005). However, plastid expression may not be appropriate for all proteins, particularly those with specific protein-folding, disulfide cross-linking, and glycosylation requirements (Ma 2000, Chargelegue et al. 2001).

Transformation methods

Delivery of transforming DNA to plastids has been achieved by particle bombardment, by treatment of protoplasts with polyethylene glycol (PEG), and by microinjection (Bogorad 2000, Heifetz 2000, van Bel et al. 2001). DNA deposited on gold or tungsten microprojectiles ~ 1µm in size projected into cells can integrate in plastid genomes. For transformation of the agronomically important gramineous plants, embryogenic cells are required which, however, contain non-green proplastids that are smaller than the projectiles used for biolistic plant transformation (Chamberlain & Stewart 1999). Following protoplast isolation, PEG treatment leads to poration of cell membranes for the entry of genetic material (Koop et al. 1996). Using syringes with extremely narrow tips, DNA material has been successfully injected into chloroplasts (Knoblauch et al. 1999). But these methods for transformation of plastids may have some drawbacks, as the biolistic delivery technique can lead to mechanical shearing of large plastids and chemical attack of tungsten (a reactive transition metal) can result in DNA modifications or cleavage (Heifetz 2002). In additon, different phenotypes have been

observed after biolistic and PEG transformation with similar genetic constructs (Heifetz 2000).

Homologous recombination

Transformation of plastids occurs through site-specific homologous recombination of plastid DNA sequences with transforming DNA carrying regions of nucleotide identity to these plastid sequences, the efficiency of recombination depending strongly on the extent of homology between donor and target regions. Multiple recombination events may take place (Maliga 2002). Foreign vector DNA is usually excluded. This is in contrast to the integration of cloned DNA into the nuclear genome which occurs randomly and often involves the cointegration of vector DNA (Kavanagh et al. 1999). The formation of episomal, plasmid-like elements in chloroplasts after transformation has been reported (Bock 2001). The plastid genome shows high recombination activity, probably linked to a RecA-mediated recombination system and, as a consequence, the genome exists in a continuous state of inter- and intra-molecular exchange (Heifetz 2000). Recombinants may exhibit multiple internal exchange events resulting in mosaic-type integration of donor DNA (Kavanagh et al. 1999). For homologous recombination, various target sites in plastid genomes can be used (Koop et al. 1996). Kavanagh et al. (1999) report about position effects, whereas according to Grevich & Daniell (2005), there is no concern about such effects because of integration in site-specific spacer regions of the chloroplast genome. Ruf et al. (2001) reported the construction of new vectors leading to high transformation frequencies of tobacco and tomato plastids. In these vectors, the selectable marker gene aadA was inserted between two transfer RNA (trn) genes providing regions of homology. This plastid targeting region seems to be highly conserved in the chloroplast genomes of dicotyledonous plants and may be used also for plastid transformation of other higher plants. The most preferred integration site seems to be the transcriptionally active spacer region within the rrn operon located in the inverted repeat regions. As the chloroplast origin of replication is close to this site, replication of foreign vectors within chloroplasts may be facilitated (Grevich & Daniell 2005). Aberrant integration and/or recombination events after integration have been observed.

Functional inactivation of photosynthetic genes and other plastid genes not essential for cell viability has been achieved by homologous recombination in *Chlamydomonas* and tobacco (Heifetz 2000, Bock 2001). Homeologous recombination seems to be possible too, as shown by plastid transformation of tobacco chloroplasts by homeologous potato plastid DNA, found to be mediated by multiple recombination events (Kavanagh et al. 1999). Homologous recombination can also be used to excise marker genes.

Inadvertent nuclear transformation

DNA transfer to plastids can lead to inadvertent nuclear transformation (Daniell et al. 2001b). This seems to be a general phenomenon in direct-gene-transfer experiments involving plastome-specific vectors, with PEG transformation perhaps being more prone to create unexpected nuclear mutations (Heifetz & Tuttle 2001). After PEG plastid transformation using two different plastome transformation vectors, only 45 % and 86 % of the spectinomycin/streptomycin resistant transformants, respectively, proved to be true plasmid transformants (Koop et al. 1996).

It was not clear, whether plastid regulatory sequences would also be active in the nuclear compartment or whether the aadA marker gene used could have been inserted downstream of an active nuclear promoter element. The perceived advantages of transforming the genome of plastids rather than the nuclear genome may thus not be realized in every case. Low-copy number integration into the nuclear genome may not be detected by Southern blots, since only event-specific characterization of the genetic structure of the transgenic insert would allow to determine the location within the cell. If inadvertent nuclear transformation occurs, it has to be assumed that transgenes from plants transformed in their plastid genomes can spread to the gene pool of other plants via normal hybridization and, therefore, such plants need not be particularly safe with regard to gene spread.

Recently, gene transfer from chloroplasts to the nucleus has been reported to occur in chloroplast-transformed tobacco (Huang et al. 2003a, Stegemann et al. 2003). Transfer of a nucleus-specific neomycin phosphotransferase (neo) gene, integrated into the chloroplast genome, was detected by screening for kanamycin resistant seedlings in 16 of about 250,000 progeny seedlings of wild type female plants fertilized with pollen from plastid transformants resulting in a frequency of about one in 16,000 (Huang et al. 2003a). Integrated sequences were of different size and, in addition to the transgene sequences, nuclear junction regions contained plastid sequences, an observation considered to provide evidence that nuclear insertion of the neo gene was not due to inadvertent nuclear transformation, as suggested by Daniell & Parkinson (2003), but was rather caused by secondary transfer of plastid sequences. Similarly, Stegemann et al. (2003) also reported about a surprisingly high frequency of gene transfer out of the chloroplast into the nucleus (1 of five million tobacco cells with chloroplasts transformed with antibiotic resistance genes). Plastid genes including transferred genes, therefore, seem to be mobile to some extent and integrate into the nucleus. In fact, there may be an ongoing mechanism for nuclear evolution through frequent acquisition of organellar DNA, perhaps facilitated by degradation of paternal plastids in tobacco pollen development (Stegemann et al. 2003, Martin 2003).

Homoplasmy and heteroplasmy

Initially, plastid transformation leads to a heteroplasmic state, where a portion of the cell's plastids has been transformed and the remainder of the plastids has still wildtype character. Iamtham and Day (2000) obtained heteroplasmic cells with different plastid recombinants after excision of the antbiotic resistance marker genes. Rice suspension cells whose plastids had been transformed by a GFP-antibiotic resistance marker fusion gene proved to be highly heteroplasmic (Khan & Maliga 1999). With 3 000-10 000 chloroplast chromosomes per cell, securing genetically stable transplastomic lines remains difficult. Therefore, efficient selection and segregeation to the homoplasmic state has proven to be a limiting factor (Heifetz 2000). The current major approach to obtain homoplasmic plants is repeated selection from transformed tissues in culture by antibiotics followed by regeneration of plants (Bock 2001). However, after release from selection pressure wildtype chromosomes „lurking" in the background may take over again. Several ways to improve the success rate of obtaining homoplasmic regenerants have been proposed by Bogorad (2001), for instance, GFP expression in plastids may be used to confirm the homoplasmic state (Stewart 2001). If gene

traffic occurred between chloroplasts, the chances and rate of stable transformation into a homoplasmic configuration could be increased.

Gene expression

Promoters derived from endogenous plastid genes have been used successfully for the constitutive expression of foreign genes in transformed plastids and, in addition, regulatory sequences derived from non-endogenous plastid genomes have also been used. The latter may help to reduce the degree of direct homology of sequences and hence the chance of unwanted recombination (Heifetz 2000). In addition to the RNA polymerase of the eubacterial type encoded in the plastid genome, plastids contain a second RNA polymerase expressed from the nuclear genome and imported into plastids. Promoters reacting specifically to this nuclear encoded RNA polymerase may be constructed too (Heifetz 2000).

Plastids seem to possess a broad range of post-transcriptional regulation (Daniell et al. 2002). Untranslated repeat (UTR) sequences of plastid RNAs act as regulatory elements for post-transcriptional control of gene expression. Transgenic tobacco lines containing different combinations of UTRs showed fivefold variation in the reporter gene mRNA level and approximately 100-fold differences in activity of the GUS protein as well as a different light inducibility (Eibl et al. 1999). RNA stability and translation efficiency in plastids are, therefore, differently affected by such UTR sequences. A 10 000 fold difference in protein accumulation was achieved by modification of translational control sequences, such as ribosome binding sites (RBS) and the N-terminal fusion of 14 amino acids of GFP (Ye et al. 2001). Post-transcriptional control of expression in plastids by RNA editing (conversion of specific C nucleotides to U in plastids) seems to play an important role too, and may be used to construct specific cassettes to function only in plastids and not in the nuclear genome, to which foreign genes could inadvertently be transferred during transformation procedures (Heifetz 2000, Heifetz & Tuttle 2001). In addition, chemically inducible trans-activation of plastid genes has been discussed. In such cases plastid gene expression could be induced on demand via a plastid-targeted transactivator protein which in turn is transcribed in the nucleus by a chemically regulated promoter (Heifetz 2000).

Expression of plastid transgenes may be different in leaves and reproductive organs, petals accumulated ~10 fold less and immature anthers ~50 fold less EPSPS than tobacco leaves (Ye et al. 2001). Chromoplasts in tomato fruit express the aadA marker transgene to ~50 % of the expression levels in leaf chloroplasts (Ruf et al. 2001). Foreign transcripts (and proteins) may accumulate in chloroplasts to far higher levels than after nuclear transformation (Daniell & Dhingra 2002). According to DeCosa et al. (2001), Bt toxin protein produced in tobacco chloroplasts accumulated to 46 % of total soluble protein in mature leaves. Staub et al. (2000) reported high yield production of metabolically active human somatotropin (growth hormone) in tobacco chloroplasts, accumulating to over 7 % of total soluble protein which is more than 300-fold higher than a similar gene expressed using a nuclear transgenic approach. Using various promoter and translational control elements for chloroplast expression of the bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), providing resistance to glyphosate, protein accumulation to >10 % of total soluble leaf protein could be found (Ye et al. 2001). It is possible, though, that high levels of production in chloroplasts might disrupt their function.

For this reason, Somerville & Bonetta (2001) suggested that it might be necessary to learn how to increase the number of chloroplasts per cell to obtain higher levels of production.

If bacterial genes are to be transferred to plants, expression in the plastid may be of advantage. Due to the similar codon usage in plastids and bacteria, a resynthesis of such genes may not be necessary (Heifetz 2000). Nevertheless, expression of the native bacterial EPSPS gene (CP4 gene from *Agrobacterium tumefaciens*) was enhanced by a factor of 1.5 if plastid-preferred codons were used (Ye et al. 2001). Foreign genes introduced into the plastid genome in a single operon regulated by a common promoter may facilitate the simultaneous introduction and expression of several genes of a pathway, in contrast to nuclear transformation with the genes usually transcribed singly. But from use of the same promoter at separate locations in the plastid chromosome no gene silencing is expected (Bogorad 2000, Bock 2001, DeCosa et al. 2001, Daniell & Dhingra 2002). To what extent protein stability contributes to the high foreign protein accumulation in transgenic chloroplasts is currently unknown (Bock 2001).

After microinjection of green fluorescent protein (GFP) DNA into individual chloroplasts, fluorescence was found in other chloroplasts too. Movement of GFP from injected chloroplasts to other chloroplasts via transient connections, known as stromules, was mentioned as likely explanation for this observation (Knoblauch et al. 1999). Such tubules, being similar to bacterial conjugation tubes may allow exchange of macromolecules between plastids occupying the same cell. Gray et al. (1999) presented evidence for GFP fluorescence in stromules interconnecting chloroplasts by confocal microscopy. Whether movement of proteins through stromules occurs naturally in plants and whether genetic material can be exchanged too, remains open (Daniell 1999, van Bel et al. 2001).

The high amount of a recombinant product produced in the plastid, likely exceeding the amount produced after nuclear transformation (Staub et al. 2000), is considered advantageous for productivity or pest control reasons. However, overabundance of a transgenic product such as Bt toxin may lead to potentially increased effects on non-target organisms or on the transplastomic plant itself, if the product showed some toxicity to non-target organisms or the plant.

3.4 Marker genes

Most selectable marker genes are bacterial genes and confer resistance to different antibiotics such as kanamycin and neomycin (nptII gene) and hygromycin (hph gene). To avoid antibiotic resistance genes, positive selectable marker genes are increasingly used for plant transformation (Penna et al. 2002). Such reporter genes include ß-glucuronidase (GUS), luciferase, green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and genes involved in anthocyanin biosynthesis. Routine utilization of GFP as a visual selectable marker gene has been reported by Kaeppler et al. (2001). By linking the gene of interest, such as a Bt toxin gene, to the GFP gene, fluorescence could even indicate the expression of the agronomically interesting gene in the field (Harper et al. 1999). GFP might be used for marking seeds, flowers, and other organs, and for monitoring of transgene movement in agronomic and ecological studies, although GFP fluorescence may be masked by phenolic

compounds produced in higher quantities in field grown plants (Stewart 2001). Another selectable marker gene could be the gene for the *E. coli* enzyme mannose-6-phosphate isomerase (MPI), converting the unusable carbon source mannose-6P to fructose-6P, thus enabling the transformants to grow on mannose as a sole carbon source (Hansen & Wright 1999, Reed et al. 2001, Penna et al. 2002). Similarly, bacterial xylose isomerase expressed in potato, tobacco, and tomato allowed the selection of transgenic plants on xylose-containing medium (Haldrup et al. 2001). Screening of transformed shoots by PCR could also permit the recovery of a workable number of transgenic plants, without the use of selectable markers (Dominguez et al 2004).

For selection of plant cells with transformed plastids marker traits are essential, most often antibiotic resistance marker genes are used such as the aadA gene (aminoglycoside 3‘-adenyltransferase), conferring resistance to spectinomycin and streptomycin (Koop et al. 1996, Daniell et al. 1998, Eibl et al. 1999, Kavanagh et al. 1999, Ruf et al. 2001), and the nptII gene conferring resistance to kanamycin (Heifetz 2000). These drugs suppress chlorophyll production and inhibit shoot formation on plant regeneration media. The transformed lines can thus be distinguished by their ability to form green shoots on bleached wild-type leaf sections (van Bel 2001 et al.). Green fluorescent protein (GFP) has been reported to function in plastids both as a transient and a stable visual marker (Khan & Maliga 1999, Heifetz 2000). To trace transformation in non-green plastids of embryogenic cells of cereal crops, Khan & Maliga (1999) developed a marker system, termed FLARE-S, that combines the aadA protein with GFP.

Genetic engineering of the chloroplast genome without the use of antibiotic selection and using the betaine aldehyde dehydrogenase (BADH) gene from spinach as a marker, instead, has been reported (Daniell 2001, Daniell et al. 2001a,b). This nuclear-encoded enzyme has been found in chloroplasts of a few plant species adapted to dry and saline environments. The selection process of transformants involves the conversion of toxic betaine aldehyde by the chloroplast-expressed BADH enzyme to nontoxic glycine betaine, which also serves as osmoprotectant. Non-transformed tissue will be killed under betaine aldehyde selection. The use of BADH selection not only provides a means of selection but potentially also an approach for enhancing stress tolerance in plants (Grevich & Daniell 2005).

The use of resistance marker genes has the disadvantage that transformed cells must be traced by stringent methods. Strong plastid expression of these marker genes may also lead to the presence of clinically important antibiotic resistance gene products in transgenic plants that could inactivate oral doses of these antibiotics commonly used to control bacterial infection in humans and animals (Daniell et al. 2001b).

Marker gene removal

Because of fears that antibiotic resistance genes derived from microorganisms, once transferred horizontally from transgenic plants to microorganisms, could increase the population of antibiotic-resistant bacteria, including pathogenic bacteria, they have come under heavy criticism in recent years. The likelihood for successful horizontal transfer of marker genes may be increased if there is more than one copy of resistance gene present in the genome. It is important to note that in homoplasmic transplastomic plants up to 10,000 copies

of the marker gene are present per cell, there may still be a few thousand copies in heteroplasmic cells. But there are other problems too. As there is only a limited number of selection marker genes useful in plant transformation, these marker genes will be introduced into many more species and cultivars than any other single transgene. In addition, as future commercial products are more likely to express complex traits, stacking of transgenes through crosses among different transgenic lines will produce plants that contain multiple copies of the same antibiotic resistance gene linked to different genes of interest. In stacking the desired traits by re-transformation, the same marker gene cannot be used for the selection of double-transformed plants. Additionally, gene silencing which is thought to be connected to the presence of multiple homologous sequences in the nuclear genome could be enhanced. Spreading of gene silencing from the marker genes to the closely linked trait genes would possibly also limit the long-term use of transgenic crops.

Several methods for removal of selectable antibiotic resistance marker genes have been described (Puchta 2003, Ow 2001, Ebinuma et al. 2001). If in co-transformation by two different constructs plasmids carrying marker genes integrate at a genome position unlinked from the sequences of interest, selectable marker genes can be removed in part of the progeny of such plants. The selectable marker gene and the gene of interest can be introduced by co-cultivation with two different *Agrobacterium* strains with one binary plasmid each, or by a single *Agrobacterium* strain carrying either two different plasmids or one plasmid with two T-DNA sequences encompassing the different genes. The frequency of co-transformation can vary from 10 % to 90 %. Co-transformation is greatly influenced by the state of the plant material, the tissue culture conditions, and the *Agrobacterium* strain. Since a proportion of the primary transformants contains a selectable marker gene, but no gene of interest, and others will have the two genes integrated at the same locus, more primary transgenic plants are needed to obtain the desired transgenic plant (Ebinuma et al. 2001). Elimination of plants carrying the marker gene may also be possible, if the marker gene is linked to a negative selectable marker gene coding for an enzyme that converts a non-toxic substance into a toxic one (e.g. conversion of N-acetyl-phosphinothricin into toxic herbicide ingredient phosphinothricin), under condition that the substance is added to the transgenic plants (http://www.biosicherheit.de/features/printvesion.php?context=&id=35).

Other approaches are based on the transfer of vectors containing the marker gene flanked by attachment regions or restriction sites of seldom cutting enzymes. Following intrachromosmal recombination between the attachment regions, somatic tissue can be identified carrying deletions of the marker gene, although with low efficiency (Zubko et al. 2000). Deletion events in genes of interest may occur as a result of illegitimate recombination (Ebinuma et al. 2001). Expression of the restriction enzyme I-SceI may induce double strand breaks in the transgenic plant and thus allow removal of the marker gene (http://www.biosicherheit.de/projekte/11.proj.html).

Transposable element systems and site-specific recombination sytems have been employed for marker removal too. The transposition method introduces either the marker or the trait gene on a transposon. Relocation of the transposon separates the two genes. The site-specific recombination system requires the expression of a recombinase protein (e.g. Cre, Flp, or R), specifically interacting with recombination sites (such as lox, FRT, or Rs) flanking the marker

gene. This interaction promotes recombination between the sites leading to removal of the marker. The recombinase gene can be introduced by crossing to another plant harboring the recombinase gene, or through a second round of transformation (Ow 2001) Excision may be more efficient and occurring earlier in development when the cre gene is introduced by re-transformation rather than by cross-pollination (Ebinuma et al. 2001). The recombinase gene could also be introduced by a viral vector that is transiently expressed and is not transferred via seed, part of the progeny of such plants will lose the marker gene (http://www.biosicherheit.de/features/printversion.php?context=1&id=57).

A method for chemical-inducible, site specific DNA excision in transgenic *Arabidopsis* plants using the Cre/loxP DNA recombination and the XVE-inducible expression systems has been developed by Zuo et al. (2001). Sequences encoding the ß-estradiol recognition sequence XVE, the selectable marker, and Cre are sandwiched by two loxP sites. Upon induction by ß-estradiol, Cre is expressed, leading to excision of these sequences from the genome which results in activation of the downstream GFP reporter gene. The cre/loxP system could, according to Keenan & Stemmer (2002), also be used to remove transgenes selectively from certain parts of the crop plant, e.g. fruit or pollen. In this case, a chemically inducible or tissue-specific promoter driving the recombinase gene would lead to the excision of a deletion cassette that is flanked by loxP sites and contains the gene of interest linked to the recombinase gene. Use of another chemical-inducible promoter, glutathione-S-transferase promoter, led to high frequencies of marker-free transgenic plants containing a single copy transgene (Sugita et al. 2000).

Drawbacks of these methods are however, that transposition activity is variable among plants and transposon excision can alter adjacent DNA sequences. Moreover, deleted fragments might reinsert into other genomic positions and recombinase/transposase proteins might cause undesirable secondary effects. The genetic segregation step required to remove the marker gene renders most methods of marker gene removal unsuited for plants that are propagated asexually or that have long generation times such as trees (Ow 2001).

Socalled multi-auto-transformation vectors can be used for removal of marker genes without crossing. In these vectors, positive marker genes such as isopentenyl transferase (ipt - leading to proliferation of transgenic cells and the differentiation of adventitious shoots) or rol (leading to “hairy roots” and abnormal phenotype of transformants) are combined with transposable elements or site-specific recombination systems, with the genes of interest placed outside these sequences. Excision of ipt or rol leads to shoots with normal phenotype without crossing (Ebinuma et al. 2001). By this method, marker free transgenic rice plants have been generated within one month (Ebinuma & Komamine 2001). Recognition sites of the site-specific recombination systems may, however, remain in the genome of transgenic plants. Particularly when many genes of interest are introduced through re-transformation using multi-auto-transformation vectors, recombination between such recognition sites could increase the chances of chromosomal recombination and rearrangement (Ebinuma et al. 2001). Instead of genes coding for plant growth regulators, genes related to organogenesis that, controlled by a strong promoter, lead to abnormal morphology, could be combined with removal systems.

As an approach to reduce the likelihood of gene spread and marker gene expression in microorganisms, the insertion of introns into antibiotic resistance marker genes has been suggested. Only eucaryotic organisms would be able to process and express such marker genes. A neomycin phosphotransferase II (nptII) gene with a potato intron inserted in its centre was shown to be processed and expressed in transgenic potato and tobacco, but not in *E. coli* and *A. tumefaciens* (Libiakova et al. 2001). In plants, introns in coding sequences may promote gene expression significantly, however, in this case an expression-promoting effect of the intron has not been observed.

Methods to remove antibiotic resistance genes from transgenic tobacco plastids have been described too (Iamtham and Day 2000). If plastids are transformed with a construct in which the selective marker is flanked by short direct repeats, the formation of different deletion derivatives by homologous recombination is allowed. The frequency of gene excision was controlled by the size and numbers of the direct repeats, whereas the pattern of gene excision was directed by the organisation of the repeats. Gene excision in transplastomic plants was a continuous process that accompanied vegetative growth and transmission through sexual crosses. The CRE-lox system can also be adapted to plastids, in such a way that the lox-flanked marker gene and the gene of interest introduced into the plastid genome are stable until CRE activity is provided from a nuclear-encoded, plastid-targeted CRE that is introduced by *Agrobacterium*-mediated transformation or by pollination (Corneille et al. 2001, Maliga 2002). However, rearrangements of the plastid genome have been detected, particularly in *Agrobacterium*-transformed clones.

Part II Traits and environmental impacts of transgenic plants

The ongoing discussion about potential impacts of GMOs on the environment reflects the general difficulty in predicting the occurrence and extent of long-term environmental effects when non-native organisms are introduced into ecosystems. The spread of the introduced sequences to other organisms of the same or another species via hybridization or horizontal gene transfer is one of the major concerns. Similarly, dispersal of transgenic organisms to natural ecosystems and the development of "superweeds" have received much attention. The possible evolution of new viral pathogens as a result of recombination between viral sequences and, particularly in the last years, the question of the stability of the transgenic sequences and their expression have also been discussed in great detail. Unintended effects of transgenes and/or their products on ecosystems will have to be dealt with on a case-by-case basis, although there are also general findings that may be of relevance in many cases. Finally, secondary effects have to be considered too. They may involve the evolution of weeds resistant to herbicides used in combination with HR crops, the evolution of pests and pathogens resistant to newly expressed insecticidal and antibiotic compounds, or altered agronomic practices or pesticide use linked to newly introduced crop systems.

Hazard for the environment will depend on the probability that a harmful event takes place (risk) and on the kind and degree of harm (Beusmann & Stirn 2001). Long term hazards to the ecosystems, i.e. species displacement and extinction, are difficult to predict because not all organisms affected may be identified, species can evolve in response to the hazard, and a nearly infinite number of direct and indirect biotic interactions can occur in nature. According to Muir (2001), if only GMOs would be released whose fitness is such that transgenes could not spread, risks would be close to zero and hazard would be irrelevant because the transgene is lost from the population. However, effects on non-target organisms and secondary effects dealing with agronomic practices or evolution of resistance would still have to be considered.

4 Gene spread

4.1 Hybridization

4.1.1 Gene flow to crops and to related species

4.1.1.1 Natural hybridization – an introduction

Via hybridization, transgenes can be transferred to non-GM plants of the same crop leading to transgene contamination of non-GM varieties or of landraces not supposed to be genetically engineered. In addition to that, many crop species are able to hybridize with wild relatives, many of them weedy species. Transgenes can thus introgress into the genomes of a range of

wild plant species, conferring new traits to them. As a result, new weed species may evolve or genetic diversity of wild native species could be reduced, perhaps contributing to their extinction (Wolfenbarger & Phifer 2000).

In recent years, numerous reports and reviews about gene spread, also called gene escape, have been published, most of them dealing with the probability of hybridization between crops and related species and the distances required for successful hybridization between pollen donors and recipients. In Europe, most studies dealing with gene transfer have been done with oilseed rape and sugar beet, as these crops originate from Europe and possess several related species.

In general, regions from which the crop species in question originated contain many wild relatives and quite often a great number of landraces that can hybridize with the transgenic crop, increasing the risk of unintended gene transfer from crop species to wild relatives. Many of the crop species now used worldwide originated from centers of biodiversity in the south. For this reason, these countries carry a particularly high risk of gene transfer if transgenic crops like corn, rice, tomato, and potato are cultivated there. If the cultivation of transgenic crops extends to many countries, the opportunity for range overlap with compatible relatives and the probability of transgene introgression into wild relatives will increase. Transfer of widely used genes, such as herbicide and insect resistance genes, will very quickly become a worldwide matter, over different geographical and climate regions and over different kinds of flora. Since the probability of gene flow is a function of the spatial scale of the introduction of GMOs, future reality of potential widespread planting will not always be mimicked sufficiently by field experiments (Wolfenbarger & Phifer 2000).

Transgene escape from GM crops can occur through different routes. Transgenes can be transferred by pollination to other crops or to wild related plants which might persist in agricultural, disturbed, or natural habitats. The transgenic plants may persist after crop harvest, possibly becoming a weed of agricultural land or they may persist in disturbed areas or natural habitats. Gene flow can also occur through seed dispersal.

Hybridization of crops with plants of the same or a related species will depend on a variety of parameters, quite often exerting influence on each other. Minimum requirements for successful hybridization are that potential hybridization mates flower at about the same time in a distance allowing pollen transfer by wind or insects and that the female plant is cross-pollinating. Factors influencing the rate of hybridization include (Schmitz & Schütte 2001, Mertens & Plän 2001, Brown et al. 2000, Chevre et al. 1999, Klöpffer et al. 1999, Pfeilstetter et al. 1998, Schütte 1998a, Eastham & Sweet 2002, Stewart et al. 2003, Ellstrand 2003, and references therein),

- flowering times and periods of crops and related species,
- distances between pollen donors and recipients
- population size of pollen donors and recipients
- shape and situation of pollen source
- weather conditions such as temperature, rainfall, humidity, wind speed and direction

- presence and foraging behaviour of insect pollinators
- pollen size, weight, and viability
- genotype and compatibility of the mating systems of crop plants and their relatives
- self-pollinating or cross-pollinating species
- survival rates and reproductive fitness of the hybrids and backcross progeny
- chromosome recombination.

Gene transfer to non-GM plants of the same crop species will depend largely on the factors dealing with distance, population size, and flowering periods of the pollen donors and recipients, presence and range of pollinators, and weather conditions. In particular, weather conditions will influence parameters such as activity of pollinators, viability of pollen, and direction and distances over which air-borne pollen can be carried. As weather conditions can change from day to day and from year to year, data gained from one day or year may not be valid for subsequent days or years. Self-pollinating species will show a lower degree of gene transfer.

Hybridization with wild plants will, first of all, depend on the presence and abundance of relatives of the transgenic crop species in a given region and the compatibility of the mating system of the crop species and its relatives. The synchrony of their flowering periods, the survival rates and reproductive fitness of the resulting hybrids and their backcross progeny are important parameters too. Chromosomal recombination may ease stable introgression of transgenes in the genomes of wild populations. Features of transgenes that increase the likelihood of their introgression into a wild relative include dominance, no association with deleterious crop alleles, and location on shared genomes and/or on homologous chromosomes (Stewart et al. 2003). Even if hybrids show low reproductive fitness, introgression may not be completely prevented since reproductive fitness can be regained by successive backcrosses (Brown et al. 2000, Snyder et al. 2000, Wang, Z. et al. 2001). Traits that permit the crop to be grown in new regions, including sites adjacent to a wild relative previously isolated, may lead to altered hybridization rates (Ellstrand 2003). Primary risks will come from crops that have undergone little domestication, that behave as weeds or have biotypes or relatives that are already weeds, and that outcross with some degree of self-incompatibility (Ellstrand 2003, Ellstrand & Hoffman 1990). For this reason, Stewart et al. (2003) recommend to use the herbicide resistance trait with care to avoid new weed problems.

In crop plants such as oilseed rape and corn, contamination of non-GM crops and seed by transgenes will be inevitable (http://europa.eu/int/comm/food/fs/sc/outcome_gmo_en.html). In fact, transgene contamination is widespread in oilseed rape seedlots collected in the US and western Canada, with levels of 0.05 % to 0.25 %, some even exceeding 2 % (Mellon & Rissler 2004, Friesen et al. 2003). But GM contamination of non-GM seed is much more widespread (www.gmcontaminationregister.org). This is of great concern for seed production and for agricultural forms such as organic farming that preclude the use of genetically modified organisms and demand zero contamination by transgenes (Bouchie 2002). Haslberger (2001) suggested to produce seeds only in areas where it has been ensured that no GMOs, or at least no GM varieties of the same or closely related species, have been grown.

There are no reliable data on spontaneous hybridizations between crops and wild plants in nature and their ecological consequences, but spontaneous interspecific crop x weed crosses may occur more frequently than reported in the literature (Darmency & Renard 1992). The number of known hybrids within an area can only be estimated, partly due to the difficulties in discerning the hybrid phenotypes. Data on cross-compatibility were usually collected specifically for crop improvement, involving often only a crop plant pollinated by pollen of a wild relative, or focusing on the potential for weedy traits to contaminate crops. As compatibility relationships are frequently asymmetrical, the effectiveness of cultivated species as pollen contributors is unknown in many cases (Ellstrand & Hoffman 1990). Seed purity data can be used to estimate the average pollen mediated gene flow from crops to wild relatives by measuring the inverse gene flow, i.e. gene flow from the wild plants to the crop (Lavigne et al. 2002). But, according to Abbo & Rubin (2000), the direction of gene flow may be more likely to occur from cultivars to the wild plants.

Many cases of introgression between wild and cultivated species in different parts of the world and in Europe have been reported. Some plant families, among them the *Poaceae* and the *Brassicaceae*, are known to show high numbers of natural hybrids (Gregorius & Steiner 1993, Bartsch et al. 1993, Gerdemann-Knörck & Tegeder 1997). At least 44 cultivated plants mate with one or more wild relatives somewhere in the world (Ellstrand 2002). According to Ellstrand (2003), among the 25 world's most important food crops 22 crops (representing 28 species) have some evidence for natural hybridization with one or more wild relatives (exceptions are peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), and sweet potato (*Ipomoea batatas*)). The 28 species with evidence for natural hybridization include annuals, perennials, herbaceous and woody plants, temperate and tropical crops, and crops grown worldwide or on a single continent. They can be cereals, vegetables, pulses, oil crops, and fruits. Extending the list to cultivated plants, Ellstrand (2003) found evidence for hybridization with related wild plants for 83 species, for 48 species evidence was more than just presence of morphological intermediaries where crop and wild relative are sympatric. Worldwide commercialisation of GM plants will therefore greatly increase the opportunity for range overlap with compatible relatives and, therefore, the probability of gene flow (Wolfenbarger & Phifer 2000).

According to a scheme developed to evaluate the probability of gene flow for some 30 important Swiss crops, the grasses *Festuca arundinacea, Festuca pratensis, Lolium multiflorum, Lolium perenne,* and alfalfa (*Medicago sativa*) are plants with the highest risk for gene flow, followed by salad (*Lactuca sativa*), carrot (*Daucus carota*), oilseed rape (*Brassica napus*), its relative *Brassica rapa* and others (Ammann et al. 1996, Jacot & Ammann 1999, Schmitz & Schütte 2001). Potato (*Solanum tuberosum*) and tomato (*Lycopersicon aesculentum*) were not considered to show a high risk of gene transfer to wild plants in Europe, as these crops originate from the Americas and have no wild relatives in Europe.

Stewart et al. (2003) proposed a somewhat different scheme, considering soybean (*Glycine max*), barley (*Hordeum vulgare*), finger millet (*Eleusine coracana*), pearl millet (*Pennisetum glaucum*), common bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*), and potato as very low risk crops. Corn (*Zea mays*), rice (*Oryza sativa*), and cotton Gossy*pium hirsutum, G. barbadense*) are supposed to be low risk crops, whereas alfalfa, sugar beet (*Beta vulgaris*),

wheat (*Triticum aestivum*), sunflower (*Helianthus annuus*), and oilseed rape would be moderate risk crops, and sorghum (*Sorghum bicolor*) was named as the only high risk crop. In a review of published literature on gene flow, Eastham & Sweet (2002) concluded that in Europe oilseed rape is a high risk and sugar beet a medium risk crop for pollen mediated gene flow from crop to crop and from crop to wild relatives, whereas corn is a medium to high risk crop for pollen mediated gene flow from crop to crop, but low risk crop for gene flow to wild species. A high risk crop for crop to crop gene transfer may also be rye, as even in distances of 750 to 1,000 m outcrossing rates higher than 0.5 % are to be expected (Feil & Schmid 2001). Since in many cases gene flow between transgenic cultivated species and their wild relatives seems inevitable, it is, according to Abbo & Rubin (2000), imperative to ensure that such crops are grown only outside the range of their wild progenitors, otherwise, the most valuable gene pools for future food supplies would be at risk.

4.1.1.2 Gene flow with distance

In the past years many field experiments have been performed to collect data on gene flow from GM crops to non-GM crops or to wild and feral relatives depending on distance. Generally, there was a high variability of hybridization frequencies, which seem to be dependent on the species in question, their pollination systems, the experimental design, biotic and abiotic conditions and distance. Summaries of published hybridization distances for crop and wild species have been provided (Schütte 1998a, Schmitz & Schütte 2001, Barth et al. 2003). In general, the frequency of hybridization decreased with increasing distance between pollen donor and recipient. However, many cases did not follow this rule.

Reasons for the high variability may be found, among others, in the experimental design, relying either primarily on pollen donors or on pollen recipients (Schmitz & Schütte 2001, Lavigne et al. 2002). In the "source" approach, hybridization frequency is studied by analysis of seeds or seedlings derived from recipient plants that are located in varying distances to the transgenic (or otherwise marked) plants, used as pollen donors. As in such experiments the number of seeds or seedlings that have to be examined increases exponentially and hybridization will be found only in plants at sites that are examined, evidence for hybridization over large distances can be hardly found and hybridization rates tend to be underestimated. In the "sink" approach, however, the marked plants serve as pollen recipients. In this case seeds that are not homozygous for the trait analysed result from hybridization with pollen from different plants. Distances for pollen transfer result from the nearest non-marked plant populations, but if pollen sources are overlooked, distances for pollen transfer may be overestimated (Schmitz & Schütte 2001). In fact, mean maximum hybridization distances found were far higher in "sink" approaches (1 960 m) than in "source" approaches (312 m).

From the analysis of such (usually small scale) studies with crop – crop hybridization Schmitz & Schütte concluded that:

- the highest frequency of hybridization in a "source" approach found was 3 % at a distance of 400 m in *Raphanus sativus*
- "sink" approaches led to high hybridization rates (e.g. squash 5 % at 1 300 m and about 13 % at 480 m , sunflower 15 % at 200 m)

- male fertile plants of oilseed rape, tobacco, potato, tomato, and sugar beet show low outcrossing rates of no more than 1 % at 100 m distance
- male sterile plants (e.g. oilseed rape) show high rates of hybridization if used as recipients
- the lowest hybridization rates were observed in self-pollinating species and in small scale experiments with barren zones
- generally, tree pollen is transported over far greater distances than crop plant pollen

According to Gliddon (1999), virtually all of the sampling methods and monitoring protocols published fail to describe the minimum levels of detection of gene flow that could be achieved using the particular protocol. A fault of experimental design could account for the very small distances that have often been reported for the spread of transgenes. He suggested being careful in using published data on isolation with distance as an element of risk assessment. In insect-pollinated species in particular, there can be a very strong effect of experimental conditions, since barren zones or borders separating potential pollen recipients from donors show a major effect on the pollen transport by insects. The nature of the plant canopy, surrounding vegetation and topography can influence patterns of pollen dispersal as wind velocity and airflow may be affected (Eastham & Sweet 2002). Gene flow might be favoured if donor populations are large and recipient populations are small, e.g. feral populations. Multiple source populations of pollen donors likely increase the rate of introduction of transgenes to wild populations (Klinger & Ellstrand 1999). Existing models of long distance pollen movement based on small scale experiments tend to underestimate gene flow.

The distribution of fields and fragmentation of habitats in agricultural landscapes will also influence gene flow. Although a large portion of pollen may follow predictable patterns of dispersal with deposition declining with distance from the source, a certain portion of pollen can be caught in updrafts, potentially traveling great distances. This may be of special importance in wind-pollinated species, in particular trees, where successful pollen transfer up to 10 km, perhaps 16 km has been reported for poplar (Strauss et al. 2002). Since large pollen sources such as crop fields interact on a regional scale to increase pollen flow, gene flow should rather be considered at the landscape level (Squire et al. 1999, Timmons et al. 1996). To detect transgenes in hybridization progeny, screening of phenotypes may not suffice since silencing of transgenes can occur. In a gene flow study with transgenic tall fescue, Wang et al. (2004) observed that simple antibiotic or herbicide selection methods produced misleading results and, therefore, recommended PCR and Southern hybridization analysis as the most reliable method.

High variability in gene flow suggests that the use of average hybridization rates leads to an underestimation of risk, since exceptional pollination events could introduce genes into wild populations at rates far exceeding those predicted from measures of average gene flow. Isolated hot-spots of transgenic hybrids could contribute disproportionally to the spread of transgenes (Klinger & Ellstrand 1999). The efficiency of interspecific crosses could be dependent on the genetic variability among the plants, as intra-population polymorphisms may exist with regard to pollen germination, receptivity of ovules to foreign pollen, embryo development and seed maturation (Darmency 2000). Outcrossing rates of transgenic HR plants may be higher than those of wild type or mutant HR plants (Bergelson et al. 1998).

4.1.1.3 Potential impacts of gene flow

For the introgression of a transgenic trait from a crop to a wild plant, a rare hybridization event may be sufficient and, as Colwell (1994) pointed out, hybrids need not be particularly fit as long as they are competent to backcross with the wild relative, a capacity many interspecific hybrids have. Fitness of hybrids could be even higher than the fitness of the parent species, cases have been identified where interspecific hybrids were bigger and more competitive than either of the parents (Darmency 2000). Heterosis effects may play a role in intraspecific hybrids between crop species and populations of that crop which had run wild.

Once transgenes moved into genomes of related plants, their frequency within wild plant populations will be influenced by conditions such as hybrid vigour and strong selection for the newly acquired traits, e.g. resistance to herbicides, pests, pathogens, or stress, or the possibility of selection against new phenotypes, if the transgenes were connected to reduced fitness. Fitness costs could be caused by pleiotropy, physiological costs of the new traits or effects of particular insertion sites within the genome and could be different in crops and wild plants, due to different genetic backgrounds (DETR 2000). Such unintended and unpredictable effects could also influence other traits, e. g. the bolting pattern in the case of hybrids between transgenic sugar beet and Swiss chard, and might go unnoticed until transgenes become established in wild populations (Ellstrand 2003). Unexpectedly, transgenic herbicide resistant *Arabidopsis thaliana* plants had slightly larger flowers and showed an increased ability to donate pollen to nearby non-GM mother plants, compared to mutant chlorsulphuron-resistant plants (Bergelson et al.1998. The underlying mechanism for this potential of enhanced outcrossing was not clear, but, according to Gressel (2000), a fitness penalty due to the target site resistance of the mutant *A. thaliana* may also be involved.

In cases where fitness costs do not seem to occur, introgression of transgenes into populations of wild plants and their persistence is possible, even in the absence of selection pressure by herbicides, pests, pathogens, or stress, as data gained from HR oilseed rape crossed with its weedy relative field mustard (*Brassica rapa*) and backcrossed three times indicate (Snow & Jørgensen 1999). Loss of genetic diversity may be a concern too, in particular, if a very beneficial, fitness increasing transgene - and its linkage group - rapidly sweeps through a wild population, thus eliminating alleles of this linkage group (Ellstrand 2003). Native diversity at these loci may be reduced, in particular in species that reproduce by selfing or asexual reproduction, whereas outcrossing species tend to break down linkage groups. Hybrids between a crop and a related wild plant can serve as a genetic bridge, delivering transgenes to species not hybridizing with the crop species directly. Introgressed populations could also serve as reservoirs that hold transgenes from crops once cultivated and deliver them back to (transgenic) crop plants grown later. This temporal aspect of transgene stacking has been rarely considered.

Introgressed transgenes, in particular, those coding for new compounds and toxins directed against pests and pathogens, can affect non-target organisms. The community of animals feeding on such wild hybrids might thus be changed. If the same pest species consumes the crop plant and the wild relative, toxin-expressing hybrids might also have an impact on resistance management intended to slow the evolution of resistance to the toxin (Ellstrand 2003).

Of particular concern would be gene spread from woody species such as trees, because trees have a very limited history of domestication and show extensive gene flow over wide distances among their populations. They produce large amounts of pollen and seeds which are easily distributed. In addition, due to the long lifespans and generation times of trees, predictions of impacts must consider very large temporal and spatial scales and thus have to deal with a high level of uncertainty about future ecological conditions (Slavov et al. 2004, Strauss et al. 2002). Baseline data with regard to several tree species, be they forest trees or fruit trees, have been collected, including a list of transgenic woody species and the genes transferred (Zoglauer et al. 2000).

4.1.2 Selected crop species cultivated in Europe

Monocot plants

4.1.2.1 Barley

Barley, originating in regions of the Near East some 10,000 years ago, has been grown in Europe for a long time. Though barley (*Hordeum vulgare*) is a mainly self-pollinating species, cross pollination can occur at rates from 1 – 10 %. Barley pollen is distributed by wind, outcrossing in a distance of 60 m has been reported. There is a range of related species in the genus *Hordeum* capable of interspecific hybridization, crosses with at least one of them (*Hordeum spontaneum*) can lead to fertile offspring (Neuroth 1997). A list of wild *Hordeum* species present in Europe has been provided by Estham & Sweet (2002). They considered the risk of transgenes introgressing from GM barley into wild relatives to be very slight. Outcrossing rates of barley landraces were found to be correlated with environmental conditions, with increased outcrossing rates under less stable environments (Parzies et al. 2000). In a recipient centred study, the hybridization rate of transgenic barley with male-sterile barley plants as recipients was found to vary from 0 – 7 %, strongly depending on distance and weather conditions with up to 3 % hybrid seeds in distance of 50 m (Ritala et al. 2002). Due to the persistence of barley seeds, transgenic volunteers could evolve in fields and contaminate subsequent barley crops (Eastham & Sweet 2002).

4.1.2.2 Grasses

For some transgenic species of forage and turf grasses, permits/notifications for field tests have been issued through USDA/APHIS (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm). Among them are economically important fescue species, meadow fescue (*Festuca pratensis*) and tall fescue (*Festuca arundinacea*), as well as ryegrasses, perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*), all of which have been placed in the highest risk category for gene transfer by Amman and coworkers (Ammann et al. 1996, Jacot & Ammann 1999). Besides being an important forage species, tall fescue is also widely used as turf in lawns, parks, football fields, and roadsides (Wang et al. 2004). *L. perenne* and *L. multiflorum* are wind and cross-pollinated and highly, but not completely, self-sterile. They

hybridize readily, resulting in good seed set and vigorous and fertile F1 progeny (Wipff 2002). Both cross also with rigid ryegrass (*L. rigidum*) which is one of the most aggressive weeds in many regions in the world. Rigid ryegrass is also one of the weed species that have evolved many herbicide resistant ecotypes, including resistance to glyphosate (www.weedscience.org). Shown by numerous plant breeding experiments over many years, there is a close relationship between *L. perenne*, *L. multiflorum*, *F. pratensis*, and *F. arundinacea*, allowing hybridization between *Festuca* and *Lolium*. Such *Festuca-Lolium* hybrids can be fertile and have the ability to backcross with either of the parents. Genetic information can probably also be transferred from one species of *Festuca* to another, although the frequency of successful hybridizations will depend on the genotypes of the parents (Wipff 2002). Wang et al. (2004) detected marker transgenes in seeds of recipient tall fescue plants that surrounded transgenic tall fescue plants in distances up to 150 m (0.96 %). Transgene frequencies were dependent on wind direction and distance, with the highest frequency (5 %) observed at 50 m in the prevailing wind direction. As neither antibiotic screening nor GUS staining of seedlings was a reliable method to detect transgenes in this pollen flow study, PCR amplification was the main method. Wang and coworkers concluded that gene silencing had occurred in the progenies.

According to Wipff (2002), creeping bentgrass (*Agrostis stolonifera* L.) has the potential to be the first perennial, wind pollinated, outcrossing transgenic crop to be grown adjacent to feral and cultivated populations of creeping bentgrass, as well as to cross-compatible perennial relatives and native species. *A.* stolonifera has competitive and ruderal features. New plants can be established both by seeds and by dispersal of stolon pieces. These traits of *A. stolonifera* can increase the risk of outcrossing, persistence, and introgression of alien genes into an adjacent population.

A. stolonifera has been documented to form natural hybrids with 6 *Agrostis* species, some interspecific hybrids (e.g. *A. stolonifera* x *A. capillaris* and *A. capillaris* x *A. gigantea*) are particularly common in nature. Interspecific hybrids have varying degrees of fertility, from sterile to as fertile as the parents, depending also on the parent genotypes (Wipff 2002). *A. stolonifera* and its related species *A. canina, A. capillaris, A. castellana, A. gigantea,* and *A. vinealis* form a complex of interpollinating, cross-compatible species that readily cross when the species are sympatric. Transgene flow from *A. stolonifera* to *A. capillaris* and *A. castellana* has been shown to occur for much longer distances than traditionally theorized (up to 1 300 m), transgenic bentgrass plants were fertile and stable (Wipff & Fricker 2001, Belanger et al. 2003). Recently, Watrud et al. (2004) reported high relative frequencies of pollen mediated gene flow from a large population of transgenic glyphosate resistant creeping bentgrass to non-transgenic sentinel and resident *A. stolonifera* and *A. gigantea* plants within 2 km. The maximum distance at which gene flow was observed, however, was 21 km and 8 km for sentinel and resident *A. stolonifera* and 14 km for resident *A. gigantea*, all plants located in primarily nonagronomic habitats. Intergeneric hybrids have also been reported between *Agrostis* and *Polypogon* species. Even if F1 hybrids may not be fertile, they can contribute significantly to plant communities because of vegetative spread by means of stolons (Watrud et al. 2004).

Bluegrass (*Poa*) is a very diverse genus of widely adapted, cool season grasses comprising about 300 species with highly variable chromosome numbers, the classification of which is often difficult because of interspecific hybridization. Kentucky bluegrass (*Poa pratensis*) is grown worldwide as forage and turf grass, the first transgenic variety has gained permit for release by USDA/APHIS in 1998. *P. pratensis* shows facultative apomixis (asexual seed formation) allowing the production of different types of progeny: maternal clones, offspring resulting from fertilization of reduced gametes, and offspring resulting from the fertilization of unreduced or reduced eggs by unreduced or reduced male gametes (Wipff 2002). Fertilization of unreduced eggs allows the incorporation of a genome into an unreduced maternal genome. If hybrids are stabilized by apomixis, then an agamic complex or "hybrid swarm" occupying large areas could develop. Facultative apomixis has been described as an efficient reproductive system in which the products of wide hybridizations can escape the penalties of sterility, increasing also the generation time for each genotype, and, therefore, the time available for gene flow due to pollen and seed dispersal (Wipff 2002). When the plants persist long enough and disperse widely enough to backcross and hybridize, then the group of plants potentially exchanging genetic material can become quite large.

4.1.2.3 Maize

Maize or corn (*Zea mays*), a mainly wind pollinated grass, originated from regions in Central America where related species exist. There are no wild relatives in Europe, but cross-hybridization with other maize varieties under agronomical conditions does occur. Maize pollen, produced in enormous quantities and viable from one to a few days, can be distributed by wind and insects over distances of several kilometers. In the Farm Scale Trials in UK, levels of gene flow decreased as expected with distance, with a rapid decline in the first 20 m form the GM crop, but thereafter the rate of decrease was greatly reduced with one positive result in a distance of 650 m (Henry et al. 2003). Isolation distances to other maize varieties recommended in breeding range from 200 to 1 000m, depending on variety, main wind direction, and seed quality (Niebur 1993, Neuroth 1997). The main wind direction, but also the shape of the fields, and the presence of hedges or woods that create turbulence are important factors, as found by Henry et al (2003). Fields providing a wide front to GM pollen and fields bordered by hedges had higher hybridization rates in relation to distance. Socalled half-distances, i.e. the distance required for pollen to drop by half, have been reported to range from 4 – 47 m (Jemison & Vayda 2001). The levels of cross-pollination, recorded between different maize varieties up to 800 m, show that cross-pollination of non-GM plants is possible beyond the isolation distance of 200 m most often recommended (Eastham & Sweet 2002). According to Feil & Schmid (2001), an isolation distance of 300 m should be sufficient to keep contamination rates of non-GM maize below a threshold of 0.5 %. Henry et al. (2005) suggested an isolation distance of about 260 m to keep values below 0.1 %. Analysing various data about hybridization rates with distance, Barth et al. (2003) concluded that at a distance of less than 800 m from the pollen source a pollination rate of more than 1 % is expected and at a distance from 800 to 1 000 m a pollination rate of more than 0.5 %. At a distance of 1 000 m the pollination rate is expected to drop below 0.5 %.

In Mexico and Central America, annual and perennial teosinte, wild relatives of maize that can hybridize with maize, and a range of maize landraces exist (Nigh et al. 2000, de Kathen 1999, Neuroth 1997, Niebur 1993). Therefore, transgenic traits that are introduced are expected to diffuse into other maize races and wild relatives. Contamination of corn landraces by transgenes (e.g. CaMV 35S promoter sequences) has been reported from several sites in Mexico, despite a ban on cultivation of transgenic corn varieties in this country since 1998 (Quist & Chapela 2001). The foreign sequences seemed to have become reassorted and introduced into different genetic backgrounds, leading to discussions about stability of transgenic sequences and, in particular, the CaMV 35S promoter sequences (Mann 2002a). Several criticisms of the paper questioned that transgenes had been reassorted and inserted into a diversity of genomic contexts, arguing that Quist's and Chapela's claims were based on artefacts produced by inverse PCR (Metz & Fütterer 2002, Kaplinsky et al. 2002). All sides, however, seemed to agree that transgenic maize is probably growing in Mexico (Mann 2002b). In 2002, Mexican agencies reported that in the states of Oaxaca and Puebla, centers of origin of maize, evidence of contamination was found at 95 % of the 23 sites tested, when screened with the CaMV 35S promoter sequences. Contamination varied from 1 % to 35 % of the crop plants, 10 – 15 % in the average, with the highest degree of contamination near main roads along which maize is sold to villagers, but the source(s) of contamination could not be elucidated (http://www.guardian.co.uk/gmdebate/Story/0,2763,686955,00.html, www.genet-info.org). Recently, Ortiz-Garcia et al. (2005) reported not to have found transgene contamination in maize seeds sampled in 2003 and 2004 from 18 localities in Oaxaca.

4.1.2.4 Rice

Rice, a staple food for half of the world's population, is grown worldwide under various climatic conditions, but most of its production comes from Asia, where Asian rice (*Oryza sativa*) has been domesticated 10 000 to 15 000 years ago (from progenitor *Oryza rufipogon*). The genus *Oryza* includes one more cultivated species, African cultivated rice (*Oryza glaberrima*), which is believed to have been domesticated from progenitor *Oryza glaberrima* in the Niger River area, and more than 20 wild species (Lu et al. 2004, OECD 1999c). *Oryza sativa* has been subdivided into the supspecies *japonica*, grown mostly in the tropical and subtropical zones, and *indica,* cultivated in the tropical to northern zones. Rice is a wind-pollinated crop with seeds produced by self-pollination. While ovules keep their viability to receive pollen for several days after maturation, pollen viability is lower, from 3 to 5 minutes (OECD 1999d) up to more than 60 minutes (Lu et al. 2004).

Most rice species, including the two cultivated species, are diploid with 2n = 24 chromosomes, but eight wild species are tetraploids with 48 chromosomes. Since *O. sativa* and *O. glaberrima*, weedy rice (*O. sativa* f. *spontanea*), and six wild rice species (*O. nivara, O. rufipogon, O. longistaminata, O. barthii, O. meridinalis*, and *O. glumaepatula*) contain the AA genome, crosses between the crop and these wild relatives result in progeny with generally high fitness (some even show a heterosis effect), but hybrids from certain crosses have reduced fertility (Ellstrand 2003, Lu et al. 2004). Morphologically intermediate plants often appear as hybrid swarms in the field. Weedy rice in Asia originates usually from hybridization between cultivated and wild rice species or from degenerated individuals of

cultivated rice. Individuals of wild rice species flower at different times which can lead to considerable overlap in flowering period in a year and flowering time in a day with cultivated rice varieties (Lu et al. 2004).

The geographical distribution of the AA genome wild rice species overlaps significantly with the cultivation areas of *O. sativa*. Frequencies of gene flow from cultivated rice to weedy red rice and to *O. rufipogon* have been reported to range from 1 % to 52 %, depending on the rice cultivar acting as the pollen parent (Ellstrand 2003, Lu et al. 2004). Recently, transfer of herbicide resistance traits from *O. sativa* to weedy red rice has been reported from the US (Scott & Burgos 2004) and from Spain (Messeguer et al. 2004). Gene transfer rates in Spain were low, at frequencies of 0.1 % and below, measured in distances up to 10 m. According to Messeguer et al. (2004), differing heights of transgenic rice and red rice plants (~15 cm taller) and short overlapping flowering periods may have been responsible for the low outcrossing frequencies observed. But despite low gene flow values, shattering and dormancy of red rice seeds, ensuring their persistence in the field, can lead to unwanted durability of transgenes, in particular, herbicide resistance traits. Given the fact that the spatial, temporal, and biological conditions for rice transgene escape are given in many rice-producing countries or regions, Lu et al. (2004) recommend a buffering isolation zone of more than 110 m or to use tall crops such as sugarcane as a buffer between transgenic rice and its wild relatives.

