

Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event?

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Abstract

Today, 12 years after the first field release of a genetically modified plant (GMP), over 15 000 field trials at different locations have been performed. As new and unique characteristics are frequently introduced into GMPs, risk assessment has to be performed to assess their ecological impact. The possibilities of horizontal gene transfer (HGT; no parent-to-offspring transfer of genes) from plants to microorganisms are frequently evaluated in such risk assessments of GMPs before release into the field. In this review we indicate why putative HGT from plants to terrestrial (soil and plant associated) bacteria has raised concern in biosafety evaluations. Further, we discuss possible pathways of HGT from plants to bacteria, outline the barriers to HGT in bacteria, describe the strategies used to investigate HGT from plants to bacteria and summarize the results obtained. Only a few cases of HGT from eukaryotes such as plants to bacteria have been reported to date. These cases have been ascertained after comparison of DNA sequences between plants and bacteria. Although experimental approaches in both field and laboratory studies have not been able to confirm the occurrence of such HGT to naturally occurring bacteria, recently two studies have shown transfer of marker genes from plants to bacteria based on homologous recombination. The few examples of HGT indicated by DNA sequence comparisons suggest that the frequencies of evolutionarily successful HGT from plants to bacteria may be extremely low. However, this inference is based on a small number of experimental studies and indications found in the literature. Transfer frequencies should not be confounded with the likelihood of environmental implications, since the frequency of HGT is probably only marginally important compared with the selective force acting on the outcome. Attention should therefore be focused on enhancing the understanding of selection processes in natural environments. Only an accurate understanding of these selective events will allow the prediction of possible consequences of novel genes following their introduction into open environments. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Horizontal gene transfer; Genetically modified plant; Risk assessment; Natural transformation; Soil bacterium; Competence

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1. Introduction

The application of gene technology as a tool in plant breeding has greatly expanded the genetic variation available to plant breeders. In principle, gene technology can take advantage of the genetic information present in all sources of DNA, and as genetic material can be modified to function in different hosts, previous strong species barriers to gene transfer can now be experimentally inactivated. After the first field trials in open environments with genetically modified plants (GMPs) in 1986, over 15 000 field trials with GMPs have been performed at different locations [1,2]. Whereas traditional plant breeding can draw support from familiarity and an inherent history of safe use, modern plant breeding based on the use of gene technology lacks such supportive assurance. Scientific as well as ethical, social and environmental concerns about potential risks and impacts associated with the use of the new technology are presented [3–8]. The use of GMPs in agri-

culture has raised concerns over the ecological impact of engineered genes as pollutants. This is based on the hypothesis that if the genes are transferred to e.g. microbial recipients, they can be disseminated into the environment. For instance, the frequent incorporation of antibiotic resistance genes in GMPs has raised questions about the possible transfer of engineered genes to indigenous microbes in soil. It is known that opportunistic pathogenic microorganisms of soil origin with novel antibiotic resistance patterns can emerge [9,213]. However, the clinical and veterinary significance of horizontal gene transfer (HGT; non-parental-to-offspring, non-sexual transfer of genes [10]) of antibiotic resistance genes in soil have not been documented.

From a scientific point of view, it is difficult to obtain a clear consensus about whether GMPs pose an actual risk to the environment, since few experimental data exist to support such a concern. However, in order to ensure the safety and public acceptability of GMPs used in open environments,

extensive regulations with the implementation of risk assessment procedures must be followed. A thorough evaluation of the effects of the inserted trait in the GMP is performed in such assessments, addressing factors such as the source and stability of the inserted gene, the ecological effects of GMPs concerning the estimation of its survival, multiplication or transfer of genetic material to other organisms, and health and food quality aspects [5,11–16]. An accurate perception of the influence of the engineered genes on natural communities is needed for an assessment of the risks of GMP field releases.

Current GMPs are commonly constructed with a limited number of chimeric gene insertions (novel genes), and knowledge of mechanisms that affect their dissemination should be enhanced along with biosafety evaluations before GMPs with multiple inserted genes or genes with new combinations of protein domains creating multifunctional proteins are introduced into open fields. Thus, in this review, we focus mainly on the possibilities of HGT from transgenic plants harboring unique combinations of genetic material as a result of the genetic engineering applied. The insertion of wild-type genes from unrelated organisms into plants should nevertheless be evaluated for HGT with emphasis on their selection.

2. Mechanisms of horizontal gene transfer between bacteria and their relevance to transfer of DNA from plants to bacteria

The possibilities of HGT from plants to microorganisms are often evaluated in current schemes for the assessment of risks of GMPs before field release. As plants do not have any identified mechanism to facilitate broad host-range gene transfer (except for pollen hybridization with related species), the possibilities and barriers of HGT from plants to bacteria have been approached within the framework of known mechanisms of HGT within bacteria. Although our present understanding of the diversity and interactions in the microbial world is still rudimentary [17,18], horizontal transfer of genetic information between bacteria has been extensively demonstrated both *in vitro* and in natural systems [19–

24]. For general discussions of HGT see [10,12,13,16,25–31]. In spite of convincing evidence for HGT, the genetic structure of bacterial populations is thought largely to consist of assemblages of clones with little genetic contact with each other [32]. These clones are thus evolving independently, mainly via mutations. This clonal mutation paradigm does not suggest the occurrence of frequent interspecies gene transfer in the evolution of bacteria, with the exception of the fast spread of genes conferring antibiotic resistance in clinical environments. On the other hand, an increasing number of studies indicate that transfer of fragments of chromosomal DNA between bacterial species is a significant mechanism for their evolution [32–41]. Moreover, it is well known that pathogenic bacteria utilize recombination to generate genetic variation during infection processes [42]. The impact of interspecies gene transfer compared with the generation of mutations by the variable specificity of the DNA replication and repair machinery on the natural evolution of terrestrial bacteria thus has to be further elucidated [43,44].

HGT between bacteria seems to depend on the number of bacterial species capable of transferring genes, factors that regulate their host range, the nature and the availability of the transferred DNA, the transfer efficiencies, and the selective forces acting on the bacterial recipients. Fig. 1 outlines the steps thought to be of importance for the stable uptake of DNA in bacteria. Three different mechanisms of HGT in bacteria have been described; these are transduction, conjugation and transformation (see Table 1 for a summary of their characteristics). It is not clear to what extent all possible mechanisms of HGT in bacteria have been identified since less than 1% of the bacteria present in the natural environment have been described at species level [45]. The known mechanisms, their relevance in terrestrial environments, and the likelihood of their mediating HGT from plants to bacteria will be briefly discussed in Sections 2.1–2.3. We focus mainly on the conditions for HGT in bulk soil. However, HGT is also likely to take place in the rhizosphere or within the digestive systems of protozoa, nematodes, insect larvae, earthworms and other areas in soil displaying high local activities of bacteria [46–48].

