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Thematic Issue on Horizontal Gene Transfer

Review article

Fate of transgenic plant DNA in the environment

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This review addresses the possible ecological effects of transgenic plants on micro-organisms in the field, hence, in the phytosphere and in the soil matrix. The important steps involved in the interaction between plant DNA and bacteria and the factors that influence the horizontal gene transfer (HGT) process will be discussed. HGT is a process in which two partners are involved, even if indirectly. In the first section, aspects concerning bacteria, such as their physico-chemical, biological and genetic characteristics, are described. Parameters affecting transgenic DNA fate in the environment are described in the second section. Subsequently, terrestrial habitats are evaluated in terms of their capacity to favor horizontal gene transfer. Finally, we focused on several studies in order to evaluate possible perturbations of soil bacterial community composition due to cultivation of transgenic plants in the field.

Keywords: GMO / bacteria / transgene / fate / microbial

GENETICALLY ENGINEERED PLANTS: HISTORY, FEATURES AND APPLICATIONS

Since the dawn of agriculture, humans have shaped the characteristics of domesticated plants in order to develop better-adapted varieties and to increase yields by taking advantage of the natural occurrence of mutants. Despite the poor understanding of the process, plant breeding was a popular activity even before the botanist Gregor Mendel in 1865 published his findings on how dominant and recessive alleles produce specific traits that can be passed to offspring (<http://www.mendelweb.org/Mendel.plain.html>). This was the first major insight into the science behind the art, and breeders soon applied the new understanding of genetics to traditional techniques of self-pollination and cross-pollination. Later, at the beginning of the 20th century, scientific advances in other disciplines, such as physics and chemistry, led to a science-based approach for the genetic modification of plants, thus providing the means for plant breeders to enhance plant genetic diversity at a faster pace.

After the discovery of deoxyribonucleic acid (DNA) in the early 1950s as the basis of life and, shortly thereafter, the determination of its molecular structure by

Watson, Crick and Franklin, genetic traits could be manipulated directly. Techniques for the insertion of foreign genes into bacteria were first developed in the early 1970s, and only a decade later, the ability to transfer foreign genes to plants via transgenesis was achieved (Comai et al., 1985; Horsch et al., 1985; Krens et al., 1982).

Thus, plant genetic engineering was added to the long list of methods that broaden the available genetic diversity of a given plant species (Belzile, 2002). The worldwide expectations generated by this technology among scientists and supporters were so optimistic that the term *Doubly Green Revolution* was introduced to describe the extent of the innovation and to summarize the potential benefits for mankind (Wisniewski et al., 2002).

Early transgenic plants were laboratory specimens, but already in the mid-1990s transgenic plants with commercially useful properties appeared on the market. They carried traits of agricultural interest, such as plant protection from insects or pests, herbicide resistance and tolerance to stress, in addition to qualities such as prolonged shelf life and enhanced nutrient content. The potential of the second generation of transgenic plants currently under development is for the production of vaccines and proteins of pharmaceutical interest. Finally, aesthetic applications might be proposed, such as ornamental bright

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colored fluorescent lawn grasses (Marvier and van Acker, 2005). The US patent database provides a view of the state of the art of commercial applications of plant genetic engineering and describes which plant-derived products might be available on the market in the near future (Dunwell, 1999).

The U.S. Food and Drug Administration web site (<http://www.cfsan.fda.gov/~lrd/biocon.html>) offers a nearly complete list of currently commercialized genetically modified plants (GMPs). Currently, the transgenic crops that are cultivated most successfully and widely are herbicide-tolerant soybean, oilseed rape, cotton and maize, and insect-resistant cotton and maize. In 2005, they were grown in a total of 21 countries, among which the United States, Argentina, Canada, Brazil, China and South Africa accounted for 99% of the planted surface. Between 1996, when the first transgenic crops were commercialized, and 2006, the global acreage devoted to these plants has increased 50-fold, from 1.7 to 102 million hectares (James, 2006).

POTENTIAL ECOLOGICAL RISKS LINKED TO FIELD RELEASE OF GMPs

Despite the increasing surface dedicated to these crops, public concerns were raised, particularly in Europe, *in primis*, on their safety in relation to human health and the environment, and also on the sustainability of this new agricultural technology and on its impacts on global agro-food production and society at large (Hails and Kinderlerer, 2003). An argument against approval of transgenic plants involves the dependence upon seeds protected by intellectual property rights and owned by major agrochemical companies, thus enriching large corporations and depriving farmers from their rights to reuse the seeds. Other reasons of dispute concern the elimination of crop and herbicide rotations, the potential for seed dispersal through contamination, cross-pollination with wild plants creating “superweeds” and the inability of the public to be adequately informed about the presence of genetically modified food (Greenpeace, 2003). During the period 1999–2004 a *de facto* moratorium on cultivation and import was in place in Europe; since then the use of GMPs in Spain, Portugal, France, Germany and in the Czech Republic is still limited to a single variety of maize (insect-resistant due to expressing the insecticidal protoxin of *Bacillus thuringiensis*).

Transgenic plants cultivated in the field can be regarded as reservoirs of transgenes, which could be released in the surrounding environment either by roots, during plant decay or by pollen (Fig. 1). Some researchers suggest that transgenic crops can create critical environmental impacts such as gene flow, rapid development

of insect resistance and effects on non-target organisms (Altieri et al., 2004; Marvier, 2001; Quist, 2004). Release of the gene product for example by root exudation (Saxena et al., 1999) might affect soil health and ecosystem functioning by altering the dynamics of microbial populations.

A specific concern stems from the fact that commercialized transgenic plants have prokaryote-derived marker sequences coding for antibiotic resistance. These are central to the controversy, since there might be a possible risk of horizontal gene transfer from plants to soil and gut micro-organisms with uncontrolled spread of antibiotic resistance genes in the ecosystem, and clinical therapy might be compromised due to increased microbial resistance to the antibiotics obtained from the consumption of food derived from transgenic crops. The validity of these arguments will be discussed below.

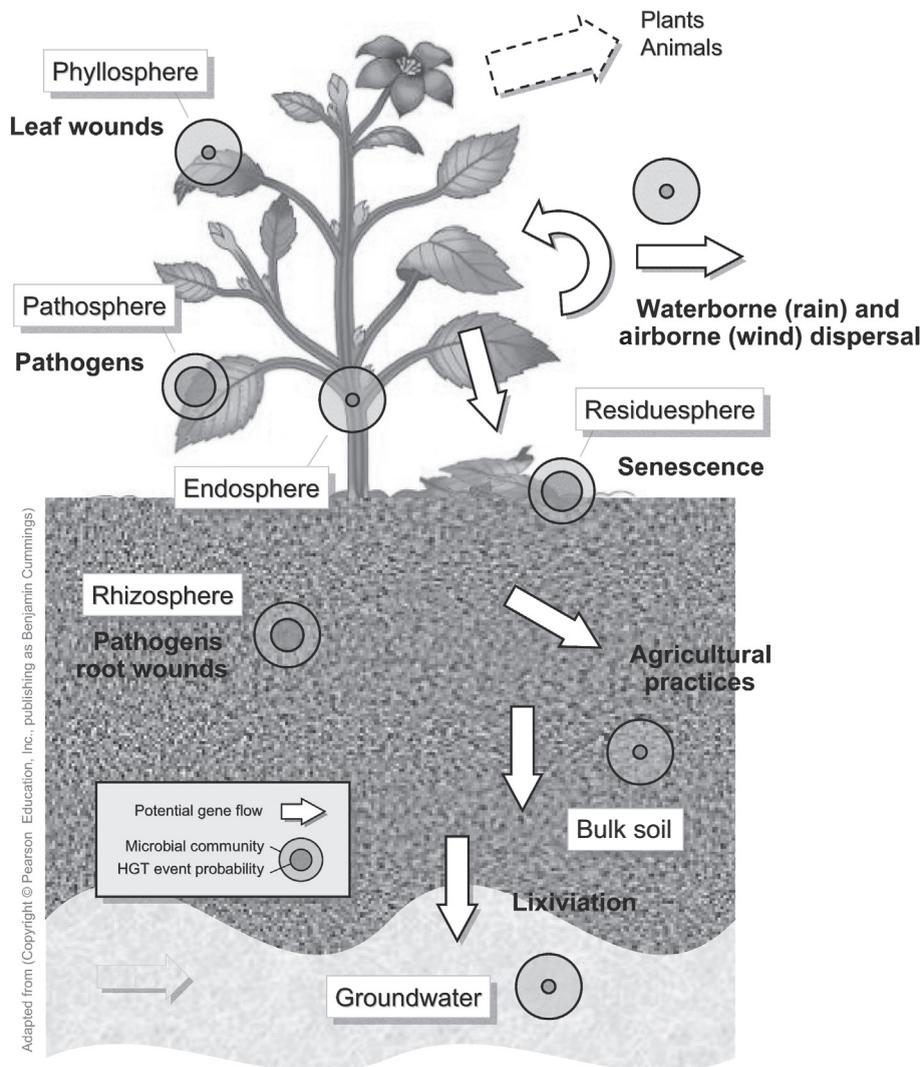
This review summarizes the current scientific studies concerning possible ecological effects of transgenic plants on micro-organisms in the field, both at the plant level (the phytosphere) and in the soil matrix. The critical steps involved in the interaction between plant DNA and bacteria, and the factors that influence the gene transfer process will be discussed. In the first section, bacteria-dependent aspects such as physical-chemical, biological and genetic characteristics will be addressed. In the second part, parameters affecting transgenic DNA fate in the environment will be described. In the third section, terrestrial habitats will be evaluated in terms of their capacity to favor horizontal gene transfer. Finally, studies that assess possible perturbations of soil bacterial community composition due to the presence of transgenic plants will be considered.

