Transfer of DNA from genetically modified plants to bacteria

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Executive summary

The numbers of genetically modified plants being constructed worldwide are continually increasing, which has brought about a debate on the possibility of uncontrolled spread of transgenic DNA through the environment. In this report, the focus is on the (interkingdom) transfer of transgenic DNA from plants to bacteria. A study of the available recent literature on Horizontal Gene Transfer (HGT) from transgenic plants to bacteria was performed and in addition, the topic was discussed in a two-day workshop with experts in the field.

HGT processes between bacteria cover conjugation, transduction and transformation. However, natural transformation is clearly the single key mechanism that can mediate the transfer of genetic material from plants to bacteria. The likelihood (frequency) of occurrence of gene transfer from plants to bacteria was found to depend on a range of ecological factors (mainly the occurrence of so-called hot spots and selection) as well as intrinsic genetic factors (e.g. sequence homology).

With respect to the ecological factors, persistence of transgenic plant DNA in the environment is an important factor to address. In soil, the fate of DNA depends on the ecological factors reigning in this environment. “Naked” transgenic DNA can be protected due to adsorption onto surfaces (e.g. quartz, clay or cell walls), but also in pollen or other tissue of transgenic plants. Although most of the DNA is commonly degraded to smaller fragments that seem insignificant for transformation, low amounts of high molecular weight (transgenic) DNA have been detected after at least four years in the field. In the gastrointestinal tract of animals, most released DNA is also degraded into smaller fragments (<200 and 400 bp), but protection against breakdown has also been observed. There is only limited knowledge on its availability for *in situ* transformation and the occurrence of natural transformation can therefore not be excluded. There is emerging evidence that larger numbers of bacteria than hitherto known are capable of capturing DNA in the environment. On the other hand, such transfer has so far been chiefly observed under rather artificial (laboratory) conditions in microcosms. A range of environmental hot spots with an increased likelihood for the occurrence of naturally-competent bacteria (able to capture DNA) has been identified. These are: the surface of soil particles, the rhizosphere, the within-plant environment, but also the gastrointestinal tract of soil arthropods and of animals/humans. Transformation processes are diverse and differ between bacterial species. Unfortunately, the environmental factors that trigger natural transformation are still poorly understood. In addition, current research has so far mainly referred to the culturable fraction of the bacterial population and there are no data on the occurrence of transfers to the non-culturable fraction.

The presence of sequences in a transgenic plant with homology to sequences in bacterial genomes is a key factor for successful transfer of DNA from plants to bacteria via natural transformation. Artificial systems, such as those based on marker rescue, have shown that natural transformation of bacteria with plant DNA can take place under natural conditions, with transformation frequencies up to 10^{-4} transformants per transgene, but only when sequences homologous between donor

DNA and the recipient genome are present. In this respect, both the insert and the DNA sequences flanking the insert in a transgenic plant are important. If these show homology to bacterial DNA, they can serve as “anchor” sequences for incoming transgenic DNA and facilitate horizontal gene transfer. There is currently limited knowledge on the occurrence of such anchor sequences and the requirements for successful insertion into the bacterial genome. Recent experiments have shown that transfer efficiencies of marker genes from transplastomic plants were higher than those in the nucleus of a plant. In the absence of any regions of homology of transgenic plant DNA with bacterial DNA, transfer to bacteria has not been reported so far (detection limit $<10^{-13}$). However, DNA that enters bacterial genomes via homologous recombination might simply not have been fixed due to the short time GM plants are present in our ecosystems.

Horizontally transferred transgenic DNA will only have an impact in bacterial communities when it is “correctly” integrated into the genome, with all bacterial regulatory sequences present (promoters, ribosome binding sites, etc), which would allow its expression. As most DNA in the environment is degraded into fragments smaller than the average size of a whole gene, the possibility that a complete gene is inserted from released DNA seems small. Nonetheless, the presence of prokaryotic-like expression signals in transgenes should be avoided to limit possible bacterial transgene expression after transformation, as well as to reduce sequence homology and thereby the possibility of recombination. In case of stable transformation, the fixation and spread of a transgenic gene in a bacterial population depends on its potential to raise the fitness of the recipient bacterium, leading to selection of the trait and proliferation of the organism. In this respect, also the factor time should be considered. Taking into account the low natural transformation frequencies and a low fitness increase of the transformed organism, it could be years before the number of transformants is above the detection limit. Hence, it is recommended that potential transfers be monitored over timeframes that allow the detection of genetic fixation (e.g. 10 years).

The selectability (positive selection) of genes in case of a transfer out of transgenic plants should be a key criterion in the assessment of the potential impact of such a transfer (cf. antibiotic resistance genes). In addition, their natural occurrence in the environment should be considered as the baseline. Many genes used in the construction of transgenic plants have a prokaryotic (bacterial) origin and, as a consequence, a natural baseline is present in the environment. The antibiotic resistance genes used as markers in the construction of GM plants are naturally widely distributed in a range of natural bacterial communities. Hence, it is unlikely that these marker sequences enhance the extant antibiotic resistance gene. However, with respect to the antibiotic resistance markers, the possibility of enhancing the transfer of the primary transgene by co-selection should be considered.

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2. Glossary and Definitions:

***aadA*:** antibiotic resistance gene conferring cells resistant to streptomycin and spectinomycin

***Agrobacterium tumefaciens*:** bacterium, which causes diseases in plants, and possesses the ability to build its own heritable information into plant DNA. This bacterium is used for genetic modification of plants.

Baseline: Current conditions of natural or human-affected systems, in which no GMO's have been used. The baseline serves as a comparator to estimate the impact of the use of GMO's (in terms of horizontal gene transfer).

Biological diversity: the number of species and their relative abundance within a given area, including also the phenotypic and genetic diversity maintained within the population of these species.

Bt: *Bacillus thuringiensis*

Conjugation: a form of gene transfer from one bacterial or yeast cell to another. Products of genes located on a small circular DNA molecule called a plasmid accomplish the transfer. The process of conjugation is found in nature and is also used in genetic modification.

Containment: the condition in which an organism or its genetic material is prevented from freely moving beyond a specific location.

Cry-proteins: cry(stal) Bt toxins naturally produced by *Bacillus thuringiensis* during sporulation, present in crystal inclusions.

EFSA: European Food Safety Authority

Eukaryote: organism characterised by the presence of a nucleus, nuclear membrane and other membrane-bound organelles (e.g. chloroplasts, mitochondria).

Genetically modified organism: an organism, with the exception of human beings, of which the genetic material has been altered in a way that does not occur naturally through reproduction and/or natural recombination.

Genome (bacteria): Total genetic information present in the chromosome, on plasmids and other extrachromosomal elements

Genome (plants): Total genetic information present in the nucleus and cellular organelles (e.g. chloroplasts, mitochondria)

Genomic islands: clusters of genes in bacterial genomes, which have been acquired by the recipient strain as a one stretch of DNA by horizontal gene transfer.

GM plant: genetically modified plant

GMO: genetically modified organism

GI tract: gastrointestinal tract

HGT: horizontal gene transfer

HFIR: Homology Facilitated Illegitimate Recombination

Horizontal gene transfer: Collective processes between prokaryotes that allow transfer of genetic transformation between cells

Hot spot: Location with enhanced transformation frequency.

hpt: antibiotic resistance gene conferring resistance to hygromycin B to bacterial cells.

***in situ*:** measurements/observations 'on the site'; in original position.

in planta: measurements/observations 'in the plant'.

***in vivo*:** measurements/observations 'in living (creature)'.

***in vitro*:** measurements/observations 'in glass'; in test-tube or laboratory experiment.

Marker gene: a gene that facilitates the identification of organisms that have taken up recombinant DNA molecules.

Micro-organism: (living) organisms that are only detectable using a microscope. In this document, this definition includes organisms such as the prokaryotes (Bacteria and Archaea), fungi and protozoa

Non-coding DNA sequences: some of these are DNA sequences that serve as spacer regions (introns) between sequences that are parts (exons) of a complete protein sequence; they are spliced out of the message (mRNA) that provides a cell with complete instructions for assembling the protein. Other non-coding sequences come in a variety of longer and shorter repetitive forms; no cellular function is known for any of them.

***nptII*:** gene conferring resistance to kanamycin and neomycin to cells

Pathogen: a specific causative agent of disease.

PCR: polymerase chain reaction.

PEG: polyethylene glycol

Plasmid: a small circular molecule of DNA that may contain a variety of genes. Found in bacteria, although many artificial ones have been made. Some are capable of conducting their own transmission from one bacterial cell to another, and may also cause other plasmids that are not self-transmissible to move between cells (mobilisation).

Plastid DNA: DNA genome of chloroplasts or mitochondria.

Primary transgene: Transgene of interest, adding a specific trait to a plant.

Prokaryote: organism characterised by the absence of a nucleus, nuclear membrane and other membrane-bound organelles.

Reporter gene: a class of marker gene where the product reacts with a chemical to produce a detectable coloured compound, fluorescence, or emits light and enables a tagged trait to be identified.

Rhizosphere: the volume of soil around a root, under the influence of that root, in which microbial activity is increased.

Risk assessment: a process of evaluating risks involving hazard identification, estimating likelihood of its occurrence and magnitude of consequences.

RNA: ribonucleic acid, chain of nucleotides typically produced using DNA as the template with ribose as sugar, four bases Adenine, Cytosine, Guanine, and Uracil.

rRNA: ribosomal RNA

Symbionts: two or more individuals that interact closely, to the benefit of one or more of the participants.

Target organism: organisms for which a specific control method has been developed.

***tetA*:** antibiotic resistance gene conferring resistance to tetracycline to cells.

Transduction: a form of gene transfer among bacteria (found in nature and also used in genetic modification). The transfer is accomplished by a bacterial virus called a bacteriophage (or just phage). After the bacteriophage has replicated (copied itself) numerous times within the host bacterial cell, it forms a protein wrapped viral particles containing its own DNA and often some parts of the host and donate the chromosomal DNA sequences to the new host, of the changing the genetic makeup of the new host.

Transgene: Foreign gene in a genome, usually inserted by genetic modification procedures.

Transformation: a form of gene transfer among, for example bacteria; the process is found in nature and also used in genetic modification. During transformation, one bacterial cell copies its DNA and releases the copy into the environment. Another cell takes up the free DNA and with some frequency exchanges it for the same region of DNA in its own chromosome. If the process brings different (variant) forms of the genes, the receiving cell is said to be transformed.

Transplastomic plants: Transgenic plants in which the transgene is located in the genome of chloroplasts (plastid) and not in the plant cell nucleus.

Wild type: the organisms as growing in the natural environment, i.e. before laboratory culture, selection or genetic modification.

3. Introduction

In the last two decades, increasing numbers of genetically modified plants have been constructed worldwide. The genes inserted in these plants include those conferring resistance to herbicides, to insects, to plant diseases and to environmental stress. Other functions include the production of pharmaceuticals, enzymes, antibiotics and amino acids (Giddings 2001; Daniell et al. 2002; Nap et al. 2003). Table 1 shows selected examples of current and potential applications. For a review of patent-protected *Agrobacterium*-mediated plant transformation protocols, see Roa-Rodriguez and Nottenburg (2003). Transgenic plants are being placed on the market at an increasing speed. The USA has taken the lead in this with several GM crops covering large areas in USA and Canada. After a period in which transgenic crops have almost been banned, the EU very recently has revived the admission process, with various transgenic crop plants currently being placed on the market or being considered for this purpose. It is expected that this increase in production will continue in the following years. Before allowing placing on the market, the European Food Safety Authority (EFSA) can be asked for advice under directive 2001/18/EC. Furthermore, EFSA can also issue opinions on transgenic plants under the food/feed directive (EC/1829/2003). An overview of current opinions of the EFSA can be found at the EFSA website:
http://www.efsa.eu.int/science/gmo/gmo_opinions/catindex_en.html.

The production of genetically modified plants has brought about a debate on the potential of these plants to cause unintended effects, concerning food safety, allergenicity and a possibly uncontrolled spread of transgenic DNA through the environment. Various studies are currently being, or have been conducted, such as ENTRANSFOOD, a European network for safety assessment of genetically modified food crops (Kuiper et al. 2004) and the EU project TRANSBAC (QLK3-2001-02242), which studies the potential for gene flow from transplastomic plants to bacteria. Also, various reports have addressed the possible impacts of transgenic plants and proposed strategies for containing the transgene within crops and limit its dispersal (Thomson 2003; Kuiper and Kleter 2003; Kok and Kuiper 2003; Renwick et al. 2003; Bennett et al. 2004). Furthermore, various reports on safety issues related to genetically modified foods can be found on the website of the World Health Organization (http://www.who.int/foodsafety/publications/biotech/en/), such as “Fourth session of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology. Yokohama, Japan, 11 - 14 March 2003” and “Meeting on the safety of foods derived through biotechnology. PAHO/WHO/INPPAZ Buenos Aires, Argentina, 20-21 February 2003”.