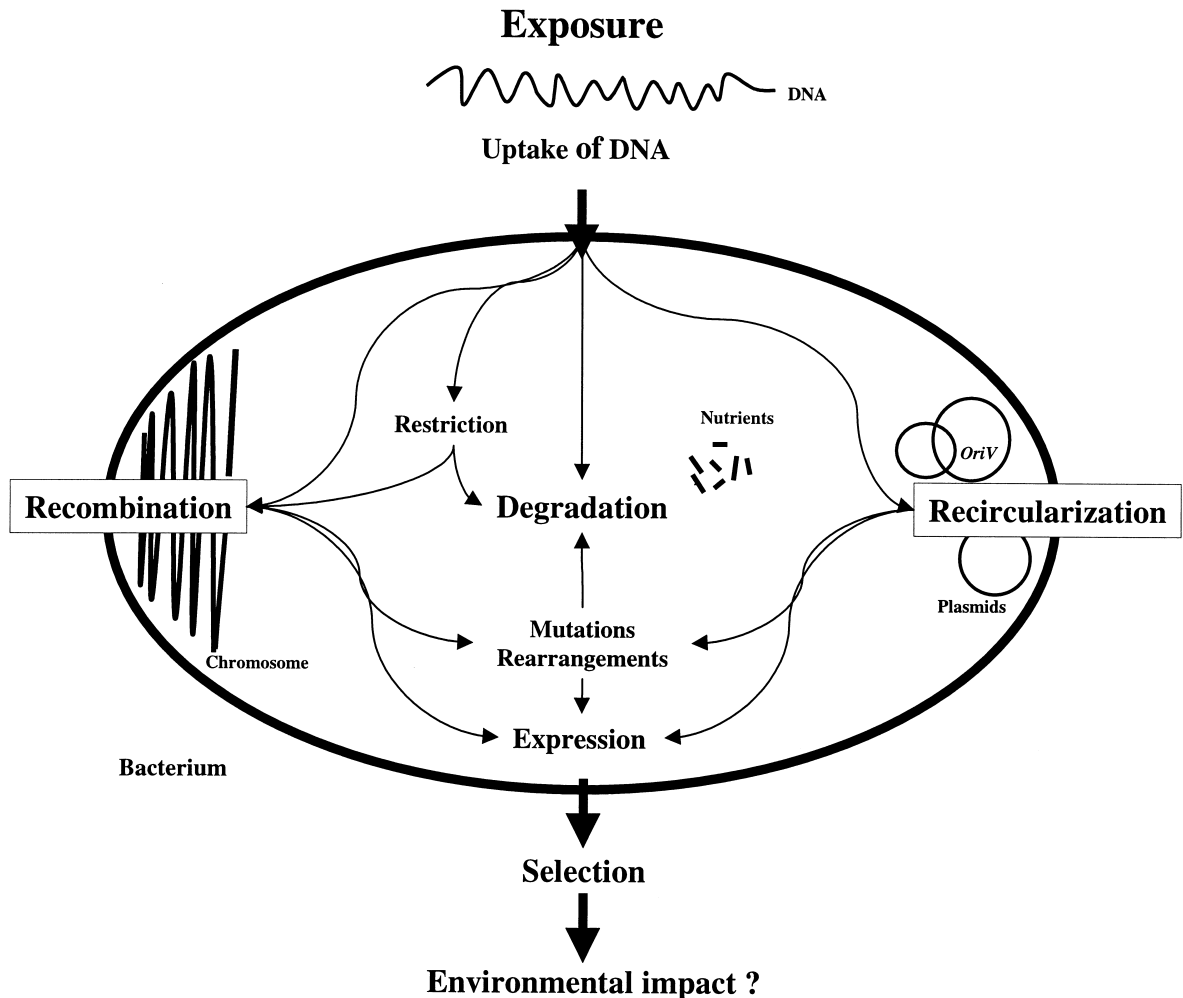


Fig. 1. Proposed fate of DNA exposed to bacterial cells expressing competence for uptake of DNA. The steps thought to be of importance for natural transformation of bacteria with genetically modified plant DNA are further described in Sections 3.1–3.5.

2.1. Gene transfer by general transduction

General transduction is a bacterial cell-virus interaction which can mediate gene transfer in the environment, e.g. on plant leaf surfaces, in soil or water (Table 1). The host ranges of bacterial viruses (bacteriophages) are considered to be generally narrow, mainly due to the requirements of surface determinants on the bacterial envelope for bacteriophage attachment. In spite of the fact that viruses such as bacteriophages have been proposed to be amongst the most numerous organisms in soils [55], the importance of general transduction as a mechanism of

HGT in soil is at present not well understood [52]. As viruses can persist in soil over time, their DNA can be protected by the protein envelope [55]. Consequently, bacteria which degrade virus envelopes might get access to released DNA from e.g. plant-transducing viruses.

Viruses that function in different species are known [56–58], but, to our knowledge, viruses that function in both plants and bacteria, and thereby possibly facilitate HGT from plants to bacteria, have not yet been identified. Such viruses should be able to attach to the different surfaces displayed by plants and bacteria and function in their heteroge-

neous cytoplasms. Although speculative, viruses infecting e.g. mitochondria, plastids or internalized bacteria in plants (exemplified by *Rhizobium* spp.) would overcome some of the constraints possessed by these heterologous environments. Such viruses need to encode functions that effect their translocation to compartments which allow expression of the viral genes.

2.2. Gene transfer by conjugation

Conjugation is a mechanism of cell-to-cell interaction which can mediate gene transfer in the environment e.g. in soil and rhizosphere, on plant surfaces, and in water (Table 1). Conjugation is known to occur frequently between bacteria and the transferable genes usually reside on plasmids. Moreover, chromosomal genes can be transferred if mobilized by a plasmid [64,65]. Conjugative gene transfer has been regarded as the most frequently occurring mechanism of HGT between bacteria [21,66–69] and plasmids belonging to the incompatibility groups incQ, P, W, N, C, and J are known to effect

transfer over a broad host range [70]. Bifunctional shuttle plasmids have been shown to be capable of encoding their transfer from *Escherichia coli* to yeast [71], and from *Agrobacterium* to yeast [72] and plants [73–75]. The virulence (*vir*) genes, encoded by plant pathogenic *Agrobacterium* Ti and Ri plasmids, are known to mediate transfer of defined fragments of these plasmids (called the T-DNA) into plant genomes during infection by a conjugation-like mechanism. Retro-transfer, the conjugative back-transfer of chromosomal or plasmid genes from infected bacteria to the donor bacteria, has been suggested to mediate chromosomal gene exchange [76]. However, similar retro-transfer of plant DNA from T-DNA infected plant cells to bacteria has not been shown to occur. It is believed that T-DNA needs to be processed and coated by specific *vir* gene-encoded proteins in the bacterium before it can be transferred to the plant cell. Since *Agrobacterium* spp. do not transfer these *vir* genes, which is necessary for T-DNA transfer upon infection of plants, the putative retro-transfer of plant genes seems unlikely.

Interestingly, plasmids have been detected in plant

Table 1
Some characteristics of the recognized mechanisms of horizontal gene transfer in bacteria

Characteristic	Mechanism of gene transfer		
	Transduction	Conjugation	Transformation
Donor organism	Bacterium infected with a bacteriophage	Bacterium harboring a plasmid or a conjugative transposon	Any organism with double-stranded DNA
Requirements to the recipient bacterium ^a	Attachment sites for binding of bacteriophage (phage receptors)	Able to bind pili from donor bacterium	Expression of competence for uptake of DNA
Vector	Bacteriophage	Plasmid or chromosome with an inserted plasmid or a conjugative transposon	Free DNA
Stability of vector DNA (in soil, water, or on plants)	Long-term stability expected due to protection of DNA in the protein envelopes of bacteriophages	Stability depends on the survival and activity of the donor bacterium	Stability of free DNA is poor due to nucleases, chemical modifications, shearing or binding to solids
Host range dependence	Presence of phage attachment sites	Binding of pili and plasmid incompatibility ^b	Ability of the DNA to integrate into the genome or recircularize into plasmids
Size range of transferred DNA (average size transferred)	≤100 kb (45 kb)	< 100 bp to 10 ⁶ kb ^c (size of bacterial plasmids)	0–> 25 kb (< 100 bp–10 kb)
Shown to occur in/on:	Soil [49–52], plants [53] and water [54]	Soil [21,59–61], plants [62] and water [63]	Soil [85–90], plants [91] and water [92,93]

^aThe recipients must be able to stabilize the transferred DNA by either homologous/illegitimate recombination into the genome, or recircularization into plasmids.