CURRENT TRANSGENIC PLANTS AND SELECTABLE MARKER GENES

Advances in molecular biology and *in vitro* cell culture led to the development of GMPs. The rapid acquisition of data on the blueprints of life of eukaryotes and prokaryotes and the cloning technology boosted the delivery of novel organisms on the market with traits for wide applications in agriculture. The first GMPs were developed using *Agrobacterium tumefaciens* mediated transformation. The gene carrying the new trait was integrated in the plant nuclear genome after cloning it into an adapted Ti plasmid. The plasmid was transferred into the *Agrobacterium*, which was then inoculated into plant tissue. This technique worked well for most of dicotyledonous species.

Another common technique to generate transgenic plants is particle bombardment or “biolistic transformation”, *i.e.*, the mechanical delivery into the nucleus of

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Figure 1. Some possible transgene transport routes from the plant to environmental bacteria (HGT = horizontal gene transfer).

plant cells of fine metal particles (gold or tungsten) that have been coated with transgene DNA.

Independent of the process used, released transgenic crops first raised concerns about the risk that *de novo* assembled genes could be disseminated in the environment by pollen, and cross hybridize with native weeds or non-transgenic crops, thus creating novel resistant species. Two recent studies documented the occurrence of cross-pollination in herbicide-tolerant oilseed rape (OSR) (*Brassica napus* L.) (Hall et al., 2000) and creeping bentgrass (*Agrostis stolonifera*) (Watrud et al., 2004) and also provided evidence that pollen can travel significant distances, up to 21 km in the case of creeping bentgrass.

Meanwhile, researchers have developed other strategies to prevent gene flow from plant to plant. For example, a breakthrough in transformation systems was the delivery of genes, through biolistic transformation, into chloroplast genomes (the plastomes) creating transplastomic plants. Not only does this process yield a higher protein content in the plant, due to the elevated copy number of plastomes per cell and of chloroplasts per plant, it also offers a confined placement of transgenes in the plastome; since the latter is maternally inherited, pollen is likely to be transgene free (Daniell et al., 2002).

All systems for creating transgenic plants require separate processes for introducing cloned DNA into

living plant cells, for identifying or selecting those cells that have integrated the DNA into the appropriate plant genome (nuclear or plastid), and for regenerating or recovering fully developed plants from the transformed cell. In order to identify transformed cells, which are usually a small percentage of the starting cells, scientists have used selectable marker genes. Therefore, genetic constructs in transgenic plants used in agriculture frequently contain prokaryotic antibiotic resistance genes and origins of replication. For the majority of commercial transgenic varieties, antibiotic- or herbicide-resistance selectable marker genes such as kanamycin or phosphinothricin resistances were used (Aragao et al., 2000; Barry et al., 1992; Miki and McHugh, 2004).

As the source of these genes is mainly bacterial (Tab. 1), the possibility exists for transgenic plant DNA to bypass the genetic barriers to horizontal gene transfer from plants to bacteria, and antibiotic resistance determinants might spread among environmental bacteria. The prokaryotic origin of chloroplasts (and thus their potential homology with bacteria) also potentially increases the likelihood for the HGT from transplastomic plants to environmental bacteria. Indeed, the prokaryotic progenitors of the current cell organelles were engulfed by a pre-eukaryotic cell in an endosymbiosis-like process (Margulis, 1981). During their gradual integration, the organellar genomes underwent a dramatic size reduction and gene transfer to the nuclear genome (McFadden, 2001).

Furthermore, genes present in currently commercialized transgenic plants are mostly derived from soil bacteria such as the herbicide-resistance gene *bar* from *Streptomyces hygrosopicus* or the insect-resistance gene *cryA* from *B. thuringiensis*. As soil is an enormous reservoir of bacteria, it is most likely that these bacteria could come in contact with DNA released from living or dead plants. Data has accumulated about some of these bacteria being capable of picking up free DNA and integrating it into their genomes; for example, in the same genus *Streptomyces*, some species were shown to have the ability to undergo transformation (Lorenz and Wackernagel, 1994).

Are these determinants susceptible to dissemination from their original environments and capable of transfer among the indigenous microbiota? Would the equilibrium of existing ecosystems be perturbed by creation of novel organisms? Would these be able to take over other species due to acquisition of an adaptive advantage, therefore, becoming more competitive than the wild species? How likely (*e.g.*, at which frequency) would this happen? And finally, are eventual perturbations detrimental or favorable to soil and plant health?

In order to address the likelihood of a gene transfer between plant and bacteria, a certain number of laboratory studies separated the different steps necessary for the

process to occur. *In vitro* studies took into account the different complex environmental parameters that could affect this process, and made use of various biological models identified as the most appropriate and likely to occur *in vivo*. The next step was the design of experimental protocols in which optimal conditions were defined with the aim of obtaining evidence of the transfer *in situ*.

HORIZONTAL GENE TRANSFER BETWEEN TRANSGENIC PLANTS AND BACTERIA

The occurrence of gene transfer between eukaryotes and prokaryotes depends on the contact between the DNA released by the plant and the indigenous bacteria colonizing the surface of the plant or dwelling in the soil. These bacteria are potential recipients of transgenes present in plant residue and root exudates. Bacteria in these habitats are numerous and diverse, and have adapted to specific niches located on the different parts of plants as epiphytes, endophytes or symbionts. In addition, many bacteria live in proximity to plants, as about 20 000 common bacterial species and 500 000 rare species could be present in 30 g of soil (Dykhuisen, 1998).

Some bacteria play key roles in decomposing plant material, either as degraders or as pathogens, potentially leading to the dissemination of nucleic acids in the environment. Clearly, the released DNA may serve as a nutritional supply, supporting microbial growth in addition to its potential for the incorporation into bacterial genomes. There are many biotic and abiotic factors that influence the availability of DNA in the environment (as discussed later). Bacteria generally inherit their genetic material from the previous generation via asexual reproduction (vertical transfer). Hence, the creation of genomic variability allowing rapid adaptation to environmental challenges occurs principally via two major mechanisms: mutation and recombination. Thus, genetic variability is generated by endogenous rearrangements such as displacement by insertion sequences (IS) or transposons, and deletions or duplications of large regions of DNA. In addition, the bacterial genome diversity stems from a mechanism that drives acquisition of new functions from foreign genes: horizontal gene transfer (HGT).