Dispersal of transgenes can be brought about by the escape of transgenic crops from the field, by hybridisation and backcrossing into related weedy species (Snow 2002) and/or by the transfer of the transgenic DNA to microorganisms. In this report, we will focus on all aspects related to the latter process, that is, the (interkingdom) transfer of genetic material from transgenic plants to microorganisms, in particular bacteria.

4. Construction of transgenic plants

To assess the potential for gene transfer from transgenic plants to bacteria, a consideration of how these plants are constructed is necessary. There are a number of methods available for the construction of genetically modified (GM) plants (Newell 2000; Halford and Shewry 2000), which are continually being improved (Twyman et al. 2003; van den Eede et al. 2004). In broad outline, the process of plant transformation comprises the application of a DNA delivery system to the host cell, the integration and proper expression of the transgene in the plant DNA, and the selection and recovery of a viable transgenic plant (van den Eede et al. 2004). Most procedures start with the construction (or modification) of genes, the so-called "primary transgenes" often derived from bacterial genomes, which will give the transgenic plant the desired trait (Table 1). During this process, the primary transgene is usually located on a (delivery) vector, together with a selectable marker gene to facilitate DNA manipulations, which is usually performed in bacteria (e.g. *Escherichia coli*). Previously, this selectable marker was often an antibiotic resistance gene, but nowadays, alternative selection systems are available (Miki and McHugh 2004). The EFSA has recently issued an opinion in which antibiotic resistance genes were classified in 3 groups. Group I contains antibiotic resistance genes, which are already widely distributed and have only minor therapeutic relevance. Therefore, this group is regarded as safe. Group II also contains antibiotic resistance genes, which are widely distributed, but which do have a therapeutic relevance to either human or veterinary medicine. Group III contains antibiotic resistance genes highly relevant to human therapy, which should be avoided in transgenic plants. For further details see the EFSA website (European Food Safety Authority 2004).

The genetic construct can be transferred to plant cells in order for the transgene to stably integrate in the plant nucleus. There are different ways to deliver the gene construct in plant cells. These include biological delivery systems (e.g. the modified Ti plasmid system of *Agrobacterium tumefaciens*) and physical delivery systems, such as microprojectile (biolistic) bombardment, polyethylene glycol (PEG)-induced DNA uptake, electroporation and microinjection of DNA into cultured plant cells. Several methods have been developed for selecting stable transgenic plants, either depending on the nature of the primary transgene or on the presence of other selectable plant marker systems. The genes *hpt* and *nptII* have predominantly been used as selectable marker genes under the control of plant promoter in transgenic plants, conferring resistance to hygromycin B and kanamycin, respectively. For a more detailed overview, see Babu et al. (2003), Dube and Thomson (2003), Newell (2000), Halford and Shewry (2000) and van den Eede et al. (2004).

Table 1. Foreign gene expression in higher plants (Adapted from Daniell at al. 2002)

Genes and use	Gene products and use
Plant traits: herbicide resistance	
aroA	Glyphosate resistance
Bar	Bialaphos resistance
Insect resistance	
Cry1Ac	*Bacillus thuringiensis* (*Bt*) toxin
Cry2Aa2	*Bacillus thuringiensis* (*Bt*) toxin
Cry2Aa2 operon	*Bacillus thuringiensis* (*Bt*) toxin
Pathogen resistance	
msi-99	Bacterial, fungal resistance
Drought or salt tolerance	
tps1	Trehalose phosphate synthase
BADH	Betaine aldehyde dehydrogenase
Amino acid biosynthesis	
EPSPS	5-enol-pyruvyl shikimate-3-phosphate synthase
ASA2	Anthranilate synthase (AS) α-subunit
Phytoremediation	
mer A	Mercuric ion reductase
mer B	Organomercurial lyase
Non-plant traits: biopharmaceuticals	
hST	Human somatotropin
HAS	Human serum albumin
msi 99	Anticancer, lytic antibiotic
proinsulin	Human insulin α, β chains
IFN α 5	Human interferon α5
Monoclonals	
Guy's 13	For dental cariers against *Streptococcus mutans*
Biomedical polymer	
gvgvp-120	Bio elastic protein-based polymer
Edible vaccines	
ctxB	Cholera toxin β-subunit
Selectable markers and reporters	
aadA	Aminoglycoside-3'-adenylyltransferase
nptII	Neomycin phosphotransferase
codA	Cytosine deaminase
BADH	Betaine aldehyde dehydrogenase
uidA	B-glucoronidase
Cat	Chloramphenicol acetyl transferase
Gfp	Green fluorescent protein
aadA:gfp	Selectable or screenable fusion protein

To ensure production of the desired protein in a transgenic plant, knowledge in respect of the integration site in the plant genome is important. Interference with the plant metabolic pathways could result in unexpected or undesired effects. In this respect, the physical delivery systems, including the biological delivery system derived from *Agrobacterium*, are not controllable. For expression of the transgene, commonly the 35S CaMV promoter, derived from the cauliflower mosaic virus (CaMV) is used, giving a high, non-regulated level of transcription. In addition, tissue-specific and environmentally regulated promoters have been used (van den Eede et al. 2004).

Whereas transgenes were formerly targeted to the nucleus of the plant cell, chloroplast transformation (yielding transplastomic plants) is now gaining more attention. Plant cells often contain high numbers of chloroplasts, each containing multiple copies of the plastid genome. For example, one diploid tobacco leaf cell contains on average 1.3×10^4 copies of plastid DNA (de Vries et al. 2004).

Furthermore, plastid DNA has a prokaryotic “look” in respect of DNA replication, RNA synthesis and DNA codon usage for translation into proteins, which gives rise to the possibility of construction of operons (gene clusters) and over-expression of foreign genes (Bock 2001; Belzile 2002; Maliga 2003; Maliga 2004) Therefore, after the construction of a transplastomic plant, up to 10.000 copies of the insert can be present per cell, dramatically increasing the expression level of the transgenic protein as compared to insertion in the nucleus (Miki and McHugh 2004; Maliga 2004). It has been suggested that the construction of a transplastomic plant could contain the transgene within the crop and limit the opportunity of gene dispersal outside a production field due to the absence of plastid DNA molecules in pollen (maternal inheritance). However, frequent translocation of plastic DNA to the nucleus has been reported. In effect, transfer of plastid-encoded traits via pollen has already been observed (Wang et al. 2004; Haygood et al. 2004).

As described below, the dispersal of transgenes and their subsequent fixation in a bacterial genome strongly depends on the presence of homologous sequences. Thus, the sequence homology of plastid DNA to bacterial DNA has consequences for the potential for gene flow to bacteria, as discussed below. Due to the prokaryotic nature of the plastid DNA (Miki and McHugh 2004; Erikson et al. 2004), the possibility of the presence of suitable homologous sequences for transgenic gene transfer is likely to be enhanced. Moreover, the total copy number of the transplastomic transgene per cell is much higher (up to 10.000 copies) than that of the nuclear transgene (up to 10 copies) (Miki and McHugh 2004).

5. Horizontal gene transfer

The transfer of genetic material from donor to recipient organisms and the subsequent stable persistence of the newly acquired genetic trait in the recipient is termed Horizontal gene transfer (HGT). HGT has been extensively described in the literature (Droge et al. 1998; Nielsen et al. 1998; Bertolla and Simonet 1999; Davison 1999; Ochman et al. 2000; Smalla et al. 2000a; van Elsas et al. 2003). It is considered to represent a key mechanism that accelerates evolution and adaptation in microorganisms, in particular bacteria. There are three major mechanisms by which the transfer of genetic information between bacteria can be established, namely conjugation, transduction and transformation.

During conjugation, a physical interaction takes place between donor and a recipient cell. This requires the presence of transfer functions, which are commonly located on conjugative plasmids or conjugative transposons (Zecher et al. 2000). Conjugative DNA transfer can even occur between different bacterial species. Conjugation is responsible for much of the HGT seen among bacteria, particularly of antibiotic resistance genes located on conjugative plasmids. This is true for both gram-negative and gram-positive bacteria (van Elsas et al. 2003). Some bacterial conjugation or mobilisable systems can also mediate DNA transfer to eukaryotic cells. For instance, the Ti-plasmid of *Agrobacterium tumefaciens* represents a natural prokaryotic-to-eukaryotic DNA transfer system that can be used to transform plant cells to generate GM crops (Tzfira et al. 2000; Dube and Thomson 2003). Contrary to the bacterial conjugation system (Mergeay et al. 1987; Top et al. 1995), retro-transfer from plant to bacterial cells has never been reported, nor does this seem to be a likely event. Therefore, although we clearly lack scientific data, it seems that conjugative transfer from plant to bacterium is not a likely mechanism that can mediate successful transgene transfer from plant to bacterium.

For DNA transfer via transduction, a physical interaction between the donor and the recipient organism is not required. Transduction involves bacterial viruses, called bacteriophages or phages. These phages tend to have narrow host ranges and there is no known example of a phage or other virus infecting both bacterial and higher organism cells (Canchaya et al. 2003). For plant cell-to-bacterial cell transduction to occur, the virus or phage should be able to recognize not only its normal bacterial host, but also cells in the GM plant. Since transduction involves phage reproduction, transfer of genes from plants to bacteria by this route would require the viral component to reproduce in the plant cell and to infect the bacterial host cell. There is no precedent for this, so the likelihood of gene transfer from plant to bacterium by this means seems to be so remote that it can be discounted (Bennett et al. 2004).

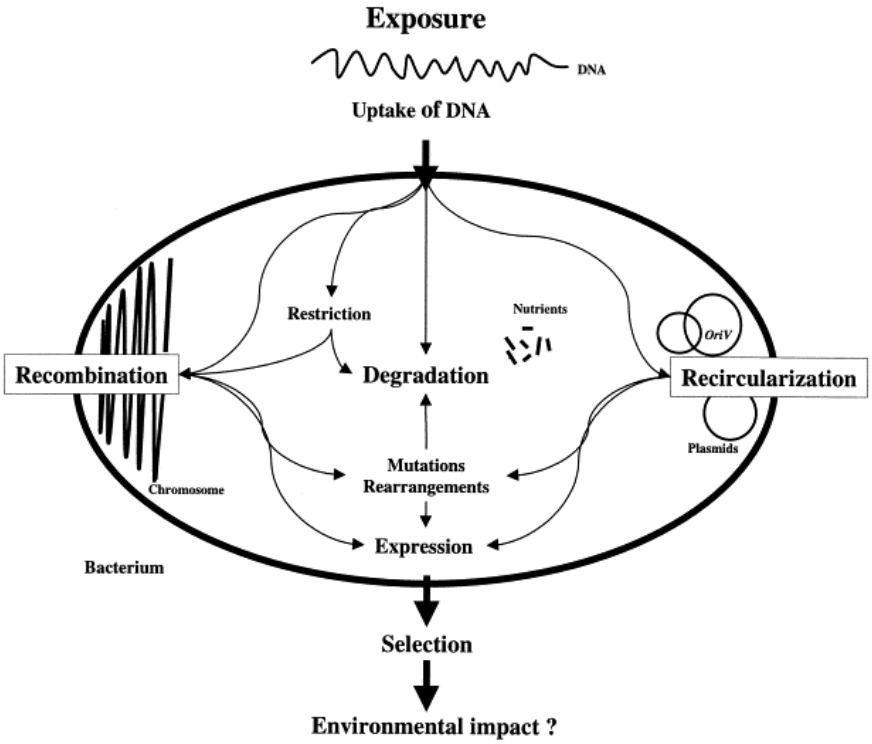

Figure 1. Adapted from Nielsen et al. 1998) Proposed fate of DNA exposed to bacterial cells expressing competence for uptake of DNA. The steps thought to be of importance for natural transformation of bacteria with genetically modified plant DNA (Nielsen et al. 1998).