^bUnless the plasmid is integrated into the bacterial genome.

^cPartial to whole genomes can be transferred if the plasmid is chromosomally integrated in the donor (e.g. HFR strains of *E. coli*).

mitochondria [77,78], and recently plastid tubules resembling bacterial pili were shown to extend from plastids [79]. The authors suggested that the tubules might take part in the transfer of DNA.

Some transposons, called conjugative transposons, are known to contain genes necessary for their transfer by conjugation, and it has been shown that the transfer rate can be increased by applying selective agents (e.g. antibiotics) for the transposons [80,81]. Phylogenetic analyses have implicated HGT in the spread of transposons [82], and trans-kingdom transposition has been shown experimentally from *Drosophila* to the protozoan *Leishmania* [83,84]. However, to our knowledge, mechanisms that support conjugative gene transfer from higher plants to bacteria are not yet known, and transposons that function in both plants and prokaryotes have not been identified.

2.3. Gene transfer by transformation

The natural uptake of naked DNA by bacteria, a process called natural transformation, has been shown to occur in the environment such as in soil, in plants and in water (Table 1). The heritable capability of natural transformation has so far been detected in approximately 43 bacterial species [94–96]. Recently, even *E. coli* was found to be naturally transformable in spring water [97]. The number of currently known transformable species is probably an underestimate due to the failure to identify conditions needed for the development of bacterial competence. Observations of local genetic variation within genes (mosaic genes) generated after recombination with DNA from related species in non-transformable bacteria also indicate the possible occurrence of transformation in these bacteria [98]. Most studies describing natural transformation have been conducted in vitro [94,99,100], and the experimental conditions used in such studies, e.g. long periods of exponential growth, unlimited balanced nutrient supply and selective clonal amplification in monocultures, are of little relevance to most natural terrestrial environments. The importance of transformation as a gene transfer process under natural conditions has thus remained unclear, but it is receiving increasing attention [24,88,89,91,95,101].

Since some competent bacterial species take up

naked DNA independently of its sequence, natural transformation theoretically facilitates HGT from plants to bacteria [102]. In Sections 3.1–3.5, the possibilities of, and potential barriers to, natural transformation of bacteria with genetic material derived from GMP will be discussed.

3. Possible barriers to horizontal gene transfer in bacteria

Several factors and barriers that restrict HGT between distantly related organisms in the environment have been proposed (Table 2). The main barriers to interdomain exchange of genes are probably transfer and establishment barriers [103]. In addition, barriers related to spatial and temporal localization of available DNA and competent bacterial cells will probably generate constraints to successful HGT. Although barriers related to the expression of the acquired genetic material (genes or DNA fragments) and subsequently the selection of the host in the environment are not strictly related to HGT, they will affect the environmental impact of a successful transfer event [104].

Table 2
Barriers to horizontal gene transfer from plants to bacteria in soil

Barrier	Process affected
Temporal and spatial	Release and stability of plant DNA ^a Exposure of bacteria to plant DNA ^a Development of bacterial competence ^b
Transfer	Uptake of plant DNA ^c
Establishment	Stabilization of plant DNA ^d
Expression	Expression of plant DNA ^e
Evolutionary	Selection of bacterial transformants

^aDNA present in crushed leaf material from GMPs was shown to be available as transforming DNA to bacteria [126].

^bBacteria that express competence under natural conditions have been detected [88,132].

^cSome bacteria have sequence-independent uptake of DNA [94,96,126].

^dGenes from GMPs may be stabilized by homologous recombination into bacterial genomes (based on the genes inserted in the plant) or by recircularization into plasmids (based on inserted plasmids in GMPs); see Table 3.

^eDue to frequent use of prokaryotic promoters in GMPs, inserted genes in plants may be expressed in bacteria [190].

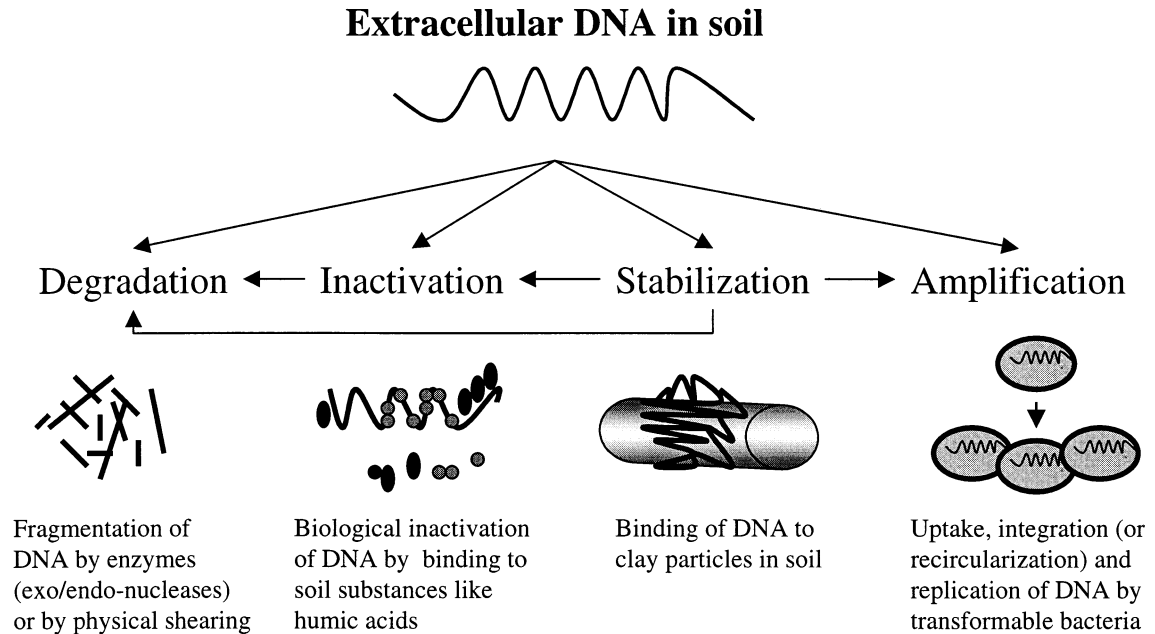


Fig. 2. Fate of DNA released into the soil environment. Most DNA released into soil is probably rapidly degraded by exogenous nucleases released by microorganisms. However, persistence of DNA in soil for extended periods has been shown [107–117].