4.1.2.5 Wheat

Common wheat (*Triticum aestivum*) is a cereal of temperate climates that originated from the Near East. Interspecific hybridization of diploid ancestors, followed by chromosome doubling, led to tetraploid and hexaploid wheat species, the hexaploid bread wheat containing the genomes named A, B, and D with 6 x 7 = 42 chromosomes. The annual crop is mainly self-pollinated, but cross-pollination does occur too, strongly depending on variety (reproductive biology) and weather conditions such as temperature and humidity (Cook et al. 1993, Neuroth 1997, reviewed by Waines & Hedge 2003). To keep contamination rates of non-GM wheat below 0.5 %, isolation distances of 25 to 50 m between GM and non-GM wheat crops have been recommended (Feil & Schmid 2001). Jacot et al. (2004) found that emasculated spikes could be fertilized up to 80 m away from the pollen source. According to data collected by Barth et al. (2003), at distances between 0 and 150 m from the pollen source, pollination of male sterile wheat may be expected to occur at a rate of 3 %, whereas in male fertile wheat pollination is expected at a rate of 1 % and of 0.5 - 1 % at distances up to 10 m and up to 50 m, respectively. After 50 m, pollen movement is low and at distances greater than 100 m, the rate of pollination is expected to be under 0.1 %. But transfer of wheat pollen to distances of 60 m up to 1 000 m has been reported (Waines & Hedge 2003, Zemetra et al. 2002). Feil & Schmid (2001) suspected that, because of their smaller size, pollen of tetraploid wheat such as *T. durum* may be transported farther than pollen of the hexaploid species *T. aestivum*, whereas Jacot et al. (2004) found lower pollen movement for durum wheat.

Traits can be transferred from the cultivated wheat to other cultivated varieties or to related wild forms. However, Eastham & Sweet (2002) described wheat as a crop with little potential

for hybridization under field conditions with related crop species currently grown in Europe, due to its reproductive biology which is characterized by mainly self-pollination and limited pollen viability. Gene spread in wheat, of course, can occur by seed movement too, through grain handling, transport, and storage, and by animals, wind and water. Wheat can also emerge as volunteer in subsequent crops and persist to a measurable level for up to five years. In Canada, pre-treatment wheat volunteer densities frequently range between 20 and 40 plants/m^2, and post-control average densities in many fields may reach 2 plants per m^2 (Van Acker et al. 2003).

Related species are subdivided into three gene pools, depending on the genomic constitution and the ease with which crosses can be performed. Crosses with members of the primary gene pool are possible in various combinations, the most successful ones occurring between hexaploids, whereas hybrids resulting from crosses between hexaploids and diploids or hexaploids and tetraploids often show sterility (reviewed by Neuroth 1997). The secondary gene pool consists of polyploid *Triticum* and *Aegilops* species that have only one part of the genome in common with wheat. The tertiary gene pool comprises diploid and tetraploid species more distantly related, e.g. species of the genera *Secale, Agropyron, Hordeum*, *Elymus,* and others. Intergeneric hybridization with *Agropyron* species has been reported, but hybrid seed set was generally low. Wild *Elymus* species may be parents in crosses with wheat cultivars too (Pfanzagl 1999). By the use of isozyme, RAPD, and microsatellite marker analysis, Guadagnuolo et al. (2001b) could not find evidence for introgression of wheat traits into bearded wheatgrass (*Elymus caninus L.*) collected from England and Austria, they did find, however, wheat specific DNA markers in an individual of sea barley (*Hordeum marinum*) from England, indicating a previous hybridization event.

Wheat cultivars show a low frequency of cross-fertilization with wild members of the secondary gene pool, in particular, *Aegilops cylindrica, Aegilops ovata, and Aegilops triuncialis* (Jacot et al. 2004). According to Ellstrand (2003), there may be at least 11 different *Aegilops* species that hybridize naturally with bread wheat and four that hybridize with durum wheat. Jointed goatgrass (*A. cylindrica*) is a major weed of winter wheat in North America (Cook et al. 1993), it has been shown to occur as an adventive in Switzerland too (Guadagnuolo et al. 2001a). In wheat breeding programs, jointed goatgrass has been used to transfer desirable genes to wheat (Zemetra et al. 2002). Because of its genetic relationship to wheat (tetraploid species with genomes C and D) and its similarity of life cycle, *A. cylindrica* cannot be controlled easily, once it has been introduced into a wheat field. To prevent contamination with this weedy species, the US seed law does not allow certification of seed from fields with even a single hybrid plant of wheat x *A. cylindrica* (Cook et al. 1993). In Oklahoma, frequency of hybrid plants in the field was found to depend also on wheat variety, with Dominator showing the highest frequency (2.08 %) (Stone & Peeper 2004).

Although believed to be mainly sterile, 2 – 3 % and up to 9 % viable seeds were found on (phenotypically intermediate) hybrid plants in the greenhouse and in the field, respectively (Mallory-Smith et al. 1999, Seefeldt et al. 1999, Snyder et al. 2000, Guadugnuolo et al. 2001a). The observed low female-fertility of the hybrids allows natural backcrossing in the field either with *T. aestivum* or *A. cylindrica*. Under field conditions, male-fertility and self-fertility may be (partially) restored after only one or two backcrosses to *A. cylindrica*, leading

to seeds that show high germination rates of about 80 – 90 %. Therefore, after only two backcross (BC) generations, traits transferred from wheat can be established in jointed goatgrass (Zemetra et al. 1998, Zemetra et al. 2002). The ability of BC_2 plants to pollinate surrounding jointed goatgrass increases the chance of transgene transfer. In plants obtained after selfcrossing of BC_2 generation, transgenes such as the HR transgene could exist in the homozygous state (Wang Z. et al. 2001).

The rapid transfer of a gene conferring resistance to the herbicide imazamox (active ingredient imidazolinone) from a winter wheat cultivar to jointed goatgrass has been shown (Seefeldt et al. 1999). Cultivation of HR wheat, therefore, could rapidly lead to HR jointed goatgrass that cannot be controlled by the respective herbicide(s), stacking of HR genes might occur too. Significant differences between populations of jointed goatgrass with regard to their hybridization rate have been observed, demonstrating the need for risk assessment on a regional scale (Guadugnuolo et al. 2001a). This may be of particular relevance for regions such as Southern Europe and the Middle East, where *A. cylindrica* grows in the vicinity of wheat fields, but also for countries of Central Europe such as Switzerland, where this adventive species was able to establish new populations. *A. ovata*, not known to be weedy, crosses freely with cultivated durum wheat in the field and, as herbarium data indicate, also with *A. cylindrica* in the wild (Jacot et al. 2004). Unreduced gametes could play a central role in transfer of genetic information from cultivated durum wheat to *A. ovata.*

Dicot plants

4.1.2.6 Fruit crops

Transgenic fruit crops such as apple or plum trees, grapevines, strawberries, and other kinds of berries may become more important in coming years, engineered to show resistance to pests, pathogens, and abiotic stress. Gene flow to non-GM crops can occur from GM crops of apple (*Malus x domestica*), plum (*Prunus domestica*), grapevine (*Vitis vinifera*), and strawberry (*Fragaria x ananassa*) (Eastham & Sweet 2002). Likewise, the reproductive characteristics of blackberry (*Rubus fruticosus*), raspberry (*Rubus idaeus*), and blackcurrant (*Ribes nigrum*) make gene flow in these species likely. These fruit crop species are native to Europe where several related wild species exist. Apples, pears, plums, including its subspecies, as well as the berry species are insect pollinated and readily visited by bees, bumblebees and a range of other insects. Pollen can thus be carried over considerable distances of several km (Zoglauer et al. 2000). In addition, attractive edible fruits produced by these crops can be distributed by animals, in particular birds, often over long distances. Therefore, seed dispersal mechanisms may pose a significant risk for gene escape at substantial distances from the cultivated fields (Abott et al. 2002). Strawberries, blackberries, raspberries, and blackcurrants also reproduce vegetatively, traits that increase the risks of spread and persistence of GM crops and their transgenes.

According to the data collected by Zoglauer et al. (2000), interspecific hybridization in the genera *Malus* (apple), *Pyrus (pear*), and *Prunus* (cherry, plum) is easily possible. Hybridization of *M. domestica* with relatives present in Europe, e.g. crab apple (*M. silvestris*) that is used in commercial orchards as a pollinator for self-incompatible apple varieties, does

occur, evidence of introgression with cultivated apple exists (Zoglauer et al. 2000, Eastham & Sweet 2002). Crosses of *M. domestica* with closely related apple species yield vigorous progeny. Wild *Prunus* species, many of which are found in hedges, copses, scrub and waste ground, include wild cherry (*Prunus avium* var *avium*), buckthorn (*P. spinosa*), and bird cherry (*P. padus*). Hybridization between the subspecies of plum and the wild relatives can occur. In addition, plum (*P. domestica*) is a frequent escape which can form fully fertile hybrids with buckthorn that can backcross with either of the parental species. The possiblity of gene flow from GM varieties of plum to wild relatives therefore seems particularly high (Zoglauer et al. 2000, Eastham & Sweet 2002).

Within the genus *Vitis* all species of grapevine can be easily crossed experimentally leading to vigorous and fertile F1 hybrids. Gene flow from GM crops to non-GM crops and wild relatives such as *V. silvestris*, a colonizer of river banks and damp woods in parts of Central Europe, could occur (Zoglauer et al. 2000, Eastham & Sweet 2002). Berries are eaten by animals, in particular, birds and transported over wide distances.

Cultivated strawberry (*Fragaria x ananassa*), derived from crosses between the two introduced American species *F. virginiana* and *F. chiloensis*, can hybridize with the parent species and wild strawberries that are native to Europe and distributed throughout the continent. Based on reports about limited hybrid viability, Eastham & Sweet (2002) expected minimal impacts by gene introgression from transgenic strawberries to wild flora. In a US study, however, fertile hybrids between *F. x ananassa* and wild *F. virginiana* have been produced that were vigorous and in many respects comparable to offspring of the wild plants, e.g. stolon number, leaf number, above-ground biomass, and fruit production, although there were some differences with the cultivars used (Abbott et al. 2002). In addition, using AFLP markers it was confirmed that *F. virginiana* populations near strawberry farms contained substantial numbers of hybrid plants displaying DNA markers from cultivars currently grown and from cultivars no longer grown in the region (Abbot et al. 2002, Westman et al. 2004). This indicates that markers from older cultivars introduced in the sixties can persist in hybrids. Cultivar markers have been found in wild strawberry populations in distances of 5 – 7 km from strawberry farms, but marker frequency was not well correlated with a population's distance from the crop fields (Westman et al. 2004). Studies of chloroplast DNA polymorphism might allow evaluation of the contribution of seed dispersal versus pollen dispersal mechanisms for gene escape.

Many variants of blackberries and raspberries are widely cultivated in Europe, both species are also found in natural habitats throughout Europe in both wild and feral forms (Eastham & Sweet 2002). Vegetative reproduction enables spreading over large areas. Hybridization events in the *Rubus* genus, comprising some 2 000 species, do occur, but hybrids often seem to be sterile. The levels of gene flow between GM and non-GM crops and wild relatives expected to occur might be difficult to predict. Eastham & Sweet (2002) recommended monitoring of GM *Rubus* varieties prior to release to determine whether outcrossing to wild species would be more likely in GM plants than in non-GM crops. Blackcurrant has numerous wild relatives distributed across Europe. Most hybridizations among the wild *Ribes* species lead to vigorous fertile F1 progeny, but hybridizations between *R. nigrum* and its related

species have not been reported in Europe (Eastham & Sweet 2002). Nevertheless, gene escape cannot be ruled out, in particular, as blackcurrant can easily escape cultivation.

4.1.2.7 Oilseed rape

Oilseed rape, also called canola (*Brassica napus*), is an annual or winter biennial member of the *Brassicaceae* mainly grown in Central and Northern Europe, Canada and parts of Asia. *Brassica* species have been cultivated for thousands of years in Asia and Europe and a number of wild species are widely distributed in these regions. Amphidiploid *B. napus* (genome AACC, 38 chromosomes) probably resulted from spontaneous hybridization between field mustard (*Brassica rapa/B. campestris*, genome AA, 20 chromosomes) and cabbage (*Brassica oleracea*, genome CC, 18 chromosomes). *B. napus* can be both self-pollinated and cross-pollinated with cross-pollination rates varying from 10 – 47 %, depending on variety and weather conditions. Oilseed rape pollen remains viable from one to several days. It can be transported by insects and wind over long distances. Isolation distances for breeding vary between 100 and 1 000 m (Renard et al. 1993, Gerdemann-Knörck & Tegeder 1997, OECD 1997, Eastham & Sweet 2002).

Wind-borne oilseed rape pollen can be found in various distances from oilseed rape fields, generally pollen concentration decreases with increasing distance, but main wind direction and speed also play an important role. To measure pollen distribution and hybridization rates, in the past years many experiments using pollen traps and bait plants have been performed, resulting in different rates of cross-hybridization of male-sterile plants in distances up to 800 m (Scheffler et al. 1993, Timmons et al. 1996, Pfeilstetter et al. 1998, Schütte 1998a, Saure et al. 1999, Downey 1999, Brown et al. 2000, Eastham & Sweet 2002). Experimental data collected in Canada indicate pollen flow from fields of transgenic HR oilseed rape to fields of susceptible cultivars to occur over distances of at least 600 m, without clear evidence of reduced outcrossing with increasing distance (Downey 1999). Outcrossing in commercial oilseed rape has been found at distances of up to 2.5 km (Rieger et al. 2002). Oilseed rape flowers are very attractive to bees and other insects such as hoverflies, sawflies, and bumble bees and attract them over long distances. Many species of these insects have been found during the flowering period of oilseed rape. Bumble bees have large foraging ranges of up to 3 km, exploit mass resources like oilseed rape and serve, besides bees, as efficient pollinators at a landscape level (Westphal et al. 2003). Since insects visiting rape fields also visit wild *Brassica* species, pollen can easily get mixed and be transferred to non-GM oilseed rape or wild *Brassica* species in the vicinity, with wild bees being the most important vectors for pollen transfer from oilseed rape to wild relatives (Osborne et al. 1999, Saure et al. 1999, http://www.biosicherheit.de/projekte/3.proj.html).

Pollination with pollen from single bees placed 800 m from a transgenic oilseed rape field resulted in GM progeny (Ramsay et al. 1999). With honeybee colonies regularly foraging up to 2 km from the hive, some pollen transfer and fertilization up to 4 km must be expected. Bees have been reported to fly to oilseed rape fields that are 5 km away, so there is potential for pollen dispersal to distances far exceeding 4 km (Ramsay et al. 1999). Bee-to-bee contact in the hive might be a major means of effective pollen dispersal through the foraging area of

the colony (Ramsay et al. 2003). Since pollination of male-sterile oilseed rape plants took place over longer distances than those flown by worker bees, other insects could be vectors for pollen transfer too, among them the pollen beetle which is known to move over long distances (Ramsay et al. 2003). Earlier studies done in UK showed that male-sterile bait plants growing at distances between zero and 4 000 m from the nearest identifiable oilseed rape pollen source were cross-pollinated (Timmons et al. 1996, Thompson et al. 1999). This is all the more important as bee hives placed close to oilseed rape crops increase seed yield by more than 45 % at a density of three hives per hectare (Sabbah et al. 2005). Low levels of fertilisation of male-sterile oilseed rape have been reported in distances of 5 km and even 26 km (Ramsay et al. 2003).

Interaction of large pollen sources on a regional scale can increase pollen flow (Squire et al. 1999), leading to the conclusion by DETR (Department of the Environment, Transport, and the Regions) that complete genetic isolation of oilseed rape, were it required, would have to be on a regional (tens of kilometers) scale, and that, under current farm practices, local contamination between crops is inevitable (DETR 2000). Ramsay et al. (2003) found that rates of cross pollination dropped rapidly over the first tens of meters from the edge of the field, but beyond that the decline was small with distance, leading to a long tail in the distribution variing between seasons and not following mathematical description. Contamination of non-GM rape seed lower than 0.3 % in a seed harvest will be difficult to warrant if GM and non-GM varieties are grown on the land of the same village (Darmency & Messéan 1999). According to Damgaard & Kjellsson (2005), increasing the width of a recipient oilseed rape field, relative to the GM donor field, can reduce the average fertilization level by GM pollen, <0.1 % may be possible if the isolation distance exceeds 100 m and the non-GM field is wider than 200 m. Generally, varieties containing male-sterile components will outcross with neighbouring fully fertile GM oilseed rape at higher frequencies and at greater distances (Eastham & Sweet 2002). According to Barth et al. (2003), at distances up to 4 000 m pollination rates of male-sterile oilseed rape are expected to exceed 5 %. The authors were unable to recommend isolation distances for keeping pollination rates in male-sterile lines below 0.5 or 1 %.

Bock et al (2002) provided evidence that production costs for oilseed rape seed would increase significantly if transgenic oilseed rape would be grown in a region at a large share and if a threshold for GM contamination of 0.3 % was to be secured. With socalled zero tolerance for GM contamination demanded by organic agriculture or a threshold of 0.1 %, seed production costs would increase even further.

Hybridization with wild relatives

B. napus can cross with a variety of *Brassica* species and wild relatives in artificial and spontaneous hybridizations. If in rare cases unreduced gametes are produced, amphidiploid hybrids may result whose ability to produce progeny will be higher than that of normal hybrids because of their regularity in meiosis. Among hybrids derived from crosses between oilseed rape and cabbage (*B. oleracea*), wild mustard (S*inapis arvensis*), or wild radish (*Raphanus raphanistrum*), such amphidiploids have been found (Kerlan et al. 1992, Darmency 1998). Controlled and spontaneous reciprocal hybridizations of *B. napus* with *B. rapa* (field mustard/wild turnip), *B. juncea* (brown mustard, genome AABB), and *B. oleracea*

are easily possible. In particular, hybridization with the outcrossing species *B. rapa*, that is also grown as a crop, does occur readily in either direction, with hybridization frequencies reported of 0 – 69 %, depending on parental genotypes/varieties, experimental design, agricultural practice, site, and population size, hybrids between herbicide resistent GM oilseed rape and *B. rapa* have been described (data and summaries of hybridizations between *B. napus* and its close relatives and techniques used can be found in Kerlan et al. 1992, Renard et al. 1993, Scheffler & Dale 1994, Mikkelsen et al. 1996, Gerdemann-Knörck & Tegeder 1997, OECD 1997, Jørgensen 1999, Eastham & Sweet 2002, Warwick et al. 2004, Chévre et al. 2004, Daniels et al. 2005, and at http://www.environment.detr.gov.uk/acre/pgs/index.htm).

A relative ranking of species by their ability to form hybrid progeny when crossed with *B. napus* placed *B. rapa* in position 1, *B. juncea*, and *B. oleracea* in position 2 and 3, respectively (Scheffler & Dale 1994, Eastham & Sweet 2002). In the UK, *B. rapa* occurs as a weed and as wild plant, particularly along river banks. Annual numbers of hybrids between oilseed rape and *B. rapa* will be in the order of tens of thousands, evidence of introgression in seed bank samples has been found (Wilkinson et al. 2003, 2004). Frequencies of transgenes in *B. rapa* populations introgressed from *B. napus* will depend on the selection pressure that is exerted against the newly acquired trait(s) and/or against aneuploids (part of the progeny of a *B. napus x B. rapa* hybrid (genome AAC, 29 chromosomes) backcrossed with *B. rapa* will be aneuploids). According to a model developed by Lu et al. (2002), selection against herbicide susceptible individuals will stabilize the frequency of an HR transgene at about 5.5 % within six generations of successive backcrosses. Under selection pressure against aneuploids, the frequency of a transgene in backcross generations will strongly depend on its location on the A or C genome, with far higher frequencies for the A genome.

Spontaneous and artificial hybridization of *B. napus* with weedy species native to Europe has been shown for wild radish/white charlock (*R. raphanistrum*), hoary mustard (*Hirschfeldia incana/Brassica adpressa*), and wild mustard/charlock (*S. arvensis/Brassica kabel*), compatibility with a range of other wild species may exist (Jørgensen et al. 1998). Wild radish and wild mustard, for instance, belong to the economically damaging weeds worldwide, wild radish seeds can remain viable in the soil for many years (Warwick et al. 2000, Snow et al. 2001). Hybridization rates seem to be higher when oilseed rape is used as female parent, being the parent with the higher ploidy level (Kerlan et al. 1992). Low rates of interspecific hybridization between GM or non-GM oilseed rape and wild radish (*R. raphanistrum*) in either direction can be found under agronomic conditions, depending very much on the female cultivar. Hybrids generally exhibited poor female fertility, but in successive backcrosses to wild radish plants reached almost the fertility and morphology of wild radish (Chèvre et al. 1997, 1999, 2000, Rieger et al. 1999, Chèvre et al. 2004). *B. napus x R. raphanistrum* hybrids could also be found on fully-fertile oilseed rape (Rieger et al. 2001).

Hoary mustard (*Hirschfeldia incana*) which is found within and on the border of fields has been shown to hybridize to oilseed rape in the field, but the low reproductive fitness of interspecific hybrids may slow introgression of transgenes into *H. incana* (Chèvre et al. 1999, Darmency & Messèan 1999, Darmency & Fleury 2000). Although Downey (1992) and Brown et al. (2000) did not obtain fertile hybrids from direct crosses between oilseed rape and wild mustard/charlock (*S. arvensis*), under experimental conditions hybridization between

oilseed rape and wild mustard seems to be possible at very low rates (Warwick et al. 2000, Chèvre et al. 2004). Moyes et al. (2002) reported, that by using hand pollination, but without the aid of artificial techniques, low rates of hybridization with *S. arvensis* can be obtained both with wild mustard as paternal or maternal parent, but the triploid hybrids did not backcross with *S. arvensis* and backcrosses with oilseed rape were less successful than control crosses. Recently, in the Farm Scale Evaluations in the UK, a hybrid derived from herbicide resistant GM oilseed rape crossed with wild mustard has been found to contain the GM construct (Daniels et al. 2005). *Erucastrum gallicum* occurs as a weed in Europe and Canada, manual hybridization with *B. napus* seems possible, but with extremely low frequencies (Warwick et al. 2003).

Since reciprocal crosses between field mustard (*B. rapa*) and wild mustard (*S. arvensis*) produced mature seed, such progeny, most likely being an amphidiploid, might act as a bridging species for gene transfer from oilseed rape to wild mustard. Having the same chromosome number as *B. napus* (2n = 38, genome AASS) and the common genome A, such offspring could add to the risk of transgene flow into weedy species. Indirect gene transfer to *S. arvensis* may also be possible through crosses with *R. raphanistrum* (Darmency 1996). *R. raphanistrum* may be a particularly important bridging species, as it has been shown to hybridize readily with radish (*Raphanus sativus*), be it the cultivated or wild form (Snow et al. 2001).

Volunteers and feral populations

Volunteers and feral populations can act as gene pools carrying over transgenes into subsequent rape crops and seed production (Eastham & Sweet 2002). Seed losses are high in oilseed rape, regardless of the harvesting method, and readily lead to volunteers in subsequent years. In Canada, average yield losses of 107 kg/ha or 5.9 % of the crop seed yield, amounting to about 3 000 viable seeds/$m^{2,}$ have been observed (Gulden et al. 2003a). This equaled approximately 20 times the normal seeding rate of 4 – 5 kg/ha. According to Squire et al. (2003), the typical seedbank population density is 100 per m^2. For this reason, germination of even a small fraction can have large impacts as an impurity in a succeeding oilseed rape crop. Rapeseed, dispersed at harvest and during transport, can resist cold temperatures and may develop secondary dormancy which allows persistance in soil for prolonged periods of time, in particular if buried at greater soil depth. Survival depends on cultivation methods, seed size and genotype (Gerdemann-Knörck & Tegeder 1997, Pekrun et al. 1998, Neemann et al. 1999 (http://www.biosicherheit.de/features/printversion/php?context=1&id=6), with genotype being the most important factor (Gulden et al. 2003b). Genotypes less rapid in germination show greater potential to develop secondary dormancy which is also favoured by osmotic stress (Momoh et al. 2002). Volunteer oilseed rape was found in 90 % of the fields surveyed in Québec, Canada, decreasing in density with years after production, but still present 4 and 5 years after production. The presence of dense stands of volunteers found before herbicide application in no-till fields indicated that oilseed rape seeds could become dormant in no-till as well as in tilled systems (Simard et al. 2002). In the UK, ferals appear to have persisted in some fields for at least ten years after an oilseed rape crop had been grown (Squire et al. 2003).

Seed generally plays an important role in spatial and temporal dispersal of transgenes, as seed is not only lost on the field during harvest but also spilled along transport corridors and can germinate years later. Oilseed rape volunteers flowering in subsequent years can produce a few thousand seeds that may be shed prior to the harvest of the following crop, such as corn, and thus replenish the seed bank. Populations of volunteer plants in crop fields may, therefore, include plants from many generations. Volunteer populations can appear sporadically, as observed in winter wheat cropping, where volunteers were absent the first year but occurred in relatively high numbers the second year of follow-on crops (Daniels et al. 2005).

Cropping systems can influence the risk of gene escape from crop to rapeseed volunteers as indicated by the model GENESYS that was developed to describe the temporal evolution of rapeseed volunteer populations and their spatial dimension, considering not only fields but also waysides and field margins where seed spills do occur (Colbach et al. 2001a,b). Simulations showed that insufficient volunteer control in a single field can lead to rapeseed volunteers not only in adjacent fields but also at locations more than 1 km away and can thus affect a whole region. According to the model, harvest pollution may exceed 1 % even after 12 years if rapeseed volunteers are poorly managed. Recently, the model has been extended to include more than one transgene and genotypic effects such as plant height and male sterility (Colbach et al, 2005, Fargue et al. 2005). In a simulation based on two years of winter wheat and one year of winter oilseed rape, with no attempt to control the volunteer population, it took 16 years after harvesting of the original crop for impurity to fall below 1 % (Squire et al. 2003).

Where different transgenic oilseed rape varieties are cropped in adjacent fields, volunteer management will become more difficult, in particular, if pest or disease resistance traits are used that may even be stacked (Eastham & Sweet 2002). For this reason, to control glyphosate-resistant canola volunteers in conservation tillage, the addition of effective broadleaf herbicides has been recommended (Rainbolt et al. 2004). Additional problems will be caused by GM contamination of the traditional seed supply. In the US and Canada, where almost all of the traditional oilseed rape varieties are contaminated with GM seed in the range of 0.05 to 0.25 % (or even higher), this phenomenon is already widespread (Mellon & Rissler 2004, Friesen et al. 2003). Van Acker et al. (2003) calculated that at a 0.25 % contamination level of an HR trait in a traditional oilseed rape seedlot, there will be 75 000 transgenic seeds/ha shattered onto the soil. If one-tenth of these seeds successfully established a seedling the following year, there would be one HR volunteer canola plant every 1.3 m^2. In fact, all 27 pedigreed oilseed rape seedlots collected in western Canada in 2002 have been found to be contaminated with herbicide resistance traits, more than half at levels above 0.25 %, three seedlots had even contamination levels in excess of 2 % (Friesen et al. 2003).

Feral descendants of oilseed rape are frequent and exist in close proximity with rape crop fields throughout the arable land of Central and Western Europe, some 30 to 40 % of feral populations could be found within 360 m of an oilseed rape field (Squire et al. 1999, Menzel & Mathes 1999, Klöpffer et al. 1999, Timmons et al. 1996). Feral plants include both volunteers within fields and other populations on field margins, soil dumps and roadsides, mostly derived from seed spills. Many of these populations are not routinely controlled. There is accumulating evidence that ferals can be found in more than one season (Theenhaus 2003)

fed by a long-lived soil seedbank. Within and around the margins of agricultural land, persistence for at least eight, possibly over ten years has been reported (Squire et al. 1999, Pessel et al. 2001). The most important factors for feral population growth and persistence are seedbank survival and dormancy. When metapopulation structure is considered, some feral oilseed rape patches may even survive for decades (Claessen et al. 2005a). Transgenes that induce changes in life history traits can increase persistence of transgenic oilseed rape ferals, Claessen et al. (2005b) cited oil-modifications and, possibly, insect resistence, as examples, whereas they predicted glufosinate-resistance to reduce persistence.

Feral populations outside fields experience a wide range of selection pressures, leading to diverse forms including individuals that flower when very small or at various times or late in the season. Related species, such as field mustard (*B. rapa*), wild radish (*R. raphanistrum*), wild mustard (*S. arvensis*), and white mustard (*Sinapis alba*), showing overlapping flowering periods, have been found in proximity to oilseed rape feral plants (Breckling & Menzel 2004), with pollen bearing insects carrying pollen of both oilseed rape and wild relatives at the same time (Menzel & Mathes 1999). Therefore, feral sites may serve as stepping stones for gene flow from transgenic crops to feral oilseed rape and to wild related species over time and space. In case of HR oilseed rape, application of the respective broad-spectrum herbicide may lead to herbicide residues on field margins, thus providing HR feral plants with a selective advantage increasing their chances of establishment and of gene transfer.

4.1.2.8 Potato

Potato (*Solanum tuberosum*) is an annual crop originating from Latin America. It is vegetatively reproduced by tubers, but can also survive by seeds. Potato can show self-pollination as well as cross-pollination, the latter occurring mainly by insects (Jacobsen & Rousselle 1993, Neuroth 1997). Cross-fertilization ratios of less than 1 % have been found in distances of 40 and 80 m (Neuroth 1997), and of 24 % in a distance of 1 m between the hybridizing potato varieties (Mc Partlan & Dale 1994). Many potato cultivars produce true potato seed that can survive under field conditions for several years, volunteers germinated from such seeds can contaminate subsequent potato crops. In recent years, reduced herbicide use, a succession of mild winters, and the use of vigorous potato varieties has increased the numbers of volunteer potatoes (Eastham & Sweet 2002). Crosses of *S. tuberosum* with the related European species *S. nigrum* and *S. dulcamara* did not result in viable offspring (Mc Partlan & Dale 1994), therefore, gene transfer from *S. tuberosum* to these wild plants does not seem very likely (Neuroth 1997, Schütte 1998a). Wild bees and bumble bees have been found to collect potato pollen, thus gene transfer by insects may be possible (Becker et al. 2000).

4.1.2.9 Sugar beet

The wild sea beet, *Beta vulgaris* L. ssp. *maritima*, growing along European coasts, is considered to be the ancestor of *Beta* beets, including sugar, fodder, and red beet, and chard. All cultivated beets and a range of wild forms are sexually compatible and give fertile offspring with each other. The biennial cultivated sugar beet develops the large root in the

first year and the seed stalk in the second year. However, seed stalk development may be induced the first year, a phenomenon referred to as bolting which seems to be favoured by low temperatures. Most wild *Beta* beets are annual, some are biennial or perennial. The annual growth habit is governed by a dominant gene that can cause considerable problems if allowed to contaminate breeding stocks or commercial seed fields, weed beets may result (Bosemark 1993, Gerdemann-Knörck & Tegeder 1997, Eastham & Sweet 2002). Pollen of sugar beet can be dispersed by insects and wind over distances of up to 5 km, possibly even 8 km. Isolation distances in seed production vary from 300 to1 600 m, however, such isolation distances may be too small to prevent pollen transfer (Gerdemann-Knörck & Tegeder 1997, Neemann et al. 1999). The various forms of cultivated *B. vulgaris* (sugar beet, red beet, and Swiss chard) naturalize as weedy forms in a variety of habitats (Bartsch & Ellstrand 1999).

Wild sea beet, the ancestor of sugar beet, exhibits a large phenotypic variation and is adapted to a wide range of different ecological niches. Populations of wild beets are found at the coasts of Europe from the Baltic Sea and the North Sea along the Atlantic to the Mediterreanean Sea, including seed production areas in Northern Italy. Populations have also been described as ruderal inland beets in France (Bartsch et al. 1993, Gerdemann-Knörck & Tegeder 1997, Desplanque et al. 1999, Driessen et al. 2000, Van Dijk 2004). Both cultivated beet and sea beet are outcrossing species, a factor which enhances the possiblity of gene flow (Eastham & Sweet 2002). Hybrids between wild beets and *Beta vulgaris* cultigens arise wherever the parental plants grow and flower in close proximity resulting in weedy beet forms. Seed production areas in Europe present a particular high risk for gene flow from cultivated beet to wild beet. Bolting beets allowed to flower and set seed in the first year may lead to seeds that can germinate the following year or years later, if buried deeper in the soil. Stable weed beet complexes that are difficult to eradicate can form quickly (Bartsch et al. 1993, Neemann et al. 1999). Experimental transfer of HR genes from sugar beet to red beet, chard, and the wild beet as well as from bolters in a field has been demonstrated (Bartsch et al. 1999, Vigouroux et al. 1999). Genetic evidence for gene flow from seed production fields of domesticated sugar beet to nearby wild beet populations exists (Desplanque et al. 1999). Genetic markers (e.g. RFLP or microsatellites) specific to cultivated sugar beet plants have been detected in weedy beet populations (Desplanque et al. 1999, Bartsch & Ellstrand 1999). Based on the analysis of seed purity data, Lavigne et al. (2002) expect that under conditions of seed production in France (isolation distance of 1 000 m) wild beets produce high numbers of seeds fathered by cultivated plants.

From these data it can be inferred that in regions where wild beet is present in the vicinity of beet fields, be they seed production or cultivation fields, gene spread from transgenic sugar beet to wild, feral, or weedy beets will occur given sufficient time. As beet seeds persists in the soil for a considerable length of time, a long-lived seed bank of transgenic beet can develop in a field sown only once with transgenic beet and even more so, if the beet crop is rotated regularly every 3-4 years (Eastham & Sweet 2002).

4.1.2.10 Woody species

According to a recent FAO (2004) study, most projects of genetic engineering of forest trees, taking place in at least 35 countries, deal with poplar, pine, eucalyptus, Liquidambar, and spruce, some work is done, among others, with birch and larch. Of the more than 225 field trials with GM trees, 150 or more have been reported from the US. Baseline data for a number of European woody species such as larch, pine, poplar, and spruce, and their reproductive biology have been collected by Zoglauer et al. (2000). Wind dispersal of pollen and seeds of trees is known to occur over distances of dozens of kilometers if not more (Slavov et al. 2004), with foliage shedding in deciduous forests affecting long-distance seed dispersal significantly (Nathan & Katul 2005).

Pollen of European larch (*Larix decidua*), a mainly cross-pollinating species, is reported to sediment rapidly, but high winds will lead to longer range pollen transport. Hybridization is possible with other larch species such as *L. kaempferi* and *L. sibirica* (Zoglauer et al. 2000). Seeds are transported by wind over distances from 20 to 240 m, by water and snow, and by animals such as birds and small mammals. Pine (*Pinus sylvestris*), a species widely distributed in Europe, produces enormous amounts of pollen that can travel over distances of several hundred kilometers (up to 1 000 km), although over 90 % of the pollen produced may settle within 100 m (Zoglauer et al. 2000). Hybrids between *P. sylvestris* and other *Pinus* species are possible. Seeds resulting from cross-pollination or self-pollination are long-lived, able to germinate after several years, even after 10 - 12 years. Old trees can produce more than 50, 000 seeds per year that are distributed by wind, birds, and small mammals. Because of their low weight and their capability of floating for several weeks they can reach distances of 1 km and more, in particular, if transported by water.

Norway spruce (*Picea abies*), a native and the economically most important conifer tree species in Europe, has been planted widely in central, eastern and northern Europe. Floral induction depends on favourable (temperature) conditions and does not occur every year. Spruce pollen, produced in large quantities, moves over long distances, in a distance of 2.5 km about half of the pollen amount at the edge of a spruce stand has been reported (OECD 1999c). Seeds are produced mainly through cross-fertilization, but also by self-ferilization. They are dispersed by wind and partly by birds and other animals. In the mountains, vertical and horizontal seed dispersal may extend over 800 m and 1 500 m, respectively (Zoglauer et al. 2000). Spruce seedlings are very shade-tolerant and can survive for decades under a closed canopy. Natural hybrids involving spruce (*var. obovata*) have been reported only from crosses with eastern Asian *P. jezoensis* and *P. koraiensis*, but spruce from different provenances hybridizes freely (OECD 1999c).

Poplar (*Populus L.*) is among the most frequently planted tree genus, because of its importance for wood pulp and other uses and its speed of growth, it is also the most commonly studied tree genus for genetic modification purposes (FAO 2004). There are more than 25 species of poplars belonging to at least five sections that occur throughout the Northern Hemisphere (Europe, Asia, North-East Africa, and North America). They hybridize extensively in nature with formation of complicated natural hybrid populations where three or more species are sympatric. Hybrids are often more vigorous than their parents. Many hybrids have also been produced by controlled crossing and some of them are now widely planted

(OECD 2000). In general, poplars are dioecious and obligatory outcrossers pollinated by wind, but insects may collect pollen in early spring too (Zoglauer et al. 2000). Gene flow occurs over long distances, due to long-range pollen transport and easy dispersal of the many small seeds that have long white, silky hairs attached to them (OECD 2000). Extensive pollen immigration into stands of *P. trichocarpa* that were isolated by up to 16 km from the nearest ungenotyped pollen source has been reported (Slavov et al. 2004). Birds, too, can be effective agents of long-distance seed dispersal. Poplar seeds have a short lifespan, they usually germinate within a few days after seedfall, or they die. Poplars sprout vigorously from the stomp and root collar, forming clonal groups that may extend up to 35 m and, in the US, cover over 40 ha (Zoglauer et al. 2000, OECD 2000).

4.1.3 Barriers to gene flow

4.1.3.1 Biological barriers

Several approaches have been proposed to reduce the risk of gene escape from transgenic crops, be they biological or physical (reviewed by Daniell 2002). If transgenic plants could produce seeds without fertilisation, a process called apomixis, such plants could be male-sterile and, therefore, pollen transfer to neighbouring plants would be minimal. In general, male-sterile plants could lead to reduced pollen transfer, but such plants can still be pollinated by exogenous pollen and produce viable progeny (Eastham & Sweet 2002).

The design of altered constructs such as “killer constructs”, by which fertile seed is prevented from being produced, known also as genetic use restriction technologies (GURT) or “Terminator” technology, has been suggested as a means to reduce the rate of introgression. The system, described in the USDA/Delta & Pine patent (EP 775212 B1 20051005) involves transfer of several genes and depends on toxin production in late embryogenesis. The promoter of a site-specific recombinase is blocked by a constitutively expressed (bacterial) repressor, but activated upon addition of tetracycline that binds the repressor. Recombination induced by the site-specific recombinase leads to excision of a sequence inserted between the late embryogenesis abundant (LEA) promotor and the toxin gene resulting in toxin production. Plant propagation, of course, occurs without activation of toxin production, the inducer tetracycline is added only before commercialization of the seed, leading to induction of the LEA promoter and aborted embryo development only in plants grown on farmers’ fields.

This “Terminator” technology has been hotly debated in recent years, as this technology would effectively prevent farmers from saving seed for replanting the next season. In addition to such socio-economic risks, ecological risks may also be connected with the integration of GURT traits (Hartmann 2002). For functioning of GURTs, conditions such as exact and stable integration of the GURT genes, precise regulation, and precise response of the system to external chemical stimulation must be fulfilled. However, GURTs do not always react in the expected manner, due to position effects, pleiotropic and silencing effects and thus the mechanisms may fail, perhaps also leading to the loss of the activity of the genes of interest. It

would also be imperative to study the chemicals used for induction individually and carefully with regard to their potential to harm human and animal health and the environment. If GURT traits would be transferred to crops of neighbouring fields, economical problems may arise because of the unintended acquisition of sterility in such seed. Transfer of GURT genes to wild species could contribute to population reduction. Similarly, male sterile plants produced by the barnase/barstar system could lead to reduced gene flow. In this case, pollen-specific expression of the barnase gene coding for a cytotoxic RNase inhibits pollen production. To inhibit background barnase activity, the barstar gene encoding an inhibitor of barnase is constitutively expressed.

In analogy to methods suggested for marker gene removal and similar to some GURT traits, Kennan & Stemmer (2002) proposed to use gene deletion cassettes comprising a tissue-specific or chemically inducible promoter that drives the expression of a site-specific recombinase linked to a separate promoter that drives the gene of interest. The entire cassette should be flanked by site-specific recombinase sites. Induction of the promoter driving the recombinase would again result in the excision of the transgenic cassette from the genome. A pollen-specific promoter driving the recombinase might be used to excise transgenes selectively from pollen, thereby reducing the potential spread of transgenes through cross-pollination of non-GM plants. However, excision may not be completely effective and parts of transgenic DNA could remain. In addition, these mechanisms may all be subject to gene silencing. Therefore, gene flow from transgenic crops to wild plants cannot be prevented with certainty.

An alternative approach might be the use of tandem constructs in which the gene(s) of interest could be flanked by socalled transgenetic mitigation (TM) genes that affect germination or plant growth (Gressel 2000) and lead to fitness disadvantages in wild relatives (Stewart et al. 2003). Genes encoding traits that alter seed dormancy, ripening, and shattering, that cause dwarfing, inhibit flower production, prevent maturation, or induce pollen sterility may be used as flanking sequences for the genes of interest. An antibolting trait could perhaps prevent biennial plants from flowering the first year, thus reducing gene transfer from biennials such as sugar beet. This approach is based on the premises that tandem constructs act as tightly linked genes and their segregation is exceedingly rare, and that such traits are neutral or positive for crops but deleterious for weeds. Ellstrand (2003) points out that, for tandem constructs to be effective, the traits conferred by the flanking alleles must be dominant relative to their counterparts in wild plants. In any case, gene flow from a GM crop to other crops or to wild relatives that are not considered weeds and should be protected from transgene introgression would not be addressed (Daniell 2002).

To restrict gene flow in transgenic forest trees, it has been suggested to block the reproductive pathway by the use of flower-specific promoters that direct the expression of cytotoxic structural genes, thus leading to the specific ablation of floral organs (Tzfira et al. 1998). Sense-antisense or promoter suppression of specific homeotic genes involved in reproductive development genes might also be used. Blocking the reproductive pathway might also redirect energy resources to vegetative growth leading to accelerated growth and yield of forest trees. However, as tree pollen and seed are of vital importance for a multitude of insects, birds, and small mammals, the replacement of natural forests by plantations of flowerless trees will have

a serious effect on the richness and abundance of these species, the foodweb, and biodiversity of forest ecosystems in general. Grafting of transgenic trunks with non-transgenic scions before reaching the reproductive state has been suggested as an additional means for transgene containment (Lev-Yadun & Sederoff 2001).

The transgene integration site(s) in the crop plant genome may be of some importance for introgression (Stewart et al. 2003). This has been discussed with respect to oilseed rape (*B. napus*) which is an amphidiploid species (genome AACC) derived from hybridization between *Brassica rapa* (genome AA) and *Brasica oleracea* (genome CC), and with respect to common wheat (*Triticum aestivum)* which is a hexaploid (genome AABBDD) having the D genome in common with the weed jointed goatgrass (*Aegilops cylindrica*). If transgenes are integrated in the C genome of *B. napus*, the likelihood of transgene introgression to *B. rapa* might be reduced (Metz et al. 1997, Lu et al. 2002). But even if inserted in the C genome, transgenes may still escape from oilseed rape (Jørgensen 1999) since recombination between the chromosomes of the different genomes can occur and thus allow transgene exchange (Darmency 2000). Introgression into C genome carrying brassicaceae might be facilitated. If in the case of transgenic wheat the transgene was inserted in the A or B genome, the chance of transgene spread to jointed goatgrass could be reduced. However, single A or B chromosomes may be permanently maintained in a jointed goatgrass genomic background and translocations between A, B, and D chromosomes may take place (Wang Z. 2001, Zemetra et al. 2002). Tomiuk et al. (2000) questioned that integration of a transgene into a certain part of the nuclear genome would in fact reduce introgression into wild plant genomes, since existing experimental results did not indicate any specific chromosomes to be safer candidates for an integration of transgenes. Backcross frequencies of hybrids derived from 12 independent oilseed rape transformation events crossed to *B. rapa* supported the hypothesis that there are few likely "safe" locations in the oilseed rape genome with regard to gene flow (Halfhill et al. 2001).

4.1.3.2 Clean and safe plastid transformation?

Plastid transformation is recommended as one particularly excellent method to reduce the risk of gene escape by hybridization of transgenic plants with relatives, be they other varieties of the crop species or wild plants (Daniell et al. 1998, Bilang & Potrykus 1998, Daniell & Varma 2000, Heifetz 2000, Daniell et al. 2001b, Daniell 2002). This has been attributed mainly to the fact that in many crop plants chloroplasts are inherited maternally and therefore pollen does not transfer chloroplasts. Gene escape via pollen spread seems to be impossible. However, there is a number of plant species which do not follow this rule strictly. The best studied plant group in this respect are the conifers, which, in general, have paternal inheritance of chloroplasts and maternal inheritance of mitochondria (Steweart & Prakash 1998, Lev-Yadun & Sederoff 2000). A low degree of maternal chloroplast inheritance has been observed in some cypress species (Shiraishi et al. 2001). Other plants, such as alfalfa, have biparental inheritance of plastids and in plants, such as tobacco, chloroplasts occasionally can be transmitted via pollen too, additional exceptions may be rye, rice and pea (Mogensen et al. 2000, Stewart & Prakash 1998, van Bel et al. 2000). For some cultivar species, the mode of chloroplast transmission is uncharacterized (Scott & Wilkinson 1999). In other cases, where

maternal transmission is the rule, chloroplasts may be transmitted by pollen under stress conditions (Cummins 1998). The nuclear-cytoplasmic relationship could also influence the transmission of chloroplasts via pollen, even in species with strict maternal inheritance of plastids (Maliga 2002). For these reasons, chloroplast transformation is far from being a universal containment strategy (Stewart et al. 2003, Stewart & Prakash 1998).

Even in cases where chloroplasts are exclusively maternally inherited, this would not necessarily mark the end of the risk assessment process. Chloroplasts can be exchanged via reciprocal hybridization occurring between the transgenic crop plant as mother plant which is pollinated by non-transgenic varieties or wild plants. In a study using naturally occurring oilseed rape chloroplast genes as markers, Scott & Wilkinson (1999) showed that such markers could transgress if oilseed rape (*Brassica napus*) was grown in a mixed stand with its close wild relative *Brassica rapa* and oilseed rape acted as the recurrent female parent. Mixed populations of feral oilseed rape and *B. rapa* can occur in the vicinity of oilseed rape fields since spillage of oilseed rape seed during harvest and transport is inevitable. Particularly if oilseed rape is rare in the population and its flowers are overwhelmed with *B. rapa* pollen, such crosses and backcrosses occur quite easily (Scott & Wilkinson 1999, Chamberlain & Stewart 1999). Nuclear-cytoplasmic relationship may be altered in hybrids resulting from interspecific crosses between transgenic plants and related wild species which, in turn, could influence the transmission of chloroplasts via pollen (Maliga 2002). Plastid transformation has also been advocated for the simultanous transfer of several genes because of the expected ease of gene stacking in plastid transformation constructs. In such cases, however, chloroplast transmission could lead to the escape of multiple transgenes. Traits coded by these stacked transgenes could thus simultaneously be expressed in previously non-transgenic varieties or wild plants.

Recent reports about gene transfer from chloroplasts to the nucleus suggest that genes integrated in tobacco plastid genomes can translocate to the nucleus at high rates (Stegemann et al. 2003, Huang et al. 2003a). The nuclear-specific neomycin phosphotransferase gene, inserted in tobacco chloroplast DNA, has been found to be integrated into the tobacco nuclear DNA at a rate of 1 in 16 000, comparable to spontaneous mutation of the nuclear DNA. It was found at different genomic locations and to be linked to chloroplast sequences other and larger than the target sequences for chloroplast transformation (Huang et al. 2003a). Based on genetic analysis and these observations, Huang et al. (2003b) argued that nuclear insertion of the neo gene was not due to inadvertent nuclear transformation, as suggested by Daniell & Parkinson (2003), but was rather caused by secondary transfer of plastid sequences. Stegemann et al. (2003) also reported about escape of tobacco plastid genetic material to the nuclear genome at surprisingly high frequencies (one in five million cells). Transfer of plastid sequences to the nucleus may occur at different rates, depending on the species and the genome size, with larger genomes having potentially higher transfer rates (Maliga 2003). It is not clear yet, whether the degradation of chloroplasts during pollen development contributes to these high frequencies of plastid DNA escape (Martin 2003, Stegemann et al. 2003). Analysis of chloroplast sequences integrated in the rice nuclear genome revealed that in the course of evolution chloroplast sequences were frequently integrated into the nuclear genome, preferably in pericentromeric regions (Matsuo et al. 2005). The authors reasoned that in a given rice field of 1 hectare, there may be hundreds of grains in which a given chloroplast

gene has been newly transferred to the nucleus. Large fragments seem to disappear faster than small fragments. Gene transfer out of chloroplasts into the nucleus may be part of an ongoing mechanism for nuclear genome evolution through frequent acquisition of organellar DNA (Stegemann et al. 2003). Whether transgenes originally located in the plastid genome will be expressed if transferred to the nuclear genome remains an open question.

4.1.3.3 Physical Barriers

Physical barriers to reduce gene flow may include isolation zones and barrier crops. Isolation zones between a GM crop and non-GM crops can be de-vegetated (barren zone) or planted with a non-insect pollinated crop discouraging insect pollinators from leaving the GM crop. The influence of isolation zones on the behaviour of insect pollinators will vary between crop types and sites and with weather conditions, therefore, the impact of isolation zones on rates of gene flow in insect-pollinated plants cannot be assessed easily (Eastham & Sweet 2002). Barrier crops of non-GM plants surrounding the GM variety can act as absorber of GM pollen and, 8 – 30 m wide, reduce the rate of gene transfer, more so than isolation zones, but they cannot completely eliminate it (Feldmann 2000, Staniland et al. 2000). As a method to reduce the risk of transgene spread, proper management of the transgenic crop to eliminate hybrid generations between crop and weed which are necessary for gene flow to occur, has been suggested too (Wang et al. 2001, Zemetra et al. 2002). Eliminating the hybrids and early backcross generations from the field would reduce the risk of transgene introgression. For that reason, seed from such fields (e.g. wheat fields infested with jointed goatgrass) would better not be replanted or sold for commercial use. Seed dormancy will affect gene spread in temporal terms, therefore the level of dormancy of hybrid seed should be determined and how this would affect management plans. In forestry, limiting the use of transgenic trees to nurseries and harvesting transgenic trees before they reach their reproductive age has been suggested to facilitate the commercial approval of transgenic trees (Tzfira et al. 1998). However, transgenic trees might flower earlier than wildtype trees, as studies done with poplars transformed with a rol gene indicated (Fladung 1997).

4.1.3.4 Tracing of transgenes

For tracing the movement of transgenes and transgenic plants, Chamberlain and Stewart (1999) suggested to tag them with a visual marker such as green fluorescent protein (GFP), if non-toxicity of GFP could be established and GFP imposed no fitness costs. Harper et al. (1999) did not find fitness costs associated with the linkage of the GFP gene to the Bt cry1Ac gene in nuclear transformed tobacco and oilseed rape. In oilseed rape that had been transformed with genes encoding GFP and Bt-protein under control of the CaMV 35S promoter, GFP fluorescence was macroscopically detectable througout the lifecycle although the location of easily detectable fluorescence changed as the plant matured (Halfhill et al. 2001). In hybrids generated by crossing the transgenic plants to *B. rapa*, GFP fluorescence intensities were significantly lower than in the homozygous oilseed rape parent plants. Monitoring of gene spread by GFP, however, may be limited in subsequent generations by the separation of the gene of interest (in this case Bt) and the GFP gene that was linked to the

former in a separate expression cassette. Therefore, the generation of single GFP-Bt fusion proteins has been suggested by these authors. Another monitoring scheme, suggested by Chamberlain and Stewart (1999), is to express GFP in the seed coat of transgenic seed, using seed-coat specific promoters which may allow sorting of non-transgenic from transgenic seed as well as tracking of seed spilled in the environment.

4.1.4 Stacking of transgenes

The recombination of different transgenes can lead to polygenic traits. This may occur in an open-pollinated plant more readily than in a self-pollinated one. As transgenes in the cultivated crops change, traits could accumulate in crops by breeding and by accidental stacking through gene flow between sexually compatible transgenic crops planted in the same area. Weeds could thus develop resistance to several herbicides with different modes of action or to pests, pathogens, and stress, as they acquired genes from consecutive generations of crops over many years (http://www.acs.ohio-state-edu/units/research/index.htm). Of course, unwanted stacking of transgenes in plants related to crop plants could also occur as a result of transgene escape from plants carrying more than one gene of interest. In particular, if plastid transformation was used to transfer and express several genes in a single operon, gene escape could lead to introgression of a whole metabolic pathway. Stacking of genes, be it intended or not and caused by contamination, could lead to multiple transgene escape from crop plants to weedy or wild relatives even in a single generation (Klinger and Ellstrand 1999). The probability of multiple gene escape may be low initially, but in the long term, it is likely that multiple transgenes will be found in wild plant populations. In rare cases, nuclear-encoded and plastid-encoded genes may even be combined. Little data is available on the effects of single transgenes in wild populations and even less for multiple transfers. Some gene silencing might occur, but in most instances expression can be expected (Senior and Dale 1999).

The potential for simultaneous escape of more than one transgene could make management and eradication efforts (of weeds or volunteers for instance) substantially more difficult. The first agronomical example of gene stacking has been reported from Canada, where oilseed rape varieties resistant to different herbicides have been grown for several years. In 1998 oilseed rape volunteers were found that carried multiple resistances to glyphosate, glufosinate, and/or imidazolinone and, therefore, could not be controlled by any of these herbicides (Downey 1999). Pollen flow has been shown to be the cause of such multiple-resistant *B. napus* volunteers (Hall et al. 2000). Based on the North American experience, a recent English Nature study comes to the conclusion that the occurrence of HR gene stacked volunteers of oilseed rape would be inevitable in practical agriculture of Europe, if different HR varieties would be grown on a large scale (Orson 2002). In the case of transgene contamination of seeds, such gene stacking could include not only approved transgenic constructs but also sequences and constructs that have not been approved in a given country. The presence of unapproved oilseed rape seeds on farmers‘ fields has been reported (http://www.nytimes.com/2002/04/16/business/16SEED.html).

4.2 Horizontal gene transfer

Horizontal gene transfer, the transfer of genes to organisms of other species by means different from sexual reproduction, is known to occur among microorganisms. It has also been suggested that horizontal gene transfer played a significant role in the evolution of microorganisms and even of higher organisms (Lorenz & Wackernagel 1994, Feng et al. 1997, Bertolla & Simonet 1999, Jain et al. 2003, Kurland et al. 2003). Under selection due to the widespread use of antibiotics in human medicine and in animal husbandry, antibiotic resistance genes have spread in bacterial populations of different environments and bacterial pathogens have acquired multiple antibiotic resistance (Smalla et al. 2000). With the advent of genetic engineering of plants the question whether horizontal gene transfer from transgenic plants to microorganisms might affect natural populations and contribute to spread of antibiotic resistance genes used as markers has received considerable attention.

Naked DNA or DNA present in cells or plant tissue, introduced into soil or sediment can persist for extended periods of several months or even years (Lorenz & Wackernagel 1994, Eckelkamp et al. 1998, Gebhard & Smalla 1999, Meier & Wackernagel 2003). Binding of DNA to minerals such as sand and in particular clay particles increases its persistence, presumably due to enhanced protection from the action of nucleases. Extracellular DNA present in microbial habitats may provide a gene pool from which bacteria competent for natural transformation can derive genetic information. Many bacterial groups and some fungi are naturally competent to take up DNA from the environment, where competence for transformation is influenced by factors such as the physiological state of the bacteria, availability of nutrients, and stress (Lorenz & Wackernagel 1994). Frequency of transformation seems to be dependent on the type of DNA (plasmid DNA or chromosomal DNA), environmental parameters such as pH and temperature, and the kind of minerals on which the DNA is bound (Schlüter & Potrykus 1996). DNA fragment size is important too, whereby smaller fragments impair the transformation potential (Meier & Wackernagel 2003). Recombination of foreign DNA with endogenous genetic material is facilitated by long stretches of homologous sequences. Transformation of bacteria by foreign DNA may not only occur in soil ecosystems, but also in aquatic ecosystems, and perhaps in the gastrointestinal tract of animals and humans, fungal species may be able to take up foreign DNA too (Schlüter & Potrykus 1996, Mullany 2000).

