An assessment of factors affecting the likelihood of horizontal transfer of recombinant plant DNA to bacterial recipients in the soil and phytosphere

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Abstract

The unintended transfer of plant transgenes to bacteria would result in the uncontrolled dispersal of the engineered genes to new hosts, and, in the case of antibiotic resistance marker genes, impede clinical treatment of bacterial infections. However the potential impact of gene transfer from plants to bacteria should be a concern for any released transgene that possesses unique characteristics in the environment.

The plant phytosphere is heavily colonised with bacteria that may be exposed to transgenic plant DNA when released from the plant. However the uptake of plant transgenes in natural populations of bacteria in the phytosphere remains to be demonstrated.

The main factors affecting a stable horizontal gene transfer (HGT) of plant transgenes to bacteria are the following: (i) availability of plant DNA to competent bacteria in the field, (ii) ability of bacteria to translocate plant DNA into the bacterial cytoplasm, (iii) stabilization of plant DNA in the bacterial recipient, (iv) expression of plant transgenes in the bacterial transformant, and (v) selection of transformants in a fitness landscape.

Analysing the likelihood of HGT of plant transgenes to soil bacteria, an impact of the *cry*, *pat*, *bar*, CaMV sequences, and EPSPS encoding plant transgenes has not been identified in the soil, with the exception of some antibiotic resistance marker genes.

The laboratory investigations that have exposed various bacterial recipients to transgenic plant DNA have confirmed the dependency of DNA sequence homology for integration (via homologous recombination), suggesting that the presence of such homologous sequences may facilitate the transfer of engineered plant genes into bacteria. Such recombination, however, has not been demonstrated.

Riassunto

Il trasferimento occasionale di transgeni dalla pianta a batteri potrebbe dare origine alla passaggio non controllato dei geni ingegnerizzati verso nuovi ospiti, e, nel caso di geni marcatori per la resistenza agli antibiotici, ostacolare il trattamento clinico delle infezioni batteriche. Comunque

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l'impatto potenziale del trasferimento di geni dalle piante ai batteri dovrebbe essere considerato seriamente per qualsiasi transgene rilasciato che possieda caratteristiche uniche nell'ambiente.

La fitosfera delle piante e' colonizzata pesantemente da batteri che in questo modo possono essere esposti al DNA della pianta transgenica qualora questo venisse rilasciato dalla pianta stessa. Resta comunque ancora da dimostrare l'acquisizione dei transgeni provenienti da piante modificate, da parte di popolazioni naturali di batteri nella fitosfera.

I principali fattori che influenzano uno stabile trasferimento orizzontale di geni dalla pianta ai batteri sono i seguenti: (i) disponibilità del DNA della pianta per batteri competenti presenti nel campo, (ii) capacità dei batteri di trasferire il DNA della pianta nel loro citoplasma, (iii) stabilizzazione del DNA della pianta nel batterio ricevente, (iv) espressione dei transgeni della pianta nei transformanti batterici, (v) selezione dei transformanti nel particolare panorama adattativo ambientale.

Analizzando la probabilita' del trasferimento di transgeni da una pianta ai batteri del suolo, non e' stato identificato alcun impatto nel suolo delle sequenze dei geni cry, pat, bar, CaMV, ed EPSPS, con l'eccezione di alcuni geni marcatori per la resistenza ad antibiotici.

Indagini di laboratorio in cui dei batteri vengono esposti al DNA proveniente da una pianta transgenica hanno confermato la dipendenza dell'integrazione dall'omologia tra sequenze di DNA (via ricombinazione omologa), suggerendo che la presenza di tali sequenze omologhe possa facilitare il trasferimento dei geni della pianta ingegnerizzata ai batteri. Tale ricombinazione non è comunque stata ancora dimostrata.

Preface

This report has been requested by the International Centre for Genetic Engineering and Biotechnology (ICGEB), Area Science Park, Padriciano 99, I-34012 Trieste, Italy, to summarize the scientific knowledge on horizontal gene transfer in soil bacteria with specific reference to the putative transfer of genes from transgenic crop plants expressing resistance to the herbicides glufosinate or glyphosate, insect resistance (Bt-toxin) or viral sequences from cauliflower mosaic virus. Although great care has been taken, errors or omissions in the text may occur. The views expressed in this publication are those of the author and do not necessarily reflect the views of ICGEB.

For additional information about the subjects covered in this report, the reader is referred to the following overviews on biosafety aspects of transgenic plants and horizontal transfer of plant transgenes to bacteria and gene transfer between bacteria: Tiedje et al., 1989, Fry and Day 1990, Heinemann, 1991, 1997, Ginzburg, 1991, Veal et al., 1992, Wellington and van Elsas, 1992, Amábile-Cuevas and Chicurel, 1993, Kidwell, 1993, Kok et al., 1994, Lorenz and Wackernagel, 1994, Paget and Simonet, 1994, Syvanen, 1994, Landsmann and Casper, 1995, Smalla, 1995, 2000, Trevors and van Elsas, 1995, Tzotzos, 1995, Harding, 1996, Schlüter and Potrykus, 1996, Kruse and Jansson, 1997, Wöstemayer et al., 1997, Yin and Stotzky, 1997, Ljungquist et al., 1998, Dröge et al., 1998, 1999, Nielsen, 1998, Nielsen et al., 1998, 2000, 2001, Nielsen and Townsend, 2001, Bertolla and Simonet, 1999, Doolittle, 1999, Davison, 1999, Campbell, 2000, Ochman et al., 2000, Feil et al., 2001.

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Executive summary[1]

The application of gene technology in plant breeding holds great promise for the future. However, concerns about possible health and environmental impacts of the technology have resulted in the enforcement of extensive governmental regulations to ensure that the novel crop varieties are as safe as their traditionally bred counterparts. The likelihood and consequences of horizontal transfer of engineered genes from transgenic plants to microorganisms are frequently evaluated in such regulatory risk assessments preceding field release. It has been proposed that the unintended transfer of plant transgenes to bacteria would result in the uncontrolled dispersal of the engineered genes to new hosts, and, in the case of antibiotic resistance marker genes, impede clinical treatment of bacterial infections. It is noted that this is a concern based on precaution since it is the overuse of antibiotics that has been identified as the major cause of the resistance problems seen today. Nevertheless, much of the discussion of potential impacts of gene transfers from plants to bacteria have been focused on the impact of antibiotic resistance markers since plant markers encoding antibiotic resistance are the most commonly occurring transgenes in commercialized genetically modified plants (GMPs). However, a similar assessment of ecological and evolutionary impact should be applied to any released transgenes that possess unique or undesired selectable characteristics in the environment.

In this report, the factors influencing the bacterial utilization of horizontally transferred DNA are assessed with emphasis on the probability of transfer of plant transgenes to bacteria. The impact of plant transgenes conferring either antibiotic, herbicide, insect or viral sequences, if transferred to indigenous bacteria in soil, is assessed based on the following factors:

1. Knowledge of the prevalence, population dynamics and transfer of naturally occurring homologues of the transgene in the environment.
2. Identification of alternative exposure sources of the transgene homologues introduced in the same environment.
3. Evaluation of selective pressure (agents) and potential fitness advantage conferred by the novel plant transgene if transferred to a bacterial host.

Moreover, knowledge gained from the experimental investigations conducted to detect the occurrence of such transfers are presented and discussed.

Chapter 2 defines horizontal gene transfer and lists recent literature that emphasize the importance of gene flow in bacterial communities. The

[1] See Nielsen et al., 2000d

known mechanisms of horizontal gene transfer between bacteria and their relevance to the transfer of recombinant plant DNA to bacteria are reviewed. The natural host ranges of both conjugation and transduction are confined mainly to bacterial donors and recipients, and further limited by the transfer and stabilization functions of the replicon of the mobile conjugative elements or bacteriophages. It appears that only natural transformation, the uptake of extracellular DNA by bacteria, could facilitate HGT from plants. The occurrence of bacterial gene transfer in the phytosphere is briefly reviewed. It is concluded that the phytosphere, including rhizosphere (plant root surfaces), aerial plant surfaces, and plant tissues, all are heavily colonized with bacteria which are candidates for exposure to transgenic plant DNA released from the plant during cell decomposition, cell lysis induced by plant pathogens, or after the mechanical disruption of plant tissue caused by insect larvae, aphids or nematodes. However, the uptake of plant transgenes in natural populations of bacteria residing in the phytosphere remains to be demonstrated.

Chapter 3 identifies the main factors that limit stable HGT of plant transgenes to bacterial recipients. Several barriers (Chapter 3.1 to 3.5) representing both environmental (DNA availability, physiological competence development, fitness advantages) and cellular parameters (e.g. sequence specific DNA uptake and factors affecting integration and expression of the translocated DNA) have been identified that may determine the probability of an unintended transfer and stabilization of plant transgenes in bacteria. In the following, each of these barriers are briefly discussed.

(i) **Availability of plant DNA to competent bacteria in the field**
Although the majority of plant DNA in decaying plants is rapidly broken down by plant or microbial DNases, fragments of plant DNA have been shown, by PCR, to persist under field conditions for several months and even up to years. The availability of this persistent DNA to competent bacteria remains to be demonstrated. The dynamics of the release, stability, and bacterial availability of plant DNA in specific microhabitats in the phytosphere such as within the rhizosphere, on leaf surfaces and in plant tissues have not been described or quantified.

(ii) **Ability of bacteria to translocate plant DNA into the bacterial cytoplasm**
As many bacteria do not discriminate between the type of DNA they translocate over the bacterial membrane, it is reasonable to assume

that competent bacteria are exposed to plant transgenes to the same extent as to other plant DNA. Although positive results have been obtained *in vitro*, the ability of bacteria to take up plant DNA that is naturally released *in situ* e.g. in soil or *in planta*, has not been shown. Information on the general composition, dynamics and succession of bacterial communities in the phytosphere of various agriculturally grown plants needs to be further determined and understood before the potential bacterial recipient populations of plant transgenes in these habitats can be identified.