3.1. Availability of DNA

Natural transformation requires access to free DNA which must be present at the time and place in which competent bacteria develop or reside. Thus, the stability of released DNA in the terrestrial environment is of great importance to the transformation process. Fig. 2 shows the possible fate of DNA released in soil. Natural transformation was previously regarded to be of little ecological relevance in soil environments because of the proposed rapid enzymatic degradation of DNA [105,106]. Evidence indicating that DNA can be stabilized by binding to mineral surfaces has, however, been presented [106–112], and both bacterial and plant DNA have been shown to persist in soil for weeks or months [95,112–117]. For instance, Widmer et al. [116] found persistence of the plant (tobacco and potato) marker gene *nptII* for 77–137 days in soil as measured by PCR. However, the persistence of chromosomal DNA in soil over time does not imply that the DNA is in a physical or chemical condition which makes it available as transforming DNA to competent bacteria that are locally present. Transformation can, for in-

stance, be inhibited by clay particles and humic acids [118,119]. However, DNA bound to sand or clay in microcosm studies has also been reported to retain or enhance its transforming ability [112,120–123]. The biological availability of DNA released into natural soil has only been demonstrated for very limited periods of time [87,90]. Nielsen et al. [87] observed that within hours after addition of purified chromosomal DNA to soil, its availability as transforming DNA to *Acinetobacter* sp. (formerly named *A. calcoaceticus*) cells disappeared. The availability of plant DNA to bacteria present in soil has not been demonstrated. However, the fate of DNA released from GMP will probably not differ substantially from that of bacterial chromosomal DNA, except for putative effects of an altered methylation pattern [124] and the different proteins attached. It may therefore contribute to the transient reservoir of genetic material to which terrestrial bacteria can be exposed. It is, however, uncertain to date if transgenic plants modify their novel genes by methylation and protein binding the same way as their native DNA. Although the natural release of DNA in soil is difficult to simulate with the experimental addition

of purified DNA, in vitro transformation of *Acinetobacter* sp. with DNA released from lysed cells was almost as efficient as with pure DNA [125]. Similar observations were reported by Paget and Simonet [89] who used crude lysate as a source of transforming DNA for *Pseudomonas stutzeri*. Recently, *Acinetobacter* sp. was shown to be able to utilize transforming DNA present in homogenized leaf material from GMP [126] (see Section 4.3).

3.2. Uptake of heterologous DNA in bacteria

Three factors are assumed to limit the uptake of DNA in bacteria. These are (i) dependence on active transfer of DNA from the donor bacterium (by conjugation) or bacteriophage (by transduction), (ii) development of competence for uptake of DNA by natural transformation (Section 3.2.1), and (iii) rapid degradation of DNA by restriction enzymes in the bacterial cytoplasm (Section 3.2.2).

3.2.1. Development of competence for natural transformation

The uptake of DNA by transformation is a genetically and environmentally controlled process. Bacterial cells that are transformable enter a physiologically regulated state of competence for the uptake of exogenous DNA [94,127–129]. Competence is usually not constitutively expressed in bacteria. For instance, in the Gram-positives *Streptococcus pneumoniae* and *Bacillus subtilis* the accumulation of a low molecular mass polypeptide in the growth medium stimulates the expression of genes involved in the development of competence, whereas in the Gram-negatives *Haemophilus influenzae*, *Pseudomonas stutzeri*, *Azotobacter vinelandii*, and *Acinetobacter* sp. competence seems to be internally regulated and is usually expressed in the late exponential or early stationary phase [94,95,129–131]. Only few terrestrial bacteria that express competence under natural growth conditions have been detected [88,132], and knowledge of environmental factors that affect the regulation of bacterial competence is scarce [23,88,133–135].

Successful translocation of DNA over the bacterial membrane may depend on the DNA sequence [68,96]. Some bacterial species are only able to take up DNA from their own species or close relatives

(i.e. homologous DNA) whereas other bacteria do not seem to distinguish between homologous DNA and DNA from more distant species (i.e. heterologous or diverged DNA). For example, uptake of DNA in *Neisseria gonorrhoea* and *H. influenzae* depends on the presence of short species-specific sequences in the DNA, while in *B. subtilis* and *Acinetobacter* sp. uptake of DNA is aspecific with regards to sequence [94,96,99,128,136]. The soil bacterium *Acinetobacter* sp. BD413 has even been shown to take up and incorporate DNA from genes inserted in transgenic plants [126,137]. The assumption that several types of transformable bacteria do not distinguish between different DNA sequences for translocation over the membrane is also supported by the observation that plasmid DNA from different hosts can be taken up in these bacteria and that the addition of non-transforming DNA together with the transforming DNA reduces their transformation frequencies.

3.2.2. Degradation of DNA by restriction enzymes

It is generally assumed that heterologous DNA that has entered bacteria is rapidly restricted by bacterially encoded restriction enzymes. The fragmentation of DNA after uptake by natural transformation is, however, questionable since restriction enzymes attack double-stranded DNA [138], while most competent bacteria are believed to generate single-stranded DNA during translocation of the DNA into the cytoplasm [94,96,100]. Correspondingly, natural transformation utilizing single-stranded DNA may not be affected by restriction enzymes [94,139]. It has been proposed that the presence of saturating amounts of DNA, or a leaky restriction barrier, can lead to successful transformation of cells that captured DNA [65,140]. Schäfer et al. [141] proposed inactivation of a restriction system active against foreign DNA after exposure of *Corynebacterium glutamicum* to stress conditions. Even if restriction enzymes are highly active in the recipient cells, DNA fragments of approximately 250 bp are expected to be present after digestion with restriction enzymes that recognize specific 4-bp sequences [142]. Larger fragments will be present after digestion by restriction enzymes that recognize longer sequences. Both types of fragments might be able to complement genes with deletions, and larger fragments might

even encode functional traits after integration into the genome.

3.3. Stabilization of heterologous DNA in bacteria

The stable maintenance of transgenic plant DNA in bacteria requires linkage to an origin of replication such as by (1) integration of this DNA into the bacterial chromosome, or (2) its autonomous replication based on the presence of replication functions and an origin of vegetative replication (*oriV*) in the DNA.

(1) Integration of genes into the genome of recipient bacteria is known to be dependent on sequence homology between the captured DNA and that of the recipient bacteria, and it seems that the degree of heterology between these sequences is the main factor determining the barrier to the stable introduction of diverged DNA in bacteria [143–148]. Indeed, Vulic et al. [148] observed exponentially decreasing recombination frequencies in enterobacteria with increasing sequence divergence of the introduced DNA. Similar observations have been reported for *Bacillus* spp. [149]. The log-linear relationship between increasing sequence divergence and the reduced number of excipients was continuous, and any drop-off effect as the degree of DNA divergence increased was not reported. Examinations of mosaic genes in *Streptococcus* and *Neisseria* isolates have revealed that recombination can be obtained between DNA with over 20% difference in sequence homology [98,142,150–154].

Bacteria with mutations in some of their *mut* genes (genes that are involved in mismatch DNA repair) show less stringent homology requirements for recombination [145–148,155–157] and an increased frequency of recombination with divergent DNA is obtained in these strains [148]. Unexpectedly, in a study of pathogenic enterobacteria (*E. coli* and *Salmonella* sp.) it was found that up to 1% of the natural isolates obtained displayed such *mut* mutations [158]. A similar frequency of these mutations was also found in commensal *E. coli* strains [147]. Thus, subpopulations of bacterial communities might display an enhanced frequency for recombination with divergent DNA, and it has been proposed that an adjustable species barrier may be present in mutator strains [44]. Indications of a lowering of the stringency for

recombination in bacteria growing under adverse conditions (e.g. in late stationary phase) have also been presented [159–161], possibly by a transient decrease in *mut* gene product activity [162]. Recently, although controversial, adaptive mutations in stationary phase populations of bacteria (possibly involving double-strand DNA breaks and recombination) have been receiving increased attention [155,161–166]. The mechanisms of homologous recombination in *E. coli*, by far the best studied bacterium, still remain to be completely elucidated [148,180], and unknown (illegitimate) recombination events occur in different organisms [181–183]. It should be noted that the construction of all GMPs is currently based on unknown (illegitimate) recombination events with random sites of insertion of the genes in the plant genome. Thus, the barriers to recombination with heterologous DNA in different organisms can be flexible, and the barriers might even depend on the environmental conditions of the host organism.