DNA from transgenic plants has been shown to be present and persist at field sites where transgenic plants have been grown and plant litter has been deposited (Gebhard & Smalla 1999, Bertolla & Simonet 1999), but sites that have not been planted with transgenic plants may nevertheless harbour transgenic DNA, too, probably due to pollen movement, as Meier & Wackernagel (2003) observed. Most recombinant DNA may be contained in plant material deposited, but free DNA, released from plant cells, has also been found (Meier & Wackernagel 2003). Recombinant DNA has also been detected in eluates from transgenic potato rhizosphere (de Vries et al. 1999). Given that bacteria thrive around plant roots, nourished by root exudates and cell debris, they may also take up and integrate genetic material released from plants. If plant DNA is introduced into bacteria, it might be exchanged with other soil bacteria, including *Agrobacteria* and their T-plasmids, which in turn might

even lead to transfer back to plant cells, thus providing a genetic link between distantly related plant species (Tepfer et al 2003).

Attempts to directly observe transfer of DNA from plants to microorganisms have not produced unequivocal results (Schlüter & Potrykus 1996, Smalla et al. 2000, Heinemann & Traavik 2003), although under optimized laboratory conditions transformation of *Acinetobacter* with transgenic plant DNA and restoration of deletions in the bacterial neomycin phosphotransferase (nptII) gene by nptII sequences derived from various transgenic plants have been observed (Gebhard & Smalla 1998, de Vries & Wackernagel 1998). Reasons for failure to detect horizontal gene transfer from plants to bacteria may be the absence of homologous sequences in the bacteria tested, the use of less efficiently transformable bacteria, or the attempt to monitor transfer of complete genes, whereas bacterial transformation might involve recombination of short DNA fragments (Smalla et al. 2000). Recently, natural transformation of *P. stutzeri* bacteria, a species widely present in soils and marine sediments and naturally transformable in vitro, with DNA extracted from environmental samples has been reported (Meier & Wackernagel 2003). DNA derived from sites planted with GM sugar beet that carried the nptII gene transformed *Pseudomonas stutzeri* bacteria containing a plasmid with an internal 10 bp deletion in its nptII gene (marker rescue transformation).

Under laboratory conditions, DNA transfer from roots and leaves of several transgenic plant species to the soil bacterium *Acinetobacter* spp. was demonstrated using again the nptII gene system as a marker (Tepfer et al. 2003). Disrupted ground leaves from *Arabidopsis*, tobacco, oilseed rape, carrot, and alfalfa produced bacterial transformants, with particularly high transformation frequencies when tobacco carrying the nptII gene in the plastid genome was used. In the latter case, as little as 0.1 g of leaves was sufficient to detect transfer of the nptII gene, resulting in an increase of transformation activity by a factor of 10^4, compared to nuclear insertions of the transgene. The addition of soil reduced, but did not prevent DNA transfer. DNA transfer was also observed from intact tobacco leaves, albeit at lower frequency, and from intact oilseed rape roots. Intact tobacco plants carrying plastid insertions of the nptII gene consistently gave transformants, whereas intact tobacco and *Arabidopsis* plants with nuclear marker gene insertions resulted in low transformation frequencies. Silent mutations in nptII from donor plants were acquired by the receptor bacteria, indicating that DNA transfer was not an artifact.

Tepfer et al. (2003) concluded that, as less than 2 g of intact roots or less than 0.1 g of disrupted leaves was sufficient to produce *Acinetobacter* transformation in the laboratory, horizontal gene transfer can occur on an evolutionary time scale, even if transfers were many orders of magnitude less likely in situ. Since transfer of long DNA fragments would be reduced by degradation and inefficient uptake, transfer of protein domains might be more likely in nature. But, as Tepfer et al (2003) and Heinemann & Traavik (2004) point out, sequence mosaicism attributed to horizontal gene transfer can be observed both within proteins and within genomes. Selective pressure would certainly play a role in the integration and maintenance of foreign DNA in bacterial populations, such selective pressures might be exerted by drought or herbicides, favouring rhizosphere bacteria that have acquired herbicide resistance genes of microbial origin which have been transferred to plants (Schlüter & Potrykus 1996). Transgene copy number and genome size will influence the likelihood of

transgene movement to soil bacteria too, as indicated by a 30-fold enhanced transformation frequency obtained with *Arabidopsis*, having a small genome, compared to oilseed rape, where the transgene may be diluted through the 17 times larger genome size (Tepfer et al 2003).

In contrast, repetition of a sequence in the plant will enhance gene transfer. This has been confirmed by the high transfer frequency observed with tobacco leaves containing plastid insertions of nptII which reflects the high number of plastid genomes per cell (Tepfer et al. 2003). In homoplasmic transplastomic plants up to 10 000 copies of the antibiotic resistance marker gene, most often the aadA gene (aminoglycoside 3‘-adenyltransferase from *E. coli*), conferring resistance to spectinomycin and streptomycin, are present per cell, in heteroplasmic cells a few thousand copies may still be present. In addition to the high copy number of transgenes, other features of chloroplast transformation increase the risk of gene transfer from plant plastids to bacteria: codon usage of genes expressed in plastids often resembles codon usage patterns in bacteria and the promoters used for plastid expression of transferred genes can be active in bacteria too, since bacteria and plastids show compatible RNA and protein synthetic machineries (Heifetz 2000, Iamtham & Day 2000, Daniell et al. 2001a,b). This holds true for antibiotic resistance marker genes and plastid transgene sequences coding for other proteins. If their products are of any use for bacteria, chances for successful horizontal gene transfer will increase.

Horizontal gene transfer might also occur to the intestinal microflora in the gastrointestinal tract of humans and animals, as studies with human volunteers that are ileostomists (individuals with the terminal ileum resected, digesta are diverted from the body to a colostomy bag) consuming transgenic soybean in their food suggest (Netherwood et al. 2004). The herbicide resistance transgene survived passage through the small bowel of ileostomists, but not through the intact gastrointestinal tract of other volunteers. Stable integration of the transgene from GM soya in intestinal bacteria was observed in three of the seven ileostomists when the microbes in the digesta had been expanded by culturing, indicating that the transformed microbes represented a minor component, presumably belonging to the 90 % of microorganisms in the intestinal microflora that remain uncultured. Gene transfer may have occurred before the feeding experiment, likely reflecting long term consumption of GM foods. From feces of humans with an intact gastrointestinal tract transformed microbes could not be cultured, leading Netherwood et al (2004) to the conclusion that the bacteria containing the GM soya transgene were viable only in the small bowel and did not survive in the large bowel with its more anaerobic environment and a higher density of competing organisms.

According to Schlüter et al. (1995), gene transfer frequencies from plants to bacteria under natural conditions are so low (about 2×10^{-17}) as to be essentially irrelevant to any realistic assessment of the risk involved with release of transgenic plants. Heineman & Traavik (2004), however, argue that horizontal gene transfer from transgenic plants to microbes could still have an environmental impact at a frequency approximately a trillion times lower than the current risk assessment literature estimates the frequency to be. They based their assumptions on experience with strains of *Streptococcus pneumoniae* with reduced susceptibility to penicillin that have evolved through successive nucleotide introductions within the last 50 years and the observation that the evolution of penicillin resistance has resulted from events

predicted to be $10^7 – 10^{19}$ times rarer than the frequency of horizontal gene transfer estimated to be occurring in the soil (10^{-17}). In addition, Heinemann & Traavik (2004) and Nielson & Townsend (2004a) proposed that current methods of environmental sampling to capture transgenes and traits in a recipient are too insensitive for monitoring evolution of horizontal gene transfer. Recurrent transfers and mosaic genes may play a significant role in horizontal gene transfer but they may be detected at extremely low frequencies only. Even if most transfer events would be deleterious to the bacterial transformant, rare beneficial events cannot be a priori excluded (Nielsen & Townsend 2004a) and the vast area cultivated with transgenic crop plants might still provide opportunities for successful horizontal gene transfer. Based on horizontal gene transfer frequencies and the size of the microbial population, Heinemann & Traavik (2004) calculated that approximately ten recombinants per 250 m^2 could be expected if the gene transmission frequency was 10^{-17}.

Analysing the sensitivity of current monitoring efforts in soil (Gebhard & Smalla 1999) and the gastrointestinal tract (Netherwood et al. 2004), Nielsen & Townsend (2004a) concluded that studies to date have examined potential horizontal gene transfer events occurring, when combined, in less than 2 g of sample material. Since rare bacterial transformants that have received transgenes might require years to out-compete wild-type bacteria, time of sampling would be crucial to useful monitoring schemes. They suggested major changes of current monitoring approaches of horizontal gene transfer in soil and the intestine, such as to include the population size of exposed bacteria, the bacterial generation time, the strength of selection acting on the transgene-carrying bacteria, and the sample size necessary to verify or falsify the horizontal gene transfer hypotheses tested. They also recommended shifting the focus of future research and monitoring efforts to the identification of bacterial genetic compositions and environmental conditions that facilitate transfer and positive selection of microbial transformants carrying transgenes acquired through horizontal gene transfer.

To reduce risks connected with potential horizontal transfer of antibiotic resistance marker genes, the European Food Safety Authority (EFSA 2004) grouped marker genes according to the medical use of the respective antibiotics. EFSA placed no restrictions on nptII (resistance to kanamycin) and hph (resistance to hygromycin) genes (group 1), but recommended that marker genes conferring resistance to antibiotics used for therapy in human and veterinary medicine such as the ampicillin (amp^r) and spectinomycin (aadA) resistance gene should not be present in GM plants placed on the market (group 2). Genes conferring resistance to antibiotics that are highly relevant for human therapy, e. g. the amikacin (nptIII) and the tetracycline (tetA) resistance gene, should not be present in plants placed on the market and used for field releases (group 3).

For risk reduction it has also been suggested to avoid bacterial sequences as far as possible, to use plant genes and promoters not active in bacterial systems, and to insert introns that could render the transgene dysfunctional in bacteria (Schlüter & Potrykus 1996, Tepfer et al. 2003). Because of their dissimilarity to bacterial DNA, plant sequences should not provide sequences of homology that facilitate recombination. But, most transgenes inserted in GM plants cultivated now are of bacterial origin, such as antibiotic resistance genes, herbicide resistance genes and insect resistance genes, e.g. the Bt toxin gene. In addition, as discussed in previous sections, transgenic plants derived from *Agrobacterium*-mediated or particle bombardment

transformation generally contain bacterial sequences such as T-DNA border, antibiotic resistance gene, and/or plasmid backbone sequences, potentially even including bacterial origins of replication. Homologous DNA can serve as a recombinational anchor facilitating illegitimate recombination of heterologous genes, increasing integration of foreign DNA by a factor of at least 10^5, when linked on one side to the non-homologous DNA (de Vries & Wackernagel 2002). The antibiotic resistance marker gene present in many transgenic plants could provide such an anchor sequence necessary to initiate transfer of adjacent transgenes. After homology-initiated recombination, additive integration of transgenes, known to occur at high frequencies in bacteria, could also take place (Nielsen & Townsend 2004b).

Since DNA fragments derived from transgenic plants that contain origins of replication might be able to reproduce independently after transfer to bacteria, the use of transgene constructs with the gene(s) of interest only is recommended (Schlüter & Potrykus 1996), but this may be feasible only in transformation protocols not relying on *Agrobacterium*. Although the use of plant promoters not active in bacterial systems is advised, the CaMV 35S promoter, known to be active in microorganisms such as *E. coli* and yeast (Ho et al. 2000b), is still the most widely used promoter in genetic engineering of plants. In addition, Jacob et al. (2002) demonstrated that plant-specific promoters (derived, besides *A. tumefaciens* and *A. rhizogenes,* from potato, tobacco, and 35S CaMV) can in fact lead to expression in bacterial species of different taxonomic groups, with some potato promoters consistently showing strong expression of the transgene, and one of them, ST-LS1, directing expression in all bacteria tested.

Most studies dealing with horizontal gene transfer have used antibiotic resistance marker genes and discussed effects expected from spread of these genes to human or animal pathogens making control of these pathogens more difficult or even impossible. However, Nielsen & Townsend (2004b) point out that novel transgenes, other than marker genes, that do not have natural counterparts require particular attention with regard to horizontal gene transfer, as these often include novel combinations of regulatory elements and toxin protein domains, derived from different species, that may differ substantially from those arising by natural evolution.

4.3 Spread of transgenic plants

Not only hybridization of transgenic crop plants with other crops or related wild plants leads to transgene escape, but also running wild and seed dispersal of crop plants. In Central Europe there are about 10 000 taxa of cultigens that are agricultural crops, fruit-trees and shrubs, ornamental and forestry plants. Foreign species are continuously introduced through world-wide trade and travel. It is difficult to assess to what extent these large numbers of cultigens exert an influence on natural ecosystems. Reversion of cultigens to wild-type forms has been observed, reversion may be facilitated in cultigens which have a low degree of domestication. There are estimates that about 1:100 of foreign plant species will naturalize and about 1:1 000 will induce undesired changes in the existing ecosystems (Bartsch et al. 1993). The running wild of cultigens may be of particular concern, if they have closely related wild plants as potential hybridization mates, Bartsch et al. provided a list of such plants for Central Europe.

About 3.5 % of the vascular plant species introduced to Germany have established themselves permanently and about 0.3 % are targets of control programs (Kowarik 1999a). Once introduced species become invasive, it will usually be both difficult and expensive to eradicate them, if possible at all (Kowarik 1999a, Shine et al. 2000).

The question whether genetic engineering causes measurable changes in the ecological performance of transgenic crop plants is of particular concern. Recombinant DNA technology makes it possible to introduce a greater diversity of genes into living organisms than traditional methods of breeding and selection and to obtain novel combinations of genetic material. GMOs are by definition "alien" insofar as they have no normal distribution and occur nowhere in the natural environment until released (Shine et al. 2000). Although genetic engineering transfers only short sequences of DNA, the resulting phenotype, which includes the transgenic trait and possibly changes in traits due to pleiotropic effects and position effects, can produce an organism novel to the existing network of ecological relationships (Wolfenbarger & Phifer 2000). Therefore, transgenic plants might have severe and irreversible effects on environmental safety by showing increased weediness or becoming more invasive of natural habitats or having selective advantages under selection pressure.

Data collected on transgenic HR oilseed rape, corn, and sugar beet indicate that they do not seem to be more weedy or invasive in natural habitats than their respective non-GM lines if selection pressure by herbicides is not exerted (Crawley et al. 1993). HR crops and insect-resistant potato, studied over a 10-year period, were also not found to be more invasive than their conventional counterparts (Crawley et al. 2001). Although, in some cases there had been differences between GM lines regarding survival (Crawley et al. 1993) and some of the transgenics were less fit than their non-GM counterparts (Crawley et al. 2001), substantial costs involved in expressing a kanamycin- or glufosinate-resistant phenotype could not be found (Crawley et al. 1993). There also seemed to be no general fitness costs associated with transgenic glufosinate-resistance when introgressed from oilseed rape into its wild relative field mustard (Snow & Jørgensen 1999). Thus the collected data provided little support for the "excess baggage" hypothesis, which postulated that release of genetically modified organisms into the environment is inherently safe due to the extra costs of carriage and expression of recombinant DNA reducing the fitness of transgenic organisms (Lenski & Nguyen 1988). Ecologists have criticized such generic safety arguments for some time, arguing that data gained largely with ecologically crippled laboratory organisms would not be valid for genetically modified organisms that were designed to be viable if not vigorous in nature (Tiedje et al. 1989, Regal 1993, Regal 1994).

Transgenic plants and their seeds or propagules may be dispersed by animals and humans or abiotically by wind and water. Birds are pretty effective in dispersing seeds from one place to another, often over long distances. Fruit-eating birds may digest only the fleshy fruit parts, allowing the seeds to pass on through the gut, thus acting as efficient dispersers. Seed-eating birds digesting seeds for their nutrient content may also act as seed dispersers, if some seeds evade destruction or if the birds die with viable seeds in their gizzards. In addition, passing through an avian alimentary system can also affect the overall viability of seeds, as dormancy characteristics and germination rate may be modified, thus diversifying the germination strategies available to a plant (Moore 2001). Seed dispersal by wind is of importance for

many species, in particular for tree species. Although, in general, only few seeds are uplifted, those that are uplifted beyond the canopy height can travel at least several hundred metres and perhaps tens of kilometres. Therefore, long distance dispersal can be as large as 1 – 5 %, making long-distance colonization more frequent than previously believed (Nathan et al. 2002).

Human activity has been responsible for most intentional or unintentional introductions of alien invasive species. In the era of globalisation, the four "Ts" – Trade, Transport, Travel, and Tourism – have sharply accelerated the rate of species movement (Shine et al. 2000), and these Ts will also accelerate the introduction of transgenic organisms to many habitats, be it intended or unintended. Secondary transfers such as transportation of soil, garden waste, tree nursery products, and seedings have alse played an important role in establishment of invasive species (Kowarik 1999a). Of particular concern will be introductions of GMOs that are being delivered as grain commodities or in food aid programs without corresponding safety assessments and measures. GM material could be spilled or used by poor farmers as seed. There are already reports about GM oilseed rape plants growing in many locations close to Japanese ports where oilseed rape was imported (www.gmcontaminationregister.org). In the past, invasive plants have been introduced by seed consignments contaminated with weedy plants (Shine et al. 2000). GM contamination of non-GM seed lots may similarly lead to unintentional introduction of transgenic plants carrying different transgenic traits.

As pointed out by several authors (Kareiva 1993, Sukopp and Sukopp 1994), an ultimately successful invader might initially fail miserably or barely persist for decades, before exhibiting considerable or even explosive population growth. Considerable time-lags can be observed between release of an organism and its invasion of other habitats as studies with woody species indicate (Kowarik 1998, Kowarik 1999b, Marvier et al. 1999). Certain traits seem to increase the potential of an organism to invade ecosystems, among them broad native range, plasticity in response to environmental factors, escape from herbivory, and rapid dispersal. Rapid dispersal is associated with traits such as short generation time, long fruiting period, large seed number, prolonged seed viability, and anthropogenic transport. More plastic genotypes might have a higher chance to perform well in a greater number of new habitats. Zoglauer et al. (2000) collected and reviewed data about the prognostic value of some 40 plant traits that have been studied by several groups with respect to their potential influence on invasivity.

Hybridization of transgenic plants with native wild species may increase genetic variation and enhance the potential for evolution and adaptation of the progeny (Auge et al. 2001). Competition for pollination can also be an important factor in plant reproduction, as work done with the invasive species purple loosestrife (*Lythrum salicaria*) and the native co-flowering congener *L. alatum* has shown (Brown & Mitchell 2001). If pollen of invasive plants would lead to reduced seed set in related native species, these natives may be threatened at distances that are too far for vegetative competition, depending on the range of pollen transfer by pollinators and wind. Herbicide resistance could provide plants with a competitive advantage in habitats that are subject to spray drift of the respective herbicides. As abundance and diversity of wild plants along field edges are affected negatively by herbicide spray drift from crop fields (de Snoo & van der Poll 1999), HR crop plants running

wild could invade habitats in the vicinity of crop fields more easily, favoured by recurring selection pressure. Likewise, transgenic plants engineered to be resistant to pathogens or insect pests could be more competitive, compared to native species.

There have been attempts to evaluate transgenes and recipient plants according to the risk they carry to show increased fitness (Sweet et al. 1999, Schmitz & Schütte 2001b). Generally, genes improving the fitness of transgenic plants by conferring tolerance to pathogens, pests, and abiotic stress will have the greatest environmental impact, but transgenes responsible for alterations of oil and starch content can also enhance fitness, if winter survival and dormancy would be improved (Schmitz & Schütte 2001b, Claessen et al. 2005b). Low impact transgenes should not noticeably change the fitness of the engineered plant, but it has to be verified on a case-by-case basis that the transgene and the transformation process did in fact not alter environmental fitness of the transgenic plant. High impact plants would be hardy, perennial, competitive, open pollinating, and prolific, have a wide range of relatives with which they hybridize and an ability to colonize a range of natural and semi-natural habitats (Sweet et al. 1999), as for example, perennial grasses and certain trees and shrubs. Medium impact plants would be open pollinating, prolific, and able to hybridize with wild relatives and to colonize a limited range of habitats, e.g. oilseed rape, oats, sugar beet, and rice. Largely self-pollinating annual or biennial plants with few hybridizing relatives such as maize or sunflower in Europe have been termed low impact plants. However, as studies done on invasive species indicate, the presence of traits thought to increase invasiveness of a non-native species does not guarantee successful invasions, likewise their absence does not preclude them (Kowarik 1998, Zoglauer et al. 2000, Alpert et al. 2000). Comparative studies have failed so far to identify simple, general predictors of invasion success, although it has been recognized that plant species that are successful invaders in one region are likely to be successful in others too (Marvier et al. 1999).

According to Alpert et al. (2000), the most likely reason, why the search for traits that underlie invasiveness has largely failed, is that invasiveness depends more upon the interaction between the characteristics of non-native species and their potential new habitats, than upon the characteristics of the species alone. Therefore, risk assessment of the spread of GMOs has to take into account that similar traits of transgenics may lead to different ecological effects in the various areas into which the transgenic plants will be introduced, depending also on the characteristics of the habitats in question. If physical conditions or predators tend to reduce seed longevity, habitats might be less invasible because dispersal is limited in time. Performance and biomass of transgenic plants may also depend on pathogens and pests present in the invaded habitat. F_1 offspring of virus and herbicide resistant sugar beet, crossed to Swiss chard, produced significantly less biomass under low virus infestation than hybrids derived from crosses of non-GM sugar beet with Swiss chard, but superior performance of transgenic F_1 hybrids was observed in habitats with a high level of virus (Bartsch et al. 2001). Success of invasive plants may also depend on the presence of insect herbivores feeding on the introduced plants (Auge et al. 2001).

Environmental stress, such as abiotic stress and low nutrient availability, may influence invasibility of habitats. Alpert et al. (2000) proposed that low stress, e.g. high amount of nutrients such as nitrogen, may favour invasive species because they are better able than

natives to take advantage of high resource availability. On the other hand, if, because of their newly introduced stress tolerance, transgenic plants would be able to survive in habitats with high environmental stress, such as salt, drought, and cold, then they might be able to invade habitats when stress is high and thus compete successfully with native stress-tolerant species. Changes in disturbance of a habitat and global change altering carbon dioxide levels and mean temperatures may also play an important role in invasibility (Dukes & Mooney 1999, Auge et al. 2001), degraded and stressed areas being at high risk (Shine et al. 2000). For instance, in areas where they co-occur, C4 species might profit more from warmer temperatures than C3 plants, because they generally have a higher optimum temperature for photosynthesis (Dukes & Mooeny 1999).

In summary, because of the many unknowns with regard to interactions between native and introduced species in a given habitat, predictions about invasiveness of transgenic organisms and invasibility of the ecosystems, into which these GMO are to be relased, are hardly possible. Future research should address critical questions such as the risk linked to spread of transgenic plants carrying traits that confer resistance to pathogens, pests and abiotic stress. This will be even more important because such traits will likely be found stacked in the same plants, either as a result of multi-gene transformation or of inadvertant hybridization.

5 Traits of transgenic plants and their environmental impacts

5.1 General considerations

Most of the genes of interest used for transformation of plants are derived from microorganisms and plants and some have been isolated from animal or human cells. Transgenes of bacterial origin generally have been adapted to plant codon usage. In future, attempts may be made to increase the range of genes by directed molecular evolution, also termed molecular breeding (Lassner & Bedbrook 2001). In this process, various parental genes are fragmented and subsequently reassembled. The resultant library of gene variants is then screened to isolate progeny with altered properties. For instance, the shuffling of DNA coding for two different enzymes of carotenoid biosynthesis pathways in *Escherichia coli* resulted in progeny that expressed a novel carotenoid not produced in nature (Schmidt-Dannert et al. 2000). Such molecular breeding may be used for the expression of enzymes with novel substrate specificities and enhanced activity or for novel proteins leading to new herbicide or pest resistance traits (Lassner & Bedbrook 2001).

Lheureux et al. (2003) compiled data on transgenic plants in the pipeline in the EU. They grouped the plants according to their likelihood to be developed within the next five years (group 1), the next five to ten years (group 2), and those likely to need more than ten years for development (group 3). Group 1 comprised traits such as herbicide and insect resistance (including stacked traits), modified starch and fatty acid content, modified flower colour and modified fruit ripening in crops such as maize, oilseed rape, soybean, wheat, beet, cotton, chicory, tomato, and potato. Basically the same crop species plus species like sunflower, fruit

trees, melon, barley, and rice were expected to be developed within ten years (group 2), they are supposed to carry traits such as resistance to fungi, viruses, and herbicides, and modified protein content, and, in addition, increased erucic acid content in oilseed rape. Transgenic plants resistant to abiotic stress factors, with enhanced yield and increased levels of functional ingredients, as well as hypoallergenic and pharma crops, and trees with modified lignin content are all expected only for the period after 2011 (group 3).

In addition to these traits, "gene usage restriction technology" (GURT) traits have been expressed in transgenic plants. GURTs, aimed at protection of intellectual property rights on transgenic plants, allow the production of plants whose seeds contain a genetically introduced mechanism that prevents (or only permits) germination when a certain chemical is applied (Hartmann 2002). Similarly, transgenic plants have been created that express quality-enhancing traits (added value traits) only in the presence of a certain chemical, or that are less resistant to diseases or unable to survive in the absence of the chemical. These GURT traits may be linked to other traits of interest to exert control over seed production and to inhibit the reuse of harvested seed by the farmer.

In risk assessment of release of these different GMOs, their potential effects on non-target organisms and on biodiversity in general have to be considered. Such an assessment has to deal with effects of the new genetic information and the traits conferred to the GMOs that are exerted on non-target organisms, be they plants, animals, or microorganisms. Some of these effects will be unintended, perhaps even unwanted. This may be due to direct effects of a new compound such as a protein conferring pest or pathogen resistance that could harm beneficial organisms in an unexpected manner. GMOs with new traits that alter growth characteristics or stress resistance could also interact in unexpected, perhaps negative ways with organisms of the ecosystems into which they are released, thus endangering biodiversity. Changes in phenotype can be caused by the genes' products directly, by unintended alterations of the biochemistry through the genes' products, and by changes caused by pleiotropic effects and/or position effects due to altered activity of endogenous genetic sequences close to the insertion site(s). Tissue culture may also contribute to phenotypic changes since it involves disorganized cell growth that can induce somaclonal variation, i.e. mutations including changes in chromosome structure and gene expression or altered methylation patterns (Bao et al. 1999, Dahleen et al. 2001, Wilson et al. 2004). However, the transformation procedure might cause greater somaclonal variation than tissue culture alone (Sharp et al. 2002).

At present it is unclear, to what extent position effects and pleiotropic effects due to multiple and scrambled insertion sites and due to possible interactions of the novel genes, their promoters, or products might lead to increased instability and unpredictable alterations of the biochemistry and phenotype of transgenic crop plants (Senior & Dale 1999, Wilson et al. 2004). Many unexpected alterations in biochemistry and/or phenotype of transgenic plants, have been described, some of them have been observed in the laboratory or greenhouse, some in the field, such as the cracking of RR soybean stalks in high soil temperatures (Coghlan 1999). Because of the complexity of plant metabolism, attempts to engineer basic metabolic pathways very likely will lead to unexpected changes in plant compounds and/or altered morphology and phenotype, as has been reported for transgenic potatoes with altered carbohydrate metabolism (Becker et al. 1998). Presumably a great deal of such unexpected

alterations has never been published, since ill-performing transformants will be removed very early in development of transgenic cultivars. If, however, unexpected effects will show up later, after release of GMOs into the field, perhaps under stress conditions, then safety concerns may arise, more so, if pleiotropic or position effects lead to toxic compounds or to new types of interactions with other organisms.

Although it is often assumed that GM cultivars are similar or even identical to the original cultivar, except for the expression of the introduced gene(s), conferring, for instance, resistance to insects or herbicides, genetically modified plants may show altered susceptibility to pests or pathogens, compared to their non-transgenic parents, as has been observed with GM cotton varieties. GM insect resistant cultivars showed a significant increase in susceptibility to root-knot nematode, a serious pest of cotton, compared with the non-transgenic parents (Colyer et al. 2000), the reason for this increase in nematode reproduction and root knot galling remained unclear. According to Benbrook (2000), transgenic Bt varieties will, in some circumstances need additional pesticide treatment, perhaps because of some pleiotropic effect on natural plant defense mechanisms.

Generally, the quantity of modifications and modfied products derived from GMOs will differ from those available through traditional breeding programs, increasing the variety of GMOs dramatically. Such an increase may collectively represent an environmental risk, given the many new interactions now possible and the limitations of predicting negative effects (Wolfenbarger & Phifer 2000). In addition, the quality of modifications and modified products will also differ from those available through selective breeding, because of the wide range of genetic sequences that can be used, basically without limit, for genetic engineering, providing a far greater range of possibilities to create desired phenotypes. Organisms novel to the existing networks of ecological relationships can thus be produced (Tiedje et al. 1989, Regal 1994).

Genetically engineered crops may not only affect plant and animal life in ecosystems, but also soil ecosystems, thereby potentially altering decomposition rates of organic matter and carbon and nitrogen levels (Wolfenbarger & Phifer 2000). Root exudation of transgenic proteins has been shown. Such exudates may retain biological activity in soil over a considerable length of time (Saxena et al. 1999). Plant engineering that leads to new or altered products in crops raises the question of potential toxicity to humans and animals when the transgenic plants are consumed. This is particularly relevant for strategies using products not normally belonging to the human or animal diet. Many of the genetic engineering approaches use genes from microorganisms, animals or plants not used as food or feed. Therefore, a thorough evaluation of toxicity is necessary (Franck-Oberaspach & Keller 1997). But even in cases where the transgenic plants or products thereof are not used as food or feed there may be impacts on non-target species and wildlife feeding on such plants.

5.2 Herbicide resistance

5.2.1 Herbicide resistance genes transferred

Most of the transgenic plants that are cultivated or tested in field experiments have been transformed to exhibit resistance to either glyphosate (Roundup) or glufosinate (Basta or Liberty), with glyphosate resistance deployed far more often then glufosinate resistance. In 2005, herbicide resistant (HR) crops such as soybean, corn, oilseed rape, and cotton occupied about 71 % of the 90 million hectares grown with transgenic crops worldwide plus an additional 11 % grown with stacked transgenes such as HR plus Bt-insect resistance (James 2006). Glyphosate is a broad-spectrum herbicide that kills plants by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), involved in the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), vitamins, and secondary metabolites. The shikimate pathway plays a critical role in normal cell function, plant growth, and disease and stress response. Resistance to glyphosate can be achieved by the expression of EPSPS from *Agrobacterium* which is, unlike the plant enzyme, not inhibited by glyphosate (OECD 1999a). A gene from the ubiquitous soil bacterium *Achromobacter*, strain LBAA, coding for the glyphosate degrading enzyme glyphosate oxidoreductase (GOX) has been used too.

Glufosinate with the active ingredient L-phosphinothricin (the D-isomer is inactive and is not converted in plant cells to the active ingredient) is a broad-spectrum herbicide too, killing plants by inhibition of glutamine synthetase which results in the accumulation of lethal levels of ammonia. Phosphinothricin-acetyltransferase (PAT) encoding genes from two species of actinomycetes, *Streptomyces viridochromogenes* and *S. hygroscopicus*, have been transferred to plants to confer resistance to glufosinate. Both PAT enzymes, encoded by the pat and bar genes, respectively, inactivate glufosinate by acetylation resulting in the main metabolite N-acetyl-phosphinothricin (OECD 1999b, Ruhland et al. 2002). They have a similar substrate affinity towards L-phosphinothricin and lead to a comparable performance in transgenic plants (Wehrmann et al. 1996). According to Nolte et al (2004), transfer of the glutamate dehydrogenase gene gdhA can also increase the resistance of tobacco to glufosinate.

Increased herbicide tolerance may also be achieved by the expression of mammalian P450 cytochromes that are known to degrade xenobiotics including herbicides. Transfer of human cytochrome CYP1A1 and CYP2B6 genes to rice has been reported to enhance metabolism of various herbicides with different modes of action and thus increase tolerance to herbicides such as atrazine, chlortoluron, norflurazon, trifluralin, or metolachlor (Kawahigashi et al. 2003, Hirose et al. 2005).

5.2.2 Environmental impacts of herbicide resistance

The cultivation of HR crops can lead to unintended effects, due to unexpected changes in plant metabolism and to the application of broad-spectrum herbicides on areas and in cultures not possible before, thus influencing soil life, weed populations, and farmland biodiversity. Effects could be related to potential toxicity of these herbicides to non-target organisms and to

a reduction in abundance and diversity of weed species (Ruckenbauer 1998, Schütte 1998c, Klöpffer et al. 1999, Schütte 2000, Schütte & Schütz 2001). Selection pressure exerted by multiple applications of the respective herbicides will lead to the evolution of resistant weed biotypes and to shifts in the weed flora to less susceptible species.

Direct effects

Glyphosate and glufosinate are non-residual herbicides that are considered to be degraded comparatively rapidly from soil, with estimated dissipation half-lifes ranging from 6 to 200 days (Alister et al. 2005) and from 1 to 25 days, respectively. They are absorbed to soil particles and believed to show low mobility through the soil profile. However, the Danish pesticide monitoring programme revealed leaching of glyphosate and its metabolite aminomethyl phosphonic acid (AMPA) from the root zone in loamy soils in average concentrations exceeding the maximum allowable concentration of 0.1 µg/l, with maximum concentrations of 5.1 µg/l and 5.4 µg/l, respectively (Kjaer et al. 2004). Leaching of glyphosate was observed within the first months of application, whereas leaching of AMPA occurs considerably longer, indicating that AMPA is retained in the soil for a rather long time. The surfactants used to improve efficacy can show considerable toxicity on their own, in particular for aquatic organisms (Diamond & Durkin 1997, Relyea 2005a). Accinelli et al. (2004) studied the effect of insecticidal toxins extracted from a commercial formulation of *Bacillus thuringiensis* subsp. *kurstaki* that have been added to glyphosate and glufosinate treated soils. They found that addition of Btk toxins lead to a significant increase of glyphosate and glufosinate persistence in both soil types tested. If such effects could be extrapolated to field conditions, it would be of significance since corn and cotton crops expressing both herbicide resistance and insect resistance due to the transfer of Bt-toxin genes have been commercialized and are now grown on about 11 % of the GMO crop area worldwide (James 2006).

Although glyphosate is the most ubiquitous herbicide around the world, there is a paucity of data about the effect on certain animal species that can come into contact with this herbicide. Recently, in laboratory and outdoor pond mesocosm tests lasting longer than usual, lethal effects of Roundup on six species of American amphibia have been found. At concentrations that have been observed in natural habitats (0.1 to 2.3 mg active ingredient/liter AI/L) Roundup killed a significant proportion of larval amphibians. LC50 values (lethal concentration to 50 % of test species) ranged from 0.6 to 2.5 mg AI/L (Relyea 2005a,b, Relyea et al. 2005). Juvenile anurans were also affected when sprayed with Roundup simulating a direct terrestrial overspray in an agricultural field (Relyea 2005b). Synergistic interactions with predatory stress may be possible too, at least in some species (Relyea 2005a). In aquatic communities consisting of tadpoles, snails, and predators, Roundup at the maximum application rate (3.8 mg AI/L) reduced tadpole richness by 70 % but showed no effect on insect predators and snails (Relyea 2005c). Lethal effects on tadpoles did not result from tadpole starvation. The surfactant POEA (polyoxyethyleneamine, a derivative of tallow) seems to play an important role in Roundup toxicity, its toxicity is higher in alkaline than in acidic water (Diamond & Durkin 1997). Varying toxicity of glyphosate to other species such as arthropods, small mammals, and fish has been reported too (Giesy et al. 2000).

As glyphosate and glufosinate exhibit antimicrobial activity, concerns relate also to potential toxicity to soil and aquatic microorganisms whose diversity and abundance could change under herbicide application. Microorganism populations show different sensitivity to glyphosate and glufosinate, some beneficial fungi seem to be more sensitive than pathogenic species. Glyphosate and glufosinate can decrease activity of nitrogen fixing bacteria such as *Bradyrhizobium japonicum,* the microorganisms that fix nitrogen in soybean plant roots, and *Rhizobium meliloti*, nitrogen-fixing bacteria in alfalfa, respectively (Schütte 1998, Meyer & Wolters 1998, Labes et al. 1999). In studies performed by Morjan et al. (2002) on entomopathogenic fungi that help combat populations of arthropod pests such as spider mite and green cloverworm, the active ingredient glyphosate alone did not have fungicidal activity. But the four species of fungi showed susceptibility when tested with field concentrations of the various Roundup formulations, the strongest response was detected with Roundup Ready-to-Use. In most cases fungicidal activity of the formulations was increased by glyphosate in a synergistic manner. This indicates that the formulation ingredients influence the toxicity of the herbicide and that active ingredient und formulations can interact in a synergistic way. HR plant material harvested three days after glyphosate application still retains herbicide activity when used as mulch (Goss et al. 2004).

EPSPS, the target enzyme of glyphosate, is essential for aromatic acid biosynthesis not only in plants but also in microorganisms. The glyphosate-insensitive EPSPS conferring resistance to transgenic plants has been isolated from *Agrobacterium* sp., strain CP4 (OECD 1999a), but not all microorganisms possess similarly insensitive forms of this vital enzyme. Therefore, questions have been raised regarding the potential impacts of glyphosate-treated feed such as corn and soybean on microorganisms that are members of the rumen microflora. This may be all the more important, as herbicide residues may be higher in transgenic HR plants because the respective herbicides can be applied at higher doses and at any time in the season. Glyphosate can accumulate in soybeans (Duke et al. 2003). The nitrogen-fixing symbiont of soybean, *Bradyrhizobium japonicum*, has been shown to be sensitive to glyphosate, varying with herbicide concentration and bacterial strain. Reddy and Zablotowicz (2003) reported inhibition of *B. japonicum* nodule development and reduction of nodule biomass by 28 % 14 days after application of certain glyphosate formulations, as well as glyphosate accumulation in nodules and a reduction of leghemoglobin content in RoundupReady (RR) soybeans by 8 % to 10 %. Glyphosate applications to young soybean plants delayed nitrogen fixation and reduced root and shoot growth, which could lead to significant yield losses, up to 25 %, in less fertile soils and/or under drought stress (King et al. 2001). The authors concluded that under glyphosate treatment nitrogen fixation is more sensitive to water deficit. In soils with sufficient humidity and soil nitrogen available, depressed nitrogen fixation showed little impact on yields. But if *B. japonicum* was damaged by continuous glyphosate application, nitrogen fixation could be affected resulting in lower crop yield.

In general, research about yield potential of HR crops has led to mixed results (Fernandez-Cornejo & McBride 2002). When compared to otherwise identical but non-GM varieties grown under similar field conditions, RR soybeans quite often show a yield drag, ranging from 1 % to about 20 %, depending on varieties and growth conditions, amounting in 1999 and 2000 to 5 – 10 % in the average (Benbrook 2001, King et al. 2001, Mertens & Plän 2001). This yield drag, observed by many agronomists and scientists, has been attributed by

Benbrook in part to reduced nodulation and nitrogen fixation, especially under stress conditions. In RR soybean field experiments with and without glyphosate application, Elmore et al. (2001a,b) did not find a significant yield difference, but compared to high-yield non-GM soybean varieties and to the non-GM sister lines, they observed a yield drag of 10 % and 5 % respectively. This would indicate that the yield suppression may result from the resistance gene and/or its insertion in the soybean genome.

Glyphosate may also increase the susceptibility of crop plants to disease, as suggested by Kremer et al. (2000) who found that frequent use of glyphosate can increase the incidence of common rhizosphere fungi such as *Fusarium solani* which can become pathogenic on susceptible plants (there are occasional reports about wheat fields treated with glyphosate having higher levels of fusarium, too (www.mycoherbicide.net/NEWS/Scientists%20Link.htm). Njiti et al. (2003), however, found no significant effects of glyphosate on root infection by *Fusarium solani* but a significant relationship with soybean genotype. Assuming, that relatively little glyphosate enters the soil as it is applied over the top of growing soybean plants, it has been argued that pathogen defense responses in RR soybeans may be altered, perhaps in combination with unanticipated response to applications of glyphosate itself (Benbrook 2001).

Aromatic amino acid levels may be decreased in RR crops, as shown for phenylalanine and tyrosine in RR soybean and RR corn respectively, perhaps due to altered expression/activity of the bacterial EPSPS in transgenic plants under stress conditions (Benbrook 2001). These aromatic acids play an important role in many vital processes in plants, such as growth, secondary metabolism, and disease and stress response, e.g. in the cascade of reactions leading to systemic acquired resistance. Therefore, if aromatic acid levels in HR crops treated with glyphosate are decreased, this may result in a less effective plant defense mechanism to pathogens or abiotic stress (Benbrook 2001, 2005). Plants more vulnerable to pathogens and pests may show heavier disease symptoms and pest infestations, which, in turn, could lead to increased application of pesticides. In RR soybeans, unexpected pleiotropic effects have been observed leading to cracked stems in high soil temperatures. This has been linked to the heightened production of lignin, making the stalks more brittle and hence more likely to crack when dry (Coghlan 1999).

Metabolic pathways of glyphosate and glufosinate in transgenic HR plants can be different from pathways in non-transgenic plants, possibly varying with different transgenic crop species. In HR crops, these herbicides and their metabolites can accumulate in the course of repeated application. Reproductive tissues may accumulate glyphosate to higher concentrations than other tissues, as results gained with RR cotton indicate (Pline et al. 2001). Toxicity concerns might arise, if reproductive tissues accumulating glyphosate are used for food and feed or if they are consumed by wildlife. If these reproductive tissues are not as resistant to glyphosate as other tissue types because of differential EPSPS expression, the accumulation of glyphosate and inhibition of aromatic acids could potentially lead to damage in the reproductive tissues too. Contents of CP4-EPSPS in RR cotton stigma, anther, preanthesis floral bud, and flower petals have been shown to be significantly lower than in vegetative leaf tissue, an observation which might provide an explanation for reports of increased boll abortion and pollination problems in glyphosate treated RR cotton (Pline et al.

2002). Pollen viability has been reported to be 38 % to 51 % lower for glyphosate treated RR cotton compared to untreated RR cotton and seed set and boll retention can be significantly reduced when the pollen donor parent was glyphosate treated for the first two weeks of flowering (Pline et al. 2003a). Application of gibberellic acid (GA) known to restore male fertility in male sterile plants could increase boll retention or seed set only partially. Glyphosate treatment also delays growth of reproductive tissues and maturity and affects boll abscision in a manner that is probably different from effects of water stress (Pline et al. 2003b). Abscision increases as the amount of glyphosate translocated to fruiting sites of HR cotton increases (Viator et al. 2003). In glyphosate-resistant corn, glyphosate negatively affects pollen viability but not pollination and seed set (Thomas et al. 2004).

Direct effects of broad spectrum herbicide application on insects have hardly been studied. In greenhouse studies with glufosinate-resistant rice, Tindall et al. (2004) observed preferred oviposition of rice water weevils (*Lisorhoptrus oryzophilus*), a rice pest, on nontreated rice, compared with glufosinate-treated rice and the parent line. They also found a 20 % reduction of larvae on glufosinate-treated rice, compared with nontreated resistant rice. Since at recommended glufosinate use rates direct toxicity was not observed, they concluded that the reduction in rice water weevil densities may result from some kind of herbicide-induced plant resistance. In field tests similar effects were not detected, perhaps due to differences in time of rice flooding and use of insecticides.

Indirect effects

Weeds, apart from reducing yields of crop plants and leading to product contamination or harvest difficulty, exert considerable positive effects on the agricultural ecosystem too. Soil cover by weeds can help to decrease erosion by wind and water. Weeds and wild plants support a range of organisms, in particular arthropods, among them decomposers, predators, and parasitoids, providing food and shelter for them (Marshall et al. 2003). Introduction of the HR system for weed control will thus influence arthropod communities. Rapid changes in food supply could be especially relevant for organisms not adapted to rapid changes of their environment, including soil organisms. The reduced biodiversity could disturb symbiotic relations with mycorrhiza and beneficial organisms that help combatting pests (Müller et al. 1998). This in turn may increase the use of pesticides other than herbicides. Weeds can also play a significant role in management of insect pests, as a certain weed density promotes species diversity of arthropods in farmland, increases abundance of beneficial insects, and decreases crop infestation with insect pests (Stöppler-Zimmer 1994, Heitefuss et al. 1994, Schütte 1998a, Schütte & Schmitz 2001). Interaction with arthropod pests can be both positive and negative, but since most interactions are species specific and change when the species involved change, general conclusions about the role of weeds in arthropod pest management cannot be drawn (Norris & Kogan 2000).

The Farm Scale Evaluations and related studies

The impact of cultivation of HR crops on farmland biodiversity has been studied in the course of the UK Farm Scale Evaluations (FSE). The project was set out to test the null hypothesis that there is no difference between the management of HR varieties of beet, spring-sown and winter-sown oilseed rape, and maize, and their comparable conventional counterparts in their

effect on the abundance and diversity of arable plants and invertebrates. The more than 260 split-field sites of the three-year study were selected to represent the range of agricultural and environmental conditions that are likely to be encountered during large-scale cropping. Taxonomic and functional groups of plants and invertebrates identified to be sensitive to changes in field management have been sampled at fixed points within fields and on field margins starting before the crop was sown and continuing into following crops (Firbank et al. 2003, Squire et al. 2003), a baseline seedbank sample was taken before the treatments were imposed (Heard et al. 2003a). Herbicide inputs and crop management by the farmers were recorded, according to Champion et al. (2003), the greatest difference between conventional and HR systems was the later application of the herbicides in HR systems.

Earlier in the season weed plant densities were higher in HR beet and spring-sown oilseed rape half-fields than in conventional half-fields, but following application of glyphosate (sugar and fodder beet) and glufosinate (oilseed rape) in HR fields, plant densities were significantly lower (Heard et al. 2003a). Weed biomass and seed rain in HR crops were between one-third and one-sixth of those in conventional fields, the lower seed rain being a direct result of the elimination of larger individuals by the later herbicide sprays in these crops. The weed seedbank density in HR crops was about 20 % lower than in conventional treatment. Recently, similar findings have been reported from a Danish 3-year study. Compared to conventional beet, a denser and more diverse weed flora was found in glyphosate-resistant, but not yet treated, fodder beet in early and mid-summer. Following glyphosate application, however, the HR fields had fewer weed species and lower weed densities, biomass, and seedrain (Strandberg et al. 2005). Application of glyphosate earlier than recommended resulted in extremely low weed diversity, density and biomass during the entire season. Strandberg et al. concluded that timing of the first glyphosate application was essential in terms of biodiversity effects.

Of the 12 weed species that had been selected in the FSE because they are among the most frequent and abundant weeds, not all reacted in a similar manner (Heard et al. 2003b).In general, dicotyledons seemed to be more affected by glufosinate and monocotyledons seemed to be more susceptible to glyphosate. Although in the short term, seedbank declines may be buffered by the relatively large size of the existing seedban, small differences in seed rain could sum up and accelerate species decline if they were sustained over several crop rotations (Heard et al. 2003a,b). The reduced weed seed production observed after effective weed control in HR fodder beet can also shift the flora towards species less sensitive to glyphosate (Strandberg et al. 2005).

The results of the winter-sown oilseed rape trials were in part similar to the results for the spring-sown oilseed rape and beet crops, but some differences were revealed too. There were no treatment effects on total weed biomass, but in glufosinate tolerant winter-sown oilseed rape significantly fewer dicot weeds and significantly more monocot weeds were found, compared to the conventional treatment (Bohan et al. 2005). On average, in HR oilseed rape, dicot weed biomass amounted only to 36 % of dicot weed biomass in conventional crops, whereas monocot weed biomass was nearly three times greater than in conventional fields. Similar results were found with seed rain. Field boundaries were not significantly affected.

Recent reports confirm that the differences in the weed seedbank persist in the following years (Black 2005).

In contrast to the results from HR spring-sown and winter-sown oilseed rape and beet, in glufosinate-resistant HR maize fields weed density was three times higher and biomass and seed rain were about 80 % higher than in conventional treatment, but as the total weed seed return was low in both treatments, the seedbank was little affected (Heard et al. 2003a). These differing results in HR maize seemed to be due mainly to the broad application of atrazine in conventional maize, a residual herbicide that has been banned in 2003 by the European Union because of its toxicity and persistence. Critics, therefore, demanded that the HR maize trials should be repeated using for conventional half-field comparison herbicides that are still allowed in the EU.

The different weed densities and biomasses observed in HR crops in turn affected invertebrates: Weed seed feeders among the surface-active invertebrates, such as certain carabids, tended to have smaller counts in HR beet and spring-sown oilseed rape, but higher counts in HR maize (Brooks et al. 2003). Omnivorous species and generalist and highly mobile predators were less affected. In all HR crops, the detritivore springtails (*Collembola*) were significantly more abundant (Brooks et al. 2003, Haughton et al. 2003, Bohan et al. 2005). This may confirm studies with HR soybean performed by Bitzer et al (2002), showing that glyphosate and glufosinate application did not result in deleterious short-term effects on the abundance of 21 springtail species. According to Brooks et al. (2003), the higher abundance of Collembola in the FSE ist most likely the result of more efficient control of initially greater weed vegetation densities by the broad spectrum herbicides which leads to additional detritus. But the authors discuss also, that if the successive use of HR crops leads to long-term decline in the abundance of weed vegetation, there would be less plant biomass to produce detritus. Long-term effects on Collembola, therefore, remain unclear.

Epigeal and aerial arthropods also showed different responses to conventional and HR crop management (Haugthon et al. 2003): Over the whole season, numbers of butterflies were 27 % and 22 % smaller in HR beet and spring-sown oilseed rape crops, respectively. Considerung that butterfly density was highest in oilseed rape (about 16 times the density in beet), the 22 % reduction appears severe. In HR beet, Heteroptera and bee numbers were significantly smaller than in conventional fields and in HR oilseed rape, spider numbers were lower. In contrast to the results with HR spring-sown crops, in winter-sown oilseed rape, the majority of invertebrate taxa did not respond to the different herbicide treatment, pollinator numbers, however, were significantly lower in HR oilseed rape, particularly in July (Bohan et al. 2005). These results indicate that weed plants within fields, in particular dicot species, are of particular importance for butterflies and bees by providing forage and for spiders by providing structural diversity.

Field margins can support a high diversity of plant species within the farmed landscapes and provide habitat and food resource for numerous invertebrates, birds and mammals. They may be the only source of nectar and pollen in arable landscapes through much of the season. For this reason, in the FSE project Roy et al. (2003) studied the effects of HR cropping on vegetation and invertebrates of field margins. In HR spring oilseed rape fields, mean plant cover, flowering and seed set were 25 %, 44 % and 39 % lower, respectively, in the

uncropped tilled margins. For HR beet, flowering and seed set in tilled margins were reduced by 34 % and 39 % respectively. These vegetation effects were mirrored in significant effects on butterflies, leading to 24 % and 18 % fewer butterflies in margins of HR spring oilseed rape and HR beet, respectively. Roy et al. assumed that the lower nectar supply in HR crop margins was the likely cause. Although for bees and other invertebrates some treatment differences were observed too, butterflies appeared to be particularly sensitive to vegetation changes. Again, the effects differed for maize, with increased plant cover (28 %) and more flowering (67 %) on HR maize margins, butterfly numbers, however, remained the same. Sampling of phytophagous, detritivorous, predatory, and parasitoid insects revealed that the invertebrate trophic groups were differently affected by contrasting herbicide regimes (Hawes et al. 2003).

The change in resource availability had knock-on effects on higher trophic levels in most cases. Herbivores, pollinators and natural enemies changed in abundance in the same directions as their resources, and detritivores such as Collembola increased in abundance under HR cropping. Later herbicide application in HR crops shifted the resources from the herbivore food web to the detritivore food web. The extent and direction of these effects would be dependent on the relative efficacy of the respective herbicide regimes and the degree of buffering provided by immigration of species from surrounding areas. Response of arthropod abundance and diversity to changes in herbicide application was also shown in the Danish study, although other factors such as microclimatic conditions, management history, field size, surrounding habitats, and, of course, insecticide use were important too (Strandberg et al. 2005). Similarly, in RR maize, increased weediness increased the acitivity-density of omnivores such as crickets and a common beetle that feed also on weed seeds, whereas other ground-dwelling arthropods such as Wolf spiders showed variable responses to weediness (Hough-Goldstein et al. 2004). A within-season indirect effect of glyphosate application has been reported for a non-target spider species that requires the support of plants to suspend its webs. Compared to controls, spider abundance in field margins was significantly lower in all different glyphosate treatments, the various doses simulating direct applications and drift (Haughton et al. 2001).

Pesticides are often transported beyond cropfields and can show considerable impacts on terrestric and aquatic ecosystems or on plant populations in the vicinity of crop fields. Glyphosate spray drift can damage adjacent non-HR crop plants and lead to shikimic acid accumulation, plant injury, and yield reductions at rates as low as 70 – 140 g/ha representing 6,25 – 12.5 % of the commercial use rate (Thomas et al. 2005). Similarly, wild plants on field margins, hedgerows, and trees growing close to arable fields may be affected (de Snoo & van der Poll 1999, Sweet 1999). Field margins and hedgerows are very important refugia for biodiversity, as they are habitats for arthropods, small mammals, and birds for food, shelter, and nesting (Marshall & Moonen 2002). As a result of spray drift, species abundance and diversity can change, since plants more tolerant to glyphosate and glufosinate will be favoured, in particular, as this selection pressure is exerted on a regular basis year after year and possibly more than once a year. If herbicides are carried to nearby forest areas, they can also damage trees and reduce bryophyte and lichen biodiversity. In a multi-year study it was shown that glyphosate application decreased abundance and species richness of bryophytes

and lichens to considerable extent, only at 4 years post treatment bryophyte diversity was beginning to recover (Newmaster et al. 1999).

It has been argued that the shift from pre-emergence to post-emergence herbicides will conserve the habitats of beneficial organisms for a longer period and thus reduce the impact on biodiversity, compared to conventional herbicides. But since glyphosate and glufosinate are most effective on weeds at their early stages of development and because lower doses are needed to control weeds, these herbicides, in fact, are recommended to be applied at comparatively early stages of weed development. Moreover, due to increasing problems with herbicide tolerant weeds or weed shifts, applications seem to be repeated (cf section on resistance in weeds). In soybean, early application of glyphosate may also have an adverse effect on crop plant growth, in particular, under limited water supply, as nitrogen fixation by the symbiont *Bradyrhizobium japonicum* can be delayed by early herbicide application (King et al. 2001).

A decline of populations of many groups of organisms associated with farmland in Europe has been observed in the past fifty years, with particularly marked declines amongst habitat specialists (Robinson & Sutherland 2002, Squire et al. 2003). Hares, a once abundant but now endangered species in many regions, depend on a diverse food supply with many different plant and weed species (Werner et al. 1999). Birds are major targets and indicators of agricultural change (DETR 1999, DETR 2000, Ormerod & Watkinson 2000, Chamberlain et al. 2002). Analysis of multi-year data from 1962 to 1995 indicate strong correlations between agricultural change and the onset of farmland bird population decline, with an observed time lag of about 6 years, implying that effects of change in habitat quality may not become apparent for several years (Chamberlain et al. 2000). Loss of weeds will show an impact on birds not only because of reduced abundance and diversity of arthropods as food source but also directly by a decline in weed seeds available for food. Based on a model of the population dynamics of lambsquarters/fat hen (*Chenopodium album*) in sugar beet with a decline rate for the seedbank of 20 % per annum, a value confirmed later in the FSE (Heard et al. 2003a,b), Watkinson et al. (2000) attempted to predict the change in plant and seed bank numbers and the resulting food supply for farmland birds such as the skylark (*Alauda arvensis*) if HR crops are grown. According to the model, the socioeconomic reaction of farmers to the new technology, i.e. speed and extent of adaptation of the HR technology, plays an important role with regard to the impacts of transgenic HR crops on weed abundance and hence on bird populations. The regional-scale consequences of farm-level decisions and the socioeconomic reaction to the new technology might be the key to predict the impacts of such herbicide-resistant crops on biodiversity (Werner et al. 1999, Watkinson et al. 2000, Firbank and Forcella 2000). From Argentina it can be learned that extension of HR systems, so popular because of their convenience, into areas that had not been cropped before endangers forests and ecosystems rich in biodiversity (Benbrook 2005, Pengue 2004).