Transformation is thought to not require any physical connection between the donor and the recipient cell, as it seems to lack a specialized DNA shuttle system, although cell-mediated transformation between bacteria has been found (Paget and Simonet 1994; Bertolla and Simonet 1999; Matsui et al. 2003). Thus, the classical case is that a bacterial cell takes up 'naked' DNA from its environment and incorporates it into its own genome (Lorenz and Wackernagel 1994). Given the natural capability of a wide range of bacteria to take up DNA, transformation seems to be the most likely mechanism that can mediate the transfer of genes from plants to bacteria. Natural transformation comprises a sequence of discrete steps, such as competence induction, DNA binding and fragmentation, DNA uptake and its subsequent stable integration into the genome of the recipient organism (Chen and Dubnau 2004). Selection acting upon the transformant ultimately determines its fate. We will focus on the various steps involved in transformation (Figure 1) related to possible transgene transfer from plants to bacteria. As indicated below, there is a

series of barriers, which the naked (or protected) transgenic plant DNA has to overcome, before it can become stably integrated into a bacterial genome.

5.1. Persistence of DNA in the environment

In most cases where there is no direct contact between plant cells and competent bacteria, one of the first potential barriers to successful transformation is the persistence of 'naked' DNA in the environment. In other words, DNA has to "survive" degradation until it is captured by a suitable recipient. There are various environments in which bacteria can be in close contact with 'naked' DNA. In particular the "hot spots" for gene transfer mentioned earlier. Our focus will be on the rhizosphere, as this is the most likely habitat in which natural bacterial communities "see" DNA from decaying plants. A second focus will be placed on the bacteria/plant-affected habitat (phytosphere).

Different abiotic factors affect the persistence of free DNA in soil, e.g. content and type of clay minerals, pH, temperature and humidity (Gallori et al. 1994; Paget and Simonet 1994; Widmer et al. 1996; Blum et al. 1997). Most data on DNA persistence have been obtained from artificial, sterile soil systems. However, reports on the persistence of DNA in non-sterile soil have also been published (Blum et al. 1997; Nielsen et al. 1997a; Gebhard and Smalla 1999; de Vries et al. 2003). Although plant DNA, including inserted genes, can be degraded during plant senescence and decay, microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil. It is supposed that the DNA released from decaying plant material can serve as a nutrient source for microorganisms. Enhanced microbial activity often coincided with an increase in DNase activity in soil (Blum et al. 1997). Investigations made by different groups have shown that, in spite of the ubiquitous occurrence of DNases, high-molecular weight extracellular DNA can be detected in many different environments, including soil (Ceccherini et al. 2003; de Vries et al. 2003). In fact, it was shown that DNA adsorbed to surfaces (e.g. quartz, clay or organic matter) is "protected", that is, shielded from attack by nucleases (Romanowski et al. 1991; Romanowski et al. 1993). Also, cell walls of lysed cells can play an important role in protecting the DNA after cell death (Paget and Simonet 1994). Long-term persistence of transgenic DNA has also been suggested to occur in pollen (de Vries et al. 2003; Meier and Wackernagel 2003b).

In different studies, long-term persistence of plant DNA inserts, suitable for transformation, was observed in non-sterilized soil systems under microcosm as well as field conditions (Paget and Simonet 1994; Widmer et al. 1996; Widmer et al. 1997; Gebhard and Smalla 1999). To detect transgenic plant DNA from the field, soil DNA was extracted directly from the soil and amplified with three different primer sets specific for the transgenic DNA. Parts of the construct under study were detectable for up to 2 years (Gebhard and Smalla 1999). Persistence of DNA suitable for transformation was even detected for up to 4 years in field soil by using a specific biomonitoring technique, containing a recombinant fusion of *nptII* with the tg4

terminator. This adapted marker rescue system specifically detected the transgenic potato DNA, without the interference of naturally occurring *nptII* genes (de Vries et al. 2003).

DNA persistence was also found during food processing (Rizzi et al. 2003; Kharazmi et al. 2003a) and in the gastrointestinal (GI) tract of animals and humans (Netherwood et al. 2004; Wilcks et al. 2004). Kaharazmi et al (2003a) showed that cooking of (transgenic) potatoes and corn resulted in a strong degradation of plant DNA (to average fragments of < 792 bp and < 585 bp, respectively). In contrast, Rizzi et al (2003) were able to detect relatively high amounts of amplifiable DNA present in various final processed food products (Rizzi et al. 2003).

Although the harsh regimes in different parts of the gastrointestinal tract (e.g. low pH, occurrence of pancreatic enzymes, DNases) are considered to damage or degrade DNA, DNA can persist for a longer time than expected, especially when embedded in plant cells (Wilcks et al. 2004). Using germ-free rats, Kharazmi et al (2003b) demonstrated a rapid decrease in transformant numbers, when DNA was incubated in saliva (from $1.6x10^7$ to $8.1x10^5$ transformants per ml) within 0.2 min. However, after 6 hours of incubation, transformants, and thus transformable DNAs, were still detectable ($8x10^1$ transformants per ml) (Kharazmi et al. 2003b). Einspanier et al (2004) studied the fate of DNA from transgenic (Cry1Ab, AmpR) maize in the GI tract of cattle. Remarkably, specific plant DNAs already decreased to 1.3-3% during the ensiling process, but transgene sequences were still detectable in the GI tract. Hupfer et al (1999) has also reported the detection of GM sequences in maize even after 7 months of ensiling (Hupfer et al. 1999), showing the persistence of GM sequences in cattle feed. Attempts to quantify the log copy genes (including CryIAb) failed, showing that only chloroplast DNA, but not recombinant low-copy genes were detectable in the GI tract of animals. However, using AmpR specific PCR-primers, an 810 bp AmpR fragment could be detected in all rumen samples examined, but these were assigned to the natural occurring bacteria carrying such genes (Einspanier et al. 2004).

In the GI tract, most DNA is degraded into smaller fragments, between 200 and 400 bp, which is smaller than the average size of a whole gene (Schubbert et al. 1998; Wilcks et al. 2004). From research in the EU-funded project GMOBILITY, it was concluded that DNA is most rapidly degraded in the small intestine of mammalian species. Within a minute, the DNA concentration drops to below the detection limit (http://www.entransfood.com/RTDprojects/gmobility/aboutgmobility.html). Although DNA is rapidly degraded in the gut, this degradation is not complete and is not instantaneous. Hohlweg and Doerfler (2001) showed that plant-associated naturally fed DNA is more stable in the intestinal tract of mice than naked DNA (Hohlweg and Doerfler 2001). Studies of an *in vitro* gastrointestinal tract model showed that DNA of ingested GM tomatoes was recovered at approximately 6% at the end of the ileal compartment (van der Vossen et al. 1988).

Overall, the data indicate that in these and potentially other ecological habitats DNA released from cells can persist for an unexpectedly long period of time in the presence of surfaces and/or cell walls.

5.2 Availability for transformation

In the early to mid 90-ties, it was amply shown that DNA protected from degradation due to binding to soil particles (quartz) could be captured by *Bacillus subtilis*, *Pseudomonas stutzeri* or *Acinetobacter calcoaceticus* cells (Romanowski et al. 1992; Romanowski et al. 1993; Chamier et al. 1993; Lorenz and Wackernagel 1994). The adsorption sites (surfaces) were even shown to stimulate the activity of bacteria, including the gene capturing activity (via competence development).

Thus, there are sites in soil and the GI tract in which (transgenic) DNA can persist for possibly a long period of time. Even if this is only a small percentage of the total released DNA, competent bacteria that colonise these sites could capture it. As proposed before, this long-term protected DNA can serve as a reservoir of genetic information, which may eventually enter the bacterial gene pool (Saxena and Stotzky 2001; Ceccherini et al. 2003). The potential consequences of this long-term persistence should be weighed when evaluating the overall impact of transfer of transgenic DNA from plants to bacteria.

5.3 Competence development

A potential barrier to successful gene transfer from transgenic plants to bacteria is the possibly limited presence in close contact with plant DNA of bacterial cells that are able to take up this DNA. The property to take up DNA is genetically determined and the ability to take up DNA sequences for genetic transformation is termed “competence” (Figure 1). Previously, it was thought that only few soil bacteria could become competent and capture DNA. However, from laboratory experiments, more than 40 bacterial species from different environments are now known to be naturally transformable (Lorenz and Wackernagel 1994). These include several bacteria prevalent in soil, such as *Bacillus subtilis*, *Acinetobacter calcoaceticus*, *Pseudomonas stutzeri*, as well as human pathogens, such as *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitis* and *Streptococcus pneumoniae*. The ability to develop competence seems to be widely distributed among very different phylogenetic groups. Some bacteria, such as the pathogenic *Neisseria spp.* are reported to be naturally competent throughout their entire life cycle (Tonjum and Koomey 1997), while in *H. influenzae* competence is induced by certain environmental stimuli, including carbon starvation and nucleotide acquisition (Lorenz et al. 1992; MacFadyen et al. 2001).

In most studies on transformation in soil, competent bacteria have been introduced in soil systems (Gallori et al. 1994; Nielsen et al. 1997c; Sikorski et al. 1998) and successful transformation has thus been demonstrated. Nielsen et al

(1997a) showed that Acinetobacter sp. BD413 remained transformable for several hours after its activation by nutrients in soil (Nielsen et al. 1997a). Recently, it was also shown that *Acinetobacter* sp. BD413 cells residing in soil become competent after addition of nutrients. The nutrient solutions used to stimulate competence development contained inorganic salts and simple compounds corresponding to rhizosphere exudates. In addition, the presence of inorganic phosphate had a strong stimulatory effect on transformation (Nielsen and van Elsas 2001). Demaneche et al. showed that transformation of a bacterium, *Pseudomonas fluorescens*, non-transformable under laboratory conditions could take place in soil (Demaneche et al. 2001b). Apparently, soil provided conditions that triggered competence development in this organism, but these conditions are as yet uncharacterised. For *Bacillus subtilis*, it was shown that cells adsorbed to quartz surfaces enter a state of enhanced competence, being able to capture protected DNA at these surfaces (Chamier et al. 1993). *Ralstonia solanacearum*, the causal agent of bacterial wilt, was reported to develop competence and to exchange genetic information even *in planta* (Bertolla et al. 1997; Bertolla and Simonet 1999).

Competence development can also be affected by the density of the bacterial cell population or by the presence of other bacterial species. *Streptococcus pneumoniae* and *Bacillus subtilis* have well-characterized pheromone- and density-regulated competence systems (Havarstein et al. 1995). Induction of competence of *Acinetobacter* sp. BD413 was observed *in planta*, when this strain was co-infected in tomato plants together with *Ralstonia solanacearum* (Kay et al. 2002a). Furthermore, the natural transformability of *Bacillus subtilis* was negatively affected as a result of interactions with protist and/or photosynthetic algae (Matsui et al. 2003). Taken together, these data show that a variety of factors influence or induce competence development, however the effects of many other natural factors remain unclear or seem species-specific.

The induction of competence and the physiology of natural transformation have been extensively studied in various gram-positive and gram-negative bacteria (Chen and Dubnau 2004) and even in extremophilic bacteria (Averhoff 2004). These studies revealed that there are different demands on the transforming DNA in relation to the recipient bacterium. A variety of transformation systems exist; both the structural components involved and their functional dynamics differ among the divergent bacterial entities (Lorenz and Wackernagel 1994; Dubnau 1999). Specifically, *Bacillus subtilis*, *Streptomyces pneumoniae*, *Acinetobacter* sp. BD413 and *Pseudomonas stutzeri* can take up any kind of DNA, whereas other bacterial species preferentially take up and integrate DNA from the same or closely related species. DNA uptake in *Haemophilus* and *Neisseria* requires short binding-motifs (DNA uptake sequences, DUS) in the incoming DNA, of 11 and 10 bp, respectively. A similar DNA uptake sequence was recently found in *Actinobacillus actinomycetemcomitans* (Wang et al. 2002). Most transformable Gram-positive and Gram-negative bacteria share a similar DNA uptake machinery, related to Type II secretion systems and Type IV pili (Dubnau 1999; Hofreuter et al. 2001; Meier et al. 2002; Claverys and Martin 2003). Under natural conditions, the bacterial cells take up

DNA as linear single-stranded (ss) DNA (Dubnau 1999). DNA is transported across the cell wall and cytoplasmic membrane. The conversion to single-stranded DNA takes place during transport through the inner membrane, which suggests the involvement of nucleases in this DNA transport (Claverys and Martin 2003; Bergé et al. 2003).