As shown in Table 3, genetic engineering of plants will result in the incorporation of DNA with homology to bacterial DNA. An increased possibility of stabilization of the GMP DNA in bacteria via homologous recombination is therefore to be expected. Random homology may also be present [102,167,168]. The minimal length of DNA homology required in *E. coli* for homologous recombination is approximately 20 bp [169], and short regions of homology can mediate recombination, which includes incorporation of adjacent non-homologous sequences [170–174]. Short repetitive sequences are commonly found dispersed in bacterial genomes [175–177], and although speculative, these may, if integrated into the genomes of GMP, also mediate the transfer of adjacent non-homologous gene sequences to bacteria. The development of the *Agrobacterium* binary bacterial artificial chromosome vector (BIBAC) that facilitates the transfer of large DNA fragments (> 150 kb) into plant genomes [178], may introduce longer sequences of prokaryotes in GMP. Recently, gene transfer to plant chloroplasts in tobacco has been reported [179]. It was estimated that between 5000 and 10 000 copies of the chloroplast genome, and hence the genes inserted into it, were present per plant cell. Genes inserted into plant chloroplasts will utilize prokaryotic ex-

pression systems, genes and selectable markers, thereby possibly enhancing by 1000–10 000-fold the numbers of engineered genes per plant cell (of possible prokaryotic origin).

Recently, a new class of mobile genetic elements in bacteria, the gene cassettes, have been described [184–187]. Gene cassettes are usually found integrated adjacent to integrons which both can mediate the expression of the gene cassettes and their movement. Each gene cassette contains a recombination site downstream of the gene, known as the 59-bp element, which is necessary for their movement by site-specific recombination [185]. Based on this element, numerous antibiotic resistance genes have been identified as functional gene cassettes. Some selectable markers in GMPs are identified gene cassettes. The use of these cassettes in GMPs may circumvent the requirements for homologous recombination-based stabilization of genes in bacteria, since chromosomal integration of the gene cassettes can be encoded by the integron.

(2) Stabilization of GMP DNA in bacteria is also feasible if the plant DNA contains replication func-

tions and a bacterial *oriV* facilitating its autonomous replication [188,189]. The construction of genetically modified monocotyledonous plants such as cereals, corn and rice is usually facilitated by electroporation or the use of particle guns which result in the integration of whole plasmids with intact replication functions. If fragments of such GMP DNA become recircularized following their uptake in bacterial recipients, they might become stabilized by a plasmid rescue-like mechanism [188,189]. Plasmid rescue is a technique developed for gene tagging and subsequent isolation of the genes from the host. It is based on insertion of an *oriV* and a selective marker into the target genome of e.g. plants. After isolation of the successfully tagged DNA and restriction enzyme digestion, in vitro self-ligation will generate circular fragments with an *oriV*. Subsequent electroporation into bacterial recipients and the use of selection will amplify the circular DNA containing the *oriV* and the marker gene within the tagged gene. Since bacteria during natural transformation usually take up plasmids as linear DNA [139], mechanisms to mediate recircularization of the translocated fragments

Table 3
Some characteristics of genes inserted in genetically modified plants

Characteristics of	Examples	Possible consequences if the genes are translocated into the bacterial cytoplasm
Inserted DNA		
Contain prokaryotic DNA sequences	Bacterial: vector (plasmid) sequences, T-DNA border sequences, repetitive sequences, promoters, protein-encoding genes, gene cassettes ^a (e.g. antibiotic resistance genes) ^a <i>oriV</i> sites from plasmids ^b	Integration into the bacterial genome (by homologous recombination) of plant inserted genes. Additive integration of adjacent non-homologous sequences (spread or change of resistance to antibiotics) Plasmid recircularization
Expression of the inserted DNA		
Genes lack introns	cDNA cloned genes ^c	Expression of eukaryotic genes if a bacterial promoter is present and active
Altered regulation and genetic background	Chimeric genes with constitutive promoters	Not known, the expressed gene product may interfere with bacterial metabolism

^aTransformation of monocotyledonous plants by use of particle gun or electroporation usually results in the integration of whole plasmids. *Agrobacterium*-mediated gene transfer inserts short border sequences in the transformed plant [188,189]. Short repetitive interspersed sequences are commonly found in bacterial genomes [175–177]. Structural genes from prokaryotes such as selectable marker genes are frequently inserted into GMPs [204]. Also, transformation of plant chloroplasts will facilitate the use of genes which are functional in prokaryotic cells [179].

^bA bacterial origin of replication (*oriV*) present in plant DNA fragments may facilitate their recircularization into plasmids in the bacterial cytoplasm.

^cMost genes inserted into GMPs are either derived from cDNA cloned eukaryotic genes or prokaryotic sources. In particular, transformation of plant chloroplasts will benefit from the use of prokaryotic genes [179].

(like plasmid rescue) must be naturally present in their cytoplasm.

In summary, homologous recombination (based on sequence homology to prokaryotic DNA introduced into GMPs), illegitimate recombination events, recombination events in bacteria with mutations or environmentally regulated transient deficiencies in their DNA repair and recombination system, and *oriV*-based plasmid rescue are all suggested to stabilize DNA from GMPs if transferred to bacteria.

3.4. Expression of heterologous DNA in bacteria

Next to the transfer and establishment barriers, failure to express the genes taken up and established in competent bacteria probably also represents a strong barrier to successful HGT from distantly related organisms. However, due to cDNA cloning procedures, eukaryotic genes inserted into GMP do not normally have introns, which probably enhances their expression if transferred downstream of promoters in bacteria. Although the promoters inserted into GMPs usually display low activity in prokaryotic hosts (e.g. the *Agrobacterium nos* and *TR1/TR2* promoters), some promoters inserted into GMP are also active in bacteria, for example the frequently used cauliflower mosaic virus 35S promoter expresses in *E. coli* [190]. Furthermore, the insertion of whole plasmids into GMP may lead to the presence of bacterially expressed vector sequences like the ampicillin resistance gene *amp* (*bla*_{TEM-1}) located on pUC19 [191]. Genes transferred to plant chloroplasts will, due to the prokaryotic-like chloroplast compartment, also contain promoters and genes that can be easily expressed in bacteria [179]. The random insertion of protein encoding sequences from GMP DNA into existing regulatory units in the genome of the bacterium can probably also mediate gene expression after HGT from plants. Such insertion may well be possible since transposon-based promoterless expression vectors have been used successfully to isolate novel promoters in bacteria [192].

Uptake and recombination with GMP DNA fragments rather than whole genes might also influence gene expression and variability in bacteria. For instance, if a deletion is restored in an antibiotic resistance gene, or its expression is upregulated after re-

combination, this would lead to a stronger antibiotic resistance in the bacterium. Similarly, recombination also may alter the specificity of the enzyme conferring the antibiotic resistance.