(iii) Stabilization of plant DNA in the bacterial recipient

The stability of translocated plant DNA in the bacterial cytoplasm would depend on its susceptibility to fragmentation and degradation by bacterial enzymes and on whether it is integrated into the bacterial chromosome or into a plasmid. The action of bacterial restriction enzymes on plant DNA is probably insignificant since DNA is likely to translocate across bacterial membranes in a single-stranded form whereas bacterial restriction enzymes recognize and cleave only double-stranded DNA. Integration of the translocated plant DNA into bacterial DNA is dependent upon the degree of sequence homology between the incoming plant DNA and that of the bacterial recipient. For the uptake of heterologous DNA sequences in *Escherichia coli*, *Bacillus subtilis* and *Streptococcus* sp., a log-linear relationship between decreasing recombination frequencies and increasing DNA sequence divergence has been established. Thus, the overall low level of sequence homology between plant and bacterial DNA may naturally limit this type of horizontal gene transfer in nature. However, as argued below, transgenic plants may have increased levels of sequence homology to bacterial genomes due to the frequent bacterial origin of the functional transgene and co-transferred cloning vector sequences.

(iv) Expression of plant transgenes in the bacterial transformant

The expression of the transferred plant transgene in the bacterial recipient would often be a prerequisite for a positive selection of the novel bacterial phenotype. Some of the promoters currently used to control the expression of plant transgenes are of prokaryotic origin and may therefore also be active in bacteria. Others are under control of plant promoters which may be non-functional in bacteria. Since most plant transgenes lack introns, there may be few constraints, other than codon usage, to the expression of plant transgenes in bacterial recipients, if transferred. This situation is expected to change as more eukaryotic promoters become available that direct expression of the

transgene to defined plant tissues only.

(v) **Selection of transformants in a fitness landscape**
As argued in chapter 5.3, the frequency of HGT from plants to bacteria will be low. Thus, positive selection (and amplification) for the acquired trait would be required to generate a significant impact in the bacterial population. The prediction of selective advantages of plant transgenes in bacterial populations is hampered by a lack of understanding of the selective pressures on bacteria in complex natural environments such as soil. Risk assessment procedures should focus on identifying factors that may contribute to the selection of plant transgenes in bacterial populations. The impact of a transferred plant transgene in bacterial populations can only be properly understood and resolved in the context of its fitness advantages.

Chapter 4 focuses on the likelihood of horizontal transfer of plant transgenes that encode resistance to antibiotics, the herbicides glufosinate or glyphosate, insects (Bt-toxin) or of cauliflower mosaic virus sequences to soil bacteria. The assessments focus on identifying the natural background level of these DNA sequences and alternative donors present in the soil. With the exception of some antibiotic resistance marker genes, an impact of the *cry*, *pat*, *bar*, CaMV sequences, and EPSPS encoding plant transgenes has not been identified in soil. A precise determination of the natural reservoirs of antibiotic resistance genes is necessary to resolve the concerns surrounding putative horizontal gene transfer (HGT) of plant-harbored antibiotic resistance markers to bacteria.

Chapter 5 outlines the investigations done to identify putative HGT events from transgenic plants to bacteria. Three different approaches have been taken to elucidate the likelihood of such transfer. These approaches apply DNA sequence comparisons, screening of bacteria from field releases, and studies of gene transfer under optimized laboratory conditions, and their results are summarized below:

(i) **DNA sequence comparisons of plant and bacterial genomes suggest few cases of horizontal transfer of plant genes to bacteria.**
Suggested transfers of genes from plants (or other eukaryotes) to bacteria are controversial, and they are estimated to have occurred several million years ago. Recent whole-bacterial genome sequencing has suggested that up to 16% of protein-encoding DNA in bacteria has been horizontally acquired, but presumably from other bacterial

donors. Thus, whereas HGT between bacteria appears to be an important mechanism for bacterial evolution, the contribution of plant genes to bacterial evolution is minor, as judged from available data.

(ii) **Horizontal transfer of plant marker genes to indigenous bacteria has not been demonstrated under field conditions.**
The 3 published studies that have investigated bacterial isolates from field sites or soil microcosms exposed to transgenic plants (harboring antibiotic resistance markers) have not been able to demonstrate the presence of the plant transgenes in the indigenous bacteria examined.

(iii) **Horizontal transfer of plant marker genes to naturally occurring competent bacteria under optimized laboratory conditions**

1. **Using recipient bacteria with no defined sequence homology to the plant transgene.**
The 4 *in vitro* studies performed, exposing the bacteria *Escherichia coli*, *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, and *Acinetobacter* sp. to plant marker genes have not been able to demonstrate such transfer. The investigations were performed both *in vitro* and in the natural habitats of some of the bacteria e.g. within tobacco crown galls or infected potato tubers. The detection limits varied from 10^{-9} to 10^{-12} (transformants per recipient) with proposed upper levels of transfer under natural conditions of 10^{-16} to 10^{-20} transformants per recipient bacterium.

2. **Using recipient bacteria with inserted sequence homology to the plant transgene.**
Several studies performed with the soil bacterium *Acinetobacter* sp. BD413 have demonstrated that this transformable bacterium is able to take up plant marker fragments if sequence homology to the bacterial recipient is present in the plant DNA. The transformation frequencies ranged from 10^{-7} to 10^{-8} (transformants per recipient) *in vitro* and in sterile soil. Similar frequencies have been reached using *Pseudomonas stutzeri*. A similar uptake of plant marker genes, based on incorporated homology to bacterial DNA, could not be shown in transformable cells of the plant pathogenic bacterium *Ralstonia solanacearum* neither *in vitro* or *in planta* during infection.

The laboratory investigations that have exposed various bacterial recipients to transgenic plant DNA have confirmed the dependency of linked DNA sequence homology for integration (via homologous

recombination). As indicated in chapter 3.3, due to the nature of the genetic modifications introduced in transgenic plants, e.g., the insertion of bacterial vector and plasmid sequences, T-DNA border sequences, bacterial or viral promoters and the choice of bacterial protein coding genes, sequence homology to prokaryotic DNA will be present. It has been suggested that the presence of such homologous sequences may facilitate the transfer of engineered plant genes into bacteria. Such recombination of plant transgenes with flanking bacterial sequence homology into naturally occurring bacterial populations has, however, not been demonstrated.

Chapter 6 provides conclusions and suggestions for the further research that is necessary to clarify possible impacts of plant transgenes in the bacterial gene pool. There are no reports of unintended transfer of plant transgenes to bacteria that are present in the environment. The main barrier to such transfer seems to be caused by the lack of sequence homology between the plant and bacterial DNA. Using purified DNA, it has been demonstrated that soil bacteria such as *Acinetobacter* sp., and *P. stutzeri* can take up and integrate plant marker gene fragments if sequence homology is present. The release of transgenic plants harboring prokaryotic DNA sequences may facilitate HGT of plant transgenes to bacteria. Few data are available on the significance of gene transfers by natural transformation in the phytosphere. However, a rare transfer event in this environment would only be environmentally significant if positively selected. The prediction of fitness advantages of bacterial transformants is hampered by the limited understanding of selection pressures in the soil environment. The assessments of a gene's selective advantages would be enhanced by knowledge of the natural occurrence and alternative exposure sources of the gene or its homologues.

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Abbreviations

HGT; horizontal gene transfer, GMP; genetically modified (transgenic) plant, PCR; polymerase chain reaction, transgene; genetically modified DNA inserted into an organism.

1. Introduction

The agricultural use of genetically modified plants (GMPs) has generated concerns over the potential environmental and ecological impact of the

engineered genes. One impact scenario is based on the hypothesis that if the plant transgenes are taken up by bacteria, the engineered genes may be disseminated into the environment with unknown consequences as the fitness advantages of these genes in new hosts are unknown. The frequent use of genes encoding antibiotic resistance as markers in GMP has strengthened these concerns based on the hypothesis that a transfer of such resistance genes to bacteria may ultimately impede clinical treatment of bacterial infections (Kruse and Jansson, 1997). Proponents of the usage of resistance encoding marker genes argue that no naturally occurring events of such transfer from the plants have been detected and that the consequences of a rare transfer would be negligible since similar genes are already present in bacterial communities and that the resistance they encode can be overcome by the use of alternative antibiotics. It has therefore been difficult to reach scientific, regulatory and public consensus about the validity of these concerns. One side argues the lack of information ensuring their safety, whereas the other side argues the lack of information demonstrating a risk. Since the use of antibiotic resistance genes are not strictly required for the production of transgenic plants, their presence in the commercialized products have been questioned and the use of alternative marker technology and minimizing the size and number of inserts have been encouraged (see Fairbairn et al., 2000).
In this report we summarize some of the scientific data and identify gaps in the information used for the current risk assessment of plant transgenes and the likelihood of transgene transfer to bacterial recipients.

2. Horizontal gene transfer – importance in bacterial communities

Horizontal gene transfer (HGT) is the transfer of genetic material by means other than normal reproductive mechanisms between individual organisms that may not be related. HGT can take place between organisms of different genotype, species, genus, kingdom and domain. Whereas HGTs between related organisms such as bacterial species, genera and divisions are facilitated at relatively high frequencies due to the presence of mobile DNA sequences or high DNA sequence homology, naturally occurring HGTs between distantly related organisms are rarely detected. Usually, HGT is unidirectional and only results in the transfer of a small part of the genome from one organism to the other. Horizontal gene transfer has been important for bacterial evolution and events of HGT between bacteria have been extensively demonstrated both *in vitro* and in natural systems (Fry and Day 1990, Veal et al., 1992, Wellington and van Elsas, 1992, Trevors and van Elsas, 1995, Yin and Stotzky, 1997, Kroll et al., 1998, Davison, 1999, Dröge et al., 1999). From recent whole-genome sequencing

of bacteria it has been estimated that up to 16 % of their protein coding DNA has been horizontally acquired (Ochman et al., 2000). More detailed analyses of DNA sequences within bacterial genes also support the notion that horizontal transfer of DNA between bacterial species is an important mechanism for bacterial evolution (Dowson et al., 1989, 1990, 1997, Spratt et al., 1989, 1992, Coffey et al., 1993, Maynard Smith et al., 1993, Bowler et al., 1994, Guttman and Dykhuizen, 1994, Nelson and Selander, 1994, Kapur et al., 1995, Feil et al., 1996, Zhou et al., 1997).