Another potential mechanism for the uptake of transgenic plant DNA by bacteria in nature might be gene transfer mediated by lightning, which was recently shown to occur under laboratory conditions (Demaneche et al. 2001a). This work was extended (Vogel, *in press*) and we now know that soil harbours so-called "lightning-competent" bacteria, i.e. bacteria that are capable of capturing DNA under conditions of lightning in soil. However, there are no convincing data so far that demonstrate the efficiency of lightning in stimulating the uptake of transgenic plant DNA.

Until recently, it was unclear whether bacteria could be transformed by plant DNA under any condition. The high content of non-bacterial DNA and the much higher methylation rate of plant DNA as compared to bacterial DNA were supposed to prevent the transfer of, e.g., antibiotic resistance genes from transgenic plants to bacteria (Nielsen et al. 1998). However, there now is evidence that natural transformation in bacteria is not restricted to bacterial DNA, but also mediates the uptake of transgenic plant DNA. Several recent reports have shown that marker genes present in plant DNA could be successfully captured by *Acinetobacter* sp. BD413 cells that had developed competence (Gebhard and Smalla 1999; Nielsen et al. 2000b; de Vries et al. 2003; Meier and Wackernagel 2003a).

Collectively, these data clearly indicate that bacteria can successfully capture plant DNA (in spite of higher methylation rates). However, they also hinted at the key role of homology as the presence of sequence homology between the marker and the target region of the bacterial genome was a strict prerequisite for transfer.

Table 2. Transformation and restoration (or integration) of antibiotic resistance genes (marker rescue) in various organisms, using various recipient plasmids and donor DNA constructs.

Organism	Marker gene	Recipient DNA	Donor DNA	Homology[a]	Transformants per resistance gene	Transformation system	References
Acinetobacter sp. BD413	*nptII*	pFG4Δ*nptII* pFG4Δ*nptII* pFG4Δ*nptII*	Chrom. DNA transgenic sugar beet (*nptII*) Chrom. DNA transgenic sugar beet (*nptII*) Chrom. DNA transgenic sugar beet (*nptII*)	Two-sided Two-sided Two-sided	$9.8x10^{-8}$ (20°C) $1.2x10^{-7}$ (30°C) BD[b]	In soil microcosms	Nielsen et al. 2000b
Pseudomonas stutzeri	*nptII*	pMR7Δ*nptII* pMR30Δ*nptII* pMR30Δ*nptII* pKT210	3.72 kb fragment of pBlue-Km1 (*nptII*) 3.72 kb fragment of pBlue-Km1 (*nptII*) 3.72 kb fragment of pBlue-Km1 (*nptII*) 3.72 kb fragment of pBlue-Km1 (*nptII*)	Two-sided One-sided One-sided None	$1.5x10^{-4}$ $2.1x10^{-10}$ $5.9x10^{-10}$ BD[b]	artificial (agar plates)	Meier and Wackernagel 2003b
Acinetobacter sp. BD413	*nptII*	pFG4Δ*nptII* pFG4Δ*nptII* pFG4Δ*nptII* pFG4Δ*nptII*	pGSFR160 Chrom. DNA transgenic sugar beet Chrom. DNA non-transgenic sugar beet Homogenate of transgenic sugar beet	Two-sided Two-sided None Two-sided	$3.15x10^{-5}$ [c] $5.36x10^{-9}$ [c] BD[b c] $1.5x10^{-10}$ [c]	artificial (*in vitro*)	Gebhard and Smalla 1998
Acinetobacter sp. BD413	*nptII*	pMR7Δ*nptII* pMR30Δ*nptII* pKT210	3.72 kb fragment of pBlue-Km1 (*nptII*) 3.72 kb fragment of pBlue-Km1 (*nptII*) 3.72 kb fragment of pBlue-Km1 (*nptII*)	Two-sided One-sided None	$0.9x10^{-4}$ $1.1x10^{-8}$ BD[b]	artificial (*in vitro*)	de Vries and Wackernagel 2002
Acinetobacter sp. JV21	*aadA*	pTH10Δ*aadA* pTH20 Δ*aadA* pRK415	Transplastomic tobacco DNA (*aadA*) Transplastomic tobacco DNA (*aadA*) Transplastomic tobacco DNA (*aadA*)	Two-sided One-sided None	$1.4x10^{-4}$ $1.2x10^{-7}$ BD[b]	artificial (*in vitro*)	de Vries et al. 2004
Bacillus subtilis	*nptII*	pMK110Δ*nptII* pMK110Δ*nptII* pMK110Δ*nptII* pMK110Δ*nptII*	pSR 8-30 pMR2 Chrom. DNA *E. coli* Sure (*nptII*) 792 bp PCR fragment *nptII*	Two-sided Two-sided Two-sided Two-sided	$1.5x10^{-9}$ $1.4x10^{-9}$ $3.8x10^{-8}$ $3x10^{-12}$	artificial (*in vitro*)	Kharazmi et al. 2002
Streptococcus gordonii	*nptII*	pMK110Δ*nptII* pMK110Δ*nptII* pMK110Δ*nptII* pMK110Δ*nptII* pMK110Δ*nptII*	pSR 8-30 pMR2 Chrom. DNA *E. coli* Sure 792 bp PCR fragment *nptII* Chrom. DNA transgenic potato	Two-sided Two-sided Two-sided Two-sided Two-sided	$8.1x10^{-6}$ $5.2x10^{-6}$ $1.8x10^{-6}$ $9.8x10^{-9}$ BD[b]	artificial (*in vitro*)	Kharazmi et al. 2003b

[a]) Presence (or not) of homology of the donor DNA to either one or two sides of the mutated marker gene in the recipient DNA construct.
[b]) BD= Below Detection limit
[c])Transformants per recipient cell

5.4 Homologous sequences

When a bacterial cell has taken up the single-stranded DNA, the DNA should become stably integrated in the bacterial genome to become fixed. The most likely way for a bacterial cell to fix the acquired primary transgene or an antibiotic resistance gene in its genome is by homologous recombination. Important factors for homologous recombination are the length of the fragment that is to be inserted, as well as the degree of homology with the target bacterial genome, but also the efficiency of the recombination machinery in the recipient organism.

In "older" work, several groups have failed to detect HGT from transgenic plants to bacteria, perhaps because of the scarcity of transgene-homologous sequences in the bacteria (Nielsen et al. 1997b) or the use of poorly-transformable bacteria (Schluter et al. 1995). Gene transfer was for instance studied using transgenic potato and the bacteria *Erwinia chrysanthemi* and *Acinetobacter calcoaceticus* (Schluter et al. 1995; Nielsen et al. 1997b). Transformation was not detected in *A. calcoaceticus* and *E. chrysanthemi* and the transformation frequencies of bacterial cells by plant DNA was estimated to be less then 10^{-16} and $2x10^{-17}$, respectively. The results thus suggested that *A. calcoaceticus* and *E. chrysanthemi* did not take up and integrate non-homologous plant DNA under natural conditions.

In later work, the ability of *Acinetobacter* sp. BD413 to capture and integrate transgenic plant DNA could be demonstrated under optimised laboratory conditions (Gebhard and Smalla 1998), using marker rescue. Typical "marker rescue" studies are performed with bacteria possessing an antibiotic resistance gene (on a plasmid) carrying an internal deletion, which serves for sequence homology and as a detection system (See also Table 2). Two-sided homologous recombination of the antibiotic resistance gene will result in restoration and resistance gene expression. Restoration of a 317-bp deletion in the *nptII* gene, resulting in the emergence of kanamycin-resistant *Acinetobacter* sp. BD413, was observed not only with purified transgenic plant DNA (carrying the intact *nptII* gene), but also with homogenates of the transgenic plant (Gebhard and Smalla 1998). Similar restorations of the *npt*II gene, with in this case a 10-bp deletion in the *Acinetobacter* sp. BD413 genome were observed after transformation of this strain with DNA from various transgenic plants (*Solanum tuberosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, *Lycopersicon esculentum*), all carrying *npt*II as a marker gene used in plant transformation (de Vries and Wackernagel 1998).

Table 3. Transformation of *Acinetobacter* sp. BD413 with plasmids providing two-sided, one-sided or no homology with DNA from transplastomic tobacco plants contain *aadA*.

Recipient plasmid	Type of homology	Transformation frequency[a]	Relative
pTH10	Two-sided	$1.4(\pm1.0)x10^{-4}$	1
pTH20	One-sided	$1.2(\pm0.14)x10^{-7}$	$0.9x10^{-3}$
pRK415	None	$\leq1.3x10^{-10}$	$\leq0.9x10^{-6}$

a. Transformants per aadA in the donor DNA (concentration 100 ng ml^{-1})

This type of recombinational rescue has now been demonstrated for several bacterial species, such as *Acinetobacter* sp. BD413 (*aadA*, Table 3) (de Vries et al. 2004), but also for *Acinetobacter* sp. BD413 (*nptII*) (Gebhard and Smalla 1998; de Vries and Wackernagel 1998; de Vries et al. 2003), *Pseudomonas stutzeri* LO15 (*nptII*), (Meier and Wackernagel 2003a), *Streptococcus gordonii* (nptII) (Kharazmi et al. 2003b) and *Bacillus subtilis* (*nptII*) (Kharazmi et al. 2002).

This type of recombinational rescue of a resistance gene embedded in a plant chromosome has also been simulated under natural conditions (Nielsen et al. 2000b). Kay *et al*. (2002b) showed that transfer can occur when a strain of *Acinetobacter* sp. BD413 containing sequences homologous to tobacco chloroplast genes and the plant pathogen *Ralstonia solanacearum* were used to co-infect transplastomic tobacco plants (Kay et al. 2002b). The *Acinetobacter* sp. BD413 strain was transformed by the plant transgene, which contained spectinomycin and streptomycin resistance genes. However, no transformants were observed when the homologous sequences were omitted from the *Acinetobacter* sp. BD413. In another study (Kay et al. 2003), gene transfer from plants to wild-type *Acinetobacter* was not detected, although *in planta* intergeneric transfer of homologous chromosomal genes between *Acinetobacter* and *R. solanacearum* occurred at significant frequencies. All these studies clearly demonstrated that the success of natural transformation using transgenic plant DNA depends on the recipient cells possessing homology to (preferably both) sequences flanking the insert (de Vries et al. 2001).

In bacteria, RecA catalyses the exchange between homologous DNA strands. RecA is involved in recombinational repair and is capable of catalysing pairing of homologous DNA at double strand breaks, but accessory proteins are also involved in these processes (McIlwraith and West 2001). For integration of foreign DNA with low or no sequence identity in bacteria, two basically different mechanisms have been identified. One relies on specific short nucleotide sequences recognized by cognate enzymes, such as transposases or integrases that can cut and paste DNA at these sites. Nucleotide sequences bordered by such sites constitute so-called mobile genetic elements, examples being gene cassettes and integrons, transposons and insertion sequences, and conjugative transposons (Arnold et al. 2003). In addition, the prevalence of “mutator cells” in natural populations of *E. coli* can have a bearing

on the fixation of plant DNA of transgenic origin in the genome (Mao et al. 1997). Mutator strains of bacteria harbour mutations that enable them to recombine at higher frequencies with DNA from more divergent species, resulting in an enhanced ability to adapt to stressful conditions. They can, for instance, develop resistance to antibiotics (Miller et al. 2004). It has been reported that various natural populations of pathogenic *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Helicobacter pylori* and *Streptococcus pneumoniae* (Chopra et al. 2003) exhibit elevated mutation frequencies. Furthermore, it has been suggested that gene transfer itself can result in the emergence of mutator strains during adaptive evolution (Funchain et al. 2001).

The second mechanism for integration of transgenic plant DNA is thought to depend on illegitimate (non-homologous) recombinational events that join DNA molecules at sites where they have only up to a few identical nucleotide pairs, as is discussed below.

5.5 Homology Facilitated Illegitimate Recombination

It has been shown that the absence of a second homologous site in the transgenic DNA in marker rescue studies strongly decreases the transformation frequency, but does not abolish recombination (Table 3; de Vries et al. 2003; Kharazmi et al. 2003b; de Vries et al. 2004; Kharazmi et al. 2002; Meier and Wackernagel 2003a). These studies have shown that, upon uptake of DNA, long stretches of heterologous DNA adjacent to a single homologous region can still be integrated into the recipient genome by a mechanism termed Homology Facilitated Illegitimate Recombination (HFIR) (de Vries et al. 2001; de Vries and Wackernagel 2002; Prudhomme et al. 2002; Meier and Wackernagel 2003a; de Vries et al. 2004). During HFIR, the homologous sequence serves as an “anchor” sequence, which allows the recombination between incoming and resident DNA. This association between the DNA molecules strongly facilitates an illegitimate recombination event acting on the heterologous parts of the molecules, probably due to the close proximity between these regions. For HFIR to occur, these so-called “anchor” sequences (short stretches of homology) are absolutely primordial.