3.5. Selection and environmental impact of bacterial transformants

Efforts to understand the selection imposed on genes transferred from GMPs should receive increased attention, since the frequency of HGT is probably of a lower degree of importance compared with the selection strength acting on the outcome [44,104]. Therefore, the low transfer frequencies anticipated for GMP to bacteria transfers should not be confounded with the potential environmental implications. If transfer of novel GMP genes (e.g. genes encoding engineered chimeric proteins) to bacteria would provide the transformants with a selective advantage or new niche occupancy, favorable conditions for amplification of the transformants and, thus, the gene should be expected. Quantifying the spread of the putative transformants by estimating the stability of the genetic material acquired and their fitness requires a thorough understanding of a multitude of environmental parameters [193–196]. However, the present knowledge of microbial ecology in soil environments is unable to predict and quantify factors in soil that affect the selection of bacterial transformants receiving novel genes. Furthermore, the conditions influencing the occurrence of HGT in the natural environment might remain unidentified in the laboratory studies of gene transfer thereby generating false estimates of transfer frequencies [60,197–199]. If the genetic material transferred does not confer any advantage to the host, the potentially very short generation times of bacteria might, however, ease the accumulation of the genetic material acquired in a bacterial population by periodic ‘hitchhiking’ [193,200]. Subsequent transfer of the gene to new hosts might result in its selective amplification. The large-scale introduction of GMP into the environment will generate a continuous exposure of bacteria to high numbers of engineered genes and may thereby enhance the probability of the amplification of these genes after integration in bacterial hosts.

3.5.1. Environmental impact: antibiotic resistance genes

The frequent use of antibiotic resistance genes as selectable markers aiding the development of GMP has raised considerable concerns regarding the possible HGT of such traits to bacteria of medical importance [201]. In general, bacterial resistance to antibiotics is believed to develop mainly by the acquisition of resistance genes from heterologous bacterial sources [202,203]. Up to 1995, genes encoding resistance to nine different antibiotics had been incorporated into plants [204]. The high numbers of naturally occurring bacteria resistant to antibiotics in the environment [24,205–211] have been used as an argument for the low impact of possible HGT of antibiotic resistance genes inserted into plants, in particular for the *nptIII* gene encoding kanamycin resistance [209,212]. However, the description of a phenotypically observed resistance pattern obviously does not address the natural presence of the antibiotic resistance genes in these bacteria. Many mechanisms can be involved in the antibiotic resistance in bacteria (e.g. reduced uptake/accumulation of antibiotics, inactivation of the antibiotics by enzymes, or modification/lack of the target site of the antibiotic [213–215]). Thus, a clearer distinction between the observed phenotype and the corresponding genotype should be made in such arguments. Few studies have

been published on the occurrence in soil environments of enzyme-mediated antibiotic resistance, the type usually integrated in GMPs [207,209,215,216]. Smalla and coworkers [117,209] frequently found kanamycin resistance in bacteria isolated from agricultural soils, without detecting the *nptIII* gene. The *nptIII* gene was found in bacteria from sewage and manure, and hence, since these materials are used as a fertilizer, routes for the introduction of this gene into soil bacteria from evolutionarily closer organisms are already present.

The selective advantage of expressing antibiotic resistance genes in soil is unclear, and an estimation of the selection of putative bacterial transformants receiving antibiotic resistance genes from GMPs is currently not possible. So far, studies have however not shown any increased survival in soil of bacterial inoculants harboring antibiotic resistance genes [217,218]. The addition of the antibiotic streptomycin to soil to generate a selective advantage for bacteria harboring the corresponding resistance gene was successful for a *Pseudomonas fluorescens* strain carrying the streptomycin resistance encoding transposon Tn5 [219]. The actual concentrations of antibiotics in natural soils are unclear to date and difficult to estimate, since, with few exceptions, antibiotics are rarely isolated from soil [220–222]. This limited detection is thought to be a methodo-

Table 4

Approaches taken for the detection of horizontal gene transfer from genetically modified plants to terrestrial bacteria

Approach	Conditions applied, analyzed or detected					
	Transferred genes detected	Bacterial diversity screened	Natural conditions applied	Sensitivity of detection	Detection of transfer mechanism	Estimation of transfer frequencies
Comparisons of DNA sequences to detect HGT ^a	Sequenced genes	Bacteria with sequence data	Yes	High ^a	No	No
Screening of bacteria from field plants or soil samples for HGT	Selectable genes	Culturable	Yes	Low ^b	No	Yes
Experimental study of HGT under optimized conditions	Selectable genes	Culturable and transformable	No ^c	High	Yes	Yes

^aDNA sequences must be available for several genes, including the gene of interest, in both evolutionarily close and distant organisms. Genes duplicate and diverge over time; hence, any claimed HGT from plants to bacteria must demonstrate a clear deviation from related or unrelated bacterial genes, and homology to the plant-encoded genes [224,225].

^bA low sensitivity is expected due to differences in cultivation requirements for soil bacteria, the level of acquired resistance to the selecting agent, efficiency of plating, and interference from the natural background of resistant bacteria to the selective agent, i.e. antibiotic resistance [206–211].

^cPotential ecological interactions and factors that are important for the transfer process are not evaluated unless natural conditions are mimicked by the use of soil-plant microcosms [256,257].

logical problem since antibiotics are presumed to bind to soil particles and thereby become biologically inactivated [210,223], the degree of binding varying with the specific antibiotic.

4. Methods for the detection of horizontal gene transfer from plants to bacteria

Possible HGT of plant DNA to bacteria in soil can be evaluated by at least three different approaches: a long-term retrospective approach comparing sequences of specific bacterial and plant genes (Section 4.1); a short-term retrospective approach based on initial phenotypic screening of putative transformants from field trials with transgenic plants harboring selectable marker genes (Section 4.2); and an experimental approach based on optimized laboratory conditions for gene transfer into culturable soil bacteria (Section 4.3). Each of these methods is different in its ability to characterize and detect HGT as exemplified in Table 4.

4.1. Detection of horizontal gene transfer from plants to bacteria by comparison of DNA sequences

A phylogenetic approach, comparing DNA sequences obtained from similar prokaryotic and eukaryotic genes, will detect HGT by revealing abnormal inheritance patterns of the genes [224]. Similarities between bacterial and plant DNA with regard to nucleotide sequence, G+C content, codon usage and protein sequence and structure can be evaluated. Comparison of DNA sequences to obtain evidence of HGT is still regarded as controversial [224,225] as it requires extensive knowledge of the DNA sequences evaluated as well as other genes in both related and unrelated organisms. Smith et al. [224] have suggested several criteria for evaluation of HGT. Briefly, some of these are:

1. sequences for proteins or genes should be available from numerous and evolutionarily distant organisms;
2. the tree relating these sequences to one another should correspond to conventional phylogeny except that one member of the group appears at a radically unexpected position;
3. the tree ought to be well rooted by sequences from appropriate species or duplicated genes whose classifications are generally accepted;
4. the case is strengthened if more than one method of tree building yields the same topology;
5. it is helpful when sequences of other molecules from the same organism yield trees with conventional phylogenetic topology;
6. the life history of the potential gene donor and acceptor organisms should involve contact between them, so transfer seems feasible.

Without the influence of HGT, several mechanisms might maintain a high level of sequence homology between species. Any claimed HGT should therefore be able to exclude these possibilities. Some of these mechanisms might be gene duplication and functional divergence of the genes (paralogous genes) within the species. Moreover, if the gene in question arose prior to the divergence of the species compared, the genes might, due to functional constraints, have been preserved (orthologous genes) over time. A gene displacement occurring simultaneously in the other compared species, leading to a paralogue displacing an orthologue, would obscure the phylogeny of the gene in question. Although functional convergence is common, convergence at the sequence level has not been proved [226], thus, high levels of sequence similarities between species are believed to be due to either vertical transfer from their ancestry, or one or several horizontal transfers from other species.