2.1 Known mechanisms of horizontal gene transfer between bacteria and their relevance to the transfer of recombinant plant DNA to bacteria

Gene transfer mechanisms in plants that facilitate horizontal transfer of genes, gene fragments or non-encoding DNA sequences to bacteria have not been identified. The assessment of the likelihood of HGT from plants to bacteria has therefore been based on the known mechanisms promoting HGT within the eubacterial division. Three mechanisms have been identified in bacteria that can mediate HGT (Lorenz and Wackernagel, 1994, Dröge et al., 1998, Davison, 1999). These mechanisms are transduction, conjugation and natural transformation. In the following, the relevance of these mechanisms in promoting putative HGT from plants to bacteria is assessed.

General transduction. The accidental transfer of DNA between bacterial cells due to bacteriophage infections, has been shown to facilitate HGT between bacterial populations residing on plant leaf surfaces, in soil or water (Saye et al., 1987, Zeph et al., 1988, Herron and Wellington, 1994, Kidambi et al., 1994, Herron, 1995). Bacteriophages generally possess a relatively narrow host range and viruses that infect both plants and bacteria have not been identified. Thus, the likelihood that transduction mediates HGT from plants to bacteria is currently assessed to be very low.

Conjugation. the transfer of mobile sequences (plasmids, transposons and mobilized chromosomal genes) between bacterial cells can mediate HGT between bacterial populations in soil and rhizosphere, on plant surfaces, and in water (Van Elsas et al., 1988, Lilley and Bailey, 1997, Troxler et al., 1997, Bjorklof et al., 1995, Normander et al., 1998). However, to our knowledge, mobile sequences that support conjugative gene transfer from higher plants to bacteria are not known, and transposons that function in both plants and prokaryotes have not been identified. However, the potential broad-host range of conjugation is demonstrated by the plant pathogen *Agrobacterium* spp. which during infection transfer defined

plasmid fragments, called T-DNA, from their Ti and Ri plasmids, into the plant genome by a conjugation-like mechanism. Since the host range of mobile plant sequences often are limited and plasmids are absent in plants (with the exception of plant organelles), the likelihood that conjugation mediates HGT from plants to bacteria is considered very low.

Natural transformation is the regulated uptake of extracellular DNA in bacteria (Lorenz and Wackernagel., 1994, Paget and Simonet, 1994). Since competent bacterial species can take up naked DNA independently of its sequence, natural transformation can in theory facilitate horizontal transfer of plant DNA to bacterial recipients the environment (Nielsen et al., 1997ab, 2000ab). Natural transformation, resulting in new selectable phenotypes has been shown to occur in soil, in plants and in water when competent bacterial recipients are exposed to bacterial donor DNA, either as purified DNA, in cell lysates or from added viable cells (Lee and Stotzky, 1990, 1999, Williams et al., 1996, Nielsen et al., 1997ab, 2000ab, Nielsen and van Elsas, 2001, Bertolla et al., 1997, 1999, 2000, Paget and Simonet, 1997, Sikorski et al., 1998). The ability to take up naked DNA by natural transformation has been detected in over 43 bacterial species distributed among all major bacterial divisions of life (Lorenz and Wackernagel, 1994, Paget and Simonet, 1994). Also the commonly used model bacterium *Escherichia coli* has been found to be naturally transformable (Baur et al., 1997). The number of known transformable species is still likely to be an underestimate due to the failure to cultivate the bacteria and to identify the conditions needed for competence to develop. Thus, transformation is currently regarded as being the most likely mechanism for the transfer of DNA from plants to bacteria, although such transfer has so far only been demonstrated in the laboratory with two species of bacteria.

The plant environment offers a highly diverse habitat for microorganisms and it is heavily colonized by microbial communities present in the rhizosphere, the phyllosphere and within plant tissues reaching densities of 10^4 to 10^8 bacteria per g plant material or soil. Also the digestive system of many plant-associated insects such as protozoa, nematodes, insect larvae, and earthworms contain high numbers of bacteria. In addition, dispersed leafs, fruits, pollen, and seeds provide additional microbial habitats.
Few studies of horizontal gene transfer in the phytosphere have been undertaken. However, both conjugation, transduction and transformation have been reported to occur between bacteria in the soil and plant environment. In sections 2.2 to 2.5 the capability of bacterial populations to mediate HGT in the plant environment will be discussed and the

potential of natural transformation of bacteria with genetic material derived from GMP host plants will be assessed. It is expected that bacteria in the phytosphere will be exposed to DNA of their host plant at various levels and quality depending on the specific habitat, as well as the growth stage and viability of the host organism.

2.2 Aerial plant surfaces

The leaves of agriculturally grown plants are colonized by bacteria (approx. 10^4 or more bacteria per g leaf material). Several studies have been conducted on gene transfer in the phyllosphere (Kidambi et al., 1994, Bjorklof et al., 1995, Lilley and Bailey, 1997, Normander et al., 1998) indicating that plant leaf surfaces may be conducive to horizontal gene transfer. For instance, conjugal gene transfer between bacteria has been observed on leaves of pear, bean, and sugar beet (Normander et al., 1998) and horizontal gene transfer from virus to bacteria by general transduction has been detected on the surface of bean and soybean leaves (Kidambi et al., 1994).

However, to our knowledge, no studies have been done to investigate the occurrence of natural transformation on aerial plant surfaces. Aerial plant surfaces may be exposed to DNA from lysed bacterial cells, transducing plant viruses and from the host plant itself during decomposition, pathogen induced lysis, or mechanical cell disruption caused by e.g. plant feeding insects, agricultural equipment, or animals. Thus, further investigations should be done to clarify the potential for horizontal gene transfer by natural transformation in this environment.

2.3 Plant tissues

Most plant tissues have been found to be colonized by bacterial endophytes ranging from 10^3 – 10^6 and even up to 10^9 bacteria per gram fresh weight tissue (Dimock et al., 1993, Hallmann et al., 1997). In addition, plant tissues infected by pathogenic bacteria can also reach high population densities (Schlüter et al., 1995, Bertolla et al., 1999, 2000). Few studies have investigated the occurrence of horizontal gene transfer by conjugation or transduction in plant tissues. Studies done on natural transformation is limited to one bacterial species. Bertolla et al. (1997, 1999, 2000) have studied conditions for natural transformation of the plant pathogenic bacterium *Ralstonia solanacearum* both *in vitro* and *in planta*. The bacterium developed competence in plant tissues, however, evidence for the stable uptake of transgenic plant DNA was not found in the experimental set-up used. As also indicated by Bertolla et al. 2000, bacterial communities residing in plant tissues are likely to be exposed to

DNA of the host plant after e.g. cell lysis caused by viral, fungal or bacterial pathogen infection or mechanical disruption of the plant cell caused by e. g. plant feeding aphids or nematodes. The natural transformability of plant pathogenic bacteria and bacterial endophytes should be further investigated as these may reach high population densities within plant tissues and become exposed to freshly released plant DNA.

2.4 Soil and on plant root surfaces

Bacterial populations residing on the root surfaces of most agriculturally grown plants are exposed to exuded organic compounds including sugars, amino acids and organic acids which can stimulate bacterial activity and gene transfer (Nielsen and van Elsas, 2000). Several studies have shown that conjugation is induced in the rhizosphere of various agriculturally grown plants (Van Elsas et al., 1988, Lilley and Bailey, 1997, Troxler et al., 1997, Kroer et al., 1998). Gene transfer by transduction has been reported in non-sterile soil (Zeph et al., 1988) and natural transformation has been described in bulk soil under non-sterile conditions (Nielsen et al., 1997ab, Sikorski et al., 1998).

Most of the studies on HGT in soil by natural transformation has been done in microcosms, however, recently Fry and coworkers (2000) reported transformants of *Acinetobacter* spp. after exposure to bacterial DNA in the rhizosphere of field grown sugar beet plants. Development of competence in the *Acinetobacter* sp. BD413 was recently also found to be stimulated by compounds present in root exudates of various agriculturally grown plants (Nielsen and van Elsas, 2000). Bacterial communities residing in the rhizosphere are likely to be exposed to plant DNA released during root decomposition e.g. from shedded root cap cells, after lysis induced by plant pathogens, or after mechanical disruption caused by insect larvae and nematodes feeding on the roots. The knowledge of the potential of natural transformation to mediate HGT of plant genes to bacterial communities residing in soil and rhizosphere is limited.

2.5 HGT between bacteria colonizing the digestive tract of plant-associated insects

The digestive system of protozoa, nematodes, insect larvae, and earthworms contain high numbers of bacteria (Thimm et al., 1998, Toyota and Kimura, 2000) and has been proposed to be a hotspot for gene transfer. For instance Adamo and Gealt (1996) detected conjugation in the gut of the *Rhabditis* nematodes, and Daane et al., (1996), reported significantly higher numbers of transconjugants in soil microcosms containing the earthworms *Lumbricus rubellus* or *Aporrectodea*

trapeziodes than in bulk soil. Schlimme et al. (1997) reported conjugation in digestive vacuoles of the protozoan *Tetrahymena pyriformis* and Hoffmann et al. (1998) reported conjugal gene transfer in the gut of the soil insect *Folsomia candida*.
Studies have not been published on the occurrence of natural transformation in the digestive system of these plant-associated organisms. The availability of plant DNA to microbial communities in these organisms should be further investigated together with a characterization of the bacterial communities present in these environments. Although not the topic of this report, it is noted that HGT of plant marker genes to intestinal bacteria of higher organisms has not been demonstrated so far (Stirn, 2000).

3. Factors affecting the likelihood of HGT from plants to bacteria in the phytosphere

To avoid deleterious effects of frequent integration of foreign DNA into the bacterial genome, many factors are thought to limit the uptake of divergent DNA. In section 3.1-3.5 we discuss these factors which focus on those that may be of particular importance for the potential bacterial utilization of plant transgenes in soil and phytosphere. The major barrier to the horizontal transfer of plant DNA into bacteria is caused by the mechanisms restricting the integration of foreign DNA in the bacterial chromosome. Both constraints to heteroduplex formation and the action of methyl-directed DNA mismatch repair enzymes (*mut* genes) have been identified as important for the discrimination of foreign DNA (Vulic et al., 1997, Majewski et al., 1998, 2000). In addition, HGT from plants may be limited due to insufficient access to DNA, a time-limited expression of competence in the recipient bacterium and mechanisms ensuring sequence-specific uptake of DNA. Moreover, a limited ability of the transformed bacterium to express the acquired genetic trait or a neutral or negative selection of the bacterial transformant may result in an evolutionary insignificant transfer event.