In summary, the HR technology can exert considerable impact on farmland biodiversity, depending in part on the degree of adoption. Most adverse impacts, however, might be caused by the use of broad-spectrum herbicides and less so by the cultivation of the HR plants as such. Despite the broad deployment of HR technology in a number of countries, comprising large proportions of soybean, corn, oilseed rape, and cotton areas, there are still considerable

knowledge gaps regarding potential toxicity of the herbicides used and environmental effects exerted. In particular, studies should aim to elucidate in detail metabolites of herbicides in transgenic plants and potential species-specific deviations of metabolism, to clarify potential impacts of HR technology on soil erosion, and to analyze toxicity and impacts of HR plants and broad-spectrum herbicides on algae, both pathogenic and symbiontic microorganisms and soil organisms under agronomic conditions (Schütte & Schmitz 2001).

5.3 Pest resistance

5.3.1 Bt toxins

In 2005, the second most dominant trait expressed in transgenic crops grown worldwide, mainly corn and cotton, was insect resistance. Of the 90 million hectares of transgenic plant, the area planted with insect resistant plants and with plants expressing both herbicide resistance and insect resistance comprised 18 % and 11 %, respectively (James 2006). All of the insect-resistant transgenic plants commercialized to date express toxin genes derived from various strains of the ubiquitous soil bacterium *Bacillus thuringiensis* (Bt). During sporulation, *B. thuringiensis* strains produce insecticidal protein crystals, also called delta-endotoxins that are, in general, specific to different insect orders such as lepidoptera, diptera, and coleoptera, and some to non-insect species like nematodes (Müller 2001, Wei et al. 2003). A few thousand strains of this bacterium, isolated from various media and from a range of geographic regions, have been described, not all of them being toxic to insects. The endotoxins constitute a family of related proteins, grouped according to their action and molecular homology (de Maagd et al. 2001), over 140 genes have been described (Crickmore et al. 1998, Hilder & Boulter 1999). They are produced by the bacteria as protoxins which are solubilized and activated by proteinases in the alkaline insect midgut. In susceptible insects the toxins bind to receptors of the midgut epithelium, the resulting increased membrane permeability leads to swelling and lysis of the cells. These receptors play a crucial role in determining susceptibility/resistance to a particular Bt toxin (de Maagd et al. 2001). Spores and protein crystals of several strains have been used as microbial insecticides since the 1940s/1950s (Schuler et al. 1999, Müller 2001).

Bt plants express the partially activated toxins, most often under the control of the CaMV 35S promoter, that confer resistance to a range of lepidopterous and coleopterous pest species. Engineering the codon usage to adapt it to plant-preferred codon usage increased expression levels of Bt genes considerably (Hilder & Boulter 1999). However, expression and efficacy of Bt toxins can vary significantly in corn varieties that carry the same transformation event, depending on the hybrid into which the Bt gene was inserted (Castro et al. 2004). Bt corn and Bt cotton express Cry1A, specific to lepidopteran larvae such as those of the European corn borer (*Ostrinia nubilalis*) and the cotton boll worm (*Heliothis zea*), respectively, whereas Bt-potato expresses Cry3A, toxic to coleopteran larvae, e.g. the Colorado potato beetle (*Leptinotarsa decemlineata*). In addition to corn, cotton, and potato, over 50 different plant species have been transformed to express Bt toxins, among them tobacco, rice, oilseed rape, alfalfa, apple, poplar, grapevine (reviewed by Jouanin et al. 1998, Schütte & Riede 1998,

Hilder & Boulter 1999, de Maagd et al. 1999, Schuler et al. 1999, Hütter et al. 2000, Schmitz & Schütte 2001c). As a strategy to improve duration of insect resistance, the fusion of two Bt toxin genes has been suggested (Tu et al. 2000), corn co-expressing two Bt toxins was shown to be protected from corn rootworms (Moellenbeck et al. 2001). Recently, Mehlo et al. (2005) proposed to engineer crops with a fusion protein combining the Bt-toxin Cry1Ac with the carbohydrate-binding domain of the ricin B-chain (RB), a lectin that binds galactose- and N-acetylgalactosamine with high affinity and plays a role in delivery of the highly toxic A-chain of ricin. Maize and rice plants transformed with the corresponding hybrid BtRB gene were resistant not only to stemborers but also to insects known to be tolerant to this Bt toxin, such as cotton leaf worm and leafhoppers. High Bt toxin expression in tobacco chloroplasts, amounting to 46 % of total protein content in mature leaves, has been reported (DeCosa et al. 2001). Bt may also show toxicity to plant-parasitic nematodes such as the cyst nematodes of the genera *Heterodera* and *Globodera* and the root-knot nematodes of the genus *Meloidogyne* (Jung et al. 1998, Wei et al. 2003). By minimizing damage from certain insects, Bt toxin expression in corn is expected to reduce infestation by *Fusarium*. However, Fusarium ear rot severity and mycotoxin (fumonisin) concentration in grain seems to be highly dependent on hybrid variety and to a lesser extent on the expression of Bt toxins (Magg et al. 2002, Clements et al. 2003).

5.3.2 Pest resistane other than Bt traits

To control insects not susceptible to Bt toxins or to pyramide insect resistance genes, genes coding for other insecticidal proteins such as proteinase inhibitors have been used. Proteinase inhibitors are polypeptides or proteins that occur naturally in a wide range of organisms, including plants, in particular, in seeds and storage tissues. They can be induced by wounding and insect attack and are part of a plant‘s natural defense system against herbivory, acting primarily as growth retardants. According to their specificity, proteinase inhibitors can be divided into four different classes, inhibiting serine, cysteine, metallo-, and aspartyl-proteases of insects. Cowpea trypsin inhibitor (CpTI) is a particularly effective proteinase inhibitor, affecting a wide range of lepidopteran and coleopteran species. Genes for different plant proteinase inhibitors, including the genes for CpTI, chicken egg white cystatin, rice cystatin, and the soybean (Kunitz) trypsin inhibitor, directed against Lepidoptera, Coleoptera, and Orthoptera, and also nematodes have been introduced into plants such as tobacco, oilseed rape, potato, tomato, rice, poplar, cotton, alfalfa, pea, and others (McManus et al. 1999, reviewed by Jouanin et al. 1998, Baghi & Mantke 1999, Hilder & Boulter 1999, Schuler et al. 1999). The expression of cysteine proteinase inhibitors (cystatins) may also confer virus resistance (Gutierrez-Campos et al. 1999) or prevent female nematodes from developing properly (Jung et al. 1998). Chicken egg white cystatin or rice cystatin inhibit not only digestive cysteine proteinases of insects, but also of some plant-parasitic nematodes (Cowgill et al. 2002a,b). Proteinase inhibitors can confer broad spectrum resistance to different insect target species (Hütter et al. 2000). But insects may be flexible enough to adapt to specific proteinase inhibitors expressed in transgenic plants and switch proteinase composition in their guts (Estruch et al. 1997). Fusion of soya cystatin and a legume lectin has been reported to delay development of the cowpea bruchid beetle (*Callosobruchus maculatus*) in a synergistic

manner, whereas a mixture of the separate proteins at the same dosage level showed only an additive effect (Zhu-Salzman et al. 2003).

The carbohydrate metabolism of insect pest species might be targeted by the expression of proteins inhibiting alpha-amylase. Genes encoding such inhibitors, derived from wheat and common bean, have been transferred to tobacco, Azuki bean, and pea (Jounanin et al. 1998, Hilder & Boulter 1999, Schuler et al. 1999, Prescott et al. 2005). Alpha-amylase inhibitors may also inhibit mammalian alpha-amylase and may confer resistance to nematodes too (Hütter et al. 2000).

Lectins are carbohydrate-binding proteins found in many plant tissues, particularly in seeds and storage tissues. They can be toxic to insects of the orders Homoptera, Coleoptera, Lepidoptera, and Diptera, some are also toxic to mammals. Many of them provide antinutrient properties in animal food. The snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) specifically binds to α-D-mannose and is variably toxic to insects, including Homoptera, Coleoptera, and Lepidoptera, but not to mammals (Bernal & Setamou 2003). Plants such as potato, tobacco, oilseed rape, and tomato have been transformed to express GNA, showing mixed results regarding resistance, however (Jouanin et al. 1998, Hilder & Boulter 1999, Schuler et al. 1999). Lectins seem to afford good insecticidal properties only when insects are exposed to lectin amounts in their diet that are far higher than Bt toxin concentrations (Estruch et al. 1997). GNA-expressing potato plants have been reported to facilitate biocontrol of tomato moth caterpillars by the parasitoid wasp *Eulophus pennicornis* (Bell et al. 2001). Expression of GNA in potato may also reduce susceptibility to nematode pests, but the level of GNA could be critical, as higher GNA expression resulted in increased numbers of nematode females (Jung et al. 1998).

Since insects contain chitin not only as an exoskeleton material but also in peritrophic membranes, chitinase activity could interfere with digestion and development of insects. As chitinase expression in insects is tightly controlled with developmental stages, constitutive expression of insect chitinases in transgenic plants may be detrimental to insect growth, because chitinase would be presented in the diet at an inappropriate time (Ding et al. 1998). In general, expression of plant chitinases in transgenic plants has not resulted in significantly increased protection from herbivory (Hilder & Boulter 1999, Schuler et al. 1999). Because of continuous regeneration of the peritrophic membrane, it might take large amounts of chitinases to effectively control target insects (Estruch et al. 1997). Ding and colleagues transferred a gene encoding the tobacco hornworm (*Manduca sexta*) chitinase to tobacco and observed reduced feeding damage and reduced growth of tobacco budworm (*Heliothis virescens*) larvae feeding on these plants. Tobacco hornworm larvae, however, were not affected. Chitinase seems to act synergistically to Bt and GNA (Hütter et al. 2000).

Secondary metabolites play an important role in protecting the plant from animal predation and pathogens. Alkaloids can interfere with insect digestion of carbohydrates or lead to reduced growth, terpenoids can act as deterrents or as neurotoxins (Franck-Oberaspach & Keller 1996, Kutchan 1997). The alkaloid tryptamine produced in transgenic tobacco, transformed with a gene encoding tryptophan decarboxylase, seems to act as antifeedant or developmental inhibitor to insect pests (Jouanin et al. 1998, Schuler et al. 1999). Increased release of the natural product cembatriene-ol, exuded from trichomes, enhanced aphid

resistance in transgenic tobacco, whose P450 hydroxylase gene was suppressed by either co- or antisense-suppression (Wang, E. et al. 2001). But because of the complexity of secondary metabolism and its interrelation with other important biosynthetic pathways, alterations of secondary metabolism could result in a range of unexpected effects.

Insect growth might also be inhibited by the introduction of viral genes such as enhancin genes isolated from insect baculoviruses. Enhancin genes encode metalloproteases that can disrupt the peritrophic membrane in the midguts of insects and thus enhance viral infection. Cao et al. (2002) suggested to express enhancin genes in transgenic plants, either alone or in combination with Bt proteins or other insect control measures. Though their tobacco transformants expressed the nptII marker gene as expected, the enhancin genes under the control of the 35S CaMV promotor exhibited only very low expression, potentially due to cryptic signals and codons unfavorable to plant expression. A few transgenic tobacco lines nevertheless could slow growth and development of *Trichoplusia ni* larvae. In vitro findings suggest that transgenic plants producing both enhancin and Bt proteins may exhibit synergistic activity against insect attack. However, such enhanced insect resistance of crop plants could extend its effects to nontarget species, since enhancin activity may not be specific only to the target species.

To increase insect resistance of crop plants or to expand the insecticidal spectrum, the combination of genes encoding different resistance proteins such as Bt, proteinase inhibitors, and alpha-amylase inhibitors has been proposed (Hütter et al. 2000), as well as the transfer of animal or bacterial genes (other than delta-endotoxin genes). This includes bacterial genes encoding cholesterol-oxidase (that oxidizes cholesterol of insect midgut membranes), isopentenyl-transferase, or vegetative insecticidal proteins derived from *Bacillus spp.*, and genes that encode animal proteinase inhibitors or spider toxins (Estruch et al. 1997, Jouanin et al. 1998, Hilder & Boulter 1999, Schuler et al. 1999, Hütter et al. 2000). Corbin et al (2001) reported that targeting of cholesterol-oxidase to chloroplasts led to phenotypically and developmentally normal tobacco, whereas expression of cholesterol-oxidase in the cytosol resulted in severe developmental aberrations, presumably because of its effect on plant sterol distribution and metabolism.

The glycoprotein avidin found in chicken egg white, sequesters the vitamin biotin. When expressed in transgenic corn, avidin is toxic to store pests and prevents their development. Toxicity seems to be due to biotin deficiency as shown by prevention of toxicity with biotin supplementation (Kramer et al. 2000). As biotin-binding proteins may be plant toxic, avidin expressing plants might not be viable unless the glycoprotein was kept separate from the plant‘s biotin stores and, for instance, targeted to vacuoles. Expression and targeting of the biotin-binding proteins avidin (and streptavidin from *Streptomyces avidinii*) to vacuoles of transgenic tobacco has been reported, leading to increased resistance to noctuid pests *Helicoverpa armigera* (cotton bollworm) and *Spodoptera litura* (cotton leafworm) (Burgess et al. 2002, Murray et al. 2002). Synergistic effects between avidin-expressing tobacco plants and a purified Bt Cry1Ba toxin have been observed. However, since avidin is also toxic to humans and animals, expression in crops destined for food and feed may lead to severe safety concerns.

5.3.3 Environmental impacts of pest resistance traits

Bt crops

Potential impacts on non-target organisms exerted by pest resistant crops, in particular Bt crops, are discussed widely. *Bacillus thuringiensis* formulations, consisting of bacterial or toxin preparations and known to be specific for insect species, have been used in agriculture for decades. These sprayed insecticides decay rapidly reducing potential effects on non-target organisms. Nevertheless, research has shown that *B. thuringiensis* formulations can have negative effects on non-target insects and natural enemy species too (Scriber 2001, Obrycki et al. 2001, Schmitz & Schütte 2001, Deml & Dettner 1998).

From the use of these formulations it has been inferred that transgenic crops expressing Bt toxins should be a comparatively safe and efficient means to combat pest species. It has been argued that transgenic Bt crops, most expressing a single Bt toxin that is specific to lepidoptera or coleoptera, may act more specifically than some of the *B. thuringiensis* formulae containing more than one Bt protoxin crystals (Schmitz & Schütte 2001c) and that problems with spray drift could be avoided.

But transgenic plants expressing Bt toxins and *B. thuringiensis* sprays differ in a number of aspects. Because of highly variable spray coverage and rapid breakdown under field conditions, both target and non-target insects encounter the *B. thuringiensis* proteins of sprays for only a short time. This is in contrast to the transgenic crops which express the Bt toxins throughout their life cycle, thereby altering the mode of exposure to target and nontarget insects. Herbivores colonizing Bt plants and predators feeding on them will be exposed to these toxins for prolonged periods and altered doses. For the sprayed insecticide to become functioning, the protoxin protein crystals have to be solubilized and activated by proteinases in the alkaline insect midgut. The activated toxins then bind to receptors of the midgut epithelium leading to increased membrane permeability and lysis of the cells. Receptors and gut proteinases seem to play key roles in determining the specificity of particular *B. thuringiensis* proteins to insect species (de Maagd et al. 2001, Müller 2001, Schmitz & Schütte 2001c, Hilder & Boulter 1999). Most Bt plants, on the other hand, express truncated, activated toxins, thus no crystal solubilization and almost no conversion from protoxin to the active toxin is necessary within the insect gut. Therefore, selectivity of Bt toxins expressed in transgenic plants cannot simply be deduced from the long record of comparatively safe use of *B. thuringiensis* insecticides. In addition, toxicity of Bt proteins in transgenic plants may be altered by plant compounds such as tannins that seem to interact with Bt toxins increasing or decreasing their toxicity to insect species. Interactions between Bt proteins and plant proteases, environmental stress or the physiological changes associated with the reduction of vegetative growth after fruiting may also influence toxicity of Bt proteins (Olsen & Daly 2000).

Bt toxin levels in corn leaves have been reported to be correlated to whole plant nitrogen concentrations, therefore, nitrogen fertility levels could influence resistance levels (Bruns & Abel 2003) and non-target impacts. Nitrogen metabolism seems to be altered in Bt crops with negative effects on yield, as studies with Bt cotton and Bt corn indicate. Bt cotton cultivars had higher nitrogen content in leaves and lower nitrogen content in bolls, resulting in

excessive vegetative and reduced reproductive growth, compared to non-Bt parent lines (Chen et al. 2005). Similarly, most Bt corn hybrids tested under natural corn borer infestation had lower nitrogen content in grain, took 2-3 additional days to reach silking and maturity and produced generally lower grain yields (up to 12 % less) and grain with higher moisture, compared to their non-Bt counterparts (Ma & Subedi 2005). Therefore, under low to moderate pest infestation, the protection of Bt crops from pest damage seems not to be realized through increased yield. Interestingly, Bt corn hybrids have a significantly higher lignin content, compared to their respective non-Bt isolines, with the Bt11 hybrid showing particularly high values (Stotzky 2000, Saxena & Stotzky 2001a). Increased lignin content might be due to a pleiotropic effect of the transgene insertion.

There is a range of laboratory and field studies dealing with non-target effects of transgenic Bt plants, sometimes using microbially derived Bt toxins (reviewed by Clark et al 2005, Lang et al. 2005, Obrycki et al. 2001a, Schmitz & Schütte 2001, Hütter et al. 2000, Schuler et al. 1999, Schütte & Riede 1998, Deml & Dettner 1998). Although government regulations often require assessment of non-target effects prior to commercial sale and widespread planting of transgenic crops, results of company laboratory or field studies have rarely been provided to the public or published in detail (Hilbeck et al. 2000). Since transformation can lead to unexpected effects such as position and pleiotropic effects in the transgenic plants, it will be essential to conduct tests not with microbially derived Bt toxins, as has been done quite often, but with transgenic plant material directly (Hilbeck & Meier 2002).

Bt toxins are expressed in green tissue of transgenic plants, but, depending on the promoter used, also in pollen, anther tissue, kernels and roots, leading to different routes through which non-target organisms can be exposed to these toxic proteins. Non-target organisms may be natural enemies of pest species such as predators and parasitoids or insect species that collect and ingest pollen or consume plant material dusted with Bt pollen or other Bt plant material. Even herbivores not susceptible to the Bt toxin expressed may pass on toxins they have ingested with Bt plant material to their natural enemies in a more or less processed form (Hilbeck et al. 1998a,b). Natural enemies could not only be affected by toxin activity passed on to them but also by sick or dying prey suffering from toxic plant material. Parasitoids inside dead lepidopteran larvae exposed to Bt usually will suffer the same fate (Agrawal 2000). If herbivores receive sublethal doses of Bt toxin, their development will be delayed and weight gain be reduced. Tri-trophic interactions between plant, herbivores and their natural enemies may be affected in additive, synergistic, or antagonistic ways. The impact of Bt pollen on wild bees remains largely unknown. Potential trophic-level effects of Bt crops on vertebrate predators that prey on lepidopteran pests should also be considered.

If lepidoptera, their predators and parasitoids are significantly reduced in Bt cropfields and adjacent margins, insect prey for birds, small mammals and amphibians might be reduced too (Obrycki et al. 2001a). These authors call for long-term studies to determine whether the widespread planting of transgenic crops creates an “ecological desert” with relatively few hosts for natural enemies, a pattern, that has been observed following the overuse of insecticides or regional planting of highly resistant crop varieties. Performance and fitness of natural enemies of pest species in Bt crop fields may also affect development of pest resistance and of secondary pests that may increase in number after the main pest species have

been targeted with Bt toxins (Saxena et al. 2002, Obrycki et al. 2001a, Schuler et al. 1999, Hilbeck et al. 1998).

Exposition to Bt toxins in pollen and plant material

Pollinators and other insects feeding on pollen may be exposed to Bt toxins expressed in pollen. They need not be damaged directly, but bees fed with Bt corn pollen may show, due to sublethal effects, enhanced sensitivity to parasites resulting in a reduction of bee numbers and bees raised, compared to bees fed with non-Bt pollen (http://www.biosicherheit.de/projekte/68.proj.html). Ingesting Bt corn pollen by adult parasitic wasps does not seem to affect their ability to parasitise corn borer larvae (http://www.biosicherheit.de/features/printversion.php?contsxt=1&id=15).

Insect herbivores other than pest species can potentially be affected by Bt expressing crops as has been shown in the laboratory for the American monarch butterfly (*Danaus plexippus*). Monarch larvae reared on milkweed leaves (*Asclepias syriaca*), their primary host plant, dusted with Bt corn pollen, suffered significantly higher mortality and grew significantly slower, compared to growth on leaves not dusted or dusted with non-Bt corn pollen (Losey et al. 1999). After this initial study several additional studies have been performed to determine whether such an effect would also relate to the field situation. It has been shown that transgenic corn pollen is naturally deposited on *A. syriaca* leaves within and adjacent to transgenic corn fields and that feeding leaf samples with 135 transgenic pollen grains/cm² to monarch larvae increased their mortality (Jesse & Obrycki 2001). Naturally occurring pollen densities on milkweed leaves have been found to be highest inside the cornfields (average 170.6 grains/cm²) falling to 14.2 grains/ cm² at 2 m outside the cornfield, highly depending on rainfall events. Inside cornfields, 95 % of leaf samples had pollen densities below 600 grains/cm², the highest value was 1400 grains/cm² (Pleasants et al. 2001). In Bavaria, average corn pollen densities on wild carrot (*Daucus carota*), an important food plant for butterflies such as swallowtail, ranged from 7.1 – 93 grains per cm^2 leaf area and 24 hours, representing from 12.4 – 63.5 % of total corn pollen reaching corn field edges (Lang et al. 2005). Total pollen densities decreased with distance, but they still could reach values up to about 90 grains/cm^2 in a distance of 10 m. According to Scriber (2001), there is a lack of data regarding the persistence of toxicity of Bt pollen (and anthers) in the soil or aquatic systems after pollen was washed off by rain.

Monarch females seem to prefer to lay eggs on a plant without pollen, but they did not show preference between Bt corn pollen and non-Bt pollen dusted on milkweed leaves (Tschenn et al. 2001). Therefore, monarch oviposition behavior will not eliminate the potential for larval exposure to corn pollen. Obrycki et al. (2001) suggested to collect data about plant species likely to be found near corn and about lepidopteran species feeding on these and related plant species, in particular, during or after the period when corn pollen is shed. Integrating distribution, phenology, and susceptibility (perhaps linked to phylogeny) might permit a ranking of the risk to specific lepidopteran species with species at particularly high risk to be identified for further testing. In Germany, analysing butterflies occurring close to corn fields showed that out of 33 day-flying and of 125 night-flying lepidopteran species 26 and 52 species may be endangered, respectively (http://biosicherheit.de/projekte/136.proj.html). Recent studies in Bavaria identified 36 day-flying lepidopteran species close to corn fields,

with 28 % of them belonging to endangered species in Germany (Lang et al. 2005). Interestingly, 47 % and 33 % of the localities of two butterfly species more closely studied, swallowtail (*Papilio machaon*) and garden tiger (*Arctia caja*), respectively, are expected to belong to agroecosystems.

The impact of corn pollen expressing Bt toxin Cry1Ab on non-target butterflies is also dependent on the transgenic event and variety, as toxin amounts in pollen vary considerably between transgenic events and varieties, reportedly ranging from 0.002 – 0.09 µg/g pollen (MON810) to 1.1 - 2.9 µg/g pollen in event 176 (Lang et al. 2005). In MON810 pollen, however, Lang et al. found significantly higher toxin levels with average values of 0.13 – 0.25 µg/g pollen. Monarch larvae exposed to low doses (22 grains/cm²] of Novartis/Syngenta event-176 pollen gained less weight than those exposed to Bt11 or Mon810 pollen, exposure to 67 pollen grains/cm² of event-176 resulted in significant reductions of survivorship and weight gain (Stanley-Horn et al. 2001). These results may be explained by the high expression level of Cry1Ab in event-176 pollen compared to Bt11 and Mon810 pollen. First instars of monarchs and other butterflies are generally more sensitive to Bt toxins Cry1Ab and Cry1Ac than older instars (Hellmich et al. 2001, Lang et al. 2005). First instar larvae of swallowtail (*Papilio machaon*) that ate within 48 hours as few as 16 grains of event-176 pollen, dusted on parsnip leaves and equivalent to about 75 grains/cm^2, showed significantly reduced survival, reduced body weight, and prolonged development, with more severe effects the more pollen grains they ate (Lang et al. 2005). Such pollen densities on host plants (7 – 93 grains per cm^2 and day) have been found in the field. Event-176 pollen also affected growth rates and larval masses of black swallowtail (*Papilio polyxenes*) and of pale grass blue (*Pseudozizeeria maha*) butterfly larvae significantly, if they had been exposed to low densities of event-176 pollen grains on their host plants, weedy plants frequently found in or around cornfields throughout the US-Midwest (Zangerl et al. 2001) or to densities of more than 20 grains/cm^2 (Shirai & Takahashi 2005). As rainfalls during field experiments reduced pollen grains on host plant leaves considerably, the potential effect of Bt event-176 pollen could be more severe under different environmental conditions. There is considerable temporal and spatial overlap between larval development of monarch and swallowtail butterflies and corn pollen deposition on host plants in and around cornfields (Oberhauser et al. 2001, Lang et al. 2005). Feeding of event-176 pollen to butterflies occurring in Germany such as peacock butterfly, large and small white, cabbage moth, and cutworm resulted also, depending on number of pollen grains, in increased mortality, slower growth, lower weight, and in part alterations of behavior in all butterflies except cutworm (http://biosicherheit.de/projekte/136.proj.html).

Although monarch larvae hatching at the onset of anthesis may be exposed to Bt pollen for periods of 12 days or more, most feeding studies examined acute and sublethal effects after two to five days of exposure. Potential sub-lethal effects due to long-term exposure, e.g. on reproductive fitness and migration ability, have, therefore, not been assessed in these studies, To study continuous exposure in the field and in the laboratory, Dively et al (2004) fed first instar monarch larvae with milkweed leaves that had natural deposits of Bt corn pollen in the range of 122 to 188 grains/cm^2/day. Mean survival to eclosion of first instars continuously exposed to Bt11 and MON810 at three to four days after initial anthesis was 23 % and 27 % lower, respectively, compared to control larvae. Exposure to Bt11 and MON810 at six to seven days reduced survival by 18 % and 25 %, respectively. In addition, continuous exposure

to Bt pollen during larval development prolonged developmental time by 1.8 days and produced consistently smaller pupae and reduced adult weight by an average of 7.9 %, again, MON810 showed stronger effects than Bt11. However, simulating the proportion of monarch butterflies in the Bt corn producing states in the US and Canada that are exposed to 50 % or more of the shed Bt pollen, the authors concluded that only a minor fraction of the breeding population of monarch butterflies will be exposed to harmful Bt corn pollen levels. Based on this consideration, Dively et al. (2004) argued that the sustainability of monarch butterfly populations would not be affected by Bt corn cultivation. Similarly, considering that event-176 corn comprised only a small proportion of Bt corn (~ 2 %), Hellmich et al. (2001) and Scriber (2001) had argued that only few butterfly larvae would have been exposed to critical lethal dosages of Bt toxin

Since in green tissue of Bt corn hybrids Bt11 and Mon810, the Cry1Ab proteins are expressed at levels more than 100 times that of their pollen (Hellmich et al. 2001), plant anther material has considerably higher concentrations of Bt toxin. In feeding studies, Bt pollen of these hybrids "contaminated" with anther material dramatically reduced weight gain and showed deleterious effects on survival of monarch larvae, compared to sifted pollen (Scriber 2001, Hellmich et al. 2001, Obrycki et al. 2001b). But it was not clear, whether the different instars of monarch (and other) butterfly larvae will be exposed to anthers, or pieces of them, in the field. Obrycki et al. observed anthers on about 80 - 90 % of milkweed plants examined in corn fields, concluding that anthers are commonly found on milkweed (and other wild plants) serving as host plants for butterflies, on soil, and all other surfaces within cornfields. Recently, Anderson et al (2004) reported about adverse effects on monarch larvae that had been fed whole Bt corn anthers. Larvae exposed to 0.9 anther/cm^2 milkweed leaf had reduced feeding, weight, and survival, and increased developmental time, compared to larvae exposed to non-Bt anthers, with first effects observed at a feeding rate of 0.3 anther/cm^2. Since anthers seem to gather preferentially in the midrib of milkweed leaves, but the most susceptible young larvae tended to avoid the midrib, the authors suggested that larvae in the field might encounter fewer anthers than larvae confined to petri dishes and that laboratory feeding trials might magnify the adverse effects of corn anthers that contain Bt toxin.

Thus, continuous exposure of non-target butterflies to natural deposits of Bt pollen on their food plants within or close to corn fields can have significant effects on larval development, larval survival, and weight and body size of pupae and adults. Event-176 and MON810 pollen may exert stronger effects than Bt11 pollen. Among these non-target butterflies may also be species already endangered. However, for monarch populations in the US it has been argued that, regardless of the impact of transgenic Bt crops, (more) significant risks to monarch butterflies may be posed by agricultural practices such as the use of very clean tillage practices, extensive herbicides and insecticides, and other crop choices affecting milkweed populations (Scriber 2001, Oberhauser et al. 2001).

Tri-trophic interactions

There is a number of studies on tri-trophic interactions and potential effects of Bt exposure to non-target organisms by predation or parasitism of herbivores consuming transgenic plant material. According to Clark et al. (2005), however, some of these studies do not permit the distinction between direct effects from Bt toxin and indirect effects of consuming suboptimal

diet consisting of sick or dying prey. Feeding studies using transgenic Bt corn expressing Cry1Ab, a susceptible prey species (*Ostrinia nubilalis*), and a non-susceptible prey (*Spodoptera littoralis*), and lacewing predators (*Chrysoperla carnea*), have resulted in significantly higher mortality of predator larvae when the prey species had been raised on Bt corn, compared to non-Bt corn (Hilbeck et al. 1998a). The authors concluded, that Bt toxin can be passed on from herbivores feeding on Bt plants to their enemies, irrespective of the susceptibility of the herbivore to the Bt toxin. *Chrysopid* larvae reared on artificial diet containing Cry1Ab toxin showed significantly higher mortality than larvae reared on control diet, indicating that Cry1Ab is toxic to *Chrysoperla carnea* at a concentration of 100µg/ml of diet (Hilbeck et al. 1998b). However, tri-trophic interactions involving Bt corn fed to thrips (*Anaphothrips obscurus*), not sensitive to Cry1Ab toxin, and the predator (*Orius majusculus*) have not resulted in significantly different predator mortality (Zwahlen et al. 2000).

When *C. carnea* larvae could choose between lepidopteran *Spodoptera littoralis* prey fed Bt corn and non-Bt corn, they showed a significant preference for non-Bt fed prey, whereas if they could choose between aphids (*Rhopalosiphum padi*) fed Bt corn or non-Bt corn, no preference could be detected (Meier & Hilbeck 2001). As the toxin has not been shown conclusively to be present in the phloem, phloem sucking aphids may not ingest Bt. *Spodoptera exigua* is known to have low susceptibility to Cry1Ac toxin expressed in cotton, but feeding *S. exigua* larvae grown for 24 hours on Bt cotton to adults of four heteropteran predator species revealed that longevity of two of them decreased significantly, wheras the other two were not affected (Ponsard et al. 2002). Generally, neonate predators are more susceptible to Bt toxins than second or third instars. But adult heteropteran predators also reacted negatively to Bt toxin, passed on to them by the less sensitive prey *S. exigua* that had been fed Bt plant material for only 24 hours (Ponsard et al. 2002).

In field studies, conducted over three consecutive years, effects of event-176 and MON810 corn on a number of predators such as lacewings, ladybirds, and hoverflies varied, depending on the year and the event, with particularly strong negative effects of event-176 in the first year and no or positive effects in year two and three, respectively (Lang et al. 2005). Abundance of spiders was significantly reduced in year one and that of predatory bugs in year one and two, with no or positive effects for spiders and bugs, respectively, in the other years. But Lang et al. (2005) point out that Bt toxin levels in corn plants can vary significantly within the season and also depend on the event, the variety chosen, and the site, thus influencing potential toxic effects on predators. Whether the unusual hot and dry summer in Germany of 2003, year three, affected the results too, remains unknown. Sisterson et al. (2004) studied arthropod abundance and diversity in Bt and non-Bt cotton fields and found more arthropods and higher diversity in row mixture plots (Bt and non-Bt) than in Bt plots, but site and plant height affected arthropod abundance too. In row mixture plots, significantly more families were sampled from non-Bt plants than from Bt plants. Based on these results, Sisterson et al. suggested growing mixed rows, but such in-field refuges may contrast efforts to slow resistance development.

Parasitoids could also be affected by hosts that have taken up Bt toxins and suffer from lethal or sublethal toxin doses. A reduction in host quality may even influence hymenopteran parasitoid sex ratios, because smaller host insects tend to receive more male eggs (Schuler et

al. 1999). By release of volatile substances following pest attack, plants attract parasitoids to deposit their eggs into pest larvae. Compared to its non-transgenic sister line, Mon810 corn exhibited differences in volatiles released with lower levels of emission and two volatile substances not produced (http://www.biosicherheit.de/projekte/23.proj.html). Whether these differences will influence parasitising pest larvae, is not known.

These studies suggest that tri-trophic effects of Bt toxins are possible, but that routes and extent of exposure to Bt toxins will differ between predators and parasitoids, depending on protein level, the species, their prey, and whether both the larval and adult stages are predatory. Predators and parasitoids not yet studied may be affected too. Besides feeding on herbivores (and perhaps carnivores), generalist arthropod predators such as mantids may also (occasionally) feed on pollen (Beckman & Hurd 2003), potentially exposing them to Bt toxins via this "vegetarian" route. Spiders could be exposed both to Bt pollen caught in their nets and to Bt toxins transmitted by herbivores collecting pollen or feeding on Bt toxin-containing plant material. Bt corn may have an effect on spider populations, but strength and direction of these effects seems to be influenced by additional (environmental) factors (http://www.biosicherheit.de/projekte/16.proj.html).

Potential impacts of Bt toxins on soil life

Since Bt proteins will be introduced into soil with plant residues after harvest and with sloughing of root cells, potential activity and stability of Bt toxins in soil may be a matter of concern. In addition, Bt toxins are released through root exudates from Bt corn (Saxena et al. 2002). Compared to application of Bt pesticides, many more soil organisms might be affected, due to incorporation of significant amounts of Bt toxin containing plant residues and because plant-expressed Bt toxins do not depend on specific cell receptors of target organisms for activity (Lang et al. 2005). Estimates about Bt toxin amount added to soils by transgenic crops are inconsistent and vary from a few grams/ha to more than 1 kg/ha for mature Bt cotton and Bt corn. For Cry3Bb1 toxin, targeted to corn root worm, estimates range from 1.3 – 5.4 kg/ha. Calculated soil concentrations of Bt toxin, therefore, range from 1.5 µg/kg soil to 2 410 µg/kg soil (Clark et al. 2005). A major source of differences in the results seems to be the lack of reliable, accurate, and universal analytical methods and the great variation in Bt protein content in various plant tissues. In general, dissipation of Bt toxins in soils is biphasic with a short lag phase, followed by a phase of comparatively rapid degradation, with the final portion (10 – 40 %, depending on the experiment, soil type, and amounts of protein) being degraded at much slower rates. In their review on environmental fate and effects of Bt crops, Clark et al. (2005) pointed out that previous methods used to investigate environmental effects of conventional pesticides are not entirely adequate for determining these parameters for Bt toxins from transgenic crops. Therefore, despite the previous evaluation of a toxic substance such as Bt toxin, it will still be neccessary to evaluate the ecological effects when it is produced by a transgenic plant.

Activity and stability of Bt toxins in soil depend on the type and amount of toxin, on temperature, pH, on soil type and humidity, and whether plant residues are left on the soil or are incorporated into the soil (Schmitz & Schütte 2001, Zwahlen et al. 2003a). Bt toxin contents can vary from site to site and from year to year, they are generally higher in rhizosphere soil than in bulk soil (Baumgarte & Tebbe 2005). Extraction efficiencies of Bt

toxins may also be dependent on soil characteristics, but in general, none of the curent methods seems to provide adequate quantification of Bt protein in natural soils (Clark et al 2005). As temperature is a major factor driving degradation processes under natural conditions, laboratory experiments conducted at constant temperatures of 20°C will not provide adequate data on the degradation of Bt toxins in the field, where mean soil temperatures may be in the order of 7 – 8°C, ranging at a farm site in Switzerland from a minimum of less than 1°C in January to about 20°C in June/July (Zwahlen et al. 2003a,b). Since after 200 and 240 days the Cry1Ab toxin could still be detected in soil in low amounts (Zwahlen et al. 2003a), soil organisms feeding on plant residues are likely continuously exposed to the Bt toxin, particularly if the field is repeatedly planted with a transgenic crop. Presence of Cry1Ab protein has been confirmed in German soils seven months after harvesting of Bt corn at an average concentration of 0.21 µg/kg soil (Baumgarte & Tebbe 2005). Ahmad et al. (2005) reported that in soil having higher amounts of clay (36 %), no Cry3Bb1 from corn rootworm-resistant corn was detected by ELISA over three consecutive years, whereas in soil having 5 % clay, low amounts were detected on all sampling occasions, but Cry3Bb1 protein released from root exudates or decaying plant residues was rapidly broken down in the soil.

Changes in levels, species composition and DNA fingerprints of soil microorganisms have been observed when leaf material of two transgenic cotton varieties expressing Cry1Ac toxin had been added to different soil types, compared to addition of the pure toxins, non-Bt cotton leaves and leaves from Cry1Ab expressing cotton (Donegan et al. 1995). Whether the different effects of the two types of transgenic leaves depended on the different Bt toxins expressed or on some other unidentified effect of the transformation process remained unclear. The change in microbial species composition, having a potential to impact soil processes, may be of ecological significance, even if a transitory stimulation in microbial populations observed with the transgenic Cry1Ac cotton leaves might not be of environmental concern. Soil type was shown to be important for the extent of quantitative and qualitative changes, with soils that contain lower levels of clay and organic matter seemingly leading to greater impacts on soil microorganims. In a multi-year field study on four different sites in Bavaria, Lang et al. (2005) observed no significant differences of microbial biomass and microbial enzyme activities in Bt corn, compared to isogenic controls.

Laboratory and field tests with adult and immature earthworms (*Lumbricus terrestris*) that ingested Cry1Ab expressing corn leaves did not show significantly different mortality between treatments with Bt corn and control corn leaves during the first 160 days. However, after 200 days a significant drop in relative weight of Bt corn-fed earthworms has been observed (Zwahlen et al. 2003b). According to Ahmad et al. (2005) and Al-Deeb et al. (2003), numbers of surface and below-ground arthropods and nematodes were not significantly affected by cultivation of Cry3Bb1 expressing corn.

The Cry1Ab Bt toxin protein has been shown to be released in root exudates from transgenic corn grown in sterile hydroponic culture, in sterile and non-sterile soil and in a natural soil in the field (Saxena et al. 1999, Saxena & Stotzky 2000). The toxin released adsorbs and binds rapidly on surface-active particles such as clay and humic substances in soil, rendering it less accessible to microbial degradation. Vertical movement of Cry1Ab toxin depends on surface

area and cation-exchange capacity of soils, larvicidal activity has been retained in binding at different depths of soil columns (Stotzky 2000). Non-Bt plants do not seem to take up toxin released to soil in root exudates of Bt corn, from biomass degradation of Bt corn, or as purified protein, as studies with non-Bt corn, carrot, radish, and turnip indicate (Saxena & Stotzky 2001b).

Rhizosphere soil samples from 12 corn hybrids (event 176, Bt11, and Mon810) contained Cry1Ab toxin 40 days after germination and were toxic to larvae of tobacco hornworm (*Manduca sexta*) (Saxena et al. 2002). Exudation of Bt toxin from transgenic corn plants apparently occurs, regardless of whether the Bt corn plants were derived from different transformation events. Cry1Ab remains toxic for insect larvae for at least 180 days, the longest time studied by Saxena & Stotzky (2001), but apparent effects on numbers of earthworms, nematodes, protozoa, bacteria, and fungi in soil could not be observed. This contrasts to the results of Donegan et al. (1995) who found quantitative and qualitative changes in microorganism populations upon addition of Cry1Ac expressing cotton leaves to soil. Bt toxin levels in soil probably are not evenly distributed, they seem to be far higher in the few centimeters of soil around roots, thus leading to high exposure of organisms associated with plant roots (Baumgarte & Tebbe 2005, Hilbeck & Meier 2002). Why Bt corn is degraded less readily than non-Bt corn, is not known, but it may be connected to the significantly higher lignin content of Bt corn hybrids, compared to their respective non-Bt isolines (Stotzky 2000, Saxena & Stotzky 2001a).

As the toxin will be released in root exudates during the entire growth of Bt corn and will be introduced to soil from pollen and anthers during tasseling and in plant biomass after harvest of the Bt crops, it could accumulate in the environment to concentrations that may enhance pest control, but that may also endanger non-target organisms and increase the selection pressure for evolution of toxin-resistant target insects (Saxena et al. 2002). In particular, decomposers and soil-dwelling arthropods such as springtails (*Collembola*) could be affected (Obrycki et al. 2001a). The toxins could also be passed to predators and omnivorous organisms living on soil surface. Consumption of plant material by decomposers may be affected by variety, energy content of plant material, lignin content, but also by expression levels of Cry1Ab in Bt corn.According to Lang et al. (2005), however, cultivation of event-176 and MON810 over three years did not change abundances of earthworms and collembola. Other soil dwelling organisms feeding on dead plant material, such as larvae of sciarid flies, may also be exposed to increased Bt toxin concentrations. Although in the field significant alterations of sciarid species compositions and feeding behavior have not been found, in laboratory studies larvae of *Lycoriella castanescens*, a sciarid abundant in fields, fed with Mon810 Bt corn litter had longer pupation times. Larvae of coleopteran predators (*Poecilius cupreus* and *Athea coriaria*) fed with sciarid larvae reared on Bt corn had longer pupation times too. Although both sciarid and coleopteran larvae ate more, they needed more time to pupate (http://biosicherheit.de/projekte/14.proj.html). Feeding of MON810 Bt corn pollen with toxin concentrations of 97 ng/g showed similar effects, but feeding of event Bt176 corn pollen, having about 30 times this amount (2 962 ng/g), did not. Therefore, the observed lower food quality of MON810 Bt corn may not have been due to Bt toxin levels only.

It is known already that some Bt toxins can be active against soil dwelling nematodes (Wei et al. 2003). In a life-cycle assay with the nematode *Caenorhabditis elegans* exposed to root soil isolated from Bt event176 and Mon810 corn, reproduction rate, egg number, and body length of *C. elegans* were significantly reduced, compared to root soil from the isogenic corn lines (Arndt 2003, Lang et al. 2005). Cry1Ab levels in root soil leading to effects on growth and reproduction of *C. elegans* were higher than 0.5 µg/kg soil. Wandeler et al (2002) found that the woodlouse *Porcellio scaber* consumed significantly less Bt corn expressing high toxin levels (Bt11) than Bt corn with low toxin levels (event176). They also found Cry1Ab toxin in the gut, body, and in significant amounts in faeces of *P. scaber* after feeding on trangenic corn, indicating that Bt toxin is still available after ingestion and excretion, perhaps even to non-target organisms not ingesting the plant material itself. Faeces-feeding organisms important for decomposition may thus also be exposed to Bt toxins. Recently, Zwahlen & Andow (2005) showed that ground beetles, considered polyphagous predators of arthropods, contained Cry1Ab toxin when collected in fields containing Bt corn residues. Potential adverse effects on ground beetle abundance, therefore, need further investigation.

As Bt corn is cultivated continuously on large areas, it seems neccessary to study potential accumulation of Bt toxins in such soils (Gray 2002, Hilbeck & Meier 2002, Adamczyk et al. 2001). This may be of special importance as, in the US, Bt corn showing high root expression levels of Cry3Bb, a toxin directed to the corn root worm complex, has been commercialized (Powell 2003) and Bt corn expressing stacked Bt genes, such as Cry3Bb and Cry1Ab to control corn root worm and European corn borer simultaneously, will follow. Whether different Bt toxins interact and whether they are also released from other Bt crop species remains to be elucidated just as the mechanism(s) of release. Using ELISA and bioassays with the susceptible species *Heliothis virescens*, Head et al. (2002) did not detect Cry1Ac protein in soil three months after the last growing season of transgenic Bt cotton (Bollgard). They reported that accumulation of Cry1Ac toxin after continuous use (3 – 6 years) of transgenic Bt cotton and subsequent incorporation of plant residues into the soil by postharvest tillage was extremely low and did not result in detectable biological activity. Yet data on the presence of Cry1Ac toxin during the growing season of Bt cotton have not been provided.

To study potential effects of Bt toxins on non-target organisms, a long-term approach is essential, since in commercial application of Bt crops the Bt toxins will be present for several weeks if not months and accumulation in the soil seems probable. Without long-term studies, sublethal adverse effects of the ingestion of plant material containing Bt toxins and resulting effects on the foodweb above and below the soil surface may not be detected. It is also vital to test various tissues of toxin-expressing transgenic plants, including pollen (mixed with anthers), and not just the purified (microbially derived) toxin. Monitoring efforts should involve a wide variety of species, including those that are unlikely to interact directly with the transgenic crop. Analyzing laboratory and field studies, Hilbeck et al. (2000) and Clark et al. (2005) concluded that protocols for ecotoxicological testing of transgenic plants must be improved. Testing protocols for chronic lethal and sublethal effects, for multi-trophic interactions and for ecologically relevant species should be included, considering life stages, feeding behavior, and predator-prey/host relationships of the organisms studied. As the variability in field studies may be large, multiple year experiments with different events and varieties, on different sites, are necessary to detect inter-generational and inter-seasonal

effects. Marvier (2002) evaluated design and statistical rigor of experiments used by industry to assess the safety of transgenic Bt crops for non-target organisms. She concluded that in most cases the number of replicates was too small to detect real effects. In addition, duration of experiments assessing the risks to non-target organisms should be extended to more accurately reflect the pattern and duration of exposure to Bt toxins under field conditions. Marvier also recommended access to (confidential) information about experimental studies of non-target effects should be improved.

To mitigate the risk of Bt toxin affecting non-target organisms, Obrycki et al. (2001) discussed various approaches. It might be possible to use only Bt varieties that do not express the Bt toxin in pollen. However, hybrids lacking Bt toxin in the pollen may not be as effective against first instar corn borers, which may feed on corn pollen before feeding on plant tissues, presenting problems for resistance management programs that rely on high mortality of target populations. To trap most transgenic pollen, it has also been suggested to create buffer zones of non-Bt corn around Bt cornfields that could perhaps also be used as a refuge in resistance management, or, a third option, simply not to plant transgenic Bt hybrids. Expression of Bt genes in chloroplasts has also been suggested, based on the observation that in many plant species pollen do not contain chloroplasts (Daniell & Dhingra 2002). However, as shown by DeCosa et al. (2001), transformation of chloroplasts may allow far higher accumulation of transgenic Bt toxins in leaf material than can be achieved by nuclear transformation. This in turn could lead to more severe impacts on non-target herbivores feeding on green parts of Bt crops and on leaf litter or on predators ingesting them.

Impacts of pest resistant plants other than Bt crops

Pest resistant plants expressing proteins such as proteinase inhibitors, α-amylase inhibitors, lectins, chitinases, or avidin probably show impacts on a broader spectrum of insects, as these proteins confer resistance to different insect species and to some nematodes (Hütter et al. 2000, Hilder & Boulter 1999, Schuler et al. 1999, Baghi & Mantke 1999, Jounanin et al. 1998). Fusion proteins consisting of a Bt toxin sequence fused to the B-chain of ricin, a lectin that binds to carbohydrate residues and seems to mediate membrane insertion of the toxin, broaden the range of target species, as reported by Mehlo et al. (2005). However, the range of non-target species affected could simultaneously be broadened. With respect to proteinase inhibitors, some target and non-target species may be able to compensate for exposure to these toxins by overproduction of digestive enzymes, by increasing consumption of plant material, or by producing novel proteinases insensitive to inhibition (Ferry et al. 2003). Toxicity of fusion proteins may be increased synergistically, not only in the expected additive manner, as observed by Zhu-Salzman et al. (2003) who fed soyacystatin fused to the legume lectin rGSII (*Griffonia simplicifolia* lectin II) to cowpea bruchid beatle larvae. By root exudation, such proteins might be released into the rhizosphere. Expression of α-amylase inhibitors in non-native hosts could lead to health concerns too, as shown by increased immunogenicity of bean α-amylase inhibitor-1 expressed in pea (Prescott et al. 2005).

To confer resistance to plant-parasitic nematodes, cysteine proteinase inhibitors (cystatins) such as chicken egg white cystatin or rice cystatin have been expressed in potato. Based on phospholipid fatty acid analysis of soil microorganisms, the transgenic potatoes have been shown to affect the structure of soil microbial community by suppressing both bacterial and

fungal community components (Cowgill et al. 2002a). It has not been determined, however, how cystatins from transgenic plants influence rhizosphere microorganims adversely. Because the changes in microbial community structure seemingly did not affect decomposition, the authors concluded that there might be a certain degree of redundancy of species within the decomposer community. But abundance of soil microarthropods and free-living nematodes has not been significantly changed. Among the insect groups most abundant in potato fields, aphids, leafhoppers and thrips have cysteine proteinase activity that might be affected by cystatins (Cowgill & Atkinson 2003). Although cystatins have been shown to reduce growth of the potato aphid *Myzus persicae*, aphids reared on the transgenic potatos were not significantly affected. Cystatin expression under the control of the 35S CaMV promoter potentially did not deliver sufficient cystatin doses to the phloem and thus to a phloem feeding aphid (Cowgill et al. 2002b). Similarly, leafhopper *Eupteryx aurata* did not respond differently to cystatin expressing potatoes (Cowgill & Atkinson 2003). According to a study commissioned by DEFRA (Atkinson 2004), potatoes expressing cystatins under the control of root-specific promotors did not affect nematode and earthworm numbers and nematode species diversity in field tests, despite the fact that several soil invertebrates were shown to possess digestive cysteine proteinases. In contrast to the results reported by Cowgill et al. (2002a,b), phospholipid fatty acid analysis indicated that soil microbes were not affected significantly or deleteriously.

The biotin-binding glycoprotein avidin, derived from egg white, is toxic to a wide range of insects representing several different families and orders, such as Lepidoptera, Diptera, Coleoptera, and Orthoptera, and to mites, and shows also toxicity to other animals and humans (Burgess et al. 2002). It acts as an anti-vitamin, whose action could be theoretically reversed by supplying additional biotin. Insects, small mammals and birds, however, are unlikely to consume additional biotin in the case they would feed on avidin expressing crop plants. If transgenic plants expressing such broad-acting toxins and anti-vitamins are commercialized, the probability of non-target effects will increase (Stewart 1999). In case of stacked pest resistance genes such effects will be even more likely, since two (or more) defensive compounds in plants act rarely, if ever, in an additive fashion (Hilbeck & Meier 2002). In such cases, interacting, possibly synergistic, effects might be expected.

There could be other effects too. Generally, resistance to pests may come at the expense of reduced fitness in an environment in which the respective herbivores are absent (Fineblum & Rausher 1995, Heil 2002). Increased levels of defensive compounds against certain herbivores could make plants even more attractive for other ones. Proteinase inhibitors expressed in transgenic plants could also influence the susceptibility of insects to Bt toxins since they might alter the activity of gut proteinases that activate certain *Bacillus thuringiensis* toxins. Patterns of tolerance/susceptiblity of insects to such Bt toxins could thus be changed. Besides direct or indirect toxicity, non-target effects could also comprise biochemical changes or alterations of insect behaviour, in particular, if volatile chemicals released by the plant and important for plant-insect interactions would be altered. Picard-Nizou et al. (1995) studied potential impacts of transgenic oilseed rape expressing chitinase on foraging behaviour of honey bees, but could not find any significant difference compared to control. A decrease in learning performance of honey bees exposed to cowpea trypsin inhibitor has been described by Picard-Nizou et al. (1997). Ingestion of low doses of proteinase inhibitors by honey bees

did not alter olfactory learning performances, but ingestion of Bowman-Birk soybean inhibitor induced new proteinases in the bee midgut, suggesting the existence of a mechanism to control proteinase synthesis (Girard et al. 1998). In particular, if higher proteinase inhibitor doses in the plant material ingested triggered a stronger hyperproduction of proteinases, the metabolic cost of which may result in increased mortality and/or decreased learning performances, impacts other than acute toxicity may be observed. Expression of such insecticidal proteins in pollen and nectar would be of considerable concern.

In cases of pest resistant plants expressing proteinase inhibitors or lectins, potential tri-trophic interactions need to be studied too. In a tri-trophic study with oilseed rape expressing rice cystatin, fed to the non-target pest species diamondback moth (*Plutella xylostella*) that utilizes no cysteine digestive proteinases, adverse effect on the multicoloured Asian ladybird (*Harmonia axyridis*) raised on *P. xylostella* have not been observed (Ferry et al. 2003). Although it has been shown that *P. xylostella* larval tissues contained cystatin at significant levels and that larvae and adults of *H. axyridis* utilize cysteine proteases, deleterious effects on ladybirds have not been detected, apparently due to upregulation of digestive proteases.

In laboratory studies, adult 2-spot ladybirds (*Adalia bipunctata*) were fed for 12 days on pest aphids (*Myzus persicae*) that colonized transgenic potatoes expressing low levels of snowdrop lectin (*Galanthus nivalis* agglutinin GNA) (Birch et al. 1999). Predatory ladybird fecundity, egg viability, and longevity significantly decreased over the following 2 –3 weeks. After switching ladybirds to feeding on aphids from non-transgenic potatoes, adverse effects were reversed. Therefore, lectins taken up from transgenic plants into aphid gut tissues may be delivered, possibly in increasing concentration, to the predatory ladybird. Since the transgenic potatoes expressed only low levels of GNA in foliar tissue and the ladybirds were exposed to the GNA-aphid diet for only 12 days, this laboratory study was not considered to be a worst case scenario. Whether reduced female longevity, reducing perhaps number of eggs laid and aphids consumed, would impact on the population dynamics of ladybirds and aphids under agronomic conditions is unknown (Birch et al. 1999). According to Down et al. (2003), another line of GNA expressing potatoes did not show significant adverse effects on development and survival of 2-spot ladybird *A. bipunctata* larvae that fed on aphids raised on these plants, although *A. bipunctata* egg viability was reduced. Additional studies with aphids, fed on artificial diet containing GNA, suggested that GNA as such had no deleterious effect upon adult longevity of ladybirds, but resulted in improved fecundity. For these reasons, Down et al. (2003) pointed to the need to screen several plant lines when investigating effects of transgenic products on non-target organisms.