In principle, any region of homology between the interacting DNA segments large enough to allow homologous recombination can provide the anchor function. Several reports have been made on the minimal length (down to 20 bp) of an anchor sequence required for homologous recombination (see also Table 4). A decrease of the length of homology from 1.096 to 311 nucleotides decreased the transformation frequency 15-fold (Meier and Wackernagel 2003a). As depicted in Figure 2, there was a strong decrease in transformation frequency of *Pseudomonas stutzeri* with decreasing length of the donor DNA, which contained an intact part of *nptII* for marker rescue. DNA fragmentation (below 1 kbp) drastically lowered the transformation potential (Meier and Wackernagel 2003b), thus indicating that large

DNA fragments (>1 kbp) are required to permit detectable transformation frequencies.

Table 4. Minimal length of an effective anchor sequence for homologous recombination as reported for several bacterial species

Length	Bacterial species	Reference
20 bp	*Escherichia coli*	Shen and Huang 1986
50 bp	*Saccharomyces cerevisiae*	Hua et al. 1997
153 bp	*Streptococcus pneumoniae*	Prudhomme et al. 2002
183 bp	*Acinetobacter* sp. DB413	de Vries and Wackernagel 2002
311 bp	*Pseudomonas stutzeri*	Meier and Wackernagel 2003a
50 bp up 121 bp down	*Chlamydomonas reinhardtii*	Dauvillee et al. 2004

With respect to the part of the heterologous DNA, all illegitimate fusion sites identified were exclusively G+C-rich microhomologies of 3-6 bp, which were often neighboured by further micro-homologies (de Vries and Wackernagel 2002; de Vries et al. 2004). DNA from a transplastomic tobacco plant with the *aadA* gene in the plastid genome was used to transform *Acinetobacter* strain JV21, containing a single sequence (1.1 kb) homologous to part of this *aadA* marker (de Vries et al. 2004) Analysis of transformants revealed that stretches of foreign DNA (on average 500 bp, but up to 2539 bp) were integrated in the genome of the recipient bacterium. De Vries et al. (2004) showed the integration of genuine transplastomic plant (non-homologous) DNA into a bacterial genome with the aid of homologous sequences located nearby. Although the exact mechanism and the nature of the micro-homologies in HFIR are not yet fully resolved, HFIR is likely to provide a pathway for bacteria to integrate (non-homologous) transgenic plant DNA (primary transgenes or antibiotic resistance genes) into their genomes.

The *de novo* establishment of a resistance gene from a transgenic plant in a bacterium at reasonably high frequencies would require that this resistance gene in the plant chromosome is flanked by DNA sequences that are essentially homologous to sequences located closely together (two-sided recombination). If only one homologous sequence is present in the bacterial genome (HFIR) the transformation frequency immediately drops. This depends strongly on the location of the transgenic DNA in the plant genome. As discussed above, the point of integration of a transgene in the nucleus of the plant is not controlled in any of the available genetic modification methods. Targeting of a transgene to plastid DNA is more controlled, but the prokaryotic look of plastid DNA increases the likelihood of the presence of homologous sequences and thus the occurrence of HFIR.

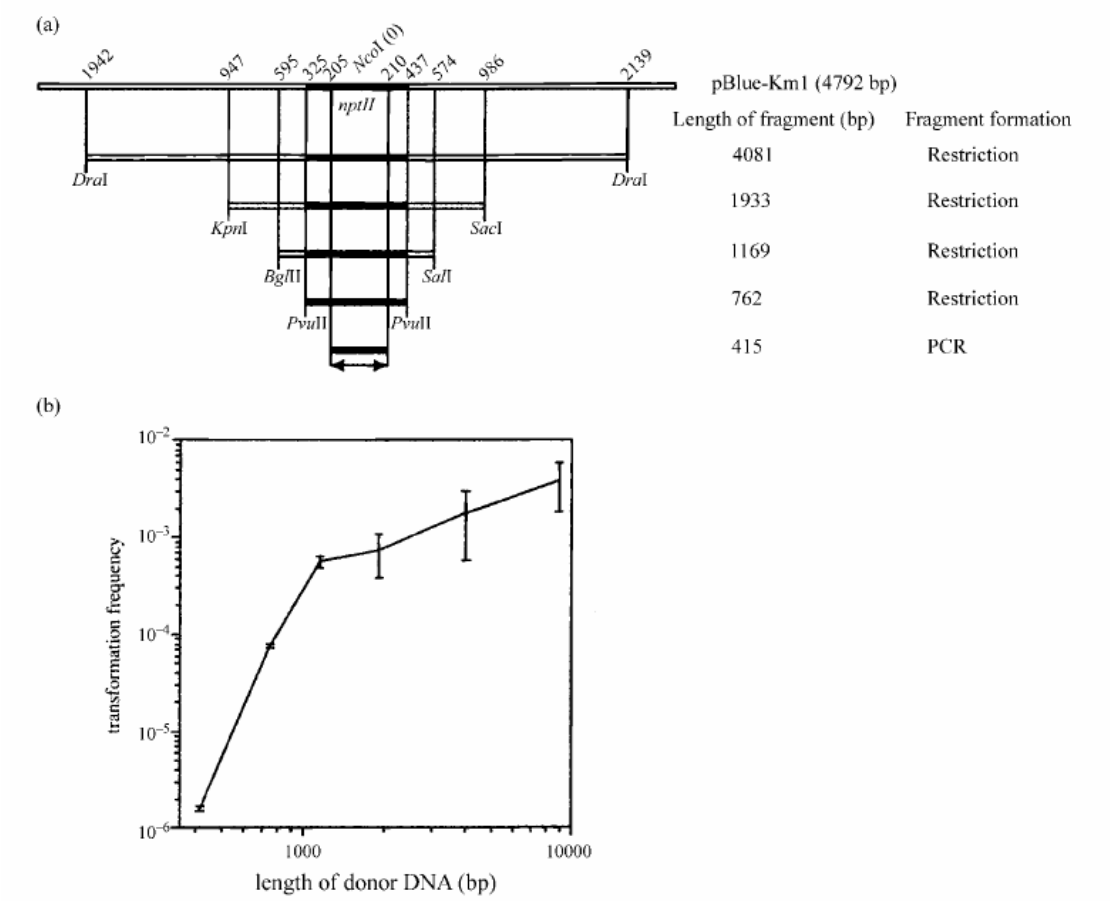

Figure 2. Adapted from Meier and Wackernagel 2003b. Dependence of the kanamycin resistance transformation frequency of *P. stutzeri* LO15 (pMR7) on the length of the donor DNA. (a) Generation of DNA fragments with *nptII*+ or the part of it covering the 10 bp deletion present in the kanamycin resistance gene (*nptII*) of pMR7 of various lengths. (b) Transformation assays contained 2.3×10^{10} DNA molecules of the various lengths. The donor DNA of 9.1-kbp length was the linearized plasmid pSR8-36 having the *nptII*+ located about one third of length away from one end. The transformation frequency is shown relative to fragment length (Meier and Wackernagel 2003b).

Nevertheless, the likelihood of HFIR mediated transfer is low, since antibiotic-resistant transformants have never been obtained (or were below detection limits) in transformation experiments using transgenic plant DNA (de Vries et al. 2004; Meier and Wackernagel 2003a)

As indicated before, a lot of transgenic plants were constructed with *nptII* as a marker gene (van den Eede et al. 2004). This antibiotic resistance gene, along with

many others, is widespread in nature due to the frequent localisation on mobile genetic elements (van Overbeek et al. 2002; Smalla and Sobecky 2002; Heuer et al. 2002a). However, these resistance genes might serve as anchor sequences, thereby facilitating HFIR and enhancing the possibility of bacteria in acquiring transgenic sequences (primary transgenes) flanking the antibiotic resistance gene used in a transgenic plant. Based on the experiments described above, usually small pieces of the foreign DNA (average 500 bp) are integrated, but larger fragments can also be acquired (de Vries et al. 2004). Relatively few studies have been performed on bacteria acquiring primary transgenes. Overall, the likelihood that a primary transgene becomes stably integrated into a bacterial genome and the transformant subsequently is clearly selected seems low, unless the gene is selectable in the bacterium.

As discussed in section 4, transplastomic plants contain much higher numbers of transgenes than GM plants in which the transgenes are inserted in the plant nuclear genome. As a consequence, soil bacteria can be exposed to high levels of transplastomic transgenes released in the environment during decay of plant tissue in soil. Also, endophytic and plant-pathogenic bacteria can be exposed to enhanced numbers of transgenes in the plastid. Both the higher degree of sequence homology and the higher number of transgenes available for transformation result in enhanced possibilities for HGT via transformation. The EU-funded project TRANSBAC (QLK3-2001-02242) assesses the gene flow from transplastomic plants into bacteria, with a special focus on the presence of possible anchor sequences by comparing the tobacco chloroplast genome with genomes of bacteria with natural competence systems. Results (forthcoming) will shed more light on this crucial aspect. Although the possibility of transgenic transformation is likely to be increased, this transfer will only have an impact when the acquired transgene increases the fitness of the transformant, resulting in positive selection of the transformant in the bacterial community (as discussed below in section 7.).

5.6 Frequency of transformation in the environment

Except for the (rare) HFIR events, the presence of sequences that are homologous between plant DNA and bacterial DNA is a common prerequisite for successful stable integration of transgenes into the genome of a recipient bacterial strain. Some attempts have been made to make an inventory of the transformation events in the field, including single-homologous sequences (HFIR) or non-homologous sequence transfer events (Gebhard and Smalla 1999). Field releases of *nptII*-containing transgenic sugar beet plants were accompanied by a study of the putative transfer of the *nptII* marker from the plant to bacteria. A total of 4000 bacterial isolates was screened, but no evidence of any transfer of the *nptII* marker gene from the plant to these bacteria was obtained (Gebhard and Smalla 1999). However, these data are limited as, based on the low transformation frequencies (Table 2), no transformants would have been expected. There was also a lack of detection of transfer of the ampicillin resistance gene from transgenic (Bt176) corn to

culturable bacteria under field conditions (Badosa et al. 2004). Research in the area of transformation and competence development has focussed on the culturable fraction of the natural bacterial communities. It may be assumed that the underlying principles of transformation also apply to the as-yet unculturable bacterial fractions. Most researchers indicate that transfer events might have occurred in these experiments, but the expected transformation frequencies would be below the detection limit, which is estimated to be about 10^{-10} to 10^{-17} (references within Table 2).

When performing transformation experiments (marker rescue), the transformation frequencies with plant DNA or plant homogenates were drastically reduced, compared to transformation with chromosomal or plasmid DNA. Bertolla *et al.* (2000) showed that the efficiency of transformation of *Ralstonia solanacearum* was directly related to the complexity of the donor DNA (Bertolla et al. 2000). The transformation frequency ($3.76x10^{-7}$) dropped tenfold when the same amount of plasmid DNA (donor, containing *aadA*) was mixed with 5 µg of tomato plant DNA. *Ralstonia solanacearum* failed to produce any detectable transformants (containing a defective *aadA* gene) when exposed to DNA extracted from genetically modified tomato plants. A similar dilution effect, although with higher transformation frequency rates, was observed with *Acinetobacter* sp. DB413 as the recipient strain. Transformation frequencies dropped from $6x10^{-3}$ to $4.1\ x10^{-6}$ when DNA extracted from transplastomic plants was used instead of plasmid DNA (Kay et al. 2002b). However, these frequencies were higher than those observed for the same recipient strain ($5.36\ x\ 10^{-9}$) by Gebhard and Smalla (1998) when they conducted transformation tests with DNA extracted from plants containing inserted maker genes in the nucleus (Gebhard and Smalla 1998).

Furthermore, when an experiment, done by filter transformation of *Acinetobacter* sp. BD413, was taken one step further and performed in sterile and non-sterile soil, transformation frequencies dropped to below detection (Nielsen et al. 2000a). Later work showed that transformation of *Acinetobacter* sp. BD413 by transgenic sugar beet DNA could be detected in sterile but not in non-sterile soil (Nielsen et al. 2000b). Also, gene transfer from tomato and tobacco plants treated with a plasmid carrying the *nptII* gene and infected by *Ralstonia solanacearum* containing regions homologous to nptII was not observed (Bertolla et al. 2000). Hence, any putative transformation events were below detection limits (transformation frequency $< 2\ x\ 10^{-10}$ (de Vries et al. 2004).