3.1 Stability and availability of plant DNA

A limited release, stability and or availability of plant DNA in the phytosphere will reduce the likelihood of HGT to indigenous bacteria by natural transformation. The majority of plant DNA present in decaying plant material is expected to be rapidly degraded by either intracellular nuclease activity or by microbial saprophytes utilizing the remaining organic material (Nygaard, 1983, Novitzky et al., 1986, Blum et al., 1997, Benedik and Strych, 1998). Several investigations have been conducted to elucidate the

presence and stability of transgenic plant DNA in soil over time:
The first field study of the persistence of transgenic plant DNA in soil was performed by Widmer et al. (1997) who investigated the stability of DNA in tobacco leaves buried in soil and in potato plant residues decomposing on the soil surface in Oregon, USA. The transgenic plant DNA was detectable by PCR in the composting tobacco leaves for up to 77 days and for 137 days in the potato litter.
Paget et al. (1998) monitored the stability of DNA in tobacco plants grown in a field site in France. Positive PCR signals where found for up to 1 year after planting.
In a similar approach, Smalla et al. (1999) monitored the stability of transgenic sugar beet (*Beta vulgaris*) in field trials in Oberviehausen, Germany. The PCR amplification with subsequent Southern blotting yielded positive signals for up to 2 years after initial farming of the sugarbeets.
Whereas these studies demonstrate the long-term stability of fragments of plant DNA in soil, which is consistent with the general turnover rate of organic material in this environment, the availability of such remaining DNA to microbial recipients remains to be determined. The detection of the physical persistence of chromosomal plant DNA over time in soil does not imply that this DNA is available to bacteria. DNA integrity and functionality may be destroyed by cleavage by mechanical forces, UV radiation, or chemically modification. The biological activity of DNA (both plant and bacterial) in soil has so far been found to be highly limited (to hours only, Nielsen et al., 1997b, 2000ab) and does not correspond with the observed prolonged physical presence of plant DNA fragments for months or year (see above). In studies of natural transformation of *Acinetobacter* sp. BD413 cells with bacterial DNA in soil, Nielsen et al., (1997b, 2000ab) observed that within few hours after addition of purified chromosomal DNA or cell lysates to soil, the transforming activity of the DNA ceased.
DNA may be released from plants after mechanical disruption of plant tissues or dried plant material or from enzymatically lysed plant cells (by pathogens). Numerous investigations have shown that clay, sand and humic particles commonly found in soil can bind and stabilize cell-free (extracellular) DNA (Aardema et al., 1983, Lorenz and Wackernagel, 1987, Ogram et al., 1988, 1994, Romanowski et al., 1991, 1992, 1993, Paget et al., 1992, Gallori et al., 1994, Blum et al., 1997, Alvarez et al., 1998, Crecchio and Stotzky, 1998a). However, it is unclear to what extent cell-free DNA would bind to mineral particles when released under natural conditions. The availability of plant DNA remaining within plant cells to bacteria present in soil has not yet been demonstrated. The effects of altered

methylation patterns in plant DNA and effects of plant cell residues on natural transformation should also be investigated further.

3.2 Bacterial uptake of DNA by natural transformation

The development of bacterial competence for transformation under natural conditions would be a prerequisite for HGT to occur. Competence development in bacteria is a physiologically regulated process and is usually expressed in response to specific growth phases or environmental stimuli (Lorenz and Wackernagel, 1994, Paget and Simonet, 1994). It is unclear to what extent bacteria express competence in soil and phytosphere (Nielsen et al., 1997b, 1998, 2000a), however, *Acinetobacter* sp. BD413 become competent when stimulated by compounds present in root exudates of various agriculturally grown plants (Nielsen and van Elsas, 2000). Fry and coworkers (2000) reported detection of transformants of *Acinetobacter* spp. after exposure to homologous bacterial DNA in the rhizosphere of field grown sugar beet plants.

Since some of the naturally transformable bacterial species can translocate DNA through the bacterial membrane independent of its origin, it seems plausible that their cytoplasm will be exposed to any type of extracellular DNA present in their surrounding. The soil bacterium *Acinetobacter* sp. BD413 has been shown capable of translocating plant marker genes over the bacterial membrane (De Vries and Wackernagel, 1998, Gebhard and Smalla, 1998, Kay et al., 2002) and a similar capability was indicated for the plant pathogen *Ralstonia solanacearum* (Bertolla et al., 2000) and *Pseudomonas stutzeri* (De Vries et al., 2001). Further studies are needed to determine which bacteria are naturally capable of taking up DNA and, moreover, the environmental factors present in the phytosphere that trigger the expression of competence in these bacteria should be identified.

3.3 Stability of translocated chromosomal DNA in bacterial transformants

The fate of translocated plant DNA in the bacterial cytoplasm would depend on its ability to escape restriction and degradation by nucleases, and to become integrated in the bacterial chromosome or a replication competent plasmid in order to be passed on in the population.

The action of bacterial restriction enzymes on plant DNA is probably insignificant since the duplexed DNA becomes single–stranded in the process of translocation across the bacterial membrane (Streips, 1991, Lorenz and Wackernagel, 1994, Palmen and Hellingwerf, 1997). Bacterial restriction enzymes recognize and cleave only double-stranded DNA (Brooks, 1987, Cerritelli et al., 1989).

Integration of foreign DNA to a bacterial origin of replication is dependent upon the degree of sequence homology between the incoming DNA and that of the bacterial recipient. For the uptake of heterologous DNA sequences in *Escherichia coli*, *Bacillus subtilis* and *Streptococcus* sp. a Log-linear relationship between decreasing recombination frequencies and increasing DNA sequence divergence has been established (Wörth et al.1994, Zawadazki et al., 1995ab, Vulic et al., 1997, Majewski et al., 1998, 2000).
The high level of DNA sequence divergence present between wildtype plant and bacterial DNA would therefore naturally limit the extent of successful horizontal gene transfers between these domains. However, transgenic plants may have localized regions of increased sequence homology to bacterial genomes. As outlined in Nielsen et al., (1998) the genetic engineering of plants often result in the incorporation of DNA with homology to bacterial or other prokaryotic organisms. These incorporated sequences may include bacterial vector and plasmid sequences, T-DNA border sequences, bacterial or viral promoters and protein encoding genes (Lorito and Scala, 1999). Since cDNA cloned genes transferred into genetically modified plants usually do not contain introns and may be capable of being expressed by a broad range of bacteria due to compatible codon usage and generally strong promoters their likelihood of uptake and expression in bacterial hosts is enhanced. Additive integration of non-homologous genes due to flanking sequence homology occurs at high frequencies in *Acinetobacter* sp. (Nielsen et al., 1997ab) indicating the possibility of uptake of plant transgenes based on linked sequence homology. The extent of sequence homology needed for additive integration of DNA in bacteria should be elucidated in detail for representative bacteria in the environment (De Vries and Wackernagel, 2002, Prudhomme et al., 2002).
In addition to the integration of DNA based on homologous recombination, illegitimate recombination events are known to occur in bacteria, albeit at lower frequencies (Ehrlich et al., 1993).

Mutators. Homologous recombination occurring in bacteria harboring mutations or environmentally induced deficiencies in their DNA repair system may increase the likelihood of uptake of foreign DNA in bacteria. Sub-populations of bacteria, called mutators, have been found at frequencies of 1-2% in natural populations of *E. coli* and *Salmonella* sp. (LeClerc et al., 1996, Matic et al., 1997). These bacteria harbor mutations, often located in the *mut* genes, that are involved in methyl-directed DNA mismatch repair that enable them to recombine at higher frequencies with DNA from more diverged species (Rayssiguier et al., 1989, Matic et al.,

1995, 1997, Vulic et al., 1997, Taddei et al., 1997). The occurrence of mutators have only been investigated for a few bacterial species and the presence and significance of mutator phenotypes in bacterial populations in the phytosphere remains unknown.
A lowered dependency of sequence homology for recombination possibly by a transient decrease in *mut* gene product activity in bacteria growing under adverse conditions have also been reported (Taddei et al., 1995, 1997, Feng et al., 1996, Rosenberg et al., 1996). Thus, within a bacterial population, the frequency of recombination with divergent DNA is probably spatially and temporarily variable according to the physiological state of the cells and the frequencies of mutator phenotypes.
To better understand the functional limits to bacterial utilization of horizontally transferred DNA and the degree of sequence divergence that would allow recombination to occur in the phytosphere (Townsend et al., 2003), the significance of gene flow in bacterial mutator populations and their prevalence in natural bacterial communities need to be further clarified.

3.4 Expression of heterologous DNA in bacterial transformants

The functionality of foreign DNA integrated in bacterial recipients may be limited by the capability of the host to utilize the encoded genetic information. The expression of the transferred plant transgene in the bacterial recipient would often be a prerequisite for a positive selection of the novel bacterial phenotype. Failure to express plant genes taken up in the bacterial cytoplasm can be due to differences in codon usage, in the transcription and translation signals and the presence of introns. The codon usage pattern in plant genes often differ from those used in many bacteria. However, due to the use of complementary DNA cloning procedures, eukaryotic genes inserted into GMP lack introns which might enhance their likelihood of expression if transferred to bacteria. Moreover, many of the promoters currently in use to control the expression of plant transgenes are of prokaryotic origin and therefore also active in bacteria. For example, the cauliflower mosaic virus 35S promoter is active in *E. coli* (Assad and Signer, 1990).
There may therefore be few constraints, except codon usage, to the expression of plant transgenes in bacterial recipients, if transferred. This situation is expected to change as more eukaryotic promoters become available that may direct expression of the transgene to defined plant tissues only.

3.5 Selection and environmental impact of bacterial transformants

Given the low frequency of HGT between divergent organisms such as

plants and bacteria that is expected to occur in the natural environment, a positive selection of the transferred genetic material would be prerequisite for establishing the new genotype in the population and for any environmental impact to be realized. The identification of a fitness advantage conferred by the novel gene upon the transformed bacteria requires a knowledge of environmental factors leading to selection and dispersal of novel genotypes. In general, current understanding of environmental factors that contribute to the selection of bacterial genotypes in nature does not allow for the identification of the selective constraints acting on a gene (putatively horizontally transferred) or a genome to evolve a particular function in a bacterial host (Hall, 1999). For instance, it is currently not possible to accurately describe the selective factors that enable the variety of bacterial species to coexist in complex environments such as soil, or how the introduction of a novel gene would affect fitness of a given bacterial recipient. Thus, the environmental causes of natural selection and factors limiting the evolutionary potential of a bacterial genome need to be understood to enable prediction of an impact of horizontally transferred genes.