Several indications of HGT from plants (or other eukaryotes) to bacteria have been reported by using DNA sequence comparisons. These refer to the transfer of the following genes: glutamine synthetase II from a eukaryote to *Bradyrhizobium japonicum* [227], glyceraldehyde 3-phosphate dehydrogenase from a eukaryote to an ancestor of *E. coli* [228], glucose 6-phosphate isomerase from *Clarkia urgulata* to *E. coli* [229], glutamyl-tRNA synthetase from a eukaryote to *E. coli* [230], and a plant leghemoglobin gene to *Vitreoscilla* [231]. Another example of a suggested horizontally transferred gene sequence is the PDZ domain [232].

The chromosomes of over 40 bacterial and archaeal species are currently being sequenced [233–235]

and the validity of the approach based on comparisons of DNA sequences to demonstrate HGT events will undoubtedly improve as these genetic data accumulate. Interpreting reported phylogenetic data with respect to HGT, it may be concluded that stable HGT from plants to soil bacteria is extremely rare if it occurs at all. However, the few HGTs suggested by comparisons of DNA sequences may indicate that plant genes transferred to bacteria do not overcome an expression barrier, that the fragments transferred are too short to be consistently detected, that subsequent rapid sequence alterations obscure the origin of the DNA, or that plant DNA does not confer any selective advantage in the recipient bacteria.

4.2. Screening of bacteria from environmental samples for horizontal gene transfer from genetically modified plants

Screening of bacterial strains in soil samples from fields or microcosms with introduced GMPs (harboring antibiotic resistance genes) has been used to detect putative bacterial transformants [236,237]. Unfortunately, only a minor fraction of soil bacteria, approximately 10%, are assessable via cultivation techniques [45]. The expected low frequency of HGT under natural conditions hampers this approach as the number of putative transformants has been suggested to be below the limit of detection. Some of the biases involved in the isolation of bacteria from the soil environment can be circumvented by analyzing total DNA extracted from soil samples, but such an approach usually fails to prove the incorporation of genes into the genomes of the bacteria [117,237].

Smalla and coworkers [114,117,237] have isolated over 5000 kanamycin-resistant bacteria from soil samples obtained from field trials with *nptII* containing transgenic sugar beets (*Beta vulgaris*) in Germany. Screening for HGT of the kanamycin resistance gene (*nptII*) from the sugar beet to naturally occurring soil bacteria was done without obtaining any positive results as confirmed by colony hybridization and PCR. See Fig. 3 for an outline of the experimental procedure used. These negative results also confirmed the low prevalence of *nptII* in culturable soil bacteria resistant to kanamycin [209].

Becker and coworkers [236] used a soil microcosm

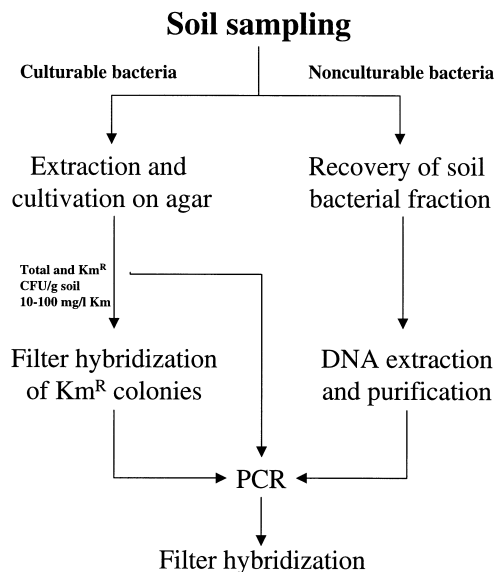


Fig. 3. The experimental approach taken by Smalla et al. [114,117,237] to detect HGT from field-grown transgenic sugar beet to indigenous bacteria. Both the culturable and the nonculturable fractions of soil bacteria were sampled and screened by PCR and filter hybridization. The primers and probes used were designed to detect the sugar beet-encoded *nptII* gene (conferring kanamycin resistance) or the *nptII* gene linked to either of two plant insert-specific genes.

to investigate possible HGT of the *nptII* and hygromycin resistance (*hph*) genes from tissue homogenates of transgenic tobacco (*Nicotiana tabacum*) into indigenous soil bacteria. Bacterial transformants were not detected in these experiments after selective plating for acquired antibiotic resistance in the bacteria followed by DNA hybridization with the specific probe.

Thus, the few analyses of HGT from GMPs to bacteria residing in soil under natural or soil microcosm conditions have not been able to show such HGT, indicating that either transfer did not occur, or the transfer frequencies and expression were too low to be detected, or the techniques applied were not appropriate for the detection of such HGT. In these studies, obviously, detection of HGT from transgenic plants to the isolated bacteria was only possible if (1) a complete DNA fragment containing both the promoter and the selectable structural gene was transferred and integrated into the bacterium, and (2) the promoter allowed sufficient expression for selection of the resistance gene. The use of selec-

tive pressure to enhance possible HGT from plants to bacteria in soil or the use of enrichment techniques of putative bacterial transformants have not been reported so far.

4.3. Experimental studies of horizontal gene transfer from genetically modified plants to bacteria under optimized laboratory conditions

To our knowledge, HGT from GMPs to bacteria has only been investigated experimentally with the hypothesis that such gene transfer takes place by natural transformation. The reported studies have all been done in the laboratory with readily culturable, Gram-negative, soil or plant-associated bacteria. The model bacteria belonged to the β (*Agrobacterium*) and the γ subgroups (*Erwinia*, *Acinetobacter*) of the proteobacteria.

Schlüter and coworkers [238] have investigated the possible (natural or artificial) transformation of the plant pathogenic bacterium *Erwinia chrysanthemi* with DNA from transgenic potato (*Solanum tuberosum*) containing a selectable ampicillin resistance gene (encoding a β -lactamase) and a bacterial origin of vegetative replication. Transformation of the bacterium was also investigated by inoculation of the bacterium directly onto potato tubers which facilitated lysis of the plant cells with subsequent possible release of plant DNA. None of the approaches reported resulted in any detectable transformants (detection limit 10^{-9}) (numbers given as transformants per bacterial recipient CFU). The potential transformation frequency after extrapolating the data to natural conditions was calculated to be below 2×10^{-17} . However, the natural competence of *E. chrysanthemi* was low (6.7×10^{-8} per 100 ng plasmid pUC19) and the presence and stability of released plant DNA with transforming activity from the lysed potato tuber tissue was not demonstrated.

Broer and coworkers [239] used the plant pathogenic bacterium *Agrobacterium tumefaciens* as a recipient for T-DNA transformed tobacco (*Nicotiana tabacum*) DNA containing a selectable gentamicin resistance gene (*accI*). This approach facilitated homologous recombination between the *A. tumefaciens* Ti plasmid and the integrated T-DNA in the GMPs, if the bacteria were able to capture GMP DNA. The experiments were also performed to take advantage

of the naturally selective conditions for *A. tumefaciens* in the plant tumors generated. However, the transformation frequency with the used GMP was found to be below the detection limit (6×10^{-12}). Development of competence for natural transformation has not been shown for *A. tumefaciens* [240] and its ability to take up linear DNA was not shown in this study.