The number of copies of target sequences, the size of the plant genome and the ratio between the transgene and the whole plant genome are all characteristics of the donor DNA, which should be considered in view of possible gene transfer events. Although transformation frequencies are likely to be low, it is difficult to predict whether interkingdom gene transfer takes place in soil and will have any significant impact in the light of the enormous amount of field-grown transgenic crops worldwide.

5.7 Selection of transformants

If stable integration of transgenic plant DNA into a bacterial genome occurs, there are several factors that influence the fixation of the transgene in the bacterial community. The spread of the transgene in a population will not only depend on the transformation frequency in terms of the chance that the event will happen at least once. It depends more strongly on the chances of selection of the newly acquired trait. A prerequisite is that the transgene is integrated in such a way, that it will be properly expressed. For a gene of eukaryotic origin, this means that introns that are possibly present in the coding region should be spliced out and that all bacterial genetic motifs required for efficient expression are present. The likelihood that the complete coding region of a non-homologous transgene, devoid of any expression motifs, would properly integrate in the bacterial genome and be expressed depends on the presence of at least one flanking region with homology to the bacterial genome. When (bacterial) expression motifs are already connected to the transgene (e.g. in the chloroplast) the probability of expression increases. Moreover, transgenic sequences should not carry elements that can be recognized by the integration sites of integrons.

With respect to the transgenic plants already constructed, it has been estimated (Al Kaff et al. 2000) that about 80% of the vectors used to transfer foreign DNA to plants carry the 35S promoter gene derived from the Cauliflower Mosaic Virus (CaMV). Despite the fact that this promoter is plant-specific and thus would not result in gene expression after a transfer to bacteria, it has recently been shown that it can be active in bacteria (Jacob et al. 2002), fungi and yeasts (Pobjecky et al. 1990) and even in extracts of human Hela-cells (Burke et al. 1990). Therefore, it is recommended that attention is paid to the construction of transgenes with reference to the regulatory sequences. A careful selection of the expression system should be made, avoiding promoter regions, which might be active in bacterial cells.

To cause an impact, the integrated transgene, by its expressed product, should have an effect on the fitness of the recipient bacterium, although the function of the expressed product or its effect on the fitness usually remains unknown. When the acquired trait is toxic to the recipient, fitness will decrease and the newly formed transformant will disappear from the community. Even if an acquired trait is not toxic and the transferred gene is not involved in any adaptive function, it will probably be lost by random mutation (Berg and Kurland 2002) and thus cause no impact on the environment. Only when there is a selective advantage linked to the transferred transgene, it will become fixed within the bacterial population, multiply and become detectable over time. However, in the presence of a selective advantage, detection of the transfer event might only come into prominence after a considerable amount of time, even up to 10 years or more, depending on the recipient population size, the structure of the bacterial community and the bacterial generation time (de Azevedo and de Araujo 2003).

This illustrates the uncertainty with respect to the long-term effects of plant transgenes that might eventually become fixed in bacterial populations. Simple detection, or even non-detection, of a transfer event, is likely to be non-informative on the ultimate fate of a transgene in bacterial populations, for which forces of selection over time are strong drivers. On the other hand, in the impact assessment process, this uncertainty should be weighed against the increasing evidence of products of HGT (even trans-kingdom) that have become fixed in bacterial genomes over evolutionary time (Doolittle 2002; Boucher et al. 2003).

6. Hot spots for horizontal gene transfer

It is known that locations exist in the environment, in which the frequency of gene transfer and subsequent selection of transformants are raised (van Elsas et al. 2003). Some sites in natural habitats have been identified as “hot spots” of gene transfer. Examples are the rhizosphere and within-plant compartments, and the digestive tracts (gastrointestinal tract) of various organisms. These environments have in common that there is a variety of different microorganisms in close contact and that the nutrients available will support high bacterial cell densities. The barriers to natural transformation described above are thought to be alleviated in these hot spots. Hence, these are important factors to address in each environment. DNA persistence, availability for transformation, the presence of competent bacteria and selection pressure for transformants depend on the respective environment and these factors should all be considered. In the next section, we briefly review the rhizosphere/phytosphere and gastrointestinal tract, as paradigms of environmental hot spots for gene transfer.

6.1 Rhizosphere and phytosphere

In soil, the rhizosphere as well as rhizoplane of plants have been recognized as sites that support high rates of HGT, especially when tested for conjugation (van Elsas and Bailey 2002; van Elsas et al. 2003). In agricultural land, the soil regularly becomes physically rearranged. There are changes in humidity due to water channels and animal/plant processes, providing many microhabitats, in which various bacteria can come into close contact with each other. In this heterogeneous setting, the rhizosphere has been considered to offer better colonisation microhabitats compared to bulk soil. Due to the presence of nutrients, various bacterial types often aggregate in biofilms or micro-colonies, which facilitates cell-to-cell contact and thereby gene transfer. Aerial plant surfaces or roots of agriculturally grown plants are typically colonized by approx. 10^4 bacteria per g leaf material to 10^8 bacteria per g rhizosphere soil (Nielsen et al. 2001). Furthermore, bacteria living on or close to plants may be exposed to DNA from the host plant itself during decomposition of plant cells and root decomposition, but also by mechanical disruption.

The rhizosphere not only provides nutrients for bacterial growth, but also compounds that stimulate natural transformation are present. Increased metabolic activity has often been observed due to nutrients exuded from the root (Davison 1999; Nielsen et al. 2001). It has also been shown that organic compounds, such as organic acids, amino acids and sugars (naturally found in the rhizosphere), could stimulate the natural transformation of *Acinetobacter* sp. BD413 (Nielsen and van Elsas 2001). A high phosphate salt level was found to allow detectable gene transfer. When using mixtures of organic compounds and high phosphate salts, based on estimated concentrations in the maize rhizosphere, the highest transformation frequencies were observed (4.4×10^{-6}).

Plants themselves (and their internal parts) can also constitute strong stimulants of transformation, likely due to their provision of favourable niches to bacteria. As indicated before, the induction of competence of *Acinetobacter* sp. BD413 was observed *in planta*, when this strain co-infected tomato plants together with *Ralstonia solanacearum* (Kay et al. 2002a).

6.2 Gastrointestinal tract

The gastrointestinal (GI) tract is the main portal of entry of foreign DNA into the body. Its epithelial lining forms the main site of contact with foreign DNA of mammals or other organisms. Since the GI tract is a postulated hot spot for HGT, it has been the objective of several HGT studies; also, guts of soil organisms, such as arthropods have been studied (Davison 1999; Jelenic 2003; Dillon and Dillon 2004). Many experiments have shown that, during passage through the GI tract, most of the orally ingested DNA seems to be completely degraded; however, a small percentage remains as fragments of a few hundred up to about 1700 nucleotides in length, which can be detected by a variety of techniques (Martin-Orue et al. 2002; Netherwood et al. 2004; Wilcks et al. 2004).

Relatively little work has been done on the possible transfer of plant transgenes to animals consuming the plant or to their commensal microflora. There is evidence that naturally-competent bacteria, e.g. *Streptococcus gordonii* (Mercer et al. 1999) or *Actinobacillus actinomycetemcomitans* (Wang et al. 2002) residing in the oral flora can capture exogenous DNA. Mercer *et al* were able to detect transformants of *Streptococcus gordonii*, which took up recombinant plasmid DNA (pVACMC1), when suspended in saliva, although no estimate on the *in vivo* transformation frequency was made (Mercer et al. 1999).

In addition, although not well studied, several bacterial species are known to be able to develop natural competence in the gastrointestinal tract (Lorenz and Wackernagel 1994). Recently, Netherwood et al reported that microbes found in the small bowel of people with ileotomies (i.e. individuals in which the terminal ileum is resected and digesta are diverted from the body via stoma to a colostomy bag) are capable of acquiring and harbouring DNA sequences from transgenic plants, but they concluded that it was unlikely that these transformed microbes would alter gastrointestinal function or endanger human health (Netherwood et al. 2004). Also, the transfer of foreign DNA fragments from the intestinal lumen into the nuclei of the cecal epithelial cells has been observed, although the nature of these cells was not determined (Palka-Santini et al. 2003).

The gastrointestinal tract of a range of animals, thus, represents another hot spot, although we clearly lack sufficient experimental data to evaluate the impact of transgenic gene transfer in this environment.

7. Impact of horizontal gene transfer from transgenic plants to bacteria

As discussed before, mass cultivation of GM crops, or spread of a transgene through cross-pollination, will certainly expand the transgenic gene pool in the environment. Moreover, gene flow into plant-associated, soil or gut bacteria will only be a relevant issue if there is a change of a, possibly arbitrary, natural baseline. The baseline should be the natural microbial communities and their gene pools and dynamics, in a given habitat. In respect of HGT, it is extremely unlikely that the conditions in the system will be beyond this baseline if fixation, expression and selection of the transgene do not take place. Hence, these three processes, already described above, are key issues in the evaluation of the potential of impact of HGT. In particular, the issue has raised concerns in respect of the prevalence of antibiotic resistance genes in the environment. However, it remains unclear whether there are enhanced opportunities for spread of transgenes, e.g. antibiotic resistance genes among human/animal bacterial pathogens. The effect (impact) of transfer of a transgene depends on the type of transgene acquired by the bacteria, but this effect will only be observed when bacteria can express this acquired transgene and potentially gain in fitness. The ecological significance of transformation of antibiotic resistance marker genes depends on the selection of the acquired trait. In addition, it should be weighed against the dissemination of the respective antibiotic resistance genes in the environment.

7.1. Baseline of antibiotic resistance genes in bacterial communities

HGT of resistance genes by conjugation or mobilisation under different environmental conditions has been well documented in bacteria. The genetic plasticity of bacteria has largely contributed to the efficiency with which antibiotic resistance has emerged, but HGT events have no *a priori* consequences, unless there is antibiotic selective pressure (Levy 1997). As a consequence of the wide-scale use of antibiotics by humans, selection has resulted in the emergence of various bacteria resistant to these antibiotics, but also has resulted in the rapid evolution of bacterial genomes. Mobile genetic elements such as self-transferable or mobilisable plasmids, transposons and integrons have played a key role in the dissemination of antibiotic resistance genes amongst the bacterial populations, and have contributed to the acquisition and assembly of multiple antibiotic resistance in bacterial pathogens (Tschäpe 1994; Levy 1997; Witte 1998). Since bacteria can circulate between different environments and geographic areas, bacteria resistant to multiple antibiotics are not restricted to clinical environments, but can easily be isolated from environmental samples and food (Perreten et al. 1997; Droge et al. 2000; Smalla et al. 2000b; Heuer et al. 2002a). Only few studies have provided data on the prevalence of antibiotic resistance genes used as markers in transgenic plants. Studies on the dissemination of the most widely used marker gene, *nptII*, in bacteria from sewage, manure, river waters and soils, demonstrated that in a high proportion

of kanamycin-resistant enteric bacteria, the resistance is encoded by the *nptII*-gene (Smalla et al. 1993). Therefore, it is unlikely that the use of *nptII* as a marker gene in plants enhances the antibiotic resistance gene pool extant in the environment. A major question here is obviously whether this gene or its product poses any hazard to the environment or human/animal health. An extended report on *nptII* has failed to identify any possible hazard on the gene and or its product, NptII (Nap et al. 1992). Fuchs et al (1993) also confirmed that ingestion of genetically engineered plants expressing the NptII protein poses no safety concerns (Fuchs et al. 1993).

Nonetheless, when a primary transgene is tandemly coupled to a resistance gene, there might be a possibility of "co-transfer" of the primary transgene to a bacterial cell, since bacterial resistance genes might serve as anchors sequences (de Vries et al. 2004). The impact of such a transfer, obviously, depends on the nature of the primary gene, but more important, on the development of a selective advantage for the recipient bacterial strain. Most studies have focussed on the spread and possible impact of the transfer of antibiotic resistance markers. Hence, knowledge on this facilitated transfer of primary transgenes from transgenic plants to bacteria in field conditions is marginal. A major limitation here may be the sensitivity of the available methodology.