Evidence that a considerable proportion of most bacterial genomes consists of horizontally acquired genes has been collected by bioinformatics analysis of sequenced bacterial genomes (Nakamura et al., 2004). Comparative genomics has shown that the high level of similarity between genes from phylogenetically remote organisms could only be the result of HGT. Horizontally transferred genes are mostly implicated in environmentally relevant functions, such as pathogenesis, antibiotic or heavy metal resistance and pollutant catabolism.

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Table 1. Common selectable marker genes used for the selection of transgenic and transplastomic plants (adapted from Miki and McHugh, 2004).

Common name	Gene	Gene product and use	Source	Genome	References
Selectable markers and reporter genes					
Antibiotics					
Neomycin	<i>neo</i> ,	Neomycin	<i>Escherichia coli</i> Tn5	nuclear	(Fraley et al., 1983)
Kanamycin Paramomycin, G418	<i>nptII</i> (<i>aphA2</i>)	Phosphotransferases		plastid	(Carrer et al., 1993)
	<i>nptII</i> (<i>aphAI</i>)		<i>Escherichia coli</i> Tn601		
Aminoglycosides	<i>aaC3</i>	Aminoglycosides-N-acetyl transferases	<i>Serratia marcescens</i>	nuclear	(Hayford et al., 1988)
	<i>aaC4</i>		<i>Klebsiella pneumoniae</i>		
	<i>6'gat</i>		<i>Shigella</i> sp.		(Gossele et al., 1994)
Spectinomycin	<i>aadA</i>	Aminoglycoside-3-adenyltransferase	<i>Shigella</i> sp.	nuclear plastid	(Svab et al., 1990)
Spectinomycin	<i>spt</i>	Streptomycin	Tn5	nuclear	(Svab and Maliga, 1993)
Streptomycin		Phosphotransferase			(Maliga et al., 1988)
Hygromycin B	<i>hph</i> (<i>aphIV</i>)	Hygromycin	<i>Escherichia coli</i>	nuclear	(Waldron et al., 1985)
Bleomycin Phleomycin	<i>ble</i>	Phosphotransferase	<i>Escherichia coli</i> Tn5	nuclear	(Hille et al., 1986)
		Bleomycin resistance		plastid	(Perez et al., 1989)
Sulfonamides	<i>sulI</i>	Dihydropteroate synthase	<i>Escherichia coli</i> pR46		(Guerineau et al., 1990)
Streptothricin	<i>sat3</i>	Acetyl transferase	<i>Streptomyces</i> sp.	nuclear	(Jelenska et al., 2000)
Chloramphenicol	<i>cat</i>	Chloramphenicol	<i>Escherichia coli</i> Tn5	plastid	(DeBlock et al., 1984)
		acetyl transferase	Phage p1cm		(DeBlock et al., 1985)
Herbicides					
Phosphinothricin	<i>pat</i> , <i>bar</i>	Phosphinothricin acetyl transferase	<i>Streptomyces hygrosopicus</i> <i>Streptomyces viridochromogens</i> Tu494	nuclear	(DeBlock et al., 1989)
Glyphosate	<i>epspsynthase</i>	5-Enolpyruvyl shikimate-3-phosphate synthase	<i>Petunia hybrida</i> , <i>Zea mays</i>	nuclear	(Zhou et al., 1995) (Howe et al., 2002)
	<i>aroA</i>		<i>Salmonella typhimurium</i> <i>Escherichia coli</i>		(Comai et al., 1988) (Della Cioppa et al., 1987)

HGT MECHANISMS

The three mechanisms for horizontal gene transfer (*i.e.*, conjugation, transduction and transformation) were described for the first time over 50 years ago (Avery et al., 1944; Griffith, 1928; Lederberg and Tatum, 1946; Zinder and Lederberg, 1952). Conjugation involves an active

process of bacterial mating, implying cell-to-cell contact through specific structures to enable DNA transfer from the donor to the recipient cell. Transduction derives from errors in lysogenic phage integration into and/or excision from the chromosome of their host or incorrect packaging of non-phage DNA into phage particles in the lytic cycle. Transformation is the uptake and stable integration

Table 1. Continued.

Common name	Gene	Gene product and use	Source	Genome	References
	<i>Cp4 epsps</i>		<i>Agrobacterium tumefaciens</i>		(Barry et al., 1992)
	<i>gox</i>	Glyphosate oxidoreductase	<i>Ochrobactrum anthropi</i>		(Barry et al., 1992)
Sulfonylureas	<i>csr1-1</i>	Acetolactate synthase	<i>Arabidopsis thaliana</i>	nuclear	(Olszewski et al., 1988)
Imidazolinones	<i>csr1-2</i>	Acetolactate synthase	<i>Arabidopsis thaliana</i>	nuclear	(Aragao et al., 2000)
Oxynils	<i>bnx</i>	Bromoxynil nitrilase	<i>Klebsiella pneumoniae subspecies ozanaenae</i>	nuclear	(Freyssinet et al., 1996)
Gabaculine	<i>hemL</i>	Glutamate-1-semialdehyde aminotransferase	<i>Synechococcus</i> PCC6301	nuclear	(Gough et al., 2001)
Cyanamide	<i>cah</i>	Cyanamide hydratase	<i>Myrothecium verrucaria</i>	nuclear	(Weeks et al., 2000)
Pharmaceuticals					
2-Deoxyglucose	<i>dog⁺1</i>	2-Deoxyglucose-6-phosphate phosphatase	<i>Saccharomyces cerevisiae</i>	nuclear	(Kunze et al., 2001)
Betaine aldehyde	<i>badh</i>	Betaine aldehyde dehydrogenase	<i>Spinacia oleracea</i>	nuclear, plastid	(Daniell et al., 2001a; Ursin, 1996)
S-aminoethyl L-cysteine (AEC)	<i>dhps</i>	Dihydropicolinate synthase	<i>Escherichia coli</i>	nuclear	(Perl et al., 1993)
	<i>ocs</i>	Octopine synthase	<i>Agrobacterium tumefaciens</i>		(Koziel et al., 1984)
4-Methyl tryptophan (4-mT)	<i>tdc</i>	Tryptophan decarboxylase	<i>Catharanthus roseus</i>	nuclear	(Goddijn et al., 1993)
Methotrexate	<i>dhfr</i>	Dihydrofolate reductase	<i>Escherichia coli</i> mouse <i>Candida albicans</i>	nuclear	(Herrera-Estrella et al., 1983) (Eichholtz et al., 1987) (Irdani et al., 1998)
Inductors					
D-xylose	<i>xylA</i>	Xylose isomerase	<i>Streptomyces rubiginosus</i>	nuclear	(Haldrup et al., 1998a)
			<i>Thermoanaerobacterium sulfurogenes</i>		(Haldrup et al., 1998b)
D-Mannose	<i>manA (pmi)</i>	Phosphomannose isomerase	<i>Escherichia coli</i>	nuclear	(Joersbo et al., 1998)
Benzyladenine-N-3-glucuronide	<i>uidA (gusA)</i>	β -glucuronidase	<i>Escherichia coli</i>	nuclear	(Joersbo and Okkels, 1996)
Reporter genes					
aadA:gfp	<i>aadA, gfp</i>	Green fluorescent protein, Aminoglycoside-3-adenyltransferase	<i>Aequaria victoria</i> <i>Shigella</i> sp.	plastid	(Khan and Maliga, 1999)

in the chromosome (or autonomous replication) of free DNA from the environment.