4. The characteristics of specific transgenes and their natural occurrence

Whereas attention to the possible impact of HGT from transgenic plants to bacteria has been focused on antibiotic resistance genes, much of the methodology and reasoning used to evaluate transfer of antibiotic resistance genes is also applicable to other types of transgenes. Here we discuss what is known of the potential impact of plant transgenes that confer antibiotic, herbicide or insect resistance or of viral DNA sequences, if transferred to indigenous bacteria in soil. The assessment of putative risks has two main components:

(i) An assessment of the prevalence of naturally occurring homologues of the transgene in the environment and identification of alternative introductions of the transgene homologues that result from human activities

(ii) An assessment of potential fitness advantages of bacterial transformants under different selection pressures

From our point of view, a rare HGT event of from plants to bacteria is only important if it results in the positive selection of the transformant. The strength of the selection will determine the eventual number of bacteria in the environment harboring the transgene and not the initial transfer

frequency, assuming transfer and expression can occur. However, because gene transfer frequencies are thought to be low, there has been little research effort into understanding the selective effects of a transgene in bacterial hosts.

4.1 Antibiotic resistance marker genes

Antibiotic resistance marker genes are probably the genes most frequently inserted into transgenic plants. This is due to the utility they provide in selecting for transformed plant cells. Genes encoding resistance to several different types of antibiotics have been engineered into plants including resistance to the clinically used antibiotics streptomycin, kanamycin, neomycin, amoxicillin, and ampicillin (Andre et al., 1986, Schrott, 1995, Kruse and Jansson, 1997, Metz and Nap, 1997). The gene encoding resistance to kanamycin (*nptII* or *aph*(3')-II) is particularly widely used (Nap et al., 1992, Redenbaugh et al., 1994). It is also possible for other antibiotic resistance genes present on bacterial cloning vectors such as the Bla_{TEM-1} or *nptIII* to be accidentally co-transferred to plants due to their presence on the transfer vector (Kruse and Jansson, 1997). Humans are exposed to both the transgenes when present in food, and to potential bacterial transformants in the soil and plant microflora by the consumption of raw vegetables and fruits. Little data is available on the ability of phytosphere bacteria to transfer antibiotic resistance genes to human pathogens and concerns have been expressed that plant-inserted markers may spread horizontally to bacteria of clinical importance (Kruse and Jansson, 1997). Overall, the contribution of natural reservoirs of antibiotic resistance markers to clinically significant resistance patterns remains to be understood.

Natural occurrence of the antibiotic resistance genes (e.g. *nptII*) in indigenous bacteria in soil. High numbers of bacteria naturally resistant to antibiotics in the environment have been reported (van Dijck and De Voorde, 1976, Kelch and Lee, 1978, Henscke and Schmidt, 1990, Schmidt et al., 1990, Nap et al., 1992, Leff et al., 1993, Smalla et al., 1993, 1994, 1997). Most of these studies describe a resistant bacterial phenotype without identification of the underlying genetic basis. Whereas some bacteria might in general not be susceptible to the class of antibiotic, many mechanisms can result in the development of antibiotic resistance. For example, a reduced uptake/accumulation of the antibiotic, inactivation of the antibiotic by enzymes (the type of antibiotic resistance inserted in transgenic plants), or a modification or lack of the target site of the antibiotic would generate resistant bacteria (Neu, 1992, Davies, 1996,

1997). It is therefore unclear to what extent a phenotypic resistance in bacteria is due to the specific enzymatic inactivation of the antibiotic as encoded in transgenic plants. For instance, whereas 50% of clinical isolates of *E. coli* that were resistant to ampicillin contained the Bla_{TEM-1} gene (used in some GMPs)(Brandt, 1999, cited in Stirn, 2000), in the soils studied, the observed kanamycin resistance has not arisen after transfer of the *nptII* gene (widely used in GMPs). The *nptII* gene could not be detected in kanamycin resistant isolates from agricultural soils even though agricultural soil typically contains high numbers of kanamycin resistant phenotypes (approx. 100 000 CFU per g of soil)(Smalla et al., 1993, 1997, Gebhard and Smalla, 1999). Few studies have been published on the occurrence of enzyme-mediated antibiotic resistance in soil, of the type integrated in transgenic plants (Van Elsas and Pereira, 1986, Henscke and Schmidt, 1990, Smalla et al. 1993, 1997, Van Elsas and Smalla, 1995). Although the natural background level of the *nptII* gene is low in soil, soil microorganisms are frequently exposed to this gene since dispersed manure contains high numbers of bacteria harboring this or similar genes (Smalla et al., 1993, Gebhard and Smalla, 1999, Aarestrup et al., 2000, Sandvang and Aarestrup, 2000). The source of the *nptII* gene present in manure is unclear.

Selection by antibiotics (e.g. kanamycin) in soil. Bacteria residing in the soil and phytosphere may already encounter considerable natural selection for antibiotic resistance because of antibiotic applications to certain crop species as well as their frequent use in livestock farming. Data are not available on the exact quantities or the specific uses of antibiotics in agriculture (GAO, 99), however, antibiotics are rarely used on commodity crops such as cotton, corn, soybeans and wheat. The antibiotics oxy-tetracycline and streptomycin are used in the treatment and prevention of diseases in fruit, vegetables and ornamental plant species (Witte, 1998, McManus, 2000). In addition, antibiotics like kanamycin, which is very stable through digestion, may be released into soil from manure collected from treated livestock (Nap et al., 1992). Indigenous soil bacteria and fungi are known to produce antibiotics (Thomashow et al., 1990, Nap et al., 1992), but only low quantities of antibiotics are expected to remain in an active form under natural soil conditions (Gottlieb, 1976, Thomashow et al., 1990, Wellington et al., 1993, Cook et al., 1995). Because natural antibiotic release in soil is thought to be local and transient, it is unclear whether this phenomenon is common enough to guarantee selection of rare bacterial transformants in the soil or phytosphere. In the soil conditions so far tested, experimental studies have not shown an increased survival of bacterial inoculants harboring antibiotic resistance genes (Recorbet et al.,

1992, van Elsas, 1992, Oliveira et al., 1995, Nielsen et al., 2000b); indicating a low level of indigenous antibiotics. However, selective advantages for a *Pseudomonas fluorescens* strain carrying the streptomycin resistance encoding transposon Tn5 (Oliveira et al., 1995), and for *Acinetobacter* sp. BD413 carrying a kanamycin resistance encoding *nptII* gene (Nielsen et al., 2000b), are observed in soil microcosms after the addition of artificially high amounts of the respective antibiotics (streptomycin or kanamycin).

Putative risks associated with horizontal transfer of antibiotic resistance genes from transgenic plants to bacteria.

In conclusion, the putative HGT of the kanamycin resistance marker, *nptII*, from GMPs to soil bacteria should be compared to exposure of soil and phytosphere bacteria to similar *nptII* sequences present in manure. When soil is exposed to manure, HGT of the *nptII* gene is probably more likely to occur from bacterial hosts in manure to soil bacteria than from GMPs. The introduction of a plant incorporated *nptII* gene is therefore likely to be insignificant for the resistance pattern seen in this environments (given the *nptII* gene is as prevalent in manure as indicated in the few studies published). Because kanamycin is not an antibiotic used in commercial crop growing, it is unlikely that kanamycin resistance genes confer a selective advantage on putative bacterial transformants in soil which are not exposed to pharmaceutically produced antibiotics. However, the high numbers of kanamycin-resistant bacteria found in agricultural soils cannot easily be explained by the current understanding of antibiotic production by soil microbes and selection expected in soil.

To evaluate the impact of other antibiotic resistance plant markers, alternative sources of resistance genes in soil and conditions under which bacteria are exposed to each antibiotic should be identified. If the antibiotic resistance genes are already abundant in agricultural soil, irrigation water or other microbial communities exposed to transgenic plants, the release of such plant transgenes would probably have little impact. Yet, the pathways taken by resistance genes into pathogenic bacteria remains to be identified.

4.2 Bt-toxin encoding genes

Bt-toxins are insecticidal proteins (delta-endotoxins) produced by the Gram-positive, spore-forming, insect pathogenic bacterium *Bacillus thuringiensis*. The bacterium contains several plasmids that encode insecticidal *cry*-proteins that show high activity on specific insect larvae (Hofte and Whiteley, 1989, Kumar et al., 1996). The toxin acts by destroying ion gradients in the insect gut lining and the insect dies 2-3 days after

exposure. The native form of the Bt-toxin is a protoxin in the bacterial cytoplasm that requires ingestion by the insect larvae and subsequent solubilization and proteolytic cleavage to produce an active toxin. In transgenic plants, codon modified synthetic *cry*-genes encoding a truncated and active form of the toxin is often used. Whereas about 50 different toxins genes have been isolated, only a few (*cry*1ab, *cry*1ac, *cry*3a, *cry*9c) have been commercialized (EPA, 2000).

Stability and impact on microbial communities of the Bt-toxin in soil. A half-life of the Bt-toxin in soil of 2-46 days has been reported for the active form of the protein (Ream et al., 1992, Palm et al., 1996, Sims and Holden, 1996). The native Bt-toxin can be degraded by sunlight and microorganisms (Koskella and Stotzky, 1997). However, Stotzky (2000a) reported recently that Bt-toxin is released from roots of genetically modified corn (*Zea mays*) and that the active form of the toxin may be more stable in soil than previously anticipated (Saxena et al., 2000, Saxena and Stotzky, 2000). They indicated that the toxin may bind to soil particles such as clay and humic acids, retain insecticidal activity, and persist for up to 243 days (Venkateswerlu and Stotzky, 1992, Tapp and Stotzky, 1995, Crecchio and Stotzky, 1998b). However, though the active form of the toxin has been shown to persist in soil over time, adverse effects on soil bacteria have not been reported in the few studies conducted (Donegan et al., 1995, 1996, Stotzky, 2000b). See EPA (2000, section IIC1-IIC195) for a more detailed discussion on the fate of Bt-toxin in soil. More research is needed in order to understand the persistence and degradation of the active form of the various *cry*-gene products in soil.