Host finding and selection by parasitoids and pests could be affected by transgenic plants too, as laboratory studies with GNA producing sugarcane, the Mexican rice borer (*Eoreuma loftini*), the primary pest of sugarcane in south Texas, and the parasitoid (*Parallorhogas pyralophagus*) indicated (Tomov et al. 2003). Parasitoid females preferentially probed, drilled, and parasitized rice borer larvae fed conventional diet. These larvae showed higher activity levels than the larvae fed on GNA sugarcane diet. Feeding on GNA sugarcane, therefore, significantly reduced attractiveness of *E. loftini* larvae as hosts to the parasitoid. Pest species potentially also discriminate between GM plants and non-GM plants. Females of *E. loftini* and of another pest, *Diatraea saccharalis*, preferentially laid eggs on conventional

sugarcane versus the transgenic cultivar (Bernal & Setamou 2003). Thus, unanticipated (pleiotropic) effects in transgenic sugarcane may have changed the balance of stimulatory and repellent chemical cues that lead to host plant acceptance (or rejection) by the pest species.

Crop plants are subject to damage by a diversity of insect herbivores that are variously susceptible to resistance mechanisms introduced by transgenes. Genetically engineered resistance to primary pests may therefore affect secondary pests to not a priori predictable, variable degrees. Altered host plant quality could influence feeding, growth, development, reproduction, and dispersal of secondary pests such as aphids (Ashouri et al. 2001). Studying the performance of potato aphids (*Macrosiphum euphorbiae*) on potatoes, transformed for resistance to the Colorado potato beetle (*Leptinotarsa decemlineata*) with the Cry3A toxin gene or a rice gene coding for the proteinase inhibitor rice cystatin I (OCI), Ashouri et al. (2001) observed negative effects of the Bt potato on aphid growth and fecundity, but positive effects of the OCI transgenic potato, improving aphid performance on the latter transgenic variety. However, on Bt potatoes, aphids showed increased flight incidence, which might enhance the risk of spreading virus diseases from infected plants and fields. Since Cry3A is not known to directly affect aphids and phloem-sucking aphids may not ingest Bt toxins (Meier & Hilbeck 2001), the reasons for these unexpected effects of Bt potatoes on secondary pests remained unclear. Similarly, it is not known, why aphids performed better on OCI potatoes. Potentially, a modification of the proteolytic profile of aphids in reaction to proteinase inhibitors might have occurred, perhaps influencing their feeding rate. These studies indicate that even simple monogenic resistance can show unanticipated results and exert considerable effects on other pest species. This observation may be of particular relevance in assessing future pyrimidal resistance created by stacking of different resistance genes such as Bt and proteinase inhibitors (Ashouri et al. 2001).

Potential impacts of insect resistant plants would be exacerbated if the respective transgenes were transferred to related wild plants, conferring them fitness advantages and affecting an even greater range of non-target organisms that rely on these plants for food. Recently, it has been shown that transfer of Bt genes from transgenic sunflower crops to wild sunflowers reduced herbivory and enhanced fecundity in backcross 1 (BC_1) wild sunflowers significantly (Snow et al. 2003). Reduced herbivory apparently caused transgenic wild plants to produce, compared to nontransgenic controls, an average of 55 % and 14 % more seeds per plant in Nebraska and Colorado, respectively. In the US, where wild sunflowers occur close to sunflower crops and hybridize readily with cultivated plants, a Bt transgene could spread quickly across wild sunflower populations conferring them a selective advantage. Repeated gene flow from neighbouring crops grown over consecutive years seems also possible. A reduction in population size of susceptible, native lepidopterans that feed on wild sunflower may be the result.

5.4 Pathogen resistance

Natural plant resistance genes (R genes) confer resistance to a number of microbial, fungal, and viral pathogens (Takken & Joosten 2000). Their expression in transgenic plants might reduce the susceptibility of crop plants to pathogens. These R genes can act in a gene-for-gene

manner, where the product of a single resistance gene in the plant specifically recognizes the product of a pathogen avirulence gene, setting off a hypersensitive response that culminates in rapid cell death around the site of infection. The spread of an invading pathogen can thus be restricted. Apoptosis-like programmed cell death seems to occur in these cases (Dickman et al. 2001). Plants can protect themselves against pathogen attack also through action of nonspecific resistance mechanisms such as physical barriers, activation of defense-related proteins, induction of viral RNA-degrading systems, or production of secondary metabolites and other natural products that confer disease resistance (Scholthof 2001). They produce a vast array of natural products such as terpenoids, phenolics, and alkaloids, many of which confer selective advantage against attack by microorganisms (Dixon 2001).

In induced resistance, pathogenesis related proteins are expressed in response to pathogen or pest attack or in response to salicylic acid and jasmonic acid treatments. Systemic acquired resistance (SAR) in plants is characterized by resistance extending to plant tissues distant from the initial infection site, persistence for weeks to months, and plant protection against secondary infection by a broad spectrum of pathogens. Systemic acquired resistance is accompanied by the induced expression of pathogenesis-related proteins such as antifungal chitinases, β-1,3-glucanases and other proteins. Several of these resistance genes, providing resistance not only to pathogens but also to pests such as nematodes (Jung et al. 1998) and aphids (Vos et al. 1998), have been cloned and divided into classes based on their structural features. Resistance to aphids may be of particular importance as these pest species quite often transmit a number of viruses to new host plants. Fitness costs, however, may be linked to induced resistance in plants (Heil & Baldwin 2002, Heil 2002, Cipollini et al. 2003). Generally, levels of resistance to viruses, bacteria, and fungi that have been observed in the greenhouse need not necessarily be the same in the field.

5.4.1 Virus resistance

Viral diseases are difficult to control since there are no pesticides available. Control of viral infections has traditionally relied on either the use of pesticides to kill insects acting as vectors for virus tansfer or the introduction of natural resistance genes through conventional breeding programs. In the past decade, many attempts have been made to increase virus resistance by genetic engineering. About a tenth of the transgenic plants tested in field trials in the US and EU in the nineties carried transgenes conferring virus resistance (Schütte & Oldendorf 2001). In most cases, virus resistance has been engineered by transforming plants with sequences derived from viral genomes. This pathogen-derived resistance (PDR) may result from the expression of a viral protein, such as a coat protein, or an RNA-mediated mechanism that appears to be analogous to post transcriptional gene silencing (Dempsey et al. 1998, Schütte 1998b, Schillberg et al. 2001). Coat protein mediated resistance has been engineered against a wide variety of viruses in many plant species. The mechanisms are varied and are not always dependent on accumulation of coat protein. Uncoating, replication, or translation of the viral genome or assembly into virions may be inhibited. In some cases, resistance has been provided only to the viral strain from which the coat protein was derived, whereas in other cases protection against related viruses has been observed too. RNA-mediated resistance may involve RNA degradation and posttranscriptional gene silencing that can target both host and

viral RNA species (Pinto et al. 1999). Specific PDR has also been engineered by expressing genes encoding viral replicase, broader resistance may be achieved by the expression of viral movement proteins. Generally, the various mechanisms through which viral genomes confer resistance are not well understood. Greenhouse results suggesting the acquisition of virus resistance after transfer of coat protein or replicase genes cannot always be confirmed in the field, as studies done with transgenic wheat indicated (Sharp et al. 2002). In this case, yield of transgenic lines was lower than that observed for the parent cultivar, both in the presence and absence of disease.

Other means to confer resistance to viral pathogens may comprise the transfer of genes encoding ribosome inactivating proteins (RIP) or proteins that recognize and degrade double-stranded RNA, the replication intermediate of many plant viruses (Dempsey et al. 1998, van Damme et al. 2001). Plant genes encoding cysteine proteinase inhibitors have been transferred too. The polyprotein encoded by the genome of (aphid-transmissible) potyviruses is processed into individual products by proteinases, among them cysteine proteinases. When expressed in transgenic tobacco plants, a rice cysteine proteinase inhibitor increased resistance to the potyviruses tobacco etch virus (TEV) and potato virus Y (PVY), but not to tobacco mosaic virus (TMV) which is not processed by cysteine proteinases (Gutierrez-Campos et al. 1999). Cysteine proteinase inhibitors might act broadly on other phytopathogenic viruses, on insects, and nematodes too, as many of these organisms rely on different types of proteinase activity.

Ribozymes, small catalytic RNA molecules that possess sequence-specific RNA cleavage activity, might also be used to increase virus resistance. Transfer of a newly constructed ribozyme, targeting the mRNA of rice dwarf virus (RDV) segment 5, to rice resulted in transgenic plants that displayed high resistance to RDV (Han et al. 2000). However, in the T3 – T6 progeny of rice, resistance phenotype segregated in an irregular pattern, indicating transgene silencing (Han et al. 2000).

Virus resistance in plants could perhaps also be achieved by expression of proteins active in antiviral defense in humans. However, constitutive expression in tobacco of human MxA protein, a member of the dynamin familiy of large guanosintriphosphatases that are tightly regulated by type I interferon and play a key role in the host defense against several RNA viruses, failed to confer resistance to a variety of RNA plant viruses (Frese et al. 2000). An alternative approach to create virus resistant plants may be the expression of antibodies or antibody fragments that bind to and inactivate pathogens in plants. Genes for full-size or single chain antibodies from hybridoma cells or derived by phage display technology (expression of a library of different antibody genes as fusion proteins on the surface of bacteriophage) have been transferred to a number of plants. Reduction in disease symptoms correlated with the amount of antibody has been observed (Ma & Hein 1995, de Kathen 2001, Schillberg et al. 2001, Fischer et al. 2001). Broad-spectrum resistance could perhaps be achieved by simultaneously expressing two to five single chain antibodies with different target specificities in appropriate compartments of transgenic plants, thus pyramiding resistance. To increase antibody stability, targeting to the endoplasmic reticulum, to the apoplast, and to the plasma membrane has been suggested. Expression of such antibodies might increase resistance to bacteria, fungi and perhaps also nematodes simultaneously. The

source of antibody genes and the plant cell compartment to which antibodies are targeted seems to influence their stability and, therefore, the degree of viral resistance.

5.4.2 Bacterial resistance

Different genetic strategies using genes of non-plant or plant origin have been proposed to generate plants resistant to bacterial diseases, among them the expression of antibodies (Schillberg et al. 2001) and the overexpression of proteins known to have antimicrobial properties. In particular, single-chain antibodies may be suitable, as, unlike complete antibodies, they need not be assembled and can be targeted to different compartments of the cell (Morgues et al. 1998). Proteins with antimicrobial properties may include lytic peptides from insects, human antimicrobial proteins such as lactoferrin or lysozyme, derived from egg-white, T4-bacteriophage, or human cells. The inhibition of bacterial pathogenicity or virulence factors and the enhanced production of reactive oxygen species or of plant defense proteins, such as thionins, antimicrobial peptides, or phytoalexins might also increase plant resistance to pathogenic bacteria (reviewed by Morgues et al. 1998, Husemann et al. 2001). Increased levels of H_2O_2 due to overexpression of glucose oxidase could enhance resistance not only to bacterial pathogens, but also to fungal ones (Kachroo et al. 2003). In using these strategies very often only mixed results have been achieved, therefore, stacking of transgenes that encode antibacterial proteins and construction of synthetic genes/molecules with enhanced expression and stability in plant tissues has been suggested (Morgues et al. 1998).

In a gene-for-gene interaction between plant R genes and corresponding avirulence (avr) genes of pathogens, plant defenses are induced via a signal-transduction pathway. If either the plant R gene or the pathogen avr gene is lacking, plant defenses are not induced in a timely and effective manner and the pathogen can colonize the plant (Dempsey et al. 1998). Such resistance (R) genes have been cloned and transferred to susceptible plants. By transforming susceptible plants with cloned R genes, novel resistant lines can be generated, though R genes isolated from one plant species may not necessarily be compatible with the resistance signaling components from another. Dempsey et al. (1998) and Morgues et al. (1998) proposed to engineer R genes that have novel resistance specificities and that recognize conserved features of a broad spectrum of pathogens. Resistance could also be enhanced by manipulation of various signaling components to allow a more effective induction of defense pathways after pathogen attack or by the enhanced production of biotic elicitors and other agents inducing disease resistance. Since constitutive expression of R genes and of genes involved in the signal-transduction pathway can interfere with plant growth, such genes might require modification or promoters inducible by pathogen attack (Dempsey et al. 1998).

Crown gall disease, resulting in economic losses in perennial crops, is caused by the soil bacterium *Agrobacterium tumefaciens* that transforms plant cells through transfer of its T-DNA. Expression of oncogenes, located on the T-DNA, causes overproduction of the plant hormones auxin and cytokinin resulting in initiation of uncontrolled cell growth and division and subsequently gall formation. Oncogenes coding for enzymes involved in auxin and cytokinin formation have been targeted with a transgene construct designed to generate self-complementary transcripts (Escobar et al. 2001). This RNA interference (RNAi)-mediated

oncogene silencing, based on mRNA sequence homology, has been reported to confer broad spectrum resistance to crown gall tumorigenesis in *Arabidopsis* and tomato, seemingly without altering plant hormone metabolism. Whether such alterations could also be feasible for perennial species where crown galls are more significant than in annual species, is not known. In perennial species even subtle changes in hormone concentration may influence plant growth und mechanisms of gene silencing seem to be more important.

Many gram-negative bacteria use substances like N-acyl homoserine lactones (acyl HSL) to modulate the expression of diverse phenotypes including virulence factor synthesis. Transcriptional regulators, allowing up- or downregulation of specific phenotypes, are activated at sufficiently high concentrations of acyl HSL. As the level of acyl HSL depends on the population density, this type of regulation is called quorum-sensing. Transgenic tobacco and potato expressing a microbial enzyme that degrades acyl HSL were described to show less soft-rot damage if infected by the plant pathogen *Erwinia carotovera* (Whitehead et al. 2001). Since affected bacteria would not be killed, such an approach might provide less selective pressure for the emergence of resistant strains. It is unclear, however, whether the enzyme could also degrade natural plant metabolites contanining lactone rings or affect beneficial soil-dwelling microorganisms.

5.4.3 Fungal resistance

Increased resistance to fungi in crop plants is a major goal of genetic engineering since pathogenic fungi lead to the most devastating plant diseases. To achieve that goal, several transgenic strategies have been proposed, including development of synthetic combinatorial libraries to design novel biologically active peptides. The use of small genes encoding antimicrobial peptides could facilitate the stacking of multiple activities in transgenic plants or even on single transgenes to allow additive or even synergistic effects in pathogen control (van der Biezen 2001). One of the strategies to increase fungal resistance could be antibody expression. Fungal proteins that are essential for pathogenesis and, in addition, fungal toxins in infected crops may thus be neutralized (Schillberg et al. 2001). Increased H_2O_2 levels might enhance resistance both to bacteria and to pathogenic fungi (Kachroo et al. 2003). Virally encoded antifungal proteins may also be used for the enhancement of resistance as shown in transgenic wheat, made resistant to a strain of devastating *Ustilago maydis*, stinking smut (Clausen et al. 2000). Dahleen et al. (2001) reviewed transgenic approaches to combat head blight caused by *Fusarium graminearum* in wheat and barley and to reduce contamination with the toxic compound deoxynivalenol produced by these pathogens.

Another approach would be the overexpression of antimicrobial proteins that kill fungi or bacteria. Such proteins and peptides can be derived from plants, certain fungi, or from insects. Antimicrobial proteins derived from plants include, among others, fungal cell wall hydrolyzing enzymes such as chitinase and glucanase, ribosome-inactivating proteins that interfere with fungal protein synthesis, and defensins that inhibit hyphal elongation or display lytic activity (van der Biezen 2001, Dahleen et al. 2001). Other proteins potentially active against fungi are thaumatin-like proteins and thionins that create pores in fungal membranes and disrupt signal transduction pathways. The defensin alfalfa antifungal peptide, if expressed

in potato, has been reported to provide agronomically useful levels of control of the fungal pathogen *Verticillium dahliae,* causing the early dying disease, not only in the laboratory but also in the field (Gao et al. 2000). In wheat, expression of genes encoding an antifungal protein, derived from the fungus *Aspergillus giganteus*, or a barley chitinase class II has beeen reported to lead to enhanced resistance to powdery mildew (*Erysiphe graminis*) and leaf rust (*Puccinia recondita*), whereas expression of a barley gene encoding the cytosolic ribosome-inactivating protein type I did not result in enhanced fungal resistance (Oldach et al. 2001). In the resistant lines, a correlation between expression levels and the number of integrated transgene copies (from one to eight!) was not obvious. Transgenic tobacco plants that synthesized alfalfa ferritin in vegetative tissues exhibited tolerance to necrotic damage caused by viral or fungal infections. They also retained their photosynthetic function under oxidative stress, indicating that the iron-binding protein ferritin could protect plants from oxidative stress (Deak et al.1999).

Cecropins (e.g. from silk moths) and melittins belong to a class of peptides, not present in plants, that cause lesions in microbial membranes. But the bee venom peptide melittin displays also hemolytic and toxic properties to animal and plant cells (van der Biezen 2001). To reduce toxicity of melittin, chimeric genes linking melittin sequences to cecropin sequences might be used. Transformation of two potato cultivars with such a chimeric gene under the control of the duplicated CaMV 35S promoter was reported to yield transgenic lines highly resistant to the pathogenic fungi *Phytophtora cactorum* and *Fusarium solani*, and the bacterium *Erwinia carotovera* (Osusky et al. 2000). However, one of the varieties, Russet Burbank, showed morphological alterations such as curly leaves and very small, branched tubers. Therefore, interference of this chimeric peptide with other components of plant metabolism may be possible.

Inhibition of fungal growth and reproduction might also be achieved by controlled generation of necrotic lesions at infection sites, analagous to the naturally occurring hypersensitive cell death. This approach relies on the expression of a cytotoxic gene such as barnase (encoding RNase) under the control of a promoter responding to pathogen attack and the constitutive expression of barstar inhibiting the cytotoxic effect of a potential background barnase expression. Some lines of transgenic potatoes carrying the barnase gene, linked to a pathogen-responsive potato promoter, and the barstar gene under CaMV 35S control have been reported to show reduced sporulation of the late-blight fungus *Phytophthora infestans* on their leaves (Strittmatter et al. 1995). Although the promoter used may respond to a broad spectrum of pathogens, including viruses and nematodes, resistance of such transgenic plants was not extended to potato viruses tested and significant effects on parasitic soil fungi and bacterial populations have not been observed (Lukow 1999). Interfering with host cell death pathways may enhance resistance to necrotrophic fungi, i.e. fungi that require host plant cell death to grow, colonize, and reproduce. Apoptosis in animals is controlled through functionally conserved genes that have been identified in human cells, in animals (e.g. *Caenorhabditis elegans*), and in viruses. Transgenic tobacco plants expressing such genes that negatively regulate apoptosis showed heritable resistance to the necrotrophic fungal pathogens *Sclerotinia sclerotiorum, Botrytis cinerea,* and *Cercospora nicotianae,* and, in addition, to a necrogenic virus (Dickman et al. 2001). From this it has been inferred that animal anti-apoptotic genes could function in plants and that such genes might be used to generate disease

resistance in crops. In transgenic tobacco lines with high expression of anti-apoptotic genes, however, morphological alterations such as variegated leaf pigmentation and male sterility have been observed.

Plant defense responses to constrain pathogen attack have been shown to be mediated by signalling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene that are not only involved in pathogen response, but in many aspects of plant development. In certain cases, there may also be antagonistic interactions between SA-dependent and JA-dependent signalling pathways. SA which plays a crucial role in triggering systemic acquired resistance, if overproduced, might confer resistance to fungal attack. Verberne et al. (2000) reported that constitutive expression of bacterial genes encoding two enzymes of the SA pathway resulted in plants that produced elevated levels of SA and a 500- to 1 000-fold increase of SA glucoside. But efficient SA production and resistance to the fungus *Oidium lycopersicon* could be observed only when both enzymes were targeted to the chloroplast. Depending on the type of pathogen and plant species, ethylen appears to have opposing roles in the progress of disease, exogenous application of ethylen can enhance resistance to fungi in some cases and decrease resistance in other cases. Constitutive expression of an ethylene-response gene in transgenic *Arabidopsis* was reported to confer resistance to necrotrophic fungi such as *B. cinerea* and to reduce resistance to the bacterium *Pseudomonas syringae* (Berrocal-Lobo et al. 2002).

Plants constitutively producing SA and SA glucoside may also show resistance to viral infection and, since SA glucoside and related compounds play an important role in plant-herbivore interactions, possbily to insect attack. On the other hand, since overactivation of one of these signaling pathways can influence other defense responses, such transgenic plants might show unexpected susceptibility to other pathogens. To limit antifungal protein expression to only susceptible parts of the plant and to minimize the metabolic drain on the host associated with over-expression, organ- or tissue-specific promoters would be needed (Dahleen et al. 2001). However, in a majority of the studies cited, constitutive promoters have been used, in most cases the CaMV 35S promoter.

5.4.4 Environmental impacts of pathogen resistance traits

Pathogen resistant transgenic crop plants are advocated because of expectations that they will lead to yield increase and to a reduction in pesticide applications, thus exerting positive effects on the environment. Potential negative effects have been discussed too, with a particular emphasis on virus resistant transgenic crops, due perhaps to the large number of virus resistant plants tested in the field (Schütte 1998b). In particular, the transfer of pathogen-derived viral genes has provoked an intensive debate about potential risks for the environment (Tepfer 1993, Farinelli & Malnoe 1996, Eckelkamp et al. 1997, Latham & Steinbrecher 2004, http://www.biosicherheit.de/projekte/115.proj.html). The main questions discussed are whether co-infection by other viruses could lead to heterologous encapsidation, i.e. nucleic acids of co-infecting viruses are packaged into heterologous coat proteins encoded by the transgene which could potentially influence virus transfer and, second, whether co-infecting viruses could recombine with transgenic virus-derived DNA, thus leading to virus

particles with new specificities and traits. Other questions are whether transgenic plants exhibit altered viral spectra or whether there will be effects on species number and population density of herbivores such as aphids.

Recombination of transgenes with infecting viruses has been shown to occur (Greene & Allison 1994). It has been argued that frequencies of recombination and transcapsidation in transgenic plants may be lower, if, as has been observed, there is less viral DNA (and mRNA) in transgenic plants than in co-infected non-GM plants. But co-infection under natural field situations may differ from co-infection of transgenic virus resistant plants in that co-infecting viruses in non-transgenic plants are unlikely to replicate simultaneously, because of their temporary, not continuous replication. In addition, expression of viral genes is limited in space and time and viruses often exhibit tissue tropisms which may not overlap with those of other viruses (Latham & Steinbrecher 2004, Schütte & Oldendorf 2001). Transgenes, in particular if controlled by a constitutive promotor such as the CaMV 35S promotor, are expressed in all tissues throughout the life cycle of transgenic crops. In most cases virus resistance relies on simultaneous repression of the transgene and virus replication by post transcriptional gene silencing (PTGS). But it has been shown that, in a general counter-defense strategy, diverse DNA and RNA plant viruses suppress gene silencing by virus-encoded suppressors, although spatial pattern and degree of suppression vary extensively between viruses (Voinnet et al. 1999). Due to such suppression of gene silencing, levels of transgene mRNA and protein may be much higer in virus-infected cells than in uninfected ones leaving the question open whether recombination is more likely between viruses or between viruses and transgenic mRNAs (Latham & Steinbrecher 2004).

Close relationship between viral transgenes and infecting viruses does not seem to be required for recombination. This can be derived from the finding that genes for movement proteins (MP) from plant viruses (tobacco mosaic virus and red clover necrotic mosaic virus) transferred to tobacco complemented the flock house virus, a virus able to replicate in both insects and plants but unable to move inside plants, to move systemically within the transgenic plant (Dasgupta et al. 2001). Interspecies hybrid viruses may be even more virulent than either parental virus as shown by synergistic interactions of a tomato virus MP gene with the cucumber mosaic virus (Ding et al. 1996). Therefore, it is not clear if recombination between closely related viruses is of more or less concern than recombination between distantly related ones (Latham & Steinbrecher 2004). Because of its recombinogenic activity, the 35S CaMV promotor is suspected to carry a particularly high risk of recombination (Ho et al. 1999, 2000, 2000b, Cummins et al. 2000).

To reduce the risk of transcapsidation and recombination, several measures have been suggested, such as avoidance of proteins known to interact with other viruses, use of the shortest possible sequences, deletion of recombinogenic sequences, use of, whenever possible, anti-sense constructs that do not result in functional products when recombined, use of genes from mild and endemic strains, and limitation of gene expression to those plant tissues that are infected by the respective viruses (Latham & Steinbrecher 2004, Schütte & Oldendorf 2001).

The main question concerning environmental effects of plants resistant to bacterial and fungal pathogens is the potential impact of transgene expression on the natural microflora. This is of

particular relevance for strategies with a wide spectrum of activities against different pathogens such as those enhancing reactive oxygen species or using antimicrobial peptides and enzymes (Morgues et al. 1998). Induction of H_2O_2 may not only increase resistance to both bacteria and fungi but, if expressed constitutively, may also affect plant metabolism and signaling networks interfering with normal growth and development (Kachroo et al. 2003). If newly expressed antimicrobial substances are released by root exudation, soil microorganisms other than the target species might get into contact with these substances (Husemann et al. 2001).

In the laboratory, De Vries et al. (1998) showed that lysozyme from transgenic T4-lysozyme expressing potatoes is released into the rhizosphere and is enzymatically active in soil solution, although at a reduced level. All strains of the target bacterium *Erwinia carotovera* and most other gram-positive and gram-negative soil bacteria tested, including some plant pathogens, turned out to be sensitive towards T4-lysozyme, soil-associated bacteria can be killed. Active transgenic DNA derived from T4-lysozyme expressing plants is also present in soil and recipient *Acinetobacter* bacteria carrying an incomplete resistance gene have been shown to take up transgenic DNA and use it to construct a complete resistance gene (de Vries et al 2001). But in field experiments, significant impacts of T4-lysozyme expressing potatoes on abundant members of the soil microflora could not be detected, although one transgenic line (with an altered phenotype) exhibited alterations in composition of bacterial populations. In general, transgenic lines had lower numbers of *Agrobacterium* and higher numbers of *Comamonas* isolates (Lottmann & Berg 1998, Smalla & Heuer 1998, http://www.biosicherheit.de/projekte/81.proj.html). Since T4-lysozyme may affect less abundant bacterial species and also some fungal species such as endophytic fungi, potential impacts on these members of the soil microflora need to be analysed too. Nitrogen-fixing *Rhizobium leguminosarum* bacteria are highly sensitiv to T4-lysozyme treatment and showed 50 % reduction of nodulation if cultured with plants of its host species *Vicia hirsuta* expressing T4-lysozyme in their roots (Broer et al. 1998). Close proximity of T4-lysozyme expressing potatoes with non-transgenic *V. hirsuta*, however, did not affect *R. leguminosarum*.

Because of their intimate relations to their host plants, symbionts or mycorrhizal fungi could be influenced by the expression of antimicrobial peptides and enzymes, be they released to the soil environment via root exudation or not. Abundance and diversity of these beneficial organisms might thus be affected (Husemann et al. 2001). The extent of genetic and ecological diversity among clones and species of mycorrhizal fungi and their respective roles in plant nutrition have only begun to be elucidated and growth and sporulation requirements of many species are not known (Bever et al. 2001). If micorrhizal fungi would be impaired by transgenic plants resistant to fungal pathogens, uptake of nutrients by plants and plant performance could be significantly reduced, with potential impacts on yields. Bacteria and fungi are also members of the soil foodweb, whose multitude of interactions is barely known. Stacking of transgenes with antimicrobial activity, as suggested by van der Biezen (2001), may complicate matters, as in such cases a multitude of bacteria and fungi may be affected simultaneously – likely leading to synergistic effects.

Some of the newly produced peptides and enzymes with antimicrobial activity potentially interfere with plant growth, if only under certain environmental conditions. Enzymes degrading bacterial lactones may also attack plant lactones (Whitehead et al. 2001) and expression of peptides and anti-apoptotic genes derived from animals has been reported to be connected with morphological alterations in transgenic plants (Osusky et al. 2000, Dickman et al. 2001). Signalling pathways are not only involved in pathogen response but also in other aspects of plant development and growth. Therefore, genetic engineering of these quite often interacting pathways may lead to unwanted side effects in transgenic plants and to altered susceptibility to insects and other pathogens. In addition, some of the antimicrobial peptides suggested to be expressed in transgenic plants, e.g. the bee toxin melittin, could display toxic activity to animals too (van der Biezen 2001), perhaps endangering animals, including wild life, that feed on such transgenic plants. Finally, as in cases of pest resistance, if transfer of genes conferring pathogen resistance to wild related plants does occur, said effects likely show up in wild plant communities and their associated microorganisms too. This could lead to fitness advantages of wild plants but also to impacts on soil life in natural habitats. Hybrids may be even less susceptible to pathogens than their parent lines, potentially due to heterosis effects, as may be inferred from preliminary results with hybrids derived from oilseed rape and field radish (http://www.biosicherheit.de/projekte/24.proj.html). In case horizontal gene transfer from pathogen resistant plants to bacteria did occur, various microorganisms might gain fitness advantages in acquiring resistance genes. This in turn could impact microbial biodiversity in soils. Because of the many unknowns about soil life and the multitude of potential effects of pathogen resistant plants, it will be particularly difficult to predict the impact on the environment exerted by such plants.

5.5 Stress tolerance

Environmental stresses, such as salinity, freezing, heat, drought, and flooding greatly influence plant productivity. Plants, continuously exposed to environmental stimuli, have adapted to varying degree to high and low temperature, mineral imbalance, excess or insufficient light, and lack or excess of water. Stress tolerance generally is a complex quantitative trait with many genes involved. Stress response mechanisms vary among different plant species and families and possibly also with the developmental stage during which a plant is subjected to stress (Wang et al. 2003b, Bohnert and Jensen 1996).

With the exception of flooding, the major biotic stresses basically result in water-deficit stress. Freezing injury in most plants and tissues results largely from the severe cellular dehydration that occurs upon ice formation, leading also to membrane injury. Freezing tolerance, therefore, must include tolerance to severe dehydration stress. Given this, one can expect that freezing tolerance may also impart tolerance to dehydration stress caused by either drought or high salinity (Thomashow 2001, Sakamoto & Murata 2002). Limitations in water availability cause a reduction, possibly inhibition of photosynthesis. If photosynthesis is impaired, but chloroplasts are still exposed to light, there is photoreduction of oxygen and concomitant production of reactive oxygen intermediates, such as superoxides and peroxides, which damage membranes and enzymes (Holmberg & Bülow 1998). High salt stress disrupts homeostasis, defined by Niu et al. (1995) as the tendency of an organism to maintain internal

steady state by the coordinate responses of its constituent components, in water potential and ion distribution both at the cellular and the whole-plant level. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest, and even death of the plant (Zhu 2001).

The complex process of adaptation to salinity and drought involves numerous changes including attenuated growth and activation/repression of a great number of genes (recently reviewed by Bartels & Sunkar 2005). Responses of the plant cell to osmotic stress may comprise ion exclusion, ion export, cell wall modifications, osmotic adjustments, and osmoprotection by osmolytes, compatible solutes such as certain sugars, amino acids, and betaines may also be produced (Sakamoto & Murata 2002). Stress-relieving genes that are transcribed may encode enzymes involved in a particular metabolic pathway, transcription factors, or proteins with specific detoxification properties. Heat shock proteins with chaperone function and late embryogenesis abundant proteins (LEA) accumulate upon water, salinity, and extreme temperature stress (Wang et al. 2003b). Enzymes with antioxidant activity, such as superoxide dismutases and peroxidases, which scavenge reactive oxygen species can be activated. Studies indicate that extensive crosstalk between transcriptional regulatory systems seems to occur and that some of the stress response genes are controlled by absisic acid (ABA), whereas others are not (Shinozaki et al. 2003).

Based on such natural stress responses, several strategies for engineering abiotic stress tolerance in plants have been suggested (reviewed by Bohnert & Jensen 1996, Holmberg & Bülow 1998, Zhang et al. 2000, Zhu 2001, Hoekstra et al. 2001, Thomashow 2001, Wang et al. 2003b, Mansour et al. 2003). Constitutive expression of a normally stress-induced gene may have serious penalties with respect to energy loss or other adverse side effects. Therefore, the most widely used promoter, the CaMV 35S promoter, which is constitutively expressed, is not a good choice for stress-related genes. For this reason, stress-inducible promoters such as promoters responsive to ABA, drought, and heat-shock would be preferred (Zhang et al. 2000). Transfer of several genes will be neccessary to achieve practical levels of stress resistance or cross-tolerance to several abiotic stresses in crops (Zhang et al. 2000), e.g by the simultanous transformation with multiple genes or the crossing of plants containing different stress tolerance genes. Manipulation of complex, metabolic or regulatory pathways involving multiple genes would be required, either by transfer of single genes having multiple stress-protective effects or of regulatory genes inducing multiple gene targets.

5.5.1 Salt tolerance

In recent years a number of attempts have been made to improve salt tolerance of crop plants by the introduction of transgenes encoding transport proteins, membrane ATPases, and transcription factors or production of osmolytes and antioxidants (reviewed by Zhang et al. 2000, Zhu 2001, Mansour et al. 2003, Wang et al. 2003). Maintenance of ion homeostasis in salt-stressed plants is linked to sodium transport by Na^+/H^+ antiport proteins, possibly also playing a role in K^+/H^+ exchange. Increased salt tolerance of *Arabidopsis* and crop plants such as tomato and oilseed rape expressing the *Arabidopsis* Na^+/H^+ antiport protein gene has been described (Apse et al. 1999, Zhang & Blumwald 2001, Zhang et al. 2001, Mansour et al.

2003, Wang et al. 2003b). The transgenics could grow in high salt condition of 200 mM NaCl. Salt content in tomato fruit and yields and oil quality of transgenic rape plants were not significantly affected by the high salinity of the medium. Questions, however, whether activation of Na^+/H^+ antiporters will confer salt tolerance, remained (Mansour et al. 2003).

Another strategy to achieve enhanced salt tolerance is to produce osmolytes that maintain cell turgor, such as polyol/sugars (mannitol, sorbitol, trehalose), amino acids (proline), and quaternary amines (glycine betaine), and ectoine, probably working through oxidative detoxification. These osmolytes are active in scavenging reactive oxygen species and are preferably targeted to chloroplasts (Zhu 2001). Compatible solutes such as glycine betaine can serve as efficient protective agents stabilizing the structures and functions of enzymes, protein complexes, and membranes against the damaging effects of salt, cold, freezing, and heat stress. Genes encoding glycine betaine-synthesizing enzymes such as bacterial choline oxidase or choline dehydrogenase have been transferred to *Arabidopsis*, tobacco, rice, and brown mustard to create stress-resistant transgenic plants (McNeil et al. 2001, Sakamoto & Murata 2001, 2002). Transgenic *Arabidopsis* plants expressing choline oxidase not only acquired resistance to high concentrations of NaCl, but were also significantly more tolerant to low temperatures during germination. Enhanced tolerance of glycine betaine producing transformants to freezing as well as to high temperatures has also been reported. However, incorrect expression of compatible solute genes often causes pleiotropic effects (necrosis and growth retardation) due to disturbance in endogenous pathways of primary metabolism (Wang et al. 2003b).

Transgenic plants overexpressing genes for enzymes that are involved in oxidative protection, such as glutathione peroxidase, glutathione reductase, and superoxide dismutase have been produced too (Wang et al. 2003). The biosynthesis of cysteine, regarded as a key limiting step in the production of glutathione, has been enhanced by the overexpression of O-acetylserine(thiol) lyase. Transgenic tobacco plants showed significant reductions of both foliar and photooxidative damage in response to oxidative stress (Youssefian et al. 2001). Overexpression of the tobacco gene for glutathione S-transferase/glutathione peroxidase enhanced growth of tobacco seedlings under salt-stress treatment (Roxas et al. 1997). The seedlings also exhibited higher tolerance to chilling, indicating the relationship between plant stress responses to salt and low temperatures. Tobacco plants that were more tolerant to salt, dehydration, and heavy metal stress as well as to oxidative stress have been achieved by the overexpression of the alfalfa aldose/aldehyde reductase, involved in a detoxification pathway (Bartels 2001).

Salicylic acid (SA), an important mediator in defense response to pathogen attack in many plant species, has been shown to modulate the plant response to several abiotic stresses too. SA seems to increase the oxidative damage generated by salt and osmotic stresses as indicated by a SA-deficient transgenic *Arabidopsis* line whose germination was less sensitive to salt and osmotic stress than wildtype germination (Borsani et al. 2001).

Transcription factors play also a significant role in stress response of plants (Wang et al. 2003b). Improved tolerance of *Arabidopsis* to salt, chilling, and drought has been reported upon transfer of an *Arabidopsis* gene encoding a stress-inducible transcription factor (Kasuga et al. 1999). This transcription factor interacts specifically with a cis-acting promoter element,

the socalled dehydration response element (DRE). Expression of the transcription factor led to overexpression of several stress-inducible genes containing the DRE or related motifs, which are expected to function in protecting cells from salt and drought stress. But if the transcription factor was constitutively expressed, transformants exhibited varying degrees of growth retardation under control conditions, suggesting that stress tolerance of these plants comes at the expense of growth and productivity. The stress-inducible promoter of the stress response protein rd29A, however, allowed high-level expression of the transcription factor under stress conditions without significant growth retardation under control conditions. Recently, increased tolerance of tobacco to salt, cold, and dehydration stress has been reported after overexpression of a stress-inducible zinc-finger protein from rice (OSISAP1 *Oryza sativa indica* stress-associated protein) (Mukhopadhyay et al. 2004).

5.5.2 Cold/freezing tolerance

In the past years, several attempts to increase cold or freezing tolerance have been described, among them increased production of sugars such as raffinose (Pennycooke et al. 2003) or overexpression of multiple dehydrin genes (Puhakainen et al 2004). Transgenic plants overproducing the compatible solute glycine betaine are more tolerant to both high salt concentrations and low/freezing and high temperatures (Sakamoto & Murata 2002). Similarly, enhanced tolerance to multiple environmental stresses, such as chilling, freezing, salinity, drought, (and paraquat toxicity), could be achieved by overexpression of spermidine which is believed to be active in scavenging of reactive oxygen species (Kasukabe et al. 2004). In addition, spermidine may also act as a regulator in stress signaling pathways, as indicated by the stronger transcription of stress-responsive transcription factors, interacting with DRE, and stress-protective proteins like rd29A in transgenic *Arabidopsis*, compared to control plants.

Cold acclimation, the process by which plants increase their tolerance to freezing in response to low non-freezing temperatures, is associated with alterations in expression of several cold regulated (COR) genes that encode hydrophilic polypeptides. COR gene expression is regulated by transcription activators binding to COR gene promoter sequences which are similar or identical to DRE elements. Therefore, overexpression of a DRE binding protein transcription factor could increase cold tolerance of transgenic plants, as described by Jaglo-Ottosen et al. (1998) for transgenic *Arabidopsis* plants. If this holds true, tolerance to low temperature and perhaps other abiotic stresses might be achieved using a single gene that can act as a transcriptional regulator which activates target genes, regardless of the type of stress (Zhu 2001). Given the difficulty of the stable transfer of several genes, prospects to alter multigenic traits in plants by „throwing such a master switch“ seem attractive (Sarhan and Danyluk 1998). The biochemical functions of the target genes are mostly unknown, but some may act in stabilization of membranes and production of protecting substances such as proline and sugars (Thomashow 2001).

Several types of evidence suggest that the plant hormone abscisic acid plays an important role in the response of plants to stress and, in particular, in cold acclimation and development of freezing tolerance. Abscisic acid (ABA) regulates diverse developmental and physiological responses, including seed maturation, desiccation, dormancy, and germination (Tamminen et

al. 2001, Tähtiharju and Palva 2001). Ectopic expression of a seed-specific transcriptional activator conferred on *Arabidopsis* vegetative tissues the ability to accumulate seed-specific transcripts in response to ABA, whose enhanced expression was correlated with increased freezing tolerance in transgenic plants (Tamminen et al. 2001). Antisense inhibition of protein phosphatases involved in the regulation of ABA signalling led to accelerated development of freezing tolerance in *Arabidopsis* (Tähtiharju and Palva 2001), thus, signalling components other than transcriptional activators could be employed to increase stress tolerance in plants. Constitutive overexpression of a cold-inducible zinc finger protein from soybean induced cold-regulated gene expression and enhanced cold tolerance of non-acclimated transgenic *Arabidopsis* and tobacco plants (Kim et al. 2001). This protein, localized in the nucleus, was shown to be ABA-inducible and possibly interacts with a transcription factor.

Frost-hardy plants may increase their freezing tolerance by initiating intercellular ice formation at subzero temperatures close to 0 °C promoting gradual dehydration of plant cells. Both the cytoplasmic freezing point and the ice nucleation point within the cells may thus be depressed (van Zee et al. 1996). The authors transferred a bacterial gene for an ice nucleation protein from *Pseudomonas syringae* into tobacco and observed increased ice nucleation activity at temperatures near 0°C, but only after low-temperature conditioning for 2 days. Expression of a membrane stabilizing antifreeze protein derived from carrot in tobacco has been described to lead to increased cold tolerance of the transformant lines (Fan et al. 2002). Altering the saturation level of membrane lipids could be another approach to increase cold or freezing tolerance of plants. Reduction of saturated fatty acids can increase the chilling resistance of higher plants, as the expression of a nonspecific cyanobacterial fatty acid desaturase in tobacco, targeted to chloroplasts by a transit peptide, has indicated (Ishizaki-Nishizawa et al. 1996).

It is important to note that stress responses of transgenic plants often act unspecifically, that is, tolerant plants may have increased tolerance not only to high salts, but also to drought, cold, and, perhaps, even to heat shock, as observed again in stress tolerant tobacco transformed with a rice gene coding for a zinc-finger protein (Mukhopadhyay et al. 2004). But overactivation of stress-responsive genes can also have deleterious effects on total plant performance, because of metabolic and energy costs and disturbance of primary metabolism (Wang et al. 2003b).

5.5.3 Heat tolerance

Chaperone functions, i.e. the capacity to assist and maintain correct folding and trafficking of cell proteins, are thought to be crucial for plants to survive abiotic stresses. For this reason, genes encoding heat shock proteins and chaperones could be used for increasing heat tolerance of plants (Zhang et al. 2000). Recently Murakami et al. (2004) reported that overexpressing the small rice heat shock protein sHSP17.7 conferred both increased thermotolerance and resistance to UV-B to rice plants. The saturation level of membrane lipids may also be important: Whereas a decrease in saturated fatty acids may increase cold tolerance, enhanced heat tolerance seems to be connected to a decrease in unsaturated fatty acids. Silencing of the enzyme omega-3 fatty acid desaturase, a chloroplast enzyme

synthesizing lipids that contain three double bonds, in transgenic tobacco and *Arabidopsis* resulted in a decrease in unsaturated lipids with three double bonds and a corresponding increase in lipids with two double bonds (Murakami et al. 2000). The reduced level of lipid unsaturation changed membrane properties of thylakoid membranes, containing the light-absorbing system, and improved the rate of photosynthesis at 40 °C and plant growth at 36°C. This indicates that the level of lipid unsaturation in thylakoid membranes may be related to cold- as well as thermotolerance of plants. However, decreased lipid unsaturation may have other effects too, such as variation in chloroplast structure (Sharkey 2000).

As discussed before, with some biochemical mechanisms in common, increased tolerance to dehydration stress in plants likely leads to an increase in cold, heat, and perhaps drought tolerance simultaneously. This seems also to be the case for the protective function of glycine betaine, that, if overproduced in transgenic plants, has been reported to lead to increased tolerance to both high and low temperatures and to high salt concentrations (Sakamoto & Murata 2002).

5.5.4 Drought tolerance

An increase in drought tolerance of transgenic tobacco has been observed after transformation with a yeast gene enabling the transgenic plants to synthesize trehalose, a disaccharide whose biophysical properties and presence in desiccation tolerant organisms suggests that it is active in osmoregulation and related to desiccation tolerance (Holmström et al. 1996). The transgenic plants showed no obvious morphological changes caused by trehalose accumulation, but their growth rate was decreased by 30-50 %. According to Gaff (1996), the reported growth retardation of transgenic plants might be connected to stomatal closure in drought stressed plants. As accumulation of reactive oxygen species under stress conditions (e.g. high light intensity, heat, drought, pathogen attack etc) depends on the availability of free iron in cells, the iron-binding protein ferritin could protect plant cells from adverse effects under oxidative stress. Transgenic tobacco plants that synthesized alfalfa ferritin in vegetative tissues retained their photosynthetic function under oxidative stress. They also exhibited tolerance to necrotic damage caused by viral or fungal infections (Deak et al. 1999). The enhanced levels of ferritin possibly sequestered the cytoplasmic free iron involved in the generation of highly toxic reactive hydroxyl radicals.

5.5.5 Soil mineral/metal concentrations

In soils becoming acidic, aluminum (Al) is solubilized into the toxic trivalent cation Al^{3+} limiting plant productivity in soils with low pH. Al-tolerant plant species such as buckwheat are known to release organic acids such as citrate, oxalate, and malate from their roots in response to Al treatment and to form Al-organic acid complexes. As an approach to increased Al-tolerance of crop plants, therefore, manipulation of organic acid biosynthesis or catabolism has been suggested. De la Fuente et al. (1997) described increased citrate synthase levels and increased Al tolerance in transgenic tobacco and papaya overexpressing a bacterial citrate synthase gene. Different results, however, have been achieved in transgenic *Arabidopsis*

expressing citrate synthase (reviewed by Feng et al. 2001). Constitutive overexpresssion of alfalfa malate dehydrogenase in transgenic alfalfa resulted in significant increases in root exudation of citrate, oxalate, malate, succinate, and acetate and increased tolerance to Al toxicity, whereas constitutive overexpression of phospho*enol*pyruvate carboxylase did not lead to increased root exudation of organic acids (Tesfaye et al. 2001). In transgenic alfalfa, Al as well as phosphorus (P) contents in roots and shoots were higher, compared to non-transgenic control, the higher P content supporting the notion that release of organic acids can improve P availability in acid soils by chelating Al from Al-P complexes.

Considering the constitutive nature of the CaMV 35 S promoter used, it was unexpected that malate dehydrogenase transcripts and protein in leaf samples of transgenic plant lines were reduced, compared to untransformed plants. When grown in soil of neutral pH, the malate dehydrogenase transgenic plants showed a reduction in biomass and a slower rate of root growth, indicating that root exudation of organic acids might impose an energy cost to plants.

High soil pH, on the other hand, can lead to reduced agricultural productivity too, since in such soils essential metal ions, especially iron (Fe), are sparingly soluble and not available to plants. A prominent iron deficiency symptom is chlorosis (yellowing from partial failure to develop chlorophyll). Mugineic acids phytosiderophores (MAs) are known to be secreted by graminaceous plants to solubilize iron in the soil. The resulting iron-MAs complexes are then reabsorbed into the root. Nicotinamine synthase and nicotinamine aminotransferase are critical enzymes in the biosynthesis of MAs from L-methionine through nicotinamine (Takahashi et al. 2001). In an effort to enhance the tolerance of rice to low iron availability in alkaline soils, Takahashi and colleagues transferred a barley genomic DNA fragment containing two nicotinamine aminotransferase genes under the control of their natural promoters. Transgenic rice plants expressing the barley genes showed a higher nicotinamine aminotransferase activity and secreted higher amounts of MAs under iron-deficient conditions, leading to enhanced tolerance to low iron availability. Whereas in barley expression of the two endogenous genes is observed only in roots, transgenic rice plants expressed them both in roots and shoots, therefore, their root-specific expression was lost.

Acidic and alkaline soils also reduce uptake of phosphorus (P), an essential nutrient for plant growth, since at low pH phosphorus forms low solubility molecules with Al and Fe and at high pH it forms sparingly soluble compounds with calcium (Ca) and magnesium (Mg). Therefore, P can be largely unavailable to plants although the total amount of P in the soil may be high. An increase in the excretion of organic acids, particularly citrate, has been proposed as a potential mechanism to enhance P uptake in alkaline soils (López-Bucio et al. 2000). These authors reported higher yield of leaf and fruit biomass in transgenic tobacco plants expressing bacterial citrate synthase and overproducing citrate, when grown in alkaline soils under P-limiting conditions. Expression of a bacterial heavy metal transporter in *Arabidopsis* has been reported to enhance resistance to cadmium, lead, and zinc and decrease uptake of these heavy metals (Lee et al. 2003).

5.5.6 Environmental impacts of stress tolerance

The development of stress resistant plants has been proposed to be one of the most important means to achieve the goal of increased food production for a growing world population. But higher cold tolerance could also be of interest in woody species, as this would allow cultivation of cold-sensitive trees in northern areas (Tzfira et al. 1998). Stress resistant plants are thought to be grown, in particular, in developing countries where the need for increased food production is greatest. Poor and degraded soils, soils with high salt content, heat, and drought are the main abiotic stressors reducing crop yield in countries of the South. However, there are doubts, whether genetically engineered plants made resistant to abiotic stress will in fact lead to increased and improved food production. To achieve higher and stable yields on soils of low productivity, low-input varieties well-adapted to local conditions will be needed. Such crop varieties should show a number of traits not neccessarily selected in high-yield varieties: low fertilizer requirement, deep rooting, vigorous growth, tolerance to pathogens and pests, genetic heterogeneity, and, finally, they should be true breeding (Schmitz 2001).

In many attempts to increase abiotic stress resistance, genes have been transferred that are supposed to confer tolerance to more than one abiotic stress factor. This is, because during normal stress response to cold, freezing, drought, and salt, plants induce genes that trigger protection against water dehydration stress in the cytoplasm (Sakamoto & Murata 2002, Thomashow 2001). Therefore, the increased production of compatible solutes such as glycine betaine or the overexpression of detoxifying enzymes or proteins with chaperone function can lead to multiple stress tolerance. Tolerance to more than one stressor has also been shown for plants overexpressing transcription factors that function as a “master switch” and regulate several cold regulated genes or for plants with an alteration in the saturation level of fatty acids. Changes in the fatty acid metabolism may lead to other metabolic effects not directly related to stress tolerance. In some cases increased stress tolerance could even extend to increased pathogen or pest tolerance. There are several reports about growth retardation in stress resistant transgenic plants, indicating that stress resistance often comes at the expense of growth and productivity, particularly under less stressful conditions (Wang et al. 2003).

These data imply that transgenic stress resistant plants very often show resistance/tolerance not only to one abiotic stress factor but simultaneously to several other ones too. The fitness of such plants in stressful environments can thus be increased considerably. Stress resistant plants may outcompete other plants if they run wild and spread to natural or semi-natural habitats. As a result, diversity and abundance of other plant species could change dramatically, leading to adverse effects on the foodweb and probably also on soil life and on biodiversity in general. Crop plants resistant to a range of abiotic and perhaps biotic stress factors are also more likely to evolve volunteers and develop into weeds that might be particularly difficult to control. In particular, cold or freezing resistance of plants and their seeds will extend the range of habitats in which crops or their progeny can survive.

Another concern is that farmers would be tempted to grow stress resistant crops in ecologically valuable areas not deemed suitable for agriculture before, thus reducing biodiversity in such areas too. These concerns may be aggravated by the prospects that, according to Zhang et al. (2000), the likely next steps to achieve practical levels of plant

resistance will be the transfer of several genes, either by simultanous transformation with multiple genes or by crossing plants containing different stress tolerance genes.

There are also attempts to increase the tolerance of crop plants to low or high soil pH and to minerals toxic to plant metabolism, such as aluminium or heavy metals. Plants have been genetically engineered for increased root exudation of organic acids such as citrate, oxalate, malate, and succinate, to render them more tolerant to high levels of aluminum in acidic soils. However, such plants may show increased levels of aluminum in roots and shoots, raising concerns about potential toxicity of this plant material if used for food or feed. Non-target organisms such as wildlife feeding on such plants could also be affected. Increased expression of a range of stress-relieving proteins or other metabolites could also affect toxicity of transgenic plants to non-target organisms, but this point has been rarely discussed in the scientific literature.

Since many of the crop plants cultivated worldwide originated from regions of the South, it is very likely that transgenic crops made resistant to abiotic stress and thought to increase yield under adverse agronomic conditions would be grown in countries where a range of related species exists. Many of these countries are also known as regions of origin of important food crops, often harbouring a great number of landraces of the particular crop. Hybridization with such related species will lead to the introduction of stress resistance genes into landraces and wild populations, providing them with a selective advantage compared to other plants of an ecosystem. Valuable genetic resources might thus be endangered and weeds might be created that are difficult to control. Gene stacking of resistance genes could make things even worse. Adverse impacts on the biodiversity of such regions would likely be the result.

5.6 Phytoremediation and Biomonitoring

5.6.1 Phytoremediation

New forms of agriculture and forestry have been proposed such as remediation of contaminated soils by transgenic plants, with a particular emphasis on utilization of root-based technologies (Gleba et al. 1999). Some plant species are naturally capable of high levels of organic compound degradation or heavy metal hyperaccumulation. At least 45 plant genera are known to contain metal-accumulating species that can accumulate copper (Cu), cobalt (Co), cadmium (Cd), manganese (Mn), nickel (Ni) selenium (Se), or zinc (Zn) up to levels 100 to 1 000 times those normally accumulated by plants (Guerinot & Salt 2001). To remove toxic substances from contaminated soils more efficiently and cheaper, enhancement of such phytoremediation capacities by genetic engineering and, in particular, by transferring microbial genes for degradative enzymes has been suggested (Rosser et al. 2001, Rugh 2001), including the use of transgenic trees (Tzfira et al. 1998). According to Tong et al. (2004), one of two main strategies for metal tolerance seeks to prevent toxic metal ions from being transported across membranes by increased binding of metal ions to the cell wall, reduced uptake, or effective pumping out of the cell. The other strategy involves detoxification of metal ions through chelation, conversion to a less toxic form, or compartmentalization into the

vacuole. Increased formation of glutathion is thought to lead to increased formation of heavy metal-binding phytochelatins and may thus enhance tolerance to cadmium and other heavy metals, allowing the use of such transgenic plants to remediate contaminated soils.

In poplars, due to the expression of a bacterial glutamylcystein-synthetase, higher levels of glutathione and higher production of cadmium-binding phytochelatins have been achieved (www.biosicherheit.de/projekte/29.proj.html). Increased synthesis of phytochelatins and metallothioneins, however, is energy consuming and might affect plant growth. Transgenic poplars forming high amounts of glutathion have a higher demand of sulfur. It is not known at present, whether enough sulfur could be provided and whether soil microorganisms would be affected.

Phytoremediation of explosives such as glycerol trinitrate (GTN) by transgenic tobacco may be possible. Plants transformed with the bacterial gene for pentaerythriol tetranitrate reductase were able to grow at high concentrations of GTN (French et al. 1999, Rosser et al. 2001). Transgenic tobacco seedlings expressing nitroreductase from *Enterobacter cloacae* were described to remove the explosive 2,4,6-trinitrotoluene (TNT), an environmental pollutant more important than GTN due to its wider use and greater toxicity, from media containing concentrations of TNT toxic to wild type seedlings (Hannink et al. 2001). The substance could not be extracted from the transgenic seedlings, it remained unclear whether TNT was completely transformed or sequestered in the plant in unextractable form. Plants are known to link xenobiotic compounds such as TNT and its highly toxic metabolites to sugars, glutathione, amino acids, and malonic acid and store these conjugates in cell compartments (e.g. vacuole or cell wall). Even if mineralization in transgenic tobacco might be shown, this could not be extrapolated to other plants, as mineralization depends on endogenous plant enzyme activities (Meagher 2001).