Of the three mechanisms, natural transformation might play the most significant role in gene transfer between transgenic plants and micro-organisms (Bertolla et al., 1999). Transduction has been viewed as largely improbable, because of the strict host specificity of

viruses, and no virus functioning in both plants and bacteria has yet been identified. Conjugation has been shown in a specific case: the unidirectional transfer of genes harbored on a conjugative plasmid between the phytopathogenic *Agrobacterium* spp. and the plant. The inverse would require that plant transgenes be carried on a circular plasmid, which is generally not the case for

current GMPs, except those in which plasmids have been cloned after linearization into the genome. In this case, recircularization of the plasmid would be required before the genetic exchange, and so far this option has been viewed as extremely improbable (Schlüter et al., 1995).

Since the discovery of natural transformation in *Streptococcus pneumoniae* (Griffith, 1928) about 90 bacterial species from water, soil and sediment have been found to be naturally transformable (de Vries and Wackernagel, 2004). Given the small number of microorganisms investigated, compared to the large number of non-cultivable environmental bacteria, and following recent identification *in silico* of apparent competence-related genes in several newly sequenced isolates (Bolotin et al., 2001; Claverys and Martin, 2003), this phenomenon could be more widespread than imagined. The 90 naturally transformable bacterial species currently identified represent only 2% of cultured prokaryotic species (de Vries and Wackernagel, 2004), which in turn represents about 1% of the estimated number of existing bacterial species (Kaeberlein et al., 2002). Therefore, studies conducted with well-characterized cultured prokaryotic species might severely underestimate the potential for such a transfer. However, there is considerable variation in the mechanism of DNA uptake, the conditions required, and the specific barriers that limit gene transfer as will be discussed further.

Genetic transformation has been commonly divided into “natural” and “artificial” transformation. Natural transformation (Lorenz and Wackernagel, 1994) is an active microbial process of DNA uptake, requiring specific genes and the development of a competence state in bacteria, while artificial transformation is a passive process requiring chemical (*i.e.*, salts) or electrical (*i.e.*, electroporation) induction; usually it is performed *in vitro* in order to introduce DNA into bacterial, fungal, plant, or animal cells. By subjecting cells to an electrical current field or a chemical treatment that creates non-permanent pores in the lipid bilayer of the cell membranes, DNA is free to move in and out of cells. Evidence is emerging that *in situ* electrical discharges in the form of lightning influence bacterial transformation and gene transfer. Thus, lightning-mediated gene transfer (electrotransformation) under natural conditions could be as important as natural transformation in driving bacterial evolution. Indeed, parameters for the *in vitro* process were found to be highly similar to those during lightning striking soil (Demanèche et al., 2001a). In addition, indigenous soil bacteria were shown to be electrotransformed by laboratory-scale lightning (Cérémonie et al., 2004, 2006). Similarly, chemical conditions such as high salt concentrations in the environment could induce a competent state (Lorenz and Wackernagel, 1994), and in one case provided evidence of *in situ* competence development;

calcium dissolved in freshwater induced transformation of *Escherichia coli* in river water (Baur et al., 1996).

REQUISITES FOR NATURAL TRANSFORMATION

Natural transformation involves several steps that must occur under natural conditions (Smith et al., 1981): extracellular DNA must be released into the environment; genetically adapted bacterial genotypes must be in close proximity; and environmental conditions (biotic and abiotic) must be favorable for the development of the physiological state of competence, which includes the adsorption of DNA to the bacterial cell surface and the uptake of DNA (Fig. 2). Then, integration in the chromosome via recombination or autonomous replication must be efficient. Finally, the acquired trait has to be expressed by the recipient bacterium. Selective pressure will determine the establishment of the new acquired phenotype and its fixation in the offspring or its disappearance.

All of these steps have been studied as separate events, mostly *in vitro*, thus providing evidence of the bacterial parameters and limiting factors of each step. The entire gene transfer process depends ultimately on fluctuating environmental conditions and correlated intrinsic cellular factors. Hence, assumptions have been made by several authors that the probability would be extremely low for the conditions mentioned above to occur, allowing a gene transfer event from plant to bacteria under natural conditions (de Vries and Wackernagel, 2004; Nielsen et al., 1998).

Successful transformation events are expressed as the ratio of the number of transformed cells to the total pool of cells exposed to DNA, *i.e.*, as a frequency of transformation. For most species studied *in vitro*, the highest transformation frequencies were obtained when bacteria were exposed to specific DNA sequences harboring a sufficient degree of homology with the recipient bacteria, and under optimum nutritional conditions, which are not necessarily the highest concentrations. For example, *Azotobacter vinelandii* had the highest transformation rates under limited nutritional conditions, showing differences imputable to alternative adaptive strategies (Lorenz and Wackernagel, 1994). Although few naturally transformable bacteria are constitutively competent, competence is usually an active physiological state; as a general rule, bacteria become competent in a growth-dependent manner. In some gram negative bacteria, such as *Haemophilus influenzae*, *Pseudomonas stutzeri*, *A. vinelandii* and *Acinetobacter* sp., competence is internally regulated and is expressed in the late exponential or early stationary phase (Lorenz and Wackernagel, 1994; Paget et al., 1998), while in gram positive *Streptococcus*

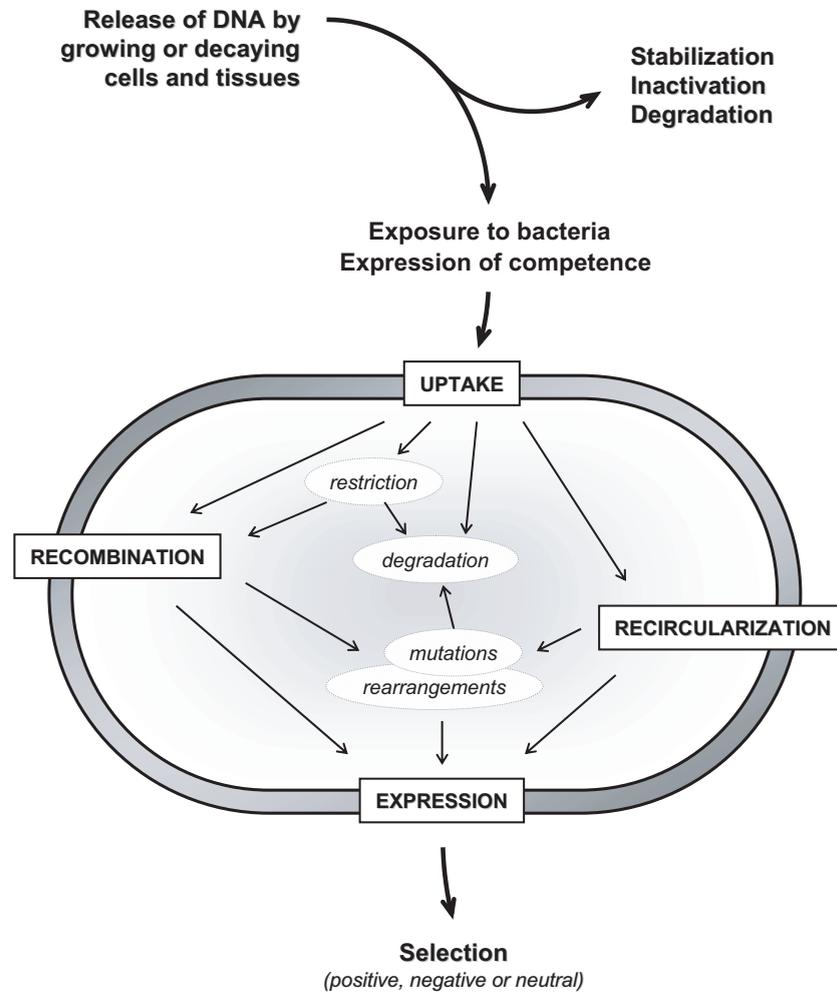


Figure 2. Natural transformation of bacteria: steps required for a successful transfer of foreign DNA into transformants. (Adapted from Thomas and Nielsen, 2005.)

pneumoniae and *Bacillus subtilis*, the accumulation of a low molecular mass polypeptide in the growth medium stimulates the expression of the about 30 to 50 genes involved in competence development (Nielsen et al., 1998). To date, only a few soil bacteria have been shown to express competence under natural growth conditions. For example, *Acinetobacter baylyi* was transformable in soil and *in planta* (Kay et al., 2002a; Nielsen et al., 1997). Compounds present in roots exudates, such as amino acids, organic acids and sugars, were proven to stimulate competence development in *A. baylyi* in soil (Nielsen and van Elsas, 2001). For this bacterium, nutrient and water accessibility is critical for producing and maintaining its competence in soil. For example, phosphate concentration and humidity were shown to boost transformation frequencies. Furthermore, experiments in soil microcosms demonstrated that *A. baylyi* remains

competent for several hours, then after reaching stationary phase (after 24 hours) its ability in acquiring host DNA is completely lost (Nielsen et al., 1997). However, the addition of a single carbon source and salt stimulates competence, which lasts for up to 6 days in soil.