Natural occurrence of the native form of the Bt-gene in soil bacteria. *B. thuringiensis* is a commonly found soil bacterium that has been isolated from many habitats including agricultural land, forest, savanna, desert, steppe, urban areas and arctic tundra (Martin and Travers, 1989). In a study of soils from 30 different countries of 5 continents, *B. thuringiensis* was isolated from 785 of 1115 samples (Martin and Travers, 1989, Meadows, 1993). Indicating a wide distribution of these strains. However, the natural occurrence and ecology of the strains harboring *cry*-genes such as the native form of the *cry*1ab, *cry*1ac, *cry*3a, and *cry*9c genes is unclear.

Putative risks associated with plant transgenes encoding the active form of the Bt-toxin. If most of the widespread *B. thuringiensis* strains harbor the native form of the *cry*-genes, then soil bacteria are exposed to *cry*-genes from sources other than transgenic plants. The *cry*-genes are

located on plasmids and *B. thuringiensis* cells can transfer genes by conjugation (Jarret and Stephenson, 1990). It is unclear whether other bacteria would be able to acquire the same pathogenicity after transfer of the *cry*-gene carrying plasmids. The restricted occurrence of the *cry*-genes to *B. thuringiensis* suggesting a fitness advantage that is confined to this species. It is, based on the above observations, suggested that soil bacteria are more likely to acquire the *cry*-genes from a *Bacillus* donor (either naturally present in soil or from the application of *B. thuringiensis*-containing biopesticides) than from a transgenic plant. It should be noted that the bacterium encode a native protein in contrast to the truncated protein with alterations in size and activity usually encoded by transgenic plants. It is unclear if the changes made in truncated *cry*-genes would alter the fitness advantages of their bacterial hosts, as compared to the native gene. On the other hand, the DNA sequence of the truncated *cry*-gene has been altered to optimize its expression in plants which will, together with the use of plant-tissue specific promoters reduce the expression level of the gene, if an unintended transfer to a soil bacterium should occur.
In conclusion, the putative HGT of plant-inserted *cry*-genes to bacteria in soil would be of little concern if indigenous soil bacteria are already exposed to the *cry*-genes of naturally occurring *B. thuringiensis* strains. Natural reservoirs of *cry*-genes and changes in fitness effects conferred by the introduced nucleotide modifications should be examined closer.

4.3 Genes encoding tolerance to glyphosate

Plant resistance to the broad-spectrum herbicide glyphosate is obtained by insertion of a bacterial gene (*aroA*) that encode 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. CP4 (Comai et al., 1985). Recently, a plant gene encoding a modified version of the EPSPS protein has also been used to provide glyphosate tolerance in corn plants (*Zea mays*, Monsanto Line GA21). EPSPS is essential for the synthesis of certain amino acids in chloroplasts. The plant EPSPS enzyme is inhibited by glyphosate, whereas the bacterial EPSPS enzyme, or the modified plant EPSPS enzyme, have reduced affinity to the herbicide and thereby confer tolerance.

Stability and impact on microbial communities of the herbicide glyphosate in soil. Glyphosate appears to be rapidly degraded by microbes in soil, even at high application rates, and without detectable adverse effects on microbial activity (Rueppel et al. 1977, Haney et al., 2000). Glyphosate is also thought to become inactivated when it binds to clay minerals in soil. Half-lives of glyphosate of 12-60 days in agricultural

soils have been reported (Cheah et al., 1998, EPA, 1998) and microbial populations, able to degrade glyphosate, have been isolated from activated sludge (Hallas et al., 1992, Heitkamp et al., 1992, Carson et al. 1997), soil (Zboinska et al., 1992, Gard et al., 1997) and fresh-water and sediment (Zaranyika and Nyandoro, 1993). There is also evidence that the un-culturable fraction of soil microorganisms degrade glyphosate (Forlani et al., 1999).

Natural prevalence of the glyphosate resistance gene. The bacterial source of the EPSPS transgene, *Agrobacterium* is a commonly found soil and plant-associated bacterium. The modified EPSPS encoding plant gene has 99.3 % sequence similarity to the gene which occurs naturally in corn, though the protein it produces is structurally different from its original form. The metabolic pathway utilizing the EPSPS enzyme is present in all plants, bacteria and fungi, therefore, this housekeeping gene and its orthologues are widely distributed in the soil environment.

Identification of putative risks associated with horizontal transfer of the glyphosate tolerance gene from plants to bacteria. Due to the wide distribution of the EPSPS gene in the environment, soil microbial communities are naturally exposed to the EPSPS encoding genes of *Zea mays*, *Agrobacterium* sp. and from other bacteria in soil. Thus, the transgene would only be an additional source of the EPSPS gene, with few nucleotide changes from the native gene, in soil. It should be noted that this assessment is based on the assumption that the differences between the transgene and the native form do not alter the fitness advantages of bacterial hosts, as compared to the available forms of the EPSPS genes. The incorporation of the plant derived EPSPS transgene would substantially reduce the likelihood of transfer of the transgene to bacteria, due to the general lack of DNA homology, and few risks (in a HGT context) can be identified from the use of this gene in plants.
In conclusion, the putative HGT of EPSPS transgenes from plants to bacteria is probably of little concern since soil microorganisms are already exposed to a wide variety of these genes from numerous organisms.

4.4 Genes conferring tolerance to glufosinate

The *bar* (bialaphos resistance) and *pat* (phosphinothricin resistance) genes have been used extensively in transgenic plants either as selectable markers, in hybrid seed production or as agronomic characters conferring resistance to the broad-spectrum herbicide phosphinothricin (Metz et al., 1998). The *pat* and *bar* genes (85% DNA homology) were isolated from

Streptomyces viridochromogenes and *S. hygroscopicus*, respectively (Metz et al., 1998), and encode phosphinotricin-N-acetyltransferase (PAT) which inactivates the herbicide by acetylation (Thompson et al., 1987). Phosphinothricin is produced by the actinomycetes *S. viridochromogenes* and *S. hygroscopicus* and interferes with the amino acid synthesis in plants by inhibition of the glutamine synthase enzyme. This inhibition triggers ammonia accumulation which results in disruption of the chloroplast structure (see Metz et al., 1997).

Stability and impact of the herbicide phosphinothricin in soil. Phosphinothricin (PPT) is reported to be degraded within days in soil although residues have been found after months (Faber et al., 1997). There is evidence that PPT degrading microbial species exist in soil (Bartsch and Tebbe, 1989, Tebbe and Reber, 1991); PPT can be used as a source of phosphorous and nitrogen by some species of soil bacteria (Tebbe and Reber, 1988, Quinn et al., 1993). Because PPT is only detected in soil up to a 10-15 cm depth it is thought that most PPT is degraded by microbes or becomes bound to soil particles near the soil surface (WSSA, 1994 cited in Metz et al., 1998, Faber et al., 1997). A study of 227 environmental bacterial isolates revealed that 37% of the bacterial species sampled showed inhibited growth at concentrations of less than 1 mM PPT (Quinn et al., 1993). However, in most cases, spontaneous mutants that were resistant to PPT arose from the sensitive populations of bacteria (Quinn et al., 1993, Kriete and Broer, 1996). An effect of plant metabolites of PPT on soil microorganisms remains to be identified.

Natural occurrence of the *bar* and *pat* gene in soil. Actinomycetes, including streptomycetes, are widely distributed in soil, compost, water and other environments (Goodfellow et al., 1988, Herron and Wellington, 1990). The specific distribution of the *bar* and *pat* genes in the environment is, however, unclear. Streptomycetes can exchange genes e.g. by conjugation (Wellington and van Elsas, 1992, Chater et al., 1997).

Identification of risks associated with horizontal transfer of the *bar* or *pat* genes from plants to bacteria. The impact resulting from a horizontal transfer of the plant-inserted *bar* or *pat* genes from GMPs to bacteria is perhaps of little concern since soil microorganisms may be exposed to these or similar genes from other streptomycetes in soil that are capable of conjugal gene transfer. It is noted that this assumption is based on the unconfirmed presumed wide distribution of the *bar* and *pat* genes in streptomycetes populations present in the environment. Because

resistance to phosphinothricin can arise rapidly in some microbial populations by spontaneous mutation, a clear fitness advantage and an environmental impact of a horizontally transferred plant transgene to soil microbial communities is not predicted.

4.5 Cauliflower mosaic virus (CaMV) sequences

The 35S promoter of the cauliflower mosaic virus (CaMV) has often been inserted into transgenic plants to control the expression of various genes because of its efficiency in heterologous genetic backgrounds (Hull et al., 2000). The CaMV contains a 8000 base pair circular double-stranded DNA molecule with one copy of the 35S promoter. Upon lysis of infected plant cells, approx. 100 copies of the virus are released (Hull et al., 2000).

Prevalence of CaMV in soil environments. The CaMV is commonly found in temperate countries where it infects cauliflower, cabbage, oilseed rape, mustard and other Brassicas and some Solanaceaous species (Tomlinson et al., 1987, Chenault and Melcher, 1994, Schoelz and Bourgue, 1999, Hull et al 2000). In a survey (cited in Hull et al., 2000, Morel and Tepfer, 2000), up to 10% of cauliflowers and 50% of cabbages on local markets in the UK were infected with CaMV. Thus, it is expected that soil bacteria are already exposed to the CaMV DNA from rotting plant residues.

Identification of risks associated with horizontal transfer of the cauliflower mosaic virus (CaMV) sequences from transgenic plants to bacteria. In conclusion, the impact of a HGT of the plant-inserted CaMV sequences to bacteria is unclear, but assumed to be low since soil microorganisms are naturally exposed to these sequences from infected plant tissues. Numerous bacteriophages are present in soil bacterial communities, so a specific fitness advantage cannot easily be identified for putative bacterial transformants receiving DNA fragments encoding the CaMV promoter sequences from transgenic plants as compared to other native viral sequences.