Mercury pollution may also be remediated by transgenic plants expressing a bacterial gene encoding mercuric ion reductase (Rugh et al. 1998). This enzyme, encoded by the merA gene, reduces highly toxic ionic mercury Hg(II) to much less toxic elemental volatile mercury Hg(0). The merA gene is a member of the bacterial mer operon which encodes genes involved in detection, mobilization, and enzymatic detoxification of mercury. Yellow poplar plantlets transformed with the merA gene have been described to grow vigorously in media containing normally toxic levels of ionic mercury and to release elemental mercury at much higher rates than untransformed plants (Rugh et al. 1998). Stability of merA expression was high when flanking regions contained sequences adapted to plant codon usage. Methylmercury can be generated from ionic mercury by the activities of anaerobic sulfate-reducing bacteria at Hg(II) contaminated areas (Rugh 2001). It has higher uptake rates and retention times than ionic mercury, shows far higher toxicity, and poses serious health risks at lower dosages. The bacterial organomercurial lyase (merB) in combination with merA converts methylmercury to elemental mercury. Transgenic *Arabidopsis* plants expressing both merA and merB have been shown to grow on 50 fold higher methylmercury concentrations than plants that express merB alone (Bizily et al. 2000).

The authors argue that elemental mercury transpiring into the atmosphere will be diluted to trace concentrations before redipositing into the terrestrial substrate since the atmospheric residence time is about two years. Rugh (2001) proposed that this pathway could be conferred

to virtually any selected, environmentally important plant with particular benefit when dominant wetland species would be developed for this application. Transfer of the native bacterial operon containing the merA and merB genes into tobacco chloroplasts has been reported to increase tolerance levels to the organomercurial compound phenylmercury significantly and protect chloroplasts from mercury toxicity (Ruiz et al. 2003). However, as elemental mercury, converted in the body to ionic mercury, is not "non-toxic", it remains open at present, whether the evaporization of elemental mercury at certain sites would not damage humans and animals. Because of such uncertainties, Watanebe (2001) doubts that, in the near future, regulatory agencies could be convinced that phytoremediation by transgenic plants was safer, cheaper, and more effective than the established alternatives and he expects only niche markets for transgenic plants for recalcitrant contaminants.

5.6.2 Biomonitoring

Monitoring the presence of landmines by transgenic plants has been suggested (Stewart & Wheaton 2001). Reporter genes such as the green fluorescent protein (GFP) gene could be linked to genes encoding enzymes for uptake and/or degradation of explosives (French et al. 1999). Helicopter-based seed pelleting and remote sensing of the fluorescence expressed in leaves of such transgenic plants would be necessary (Stewart and Wheaton 2001).

A transgenic approach to biomonitoring may be based on the integration into the plant genome of a marker gene of known sequence that will serve as a target for mutagenic influences. To study the genetic effects of heavy metals, such as Cd, lead (Pb), Zn, Cu, and arsenic (As), a transgenic plant-based assay has been developed by Kovalchuk et al. (2001a). *Arabidopsis* plants carrying a glucuronidase (GUS) marker gene either with a point mutation or as a recombination substrate were used to monitor the frequency of somatic point mutations and homologous recombination. Plants growing on contaminated media exhibited a pronounced uptake-dependent increase in the frequencies of both somatic intrachromosomal recombination and point mutation. To allow detection and to reduce the possibility that selection processes influence the frequency of particular mutations, the transgene should encode an activity that is easy to visualize and that is not essential for the plant. Markers such as GUS, luciferase, and GFP have been suggested. Strong promoters active in the tissue chosen would be needed. Besides for monitoring of heavy metals, such transgenic plants might be used for the evaluation of genotoxicity of ionizing and UV radiation, of organic substances such as polycyclic aromatic hydrocarbons, and of mixtures of compounds (reviewed by Kovalchuk et al. 2001b).

5.6.3 Environmental impacts of phytoremediation and biomonitoring

Transgenic plants destined for phytoremediation could, of course, also transfer their new traits to wild relatives, thus conferring to them traits that may be of advantage under these specific conditions, e.g. heavy metal accumulation in soils. Poplars engineered to bind cadmium more efficiently by producing high amounts of glutathion need higher amounts of sulfur. Increased uptake and accumulation of cadmium and increased demand of sulfur may affect mycorrhizal

fungi. Mycorrhiza rates have been found to be significantly higher with transgenic trees (http://www.biosicherheit.de/projekte/29.proj.html). Of particular concern might be the deployment of transgenic plants that express genes for remediation of mercury-contaminated soils (Rugh 2001). In these cases, elemental mercury could be released into the atmosphere, adding to the levels already released through a number of industrial processes. It has been argued that the Hg pollution through bioremediation would be negligible in comparison to fossil fuel burning and incineration. However, although elemental mercury may remain in the atmosphere for up to two years, it will finally precipitate with rain and snow, on agricultural soils as well as on soils of natural ecosystems, on oceans, surface water, and also on the arctic and antarctic region. The precipitated elemental mercury is converted again to ionic and organic mercury. Phytoremediation of this sort may thus lead to dispersion and redistribution of mercury from contaminated soil sites to other less contaminated areas. As mercury is a highly toxic metal that can also become accumulated in the food chain, particularly in aquatic food chains, its release should not be increased by the use of transgenic plants extracting it from Hg contaminated areas.

5.7 New growth characteristics

5.7.1 Alteration of morphology

Dwarfing of grain crops may lead to increased grain yield at the expense of straw biomass, as the introduction of new wheat varieties in the 1960s and 1970s has shown. These wheats are short because they show an altered response to the plant growth hormone gibberellin. Mutant dwarfing allelles found in wheat, maize and *Arabidopsis* are orthologues encoding proteins that are altered in a gibberellin signalling domain. Transformation of rice plants with an *Arabidopsis* dwarfing allele resulted in plants with dwarfed phenotype (Peng et al. 1999).

Recently, it has been suggested to engineer the major food crops of cooler climates, wheat, rye, and barley, such that they should develop cobs like maize, thus increasing yield. Of the major genetic factors that may have been involved in the evolution of the branched and grass-like maize progenitor teosinte to cob-bearing maize, three have been characterized. These genes seemingly act to change the architecture of the plant from branched to the single-stalk form, to eliminate the hard casing of the teosinte kernels, and to contribute to the production of kernels around the entire circumference of the ear (Lev-Yadun et al. 2002). Transfer of such genes to wheat, rye, and barley might result in new types of crops.

Agrobacterium rhizogenes-mediated transformation causes a range of phenotypic changes that are usually regarded as undesirable. In the horticultury industry, however, some morphological alterations such as dwarfing or altered flowering could be regarded as desirable. Hairy root regenerants may show shorter stature due to reduced internode distances, altered root architecture, and increased rooting. Increased leaf and branch production and increased production of essential oils have been observed (Christey 2001). In addition to alterations in vegetative morphology, plants transformed with *A. rhizogenes* can show alterations in their life cycle. Flowering may be accelerated, delayed, or even inhibited, flower

number can be increased. Perrennial or biennial plants may become annual or loose their flower capacity, also precocious flower formation without the need of vernalization seems to be possible. Increased rooting capacity may be of particular interest for the rooting of cuttings from woody plants.

More specific alterations of morphology and/or development can be obtained by the transfer of single rol genes. rolA is reported to cause leaf wrinkling, shortening, and reduced flowering. In tobacco, its promoter has been shown to be active throughout the vegetative and floral phases, but it is silent in seeds (Guivarc'h et al. 1996a). rolB may be responsible for increased auxin sensitivity and increased rooting, for example in woody plants transformed with rolB (Sedira et al. 2001). The rolC gene has been used widely for the alteration of plant morphology. Expression of rolC can result in a wide variety of morphological and developmental changes, including dwarfness, shorter internodes, increased branching, reduced flower size, lack of apical dominance, reduced fertility, reduced chlorophyll content, and altered leaf and stem size leading to a generally smaller and more bushy phenotype (reviewed by Christey 2001). The rolC protein is a β-glucosidase capable of hydrolysing *in vitro* cytokinin O-and N-glucosides, thus liberating free cytokinins from conjugated forms. Histological changes underlying the macroscopical modifications of growth habit in rolC transgenic plants appear also in areas far away from the site of expression of the rolC gene, indicating a diffusible agent (Guivarc'h et al. 1996b). Phenotypic alterations seem to increase in severity with increasing rolC transcript abundance. Since increased rooting and dwarfing may be of interest for rootstocks of fruit trees, the formation of transgenic rootstocks for apple, orange, pear, and cherry has been attempted (Christey 2001). Trees such as aspen have been transformed with rolC resulting in traits such as dwarfness, breaking of apical dominance, reduced growth rate, and altered leaves, changes in wood formation have also been noted (Tzfira et al. 1998).

Since rol genes affect many developmental processes, specific alterations will be difficult to achieve. As expression of the rolC gene also influences the hormone metabolism of transgenic plants, other biosynthetic pathways and developmental characteristics may be affected too. For instance, rolC expressing poplars had altered contents of saccharose. Transgenic poplars have been shown to be more susceptible to frequently occurring fungal diseases of aspen, with pathogen attack linked to levels of fructose and glucose in leaves (http://www.biosicherheit.de). Similarly, infestation by sooty mould, fungi that use leaf sugars, has been linked to sugar contents in leaves. Phenotype loss has been observed in transgenic poplars, caused by gene silencing and independent segregation (Fladung et al. 1997).

5.7.2 Growth/flowering acceleration

Acceleration of germination, growth, and flowering has been a goal of plant breeding for some time. Genetic engineering may provide means to influence some of these processes. Constitutive expression (CaMV 35S promoter) of floral control genes from *Arabidopsis* such as LEAFY and APETALA1 in transgenic plants has been reported to lead to premature flower formation in *Arabidopsis* and aspen, in poplar, abnormal flowers have been formed (Mandel

& Yanofsky 1995, Weigel & Nilsson 1995). LEAFY may encode a developmental switch that converts shoot meristems into flower meristems and APETALA1 seems to act downstream of LEAFY to specify meristem identity. Transgenic plants overexpressing both LEAFY and APETALA1 show transformations of apical and lateral shoots into flowers. This results in alterations of the vegetative rosette, including leaf curling, and in a significant reduction of the time to flowering. LEAFY expression in rice, again under the control of the CaMV 35S promoter, caused early heading in transformants, with a heading date 26 – 34 days earlier than in wildtype plants, but some yield penalty and panicle abnormalities have been observed (He et al. 2000). On the other hand, a late-flowering and highly branched phenotype has been observed in transgenic rice transformed with *Arabidopsis* genes controlling the timing of phase transition from shoot to flower (Nakagawa et al. 2002).

To control tuber sprouting in potato may be of considerable economic importance, since suppression of sprouting during storage would provide more flexibility to the potato industry and stimulation of sprouting could be of advantage in situations where it is desirable to minimize the dormancy period. Acceleration of tuber sprouting by 6 – 7 weeks has been observed in potato transformed with a bacterial pyrophosphatase gene controlled by the tuber specific patatin promoter (Farré et al. 2001). As inorganic pyrophosphate plays an important role in starch degradation and sucrose biosynthesis as well as in an array of biosynthetic reactions, increased activity of phyrophosphatase may exert an effect not only on tuber sprouting but also on other metabolic processes. The transgenic potato lines showed no changes in plant growth and tuber size or number, but the density of both developing and mature tubers, a direct and reliable indication of biomass production, was found to be significantly reduced, due primarily to lower starch contents, reduced by 30-40 %.

Trees are characterized by prolonged juvenile phases, extending over several years, during which the trees are unable to produce flowers and fruits. The extended maturation period of fruit and nut trees makes breeding of new strains time-consuming and difficult, as traits such as yield and quality of fruits cannot be assessed during this time. Therefore, reduction of generation time in trees could have significant economic implications for the tree fruit sector (Egea-Cortines & Weiss 2001). Transformation of citrus with flower promoting genes from *Arabidopsis*, controlled by the 35S CaMV promoter, resulted in citrus plants with a shortened juvenile period and flower and fruit production as early as the first year (Peña et al. 2001). Citrus plants constitutively expressing both the LEAFY gene and the APETALA1 gene showed a reduction of juvenile traits and early flowering. Flowers induced by LEAFY and APETALA1 in citrus were normal and fertile, in contrast to the abnormal flowers produced by LEAFY in transgenic poplar (Peña et al. 2002). Whereas LEAFY transgenics often showed growth inhibition and alterations of leaf size and form as well as a weeping growth habit, apparently correlated to the level of accumulation of LEAFY transcript, APETALA1 transgenics displayed more normal growth.

5.7.3 Lignin alteration/reduction

Global need for wood products (e.g. timber for construction, paper and pulp, or energy) will most likely not decrease in the near future. To lower the pressure on existing forests it has

been suggested to increase forest productivity by genetic engineering of forest trees (Tzfira et al. 1998). Forest-tree genetic engineering could aim at altered trunk characteristics and wood quality as well as altered root-system and tree-canopy performance and increased pest and stress tolerance. Since lignin must be removed in pulp and paper production, reducing the lignin content or altering its composition to lignins that are more easily extractable have been considered to be beneficial to the pulping process. Lignin, a key component of all vascular plants, is a complex phenolic polymer that is thought to result from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors or monolignols giving rise to p-hydroxyphenyl, guaiacyl, and syringyl units of the lignin polymer which differ in the extent of methoxylation. This variety of subunit substitution patterns can lead to highly heterogenous lignins varying with tissue type, location within the plant, developmental stage, environmental influence, and species (Campbell & Sederoff 1996, Wu et al. 2000, Vogel & Jung 2001). Because of their insolubility and complexity, lignins play an important role in plant defense. The lignin-biosynthesis pathways seem to be interrelated to the pathways leading to other carbohydrates, to flavonoids, and to growth-stimulating activities (Hu et al. 1999).

Several attempts have been made to alter the activity of enzymes crucial in the lignin biosynthesis pathways. Cinnamyl alcohol dehydrogenase (CAD) is thought to be a key enzyme in the lignification pathway because it catalyzes the last step in the biosynthesis of the lignin precursors, which is the reduction of cinnamylaldehydes to cinnamyl alcohols. In transgenic poplar, antisense suppression of CAD has led to lignin more easily extractable but the transgenic plants showed red xylem (Campbell & Sederoff 1996, Tzfira et al. 1998). Antisense suppression of O-methyltransferase (OMT), involved in syringyl lignin precursor synthesis, has resulted in reduction of the syringyl:guaiacyl ratio and in the formation of a novel lignin component (Tzfira et al. 1998).

4-coumarate:coenzyme A ligase (4CL) converts precursors such as coumaric acid into their corresponding thioesters for the formation of syringyl or guaiacyl monolignols. Downregulation of 4CL in transgenic aspen (*Populus tremuloides*) by antisense inhibition has been reported to lead to plants that show substantially reduced levels of lignin (up to 45 % reduction depending on the level of enzyme expression) and a significant increase of cellulose which altered the cellulose:lignin ratio in the most severely downregulated lines from 2 to 4 (Hu et al. 1999). 4CL suppression did not alter the syringyl:guaiacyl ratio or promote the incorporation of unusual monomeric units into lignin, it led, however, to an increased integration of precursors into the nonlignin cell wall constituents. In addition to these observed changes, ten month old plants of transgenic lines with suppressed 4CL activity showed thicker stems, longer internodes, larger leaves, and an overall increased stem and root growth. Reduction of carbon flow into the lignin pathway may have increased the availability of carbon for cellulose deposition. Therefore, suppression of lignin synthesis could be used as a strategy to increase cellulose deposition in woody species. Recently, cotransformation of poplar with an antisense 4CL gene and a sense gene for coniferaldehyde 5-hydroxylase, an enzyme playing a key role in limiting syringyl monolignol biosynthesis, resulted in plants with up to 52 % less lignin, a 64 % higher syringyl:guaiacyl ratio and 30 % more cellulose (Li et al. 2003).

Generally, inconsistent results have been obtained after antisense suppression of enzymes involved in lignin biosynthesis in trees and herbaceous plants such as tobacco (Vogel & Jung 2001). As many of the enzymes and reactions in the lignin-biosynthetic pathway and their relationship to other carbohydrate pathways in different forest trees are still unknown, impacts on quantity, composition, and localization of lignin and other vital plant constituents are possible. However, such impacts will be hard to predict, in particular, because of the high environmental heterogeneity of sites on which trees are growing. In addition, since lignins also serve important functions in plant resistance to pests and pathogens (Campbell & Sederoff 1996), altered lignin content and composition may influence the ability of forest trees to resist pest and pathogen attack.

Improved conversion or digestibility of forages for ruminants can increase the digestible energy intake of animals leading to increases in per animal milk or meat production (Vogel & Jung 2001). Cell wall digestibility of herbaceous plants has been attributed partly to lignin content and composition. Therefore, it has been suggested to alter lignins in forage grasses and herbaceous plants. Studies undertaken with antisense O-methyltransferase (OMT) and CAD transgenic tobacco or alfalfa, however, did not give consistent results with regard to digestibility. Cell wall digestibility may also vary with environmental conditions, as inferred from antisense CAD transgenic lines that showed increased digestibility when grown in the greenhouse but not when grown under field conditions. In addition, transgenic herbaceous plants that exhibited significant reductions in lignin content showed some abnormal growth and development possibly associated with a weakened vascular system (reviewed by Vogel & Jung 2001). Reduced lignin concentration or increased digestibility could result in increased winter mortality or reduced biomass, as studies done with alfalfa and three grass species that had been bred conventionally for reduced lignin content, indicated (Casler et al. 2002).

5.7.4 Yield increase

Plant biotechnologist also aim to modify photosynthesis to achieve increases in net carbon gain in crop plants such as wheat or rice that assimilate carbon through the C3 pathway of photosynthesis. In C3 photosynthesis, oxygen inhibition leads to a reduction in photosynthetic efficiency under current atmospheric conditions, due to the oxygenase reaction of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the subsequent loss of CO_2 from photorespiration. Plants such as maize that show C4 photosynthesis overcome oxygen inhibition by concentration of CO_2 at the site of Rubisco, thus suppressing its oxygenase activity and the associated photorespiration. A special leaf anatomy, the characteristic “Kranz” anatomy, seems to be crucial for concentration of CO_2. Since in the C4 pathway phosphoenolpyruvate carboxylase (PEPC) plays an important role in fixation of CO_2, Ku et al. (1999) transformed rice plants with an intact maize gene encoding C4 specific PEPC. High levels of expression of the maize PEPC accounting for up to 12 % of total soluble protein in transgenic rice plants have been reported. Although the transgenic rice plants with high levels of expression of the maize C4 enzyme exhibited reduced sensitivity of photosynthesis to oxygen, their photosynthetic rates were comparable to those of the untransformed rice cultivars under atmospheric conditions. As enzymes involved in C4 photosynthesis may also play a role in plant defense responses to biotic and abiotic stress, overexpression of C4

enzymes in C3 plants might confer enhanced tolerance under stress conditions (Ku et al. 1999). In addition, changes in leaf structure would be neccessary.

In the Calvin cycle, the primary pathway for carbon fixation, levels of fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) are very low compared to levels of other enzymes involved. It has been proposed that FBPase and SBPase are at important strategic positions in the Calvin cycle to determine the partitioning of carbon to end products. Overexpression of genes encoding these enzyme activities, therefore, might increase photosynthetic capacity (Miyagawa et al. 2001). For this reason, a gene from cyanobacterial cells encoding a unique enzyme, fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase), that can hydrolyze both FBP and SBP with almost equal specific activity, has been transferred to tobacco. Since the gene encoding FBP/SBPase has no homology to FBPase and SBPase genes derived from higher plants, it might not be subjected to gene silencing. Overexpression of cyanobacterial FBP/SBPase, targeted to tobacco chloroplasts, enhanced both photosynthetic capacity and carbohydrate accumulation under atmospheric conditions, and accelerated growth rate (Miyagawa et al. 2001).

Bogorad (2000) and van Bel et al. (2001) suggested modifications of plant metabolism, such as enabling plants to fix nitrogen and improving photosynthetic CO_2 fixation using plastid transformation. Since nitrogen fixation requires large amounts of energy, it has been proposed ATP producing chloroplasts may be suitable sites for engineering such capacities into plants. Improving CO_2 fixation would require the coordinated modification of the photosynthetic activity of plants which does not seem to be an easy task.

5.7.5 Environmental impacts of new growth characteristics

Transgenes altering growth characteristics such as fruiting patterns or crop architectures may have implications for pest population dynamics and pest control by parasitoids and predators, as pest organisms and beneficial organisms could be influenced by premature flowering and fruiting or by altered plant shape (Way & van Emden 2000). Altered growth characteristics are often connected to alterations of hormone pathways that in turn influence other biosynthetic pathways. In rolC expressing poplars showing shorter stature and small and bright green leaves, altered contents of saccharose have been detected. Compared to control plants, transgenic lines have been more susceptible to pathogenic fungi in the field. Degree of fungal infection was apparently correlated to levels of fructose and glucose formed in the transgenic poplars. Sooty molds that use sugars without being pathogens were more prevalent on those transgenic lines that produced higher amounts of fructose and glucose, laboratory studies confirmed these field observations (http://www.biosicherheit.de/projekte/105.proj.html).

Because lignins play an important role in plant defense reactions (Campbell & Sederoff 1996), changes in lignin content and composition may also alter the ability of plants to resist pest and pathogen attack in ways not foreseeable easily. Trees having reduced lignin contents might also exhibit reduced mechanical strength and altered water conducting properties. As the lignin-biosynthesis pathways seem to be interrelated to a number of other pathways (such as carbohydrate and flavonoid pathways) and to growth-stimulating activities (Hu et al. 1999),

in transgenic plants producing altered lignins substances other than lignins could be changed in quantity and quality, potentially exerting impacts on soil life, arthropods and other animals. Plant material that has lower lignin content could also be degraded more rapidly, changing decomposition processes in soil. In crop plants such as maize, sorghum, and perennial forage, a reduction in lignin content generally leads to yield depression. Long-term survival in some perennial species and time to flowering may also be affected (Pedersen et al. 2005). Reduced lignin concentration or increased digestibility could also increase winter mortality (Casler et al. 2002). Gene spread from transgenic trees via pollen and seed would be inevitable, unless GM tree cultivation was tightly controlled to inhibit GM tree flowering and fruiting. Transfer of traits altering lignin content and composition to wild relatives would almost certainly affect break down of plant material and could influence decomposition processes in soil in unpredictable ways.

If traits leading to reduced generation times will be transferred to related wild plants, they might confer a fitness advantage, as plants possessing such traits could grow under less favourable climatic conditions with shorter growing seasons or could produce two generations in areas normally sustaining only one. If, in future, enzymes involved in C4 photosynthesis might be successfully expressed in C3 cereal plants, they might confer not only increases in net carbon gain but also enhanced tolerance to biotic and abiotic stress conditions (Ku et al. 1999). Since most cereal species have a range of wild relatives, some of them weeds that can cross with the cultivated species, fitness enhancing traits could be easily transferred to wild grasses. Because of increased fitness, crop plants expressing such traits likely carry a greater risk of running wild or becoming volunteers.

5.8 Production of new or altered substances/secondary metabolites

5.8.1 Production of new or altered substances

Genetic engineering allows the production of new substances not usually synthesized in plants or in a particular crop species, often termed molecular farming. Levels and composition of a range of basic plant products such as amino acids, proteins, fatty acids, and carbohydrates can be modified (Mazur et al. 1999, Somerville & Bonetta 2001). Such new or altered products could be used in food or non-food production. The deployment of transgenic plants might also be a component of „green chemistry“ efforts, but metabolic engineering of multi-step pathways and significant use of primary plant metabolites will likely be required (Slater et al. 1999, Poirier 1999). Since field crops producing large amounts of starch but comparatively low amounts of protein, such as potato and sugar beet, are more productive than oil producing plants, Somerville & Bonetta (2001) argued, it would be useful to learn how to modify these plants so that they accumulate oil rather than starch. To accomplish this, the authors suggested alteration of the cellular identity of root or tuber cells so that they take on the identity of oil producing cell types. In cases where excretion of new substances from plant tissue would be of interest, the accumulation potential of trichome glands may be exploited to produce natural products (Wang et al. 2001).

Attempts to alter carbohydrate quantity and quality by antisense inhibition of the enzyme granule-bound starch synthetase (GBSS) have led to transgenic potatoes producing amylopectin only. A range of transgenic potato lines with other alterations of their carbohydrate metabolism, such as production of altered starches and fructan have been developed and field tested too (Becker et al. 1999, Herbers & Sonnewald 1996, http://www.biosicherheit.de/projekte/). Selected fatty acid production for food, feed, or non-food products is one of the goals of plant biotechnology (Mazur et al. 1999). Recently, expression of up to five different enzymes derived from fungi, algae, and plants active in fatty acid metabolism has been reported to lead to production of very long-chain polyunsaturated fatty acids (omega-3 and omega-6 fatty acids) in transgenic *Arabidopsis*, linseed, and soybean (Qi et al. 2004, Domergue et al. 2005). These polyunsaturated fatty acids are known to be important for human health and are present in high concentrations in fish. Commercial production of such transgenic crops is supposed to relief oceanic fish stocks.

Stearate (18:0), used in large amounts for the production of shortening and margarine, is currently produced by hydrogenation of plant oils leading to significant amounts of *trans* fatty acids (due to their enzymatic stereospecificity plants synthesize only *cis* fatty acids). To avoid the generation of *trans* fatty acids which have been associated with health risks, the creation of oil crops capable of accumulating high levels of stearate has been suggested. Transfer of a gene encoding an acyl-acyl carrier protein (ACP) thioesterase from *Garcinia mangostana* led to increased amounts of stearate in oilseed rape. By site-directed mutagenesis of ACP thioesterase, stearate content could be increased even more (Facciotti et al. 1999). In soybean, higher levels of monounsaturated oleic acid (18:1) have been achieved by cosuppression of endogenous desaturase and levels of other fatty acids have been altered by genetic engineering of crops such as oilseed rape (Mazur et al. 1999). However, overexpression of a *Cuphea hookeriana* thioesterase specific for synthesis of the medium-chain fatty acids octanoic acid (8:0) and decanoic acid (10:0) in oilseed rape did not result in efficient incorporation of these fatty acids into seed lipids, although total 8:0 and 10:0 acyl CoA pool size had been increased (Larson et al. 2002). Similar results have been obtained for an oilseed rape line overexpressing a thioesterase specific for dodecanoic acid (12:0). Thus the high proportion of medium chain CoAs in the cytosol was not translated into a high proportion of these fatty acids in the storage lipids, perhaps because medium chain acyl CoAs are not efficiently utilized for lipid synthesis. Larson et al. (2002) postulated that, if individual acyl CoAs accumulate above a certain threshold, feedback mechanisms or catabolism may limit the transfer of acyl CoAs into seed lipids, leading in effect to a futile cycle of fatty acids.

During the past decade, methods have been developed to modify grain crops such as wheat and rice, allowing the introduction of new traits into these important food crops. Gluten, a continuous proteinaceous network that is formed in the wheat dough from seed proteins called prolamins, is a major determinant of dough and breadmaking quality. Various targets for genetic modification of gluten properties according to the needs in bread, pastry, or noodle production have been suggested (Vasil & Anderson 1997). Modifying grain texture may be another goal of genetic engineering leading to reduced milling costs and enhanced digestibility. Transfer of wheat genes for puroindolines, believed to play critical roles in wheat grain texture, to rice has been reported to lead to enhanced softness of rice grains

(Krishnamurthy & Giroux 2001). Expression of such puroindolines may also confer pathogen resistance to rice, as these proteins have shown strong antimicrobial properties in vitro.

For industrial or medical purposes biomaterials such as spider silk proteins may be of interest since they show high tensile strength and elasticity (Moire et al. 2003). Recombinant spider proteins, encoded by synthetic genes and showing >90 % homology to native spider silk, have been reported to accumulate in transgenic tobacco and potato leaves and tubers, respectively, up to a level of about 2 % of total soluble protein (Scheller et al. 2001). Expression in other plant organs, such as seeds, may provide higher protein amounts.

The use of transgenic plants for the production of biodegradable polymers, such as PHAs, polyesters of hydroxy acids that are synthesized by a number of bacteria, may lower production costs (Poirier 1999, Somerville & Bonetta 2001, Moire et al. 2003). The most widespread and well-characterized PHA, poly(3-hydroxy-butyrate) (PHB) is stiff and brittle and, therefore, not well-suited for many commodity products. Since levels in transgenic plants have been low, efforts aim at the creation of transgenic plants synthesizing polymers with better characteristics and higher yields. By expressing four distinct transgenes of bacterial origin and targeting the enzymes to plastids, Slater et al (1999) hoped to produce the potentially more useful polymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in plastids of *Arabidopsis* and oilseed rape seed, but levels were low and not commercially viable. The reasons for low polymer levels are unknown, perhaps modifying the isoleucine biosynthetic pathway created a metabolic burden on the plant or the transformation method, using two vectors, translated into insertional effects. In general, increased levels of PHB, produced in the cytoplasm or in chloroplasts, seem to be correlated with negative effects such as stunted growth and leaf chlorosis (Moire et al. 2003).

5.8.2 Iron and mineral fortification

Genetic engineering of food plants has been proposed to increase nutrient and mineral content and to create designer crop plants that require minimal fertilizer input, but provide maximal nutritive value. The completion of model plant (*Arabidopsis)* and crop plant (e.g. rice) genome sequencing allows the identification of numerous genes encoding transporter and storage proteins for minerals that could be used for increasing the mineral content of plants (Sussman 1999).

Iron (Fe) deficiency is a nutritional problem afflicting a considerable portion of world population, particularly in regions where diets are based primarily on vegetables. To achieve iron fortification of food crops, the transfer of genes for the Fe-binding protein ferritin (present in animals, plants, and bacteria) seemed promising. Ferritins are multisubunit proteins that form hollow spheres storing up to 4 500 Fe atoms in a soluble, nontoxic, and bioavailable form (Drakakaki et al. 2000). Expression of the soybean ferritin gene under the control of the rice glutelin promoter in transgenic rice led to endosperm-specific enrichment of the iron content (Goto et al. 1999). The authors speculate that the iron content in a meal-size portion of transgenic ferritin rice (about 5,7 mg Fe/150 g DW) would be sufficient to provide 30-50 % of the daily adult iron requirement (13-15 mg Fe). Drakakaki et al. (2000) observed increased Fe levels in vegetative tissues of wheat and rice transformed with the

soybean ferritin gene controlled by the constitutive maize ubiquitin-1 promoter, but not in seeds. Although mRNA levels were similar, ferritin protein and iron levels between wheat and rice tissues were different, indicating that results from one cereal cannot be extrapolated to another species. Some of the ferritin-expressing rice lines showed chlorosis in older leaves and severely reduced fertility. Ferritin expressed ectopically may also lead to reduced susceptibility of transgenic plants to oxidative damage and pathogens. Expression of ferritin has, therefore, been suggested to improve stress and pathogen tolerance and at the same time increase Fe content in food crops. With respect to ferritin expression, Briat (1999) pointed out that ferritin overaccumulation in transgenic tobacco leaves leads to excessive Fe sequestration and the activation of Fe transport systems.

Additional strategies suggested for iron fortification include the development of transgenic plants that mobilize minerals in the rhizosphere more easily by releasing organic acids such as citrate, capable of mobilizing iron, or by releasing phytosiderophores that chelate soluble Fe present at low concentrations in soils (Guerinot & Salt 2001, Grotz & Guerinot 2002). Another strategy might be to transfer genes encoding divalent cation transporters capable of transporting Fe from the rhizosphere into the root. As these transporter proteins also mediate the transport of other cations such as manganese (Mn), zinc (Zn) and cadmium (Cd), plants overexpressing such transporters may show unwanted accumulation of metals other than iron, leading to potentially toxic metal levels in food and feed. Acording to Grotz & Guerinot (2002), the overexpression of ferritin should be coupled with the expression of modified versions of the Fe(II) transporters that might show different specificities for Fe and Cd. Guerinot & Salt (2001) described food fortification and phytoremediation to be the two sides of the same coin.

Decreasing phytate content in grains by the overexpresssion of phytase has been proposed to overcome malnutrition and to reduce pollution. Phytate (myo-inositol(1,2,3,4,5,6)-hexakisphosphate), the major storage form of phosphorus in seeds, is broken down during germination by endogenous phytase enzymes. It is a strong chelator of cations such as calcium, magnesium, iron, copper, and zinc, and forms salts that are largely excreted by humans and non-ruminant animals, reducing mineral uptake. Grains overexpressing fungal phytase may therefore help to alleviate iron and zinc deficiency and to reduce phosphorus excretion in animal waste (Raboy 2001, Grotz & Guerinot 2002, Brinch-Pedersen et al. 2002). Additional strategies for increased phosphate and mineral uptake and bioavailability may involve antisense approaches to block genes involved in phytic acid biosynthesis and engineering of plants to secrete phytases from their roots for mobilization of phosphate soil reserves (Brinch-Pedersen et al. 2002). Lucca et al. (2001) suggested to combine in rice the ferritin approach with the expression of *Aspergillus fumigatus* phytase and overexpression of the endogenous cystein-rich metallothionein. As fungal phytase expressed in rice showed low thermostability, crops for which long cooking is required, would not benefit from such an approach.

Lowering phytate content in grains and other plant material can lead to lower yields and have other unwanted effects. As phytate, besides serving in phosphorous and mineral storage, probably also plays a role in other cellular functions, e.g. in ATP metabolism, DNA double-strand repair, and mRNA export from the nucleus (Raboy 2001), reducing its content may

affect the plant metabolism considerably. Recently, Oltmans et al. (2005) reported about significantly reduced seedling emergence in non-transgenic soybean lines with reduced phytate content. And phytate in crops seems to have beneficial health effects too, for example as an anti-cancer agent and anti-oxidant (Raboy 2001, Brinch-Pedersen et al. 2002).

5.8.3 Secondary metabolite production

Plants produce a wide array of socalled secondary metabolites in a species specific manner with only early parts of most pathways being common to most plants. These substances are involved in pathogen and pest resistance (Dixon 2001), attraction to pollinators, interaction with microorganisms, including symbiontic ones, and determine the quality of food (e.g. taste, color) and ornamental plants (e.g. smell, flower color). More over, secondary metabolites are known to function as vitamins (e.g. tocopherol, vitamin E) and show health improving effects, such as flavonols acting as antioxidants, and glucosinolates and some carotenoids thought to be protective against certain kinds of cancer. Others function as flavors, fragrances, pesticides, dyes etc. Secondary metabolites such as nicotine are of great commercial importance, other alkaloids are used as drugs or as antibacterial agents. Isoflavones such as genistein and daidzein may act as phytoestrogens and therefore be used for treatment and prevention of hormone-related disorders in humans (Humphreys & Chapple 2000).

This has led to growing interest in secondary metabolism and the means to alter it by genetic engineering (Verpoorte & Memelink 2002). Alteration of secondary metabolites may aim at improving resistance against pests or diseases by increasing the amount of endogenous defense compounds, introducing novel compounds toxic to the pest organisms and/or pathogens, or acting as repellants (Dixon 2001). Phytochemicals that are extracted from the plant or that raise the quality of the product could be increased in their levels, undesired (toxic) compounds in food and fodder may be reduced or removed. The use of antibodies to modulate specific enzymes in the secondary metabolism of plants with the aim of reducing the amount of certain compounds has been suggested too (Verpoorte et al. 2000). Since, however, in most cases very little is known about the biosynthesis of these compounds (perhaps with the exception of flavonoids and anthocyanins), transgenic plants with altered secondary metabolism are still rare (DellaPenna 1999, Verpoorte et al. 2000, Verpoorte & Memelink 2002).

Since secondary metabolites or new compounds expressed in plants can be toxic to man and animals, for risk assessment extensive metabolic profiling of the transgenic plant would be essential. However, as Verpoorte et al. (2000) point out, no universal methods exist for making the complete profile of secondary metabolites in a plant, with chances being high that minor compounds will not be observed. In addition, secondary metabolism is species specific, only the first steps of the pathways leading to specific products will be common to other pathways.

In many plants, compartmentation plays a major role in regulation of these pathways requiring different cellular compartments or cell types for production and storage of compounds and intermediates, a fact which has some implications for the choice of promoters driving the genes of interest. In cases where increased levels of secondary metabolites might

be toxic to the cell, the use of inducible promoters has been suggested. Differences in transport of new compounds in heterologous systems may exist too.

Depending on the objective to produce more or less of a certain compound or to synthesize a new one, strategies will have to be different (Verpoorte et al 2000). Both single and multiple genes have been transferred, sometimes leading to effects on pathways other than the ones desired. In transgenic *Arabidopsis* overexpressing the Y-tocopherol methyltransferase, the large pool of Y-tocopherol was converted to alpha-tocopherol, leading to a nine-fold increase in vitamin E activity (Grusak 1999). Transgenic tobacco plants overexpressing a tobacco branching point enzyme of the alkaloid synthesis pathway showed increased nicotine content and altered levels of other alkaloids, whereas transgenic tobacco lines with cosuppression of the enzyme produced very small amounts of nicotine, as expected, but they showed pronounced changes in phenotype and reduced fertility (Sato et al. 2000).

Branches of the phenylpropanoid pathway of secondary metabolism lead to anthocyanin pigments, lignins and isoflavones. Production of the isoflavone genistein, normally present in legumes such as soybean, has been observed in the non-legume *Arabidopsis thaliana*, transformed with the gene encoding soybean isoflavone synthase, leading to the conclusion that naringenin which is an intermediate of the anthocyanin pathway seems to be also available as substrate for the introduced isoflavone synthase too (Jung et al. 2000). The expression of petunia chalcone isomerase in tomatoes resulted in fruit containing increased flavonol levels (Muir et al. 2001). In attempts to improve the market properties of flax oil, Lorenc-Kukuła et al. (2005), transformed flax to simultaneously overexpress chalcone synthase, chalcone isomerase, and dehydroflavonol reductase. They reported about significantly increased phenolic compounds and antioxidant capacity in transgenic flax which was accompanied by higher resistance to *Fusarium* species, an increase in monounsaturated fatty acids and lignans, and higher yield and earlier flowering date. The overexpression of dehydroascorbate reductase, an enzyme responsible for regeneration of ascorbic acid (Vitamin C) has been reported to increase vitamin C content in maize and, unexpectedly, led to an increase in the glutathione level (Chen et al. 2003).

Carotenoids are natural pigments that act as potent antioxidants and, as vitamin A precursors, are also essential compounds for the visual system. More than 600 different carotenoids are produced by microrganisms and plants (Schmidt-Dannert et al. 2000). Increased carotenoid levels, but also unexpected changes in content, composition and site of carotenoids produced have been observed in transgenic plants transformed with bacterial and plant genes encoding enzymes of the carotenoid pathways, for instance in tomato (Römer et al. 2000) and in tobacco (Mann et al. 2000). In some cases new not yet analysed carotenoids have been found. In generation of “golden rice”, bacterial and plant genes have been transferred leading to an increased ß-carotin content, but also to a variable carotenoid pattern (Ye et al. 2000). In future, molecular breeding of carotenoid biosynthetic pathways may be used to create new carotenoids not produced in nature as has been reported for *Escherichia coli* (Schmidt-Dannert et al. 2000).

The use of regulatory genes, encoding e.g. transcription factors, has also been proposed for the alteration of secondary metabolism, since such regulatory genes may act as master switches for (part of) a complete pathway (Verpoorte et al. 2000). To reduce levels of

undesired compounds such as toxins or antifeedants (e.g. cyanogenic glucosides or glucosinolates), antisense gene suppression (or sense gene suppression) of a step of the pathway has been attempted – with mixed success.

However, as plants contain many genes encoding transcription factors that, in addition, show combinatorial interactions, simple overexpression of a single transcription factor may not lead to the desired result but rather to unexpected alterations in secondary metabolite composition. According to Verpoorte et al. (2000), little attention has so far been paid to the effect of blocking a secondary metabolite pathway through cosuppression or antisense genes on the accumulation of other secondary metabolites.

5.8.4 Environmental impacts of new substances and secondary metabolites

In general, plants modified to produce altered or new compounds that can be used by pest species may be more susceptible to pest attack, which could be particularly relevant for transgenic plants producing increased amounts of carbohydrates or fatty acids. Schmitz (2001) reported about studies addressing this question in corn and oilseed rape. In the rhizosphere of potatoes producing only amylopectin, bacteria using substrates with high molecular weight have been found to increase and occurrence of *Pseudomonas* bacteria has been altered. For fungal populations differing compositions at certain time points have been described too, with a tendency to higher proportions of *Fusarium*-like fungi (http://www.biosicherheit.de/projekte/77.proj.html). In fructan producing potatoes that have higher levels of soluble sugars, variations of susceptibility to plant disease have been observed under controlled laboratory conditions, but not in the field. Fructan producing potatoes exhibited shorter shoots and prolonged juvenile development which may impact their competitive ability in the field. This in turn might require more stringent weed control. Survival in winter, germination and reaction to drought stress appeared unchanged (http://www.biosicherheit.de/projekte/9.proj.html). If alterations in level and composition of sugars, starch, and oil did increase freezing tolerance and dormancy, dispersion of such transgenic plants might be enhanced spatially and temporally (Schmitz 2001).

Expression of other traits may result in unexpected effects such as increased pathogen resistance, as has been suspected of ferritin-producing rice (Drakaki et al. 2000) or for rice with enhanced softness (Krishnamurthy & Giroux 2001). The increase in antioxidant capacity in transgenic flax was accompanied by higher resistance to fungal pathogens, ~3 weeks earlier flowering, and increased yield due to higher seed weight and higher seed number (Lorenc-Kukuła et al. 2005). If such traits were transferred to wild relatives, fitness and fecundity of their progeny could be enhanced substantially.

New substances could be released through root exudation into the soil, perhaps also into groundwater and aquifers and could provide new substrates for soil microorganisms – with unknown effects up to now. Gene transfer from crops carrying these traits to wild relatives could result in introgression of undesired traits into the genomes of wild plants, potentially also affecting natural ecosystems.

In cultivation of transgenic plants that produce new or altered compounds not destined for food or feed, a great concern would be to keep such plants and their products separate from the food chain. Plants supposed to produce plastic polymers, spider fibre, or other material destined for industrial use would fall into this group. Hybridization and commingling with crop varieties of the same species would have to be excluded, a task that will be extremely difficult for most crops used as food or feed, given the experience gained with contamination up to now and the StarLink case in the US in 2000 (Andow et al. 2004). Seed of non-GM plants would also have to be kept free from contamination by such transgenic seeds – a problem even more pressing than in the case of transgenic plants intended for food and feed. On the other hand, Moire et al. (2003) argue, that the large-scale agricultural production of polymers, e. g. polyhydroxy acids (PHA), likely will only be viable through the recovery of all other valuable components of the crop. This would mean that in the case of an oil- or carbohydrate-producing crop such as oilseed rape or sugar beet, both polymer and oil or sucrose, respectively, would have to be recovered. To avoid contamination of the food or feed chain by such polymers might become very difficult in such cases.

Safety concerns may also exist with other transgenic crops, even those that have been modified to enhance their nutritional quality. The data collected so far suggest that engineering of secondary metabolite biosynthesis pathways can lead to unintended changes in the content of other secondary metabolites. Knowledge about individual pathways and the many connections between different pathways of secondary metabolism is very restricted (Verpoorte et al. 2000, Sandmann 2001). Even in the case of carotenoid synthesis, the biochemistry of which has been well established over the past decades, connections of carotenogenesis to other metabolic pathways may not be known. Unexpected effects upon transformation of plants with carotenoid pathway genes have been observed. The “golden rice” project is such an example, where several genes have been transferred to lead to ß-carotin production in rice endosperm (Ye et al. 2000).

Knowledge about pathways and their regulation, the enzymes involved, and connections to other metabolic pathways is even more restricted in the case of alkaloids and other secondary metabolites. Unexpected effects of genetic engineering may be more pronounced. As a result, transgenic plants could show variations of phenotypes, growth characteristics, and fertility as well as altered susceptibility to pests and pathogens. In addition, secondary metabolites new to a plant species or produced in altered quantities may prove to be toxic for the transgenic plant and/or to organisms the plant usually interacts with, such as soil organisms, arthropods, and higher animals. As secondary metabolism is species specific, data gained with one species need not be valid for another species. Secondary metabolites, e.g. alkaloids, can also be toxic to humans and lifestock, or they can potentially exhibit mutagenic activity.

Burkitt (2001) cautioned that food plants artificially enriched in flavonols and related polyphenols may have to be studied carefully with respect to potential mutagenic activity of these compounds. Extensive metabolic profiling of the transgenic plant and its products would be essential (Burkitt 2001, Verpoorte et al. 2001). But universal methods for making a complete analysis of secondary metabolites do not exist, compounds present in lower concentrations might not be found. The transformation of soybeans to increase contents of tocopherol (vitamin E), a powerful antioxidant thought to protect cells and tissue from

damage by neutralizing free radicals (Sattler et al. 2004), may not be “the” solution for increasing human (or animal) health, as recent reports about potential harm to human health by excess vitamin E indicate (Gould 2004).

Grain enriched in iron has been proposed to alleviate iron deficiency, but, if for that reason cation transporting proteins, capable of iron transport from the rhizosphere into the root, are expressed in food crops, then toxic cations could also be accumulated (Briat 1999). Since activation of a ferrous iron transporter seems to be responsible for cadmium (Cd) loading of pea plants and iron transporter proteins also mediate the transport of other cations such as manganese (Mn), zinc (Zn) and Cd, plants overexpressing such transporters may show unwanted accumulation of metals other than iron, leading to potentially toxic metal levels in food and feed. With respect to ferritin expression, Briat (1999) pointed out that ferritin overaccumulation in transgenic tobacco leaves leads to excessive Fe sequestration and the activation of Fe transport systems.

But even if in a single case all changes in level and composition of secondary metabolites that are of importance for human health could be analysed, this would not neccessarily mean, that cultivation of such a transgenic plant would be safe for animals and the environment as such. Pollinators, phytophagous, predatory, and beneficial insects, as well as mammals and birds, perhaps also soil organisms, could still be affected by the altered composition of secondary metabolites in plants they were originally adapted to. Communication with other organisms via volatile substances could be impeded. New types of interaction of such plants and their environment will likely occur, potentially leading to adverse impacts on biodiversity. Again, if transgenic crops altered in secondary metabolism can hybridize with wild relatives, secondary metabolism of wild plants will also be affected, and this will be all the more unpredictable, as different genetic backgrounds will influence the expression of the transgenes and reactions of enzymes and substrates.

5.9 Gene pharming

In the past years numerous attempts have been made to engieneer crop plants such that they could be used as production plants – or bioreactors – for medical and pharmazeutical products. Compared to the production of pharmaceuticals in bacterial or mammalian systems, the use of transgenic plants may have potential advantages particularly from a financial point of view. Daniell et al. (2001c) cited as reasons for using transgenic plants,

1. Plant systems could be more economical than industrial facilities using fermentation systems.

2. Technologies for harvesting and processing plants and their products on a large scale are available.

3. Purification requirements could be eliminated when the plant tissue containing the recombinant protein is used as a food (e.g. edible vaccines).

4. Plants could be directed to target proteins into intracellular compartments in which they are more stable, or even to express them in certain compartments such as plastids.

5. Amounts of recombinant products can approach industrial-scale levels.

6. Health risks arising from contamination with potential human pathogens or toxins could be minimized.

The range of potential plant hosts available and the proposed stability of transgenic lines in seeds have been cited as additional attractions to this technology. Ma and Hein (1995) speculated on the use of transgenic seeds containing medicinal compounds that might be exported to deprived areas of the world. These could then be grown within the existing agricultural infrastructure rather than require the funding of new pharmaceutical factories. In addition, genetic recombination by sexual crossing of transgenic plants was recommended as a simple method to introduce new genes or to accumulate multiple foreign genes into plants.

By addition of signal sequences, recombinant proteins can be targeted to the endoplasmic reticulum and for secretion into the apoplastic space. This is expected to inhibit interference with cellular functions, to protect from intracellular processing, to increase accumulation levels, and to facilitate extraction (Ma & Hein 1995, Giddings et al. 2000, de Kathen 2001). To overcome the problem of extraction and purification of proteins from biochemically complex plant systems, secretion based systems such as rhizosecretion, relying on the ability of plant roots to exude compounds, have been proposed (Borisjuk et al. 1999, Gleba et al. 1999). Chloroplast transformation could lead to high expression levels of pharmaceuticals (Fischer et al. 2004, Stoger et al. 2002). Oleosins (small, abundant seed-specific proteins embedded in the phospholipid monolayer of seed oil bodies) may serve as carriers for recombinant proteins and ease purification by centrifugation, as was shown for the hirudin-oleosin fusion protein expressed in oilseed rape (Parmenter et al. 1995). The use of pollen-specific promoters could direct the expression of recombinant proteins to pollen which are collected by insects such as bees. Recombinant proteins may then be „harvested“ from honey. To exclude pollen transfer and escape of insects such „production systems“ would have to be located in safe glasshouses (de Kathen 2001).

For the expression of certain biopharmaceuticals (e.g. vaccines), Daniell et al. (2001c) suggested to use seed crops, e. g. cereals, as target species, since these crops produce high levels of proteins which are stable during storage. Expression in seeds and tubers under the control of seed- or tuber-specific promoters would also allow processing of biopharmaceuticals in facilities distant from the field and continually all year, rather than for just a few large batches. On the other hand, using green tissue (of tobacco, soybean, lettuce, or alfalfa) would have the advantage of sheer productivity and may provide an alternate use for the hazardous crop tobacco. However, purification of recombinant proteins from plants such as tobacco would require removal of a variety of metabolites, including nicotine, which could be inefficient and expensive. Therefore, after surveying various crops for potential protein recovery, economics of production, and established technologies for transformation and breeding, many of the companies developing transgenic plant expression systems have chosen corn (Giddings et al. 2000, Streatfield et al. 2002). In tobacco, transient expression based on the use of plant viruses as expression vectors may also be feasible (Fischer et al. 2004).

Possible strategies for genetic engineering of glandular tissue development and metabolism, aimed at production of commercially important chemicals have been reviewed by McCaskill & Croteau (1999). Fischer & Emans (2000) and de Kathen (2001) compared characteristics of the different production systems for recombinant proteins, including transgenic plants, plant viruses, yeast, bacteria, human and animal cell cultures, insects and baculoviruses, and transgenic animals with regard to cost, quality, and application. In his databank search, de Kathen (2001) found that only about 10 % of the applications were filed by public research institutions and that in 75 % of the applications details on the constructs used were classified as „confidential business information", meaning that publicly available data are quite limited. Transformed plant species include potato, tobacco, tomato, spinach, lettuce, soybean, cowpea, alfalfa, lupin, corn, rice, barley, wheat, rapeseed, *Arabidopsis*, sugarbeet, banana, and cucumber. Features and lists of transgenic plants expressing biopharmaceuticals (antibodies, vaccines, and other proteins for human health) including the source of transgenes and intended uses (Fischer & Emans 2000, Giddings et al. 2000, de Kathen 2001, Daniell et al. 2001c, Carter et al. 2002, Stoger et al. 2002, Ma et al. 2003, Warzecha & Mason 2003, Fischer et al. 2004) and of companies that develop and market biopharmaceuticals (Tokar 2001) have been published.

5.9.1 Antibodies

In contrast to bacterial expression systems, plants have the ability to assemble immunoglobulins (Giddings et al. 2000). Applications such as passive immunization with antibodies produced in transgenic plants may exploit the potential for scaleup to agricultural proportions (Ma 2000). Antibodies expressed in plants (also dubbed „plantibodies") include, among others, fully assembled whole immunoglobulins, antigen-binding fragments of immunoglobulins, and synthetic single-chain variable fragment gene fusions (scFv). To date, several antibodies have been made that are potentially useful as human therapeutics. One of these, a tobacco-produced antibody against a surface antigen of *Streptococcus mutans*, which is a causal agent of tooth decay has been tested in humans by topical application to teeth (Fischer & Emans 2000, Giddings et al. 2000, de Kathen 2001, Daniell et al. 2001c, Carter et al. 2002, Stoger et al. 2002, Warzecha & Mason 2003, and references therein). Other antibodies, produced in soybean, were directed against herpes-simplex-virus (Zeitlin et al. 1998) and, expressed in rice and wheat, against tumor cells.

Plant-specific N-glycosylation can limit the use of recombinant glycoproteins of mammalian origin since effector functions and stability of antibodies depend at least in part on their correct glycosylation. In addition, there is concern about the potential allergenicity of antibodies, glycosylated in a plant-specific manner, when used as human therapeutics (Daniell et al. 2001c, de Kathen 2001).

To humanize plantibodies, the gene for human ß1,4-galactosyltransferase (GalT), neccessary for conversion of typical plant N-glycans into mammalian N-glycans, has been transferred to the tobacco genome (Bakker et al. 2001). In some of the transgenic plants the human enzyme was functional, leading to modification of the glycosylation machinery. Despite the numerous plant proteins that had apparently acquired N-glycans with terminal ß1,4-galactose residues,

unusual to plants, obvious changes in the physiology of the transgenic plants have not been observed. Crossing of GalT tobacco plants with plants expressing the heavy and light chain of a mouse antibody resulted in the expression of a plantibody exhibiting partially galactosylated N-glycans, about as abundant as when the same antibody is produced by hybridoma cells. Compared to simultaneous transformation, sexual crossing may result in a higher yield of recombinant antibodies due to coordinate expression and assembly of the individual heavy- and light chain (Warzecha & Mason 2003). The developmental stage of the plant tissue producing heterologous proteins may influence the pattern of glycosylation, as analysis of transgenic tobacco expressing a mouse immunoglobulin G antibody indicates (Elbers et al. 2001).

5.9.2 Vaccines

The transient or stable production of edible vaccines in transgenic plants that can be eaten raw, such as tomato, lettuce, and banana, has been proposed to be an economical and attractive alternative for human or animal vaccine production, compared to cell culture systems. Such vaccines could be administered easily, without a needle and syringe and would not require refrigeration, a fact that might be important in many developing countries with a lack of specific facilities (Giddings et al. 2000, Ma 2000, Chargelegue et al. 2001, Warzecha & Mason 2003). Other crops, e. g. potato and corn, might be suitable too. Streatfield et al. (2002) suggested corn should be used, because of the established technologies for corn transformation and breeding and for processing of corn seed into a palatable form. By optimizing codon usage for plants and by using leader and specific polyadenylation signals, the expression of vaccine components in transgenic plants could be increased. Favored locations for the expression of selected subunit vaccine components are the cell surface, the endoplasmic reticulum, and the Golgi body (Richter et al. 2000, Daniell et al. 2001c). Multivalent (multiple antigen epitopes against different serotypes of a pathogen species) or multicomponent vaccines (antigens from different pathogen species) might be produced either by co-transformation or by sexual crossing (Carter et al. 2002).

A number of vaccines has been generated against different animal and human viruses (such as rabies virus, foot-and-mouth disease virus, hepatitis B virus, human cytomegalovirus, Norwalk virus) and bacterial enterotoxins of V*ibrio cholera* and enterotoxigenic *E. coli.* A few of these vaccine candidates have been tested in clinical trials or in animal trials. For example, vaccines produced in potato and lettuce and directed against hepatitis B virus, Norwalk virus, and pathogenic *E. coli* LT-B have shown immunogenic properties upon oral administration to volunteers or in clinical trials (reviewed by Fischer & Emans 2000, Giddings et al. 2000, de Kathen 2001, Daniell et al. 2001c, Tacket et al. 2000, Carter et al. 2002). Cholera toxin B (CTB) has been expressed in transgenic tomato leaves and fruit (Jani et al. 2002), the CTB subunit pentamer expressed in potato tuber tissues was reported to induce specific antibodies in orally immunized mice (Arakawa et al. 1998a). Cooking of the transgenic potatoes, however, led to a reduction of CTB of about 50 %. The B subunit of the heat-labile toxin (Lt-B) of enterotoxigenic strains of *E. coli* has been reported to be expressed in corn seed up to 1,8 % of total soluble protein, if targeted to the cell surface, and to be increased further by breeding (Streatfield et al. 2002). In addition, the Lt-B pentamer stored in

corn seed was reported to be much more resistant to heat than the pure protein. Fusion antigens, consisting of CTB subunit, enterotoxic rotavirus and enterotoxigenic *E. coli* antigens, were assembled into cholera holotoxin-like structures upon expression in transgenic potato tubers (Yu & Langridge 2001). Orally immunized mice generated significant levels of antibodies and about three quarters of their neonate offspring showed reduced diarrhea symptoms following rotavirus challenge, but protective activity of the vaccine against enterotoxigenic *E. coli* and cholera has not been reported

Expression levels are generally low and sometimes variable between different plants of the same line or even within the same plant, for example between different tubers of the same potato plant (Mor et al. 1998). There are other major challenges regarding the control of dose and quality, the requirement of adjuvants (probably required also for oral vaccines), and the overall control of the immune response. The chosen antigen may also show instability in the stomach and intestine, leading to considerable variation of the actual dose delivered to the gut between subjects (Streatfield et al. 2002). Ma (2000) and Warzecha & Mason (2003), therefore, proposed that plant-based vaccines would be unlikely to be delivered by consuming fresh produce, but rather by processed, uniform packaged products.