Demanèche et al. (2001c) demonstrated that *A. tumefaciens* and *Pseudomonas fluorescens* are capable of incorporating plasmid DNA 72 hours after their inoculation in soil microcosms. Similarly, *in planta* nutritional conditions may be favorable for bacterial development, and could thus constitute an environment that fosters competence. The phytopathogen *R. solanacearum* became competent during colonization of plant tissues, and its rapid multiplication *in planta* was correlated to an exponential growth phase *in vitro* (Bertolla et al., 1999).

A number of recent studies on bacterial biofilms demonstrate the role of cell signalling (quorum sensing)

between bacterial aggregates in driving the development of competence and gene transfer (Li et al., 2001, 2002). Furthermore, HGT via natural transformation was particularly enhanced in these biofilm-associated communities (Molin and Tolker-Nielsen, 2003); hence, several researchers hypothesize that bacteria living in aggregates on the different surfaces of the plant could be favorable candidates for HGT (van Elsas et al., 2003). Although DNA is degraded in the environment, the probability that sufficient amounts of intact DNA of microbial or eukaryote origin remain available in the environment due to metabolic turnover is not negligible. On the other hand, *in situ* soil conditions are not always conducive to metabolically active bacteria, due to nutrient limitation (oligotrophy). However, soil is heterogeneous and composed of a multitude of nutrient-rich microhabitats, such as the plant rhizosphere, the residuesphere, the gut of insects, earthworms and protozoans, which could foster competence development (van Elsas et al., 2003).

BARRIERS TO NATURAL TRANSFORMATION

In order to successfully transform a bacterial cell, free DNA must have certain features allowing it to pass a number of stages before its stable integration in the recipient genome (Fig. 2). Assuming that conditions for a physical encounter between the bacterial cell and an intact transgene-derived molecule are met, we can delineate two further barriers from the bacterial side: the first concerns the uptake of the DNA into the cell, the second is the stable internalization of the new trait in the genome.

Uptake of DNA into the cell

The first step of the transformation process of bacteria depends on the adsorption of extracellular DNA to the cell wall. This process varies among bacterial species. For some bacteria, the mechanism requires a short nucleic acid recognition sequence of 10 bp (*Neisseria gonorrhoeae*) or 9 bp (*Haemophilus influenzae*) in the donor DNA, and this strategy has been proposed to optimize gene transfer between cells belonging to the same species and to prevent the uptake of foreign DNA (Smith et al., 1995). Other naturally competent bacteria are transformable by any type of DNA, for example *Bacillus subtilis* takes up *E. coli* DNA, phage T7 and various plasmids, while *Streptococcus pneumoniae*, *Synechococcus* and *Acinetobacter* acquire DNA from any sources (Lorenz and Wackernagel, 1994). Once DNA enters the cell, it could be affected by restriction-modification (RM) systems, which degrade certain foreign DNA sequences while modifying host DNA by methylation, thus protecting it from the RM system. This mechanism reduces the incorporation of new sequences (Bickle and Kruger,

1993). RM systems are found frequently in the genomes of naturally transformable species (Kobayashi, 2001).

RM systems were certainly involved in the dramatic drop in the transformation efficiency of *Streptomyces avermitil* (Macneil, 1988) and *Pseudomonas stutzeri* (Berndt et al., 2003) when they were exposed to donor DNA that had been propagated in *E. coli* instead of the respective recipient species. However, with three naturally transformable bacteria (*Bacillus subtilis*, *H. influenzae* and *Streptococcus pneumoniae*), a strong effect of restriction on transformation frequency was not seen (Bron et al., 1980; Lacks and Springhorn, 1984; Stuy, 1976). During natural transformation, only a single strand of the extracellular DNA is transported into the cytoplasm of competent cells (Dubnau, 1999) and restriction enzymes generally recognize and cleave double-stranded DNA (Redaschi and Bickle, 1996), although now evidence exists that single-stranded DNA can also be cleaved (Ando et al., 2000). In addition, conditions such as the presence of saturating amounts of extracellular DNA or restriction systems inefficiency could promote natural transformation by overwhelming RM systems (Bickle and Kruger, 1993). Hence, the role of DNA restriction on the integration of foreign DNA must be carefully assessed.

DNA homology: the antagonistic role of the mismatch repair system and the SOS system

The major barrier to the integration of foreign DNA into the genome is probably the last step: the stable insertion of the incoming DNA either by autonomous replication for plasmid molecules or by genomic integration of linear DNA via homologous recombination (de Vries and Wackernagel, 1998, 2002; Meier and Wackernagel, 2003a).

The size of DNA also influences the success of natural transformation. Larger DNA strands tend to be less well transported into cells. Following uptake, the efficiency of integration of donor DNA in the recipient genome via homologous recombination depends on the presence of homology with resident DNA. Several studies report that a log-linear decline in the nucleotide sequence identity between donor and recipient leads to a significant decrease in the integration of donor DNA by homologous recombination (Lorenz and Sikorski, 2000; Majewski et al., 2000; Rayssiguier et al., 1989; Roberts and Cohan, 1993; Zahrt and Maloy, 1997; Zahrt et al., 1999).

However, recombination was found to occur *in vitro* even if the degree of homology between donor and recipient DNA was very low, as in homology-facilitated illegitimate recombination (HFIR) where, in paradigmatic cases homologous regions of only 3–8 bp were sufficient to produce integration of longer foreign DNA fragments in *Acinetobacter* BD413 (de Vries and Wackernagel,

2002) and of 3–10 bp in *S. pneumoniae* (Prudhomme et al., 2002). However, the frequencies of transformation *in vitro* were up to six orders of magnitude lower than those determined by transforming these strains with entirely homologous donor DNA.

The recombinational process is under the control of two main antagonistic systems, the mismatch repair system (MRS) and the SOS system, which mediate the integration of heterologous DNA. The MRS, in addition to correcting replication errors and base modifications, acts as a strong recombinational barrier by preventing the formation of heteroduplex molecules, in order to maintain a level of genetic stability in the cell. This genetic barrier might be countered by the SOS system during stress response (such as to DNA damage). The latter, catalyzed by the RecA protein (Otero and Hsieh, 1995), could promote recombination between divergent stretches of DNA (Matic et al., 1995). Hence, genetic barriers are not inexorably fixed and should be also viewed in a dynamic context.