Most of the transgenes examined here also occur naturally in some strain of soil bacterium or are already introduced into the soil environment from other sources (e.g. manure or in infected plant residues). Bacteria are capable of conjugation, transduction, natural transformation and mobile or mobilizable sequences can facilitate dissemination of the gene in question. Where natural forms of the transgene are abundant, soil bacteria would be more likely to acquire the gene in question from a bacterial donor than from a transgenic plant. Few impacts can be identified for the evaluated

genes, however, this conclusion is based on two assumptions;
1. That the native forms of the genes are as abundant in the environment as the distribution of species in which they have been found.
2. That the engineered genes would confer the same fitness to the host as the native gene.
Both these assumptions should be verified.

5. Investigations of HGT from transgenic plants to bacteria

The likelihood of horizontal transfer of recombinant plant DNA to bacterial recipients in the phytosphere can be evaluated by at least three different approaches (Schlüter and Potrykus, 1996, Nielsen, 1998, Nielsen et al., 1998, Dröge et al., 1998, Bertolla and Simonet, 1999, Smalla et al., 2000):

1. Comparisons of available DNA sequences of bacterial and plant origin to reveal recent events of horizontal transfers.
2. Screening of bacterial isolates, or total-DNA of the bacterial fraction of the phytosphere for the acquisition of transgenes from field grown transgenic plants.
3. Investigations of HGT from transgenic plants to defined model bacteria under optimized laboratory conditions.

Each of these approaches differ in their ability to detect and characterize putative events of HGT with respect to the type of genes transferred, the bacterial diversity screened, to the extent the growth conditions applied reflects natural circumstances, the sensitivity of detection of putative events of HGT, reproducibility, ability to characterize the gene transfer process, and ability to estimate gene transfer frequencies (Nielsen et al., 1998). In chapter 5.1-5.3, we briefly summarize the available literature describing these approaches.

5.1 Comparisons of known plant and bacterial DNA sequences for an assessment of recent horizontal transfer events.

Comparative sequence analyses can infer events of HGT based on similarities in nucleotide and protein sequence, abnormal inheritance pattern of a particular gene, and discrepancies in G/C content and codon usage (Smith et al., 1992, Doolittle, 1997, 1999, Ochman et al., 2000).
Some cases of naturally-occurring HGT from plants (or other eukaryotes) to bacteria has been inferred based on comparative DNA analyses (see Dröge et al. 1998, Carlson and Chelm, 1986, Froman et al., 1989, Doolittle et al., 1990, Lamour et al., 1994). Based on available DNA sequences and to the extent these have been analyzed with appropriate methodology,

HGT from wild-type plants to bacteria appear to be infrequent and evolutionary successful events are probably rare when seen in the time perspective of modern agriculture. However, as noted in chapter 3.3, the insertion of genetic material of prokaryotic origin in transgenic plants may change the frequencies of gene exchange between plants and bacteria that have similar DNA sequences based on enhanced expression and sequence dependent integration (homologous recombination) of DNA. The extent to which selected events of HGT in the evolutionary past can aid in the understanding of putative HGT from of novel plant genotypes is unclear.

5.2 Screening of bacterial isolates or total-DNA of the bacterial fraction of the phytosphere for the acquisition of transgenes from field grown transgenic plants.

Several groups have screened bacterial isolates from GMP field sites or soil microcosms to detect bacterial transformants after putative horizontal transfer of antibiotic resistance marker genes (Table 1);

1. Becker and coworkers (1994) investigated HGT of the *nptII* and hygromycin resistance (*hph*) genes from transgenic tobacco into indigenous bacteria present in a soil microcosm. Bacterial transformants could not be detected in these experiments after selective plating for antibiotic resistance and DNA hybridization with a probe specific for the plant insert.

2. Over 4000 kanamycin resistant bacteria were isolated from soil samples obtained from field trials with *nptII* containing transgenic sugar beets (*Beta vulgaris*) by Smalla and coworkers in Germany (Smalla et al., 1994, Gebhard and Smalla, 1999). However, colony hybridization and PCR amplification revealed that the plant inserted kanamycin resistance gene (*nptII*) had not transferred to these bacteria. Also direct analyzes of the bacterial DNA fraction isolated from the soil indicated that the observed kanamycin resistance was due to other mechanisms than nptII encoded resistance.

3. Similar negative results were also obtained by Simonet and coworkers in France (Paget et al. 1998) who investigated gentamicin resistant bacteria isolated from a field site with tobacco harboring the *aacC1*gentamicin resistance gene. No evidence could be found that the resistance gene had been transferred to the 600 gentamycin resistant colonies of soil bacteria that were analyzed.

The field studies performed do not indicate the level of detection per g of soil nor the sample size from which the analyzed resistant colonies arose. It is likely that only a fraction (of unknown size) of the indigenous bacteria in soil which might acquire plant DNA through HGT would express the specific trait selected for in the cultured bacterial fraction. Since only a minor fraction of soil bacteria (10% or less) respond to laboratory cultivation (Hugenholz and Pace, 1996, Pace, 1997) and even fewer bacteria on the limited number of media and cultivation techniques used in these studies, this method may underestimate the rate of HGT.
Only a small percentage of the soil microflora were assessed for their capability of uptake of plant transgenes in this approach and the natural background of antibiotic resistant phenotypes often interfere with the analyzes of the putative HGT of plant marker genes. Alternatively, the cultivation-independent approaches encompass the complex recipient structure found in natural bacterial communities, however, this technique has a limited ability to verify HGT of a plant transgenes into a bacterial genome.

5.3 Investigation of HGT from transgenic plants to defined model bacteria under optimized laboratory conditions.

The putative horizontal transfer of marker genes from plants to defined model bacteria has been investigated by several groups. With the exception of *Escherichia coli*, all of these studies have been performed with naturally transformable soil and plant-associated bacteria as recipients for chromosomally inserted plant marker genes. Below we summarize the published studies (Table 1). In addition, several unpublished studies have probably been conducted by companies, e.g. the study cited in FDA, 1998.

1. Broer and coworkers (1996) studied HGT from T-DNA transformed tobacco to the plant pathogenic bacterium *Agrobacterium tumefaciens*. This approach facilitated homologous recombination between the Ti-plasmid harbored by the recipient *A. tumefaciens* and the T-DNA integrated in the GMP. Despite naturally selective conditions for *A. tumefaciens* in the infected plant tumors, transfer of the plant harbored gentamycin resistance (*accI*) gene could not be detected in the recipient bacteria. The transformation frequency was found to be below $<6 \times 10^{-12}$ transformants per recipient bacterium.

2. In an unpublished study cited in a FDA draft guidance document (FDA, 1998) investigation of possible HGT of an ampicillin resistance gene

from a insect-resistant corn line to competent *Escherichia coli* cells was described. Apparently, transformants were not detected by the crop developer conducting the studies and "the experiments showed that transformation did not occur above a frequency of 1 in 6.8 x 10^{-19}"(FDA, 1998). To experimentally verify this detection limit, assuming a high density of 10^{14} colony forming units of *E. coli* per liter of media, would require screening of bacterial cells from 10^{6} liter of culture. The amount of DNA used in the experiments were not given.

3. Schlüter and coworkers (Schlüter et al., 1995) investigated HGT from transgenic potato to the plant pathogenic bacterium *Erwinia chrysanthemi*. Transformation of the bacterium was investigated by inoculation of the bacterium directly onto potato tubers which facilitated lysis of the plant cells with subsequent possible release of plant DNA containing a selectable ampicillin resistance gene (encoding a ß-lactamase) and a bacterial origin of vegetative replication. However, no transformants could be detected in these studies and the detection limit was $<10^{-9}$ transformants per bacterial recipient. The authors concluded that under natural conditions the more realistic frequencies were below 2 x 10^{-17} transformants per recipient bacterium.

4. Nielsen and coworkers (Nielsen et al., 1997c) investigated HGT from *nptII* containing transgenic sugar beet and potato to the soil bacterium *Acinetobacter calcoaceticus* (Baumann, 1968, recently renamed *Acinetobacter* sp.) on agar-plates in vitro. High transformation frequencies of 10^{-2} transformants per recipient bacterium can be reached when exposing this recipient to homologous chromosomal DNA *in vitro*. However, transformants could not be detected under the optimized conditions even when excess plant DNA was removed. The frequency of successful transformation in these experiments was concluded to be below 10^{-11} transformants per recipient. Under natural conditions, the transformation frequency was estimated to be below 10^{-16} transformants per recipient. It was concluded from this study that the presence of sequence homology or a stabilizing sequence like an origin of replication was required for stable maintenance of the translocated DNA in the *Acinetobacter* sp. strain used.

As discussed in section 3.3, the integration of translocated DNA in bacterial recipients depends on the degree of sequence homology between the recombining DNA strands. By introducing sequence

homology between transgenic plant DNA and a bacterial recipient, two recent studies have been able to detect HGT of plant harbored DNA by homologous recombination of a *nptII* gene into the recipient bacterium *Acinetobacter* sp. In these studies the *Acinetobacter* sp. cells were able to access purified plant DNA and restore a partially deleted (10-317 bp) bacterial *nptII* gene with the information present in a plant DNA harbored *nptII* gene. (Gebhard and Smalla, 1998, De Vries and Wackernagel, 1998). Smalla et al. (1998) obtained restoration of a 317 bp deletion in the *nptII* gene at a frequency of 5.4×10^{-9} after exposing the recipient bacterium to transgenic sugar beet DNA *in vitro*. Similarly, another group, (De Vries and Wackernagel, 1998), obtained restoration of a 10 bp deletion in the *nptII* gene at transformation frequencies of up to 3.5×10^{-8} after exposure of the bacterium to transgenic DNA purified from either potato, tomato, tobacco, oilseed rape, or sugar beet plants.
Subsequently, it was also shown that uptake of a plant marker gene in *Acinetobacter* sp., based on homologous recombination, also can occur in sterile soil microcosm (Nielsen et al., 2000b) at frequencies of 10^{-7} to 10^{-8} per bacterial recipient after the addition of nutrient solutions. Studies have also shown that this bacterium can access genetic material present in bacterial cell lysates indicating that the presence of cell material such as membranes and cytoplasmic residues are not inhibitory for natural transformation to occur (Nielsen et al 2000a).