7.2 Impact of transgenic plants on bacterial community structure and consequences for horizontal gene transfer processes

The effect of the transgenic plant itself on its environment will have implications for the likelihood that plant-associated bacteria become competent and capture DNA. For instance, naturally-competent bacteria could be selected by the transgenic plant on the basis of specific exudates or niches, which might enhance the potential transformation frequency. Alternatively, competence development could be stimulated in plant-associated bacteria by the transgenic plants. However, we currently lack information on the likelihood and impact of these two possibilities. We do have data, though, as to how transgenic plants modify the community structures of plant-associated bacterial communities. Several such studies have been performed. Some reported no changes in the culturable fractions of the community (Lottmann and Berg 2001; Schmalenberger and Tebbe 2002; Wu et al. 2004), whereas others did observe effects (Germida et al. 1998; Siciliano and Germida 1999; Tesfaye et al. 2003). The observed changes in the bacterial community structures associated with genetically modified plants, however, were minor compared with other, 'normal' sources of variation (Kowalchuk et al. 2003). Furthermore, these changes were ephemeral and did not persist into the next growing season (Dunfield and Germida 2003). Heuer et al (2002b) reported that the effects due the T4 lysozyme release from potato roots were minor compared to environmental factors related to season, field site or year (Heuer at al. 2002b).

Also, relatively little is known about the impact of transgenic plants that express insecticidal genes [such as δ-endotoxins of *Bacillus thuringiensis* (*Bt*)] on the

bacterial community structures, whereas transgenic plants with introduced *Bt* genes have been deployed in several crops on a global scale. Recently, Blackwood and Buyer (2004) concluded that the effects of transgenic *Bt* corn in a short-term experiment were small and probably not biologically important and that longer-term investigations are necessary.

8. Comparative genome analysis

Since the presence of homologous sequences seems to be the major issue when considering HGT from plant to bacterium, a detailed analysis of the genomes of bacteria and plants might yield more insight in the critical factors that determine the likelihood of transgene transfer. The growing availability of whole genome sequences of various organisms (Eukaryotes, Bacteria, Archaea) has made it possible to study HGT in a historical perspective. Genome-scale comparisons provide evidence that large portions of bacterial genomes have undergone gene transfer between major bacterial lineages (Ochman et al. 2000; Jain et al. 2003) and that “genomic islands” exist in bacterial genomes (Daubin and Ochman, 2004; Fraser 2004). It has been suggested that the eukaryotic genome could also be seen as a mosaic of eukaryotic and prokaryotic sequences, with contributions from ancestral Archaea and bacteria (Martin and Muller 1998). Moreover, many products of trans-kingdom HGT may have become fixed in bacterial genomes over evolutionary time (Doolittle 2002; Boucher et al. 2003).

On the other hand, other researchers argue that only a limited number of recent HGT events from eukaryotes to bacteria have so far been shown or suspected. Considering the huge population sizes of bacteria and their promiscuity with living or dead eukaryotic cells, adsorption and integration of eukaryotic DNA could have been widely occurring. Kurland et al. (2003) argued from a detailed genome analysis that the frequency of current HGT events might have been overestimated and that HGT transfer possibly has only been relevant in primitive genomes.

Genome sequence comparisons to detect HGT get obscured by adaptive molecular processes (amelioration), which constantly take place in (bacterial) genomes. Autochthonous genes are adjusted to new functions or new environments, and newly-acquired genes are adapted to fit into the genome of the recipient strain, e.g. by nucleotide substitutions. These occur to adapt the acquired gene to the preferential codon usage of the novel host. In respect of transfer of a transgene, an acquired transgene can get lost again, or even cycles of gain-loss-gain can occur. To become fixed and actively expressed in a prokaryote, the plant DNA must be devoid of introns in order for the product to be active, and the transferred gene should be full-length or at least a complete functional domain. Genome-based assessments of interkingdom DNA transfers indicate that, over evolutionary time, 3% of the genes of most free-living bacteria have been acquired from Archaea and Eukaryotes (Ochman et al. 2000), which indicates that such interkingdom transfer has the potential to be evolutionarily successful.

Other studies on specific eukaryotic-prokaryotic transfers indicated the following. Database searches with metazoan α2-macroglobulin sequences revealed sequences homologous to this eukaryotic gene in bacterial proteomes. It was concluded that the gene had probably been transferred out of metazoan hosts and then spread widely through various colonizing bacterial species by over ten

independent HGTs (Budd et al. 2004). A few bacteria exhibit an α-amylase, which has high sequence similarity with counterparts from animals (Janecek et al. 1999). Screening the bacterial genomes available, three novel cases of such eukaryote-prokaryote transfers have been found. The three refer to unrelated bacterial phyla. It was proposed that all the animal-like α-amylases in Bacteria result from repeated HGT from animals (Da Lage et al. 2004).

Genome sequence analysis also revealed that DNA from organelles in eukaryotic cells has constantly been bombarding the nucleus since the emergence of the organelles. Recent experiments have shown that DNA is transferred from organelles to the nucleus at frequencies much higher than previously thought. Endosymbiotic gene transfer is a ubiquitous, continuing and natural process that pervades nuclear DNA dynamics (Timmis et al. 2004). Direct transfer of chloroplast DNA to the nucleus has also been observed in tobacco (Huang et al. 2003).

It is therefore clear that HGT from eukaryotes to prokaryotes as well as from prokaryotes to eukaryotes over evolutionary time is a fact of life. It is most likely that what we see today is the result of (early) transfer events followed by periods of selection and eventual mutations. It is also likely that the chances for gene-gene contact have been high from early times on. Hence, any potential for fixation of a transgene from a current GM plant in a bacterial population should be weighed against the evolutionary picture.

9. Concluding remarks

The topic of horizontal gene transfer between bacteria has been extensively studied and an immense amount of reports have been published. Conjugation, transduction and transformation have been pinpointed as gene transfer mechanisms with ecological relevance. Concerning gene transfer from plants to bacteria, only natural transformation has been pointed out as the single key mechanism that can mediate such a transfer. The frequency of occurrence of such transfers was found to depend on a range of ecological as well as intrinsic genetic factors.

With respect to the ecological factors, the persistence of transgenic plant DNA in the environment is an important issue to address. In soil, the fate of DNA depends on various ecological factors. For instance, “naked” transgenic DNA can be protected due to adsorption onto surfaces or interactions with cell walls. Protection of transgenic DNA has also been observed in the gastrointestinal tract. Although in these environments most of the DNA is commonly degraded to smaller fragments that seem insignificant for transformation, low amounts of high molecular weight (transgenic) DNA can be detected.

The list of bacteria that can be naturally competent (capable of capturing DNA) in the environment is growing, but actual transfer of transgenic DNA to such bacteria has only been observed under rather artificial conditions in microcosms. In natural environments, a range of hot spots have been identified, in which an increased likelihood for the occurrence of naturally-competent bacteria exists. These are the surfaces of soil particles, the rhizosphere and the within-plant environment. Also the gastrointestinal tracts of soil arthropods and of animals/humans has been indicated to represent a hot spot.

The presence of sequences in a transgenic plant with homology to sequences in bacterial genomes is a key factor for successful transfer of DNA from plants to bacteria via natural transformation. The frequency of transformation clearly increases with increasing length of homologous sequences, as was demonstrated in marker rescue studies. However, there is limited knowledge on the occurrence of such homologous (anchor) sequences in transgenic plant DNA. In the absence of any regions of homology of transgenic plant DNA with bacterial DNA, transfer to bacteria has not been reported so far. In case of stable transformation, the fixation and spread of a transgenic gene in a bacterial population depends on its potential to raise the fitness of the recipient bacterium, leading to selection of the trait and proliferation of the organism. In this respect, also the factor time should be considered. Taking into account the low natural transformation frequencies and a low marginal fitness increase of the transformed organism, it could take years before the number of transformants selected is above the detection limit. Hence, monitoring schemes to assess these potential transfers should be long-term.

Despite the persistence of a fraction of released transgenic DNA, the presence of homologous sequences and the occurrence of naturally-competent

bacteria in the environment, natural transformation has not been detected *in situ*. However, although the knowledge on actual *in situ* transformation is thus very limited, it can not be ruled out that transgenic DNA that entered bacterial genomes might simply not have been fixed in the population due to the short time GM plants are present in our ecosystems.

10. Conclusions of workshop

A two-day workshop (April 22-24, 2004) was held, with the focus on transgenic gene transfer from plant to bacterium. During this workshop, fourteen experts in the field presented their latest results on the work with transgenic plants as well as bacteria, addressing some of the processes described above. Evaluation of the direct transgene transfer from plants to bacteria was the main topic, but also other transfer events, such as bacterial conjugation, were discussed. Furthermore, the possible impact of transgenic plants on the environment (bacterial communities) was discussed. This topic has only briefly been mentioned in this report. A more detailed report on this workshop and the presentations are provided in the appendix of this report.

From the workshop, it was concluded that the presence of sequences homologous between plant and recipient bacterium was the most important genetic prerequisite for a successful recombination event, fixing the transgene into the bacterial genome. Special emphasis was placed on the effects of transplastomic constructs. Although satisfactory data on the presence of sequences in the chloroplast genome with substantial homology to relevant (transformable) bacteria are lacking, the prokaryotic nature of the DNA flanking the transgene was expected to facilitate homologous recombination by the provision of anchor sequences. For instance, the adventitious presence of a 59-bp element that serves in the interaction with integrons might facilitate integration in a site on the bacterial genome that facilitates expression.

Most data on transformation frequencies have been obtained from studies using genes with sequences homologous between plants and bacteria, but hardly any information on non-homologous (illegitimate) recombination has been obtained. Although such non-homologous transgenic transformants have never been obtained from the environment, the possible occurrence of such an event cannot be excluded on the basis of recent evidence. The current detection methods are probably insufficiently sensitive and events are thus often below the detection limit. Sufficient time for evolution of putative transformants should be allowed; hence, long-term monitoring is required in order to allow putative selection and growth of transformants above the detection limit.

There is still limited knowledge on the occurrence of transformants and the factors involved in the development of competence in various natural environments. Recent research indicates that induction of competence in bacteria in natural habitats might be a much more common mechanism than previously anticipated. Various hot spots for enhanced gene transfer are present in the environment, ranging from the rhizosphere to the gut of soil organisms, arthropods and animals/humans. Most data have been obtained from culturable strains from these environments. We do not yet have the appropriate technology to study the (transgenic) transfer events that involve

the major fraction of a natural bacterial community, namely the non-culturable fraction.

Current methods used to study natural transformation are mostly based on the use of antibiotic resistance markers (marker rescue studies). However, the data obtained are sometimes difficult to interpret, because of high background levels due to the natural prevalence of these resistance genes in the environment. Development of alternative detection systems based on, for instance molecular detection techniques, was recommended.

Due to the limited knowledge on gene transfer frequency and competence, it is difficult to assess the impact of transgenic gene transfer on the environment. An impact of transgenic gene transfer can only be expected if the acquired transgene will give the recipient cell an increased fitness and the trait is selected. However, based on the growth characteristics of individual organisms, as well as population dynamics, a considerable amount of time, maybe up to ten years, might be needed before this trait will be above the detection limit.

No data on the transfer of primary plant transgenes (e.g. herbicide resistance) were presented during the workshop and hardly any exist in literature. Some work has been done on the stability and persistence of the product of Cry-proteins (Bt toxins) in the environment, but bacterial transformants carrying a *cry*-gene obtained by HGT have never been isolated from the environment. Possibly stable transfer events of primary transgenes and subsequent expression would depend on the presence of homologous sequences and suitable expression signals. It was presumed that the likelihood of such an event was low, but that it could not be excluded. More detailed studies on this topic were recommended.

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Appendix I: Overview presentations Workshop

Thursday, April 22
Folkert Faber: Opening Workshop

Session 1: Horizontal Gene Transfer events by Transformation.
Dr. Sarah Turner: Gene transfer in structured environments
Dr. Kornelia Smalla: Horizontal gene transfer from transplastomic or transgenic plants to bacteria - what can be predicted?
Dr. Kaare Nielsen: Monitoring horizontal gene transfer from GM-plants: current limitations and possibilities
Group-discussions and plenary session (Hans van Veen).