IN SITU TRANSFORMATION OF BACTERIA BY PLANT DNA

Recombination between eukaryotic DNA and prokaryotic DNA was believed to be impossible due to differences in DNA structure (exon-containing DNA complexes with proteins) and higher methylation rate of eukaryotic DNA, until it was observed in *A. baylyi* under optimized conditions *in vitro* (Gebhard and Smalla, 1998). These conditions included the development of plants in which selectable marker genes were flanked by homologous sequences, in order to promote homologous-recombination-mediated integration in the recipient host (de Vries et al., 2001). A further step was to determine if such transfer could occur *in situ*, *e.g.*, in the phytosphere or in soils cultivated with transgenic plants. Several studies addressed HGT from plant to bacteria *in situ* using different experimental models of recipient bacteria and transgenic plants. These include nuclear-modified potatoes, sugar-beets, tomatoes and tobacco expressing the *nptII* gene (Bertolla et al., 2000; Gebhard and Smalla, 1999; Nielsen et al., 2000b) or transplastomic tobacco plants in which the *aadA* gene was cloned in the plastid DNA (Kay et al., 2002b). However, no gene transfer could be detected when nuclear-transgenic tomato and tobacco plants were inoculated with *R. solanacearum*, although the agent of bacterial wilt had been found capable of developing a competence state and to exchange genetic material *in planta* (Bertolla et al., 1997, 1999). Reasons for this failure were imputed to the low efficiency of transformation of the recipient strain, and to the strong dilution of the transgene in the plant DNA in nuclear-transgenic plants (Bertolla et al., 2000). When a transplastomic tobacco, containing up to 10 000 copies of

transgene per cell (Daniell et al., 2002), was co-infected with a mix of *R. solanacearum* and *A. baylyi*, the latter was able to develop competence in infected tissues, and to acquire and express the transgene (Kay et al., 2002b). Hence, HGT occurred *in situ*, albeit under optimized conditions of homology between plant and bacterial sequences (Kay et al., 2002b). For the soil environment, a microcosm-based study revealed that HGT occurred when sterile soil microcosms were inoculated both with plant DNA and bacteria already in a competent state. No transfer could be detected under similar conditions when non-sterile soil microcosms were used (Nielsen et al., 2000b). To date, the occurrence of HGT in fields planted with GMPs could not be demonstrated (Gebhard and Smalla, 1999). However, crucial aspects in the cited study such as the sampling size and the detection method employed (efficiency of selection) were found to be inadequate to detect those rare transformants that could potentially be present among the high proportion of indigenous soil antibiotic resistant bacteria (Nielsen and Townsend, 2004; Ray and Nielsen, 2005).

KEY PARAMETERS AFFECTING TRANSGENE DNA AVAILABILITY (FATE OF TRANSGENE DNA IN THE ENVIRONMENT)

Nucleic acids are ubiquitous on Earth and are found in environments such as fresh and marine water, sediment, soil and the terrestrial subsurface (Trevors, 1996). DNA from plants, animals and micro-organisms is released into the environment after cellular lysis, but can also be actively excreted by several bacterial species, such as *Bacillus subtilis*, *E. coli*, *Acinetobacter* sp. and some species of *Pseudomonas* (Lorenz and Wackernagel, 1991; Paget and Simonet, 1994). Furthermore, DNA release also occurs during the formation of bacterial biofilms (Spoering and Gilmore, 2006). Microbial DNA concentrations in the soil environment can range from 5 to more than 35 $\mu\text{g}\cdot\text{g}^{-1}$ of dry soil (Ceccherini et al., 1998; Frostegard et al., 1999; Hastings et al., 1997). During their life cycle, plants also synthesize and replicate DNA, which is released into the soil throughout vegetative growth by root cap cell lysis and root turnover. During anthesis, pollen can contribute by adding nucleic acids to soil and water. Moreover, plant tissue decomposition (*e.g.*, following physiological maturity of crops) or enzymatic degradation of cell structures by pathogens (Ceccherini et al., 2003) also release DNA into the environment. One key factor controlling bacterial transformation frequencies in soil is the availability of incompletely degraded and biologically active DNA after its release from dead (or living) cells.

PERSISTENCE OF NUCLEIC ACIDS IN THE ENVIRONMENT

Since the beginning of use of transgenic plants in the field, interest in the fate and detection of transgenic DNA in the environment after plant removal has grown (Gebhard and Smalla, 1999; Romanowski et al., 1993; Widmer et al., 1996, 1997). A wealth of studies have been published that relied on the inoculation of soil microcosms with plant DNA originating from various sources, such as purified genomic DNA (chromosomal or plasmid) (Gebhard and Smalla, 1999; Romanowski et al., 1993; Widmer et al., 1996), bacterial lysates (Nielsen et al., 2000b), plant leaves (Widmer et al., 1997) and pollen (Meier and Wackernagel, 2003b). All of them have addressed DNA degradation in soil.

The persistence of extracellular nucleic acids in the environment seems to be influenced by a number of biotic and abiotic parameters, which favor their protection or which induce their degradation. DNA is altered by physical agents (heat, ultraviolet light), chemical factors (pH, reactive oxygen species, heavy metals, etc.) and by enzymatic hydrolysis by plant or microbial nucleases during senescence or apoptosis. DNase-producing bacteria in the soil and water environment account for more than 90% of the heterotrophic bacteria present (Greaves and Wilson, 1970). However, because nucleic acids are chemically reactive, due to their negative electric charge, they can form complexes with equally reactive environmental constituents. Several studies have been done on the interaction of nucleic acids with minerals, and are concordant on the protective role that some materials have on this anionic polymer (Demanèche et al., 2001b; Lorenz and Wackernagel, 1994; Romanowski et al., 1991). Thus, the binding of DNA to minerals provides protection against both eukaryotic and prokaryotic DNases (Ahrenholtz et al., 1994; Khanna and Stotzky, 1992; Paget et al., 1992; Pietramellara et al., 2001).

Quartz sands, clay minerals, feldspar and heavy metals were shown to be binding substrates. In addition, organic compounds, such as humic acids, were shown to complex DNA (Crecchio and Stotzky, 1998) and proteins and polysaccharides, which are present in cellular debris, may also protect DNA from enzymatic degradation (Nielsen et al., 2000a). The protective role offered by these compounds relies on adsorption; namely, the existing electrostatic repulsion between the anionic polymer (DNA) and the negatively charged surfaces of minerals is hampered by the presence of cations. The ubiquitous presence of multivalent cations in the environment and their role as mediators of DNA adsorption to surfaces is probably the reason for the binding of DNA to soils and sediments (Lorenz and Wackernagel, 1994).

Several factors influence adsorption in addition to the type of minerals and mono- or divalent cations present.

These factors include pH, soil water content, DNA tertiary structure and size (Franchi et al., 1999; Vettori et al., 1996). DNA adsorption on clay minerals is more efficient when free water activity is low and, consequently, cation concentration is elevated, but also when pH is low, because silicates cease being negatively charged below pH 5. DNA conformation is also important, because the higher the number of the degrees of freedom of the molecule, the higher the possible number of bonds to the sorbant; thus, linear and relaxed circular DNA adsorb better than covalently closed DNA (Melzak et al., 1996; Poly et al., 2000). Both chemical (cation bridging) forces and physical forces (van der Waals) are responsible for the high binding capacity of DNA to minerals (Ogram et al., 1988). The protection from nuclease degradation is not fully understood, but clay particles adsorb DNA molecules and DNases, thus physically separating the enzyme from its substrate (Demanèche et al., 2001b; Paget et al., 1992). The electrostatic attractions between DNA and clay occur over the entire length of the linear DNA molecule, while for supercoiled molecules, electrostatic attraction seems lower, thus, leading to molecules with higher sensitivity to DNases (Poly et al., 2000). Pietramellara et al. (1997) observed that the adsorption of DNA by two types of clay, such as homoionic montmorillonite and kaolinite, was affected neither by base composition nor by the morphology of the ends of the DNA molecule (blunt or cohesive). In addition, the importance of the phosphate bonds of the DNA molecule in the process of DNA binding to minerals was confirmed.