5. Bertolla et al. (2000) investigated putative HGT of tobacco or tomato transgenes to the plant pathogenic bacterium *Ralstonia solanacearum* *in vitro* using purified DNA and *in planta* during infection. However, they were not able to demonstrate *in vitro* nor *in planta* that the partially deleted *nptII* gene in the *R. solanacearum* recipient could be restored after exposure to *nptII* containing plants or its DNA. Transgenic plants of tobacco and tomato harboring a *R. solanacearum* *popA* gene with internally inserted streptomycin resistance (*aadA*) and gentamycin resistance (*aacC1-IV*) genes were also used as DNA donor. The additive integration of these two antibiotic resistance genes into the recipient *R. solanacearum popA* gene was facilitated by the flanking bacterial *popA* sequences (of 719 and 907 bp) in the transgenic plant DNA. However, transformants were not detected using this approach. The transformation frequencies of *R. solanacearum* with the plant DNA donors used were reported to be below 1.6×10^{-9} *in vitro* and 4.4×10^{-9} *in planta* per bacterial recipient. The authors suggested the presence of excess plant DNA to interfere with the transformation process.

6. For recent studies see also De Vries and Wackernagel, 2001 and Kay et al., 2002.

It seems relevant to conclude that transfer of plant marker genes to bacteria can only occur at very low frequencies even under highly optimized conditions. This is due to the dilution effect caused by the relative size of the plant genome to the bacterial genome (Bertolla et al., 2000). It can be estimated, based on the number of DNA binding sites on the bacterial cell membrane and the size of translocated DNA fragments (see Palmen and Hellingwerf, 1997), that more than 5000 bacteria are needed to take up the amount of DNA present in a single plant cell. Thus, the highest transformation frequencies obtainable at a given time (given no deleterious fragmentation of the marker gene during uptake and 1 transgene copy per plant cell) is 2×10^{-4} transformants per bacterial cell. However, since not all bacteria in a population express competence at a given time the maximum frequencies drops to 2×10^{-7} (if 10^{-3} competent bacteria per recipient bacterium, e.g. *Acinetobacter*) to 2×10^{-11} (given 10^{-7} competent bacteria per recipient bacterium, e.g. *Erwinia*, *Ralstonia*, *Agrobacterium*). These calculations are consistent with the maximum transformation frequencies observed; see above. Our calculations are based on a given time frame, a prolonged time perspective and variable environmental conditions should be integrated in further estimates since bacterial cell division and growth conditions would alter the number of bacterial recipients and their ability to integrate DNA.

Most of the model bacteria studied in this approach differed in their ability to develop competence and express the selectable trait. For instance, the natural competence of *E. chrysanthemi*, *A. tumefaciens* and *R. solanacearum* was generally low. Moreover, with the exception for the DNA availability to *R. solanacearum* during infection (Bertolla et al., 2000), the presence and stability of released plant DNA with transforming activity in the lysed potato tuber tissue or in crown galls have not been well documented. Also the expression level of the kanamycin resistance gene in the *Acinetobacter* recipient was low, providing a low degree of resistance. It should be noted that no technique other than culturing in the presence of antibiotics, has proven successful in demonstrating HGT from plants to bacteria.
The diversity and interactions of bacterial communities present in natural plant habitats are usually not reflected in the results obtained when using defined model bacteria to assess the likelihood of HGT from transgenic plants. On the other hand, the utilization of model bacteria as putative

recipients of selectable marker genes can facilitate the identification of important factors determining the likelihood of such events to occur. From the studies performed with homologous sequences present, it can be concluded that plant DNA is accessible to competent bacterial strains both *in vitro* and in sterile soil and that the stability of the transferred DNA depends on the degree of sequence homology to the bacterial recipient. As discussed in section 3.3, the inserts in transgenic plants often harbor prokaryotic genes thereby increasing the level of DNA homology between plants and bacteria.

Table 1. Experimental studies undertaken to investigate unintended transfer of plant marker genes to bacteria (modified from Nielsen et al., 2000).

Reference	Transgenic plant material (plant marker gene)	Recipient bacterium	Transfer detected (frequencies)[a]
Becker et al., 1994	Soil microcosm with tissue homogenate of tobacco *(nptII, hph)*	Indigenous soil bacteria	No
Broer et al., 1996	Infected tobacco galls *(accl)*	*Agrobacterium tumefaciens*	No ($<6 \times 10^{-12}$)
Schlüter et al., 1995	Infected potato plants and tubers (*amp*, oriV)	*Erwinia chrysanthemi*	No ($<10^{-9}$ estimated below 2×10^{-17})
Nielsen et al., 1997	Purified potato and sugarbeet DNA (*nptII*)	*Acinetobacter* sp.	No ($<10^{-11}$ estimated below10^{-16})
Described in FDA, 1998	Transgenic corn DNA (ampicillin resistance gene)	*Escherichia coli*	No ($<1.5 \times 10^{-20}$)
Gebhard and Smalla, 1998	Purified potato and sugarbeet DNA (*nptII*)	*Acinetobacter* sp.	Yes (5.4×10^{-9}) Integration facilitated by DNA homology
De Vries and Wackernag el, 1998	Purified potato, tobacco, sugarbeet and oilseed rape DNA (*nptII*)	*Acinetobacter* sp.	Yes (3.5×10^{-8}) Integration facilitated by DNA homology
Paget et al., 1998	French field grown tobacco plants (*aacl*)	Indigenous soil bacteria	No
Gebhard and Smalla, 1999	German field grown sugarbeet (*nptII*)	Indigenous soil bacteria	No
Nielsen et al., 2000	Purified sugarbeet DNA added to sterile soil microcosms (*nptII*)	*Acinetobacter* sp.	Yes (1.4×10^{-8}) Integration facilitated by DNA homology
Bertolla et al., 2000	Purified DNA and infected plants of potato and tomato (*aada, aac1-IV, nptII*)	*Ralstonia solanacearum*	No ($<1.6 \times 10^{-9}$ *in vitro* and $<4.4 \times 10^{-9}$ *in planta*)

[a] Frequencies given as the number of transformants per recipient bacterium.

6. Conclusions and further studies

The assessment of the likelihood and environmental impact of an unintended transfer of a plant transgene to a bacterium is based on an understanding of bacterial genetics and natural selection drawn from;

(i) Analyses of bacterial genomes and bacterial gene phylogenies
(ii) Empirical studies and observations of horizontal gene transfer in bacterial communities
(iii) Experimental studies of cellular barriers to the uptake of divergent DNA in bacteria
(iv) Identification of environmental barriers to the access to transforming DNA in bacteria
(v) Comparisons of sequenced plant and bacterial DNA to reveal transfer of specific sequences
(vi) Field studies performed to detect gene transfer from transgenic plants to bacteria
(vii) Experimental studies of gene transfer from transgenic plant to specific bacteria
(viii) Understanding of natural selection in bacterial populations
(ix) Theoretical prediction of fitness effects of a transgene in bacterial communities based on an understanding of the gene ecology of the native homologue
(x) The absence of reported adverse effects from the large number of field releases performed

Further research in several areas is needed to improve the science-based risk assessment of novel plant genotypes.
Although the capability of bacteria to take up extracellular DNA was discovered in 1944 (Avery et al., 1944), gene transfer by natural transformation has remained a poorly understood process in most environments. There is, for instance, a clear lack of knowledge on the occurrence, extent and importance of gene transfer by natural transformation in the phytosphere, the digestive system of soil invertebrates or in the digestive tract of farm animals and humans. To better understand the importance of natural transformation in relation to bacterial adaptation, environmental factors that affect horizontal gene transfer needs to be identified and the level of competence development in bacterial communities determined.
Although fragments of plant DNA have been detected after 2 years of plant cultivation, it is clear that the probability of functional transfer to

bacteria is inversely related to the time DNA is exposed in the environment. It is uncertain to what degree plant DNA is available as genetic information to bacteria in the phytosphere or in the gut during digestion since the degradation kinetics of DNA in different microhabitats remains to be determined.

The main barrier to gene transfer between transgenic plants and bacteria appears to be caused by lack of DNA sequence homology. Since most transgenic plant inserts include some prokaryotic DNA sequences, homologous recombination may occur in bacteria which harbor similar sequences. Further studies should be done to assess the degree of sequence homology required for the stable integration of plant transgenes in bacterial genomes. Moreover, the prevalence of mutator subpopulations, their recombination frequencies with divergent DNA, and their role in bacterial evolution may be particularly necessary for the prediction of horizontal gene transfer from GMPs to soil bacteria.

No peer reviewed data is available that has documented the unintended transfer of plant transgenes to bacteria that are naturally present in the environment. This observation is based on a small number of published investigations; <20 experimental studies of <10 model bacteria and 3 studies of indigenous soil bacterial populations. It is obvious that these studies are not representative of the diversity of bacterial communities present in natural environments. However, they support the assumption that the likelihood of transfer of plant DNA to soil bacteria is extremely low. The fact that HGT from plants to bacteria is apparently so rare that it has not been detected, suggest that the transfer barriers are effective and that it will be challenging to study this type of gene transfer unless the transformants encounter an environment in which they have a strong selective advantage.

An uncertainty underlying current risk assessment of the impact of unintended HGT from plants to bacteria is caused by a poor understanding of selection pressures acting on bacteria in the natural environment. It is expected that most plant transgenes, if inserted in bacteria, would confer a neutral or negative effect on the reproduction of those bacteria carrying the new gene.

Positive selection of a rare transformant may generate an unintended environmental impact which cannot be predicted from the low transfer frequencies that are emphasized in current risk assessments. Thus, the ability to assess fitness effects of transgenes if transferred to unintended hosts should be strengthened. With the exception of certain antibiotic resistance markers, a fitness advantage of plant transgenes in bacterial recipients has not been predicted. For instance, in the absence of

kanamycin, it is unlikely that a transformed *nptII* carrying bacterium would have sufficient advantage to become common in the soil environment.
An evaluation of selective advantages of plant transgenes (given they are directly derived from another organism) in bacterial recipients would be enhanced by knowledge of the distribution of selective agents, natural background levels and reservoirs of the gene. If alternative sources of the plant transgene can be identified in the environment, it is expected that bacterial communities are already exposed to this gene, and would have taken it up if providing a selective advantage. Thus, the identification and description of natural reservoirs of homologues to plant transgenes (most often genetic traits found in various environmental organisms) would reduce some of the controversy surrounding the release of transgenic plants today and allow a rational decision to be made on the relative risk of the transgene.

7. References

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