5.9.3 Proteins as pharmaceuticals

Biopharmaceuticals and human proteins that have been expressed in transgenic plants (mostly tobacco, potato, and tomato) include human serum albumin, lactoferrin, alpha-lactalbumin, and ß-casein, human hemoglobin, interleukin, α- and β-interferon, somatotropin, granulocyte-macrophage colony-stimulating factor, alpha-1-antitrypsin, lysozyme, collagen, and others. Expression was generally very low (data on production systems can be found in Fischer & Emans 2000, Giddings et al. 2000, de Kathen 2001, Daniell et al. 2001c). Higher expression levels could potentially be achieved by secretion to the apoplast in potato tubers (Farran et al. 2002) or by the expression in chloroplasts, as has been shown in tobacco, where chloroplasts expressed human somatotropin in a soluble biologically active form up to a level of about 7 % total soluble protein, several hundred fold higher than expressed by a similar gene using nuclear transformation (Staub et al. 2000). Other examples for transgenic production of human proteins include a potato-produced cholera toxin B subunit-insulin fusion protein that is reported to suppress development of autoimmune diabetes in mice by directed binding to the gut-associated lymphoid tissue (Arakawa et al. 1998) and oilseed rape transgenic for hirudin reported to be grown commercially in Canada by SemBioSys (Giddings et al. 2000). Controlled by the tuber-specific patatin promotor, variable expression of human serum albumin in potato has been observed, the highest values ranging up to 0.2 % of total soluble tuber protein, with double transgenics showing generally lower contents (Farran et al. 2002).

Production of human milk proteins in transgenic plants has been suggested as a cost-effective means to improve infant nutrition and prevent gastric and intestinal diseases in children (Chong et al. 1997). Human lactoferrin, an iron-binding glycoprotein with both antimicrobial and immune regulatory activity, has received considerable interest as a pharmaceutical potentially to be produced in transgenic plants. The expression of the lactoferrin gene under the control of the enhanced CaMV 35S promoter fused to the auxin-inducible mannopine-

synthase P2 promoter resulted in lactoferrin levels in potato tubers of about 0.1 % of total soluble plant protein (Chong & Langridge 2000). Antimicrobial activity of potato tuber extract against different human pathogenic bacterial strains was observed. Lactoferrin production in transgenic crop plants might therefore be an example for the transition from biopharmaceuticals to nutraceuticals, a transition that will be observed in future more often (de Kathen 2001).

5.9.4 Safety aspects

Safety aspects relate to the safety of the biopharmaceutical derived from transgenic plants when it is used as intended as well as in un-intended use by humans and animals. Expression of proteins in heterologous systems can lead to defective amino acid sequences, premature chain termination, and aberrant processing. Chances for defective translation may be higher when the transferred DNA sequences do not correspond to the codon usage of the recipient organism. Plant chaperones may be unable to fold the heterologous proteins correctly, particularly under stress conditions such as heat and drought or pest and pathogen attack. Alternatively, chaperones suited for correct folding of large and complex proteins may be lacking altogether. Changes of protein conformation can lead to altered physicochemical properties of proteins. Therefore, proteins of human origin produced in transgenic plants may not be completely identical in amino acid sequence and structure to the corresponding human protein, as reports about biopharmaceuticals with altered activity or shortened derivatives indicate (de Kathen 2001). β-casein expressed in potato was smaller and less phoshorylated than β-casein from human milk, possibly lacking a leader sequence (Chong et al. 1997). The physiological state of the transgenic plant (e.g. senescence) might also have an impact on integrity and glycosylation states of therapeutic proteins (Stoger et al. 2002).

Most therapeutic proteins require posttranslational modification such as proteolytic cleavage and glycosylation for correct functioning (Gomord & Faye 2004). Correct glycosylation of e.g. antibodies may not be achieved by the plant-specific N-glycosylation machinery. Plant-derived recombinant proteins tend to lack the terminal galactose and sialic-acid residues normally found in mammals (Ma et al. 2003), they will rather be glycosylated in a plant-specific manner. In addition, the homogeneity of the glycosylation of plant-made pharmaceuticals can differ from one expression system to another and from one developmental stage to another within a single plant (Gomord & Faye 2004). In contrast to long-standing experience with glycosylated natural plant proteins in food, experience with the oral application of human-encoded proteins combined with plant N-glycan epitopes is lacking. To what extent human glycosylation can be achieved in transgenic plants and whether such “humanized” products could help solving these problems remains open. There is also concern about the potential allergenicity of plantibodies when used as human therapeutics (Daniell et al. 2001c, Carter et al. 2002). β(1,2)-xylose and α(1,3)fucose residues are constituents of glycol-epitopes of some plant allergens that cause immune reactions (Gomord & Faye 2004). Accidental ingestion of autoantigens by disease-free individuals might actually exacerbate autoimmune disease, Carter et al. (2002), therefore, suggest to make transgenic “medicinal” plants identifiable by the addition of genes encoding different-colored plant pigments.

Whether differences in glycosylation and protein conformation are of significance in every single case and create safety concerns is presently unknown. According to de Kathen (2001) data on unintended alterations of antibody and vaccine proteins are hardly available, studies are rarely undertaken or published.

5.9.5 Environmental impacts of gene pharming

Environmental effects of gene pharming can be related both to the recombinant pharmaceutical product and to the transgenic plants that may acquire new unexpected traits. Transient expression of pharmaceuticals via plant viruses in the field could also create environmental concerns (Warzecha & Mason 2003), since genetically engineered plant pathogens might be transferred to other plants und potentially recombine with viruses naturally infecting these plants. Pharmaceutical proteins newly expressed in plants can show cryptic functions leading to unexpected changes in plant metabolism. Transgenic tobacco transformed with the hemoglobin gene from the bacterium *Vitreoscilla* exhibited enhanced growth with 80 – 100 % higher dry weight after 35 days and significantly reduced germination and growth times. Transgenic plants contained on average 30 - 40 % more chlorophyll and showed altered secondary metabolite production. In particular, nicotine content was increased by a third and the level of the alkaloid anabasine was decreased substantially (Holmberg et al. 1997). The authors suggest that increased oxygen content in the transgenic plant material may enhance the respiration rate and thus be responsible for enhanced germination and growth. If oxygen was a limiting substrate in secondary metabolism of plants, increased oxygen availablility could lead to redistribution in accumulation of secondary metabolites such as nicotine that requires oxygen in its final synthesis. Thus expression of heterologous proteins leading to increased oxygen availability, possibly results in altered concentrations of plant secondary metabolites. In analogy to alterations of secondary metabolism, (unintended) alterations of these pathways could also affect other plant traits such as fitness traits and susceptibility to pathogens and pests, as alkaloids are important mediators in resistance (de Kathen 2001). Avidin, for instance, has been shown to have insecticidal properties endowing plants that produce it with increased pest resistance (Kramer et al. 2000). But non-target and beneficial insects could be harmed too if they feed on avidin-expressing plants or consume insects that have fed on them. Altered susceptibility to pathogens could be connected to other transformations as well, as indicated by transgenic tobacco expressing human lactoferrin that showed delayed onset of symptoms after infection with *Ralstonia solanacearum*, a plant pathogenic bacterium (Mourgues et al. 1998).

Biopharmaceuticals usually elicit responses at low concentrations and may be toxic at higher ones. Some have physicochemical properties that might cause them to persist in the environment or bioaccumulate in living organisms, possibly damaging non-target organisms, particularly if they are environmentally persistent, lipophilic molecules that can pass through cellular membranes (Giddings et al. 2000). Interferone and related proteins are very potent substances and could lead to unwanted effects if transgenic plants expressing them are ingested unintentionally by humans or animals. There is little knowledge about the effect of potent pharmaceuticals released into the environment and even less about the possible

interactions of such substances and the plants expressing them with soil life, arthropods, small mammals, and birds. Pharmaceuticals active in low doses could reach aquatic ecosystems and be transferrd to surface water and groundwater, potentially showing up in drinking water too. It is unknown at present, to what extent the food web will be affected by the culture of biopharmaceutical-expressing plants and whether such plants would show enhanced fitness. As preventive measure it has been suggested to induce expression of pharmaceuticals post-harvest, to restrict transgene expression to particular organs (e.g. seeds) or developmental stages, or to express fusion proteins that are active only after cleavage, as shown for the oleosin-hirudin fusion protein expressed in oilseed rape (Parmenter 1995, Giddings et al. 2000, Ma et al. 2003).

Of major concern is the potential of gene transfer from pharm crops to other plants by hybridization with food and feed crops or wild relatives, but also the potential contamination of neighbouring or succeeding crops. Physical isolation measures such as geographical isolation of breeding and cultivation sites, differential planting seasons, the use of dedicated planting and harvesting equipment have all been suggested as additional safety levels. Genetic containment systems may include male-sterile and transplastomic plants, "Terminator systems", and the use of varieties easily identified by their pigmentation such as white tomatoes or maize (Mascia & Flavell 2004, Ma et al. 2003). Transgenic plants expressing biopharmaceuticals might be grown predominantly, if not exclusively, under greenhouse containment, particularly if outcrossing food crops should be involved (de Kathen 2001). Underground molecular pharming in mine shafts, reported recently, may be one such possibility (Neugebauer 2005). As the isolation of crops producing pharmaceuticals and other potentially dangerous substances must include all aspects of the development and production processes, from breeding and testing to commercial production, this will involve many procedures and much additional cost. The perceived economic advantages of biopharmaceutical production in plants in terms of low cost and large-scale production (Giddings et al. 2000, Ma et al. 2003, Mascia & Flavell 2004), however, might be considerably reduced if culture of such plants has to occur under more stringent conditions than previously assumed. More stringent culture conditions would most likely also preclude local production of edible vaccines in the developing world.

5.10 Concluding remarks

In summary, there are many possible ways through which transgenic plants can exert (positive and negative) impacts on the environment and on biodiversity in general, and these impacts will vary spatially and temporally from GM plant to GM plant and from region to region into which these plants will be introduced. The number of introduced plants, the frequency of introduction, and the size of cultivated areas will play a significant role too. Impacts will depend not only on the genes and traits that have been transferred but also on the plant species transformed, their growth, reproduction, and dispersal characteristics, their interactions with other organisms, and their cultivation and distribution patterns. Uninteded alterations of gene activity and plant physiology have to be taken into account too, be they due to position effects of transgene integration and/or pleiotropic effects of transgene products. It is well known that gene activity strongly depends on the genomic context into which the transgenes have been

transferred and on environmental stimuli that influence gene regulation. Epigenetic processes that regulate gene activity in organisms through interaction between genes, external and internal environment have received more attention recently. Without the need to alter DNA sequences different individual phenotypes can result from these processes, making predictions about environmental impacts of transgenic plants even more difficult.

It should be noted here that when the European Parliament and the Council decided on the EU Directive 2001/18 on the deliberate release into the environment of GMO they acknowledged that transgenic organisms can exert a range of effects on the environment, some of which may be irreversible. They also laid down that the precautionary principle should be followed in the environmental risk assessment of the release of genetically modified organisms. Directive 2001/18, therefore, requires that in GMO risk assessment not only direct and immediate environmental effects be considered, but also indirect and delayed effects as well as cumulative and long-term effects relating to accumulated effects on flora, fauna, soil functions, biodiversity, the food/feed chain, human and animal health, and resistance problems.

It often takes years until environmental impacts and effects on biodiversity induced by the introduction of a new organism or a change in agricultural production systems become visible. It may even take longer till such effects are widely recognized and accepted. To find remedies will be difficult, if not impossible. Decades and centuries of experience with foreign species in new habitats remind us that our capacity to predict ecological impacts of introduced species as well as our ability to predict long-term and higher-order interactions are extremely limited (Wolfenbarger & Phifer 2000). In addition, unidentified impacts may exist. Even if key experiments might allow certain conclusions, the complexity of ecological systems undoubtedly will not allow prediction of future effects with certainty, the more so, as chance happens to play an important role for many interactions in ecosystems.

6 Secondary environmental impacts of transgenic plants

6.1 Evolution of resistance in weeds

6.1.1 Herbicide resistant weeds

Reliance upon herbicides as the primary method of weed control in cropping systems will inevitably lead to the appearance of HR weeds as data collected in the past decades have shown. Herbicide resistance in weeds has increased dramatically in the past 30 years, with the first report about a weed resistant to the synthetic auxin dichlorophenoxyacetic acid (2,4-D) in 1957 (Heap 2000). The time period for the evolution of herbicide resistance can be as short as three years from commencement of herbicide use (Powles & Preston 1995). Plants can develop various mechanisms of herbicide-resistance, such as target site insensitivity, target site overproduction, herbicide detoxification, reduced herbicide entry, reduced herbicide translocation, and changes in the intracellular compartmentation of herbicides (Holtum and Powles 1992, extensive literature listed in http://www.weedscience.org). As of September 2005 a total of 304 herbicide-resistant weed biotypes of 182 species (110 dicots and 73 monocots) were recorded (http://www.weedscience.org).

Most of these resistant weed biotypes show resistance to ALS inibitors (herbicides inhibiting acetolactate synthase, 93 cases) and triazines (atrazine and others, 65 cases), but in total eight weed species resistant to glyphosate are listed too: the monocots rigid ryegrass (*Lolium rigidum*), goosegrass (*Eleusine indica*), and Italian ryegrass (*Lolium multiflorum*), and the annual broadleaf species horseweed (*Conyza canadensis*), hairy fleabane (*Conyza bonariensis*), common ragweed (*Ambrosia artemisiifolia*), and buckhorn plantain (*Plantago lanceolata*). Such ecotypes have been described at several sites after repeated glyphosate application in orchards, vineyards, cropland, wheat and soybean. Recently, an additional case, glyphosate resistant Palmer amaranth (*Amaranthus palmeri*) infesting about 230 ha of RR cotton in Georgia, has been confirmed (http://www.weedscience.org). Glyphosate-resistant rigid ryegrass was first found in 1996 in Australia, meanwhile 41 different sites are known (Wakelin et al. 2004). Other biotypes have been described since then in California and South Africa, one of them showing resistance to two other herbicide groups with different modes of action (MOA) as well. Glyphosate-resistant ecotypes of goosegrass (multi-resistant) have been observed for the first time in Malaysia (1997), ecotypes of Italian ryegrass showed up in Chile (2002), Brasil (2003), and the US (2004). Glyphosate-resistant buckhorn plantain and hairy fleabane were found in South Africa (2003), hairy fleabane also in 2004 in Spain (Doll 2000, Heap 2000, vanGessel 2001, http://www.weedscience.org).

Resistant horseweed has been observed in soybean for the first time in 2000 in Delaware. Such biotypes have become an increasing problem in a number of other US states such as Kentucky, Tennessee, Indiana, Maryland, Missouri, New Jersey, Ohio, Arkansas, Mississippi,

North Carolina, and Pennsylvania infesting up to 90.000 ha of soybean fields and some cotton fields, one biotype is also resistant to ALS inhibitors (http://www.weedscience.org, Robinson 2002, Trainer et al. 2005). In 2004, glyphosate resistant common ragweed has been observed for the first time in soybean crops in Missouri. Varying tolerance to glyphosate has been reported for some weed cultivars such as lambsquarters (*Chenopodium album*) and red fescue (*Festuca rubra*) (Mortimer 1993), and also for field bindweed (*Convolvulus arvensis*) (VanGessel 2001), dead nettle (*Lamium purpureum*) (Heard et al. 2003b), and tropical spiderwort in HR cotton (*Commelina benghalensis*) (Culpepper et al. 2004).

No glufosinate-resistant weed biotypes have been recorded so far (http://www.weedscience.org), though weed species with lower sensitivity to glufosinate such as dead nettle (*Lamium purpureum*), common fumitory (*Fumaria officinalis*), or violet (*Viola arvensis*) are known (Champion et al. 2003, Heard et al. 2003b, Jansen et al. 2000, Hommel & Pallutt 2000, Nap and Metz 1996). Glufosinate seems to be less effective on monocot weeds, particularly as they become larger (Bohan et al. 2005). Poplars, transformed for elevated level of glutamine synthetase (the target enzyme of glufosinate) to enhance nitrogen utilization, have been found to be more resistant to glufosinate (Gressel 2000).

Generally, glufosinate and glyphosate are considered low risk herbicides for the evolution of herbicide-resistance. Mutations at the substrate binding site of glutamine synthetase, the target enzyme of glufosinate, were thought to result in low-fitness biotypes or to be lethal (Böger 2000). Development of resistance to glyphosate was thought unlikely due to its chemical structure, mode of action, lack of residual activity and lack of soil persistence. In addition, limited uptake of glyphosate from the soil by plants and its use pattern were cited as reasons, why application of glyphosate was supposed not to lead to evolution of resistance in weeds (Jasieniuk 1995, Heap 2000, Baylis 2000). But, according to Robert & Baumann (1998), it seemed foolish to assume that resistance to a herbicide with a single biochemical target in vivo would not develop, given time. Since public concern about the ability of plants to develop resistance to glyphosate has been assuaged, “isolated” incidences of spontaneous glyphosate resistance may have gone unnoticed. Prior to the introduction of HR crops, glyphosate was applied in combination, or in sequence, with other herbicides that reduced pressure for selection of resistant biotypes. With the advent of HR technology and the widespread use of RR soybean, however, many no-tillage fields have been treated only with glyphosate (VanGessel 2001).

The resistant biotypes of *L. rigidum*, *E. indica*, and *C. canadensis* are at least eight to twelve fold more resistant to glyphosate than susceptible ones (Pratley et al. 1999, Feng et al. 1999, Lee & Ngim 2000, VanGessel 2001, Koger et al. 2004, Trainer et al. 2005), the resistant biotypes of *L. multiflorum* have been shown to be two to six fold more resistant (Perez & Kogan 2003). Possible mechanisms of resistance include the overexpresion of the target enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), a different sensitivity of EPSPS to glyphosate, and reduced glyphosate translocation. The reported doubled level of EPSPS could explain (at least in part) glyphosate-resistance in *L. rigidum* biotypes (Gressel 2000). The glyphosate resistance in some *E. indica* biotypes has been shown to be due to an altered binding site preventing glyphosate from binding, a mechanism that was considered unlikely to confer resistance to glyphosate to weedy plants (Jasieniuk 1995). Point mutations

in a 279 bp region of the EPSPS gene analysed lead to a proline to serine or to threonine substitutio, respectively, at amino acid 106 of the EPSPS enzyme (Ng et al. 2003). However, EPSPS of another resistant goosegrass biotype did not show this kind of substitution indicationg that resistance may also be conferred by other mutations. Glyphosate resistance in goosegrass and horseweed seems to be governed by an incompletely dominant, single gene located in the nuclear genome (Ng et al. 2004, Zelaya et al. 2004). Altered cellular transport of glyphosate such as reduced translocation to the critical meristematic zones can also be responsible for increased resistance, as shown for resistant rigid ryegrass biotypes from Australia (Wakelin et al. 2004). Feng et al. (2004) reported about altered translocation in resistant biotypes of horseweed from Delaware and Tennessee too. Glyphosate loading into the apoplast and phloem was delayed and reduced and reduced plastidic import resulted in less-efficient EPSPS inhibition. Wakelin et al. (2004) suggest that a phosphate pump may play a role in glyphosate translocation across the plasmalemma, but exact mechanisms are not known.

Based on these observations, it is reasonable to assume that resistance to glyphosate will also develop in other weed species, if this herbicide is used regularly on a considerable proportion of crop fields. Gressel (1996) pointed out that there are few constraints to weeds evolving resistance to glyphosate and that weeds possess the biological characters that allow resistance evolution in most management systems relying on a single pesticide.

Resistant weed biotypes need not be poorer competitors than susceptible ones. No fitness differential between susceptible and resistant biotypes of *Lolium rigidum* could be detected (Mortimer 1993). Emergence of herbicide-resistance may occur more or less simultaneously in different locations. Thus widespread herbicide-resistance need not be a consequence of a spread from a few initial sites but may rather result from independent evolutionary events on a geographically large scale. According to Mortimer (1993), pollen transfer can also contribute to spread of herbicide-resistance, as is suggested by the detection of resistant *L. rigidum* biotypes in unsprayed areas adjacent of sprayed farmland (although, some selection pressure may also be exerted by herbicide spray drift from fields nearby).

To delay resistance evolution in weeds, the combination and rotation of weed management methods have been recommended (Buhler 2002, Ghersa et al. 2000, Kropf & Walter 2000, Bastiaans, et al. 2000, Heap 2000, HRAC 2000, Ballare & Casal 2000, Canola Connection 2000, Long 1999). Essential measures would be:

- crop rotation changing the composition of weed populations
- reduced herbicide use and rotation of herbicide mode of action (MOA) reducing selection pressure
- rotation of cultural practices reducing reliance on herbicides
- alternating sowing times giving crops a competitive advantage over relevant weeds
- “integrated pest management” (IPM) adapted specifically for weed management
- more elaborate scouting, getting better knowledge about the kind of weeds

- manipulation of light environment during tillage reducing seedling emergence
- additional measures: i.e. cover crops, mixed cropping, fallow

Among the various reasons cited by Fernandez-Cornejo & McBride (2002) to explain the rapid adoption of RR crops despite mixed or even negative financial impacts, the efficiency of glyphosate to control weeds resistant to other classes of herbicides seems to be the least important one. But in fact, transgenic HR plants are perceived to give new options to farmers for weed control. Increasing problems in US soybean culture particularly with acetolactate synthase (ALS) inhibitor-resistant weeds favour the introduction of transgenic glyphosate-resistant soybeans. The rapid and widespread adoption of RR soybean varieties in the US and Argentina, therefore, seems to be connected with increasing problems in soybean cultivation to control resistant weeds effectively. Farmers apparently hope to control a broad spectrum of weeds by another herbicide mode of action whose application is simple and efficient. But if they only rotate the herbicide mode of action without applying rotation of crops and rotation of cultural practices at the same time, glyphosate-resistant weed biotypes likely will evolve within a short time and a weed shift may occur similar to the one occurring with the continuous use of ALS inhibitors (in the US and Canada, weeds resistant to ALS inhibitors comprise the largest group of herbicide-resistant weed biotypes, http://www.weedscience.org). This might happen the sooner, the larger the areas planted with glyphosate-resistant crops will be, these crops being sprayed year after year with multiple herbicide applications per season in many cases (Freudling 1999b, 2004 Darmency 1996), particularly in no-till systems. Including the recommended pre-plant application of glyphosate as a burndown herbicide (Benbrook 2001, Pro Farmer 2000, IPM Newsletter 1999), weed populations in soybean could be treated up to three times within one season. In a crop rotation with soybean and corn or soybean and cotton, all crops being glyphosate-resistant, the selection pressure on weeds will be even stronger. Glyphosate-resistant horseweed (*Conyza canadensis*), described in 2000 in several sites in Delaware, USA, has been found in fields planted with RR soybean, where glyphosate was used in 1999 and 2000 exclusively for preplant and in-crop weed control. Prior to 1999, glyphosate had been used in these sites infrequently for preplant weed control (VanGessel 2001).

If in combination with application of non-selective herbicides the transgenic HR crops are grown on a large scale, selection will occur on a much broader spectrum of weed flora in cereals and broadleaved crops. In 2005, about 87 % of the US soybean crops have been reported to comprise RR-varieties (USDA 2005) and more than 90 % of soybean acreage in Argentina was supposed to be grown with RR soybean. Because non-selective herbicides can be applied on these HR crops any time, selection can act indifferently on fall, spring, and summer weed communities, which was previously not the case for most selective herbicides (Darmency 1996). The development of resistant weed biotypes seems inevitable under these circumstances.

6.1.2 Cross resistance and multiple resistance

Strong selection by the extensive use of two or more herbicide modes of action (MOA) can lead to the rapid evolution of weeds resistant to more than one MOA, making weed control by

chemical means very difficult. In cross-resistance, the resistance mechanism confers resistance to more than one MOA, with basically two broad cross resistance categories, target site resistance and non-target site resistance. Multiple resistance, in contrast, is defined as the expression of more than one resistant mechanism within individuals or populations (Powles and Preston 1995).

Target site cross resistance occurs when a change at the biochemical site of action of one herbicide also confers resistance to herbicides from a different chemical class, but inhibiting the same site of action in the plant (Powles and Preston 1995). Non-target site resistance on the other hand is conferred by mechanisms other than resistant enzyme target sites. The ability to resist numerous unrelated herbicide chemistries as a result of enhanced herbicide metabolism is the least predictable and most challenging resistance problem (Heap 2000). The case of rigid ryegrass (*Lolium rigidum*) shows that a range of resistance mechanisms can be selected in a single species (http://www.weedscience.org). The traits include target site insensitivity, enhanced metabolism and variations in membrane properties (Holtum & Powles 1992). Biotypes of *L. rigidum* in Australia show resistance to the majority of herbicides currently used. This species provided also the first example of a weed demonstrating cross-resistance to multiple herbicide chemistries.

Multiple resistant plants may possess from two to many distinct resistance mechanisms, involving both target site and non target site resistance mechanisms, which provide resistance to one (Matthews et al. 2000) or to more herbicides or herbicide classes. Such multiple resistant weeds have been reported from several regions including Europe (Niemann 2000). Glyphosate-resistant goosegrass (*E. indica*) and horseweed (*Conyza canadensis*) have been described to show resistance also to ACCase inhibitors, herbicides inhibiting acetyl-coenzyme A carboxylase and to ALS inhibitors, herbicides inhibiting acetolactate synthase, respectively (http://www.weedscience.org). In addition, it is important to note that the Australian glyphosate-resistant rigid ryegrass (*L. rigidum*) biotypes, besides being 9- to 10-fold more tolerant to glyphosate than susceptible biotypes, also exhibit a 3-fold higher tolerance to diclofop-methyl, a member of the ACCase inhibitors. South African glyposate-resistant rigid ryegrass exhibits even resistance to two other herbicides (paraquat and ACC inhibitors). Resistance to one herbicide seems to facilitate development of resistance to another one, raising implications for weed management in preventing resistance to broad-spectrum herbicides such as glyphosate (Pratley et al. 1999). Multiple resistance is presumed to occur through accumulation of resistance mechanisms as a result of gene flow between individuals with different resistance mechanisms. According to Holtum and Powles (1992), more resistance traits will be selected more rapidly when large weed populations are sprayed, concentrating the traits in the surviving weed population.

6.1.3 Weed population shifts

Even more of a concern than weed resistance may be weed shift, when the species most susceptible to the herbicide decline over time, while less susceptible species build up with prolonged use of the particular herbicide (Holt, 1994, Owen 1999, Benbrook 2001, Benbrook 2004, additional information at http://www.weeds.iastate.edu/). Such population shifts can

occur rapidly. Problems controlling certain weeds by glyphosate have been reported from parts of the USA and from Argentina, relating to common waterhemp (*Amaranthus rudis*) populations (Firbank & Forcella 2000, Pengue 2004, Benbrook 2005). For season-long control of hemp sesbania (*Sesbania exaltata*) sequential applications of glyphosate are needed (Norsworthy & Oliver 2002). Velvetleaf (*Abutilon theophrasti*) populations can increase under RR systems (Hartzler 2000), other weeds such as horseweed (*C. canadensis*), yellow nutsedge (*Cyperus esculentus*), and nightshade (*Solanum americanum*) have become more common and difficult to control (Benbrook 2001). Ivyleaf morningglory (*Ipomoea hederacea*) and shattercane (*Sorghum bicolor*) seem to be less sensitive to glyphosate or avoid applications by late emergence (Hilgenfeld et al. 2004). Weed community changes associated with the continuous and exclusive use of glyphosate in soybean have been reported from Argentina, too, where 18 species were of decreasing and 37 species of increasing importance (Vitta et al. 2004). In particular, perennial species such as *Cirsium arvense* and couch grass (*Agropyron repens*) could become more abundant (Schütte & Schmitz 2001).

The more often a specific herbicide is applied on the same field, the more rapidly a weed shift will occur to species that are less susceptible. Weeds could also emerge late or over extended periods of time, meaning outside the window of time when glyphosate applications will lead to good control. Some of the weeds becoming more important seem to adapt more easily by showing a great variety of life forms and growth cycles (Hilgenfeld et al. 2004, Vitta et al. 2004). Reduced weed seed production after effective weed control in HR crops may also contribute to the shift to less sensitive species (Strandberg et al. 2004). In crop rotation with corn and sugar beet, both crops resistant to the same herbicide, similar weed population shifts can occur since these two crops have many important weed species in common. In fact, German studies showed that among the 20 most important weed species of corn, sugar beet, and oilseed rape, 10 species are identical (Petersen & Hurle 1998). These species will be put under strong selection pressure, particularly if in certain types of crop rotation with cereals, oilseed rape, sugar beet, and corn the same herbicide, be it glyphosate or glufosinate, is used almost every year or even several times a year.

From these data it can be inferred that repeated use of broad-spectrum herbicides such as glyphosate will inevitably lead to evolution of resistant weed biotypes, some of which will show multiple herbicide resistance, and to a shift in weed populations to less susceptible species. Farmers, accustomed to control weeds solely by herbicides, will be tempted to use additional herbicides in higher quantities to control “nasty” weeds. Analysing USDA data about herbicide use in soybean in 1998 in detail, Benbrook (2001) concluded that as a result of weed shifts and slipping efficacy of glyphosate in the control of some weeds, most farmers growing RR soybeans now apply one to three additional active ingredients. These can include 2,4-D in a pre-plant or at plant tank mix to control broadleaf weeds or other products for residual grass control. Meanwhile many combination products are specifically marketed for RR soybean producers. To control glyphosate-resistant horseweed in cotton, the use of various herbicide combinations has been suggested too (Robinson 2002).

To reduce possible adverse agronomical and environmental impacts by HR crops, a range of measures has been recommended such as cultivation of only one HR crop in crop rotation, efficient control of volunteers in following crops, and last but not least, rotation of weed

control methods (Schütte & Schmitz 2001). Because of the evolution of resistant weeds and weed shift in HR systems, efficient herbicide resistance management will certainly be neccessary, but little attention has been paid to this issue till now. For these reasons, alternative methods of weed control, not relying on herbicides, will have to be developed and put into practice.

6.2 Resistance in pests

6.2.1 Evolution of resistance to Bt toxins

Widespread use of pest resistant crops that express toxins in all tissues throughout the season resulting in a level of efficacy of >90 % will place high selection pressure on the targeted pest species which could lead to the evolution of resistant pest populations. In the past, many insect pest species have become resistant to a wide variety of chemical insecticides extensively used. In response to extensive use of sprays of *Bacillus thuringiensis*, resistant populations of the diamondback moth (*Plutella xylostella*), a pest species known for ready development of resistance to insecticides, have been observed in open field situations in Florida, Hawai, Japan, the Philippines, Central America, and China (Tang et al. 1999, Tabashnik et al. 1997). Populations from Hawai and Pennsylvania have been reported to share a genetic locus with a recessive mutation associated with reduced toxin binding that confers extremely high resistance to the Bt toxins Cry1Aa, Cry1Ab, Cry1Ac, and, in addition, to Cry1F and Cry1J (Tabashnik et al. 1997). Independent development of this type of resistance in the two strains seems likely. Since the strains had not been exposed to the latter two toxins, resistance to these Bt toxins might represent cross-resistance. In contrast, in a population from the Philippines multilocus control and a narrower spectrum of resistance has been observed, with dominant inheritance of resistance to Cry1Aa and Cry1Ac.

In the laboratory, selection of resistance to Bt toxins has been achieved in field-collected populations for several other pest species such as European corn borer (*Ostrinia nubilalis* Hübner), pink bollworm (*Pectinophora gossypiella*) (Tang et al. 1999, Zhao et al. 2001, Bolin et al. 1999, Carriére et al. 2001b,c), and cotton bollworms (*Helicoverpa armigera/Helicoverpa zea*) (Akhurst et al. 2000, Burd et al. 2003). Bt toxin Cry1Ac and Cry1Ab that are also expressed in a number of transgenic plants could successfully be used for selection of resistant populations. In several cases, resistant populations of European corn borer could be selected after only seven to nine generations (Bolin et al. 1999, Chaufaux et al. 2001), implying that resistance to these Bt toxins can evolve in populations derived from field collections from countries such as the US, France, Switzerland, and Italy. Although the level of resistance may fluctuate from generation to generation, selected strains showed significant decreases in toxin susceptibility. Fluctuating resistance levels may be attributed to multiple genes with relatively small resistance effects that may not follow the expected simple inheritance pattern of a single recessive resistance gene. In some cases, resistance to Bt toxins may be incompletely recessive (Bird & Akhurst 2004).

Comparing selection for insecticide resistance in the field with selection in the laboratory, Groeters & Tabashnik (2000) concluded that major genes played a dominant role in resistance selection, with and without fitness costs and refuges, and that the intensity of selection, rather than the number of genetic loci conferring resistance, is central in determining rates of resistance evolution. From the ease with which resistant corn borer and cotton bollworm strains could be selected, it has been inferred that partial resistance to Bt toxins must be relatively common among natural populations (perhaps up to a ratio of 1:1000), with some variation in the ability of populations to develop resistance (Chaufaux et al 2001, Akhurst et al. 2000). Recently, out of 583 and 646 screened *Helicoverpa zea* bollworm progeny from females collected in the field, Burd et al. (2003) found one individual each that appeared to carry a major gene for resistance to Cry1Ac or to Cry2Aa respectively. The gene frequency for resistance to Cry1Ac and Cry2Aa has been estimated at 1/2332 and 1/2548 respectively. Genetic analysis showed that resistance to Cry1Ac is inherited as a dominant or incompletely dominant trait. Other females tested seemed to carry additional minor resistance genes. In a laboratory-selected population of the cabbage looper (*Trichoplusia ni* Hübner), resistance to Cry1Ac has been reported to be monogenic, autosomal, and incompletely recessive (Kain et al. 2004).

In some cases varying levels of cross-resistance to more than one Bt toxin could be observed (Bolin et al. 1999, Zhao et al. 2001). This may be especially important in the case of pests that are notorious for rapidly evolving resistance, such as the diamondback moth, or for polyphagous pest species that attack different host crops expressing Bt toxins. Under laboratory selection on Cry1C protoxin and on transgenic broccoli expressing the Cry1C toxin, extensive cross-resistance to Cry1C and to Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1J toxins could be observed in a strain of diamondback moths that was derived from a field selected Cry1A-resistant population, crossed twice to susceptible moths before selection (Zhao et al. 2001). According to the authors, the gene(s) conferring resistance to Cry1C segregated independently from Cry1Ac resistance in this strain. In the study done by Burd et al. (2003), bollworm progeny that performed better on Cry1Ac diet also performed better on Cry2Aa diet.

The mechanisms of resistance seem to vary. In field-selected diamondback moth, a change in the midgut toxin receptor appears to prevent binding of the toxin to the gut, thereby protecting from pore formation by the Bt toxin (Tang et al. 1999). A similar resistance mechanism has been reported for cotton bollworm resistant to Cry1Ac (Akhurst et al. 2000). Tang and coworkers point out that, in contrast to possible behavioral mechanisms, such as avoidance or abandonment, this physiological resistance would be less likely to be overcome with molecular methods that increase toxin expression, once resistance has reached detectable levels in the field. Other possible resistance mechanisms might include altered activity of gut proteases, inhibition of structural alteration of the toxin-receptor complex and perhaps ionic compensation mechanisms that inhibit cell lysis (Akhurst et al. 2000). These authors expect binding site resistance to be narrow spectrum, not extending to resistance to Bt toxins of other classes.

The gene conferring resistance to Cry1Ac toxin in laboratory-selected populations of tobacco budworm (*Heliothis virescens*) has been shown to be a member of the cadherin-superfamily

(*Heliothis virescens* cadherin-like protein Hev-CaLP), disrupted by retrotransposon-mediated insertion leading to a null-allele (Gahan et al. 2001). The normal function of this gene is unknown, although other cadherins are involved in cell adhesion. It is not essential for life, because resistant insects are viable and fertile under laboratory conditions. Whether the absence of this cadherin confers a fitness disadvantage in the field remains unknown. Individual matings of homozygous resistant virgin females to field-caught males led to progeny of 3 out of 1 025 males that did grow on Cry1Ac diet, since they had inherited the resistance allele from their mother and a field-derived resistance allele from their father (Gahan et al. 2001). The authors point out that any allele with a molecular lesion somewhere in the HevCaLP gene preventing it from functioning as a lethal target would give the same result, because it is a null allele. Recently, Tabashnik et al. (2004, 2005) showed that recessive alleles *r*1, *r*2, and *r*3, *r*1 and *r*2, and *r*1 and *r*3 of the cadherin (BtR) gene occurred in the resistant strains APHIS-98R, SAF97-R, and MOV97-R of pink bollworm (*Pectinophora gossypiella*), respectively, and were associated with resistance to Cry1Ac. The latter two strains originated from locations 500 km apart and had been selected for resistance independently in the laboratory. Therefore, PCR-based detection of these three known cadherin resistance alleles or of the HeCaLP gene may be used for monitoring of resistance to Cry1Ac in field populations of pink bollworm or tobacco budworm, respectively. However, insects can have more than one mechanism of resistance, therefore, a single test would not suffice (Stoksta 2001).

Another mechanism of resistance to Bt toxins has been described in nematodes that are normally susceptible to some of the Bt toxins. Lack of the protein encoded by bre-5 (a putative ß-1,3-galactosyltransferase) in the *Caenorhabditis elegans* intestine led to resistance to the Bt toxin Cry5B (Griffitts et al. 2001). This protein may function in forming a carbohydrate structure on proteins or lipids that is necessary for toxin binding at the gut surface. In the absence of bre-5-dependent carbohydrates, Bt toxin cannot bind, resulting in resistance. Interestingly enough, resistant animals showed also resistance to another Bt toxin of the same subgroup, Cry14A, that is 34 % identical to Cry5B and is toxic to nematodes and insects. Therefore, this resistance mechanism may be applicable to other Bt toxins as well and may also be relevant for insects. According to Griffitts et al. (2001), loss of a carbohydrate-modifying enzyme can be particularly dangerous and more threatening than the mutation of a single receptor, since loss of a single general modfier could affect the binding of multiple Bt toxins to multiple receptors, leading to a high level of resistance to a single toxin and cross-resistance to other toxins as well.

Fitness of resistant individuals is of importance for evolution of resistance to Bt toxins. Tang et al. (1999) could not find significant differences in survival, weight gain, and oviposition of the field-selected resistant diamondback moth grown on Cr1Ac expressing broccoli compared to susceptible moths grown on non-Bt plants, indicating that such field-selected resistance traits need not reduce fitness. In other studies, laboratory-selected resistance to Cry1Ac expressing cotton was associated with reduced survival and higher overwintering costs of pink bollworm (*Pectinophora gossypiella*) on non-Bt cotton, whereas development time was unaffected (Carriére et al. 2001b,c). Maternal effects transmitted by moths that had eaten Bt as larvae affected egg development and/or adult fertility. Fitness costs in resistant strains APHIS-98R, MOV97-R, and SAF97-R were recessive and did not depend on the non-Bt

cotton cultivar (Carriére et al. 2005). In experiments designed to associate resistance to Cry1Ac and the cadherin genotype, however, the pink bollworm strains MOV97-R and SAF97-R did not show lower survival on non-Bt cotton (Tabashnik et al. 2005), but sample sizes were relatively small on non-Bt cotton. In cotton bollworm (*Helicoverpa zea*) resistant to Cry1Ac, homozygous resistant individuals were less fit than heterozygous ones (Daly & Olsen 2000). Resistant *Helicoverpa armigera* larvae feeding on young Bt cotton plants showed a significant developmental delay of up to 7 days, and a reduction in survival to pupation by about 50 %, compared with the susceptible strain on non-Bt cotton (Bird & Akhurst 2004). Postdiapausal adults from the resistant strain survived less well than susceptible ones, indicating that there was also a nonrecessive overwintering cost associated with the Cry1A resistance.

Loss of resistance in the absence of selection pressure, as has been observed in European corn borer populations selected for resistance to Cry1Ab, may also be connected to fitness costs of the resistance trait (Bolin et al. 1999). On the other hand, resistant insects may be able to use Bt toxins as a supplementary food protein, as suggested by higher pupal weight and less development time of resistant diamondback moths fed with Cry1Ac treated leaves compared to untreated leaves (Sayyed et al. 2003).

6.2.2 Resistance management

To delay development of resistance to Bt toxins, many scientists and the US Environmental Protection Agency (EPA) recommend a strategy with two components: high-toxin dose and refuge (Alstad & Andow 1995, EPA 1998). The high-toxin dose implies that heterozygous individuals that carry one gene for resistance would be killed by Bt expressing plants. The dose has been defined as 25 times the concentration needed to kill susceptible larvae (EPA 1998). Refuge refers to non-Bt crops that are planted adjacent to or within a transgenic cropfield. The intention is that homozygous susceptible insects feeding and developing on non-Bt plants will be able to interbreed with rare resistant individuals producing heterozygous susceptible progeny. Resistance alleles in the insect population should thus be diluted. For this resistance management strategy to work, it is essential that resistance traits are functionally recessive, i.e. mortality of heterozygotes is similar to that of susceptible homozygotes. However, as discussed in the previous section, in some cases resistance in insects to Bt toxins may exhibit dominant or incompletely recessive inheritance. For Australian cotton bollworm it has been shown that heterozygous resistant (RS) cotton bollworm individuals had greater survival than susceptible ones on Bt cotton and thus seem to be intermediate between the susceptible (SS) and fully resistant (RR) individuals, not recessive as had been assumed (Daly & Olsen 2000). Recently, Burd et al. (2003) reported dominant or incompletely dominant inheritance of resistance to Cry1Ac in a bollworm (*H. zea*) family selected from field collected females. According to these authors, heterozygotes could survive on transgenic cotton plants which were expressing the Cry1Ac toxin at much higher levels than the discriminating dose used in the study.

For resistance management it is equally important that toxin levels in transgenic plants are high enough to kill such heterozygous pest individuals and that refuges are large enough to

provide a sufficient quantity of homozygous susceptible adult insects that could mate with resistant individuals. Susceptible and resistant insects should also be ready to mate at about the same time. Generalizations regarding resistance management are difficult because of the complex, often unknown, interactions of population genetics and population dynamics in the various pest species and dynamics of crop maturation and toxin production (Onstad & Gould 1998a, Caprio 2001). Bourguet et al. (2000) pointed out that the concept of dominance in insecticide resistance generally used may have some drawbacks, as it often refers only to the toxin concentration required to give a particular mortality and/or to the mortality at a particular toxin dose. It should, however, be based on fitness of individuals in a treated area, taking into account genetic background and environmental conditions.

Evolution of resistance in field situations may depend on a number of factors such as the intensity of selection, size and arrangement of refuges, randomness of mating and adult behavior, population regulation by insecticides in refuges, grower acceptance of large pest populations in refuges, and seasonal changes in dispersal and habitat quality (Caprio 2001, Ives & Andow 2002). Prolonged development of resistant insects, as observed in the laboratory, might lead to non-random mating among resistant insects from Bt crops, thus creating a disproportionately high number of homozygous resistant insects (Liu et al. 1999, Crawley 1999, Akhurst et al. 2000). Bird & Akhurst (2004) stress that developmental asynchrony between resistant and susceptible insects has the potential to increase the likelyhood of temporal separation of the genotypes, thus making non-random mating between resistant and susceptible populations more likely. If a high-toxin dose cannot be achieved and a small fraction of heterozygous and perhaps even homozygous susceptible corn borer neonates survived on transgenic corn, then, according to simulations by Onstad & Gould (1998b), resistance to Bt toxin can develop in a fraction of the time required under the assumption of a successful high-dose that kills all heterozygous neonates.

In their simulation for resistance development in the European corn borer (*O. nubilalis*), Alstad & Andow (1995) demonstrated the usefulness of a patchwork of Bt corn and non-Bt corn, compared to uniform plantings of transgenic corn. Since in their 1:1 ratio of Bt and non-Bt fields corn borer damage in the unprotected fields could be high, they suggested to apply a preference-based approach and restrict Bt toxin expression to the earliest corn varieties that are most preferred by corn borer females for oviposition. These early corn fields could act as trap crops, reducing insect density on less mature varieties of later plantings, in particular non-Bt corn. According to the authors, proportion and arrangements of Bt and non-Bt fields could be adjusted to the corn phenologies and corn borer dispersal patterns in different regions, taking into account the flight range of adult corn borers.

The debate is still going on regarding size and position of such refuges. A proportion of 20 % non-Bt corn has been mandated by EPA, if the refuge is treated with insecticides and 5 % if the refuge remains untreated. However, not all farmers seem to plant these refuges, as 2003 figures with nearly one-fifth of farmers in the US midwest flouting the rules indicated (Clarke 2003). Efficacy of mixed plantings would in part depend on insect behavior. Oviposition behavior of lepidopteran females does not seem to be influenced by the type of the crop. In oviposition choice tests, females of diamondback moth and European corn borer did not discriminate between Bt expressing crops and non-Bt crops (Tang et al. 1999). In laboratory

experiments, larvae of bollworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) selectively fed on nontreated diet, compared with diet treated with Cry1Ac toxin (Gore et al. 2005), in the field however, they do not seem to discriminate between Bt and non-Bt plants in mixed cotton stands (Halcomb et al. 2000). Bt corn pollen shed on non-Bt corn grown nearby does not seem to have an impact on survival of European corn borer on non-Bt corn, thus, alternating Bt corn with non-Bt corn has been suggested as an approach for resistance management (Pilcher et al. 2001). According to Onstad & Gould (1998b), however, separate refuges planted adjacent to the transgenic crop would be superior to seed mixtures for delaying resistance. Modeling the spatial and temporal location of refugia, Cerda & Wright (2004) found that, among others, refugia that are smaller, are positioned at random in the field, comprise less than 10 %, and that are sprayed with insecticides increased the frequency of resistance alleles. Temporal refugia, i.e. rotation with a non-Bt host crop, led to a decline in the frequency of the resistance allele.

Since the corn borer has a host range of >200 plants, non-corn plants such as weeds growing on field borders or other crop species might also function as refuges. But, according to Losey et al. (2001), non-corn hosts appear unlikely to provide a substantial number of corn borer individuals susceptible to Bt toxin compared to the number expected from the 20 % planting refuge mandated by EPA registration of Bt corn. Modeling the development of resistance by stalk-boring moths in Bt corn, Guse et al. (2002) found that when the expression of the resistance allele is not dominant, behavior of adult moths appears to influence resistance movement more than refuge size. Similarly, using a theoretical model, Andow & Ives (2002) concluded that changing the movement patterns of corn borer moths between Bt corn and non-Bt corn (e.g. by planting small sites of non-Bt corn within Bt corn fields or by use of pheromones luring susceptible males into Bt fields) could be particularly effective at prolonging the efficacy of Bt corn through adaptive resistance mangament.

Based on the observation that a high proportion of the European corn borer (*O. nubilalis*) eggs oviposited on non-corn host plants apparently does not complete development on those plants (many more tunnels than corn borer larvae were found), Losey et al. (2001) raised the question, whether such non-corn plants would function as “nursery plants” for the European corn borer, supporting partial, but not complete development. If late-instar larvae which can survive much higher doses of Bt toxin, disperse to corn plants from nursery plants near cornfields, partially resistant individuals might survive and thus decrease the effectiveness of the resistance management program. Considering that toxin levels in Bt crops generally decrease with plant maturation, survival of late-instars might be even more likely. For polyphagous pests such as *Helicoverpa zea* (known as bollworm, corn earworm, or tomato fruitworm) refuges provided by other non-Bt crops or wild plants may be important too. Gould et al. (2002) found that non-Bt corn in Mexico and the US corn belt appear to serve as an important refuge for this pest and are probably more critical to resistance management than the relatively small cotton refuge (5 % unsprayed/20 % sprayed) mandated by the EPA for Bt cotton.

Variance in toxin expression over time or space will have an impact on resistance management. If the toxin titer dropped gradually from a high dose that could kill all heterozygous and homozygous susceptible larvae to a level that could kill only (or debilitate)

completely susceptible homozygous individuals, adaptation to the Bt toxin could be much faster than if the concentration remained high (Onstad & Gould 1998a). Variation in expression levels of Bt toxin genes in various Bt crops seems common (Greenplate 1999, Walker et al. 2000, Stanley-Horn et al. 2001, Hilbeck & Meier 2002), in part depending on the promoters used, on transformation events (Tang et al. 1999), on tissue type, on plant age, on crop variety, and on environmental conditions. Under field conditions, varying Bt toxin levels in Bt cotton have been reported for both American, Australian, and Chinese cotton varieties with decreasing Bt toxin levels as the plant ages (Greenplate 1999, Adamczyk et al. 2001, Wan et al. 2005) and higher survival of cotton bollworm (*Helicoverpa armigera*) larvae in mature cotton (Olsen & Daly 2000, Olsen et al. 2005). Daly & Olsen (2000) predict that, because of their partial resistance, heterozygous resistant cotton bollworm larvae may survive on plants at some later stages of plant growth or in some older larval instars.

In tests done by Walker et al. (2000), not all Bt corn hybrids provided the same protection against corn borer injury, depending also on the instar tested, as third and fourth instars are more tolerant to Bt toxins than neonates. Bt corn event 176 is known to produce not enough toxin to kill all homozygous susceptible individuals during the second generation of infestation by the corn borer (Bourguet et al. 2000). Various parts of maize leaves and cotton bolls have different amounts of Cry1A toxin, as shown by Abel & Adamczyk (2004). The green maize leaf parts had higher concentrations than yellow-green and white-yellow tissues and cotton boll tips where the flower remained attached (and the chlorophyll content was visibly lower) had significantly lower toxin levels. Incidentally, these boll tips are often the site where corn earworms (*Helicoverpa zea*) penetrate cotton bolls. Reasons for the decreased and differential expression in different tissue types, particularly in tissue with low chlorophyll content, are not fully understood. Environmental and physiological factors that effect photosynthesis and total levels of protein are likely to affect the level of a transgene product too. As leaf and stem tissue of corn contribute a significant amount of nitrogen to grain production, nitrogen is lost from these tissues after flowering, therefore, an exotic nitrogenous toxin like the Bt protein might incur significant decomposition during this period in leaf and stem tissue (Abel & Adamzyk 2004, Onstad & Gould 1998a).

In general, efficacy of Bt toxin in Australian cotton cultivars is high until the plants start flowering and then it declines. Late season damage in commercial Bt cotton fields under high pest pressure and insecticide application have been reported (Olsen et al. 2005). Olsen et al. found that the substantial developmental decline in efficacy of field-grown Bt cotton was associated with reduced cry1Ac RNA transcript and Bt toxin levels. The reduction in cry1Ac transcripts was, according to Olsen et al. (2005), most likely due to a failure of the 35S promoter in postsquaring cotton. Wan et al. (2005) and Kranthi et al. (2005), studying Chinese and Indian Bt cotton hybrids, also reported about significant variation of Cry1Ac and Cry1Ab toxin levels over time, with lower levels later in the season and in ovules and bolls, compared to first growth stages and green plant tissue. This might allow survival of pink bollworm (*Pectinophora gosssypiella*) and bollworm (*Helicoverpa armigera*) that feed mostly on bolls. In addition, toxin expression in Indian Bt cotton varied from hybrid to hybrid, some of them may not even express Cry1Ac (Kranthi et al. 2005), leading to a lack of high dose in late season which will increase the likelihood of resistance development.

Toxicity of Bt cotton to pest species may not be simply related to the amount of Cry1Ab or Cry1Ac produced, but also to physiological alterations and environmental influences (Olsen et al. 2005). As cotton plants mature, there is a change in the level of secondary compounds, such as phenolics and terpenoids, which may alter the toxicity of Bt proteins to lepidopteran species, both positively and negatively (Olsen & Daly 2000, Greenplate 1999). Cry1Ac mixed with leaves from fruiting versus younger presquare non-transgenic plants was significantly less toxic (Olsen & Daly 2000). Plant-toxin interactions in fruiting cotton, therefore, seem to reduce the toxicity of Cry1Ac proteins, perhaps linked to the increased production of tannins, although the latter compounds are unlikely to be the only factors. For these reasons it will be essential that ecological studies and field bioassays consider the entire period of time over which the insects may feed upon the crop, studying not only neonate larvae, as insects feeding and surviving on a crop during its senescence may have important consequences for the development of resistance in pests (Onstad & Gould 1998a, Losey et al. 2001).

Production levels of Bt toxins in transgenic plants may be affected not only by plant senescence but also by altered CO_2 concentrations that are expected to rise in the course of climate change. Elevated atmospheric CO_2 will cause plants to grow faster resulting in lower nitrogen content per unit of plant tissue and higher carbon to nitrogen (C/N) ratios. Experiments with commercially available transgenic Bt cotton grown under ambient and elevated CO_2 concentrations and tested in bioassays and quantitative enzyme-linked immunosorbent assays, indicated reduced Bt toxin production in elevated CO_2, in particular in plants growing in nitrogen limited systems (Coviella et al. 2000). Such an effect may also be relevant for other proteinaceous compounds conferring pest resistance such as lectins or inhibitors of proteinases and amylases.

Tri-trophic interactions and behaviour of enemies of pest species may influence the evolution of resistance too. As parasitoids are attracted to damaged plants, they may be an agent for slowing the evolution of resistance to Bt toxins in pest species. In Bt crop fields parasitoids might preferentially be attracted to either resistant larvae on Bt plants or to susceptible larvae on on-Bt plants, thus eliminating resistant individuals (Agrawal 2000). Selective feeding behavior of predators may influence the biocontrol capacity for Bt toxin containing prey, even if the prey is not sensitive to the Bt toxin expressed in transgenic crops (Meier & Hilbeck 2001).

Separate planting of refuges does not add significant costs to total labor and variable costs regardless of their configuration in strips or blocks (Hyde et al. 2001), considerations regarding physical separation and identity preservation or, perhaps, non-GM buffer zones as pollen traps might be of greater significance. For proactive resistance management, monitoring of resistant pests will be important, different approaches for sampling techniques, monitoring frequencies, and scouting of pest species have been suggested taking into account potential costs (Bolin et al. 1998, Andow & Ives 2002). In addition to that, a comparatively simple tactic worthy of evaluation is killing the European corn borer in the stubble of transgenic fields by chopping and ploughing to reduce the number of individuals carrying the resistance allele in the total population (Onstad & Gould 1998b). In fact, practicioners recommend for years to use, wherever possible, chopping and ploughing of corn stubble for control of the European corn borer. This mechanical method may achieve even higher levels

of corn borer control than insecticidal toxins and reduces simultaneously infection by pathogenic fungi such as fusarium (Anonymous 1996, 2000, Freudling 1999a), as burying fusarium-infested crop residues deeper in the soil can effectively reduce fusarium populations (Yi et al. 2002).

6.3 Resistance in pathogens

Durability of genetically engineered pathogen resistance of plants may be low because pathogens such as viruses, bacteria and fungi are known to adapt very rapidly to selective forces. In particular, if resistance mechanisms are based on single genes, they may be overcome easily. Avirulence (avr) genes of bacteria and fungi mutate frequently and so resistance mediated by the integration of the corresponding resistance (R) gene can be overcome (Morgues et al. 1998).