BIOLOGICAL POTENTIAL OF DNA

Even if DNA is adsorbed tightly to minerals, it can still react with enzymes, be amplified by PCR (Vettori et al., 1996), and transform bacteria (Gallori et al., 1994; Paget et al., 1992). The biological activity of DNA introduced into soil was demonstrated in two recent studies (Demanèche et al., 2001b; Sikorski et al., 1998), thus confirming previous results (Graham and Istock, 1978, 1979) that showed that bacterial transformation can take place in soil. The ability of DNA to transform bacteria (integration and expression) is dependent on the integrity of the DNA molecules. If the molecule is a plasmid, it has to be intact in order to replicate in the host cell, whereas if it is linear DNA, homologous sequences must not be damaged for homologous or homeologous recombination to occur. Adsorption and protection of DNA are partial and reversible, and when environmental conditions change, DNA could be desorbed and become accessible to bacteria and to DNases. Extracellular DNA appears to be degraded as a function of the microbial activity in an environment. Despite the DNA degrading capacity of microbial habitats, DNA persists and is detected by PCR and

hybridization long after it has been seeded in soil. For instance, DNA from transgenic sugarbeet (*Beta vulgaris* L.) was detected up to two years after harvest (Gebhard and Smalla, 1999).

When transgenic tobacco DNA was subjected to conditions simulating the decaying process in nature (Ceccherini et al., 2003), most of the intracellular DNA of the plant was degraded by plant nucleases or enzymes within 72 hours. However, the remaining DNA displayed a biological activity by transforming a competent bacterium originating from soil *in vitro*. Even if only a minor fraction of plant DNA escapes degradation and reaches the soil, its fate will depend on the soil micro-organisms present. They could utilize DNA either as a source of nutrients or internalize it *via* natural genetic transformation. On the other hand, DNA might bind to soil colloids and be protected from degradation. Because bacteria are heterogeneously distributed in soil, the probability of contact between them and nucleic acids is dependent in part on DNA mobility. Water might help the dissemination of transgenes from plant debris through soil. Antibiotic resistance genes, whether circular or linear, were shown to move through water-saturated soil columns, albeit degradation occurred and was dependent on the time of incubation of nucleic acids in soil (Poté et al., 2003). The DNA remaining in the effluent was biologically active, indicating that water-saturated soil and groundwater could harbor and transport functional DNA. In addition, when roots of transgenic soybean and corn plants were flushed with water, significant amounts of root-derived transgenes were found in the leachates (Gulden et al., 2005). These results suggest the possibility that biologically active transgene DNA can be released from plants into the soil environment despite rapid degradation of unprotected naked DNA.

THE PHYTOSPHERE AND “HOT SPOTS” FOR HORIZONTAL GENE TRANSFER

The majority of natural environments, soils and aquatic systems, are often characterized by low availability of resources for microbial growth, which can severely limit population density and activity. This restricts microbial processes dependent on density and activity, such as all HGT mechanisms. However, a number of specific sites in these natural habitats, mostly related to soil, plant and surfaces, have been shown to provide favorable conditions for bacterial colonization and growth, resulting in the occurrence of locally enhanced densities of active microbial cells. These sites are often conducive to HGT processes and have been considered “hot spots” for bacterial gene transfer activity (van Elsas and Bailey, 2002). However, since they are heterogeneous and vary both in time and space, local conditions at each point will control

Table 2. Environmental hot spots conducive to gene transfers. These hot spots are sites of enhanced gene transfer activity, which is often based on an increase of cell densities, of cell-to-cell contacts, of cellular movement or activity (modified from van Elsas and Bailey, 2002).

Habitat	Hot spot	HGT mechanism
Soil	Rhizosphere and plant tissue	Conjugation Transformation
	Phyllosphere	Conjugation
	Manured soil	Conjugation
	Guts of soil animals	Conjugation
	Epilithon	Conjugation
Aquatic	Sewage/sludge	Conjugation
	Sediment	Conjugation Transformation
	Water	Conjugation Transformation

HGT rates. Both abiotic (*e.g.*, temperature, pH, moisture content, nutrient availability) and biotic (grazing, predation, competing or syntrophic organisms and plants) factors can affect the microbial environments of these “hot spots”. A range of “hot spots” for HGT in soil and aquatic environments is presented in Table 2.

The presence of large mineral surfaces and organic constituents in soil plays a key role in determining the physiological status of soil-dwelling bacterial cells. Soil is a heterogeneous system with gas, liquid and solid phases (Nannipieri et al., 2003). As pointed out above, clay-organic matter complexes are important sites for soil micro-organisms due to their negatively charged surfaces and enhanced nutrient availability (Pietramellara et al., 2001; Smiles, 1988). Water availability in soil is an important factor driving microbial activity. In soil, bacterial cells occur mainly adsorbed to surfaces and are often present as microcolonies with restricted movement and contact with other colonizers located elsewhere. Hence, most bacterial cells in soil can interact only with partners in their immediate vicinity unless they are transported by water.

In spite of the nutrient-poor status of many soils, nutrients can become concentrated in different locations such as the rhizosphere – the layer of soil influenced by root metabolism – (Hiltner, 1904), the decaying organic material of animal or plant origin (the residuesphere), and the gut of soil animals like earthworms. The rhizosphere of many plants represents a region with relatively high concentrations of organic carbon and nitrogen, phosphorus and sulfur. In addition, water flow in soil induced by plant roots may enhance bacterial movement. Therefore, microbial growth induced by the presence of nutrients and water flow can promote cellular activities and

cell-to-cell contacts (De Leij et al., 1995). In addition, *in vitro* transformation was found to be enhanced by a range of different compounds exuded by plant roots into soil (Nielsen and van Elsas, 2001).

The phyllosphere (aerial plant parts) (Normander et al., 1998; Waipara et al., 2002) and the residuesphere (interface soil-plant residues) (de Liphay et al., 2001; Sengelov et al., 2000), can also provide nutrient-rich surfaces, resulting in high microbial activity. The phyllosphere of plants has been shown to be conducive to conjugative plasmid transfer (Björklöf et al., 1995) and to transduction (Mendum et al., 2001; Stephens et al., 1987). Thus, globally, soil contains physical barriers to cell-to-cell contacts and nutritional limitations, while providing local rich nutrient microenvironments. HGT rates in soil are certainly affected by the combination of these phenomena.

Other parts of the plant, such as the spermosphere, the endosphere and the pathosphere, can also sustain consistent densities of bacteria and allow HGT. In the pathosphere, during the co-infection of tobacco plants with the plant pathogen *R. solanacearum* and *Acinetobacter* BD413, *Acinetobacter* was able to incorporate plant-derived DNA (Kay et al., 2002b).

EFFECTS OF GENETICALLY MODIFIED PLANTS ON MICROBIAL COMMUNITIES

Transgenic plants might affect soil microbial communities directly and indirectly. Among the potential direct effects are changes in soil microbial activity due to differences in the amount and composition of root exudates, changes in microbial functions resulting from gene transfer from transgenic crops, and alteration in microbial populations because of the effects of management practices for transgenic crops such as pesticide applications, tillage and application of inorganic and organic fertilizers (Motavalli et al., 2004). Transgenes that enter the soil after the decomposition of plant litter will be concentrated differently in the soil profile based on tillage type (Angle, 1994). Transgenes have been shown to be released directly from the plant roots from sloughed and damaged root cells as well as through root exudation. Transgenic *Bt* maize (*Zea mays* L.) was found to release the *B. thuringiensis* insecticidal endotoxin from its roots (Saxena and Stotzky, 2000). Incorporation of transgenic plant products in the soil could alter soil microbial biodiversity due to variable responses by microorganisms to the novel proteins.

Among indirect effects of transgenic crops on microbial mediated processes, the effects of changes in the amount and composition of transgenic crop residues are the most often cited. For example, reductions in corn

borer damage or differences in the composition of the residues may increase the amount of undamaged, low N-containing residues remaining on or in soil after harvest due to the expression by the plant tissues of *Cry* genes coding for the endotoxin. This might reduce the rate of decomposition and nutrient mineralization. Consequentially, the following crop might grow slower due to reduced nutrient availability (as suggested by Boyle et al., 2001). These potential effects are complicated by the possible decrease in soil erosion due to the higher amount of crop residues left in the soil. Furthermore, the higher productivity of the culture conferred by the self protection from the pest also means higher carbon sequestration (Motavalli et al., 2004), which is important in carbon turnover and would balance its biogeochemical cycle.