Nielsen and coworkers [241] used the soil bacterium *Acinetobacter calcoaceticus* (recently renamed *Acinetobacter* sp.) as a recipient of DNA from transgenic sugar beet and potato DNA, both containing the *nptII* gene. The transformation conditions for the *Acinetobacter* sp. were optimized with homologous chromosomal DNA, and a transformation frequency of 10^{-2} transformants per recipient was obtained in vitro. This level probably represented its maximum experimentally obtainable transformation rate [94,137]. It was found that sequence homology or a stabilizing sequence like an origin of replication on the plasmid DNA was required for stable maintenance of the genetic material in the *Acinetobacter* sp. transformants [241]. Natural transformation in *Acinetobacter* sp. can be effectively blocked by competing DNA ([242], Nielsen and Gebhard, unpublished results). Therefore, the inhibitory effect of excess plant DNA was alleviated by enriching the GMP DNA with the integrated *nptII* gene before transformation. However, transformants were not detected with the use of this plant DNA. Moreover, the expression level of the kanamycin gene was low in the bacterium [241]. Using plant DNA, the detection limit was found to be 10^{-11} and the transformation frequency was estimated to be below 10^{-16} under natural conditions. The latter is due to lower concentrations and activity of DNA, suboptimal cell growth and competence development in *Acinetobacter* sp.

Two recent studies [126,137], also using *Acinetobacter* sp. as the recipient for transgenic plant DNA, employed introduced homology between the transgenic plant DNA and plasmid DNA in the recipient bacterium, to show the uptake of plant DNA by natural transformation. Both studies used restoration of a non-functional bacterial *nptII* gene (with an internal deletion) based on homologous recombination with transgenic plant DNA containing a functional *nptII* gene.

Smalla et al. [126] obtained restoration of a 317-bp deletion in the *nptII* gene at a frequency of 5.4×10^{-9} after exposing the bacterium (0.1 ml of a 5×10^8 CFU/ml culture) to transgenic sugar beet DNA (5 μ g) and selection on kanamycin. Also, plant leaf homogenates were shown to yield transformants with a frequency of 1.5×10^{-10} . De Vries and Wackernagel [137] obtained similar transformation frequencies (with an average of 3.5×10^{-8} using 3 μ g DNA and 20 ml of a 2.5×10^8 CFU/ml bacterial culture) of restoration of a 10-bp deletion in the *nptII* gene after exposure of the bacterium to transgenic DNA from *Solanum tuberosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, and *Lycopersicon esculentum*. Thus, the main barrier to HGT of chromosomal DNA from plants to *Acinetobacter* sp. seems indeed to be the lack of sequence homology between the introduced plant gene and the bacterial chromosome. As indicated in Table 3, GMPs, due to the genetic modifications, frequently possess sequence homology to prokaryotic genes.

With the exception of the aforementioned studies using artificially introduced homology between the DNA of the plant donor and the recipient bacterium, to our knowledge experimental evidence demonstrating HGT of heterologous genes from GMPs to naturally occurring soil or plant-associated bacteria is currently not available. The main reason for this may be that a suitable detection system has not been developed until now, or that attempts to monitor such events have focused on transfer of functional and expressed genes [238,239,241] instead of shorter DNA fragments [126,137]. The evolutionary aspects of the partial removal of a strong genetic barrier between plants and bacteria by the introduced prokaryotic homology in GMPs should be further addressed. Arguments have been raised against this concern pointing out that genetic homology to prokaryotes in plants is already present in the plant mitochondria and chloroplasts. It is possible that a frequent transfer of genes from, for instance, plastids, to plant chromosomes could generate sequence homologies between plant and prokaryotic genomes. However, widespread sequence homology to prokaryotic DNA has not yet been found in plant chromosomes. Plastids and their genes transferred to the host nucleus have diverged in sequence and function from their proposed ancestor during 10^9

years [243,244]. Transfer of genes from mitochondria to the *Saccharomyces cerevisiae* chromosome has been reported at a frequency of 10^{-5} cell⁻¹ generation⁻¹ [245]. Transfer in the opposite direction, which is the direction we are concerned with, was at least 100 000-fold less efficient, and not detected.

5. Horizontal gene transfer from plants to other plant-associated organisms

Although transfers of genetic material from plants to organisms other than plants have been claimed to be absent [246,247], HGT has been reported from plants to plant-associated fungi. Fungi are known to be transformable [248] and uptake of DNA from the host plant (as confirmed by hybridization to plant specific genes) has been claimed for *Plasmodiophora brassicae* [249,250]. Also, the GMP-harbored hygromycin gene (*hph*) was reported to be taken up by *Aspergillus niger* [251]. Stable integration and inheritance of the plant DNA in the genome of these fungi has, however, not been substantiated by experimental evidence.

6. Concluding remarks

The aforementioned (Section 4.1) cases of HGT from plants (or other eukaryotes) to bacteria, suggested after comparisons of DNA sequences, all lack experimental confirmation, since both field and laboratory studies have been negative. The exception is formed by the homology based transformation systems mentioned in Section 4.3. Together, these observations suggest that the frequencies of successful HGT from plants to bacteria may be extremely low. Given the limitations of the studies performed, related to detection of transfer of functional and expressed genes rather than fragments of DNA, it seems that stable HGT from naturally occurring plants to bacteria is more likely to become apparent within million of years rather than within the time scale GMPs are grown. Depending on the evolution rate [252,253], the most recently claimed HGT from a plant to a bacterium probably occurred over 10 million years ago [229]. It seems relevant to conclude

that evolutionarily successful HGT events from plants to soil or plant-associated bacteria are rare and that HGT is more likely to occur between organisms that are more related in DNA sequence [98]. However, it should be emphasized that the above conclusions are based on indications found in the scientific literature and a small number ($n < 10$) of experimental studies. The few direct investigations of HGT from plants to bacteria are probably insufficient to conduct risk assessments of environmental effects related to the approximately 15 000 field trials of GMPs. The majority of field trials have focused on the efficiency and functionality of GMPs from an economic perspective [254]. At the same time, these field trials have failed to increase the knowledge of possible HGT events in the environment. The general lack of research focusing on HGT, and the as yet limited understanding of bacterial genetics, ecology and selection in natural environments [255] suggest that several areas should be addressed in the future to improve the quality of risk assessment of novel genotypes. Particularly, the in situ functionality of barriers to HGT in bacteria should receive increased attention by focusing on the following:

1. The regulation of competence for natural transformation and its expression in natural bacterial communities, and the frequencies and significance of such gene transfer (of both genes and fragments) to bacterial adaptation.
2. The importance of environmental factors in the regulation of HGT under natural conditions.
3. The conduciveness of bacterial mutants (such as DNA repair mutants with lowered stringency for recombination) to transformation with heterologous DNA and the significance of such mutator subpopulations to the natural plasticity of bacterial communities.
4. The effects of strong selective pressure on horizontal transfer of genes which confer a selective advantage to the host. (Present studies of HGT by natural transformation have mainly been done without selective pressure during the exposure time with DNA. Studies which incorporate selective pressure during the exposure of competent bacteria with selectable DNA should be designed.)

5. The factors influencing selection of bacteria present in natural environments such as soil.

Although it is important to elucidate the mechanistic features and frequencies of gene transfer to understand its significance to genetic variability in bacteria, we strongly emphasize the need to enhance the knowledge of selection processes that occur in natural environments. Only an accurate perception of the selective advantages expressed by novel genes will allow the prediction of possible consequences following their introduction into open environments.

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