6.4 Herbicide use

In some countries such as USA, Canada, and Argentina, HR crops have been grown on a large scale, comprising more than 80 % of a crop like soybean. HR corn, cotton, and canola are also grown on large areas. Companies developing HR crops have argued that wide adoption of HR technology will lead to reduced herbicide application in both number and doses of herbicides applied. Farmers would have the opportunity to deploy reduced tillage systems, thereby reducing soil erosion. However, reduced tillage systems are more reliant on herbicides in keeping weeds under control. Detailed analysis of USDA data on herbicide use in soybean showed that no-till systems required about one additional herbicide active ingredient in contrast to conventional/conservation tillage systems and between 10 – 20 % more total herbicide per acre (Benbrook 2001). Despite claims that overall rate of herbicide use in RR crops would significantly decline (Gianessi et al. 2002, Fernandez-Cornejo &McBride 2002), RR soybeans, in fact, require more herbicides than conventional soybeans in most cases (Mertens & Plän 2001, Benbrook 2001, Fernandez-Cornejo & McBride 2002). Comparisons of herbicide use on soybeans in 1995 and 1998 showed an average increase of herbicides applied, in fewer applications, due to a strong increase in glyphosate and smaller increases in 7 other herbicides, accompanied by declines in 16 other herbicides (Wolfenbarger & Phifer 2000). Field-level comparisons of the total pounds of herbicide active ingredient applied also revealed that, on the average, on fields planted to conventional soybeans and not treated with glyphosate, there were about 11 % less herbicide applied than on RR fields, with considerable variations in different soybean growing states (Benbrook 2001). Based on annual USDA data about pesticide use and GM crop cultivation in the US, Benbrook (2004) calculated that cultivation of HR crops increased herbicide use from 1996 to 2004 by about 62,500 t, most of this increase being due to RR soybeans. Because of fierce competition, herbicide prices have been lowered significantly, which encouraged heavier reliance on herbicides. In Argentina, where RR soybeans are grown on over 90 % of the soybean acreage, glyphosate use has increased significantly from 820 t in 1996/97 to 45 860 t in 2003/04, amounting to a staggering 56 fold increase (Benbrook 2005). This increase may be explained partly by the great extent of no-till systems treated with a burndown application of glyphosate, but also by

the increase in RR soybean acreage from 0.4 million ha in 1996/97 to 14.1 million ha (a 35 fold increase).

As discussed previously, the evolution of glyphosate-resistant weed biotypes and weed shifts will lead to additional applications of glyphosate and of herbicides with different modes of action. If different crop species resistant to the same broad-spectrum herbicide are cultivated in rotation, crop rotation does not imply different weed control programs anymore, then these herbicides will be used in larger quantities on larger areas. Therefore, if weed control is consistently relying on herbicides with HR systems, the change to more sustainable agriculture systems with less pesticide input will increasingly become difficult.

Another concern relates to the effects of volunteers and feral plants, with special emphasis in oilseed rape, that show up in fields and field margins, leading to weed problems in subsequent crops (Orson 2002). In particular, if gene stacking of more than one HR gene occurred, control of such volunteers will most likely entail the application of additional herbicides, not only on fields but also on field margins where oilseed rape volunteers and feral plants have been found to be common. In fact, to control RR oilseed rape volunteers, the addition of effective broadleaf herbicides has been recommended (Rainbolt et al. 2004). Such developments will contradict government programs aimed at reducing dose and number of herbicide applications in agriculture and at increasing biodiversity by protecting field margins from spraying. This may be of special importance in Europe, where farming takes place in the countryside and crops are close to seminatural habitats and where farming must coexist with nature conservation (DETR 1999).

6.5 Insecticide Use

According to US sources, cultivation of insect resistant crops such as Bt cotton has resulted in significant reductions of chemical insecticides applied (Gianessi et al. 2002, Ferndandez-Cornejo & McBride 2002) and with the introduction of other insect resistant crops similar results are expected. If Bt crops impose high mortality on specialist pests such as pink bollworm (*Pectinophora gossypiella*), long-term suppression of these pests may occur, leading perhaps to a reduced use of insecticides (Carriere et al. 2003). However, pest species that are generalists or have high reproduction rates are less likely to suffer from long-term suppression. Moreover, reductions in insecticide use can be expected only in those insecticides previously used to treat the pests targeted by the Bt toxin. For this reason, in agriculture relying on pesticides, the use of conventional insecticides targeting insects not affected by the toxin will continue apace. In addition to that, before the commercial introduction of Bt corn in 1996, the European corn borer was only partially controlled using chemical insecticides in US agriculture (Fernandez-Cornejo & McBride 2002), probably because infestation levels vary from year to year and larvae have to be killed before they move into corn stalks. In Europe too, corn borer infestation is controlled only partially by chemical insecticides, more often it is controlled by mechanical chopping and ploughing the stubbles of corn plants and also by the application of parasitoids (Klöpffer et al. 1999).

Bt toxins are intended to control important pests such as the European corn borer or lepidopterous pests of cotton, but they give no or only partial control of non-target insect

pests, such as heteropterous pests of Bt cotton. Therefore, pests that have been suppressed incidentally by broad spectrum insecticides will almost certainly still be controlled by chemical insecticides.

In China and India, Bt cotton may not even be potent enough to control cotton bollworm adequately, as Men et al. (2005) and Kranthi et al. (2005) found. Later generations of *H. armigera* bollworms were greater than first generations, possibly because survival of larvae increased as the plants matured leading to lower toxin levels. Bt hybrids grown in India express less than the critical levels of Cr1Ac in bolls required to kill bollworms later in the season (Kranthi et al. 2005). Bt cotton hybrids in India may therefore require more supplemental insecticide sprays. Jayaraman (2005) quoted a Monsanto speaker as saying "Bollgard cotton is not a panacea. Farmers are directed to scout their Bollgard fields for insects and supplement the protection by treating with an insecticide whose amount varies from country to country". Mirid populations on Bt cotton in China exceeded the pesticide action threshold and received insecticide applications in two of three years, whereas mirid populations on non-Bt cotton did not need extra insecticide applications – they may have been reduced by insecticides targeting other pests. Consistently larger leafhopper populations on Bt cotton, exceeding the pesticide action threshold, indicated that Bt cotton may be more suitable for leafhoppers which could in turn increase the need for insecticide applications (Men et al. 2005). Over their three-year experiment, conventional cotton received fewer insecticide applications than Bt cotton.

A survey of Chinese cotton farmers indicated that farmers' knowledge of pests and their natural enemies was inadequate. Farmers still sprayed pesticides intensively on Bt cotton with an average of 12.7 applications per season, with some of the sprayed pesticides including Bt formulations, a practice that enhances the risk for resistance development to Bt toxin in the cotton boll worm (Yang et al. 2005). In the US, it became evident that correctly timed conventional insecticides, notably pyrethroids, are needed on US Bt cotton, not only to control a new spectrum of pests such as stink bugs but also against the target pest bollworm that may survive on Bt cotton of more mature stages (Way & van Emden 2000). Therefore, if integrated pest management will not be applied more successfully in conventional agriculture, cultivation of insect resistant crops may not lead to significant reductions in insecticide use in the long run.

7 Summaries

7.1 Summary

The objective of this study was to carry out a survey of the current international status of research on the environmental impacts of genetically modified plants and to combine it with a scientific assessment of gaps and limits of biosafety research. The results concerning the environmental impacts of genetically modified plants were compiled, considering first methods of genetic engineering and the risks associated with them, before considering general phenomena such as gene spread by hybridization, horizontal gene transfer and spread of GMOs. Traits transferred to plants and their potential direct and indirect environmental impacts are discussed in later chapters. Concerns such as potential impacts on biological diversity and on resistance evolution and pesticide use are covered as well.

In recent years, cultivation of genetically modified plants has increased considerably with about 90 million ha in 2005 planted with transgenic crops, most of them carrying herbicide resistance and insect resistance traits expressed in soybean, corn, cotton, and oilseed rape. Although plants carrying these agronomic traits based on the transfer of single bacterial genes will likely make up most of the GMO market in the next few years, other transgenic plants expressing more complex traits are being developed and may reach the market too. Many transformations are aimed at expression of new traits such as pathogen and stress resistance, alteration of growth characteristics and secondary metabolism, production of new substances and pharmaceuticals, and perhaps bioremediation. In order to achieve alteration of complex metabolic pathways, in a number of cases several genes have been transferred.

Assessment of environmental impacts of transgenic plants has to deal with many plant species and an enormous range of new genes and traits that are being transferred to organisms where they have not been expressed previously. Risk analysis for transgenic traits common in commercialized crops, such as herbicide resistance and insect resistance, is not an easy task, but future trait-species combinations will make risk analysis even more demanding. Stacking of genes, either by deliberate transformation with multiple genes, by crossing of transformants, or by unintended combination through hybridization in the field will complicate matters even more.

The effect of a transgene, integrated into the recipient's genome, depends among others upon the number of integrated copies, the site(s) of insertion and the level of expression. The techniques used for transfer of DNA sequences to plants depend mostly on the soil bacterium *Agrobacterium tumefaciens* and on microprojectile bombardment. Although widely believed that, in general, gene transfer is precise and transgenes are integrated in a single copy at a single integration site, there is growing evidence that very often this does not hold true. In both kinds of plant transformation, multiple copies of transgenes have been inserted, either at a single insertion site or at multiple sites. Transgene sequences can be duplicated, truncated,

inverted, and recombined leading to complex patterns of DNA integration. In cases where integration sites have been analysed in detail, major rearrangements comprising transgene sequences interspersed with genomic sequences have repeatedly been found. In addition, along with the transgene sequences intended, plasmid backbone sequences can also be transferred, introducing perhaps bacterial sequences including additional antibiotic resistance genes, origins of replication, and/or recombinogenic sequences, but the presence of superfluous DNA has not been routinely assessed. To remove antibiotic resistance marker genes from transgenic plants, several approaches have been proposed. Promoters used in plant transformation may not be as species and tissue specific as previously thought. To increase protein yields and to reduce gene spread via pollen, transformation of chloroplasts has been advocated. But integration of transgenes in the chloroplast genome will not inhibit gene transfer from transgenic plants in every instance. High copy number of antibiotic resistance marker genes, similar codon usage and promotors active in chloroplasts and bacteria increase the risk of horizontal transfer of antibiotic resistance genes from transplastomic plants to bacteria.

Gene transfer from transgenic crops to non-transgenic crops and to related wild plants has been shown to occur, with cross-pollinating species exhibiting far higher hybridization frequencies than self-pollinating ones. The rate of transfer will depend on presence of potential hybridization mates, distances, and population sizes of pollen donors and pollen recipients, weather conditions that influence also foraging behavior of pollinators, and compatibility of the mating system. Basic data for the potential of gene transfer for selected crop species cultivated in Europe have been provided. In general, crops originating from a specific region will have a range of compatible wild relatives in that region, increasing the potential of gene transfer. For Europe, therefore, the cultivation of transgenic crops such as oilseed rape, sugar beet, forages, some vegetables, fruit species, and trees would be of great concern. Worldwide commercialisation of GM plants will increase the opportunity for range overlap with compatible relatives and the probability of gene flow. Physical and biological barriers to gene flow may only reduce the risk of transgene introgression, but not eliminate it.

Horizontal gene transfer is known to occur among microorganisms, whether transgenes from GM plants will be transferred to microorganisms is a matter of debate. DNA from transgenic plants has been shown to be present and persist in fields where GM plants have been grown, but also on sites where transgenic material has been introduced from the outside, e.g. by pollen movement. Attempts to directly observe gene transfer from plants to bacteria produced mixed results, under laboratory conditions, however, transformation of bacteria with transgenic plant DNA and plant material has been observed. According to a recent report, gene transfer from GM food to the intestinal microflora may occur under certain circumstances. Transfer frequencies may be enhanced by sequence homology, high copy number of transgenes, a small genome size of the DNA donor, and by selection for the transgene acquired. The role of bacterial sequences present in transgenic plants and the increased risk for plastid-transformed plants to lead to horizontal gene transfer is discussed. Despite the low frequencies expected for horizontal gene transfer from GM plants to microorganisms, environmental impacts may not be zero.

Transgene spread can also occur by running wild and seed dispersal of transgenic plants. As recombinant DNA technology allows the introduction of a great diversity of genes into living organisms, novel combinations of genetic material and traits are possible leading to organisms that are novel to the existing network of ecological relationships in the recipient habitats. Concerns have been expressed that transgenic plants may show increased weediness or become more invasive of natural habitats thus exerting severe and irreversible effects on biodiversity. If transgenes and recipient plants are evaluated according to the risk they carry to show increased fitness, traits such as resistance to pests, pathogens, and stress, and enhanced growth are considered candidates to increase the competitivity of plants. Therefore, these traits are of special concern, particularly as they may also be stacked or combined with other traits. Recipient plants being hardy, perennial, competitive, prolific, and with an ability to colonize a range of natural and semi-natural habitats would be high impact plants with regard to spread. However, simple predictors of invasion success of transgenic plants cannot be identified, as invasion success also depends on specific characteristics of the habitats into which the transgenic organisms are being released. Therefore, data gained from the release of a transgenic plant into one habitat may not be valid for other habitats.

Environmental impacts of transgenic plants depend to a great extent on the traits they carry and whether the new phenotypes interact in new, potentially adverse ways with the organisms present in the respective ecosystem. Acquisition of new phenotypes in transgenic plants intended by genetic engineering may be accompanied by undesired and unexpected alterations of phenotypes. Position effects due to transgene integration and multiple and scrambled integration sites can change the activity of transgenes but also of endogenous genes. Pleiotropic effects caused by the gene products directly or by unintended alterations of plant biochemistry through the gene products may also lead to a change of phenotypes in unexpected ways, potentially only in the field under certain environmental and stress conditions. Organisms the plant usually interacts with can be unintentionally affected by such phenotypic changes, be they intended or unintended.

Herbicide resistance, the most dominant trait in transgenic crops cultivated, can affect plant growth and soil life in part via the application of the broad-spectrum herbicides that are active not only against plants but also against microorganisms. Effective removal of weeds reduces biodiversity of wild plants in agroecosystems, thus endangering soil organisms, arthropods, birds, and other animals depending on wild plant diversity. Widespread and repeated use of herbicides will inevitably lead to the evolution of herbicide resistance in weeds and to shifts in weed population, with less susceptible species building up. Evolution of resistance to glyphosate has been recorded for eight weed species. To control resistant weeds, doses and numbers of herbicides are usually increased, therefore, the widely expected reduction in herbicide application has not come true.

All transgenic insect resistant cotton and corn crops cultivated express *Bacillus thuringiensis* (Bt) toxin genes. Due to the expression of activated toxins in transgenic plants and the presence of the toxin during the whole vegetation period in all plant tissues, insects may be exposed to these toxins through different routes, compared to exposition to the previously used *B. thuringiensis* preparations. Bt toxins expressed in transgenic plants do not seem to be as specific as expected. Therefore, organisms other than the target pest species may be

affected by pollen or other plant material, as shown in a number of studies with non-pest butterflies, such as the monarch or swallowtail butterflies, or beneficial insects such as the lacewing that may even be affected in a tritrophic interaction by feeding on prey reared on Bt crops. By root exudates Bt toxins can be released into soil, binding to soil particles reduces degradation and may retain their toxicity for months. Bt plant material may degrade less rapidly, thus prolonging the time available to impact non-target soil-dwelling organisms. Therefore, to study potential effects of Bt toxins on non-target organisms, a long-term approach will be necessary, taking into account life stages, feeding behavior and predator/prey relationships of non-target organisms. Deployment of insect resistant plants expressing insecticidal substances other than Bt toxins may show impacts on an even broader spectrum of insect species and non-target organisms, because these substances generally act less specific than Bt toxins. Potential impacts of insect resistant plants would be exacerbated if the respective transgenes were transferred to related wild plants, conferring them fitness advantages and affecting an even greater range of non-target organisms that rely on these plants for food.

Past experience with insecticide application (including application of *B. thuringiensis* preparations) indicates that selection pressure exerted by the widespread use of pest resistant crops expressing toxins in all tissues throughout the season likely leads to the evolution of pest populations resistant to the respective toxin. To delay development of resistance to Bt toxins, the high-toxin dose and refuge strategy has been recommended. Prerequisites for functioning of this strategy are recessive inheritance of resistance genes in pest populations, expression of high enough doses of Bt toxin in transgenic plants to kill heterozygous individuals, and presence of large enough refuges to provide a sufficient quantity of homozygous susceptible adult insects that could mate with resistant individuals. But these conditions may not be met in the field. At present it remains open, whether due to widespread cultivation of insect resistant crops, insecticide use will decrease in the long run.

Resistance to pathogens such as viruses, bacteria, and fungi is expected to lead to a reduction in pesticide application and, therefore, to less environmental impact due to toxic substances. But there may be also adverse environmental impacts linked to genetically engineered pathogen resistance. In particular, virus resistance conferred by the transfer of viral genes may result in heterologous encapsidation and/or recombination of viral sequences leading perhaps to virus particles with new specificities. Anti-bacterial and anti-fungal substances could interact also with neutral or beneficial microorganisms, potentially affecting symbiotic relationships with nitrogen-fixing bacteria and mycorrhizal fungi. Soil biodiversity and function may be impeded. Some of the newly produced antimicrobial substances could also interfere with plant growth or be toxic to animals. In case horizontal gene transfer would occur, microrganisms might gain fitness advantages, again leading to impacts on soil biodiversity. Expression of pathogen resistance traits may result in increased fitness of the transgenic plants, potentially enabling them to spread to other habitats. Transfer of such resistance genes via hybridization could impart increased fitness to the progeny of such crosses too.

In many attempts to increase resistance to an abiotic stress factor such as salt, cold, heat, and drought, transgenic plants did show an altered response to a number of stresses. This is

because the major abiotic stresses basically result in water-deficit stress and plant stress responses to salt, cold, heat, and drought often involve similar pathways. Fitness increases of such transgenic plants may allow them to spread and out-compete other plants, particular under stressful conditions. As a result, diversity and abundance of other plant species may change exerting adverse effects on biodiversity. Hybridization with related wild species will lead to the introduction of resistance genes into wild populations, providing them with a selective advantage, compared to other plants of an ecosystem, perhaps creating weeds that are very difficult to control. If stress resistant crops are grown in ecologically valuable areas not deemed suitable for agriculture before, biodiversity may be reduced in such areas too. Plants engineered for increased root exudation of organic acids to render them more tolerant to high levels of aluminum in acidic soils, may accumulate aluminum in roots and shoots, raising concerns about potential toxicity of plant material for humans, lifestock, and wild animals.

Genetic engineering has been used to alter growth characteristics in plants such as plant morphology and development, and lignin content and composition. Altered plant stature, alteration of lignins, accelerated flowering and delayed senescence are thought to increase productivity or ease pulp and paper production. Yield increase by the transfer of genes involved in the C4 photosynthesis to C3 plants has also been attempted, but with limited success so far. In transgenic plants engineered for alteration of basic metabolic processes, many pleiotropic effects likely will occur. Desired morphological and developmental changes could be accompanied by undesired effects including disturbance of flower and root development, and altered susceptibility to pests and pathogens. In transgenic plants producing altered lignins, degradation properties and substances other than lignins may be changed. Transgenics with enhanced growth, accelerated flowering, and delayed senescence will have a higher probability to spread and survive than their non-transgenic sister lines. If transferred to related wild plants or weeds, such traits might increase their fitness and invasion potential too.

A range of new/altered substances can be produced in transgenic plants, leading to altered composition of proteins, fatty acids, carbohydrates, to new substances such as plastics or fibers, and to altered secondary metabolites. In particular, crops with enhanced vitamin and mineral content have been advocated to provide more nutritious food and help combat vitamin deficiencies that are prevalent among poor people with limited food supply. These „second generation“ crops, compared to „first generation“ herbicide resistant and insect resistant crops, are also thought to increase acceptance of genetically modified food by consumers. But engineering the biosynthesis pathways of secondary metabolites will likely lead to unexpected changes in the content of other compounds in the plant, some of which may be toxic to humans and animals. Variation of phenotypes, growth characteristics, fertility, and susceptibility to pests and pathogens may also be observed. Secondary metabolites new to a plant species or produced in altered quantities can prove to be toxic for organisms the plant usually interacts with, such as soil organisms, arthropods, and higher animals. If transgenic crops altered in secondary metabolism can hybridize with wild relatives, secondary metabolism of wild plants might also be affected, but different genetic backgrounds that influence transgene expression and reactions of enzymes and substrates will make outcomes even less predictable. Transgenic plants producing new or altered compounds not destined for food or feed such as plastic polymers or spider fibre and their products would have to be kept

separate from the food chain and hybridization. Commingling with crop varieties of the same species would have to be excluded.

In recent years, plant transformation has been proposed to open up new avenues of cheap mass production of antibodies, vaccines, and human proteins. Transformed plant species include many food plants such as corn, potato, tomato, banana, spinach and others. Proteins of human origin produced in transgenic plants may not be completely identical in amino acid sequence and structure to the corresponding human protein and plant-specific glycosylation of antibodies could limit their use. Cryptic functions of pharmaceutical proteins newly expressed in plants may show up, leading to unexpected changes in plant metabolism. Biopharmaceuticals usually elicit responses at low concentrations and could be toxic at higher ones creating concerns if transgenic plants expressing them are ingested unintentionally by humans or animals. They may persist in the environment or accumulate in living organisms, possibly damaging non-target organisms. It is unknown at present, whether these substances will be released via root exudates into the soil or reach surface and ground water and whether such plants would show enhanced fitness. Gene transfer to food or feed crops or to wild relatives might lead to problems with unwanted contamination of food and feed. Epression of pharmaceuticals in wild plants could endanger soil life and wildlife. Pharmaceutical producing plants may show up as volunteers in subsequent crops potentially also contaminating the food chain. To preclude this, stringent measures of separation and control of fields in subsequent years would be required.

7.2 Zusammenfassung

Ziel der vorliegenden Studie war, einen Überblick über die internationale Forschung zu Umweltwirkungen gentechnisch veränderter Pflanzen zu gewinnen und Lücken und Grenzen der biologischen Sicherheitsforschung aufzuzeigen. In einem ersten Schritt wurden die Methoden der gentechnischen Veränderung von Pflanzen und die damit verknüpften Risiken behandelt, bevor es um allgemeine Phänomene wie Hybridisierung, horizontaler Gentransfer und Ausbreitung von gentechnisch veränderten Organismen (GVO) ging. In Pflanzen übertragene neue Eigenschaften und deren potentielle direkte und indirekte Effekte auf die Umwelt und die biologische Vielfalt werden in folgenden Abschnitten diskutiert. Abschließende Kapitel befassen sich mit der Entwicklung von Resistenzen und den Auswirkungen auf den Pestizideinsatz.

In den vergangenen Jahren nahm der Anbau transgener Pflanzen weltweit stark zu und erreichte eine Fläche von 90 Millionen ha im Jahr 2005. Fast alle angebauten transgenen Nutzpflanzen sind herbizid- oder insektenresistent, wobei im wesentlichen Soja, Mais, Baumwolle und Raps als gentechnisch veränderte Varianten angebaut werden. Transgene Pflanzen, deren neue agronomische Eigenschaft auf der Übertragung einzelner Gene beruht, werden voraussichtlich auch in den nächsten Jahren den GVO-Markt beherrschen, doch GVO mit komplexeren Eigenschaften sollen künftig ebenfalls Marktreife erreichen. Angestrebt wird die Expression neuer Eigenschaften, darunter Krankheits- und Stressresistenz, wie auch die Veränderung von Wachstumseigenschaften und Sekundärstoffwechsel, die Produktion von neuen Inhaltsstoffen und Pharmazeutika und möglicherweise sogar der Einsatz in der

Altlastensanierung. Zur Beeinflussung komplexer Stoffwechselwege werden zunehmend mehrere Gene transferiert.

Die Abschätzung von Umweltwirkungen transgener Pflanzen ist eine sehr komplexe Aufgabe, sind doch viele Pflanzenarten und eine enorme Anzahl neuer Gene und Eigenschaften zu betrachten, die in Organismen übertragen werden, in denen sie bislang nicht exprimiert wurden. Ist schon die Risikoanalyse für die derzeit in GVO vorherrschenden Eigenschaften der Herbizid- und Insektenresistenz nicht einfach, werden die für die Zukunft geplanten Kombinationen von Eigenschaften und Pflanzenart noch sehr viel größere Herausforderungen an die Risikoanalyse stellen. Die zu erwartenden Transgen-Kombinationen, seien sie durch Transformation mit mehreren Genen, die Kreuzung von Transformanten oder durch unabsichtliche Hybridisierung im Freiland entstanden, werden die Abschätzung von Umweltwirkungen weiter erschweren.

Die Wirkung eines im Empfängerorganismus eingebauten Transgens hängt unter anderem von der Kopienzahl, dem(den) Integrationsort(en) und dem Expressionsniveau ab. Der Transfer von DNA-Sequenzen in Pflanzen erfolgt zumeist mit Hilfe des Bodenbakteriums *Agrobacterium tumefaciens* oder des Partikelbombardements. Entgegen der verbreiteten Ansicht, der Gentransfer sei präzise und Transgene würden in Einzelkopien an einem einzelnen Integrationsort eingebaut, gibt es zunehmend Belege, dass dies nicht so sein muss. Für beide Transformationsverfahren wurde gezeigt, dass häufig mehrere Kopien der Transgene eingebaut werden, entweder gemeinsam an einem Einbauort oder auch an mehreren Orten. Transgensequenzen können verdoppelt, verkürzt, invertiert und neu rekombiniert werden und zu komplexen Integrationsmustern führen. Bei detaillierter Untersuchung der Integrationsorte wurden wiederholt umfangreiche Umlagerungen festgestellt, die sowohl die Transgensequenzen als auch genomische Sequenzen umfassten. Zusätzlich zu den gewünschten DNA-Sequenzen können auch Plasmidsequenzen mit transferiert werden. Hierdurch werden bakterielle Sequenzen, die weitere Antibiotika-Reistenzgene, Replikationsursprünge und rekombinationsfähige Sequenzen umfassen können, übertragen, doch wurde die Präsenz solch überflüssiger DNA bislang nicht routinemäßig untersucht. Um Antibiotika-Resistenzgene aus transgenen Pflanzen wieder zu entfernen, werden verschiedene Methoden vorgeschlagen. Die bei der gentechnischen Veränderung von Pflanzen eingesetzten Promotoren sind möglicherweise nicht so art- und gewebespezifisch wie angenommen. Um die Genexpression und damit die Menge des synthetisierten Proteins zu erhöhen und den Gentransfer via Pollen zu verringern, wird teilweise die Transformation von Chloroplasten empfohlen. Doch auch die Integration von Transgenen ins Chloroplastengenom kann den vertikalen Gentransfer, also die Auskreuzung von GVO, nicht in jedem Fall verhindern. Zugleich erhöhen hier die hohe Kopienzahl der Antibiotika-Resistenzgene, die den Bakterien ähnliche Kodonnutzung, und Promotoren, die sowohl in Chloroplasten als auch in Bakterien aktiv sind, das Risiko eines horizontalen Gentransfers von Antibiotika-Resistenzgenen aus plastidentransformierten Pflanzen in Bakterien.

Gentransfer von transgenen Nutzpflanzen auf nicht-transgene Nutzpflanzen und verwandte Wildpflanzen wurde beobachtet, wobei fremdbefruchtende Arten sehr viel höhere Auskreuzungsfrequenzen zeigen als selbstbefruchtende. Die Transferrate hängt unter anderem ab vom Vorkommen potentieller Kreuzungspartner, von Entfernungen und Populationsgrößen

der Pollenspender und –empfänger, der Kompatibilität der Kreuzungssysteme und natürlich vom Vorkommen befruchtender Insekten und lokalen Bedingungen wie Witterung und Topographie. Für ausgewählte Nutzpflanzenarten Europas wurden Basisdaten für eine potentielle Auskreuzung zusammengestellt. Generell gilt, dass aus einer bestimmten Region stammende Nutzpflanzen in dieser Region auch verwandte Wildpflanzen als potentielle Kreuzungspartner haben werden, was die Wahrscheinlichkeit für einen Gentransfer erhöht. In Europa wird deshalb der Anbau transgener Nutzpflanzen wie Raps, Zuckerrübe, Gräser und Grünfutter, bestimmter Gemüsearten, Obstarten und Bäume ganz allgemein sehr kritisch gesehen. GVO-Durchwuchs in Folgejahren (z. B. beim Raps) verlängert die Zeiträume für den Gentransfer. Bei weltweiter Kommerzialisierung transgener Pflanzen werden GVO und kreuzungsfähige Wildpflanzen häufiger gemeinsam vorkommen, was wiederum eine Auskreuzung wahrscheinlicher werden lässt. Physische und biologische Barrieren für den Gentransfer mögen das Risiko einer Einkreuzung reduzieren, können es aber nicht völlig ausschalten.

Unstrittig ist das Vorkommen von horizontalem Gentransfer unter Mikroorganismen, die Frage eines horizontalen Gentransfers von Pflanzen auf Mikroorganismen wird diskutiert. DNA aus transgenen Pflanzen wurde in Böden, auf denen transgene Pflanzen wuchsen, nachgewiesen, wie auch in anderen Flächen, in die sie von Außen, möglicherweise durch Pollen, eingetragen wurde. Versuche, den direkten horizontalen Gentransfer von Pflanzen auf Bakterien zu beobachten, ergaben keine eindeutigen Ergebnisse, unter Laborbedingungen konnte die Transformation von Bakterien mit transgener Pflanzen-DNA und Pflanzenmaterial jedoch gezeigt werden. Entsprechend neueren Berichten scheint Gentransfer von GVO-Nahrungsmitteln auf die Darmmikroflora unter bestimmten Voraussetzungen möglich zu sein. Bedingungen wie Sequenzhomologie, hohe Kopienzahl der Transgene, geringe Genomgröße des DNA-Spenders und Selektion für die übertragenen Transgene könnten die Wahrscheinlichkeit für horizontalen Gentransfer erhöhen. Die Rolle der in transgenen Pflanzen vorhandenen bakteriellen Sequenzen wird diskutiert. Obwohl horizontaler Gentransfer von transgenen Pflanzen auf Mikroorganismen als sehr seltenes Ereignis eingeschätzt wird, sind Umwelteffekte nicht völlig ausgeschlossen.

Transgene können auch über verwilderte transgene Pflanzen und die Verbreitung von Samen in andere Areale gelangen. Durch die Einführung der unterschiedlichsten Gene entstehen Organismen mit Kombinationen von genetischem Material und Eigenschaften, die für das bestehende Netzwerk ökologischer Wechselwirkungen in den aufnehmenden Habitaten neuartig sind. So wird diskutiert, ob transgene Pflanzen verstärkt Unkrauteigenschaften zeigen oder in natürliche Ökosysteme eindringen können und schwere und irreversible Effekte auf die biologische Vielfalt auslösen. Transgene und Eigenschaften, die eine Resistenz gegen Schädlinge, Krankheitserreger und Stress oder verstärktes Wachstum mit sich bringen, können die Fitness der transgenen Pflanzen erhöhen und gelten deshalb als besonders problematisch, zumal sie auch in Kombination auftreten können. Mehrjährige Empfängerpflanzen, die widerstands- und wettbewerbsfähig sind, sich stark vermehren und verschiedene natürliche und semi-natürliche Habitate besiedeln können, zählen zu Hochrisiko-Pflanzen in Bezug auf die Ausbreitung. Allerdings gibt es keine verlässlichen Parameter für die Vorhersage eines Invasionserfolgs transgener Pflanzen, da der Invasionserfolg auch von den spezifischen Charakteristika der aufnehmenden Habitate

abhängt. Deshalb sind Daten, erhoben an einer transgenen Pflanze in einem Habitat, nicht notwendigerweise auf andere Habitate zu übertragen.

Umweltwirkungen transgener Pflanzen sind bestimmt durch die neu eingeführten Eigenschaften und die Möglichkeit der neuen Phänotypen, in neuartiger, potentiell negativer Weise mit den Organismen der aufnehmenden Ökosysteme zu interagieren. Der Erwerb neuer gewünschter Eigenschaften in transgenen Pflanzen kann darüber hinaus von unerwünschten und unerwarteten Veränderungen des Phänotyps begleitet werden. Positionseffekte, bedingt durch die Transgen-Integration und Sequenzumlagerungen der (multiplen) Integrationsorte, können die Aktivität der Transgene, aber auch die der endogenen Gene ändern. Pleiotrope Effekte, bedingt durch die Genprodukte selbst oder deren Beteiligung an pflanzlichen Stoffwechselvorgängen, können ebenfalls zu unerwarteten phänotypischen Veränderungen führen, die möglicherweise erst unter bestimmten Umwelt- und Stressbedingungen auftreten. Organismen, mit denen die jeweilige Pflanzenart normalerweise interagiert, könnten durch den neuen Phänotyp beeinflusst werden.

Die Herbizidresistenz, die häufigste Eigenschaft kommerzialisierter GVO, beeinflusst Pflanzenwachstum und Bodenleben durch die Applikation der Breitband-Herbizide, die nicht nur gegen Pflanzen wirken, sondern auch gegen Mikroorganismen. Die effiziente Beseitigung der Unkräuter verringert die Artenvielfalt der Wildpflanzen in Agrarökosystemen, was wiederum Arthropoden, Bodenorganismen, Vögel und andere Tiere, die auf eine vielfältige Pflanzenwelt angewiesen sind, beeinträchtigt. Breiter und häufiger Einsatz von Herbiziden führt unausweichlich zur Entwicklung herbizidresistenter Unkräuter und verändert die Artenzusammensetzung der Unkrautpopulationen, wobei sich weniger empfindliche Arten durchsetzen. Bei acht Unkrautarten wurde inzwischen eine Resistenz gegen Glyphosat nachgewiesen. Zur Bekämpfung resistenter Arten werden in der Regel höhere Herbiziddosen in Mehrfachapplikationen ausgebracht. Die vielfach angekündigte Reduktion des Herbizideinsatzes hat sich deshalb nicht eingestellt.

Alle transgenen insektenresistenten Baumwoll- und Maispflanzen im kommerziellen Anbau exprimieren *Bacillus thuringiensis* (Bt)-Toxingene. Die Expression aktivierter Toxine in allen pflanzlichen Geweben und während der gesamten Wachstumsperiode führt zu neuen Möglichkeiten der Exposition für Insekten, die sich von den Expositionspfaden unterscheiden, wie sie für bislang eingesetzte Bt-Präparaten bekannt sind. Bt-Toxine in transgenen Pflanzen sind nicht so spezifisch in ihrer Wirkung wie erwartet. Deshalb können Organismen, die nicht zu den anvisierten Schädlingen gehören, durch Bt-Pollen oder -Pflanzenmaterial ebenfalls geschädigt werden, wie eine Reihe von Studien mit Nichtziel-Schmetterlingen, z. B. Monarch oder Schwalbenschwanz, nahe legt. Nützlinge wie die Florfliege können sogar mittelbar beeinträchtigt werden, indem sie Beute fressen, die ihrerseits mit Bt-Pflanzen gefüttert wurde. Bt-Toxine werden über Wurzeln in den Boden abgegeben, die Bindung an Bodenpartikel verlangsamt ihren Abbau und stabilisiert die Toxizität über Monate. Bt-Pflanzenreste scheinen, verglichen mit Resten von nicht-Bt-Pflanzen, langsamer abgebaut zu werden, was die Zeitspanne, innerhalb derer Bodenorganismen den Toxinen ausgesetzt sind, verlängert. Um potentielle Effekte von Bt-Toxinen auf Nichtzielorganismen zu erfassen, sind deshalb auf mehrere Jahre angelegte Studien erforderlich, die unterschiedliche Entwicklungsstadien, Ernährungsgewohnheiten und Beute-Räuber-Beziehungen von Nichtzielinsekten

berücksichtigen. Insektenresistente Pflanzen, die andere Abwehrstoffe als die Bt-Toxine exprimieren, beeinflussen möglicherweise sogar ein breiteres Spektrum von Insekten und Nichtzielorganismen, da diese Substanzen im allgemeinen noch weniger spezifisch wirken als Bt-Toxine. Gelangen Resistenzgene durch Kreuzung in Wildpflanzen, würden die potentiellen Umweltwirkungen insektenresistenter Pflanzen verschärft, da eine höhere Fitness dieser Pflanzen zu erwarten wäre und eine weitaus größere Vielfalt an Nichtzielorganismen den Toxinen ausgesetzt sein könnte.

Die Erfahrungen mit dem Einsatz von Insektiziden (einschließlich *B. thuringiensis* Präparate) lehren, dass der Selektionsdruck, der durch den breiten Anbau insektenresistenter Pflanzen (die in allen Pflanzenteilen während der gesamten Vegetationsperiode ein Insektizid bilden) ausgeübt wird, mit hoher Wahrscheinlichkeit zur Entwicklung resistenter Insekten führen wird. Um die Entwicklung von Resistenzen gegen Bt-Toxine bei Schädlingen zu verlangsamen, wurde die Hochdosis-Refugien-Strategie empfohlen. Voraussetzung für das Funktionieren dieser Strategie sind eine rezessive Vererbung der Resistenzgene in Schädlingspopulationen und die Bildung einer ausreichend hohen Bt-Toxindosis in transgenen Pflanzen, sodass heterozygote Individuen getötet werden. Zusätzlich müssen die Refugien aus nicht-Bt-Pflanzen so groß sein, dass sich genügend homozygot-empfindliche Individuen entwickeln können, die als Kreuzungspartner für die resistenten Individuen in Frage kommen. Doch diese Bedingungen sind im Freiland möglicherweise nicht immer erfüllt. Derzeit ist offen, ob infolge eines breiten Anbaus insektenresistenter Nutzpflanzen der Insektizideinsatz langfristig tatsächlich zurückgehen wird.

Die gentechnisch verliehene Resistenz gegen Krankheitserreger wie Viren, Bakterien und Pilze soll zu einer Verringerung des Pestizideinsatzes und zu mehr Umweltverträglichkeit führen. Doch auch negative Umwelteffekte sind nicht ausgeschlossen. Insbesondere die durch die Übertragung viraler Sequenzen erzeugte Virusresistenz könnte zu heterologer Enkapsidierung und/oder Rekombination viraler Sequenzen und zur Entstehung von Viren mit neuen Eigenschaften führen. Substanzen, die gegen krankheitserregende Bakterien und Pilze gerichtet sind, könnten auch mit neutralen oder nützlichen Mikroorganismen interagieren und so vielleicht sogar Symbiosepartner wie die Stickstoff-bindenden Knöllchenbakterien oder Mycorrhizapilze beeinträchtigen. Negative Effekte auf Artenvielfalt und Funktionen des Bodenlebens wären nicht ausgeschlossen. Denkbar ist auch, dass einige der neu gebildeten antimikrobiellen Substanzen das Pflanzenwachstum beeinflussen oder sich als toxisch für Tiere erweisen. Über horizontalen Gentransfer solcher Resistenzgene würden Mikroorganismen unter Umständen Wettbewerbsvorteile erwerben – mit unbekannten Effekten auf das Bodenleben. Die Ausprägung von Resistenzeigenschaften kann die Fitness der transgenen Pflanzen erhöhen und damit ihre Fähigkeit zur unkontrollierten Ausbreitung. Die Kreuzung mit Wildpflanzen würde auch deren Nachkommen potentiell eine erhöhte Fitness vermitteln.

Bei Versuchen, die Resistenz von Pflanzen gegen einzelne abiotische Stressfaktoren wie Salz, Kälte, Hitze und Trockenheit zu erhöhen, zeigten die transgenen Pflanzen häufig auch eine veränderte Reaktion gegen anderen abiotischen Stress. Die Beobachtung, dass abiotischer Stress im Wesentlichen zu Wasserdefizit führt und in die pflanzliche Reaktion auf Salz, Kälte, Hitze und Trockenheit häufig ähnliche Reaktionswege involviert sind, mag diese Effekte

erklären. Die erhöhte Fitness solcher transgener Pflanzen könnte vor allem unter Stressbedingungen ihre Ausbreitung begünstigen und negative Wirkungen auf die biologische Vielfalt mit sich bringen. Verwandte Wildpflanzen, durch Kreuzung in den Besitz entsprechender Resistenzgene gelangt, würden sich unter Umständen zu schwer kontrollierbaren Unkräutern entwickeln. Sollten stressresistente Nutzpflanzen in ökologisch wertvollen Gebieten angebaut werden, die bislang für eine agrarische Nutzung ungeeignet sind, wäre eine Beeinträchtigung der Artenvielfalt auch in diesen Arealen zu befürchten. Pflanzen mit erhöhter Wurzelausscheidung von organischen Säuren, die tolerant gegen hohe Aluminiumgehalte in sauren Böden sein sollen, könnten Aluminium auch in Wurzeln und Spross akkumulieren, was wiederum Fragen nach der potentiellen Toxizität solcher Pflanzen für Menschen, Nutztiere und Wildtiere aufwirft.

Die Veränderung von Wachstumseigenschaften ist ebenfalls ein Ziel der gentechnischen Veränderung von Pflanzen. Eine veränderte Pflanzengestalt, früheres Blühen und verlangsamte Alterung sollen die Produktivität erhöhen, weniger Lignine oder deren andere Zusammensetzung die Zellstoff- und Papierproduktion vereinfachen. Durch den Transfer von Photosynthese-Genen aus C4 Pflanzen (beispielsweise Mais) in C3 Pflanzen (Reis, Weizen, Tabak etc.) wird eine Ertragssteigerung angestrebt, bislang allerdings mit mäßigem Erfolg. Die Veränderung von zentralen und vernetzten Stoffwechselprozessen führt mit hoher Wahrscheinlichkeit zu zahlreichen pleiotropen Effekten. Erwünschte Veränderungen von Morphologie und Wachstum können von unerwünschten Störungen der Blüten- und Wurzelbildung oder veränderten Reaktionen auf Schädlinge und Krankheitserreger begleitet werden. Eingriffe in den mit mehreren anderen Stoffwechselwegen verknüpften Ligninstoffwechsel können dazu führen, dass sich die Zusammensetzung der pflanzlichen Biomasse über den reduzierten Ligningehalt hinaus ändert und den Abbau abgestorbenen Pflanzenmaterials beeinflusst. Zu erwarten ist, dass verstärktes Wachstum, beschleunigtes Blühen und verzögerte Reifung beziehungsweise Alterung die Wettbewerbsfähigkeit und damit einen potentiellen Invasionserfolg der transgenen Pflanzen erhöhen. Ähnlich wie im Falle schädlings-, krankheits- oder stressresistenter Pflanzen wäre der Transfer derartiger Eigenschaften auf Wildpflanzen besonders problematisch.

Als zukunftsweisend werden vielfach transgene Pflanzen mit veränderter Zusammensetzung von Proteinen, Fettsäuren oder Kohlehydraten, mit neuen Produkten wie Kunststoffen oder Fasern oder mit veränderten sekundären Inhaltsstoffen bezeichnet. Besonders Pflanzen mit einem erhöhten Vitamin- und Mineralstoffgehalt sollen hochwertigere Nahrung liefern und Vitaminmangelkrankheiten, die unter armen Bevölkerungsgruppen in Entwicklungsländern verbreitet sind, lindern. Diese Nutzpflanzen der „zweiten und dritten“ Generation (im Vergleich zu herbizid- und insektenresistenten Pflanzen der „ersten“ Generation) sollen zudem die Akzeptanz für gentechnisch erzeugte Lebensmittel bei der Verbraucherschaft steigern. Doch der gentechnische Eingriff in die Biosynthesewege des Sekundärstoffwechsels führt mit hoher Wahrscheinlichkeit zu unerwarteten Veränderungen im Gehalt anderer pflanzlicher Inhaltsstoffe, manche davon toxisch für Mensch und Tier. Auch Veränderungen von Phänotyp, Wachstums- und Fortpflanzungseigenschaften sowie der Reaktion auf Schädlinge und Krankheiten treten unter Umständen auf. Neue oder in veränderter Menge gebildete Sekundärmetabolite können sich als toxisch für Organismen erweisen, die normalerweise mit der Nutzpflanze interagieren, seien es Bodenorganismen, Arthropoden

oder höhere Tiere. Kreuzen sich transgene Pflanzen mit verändertem Sekundärstoffwechsel mit Wildpflanzen, ist damit zu rechnen, dass auch der Sekundärstoffwechsel der Nachkommen verändert wird. Da unterschiedliche genetische Hintergründe die Expression der Transgene und die Reaktion von Enzymen und Substraten beeinflussen, lassen sich mögliche Wirkungen hier nicht vorhersagen. Transgene Pflanzen, die neue oder veränderte Inhaltsstoffe für die industrielle Nutzung wie etwa Plastikpolymere oder Spinnenseide bilden (und deren Produkte), müssten strikt getrennt von Nahrungs- und Futtermittelpflanzen angebaut und verarbeitet werden. Durch entsprechende Vorkehrungen wäre dafür zu sorgen, dass keine Hybridisierung und Vermischung mit anderen Pflanzen erfolgen kann.

In den letzten Jahren wurde verstärkt dafür geworben, transgene Pflanzen zur billigen Massenproduktion von Antikörpern, Impfstoffen und Humanproteinen zu nutzen. Unter den zu diesem Zweck transformierten Pflanzenarten befinden sich zahlreiche Nahrungspflanzen wie etwa Mais, Kartoffel, Tomate, Banane und Spinat. In Pflanzen gebildete Proteine humanen Ursprungs entsprechen aber in ihrer Aminosäuresequenz und Struktur nicht unbedingt dem korrespondierenden menschlichen Protein, zudem könnte die pflanzenspezifische Glykosylierung den Einsatz von Antikörpern beschränken. Nicht ausgeschlossen ist, dass verborgene Funktionen pharmazeutisch wirksamer, neu in Pflanzen gebildeter Proteine auftreten und den Pflanzenstoffwechsel beeinflussen. Biopharmazeutika wirken üblicherweise bereits in geringer Dosis; die höheren in Pflanzen auftretenden Konzentrationen könnten toxisch für Mensch und Tier sein, wenn solche Pflanzen unbeabsichtigt aufgenommen werden. Unbekannt ist derzeit, ob solche Substanzen in der Umwelt persistieren und sich in Organismen anreichern, ob sie über Wurzelausscheidungen in den Boden gelangen und Gewässer erreichen können oder ob sie die Fitness der transgenen Pflanzen verändern. Ein Gentransfer in Nahrungs- und Futtermittelpflanzen würde zu unerwünschter Kontamination der Nahrungsmittelkette führen, Einkreuzung und Expression von Pharmagenen in Wildpflanzen könnten Wildtiere und möglicherweise das Bodenleben gefährden. Da Pharmazeutika produzierende Pflanzen auch als Durchwuchs in nachfolgenden Kulturen die Nahrungsmittelkette kontaminieren könnten, wären strikte Kontrollmaßnahmen auch in Folgejahren zwingend.

8 Appendices

8.1 Glossary

Apoptosis
Programmed cell death
Bt crop
Insect resistant crop variety that produces an insecticidal toxin originally found in various strains of the soil bacterium *Bacillus thuringiensis*.
Bt toxin
Insecticidal protein produced by the soil bacterium *Bacillus* thuringiensis, genes for Bt toxins have been transferred to crop plants. Bt toxins differ with respect to their specificity for insect orders such as butterflies or beetles.
C3 plants
In C3 plant photosynthesis, the first product of carbon fixation in the chloroplast is the 3-carbon compound 3-phosphoglycerate. Most crops are C3 plants.
C4 plants
In C4 plant photosynthesis, the first product of carbon fixation in the chloroplast is a 4-carbon compound such as oxaloacetate. C4 plants exhibit higher rates of CO_2 assimilation, examples are maize and sugarcane.
Chaperone
Proteins that assist and maintain correct folding and trafficking of cell proteins. Many chaperones are heat shock proteins, i.e. proteins expressed in response to high temperatures.
Construct
Genetic sequences linked together into a unit that can be transferred to recipient organisms. Constructs typically include one or more genes for new traits (genes of interest), a marker gene, and regulatory sequences such as promoters and terminators. Also called transgene construct or gene construct.
Cross-pollination
Pollen transfer between different individual plants. Corn and oilseed rape are predominantly cross-pollinating.
Cry toxins
Insecticidal toxins from *Bacillus thuringiensis*, e. g. Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1C, Cry1F, Cry1J, Cry2Aa, Cry3A, Cry3Bb, Cry5B, Cry14A
Event
Plant line derived from the random insertion of a construct into the plant genome. Each insertion results in a different event, even when the same construct has been transferred.
Fertilization
Combining of pollen (male sex cells) with female sex cells of flowers to produce plant embryos. Formation of seeds (which contain embryos) is triggered.
Gene
Functional unit of DNA sequences usually carried on chromosomes and passed on to offspring. In general, genes code for proteins. Differential splicing of the transcribed mRNA can lead to different proteins.

Gene expression
Production of (mRNA and) proteins coded for by the gene(s) in question.
Gene pharming
Production of pharmaceuticals in transgenic plants, coined from pharmaceutical and farming.
Gene pool
The gene pool of a crop comprises all genes of all the varieties of a crop, including landraces and wild relatives that can hybridize with the crop.
Gene silencing
Inactivation of genes, can act on the transcriptional level (gene is not accessible to transcription machinery) and post-transcriptional level (mRNA is selectively destroyed) in order to regulate cellular gene activity. Gene silencing may also act as protective measure against foreign DNA (e. g. viral genes and transgenes).
GENESYS
Computer model developed to describe the occurrence and distribution of volunteers in GMO cultivation.
Genome
Full set of genes and associated DNA characteristic of an organism.
Genotype
Genetic constitution of an organism.
Glycosylation
Attachment of sugar molecules to proteins. Many proteins require glycosylation for correct functioning, the glycosylation pattern may also influence the allergenicity of a protein.
GMO
Abbreviation for genetically modified organism(s). The terms GEO for genetically engineered organism(s) or LMO for living modified organism(s) are used too.
Heterologous encapsidation
Nucleic acids of viruses infecting virus resistant plants are packaged into coat proteins encoded by the transgene (heterologous proteins). This could influence virus transfer.
Heterosis
Superior performance of hybrids, compared to (homozygotic) parent lines, also known as hybrid vigour.
Heterozygotes
Organisms that carry a specific gene or mutation on only one chromosome of the chromosome pair.
Homozygotes
Organisms that carry a specific gene or mutation on both chromosomes of the chromosome pair.
Horizontal gene transfer
Gene transfer from one organism to an organism of a different species without sexual propagation.
HR crop
Crop variety that expresses the herbicide resistance trait. Toxic broad spectrum herbicides such as Roundup or Liberty can be applied without damaging the HR crop.
Hybrid
Offspring of two parent plants that differ from one another in one or more genes. Hybrids do not breed true.
Hybridization
Crossing of parent plants that differ from one another in one or more genes.
Integration site

Genome site into which the transgene has been inserted. Multiple integration sites are possible.

Marker gene

Gene coding for a selectable marker such as an antibiotic resistance trait that protects from the action of antibiotics. Marker genes are used in plant transformation to ease selection of the few plant cells that have integrated foreign DNA.

Phenotype

Visible appearance of an organism.

Phytoremediation

Remediation of areas contaminated by heavy metals or other toxic compounds by use of (transgenic) plants.

Plasmids

Small circular DNA sequences found in bacteria. They can be used as vehicles for the transfer of genes.

Pleiotropic effects

Unintended effects caused by the gene product directly or by the alteration of plant biochemistry through the gene products.

Pollination

Transfer of pollen by wind or insects from male part of a flower to the female part. If pollen and the female part are compatible, pollination is followed by fertilization.

Position effects

Unintended effects due to transgene integration and scrambling of the integration sites.

Promoters

Regulatory sequences of DNA that control the activity of genes. Promoters act species and cell type specific and determine the strength of gene activity. Their activity can naturally and artificially be modified by enhancing elements called enhancers.

Self-pollination

Transfer of pollen from male part of a flower to the female part of a flower on the same plant. Soybean is a predominantly self-pollinating crop.

Somaclonal variation

Genomic and phenotypic changes seen in plants derived from tissue culture.

Stacked traits

Two or more transgenes are expressed in a genetically modified plant, such as maize carrying both a Bt toxin gene and a herbicide resistance gene.

T-DNA

Transfer DNA derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*, bordered by left and right repeat sequences. T-DNA enables transfer of foreign DNA to random locations of the plant genome.

Telomeric region

Highly repetitive DNA sequences at the ends of chromosomes.

Ti-plasmid

Tumor-inducing plasmid of *Agrobacterium tumefaciens*, containing genes for DNA transfer to plant cells. When Agrobacteria infect plants, gene transfer leads to plant tumor formation by altering the hormone balance in the plant cell.

Transformation

Natural or artificial uptake of foreign DNA by bacteria or other organisms. Bacteria can take up naturally DNA from the environment. Artificially DNA can be transferred to organisms such as plants by the help of *Agrobacterium tumefaciens* or by microprojectile bombardment, generating a genetically modified organism.

Transgene

Gene transferred to an organism through genetic engineering.

Tri-trophic interaction

Interaction between three trophic levels of organisms, e.g. effects exerted by a transgenic plant (first trophic level) on a herbivore (second trophic level) and on predators and parasitoids (third trophic level).

Variety

Subgroup of plants within a species. Its genetic makeup and characteristics distinguish it from other varieties of the species.

Volunteers

Crop plants that emerge from lost seed in succeeding years. Oilseed rape is a ready volunteer.

8.2 Abbreviations

aadA	aminoglycoside 3‘-adenyltransferase gene
ABA	abscisic acid
AFLP	Amplified Fragment Length Polymorphism
ALS	acetolactate synthase
AMPA	aminomethyl phosphonic acid
APHIS	USDA’s Animal and Plant Health Inspection Service
BADH	betaine aldehyde dehydrogenase
bar	gene for PAT from *Streptomyces hygroscopicus*
CAD	**c**innamyl alcohol dehydrogenase
CaMV	cauliflower mosaic virus
COR	cold regulated gene
CTB	cholera toxin B
2,4-D	dichlorophenoxyacetic acid, synthetic auxin
DEFRA	UK Department for Environment, Food and Rural Affairs
DETR	former UK Department of Environment, Transport and the Regions
DRE	dehydration response element
EFSA	European Food Safety Authority
EPA	US Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FBPase	fructose-1,6-bisphosphatase
FISH	fluorescence in situ hybridization
FSE	farm scale evaluation, GMO trial programme in the UK
GFP	green fluorescent protein
GNA	*Galanthus nivalis* agglutinin, snow drop lectin
GOX	glyphosate oxidoreductase
GTN	glycerol trinitrate
GURT	genetic use restriction technology
GUS	ß-glucuronidase
HGT	horizontal gene transfer
HR	herbicide resistance/herbicide resistant
HSL	Homoserine lactones
JA	jasmonic acid
LEA	late embryogenesis abundant

LL	LibertyLink
MA	mugineic acids
MOA	mode of action (of an herbicide)
NOS	nopaline synthase gene terminator
npt	neomycin phosphotransferase gene
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PAT	phosphinothricin-acetyltransferase
pat	gene for PAT from *Steptomyces viridochromogenes*
PCR	polymerase chain reaction
PDR	pathogen-derived resistance
PEG	polyethylene glycol
PEPC	phosphoenolpyruvate carboxylase
PHA	polyhydroxy acid
PHB	poly(3-hydroxy-butyrate)
POEA	surfactant polyoxyethyleneamine (a derivative of tallow)
PTGS	post transcriptional gene silencing
PVY	potato virus Y
RAPD	random amplification of polymorphic DNA
RDV	rice dwarf virus
RFLP	restricted fragment length polymorphism
RIP	ribosome inactivating protein
RNAi	RNA interference
RR	RoundupReady
SA	salicylic acid
SAR	systemic acquired resistance
SBPase	sedoheptulose-1,7-bisphosphatase
S/MAR	scaffold/matrix attachment region
TEV	tobacco etch virus
TMV	tobacco mosaic virus
TNT	2,4,6-trinitrotoluene
USDA	United States Department of Agriculture
UTR	untranslated repeat (sequence)

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