Several examples demonstrate changes in the composition of soil microbial communities in the agricultural area cultivated with transgenic crops. For instance, it has been suggested that targeting transgenic traits affecting plant nutrient acquisition in nutrient-limited environments, such as genes causing increased root exudation of organic acids, may reduce the activity of bacterial communities due to a decrease in pH in the rhizosphere (Motavalli et al., 2004). That introduction of transgenic disease-resistant trees might affect mycorrhizae and other soil biota important in decomposition and nutrient cycling in forest soils has also been suggested (Boyle et al., 2001). The demonstration that transgenic plants could alter their microbial environment was based on the legume species bird's-foot trefoil (*Lotus corniculatus* L.) engineered to produce opines (low molecular weight amino acid and sugar conjugates that can be used as growth substrates by a few of the root-associated bacteria) (Oger et al., 1997). The population densities of the opine-utilizing bacteria were 80 times higher than in non-transformed plants, while the number of cultivable bacteria was not significantly different. A recent study showed that the effect was independent of opine, plant and soil (Mansouri et al., 2002).

Considerable debate concerning the effects of transgenic plants on microbial communities is due in part to the diversity of plants, microbial indicators, and short- or long-term statistical significance. In addition, other factors could bear different potential interpretations. Investigations on the effects of GMPs on the composition of microbial communities indicate that alterations in the microbial diversity would be less significant than environmental factors such as sampling date and field site (Dunfield and Germida, 2001, 2003). Spatial and temporal effects were also demonstrated in the terminal restriction fragment length polymorphisms (T-RFLP) patterns associated with the rhizosphere of an herbicide-resistant transgenic potato (Lukow et al., 2000). Similarly, a field study conducted with transgenic potato expressing T4

lysozyme concluded that differences observed between the community structures of rhizobacteria associated with transgenic lines and those of the control isogenic line were negligible if compared to environmental factors (Heuer et al., 2002). Microbial communities associated with glufosinate-tolerant transgenic maize were not different based on the SSCP-PCR (Single Strand Conformation Polymorphism PCR) patterns from the communities linked to wild-type maize plants (Schmalenberger and Tebbe, 2002).

Yet many reports suggest there are differences in microbial communities associated with transgenic plants. Carbon utilization patterns and fatty acid methyl ester profiles (FAME) of the microbial communities associated with the roots of a genetically modified OSR variety were different from those of the conventional varieties. Moreover, the representative bacteria of the culturable microbial community associated with a genetically modified OSR variety was significantly different from that of two conventional OSR varieties tested (Siciliano and Germida, 1999). Different populations of *Rhizobium leguminosarum* bv. *viciae* were hosted by transgenic glufosinate-tolerant OSR and its non-transgenic counterpart (Becker et al., 2001), and differences between the physiological and genetic profiles of the rhizosphere community of representative microbial species were detected between parental and transgenic alfalfa (*Medicago sativa* L.) (Di Giovanni et al., 1999; Donegan et al., 1999) or transgenic OSR (Gyamfi et al., 2002). The impact of two transgenic *Bt* and one wild-type maize line and their residues was evaluated by a multiphase approach on bulk soil and rhizospheric eubacterial communities, on the arbuscular mycorrhizal fungus *Glomus mossae* and on soil respiration (Castaldini et al., 2005). Results from microcosm and greenhouse experiments showed differences in rhizospheric eubacterial and culturable rhizospheric heterotrophic bacterial communities associated with the three maize lines and a lower level of mycorrhizal colonization for one transgenic cultivar compared to the wild-type line. Most of these studies are of relatively short duration, although one such study lasted over 4 months. Opine-producing transgenic *Lotus corniculatus* from the field harbored apparently significant populations of mannopine utilizers, which decreased very slowly, and after 4 months still produce elevated counts. However, the bacterial populations isolated from soils that had been planted with transgenic or wild-type plants were very similar after the next fallow, and thus demonstrated no long term effects (Oger et al., 2000).

These different studies demonstrate that the alterations in the microbial diversity associated with GMPs and with the non-transgenic counterpart, with changes in the agroecosystem (*e.g.*, utilization of a certain

agronomic practice, utilization of a particular compound) and with season cannot be easily categorized (Dunfield and Germida, 2004; Griffiths et al., 2006).

CONCLUSIONS

Interkingdom HGT from transgenic plants to bacteria has been demonstrated to occur *in situ*, but only under optimized conditions where homologous genetic regions were present in recipient strains. In specific niches where microbial densities as well as conditions are appropriate for development of competence (hot spots), such transfer becomes measurable, albeit occurring at low frequencies (Kay et al., 2002b).

Small fractions of transgenes escape degradation, move into soil and persist in the environment. Further research needs to be done in order to gain better insight into ecological aspects of HGT. For example, experimentally or theoretically determined transfer frequencies should be viewed in a context where little is known about the growth and environmental fitness of bacteria transformed by transgenic plant DNA in the environment. What would be the evolutionary potential of these micro-organisms? Is the transfer event fixed only if it confers a selective advantage? The answer may be different for each transgene. Generally, successful events occur on an evolutionary time scale (de Vries and Wackernagel, 2004), and require a selective pressure if cells transformed by the recombinant DNA are to multiply and establish. Up to now, quantification of HGT events has been performed with cultivation-based approaches (selective plating), which give little information on the survival of transformants in natural settings. In addition, actual quantitative data obtained from *in situ* studies (transformation frequencies) should be viewed as partial, due to the lack of discrimination between original events of transformation and clonal multiplication of these events, hindering their accurate determination. Such questions should be addressed in future investigations.

Discussion of the impact of transgenic plants on environmental bacteria needs to include that antibiotic resistance genes, often located on mobile genetic elements, already exist in the environment and are widely dispersed in the microbial community, due in part to massive utilization of antibiotic compounds in animal and human health care over the last 50 years and to the natural production of these antibiotics. Constantly high selective pressure on pathogens ensures (in exchange) the spread of resistance determinants *via* HGT amongst pathogens, and is responsible for the loss in efficiency of a growing number of antimicrobial molecules (Davies, 1994). A further element deserving consideration is that spontaneous mutation could drive the acquisition of antibiotic-resistant phenotypes with frequencies close to

or even higher than those formed by natural transformation (Goldstein et al., 2005). Even if promising technological advances will eliminate selectable marker genes from the plant product through selectable marker-free chloroplast engineering (Daniell et al., 2001a, 2001b) or recombinase-mediated excision (Ow, 2002), the other functional genes present in the latest generation of transgenic crops, such as “pharmaplants”, will need to be assessed with respect to their potential effects on the environment.

Nonetheless, the first released GMPs carrying selectable marker genes still represent the most valuable model available for studies of interkingdom gene transfer, its role in the evolution of bacteria, and its influence on the ecology of microbial communities. Some evidence of transgenic plants' influence on the composition of the plant-associated microbial communities has emerged in a few studies. Effects were found for a variety of plants fitted expressing different transgenes. However, these effects were shown to depend on field site, seasonal variation and the technique chosen to assess the community. The changes in microbial communities associated with growing transgenic crops are relatively variable and transient, in comparison with some other well-accepted agricultural practices such as crop rotation, tillage, herbicide usage and irrigation. Since minor alterations in the diversity of the microbial community might affect soil health and ecosystem functioning, the impact that a plant variety may have on the dynamics of rhizosphere microbial populations, and hence, plant growth and health, as well as ecosystem sustainability requires further study. The extent of differences observed (if validated) might be negative or positive and this should be studied with the long-term effects of GMPs in rotation (considering each type of GMP as a specific individual with specific properties). Comparisons should be also made with other acceptable changes in agroecosystems, such as growing a novel non-transgenic plant or the use of new agronomic practices. Ongoing and future environmental evaluation of the diversity of transgenic crops under development may pose a unique scientific challenge that, in addition, may provide an opportunity for an improved understanding of soil and plant microbial ecology.

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