Session 2: Gene transfer in the soil and plant-microbe environment
Dr. Peter Bennett: A clinical perspective of use of bacterial antibiotic resistance genes in development of GM crops: What is the risk?
Dr. Pascal Simonet: Gene transfer between plants and bacteria. From *in vitro* studies to field experiments
Dr. Andrew Lilley: Fitness and plasmid transfer in plant habitats:
Dr. Leo van Overbeek: The effect of genetically modified organisms on the plant-associated microbial community
Dr. Holger Heuer: Evolution of IncP-1 plasmids
Group-discussions and plenary session (Pascal Simonet)

Friday, April 23

Session 3: Gene transfer related to medicine, food and other host-interaction environments

Dr. Daniele Daffonchio: Development and use of new Acinetobacter-based biosensors to study horizontal gene transfer in different environments
Dr. Henk J.M. Aarts: Dietary exposure to recombinant DNA
Dr. Christoph Tebbe: Can insect guts be regarded as hot spots for horizontal gene transfer?
Group-discussions and plenary session (Daniele Daffonchio).

Session 4: Effects of transgenic plants and gene transfer: Impact and risk assessment

Dr. George Kowalchuk: Effects of GM plants on soil ecosystems: research, risk assessment and knowledge gaps
Dr. Eric Smit: Beyond gene transfer: ecological impact of new phenotypes on rhizosphere and intestinal tract communities
Dr. Asa Frostegard: Transgene survival and impact, dependency on function and recipient organism
Group-discussions and plenary session (Peter Bennett).

Appendix II: Schematic overview of topics covered during the Workshop

A two-day workshop (April 22-24, 2004) was held with the focus on transgenic gene transfer from plant to bacterium. During this workshop, fourteen experts in the field presented their latest results on the work with transgenic plants as well as bacteria, addressing some of the processes described in this accompanying report. Evaluation of the direct transfer of transgenic DNA from plant to bacteria was the main topic, but also some related aspects were discussed. From the 3 possible means of DNA transfer mechanisms concerned in HGT, conjugation and transduction were excluded and transformation was considered the only possible route for transfer of DNA from plants to bacteria. All possible factors of such a transfer were discussed. In addition, possible impacts of GM plants on the environment (bacterial communities) were addressed.

Persistence of DNA:

Although most DNA, including transgenic DNA, is quickly degraded in the environment (e.g. gastrointestinal tract, soil or decaying plants), a low amount of DNA will persist over time, as has been detected with molecular tools. This long-term persistent DNA, often protected due to absorbance onto quartz, clay particles, and cell wall material or even in pollen, is protected against further degradation. Whether the relatively low amount of remaining DNA is significant for transformation is not known, but this probably depends on the ecosystem (e.g. the presence of competent bacteria, selective pressure, etc.). However, marker rescue studies clearly demonstrated the ability of this DNA to transform bacteria.

Competence (uptake of DNA):

Most available information on competence development is derived from studies on model organisms, but we do not know whether the same principles apply to the bacteria in the ecosystem. Still a large fraction of the natural bacterial community remains unculturable and hence no information on these bacteria is present. Nevertheless, the presence of competent bacteria in various environments (soil, *in planta* and *ad planta*) has been demonstrated and various conditions, such as the presence of root exudates, but also inorganic phosphate, can stimulate competence development. It has been speculated that stress might also induce competence, but this is not a general rule for all bacteria. In this respect, plant DNA is often taken up by bacteria for nutrients. It has been suggested that integration of foreign DNA into the bacterial genome merely occurs accidentally, depending on both the presence of the necessary recombinational machinery in the recipient bacterium and on the nature of this foreign DNA itself. Not only the length of this DNA, but also the presence of sequences homologous to the bacterial genome play an important role.

Homology:

The presence of homologous sequences has been considered to represent a key factor in a successful transformation event. It has been proven that transgenic (chromosome) as well as transplastomic plant DNA can transform competent cells, but only when the recipient cell provides DNA homology. Recent experiments have shown that transformation efficiencies of transgenes from transplastomic plants were higher compared to a transgene targeted to the nucleus of a plant, probably caused by the high copy number of the transgenes in plastids, as well as the occurrence of prokaryotic-like sequences (anchor sequences).

All transformation studies presented so far have made use of marker rescue systems, in which (competent) bacteria contain homologous sequences, usually antibiotic resistance genes, enabling homologous recombination of incoming DNA to occur. Reducing the length of this homologous sequence clearly reduced the transformation frequency. Furthermore, Homology Facilitated Illegitimate Recombination experiments clearly lowered transformation frequencies, but did not abolish transformation, showing that specific microhomologies could also serves as recombination sites. It is been suggested that non-homologous recombination events also will take place in environment, but that the corresponding transformation frequencies are so low, that they are below the detection limit of current available detection methods.

Detection methods:

All available evidence suggests that HGT from GM plants to microbial populations occurs at very low frequencies, but current methods are not able to quantify HGT under natural conditions at this moment as rates of transfer will be extremely low. Critical to the detection of HGT is the use of assay systems that are sensitive enough to detect even these very rare events. Some culture-based methods (typical detection limit of 10^{-8} – 10^{-11} HGT events per bacterium) may exceed expected rates of HGT (10^{-16} – 10^{-17}) by several orders of magnitude. The introduction of new selection methods and culture-independent assays should improve the detection limits, and also reveal some information on the non-culturable fraction.

At the moment, adequate model genes are hard to find, since no background signals of this gene should be present in the environment. Furthermore, expression of a model gene should be easy to detect and it should be known to what degree the gene confers an advantage to the recipient organism, enhancing survival and growth. The development of such a new model system, using the *linA* gene responsible for the first step in lindane degradation, was discussed. This gene seems to apply to most of the qualifications mentioned above.

Although laboratory experiments have demonstrated HGT from plants to microorganisms, the conditions used within such experiments are designed to be far more conducive to HGT than those found in the field. For instance, the use of highly competent recipient strains, such as *Acinetobacter sp* BD413, dramatically increased the likelihood of HGT. It has been suggested that organisms, which live in close contact with plants, such as the epiphytic bacterial community, might serve as a better model system. Even though experimental setups would be designed, enabling us to measure transformation frequencies as low as 10^{-17}, an even more important factor should be addressed, to which a strong emphasis was put during the workshop, namely the selection of the transformants.

Selection/Fitness:

The selective force and time passed to fix the newly acquired gene into a population might be more important factors than the transformation frequency per se. When a recipient bacterium stably integrates a transgene from a GM plant into its genome, this newly acquired gene only will get positively selected when it is correctly expressed and expression increases the fitness of the recipient strain. Correct expression sometimes depends on random genetic rearrangement events. Once a rearrangement combination is optimal, selection of the construct becomes important.

Population dynamics in the bacterial community, in which the transformed bacterium resides, usually determines the fate of the newly fixed transgene. In the absence of positive selection, most horizontally transferred genes will be lost. The indigenous microflora limits the growth of “invaders” by competition for substrates. However, it has also been demonstrated that the absence of selective pressure does not always result in the loss of a newly acquired trait, as was seen for bacterial strains receiving plasmids carrying antibiotic resistance genes, which stayed in the community, despite the absence of the accompanying antibiotics.

Whenever the newly acquired gene provides an increased fitness, detection of the transfer event might only come into prominence after a considerable amount of time, even up to 10 years or more, depending on the recipient population size, the structure of the bacterial community and the bacterial generation time. The presence of a selective advantage highly depends on the nature of the transgene, but also on the environment in which the recipient strain is localized.

Several environments have been identified in which the frequency of transformation might be higher compared to other locations. In general, each site in which an active bacterial population comes into contact with (high amounts of) free DNA might be considered to represent such a hot spot. The rhizosphere, and more broadly the phytosphere, were considered to be such hot spots, in which bacterial pathogens or saprophytes colonizing the GM plants get close to the plant DNA (this extends to bacteria co-colonizing with pathogens and even the endophytes). Furthermore, the gastrointestinal tract, rotting plant material and spoiled food (by pathogens) have been mentioned. Nevertheless, in case of a transfer event, for

instance in the field of GM maize or in the rumen of cattle fed with GM-maize, the impact will subsequently depend on what the field will be used for or how the cattle will be treated, in order for the new trait to become selected.

Impact

Impact assessment depends on the prediction whether a transformed gene will be fixed or lost in the bacterial population. As mentioned before, selection is an important factor, which is determined by the nature of the transgene. In order for a bacterium to obtain a complete gene, a minimum length of 150 – 6000 bp is required. In order to obtain a functional regulatory element, 100 – 150 bp is sufficient. The evaluation of a possible impact of a transgenic transfer should be considered in the light of the baseline level of such a gene in the environment. This is certainly the case for the antibiotic resistance marker genes, on which most impact assessment studies have focussed, studying the transfer from GM plants to bacteria. During the workshop is was suggested that the possibility of such a transfer from GM plant to bacterium is slight and that it would be more likely that the bacterium receives a resistance gene from the bacterial population itself, due to the abundant prevalence of these type of genes in the environment. For instance, gentamicin and streptomycin resistance genes have been detected in most of the environmental samples analysed in one study sponsored by the EU. The use of these antibiotic resistance genes in GM plants therefore was considered not to be a problem, but other resistance markers used in human (and veterinary) health care, should be avoided.

In theory, even a low frequency transformation event can have an important impact if selection is strong. The novelty of a trait may also influence its potential impact, as completely novel genes might give rise to new genetic variants that are not possible within the normal genetic pool of the system. However, we are currently not fully able to predict whether such a transferred transgenic gene will be naturally selected. For most of the potential new GM plants, we have no reference in plant-soil sciences to predict possible effects, when the transgene will be transformed to a bacterium.

Effect of GM plant on a plant-associated bacterial community

This topic had indirect relevance to the topic of direct transfer of transgenic DNA from GM plants to bacteria, as shifts in plant-associated communities affect relative dominances of highly-transformable bacteria in this environment. Hence, it was discussed during the workshop. Whenever a GM plant would stimulate or change the bacterial population in such a way, that a larger fraction of the population would be competent, the transformation frequency and thus the likelihood of gene transfer would increase.

Using molecular fingerprinting techniques, it has been shown that plants do select for distinct bacterial populations, which generally are maintained over the

growing season. Effects of commonly-used GM plants on the plant-associated communities have also been observed, although the extent depends on the season and is stronger in the beginning. Furthermore, after each growing season the observed effects disappeared. In that respect, bacterial communities are affected by both transgenic and non-transgenic plants and no major differences in antagonistic populations near (in) wt and GM potato lines were observed. It remains unclear whether the changes in bacterial populations with the different levels of interaction with the plant (functional groups) influences the likelihood of gene transfer from plant to bacteria, since we simply do not know the functions of the dominant, non-culturable bacterial populations in the rhizosphere.

Changes in bacterial community composition have also been observed in the gastrointestinal tract of rats, due to a diet of GM food. However, each organism (rat) has a different microbial community and variability of the bacterial community between animals is often higher than the variability induced by the diet in each animal. Furthermore, the observed effects depend on the environmental conditions, these effects are small and transient and could also have been caused by other disturbances.

Historical perspective of HGT

A topic only sparsely covered during the workshop was the historical perspective of HGT in the light of current comparisons of fully sequenced genomes of various organisms. It has been proposed that evolutionary HGT events between plants and microorganisms have left a mosaic nature of microbial genomes (*i.e.* bacteria containing genes of supposed eukaryotic origin). However, it remains unclear how much of the bacterial genome is derived from plants. This topic should be addressed more closely, since such genes might serve as anchor sequences. Successful transformation of short regions (e.g. function domains/motifs), with subsequent selection and rearrangements are difficult to track down. Nevertheless, there is a dispute over the occurrence of recent transfers (e.g. the last decade), since none of them have been reported, not even in the large areas in USA and Canada covered with GM plants. A possible explanation might be that they simply have not been detected, since they were not positively selected (yet) and remain below the detection limit.

Final remarks:

It was stated that the occurrence of HGT from GM-plants to the intestinal microflora, or to the plant associated bacterial community, is maybe not zero, but should be considered as a very rare event, implicating that transfer of transgenic DNA from plants to bacteria does happen. However, this should not be a political issue, since we think that such HGT events will not impose a threat. There should be a public awareness, that HGT processes are natural, normal processes, which have

developed in evolutionary time long before the construction of the first GM plants. The public should be informed, with quantifiable data, of possible transfers from GM plants to bacteria in the light of these natural HGT processes. For this, we still need a lot of research, since current detection methods are not adequate. In addition, current knowledge is based on models, which are sometimes irrelevant and based on worst-case scenario's (marker rescue studies). Complete data on the (effects of the) community structure (non-culturables; functional groups) are lacking and impact assessment should also address baseline values (e.g. occurrence of antibiotic resistance genes in the environment, often located on genetic mobile elements) and fixation rates of a transfer event into a bacterial population.

Appendix III Participants to the Workshop, April 22-24, 2004

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