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The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants

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Abstract

In 2000, the thematic network ENTRANSFOOD was launched to assess four different topics that are all related to the testing or assessment of food containing or produced from genetically modified organisms (GMOs). Each of the topics was linked to a European Commission (EC)-funded large shared cost action (see http://www.entransfood.com). Since the exchange of genetic information through horizontal (lateral) gene transfer (HGT) might play a more important role, in quantity and quality, than hitherto imagined, a working group dealing with HGT in the context of food and feed safety was established. This working group was linked to the GMOBILITY project (GMOBILITY, 2003) and the results of the deliberations are laid down in this review paper. HGT is reviewed in relation to the potential risks of consuming food or feed derived from transgenic crops. First, the mechanisms

Abbreviations: *A. calcoaceticus*, *Acinetobacter calcoaceticus*; *A. rhizogenes*, *Agrobacterium rhizogenes*; *A. tumefaciens*, *Agrobacterium tumefaciens*; APH, aminoglycoside-3′-phosphotransferase; a_w, water activity; *B. subtilis*, *Bacillus subtilis*; BAC, bacterial artificial chromosome; BMV, brome mosaic virus; C, cytosine; C and N, carbon and nitrogen; *C. jejuni*, *Campylobacter jejuni*; CaMV, cauliflower mosaic virus; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; DPAD, electric discharge particle acceleration device; dsDNA, double-stranded DNA; *E. coli*, *Escherichia coli*; *E. faecium*, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*; EC, European Commission; ENTRANSFOOD, European network on the safety assessment of genetically modified food crops; EU, European Union; G, guanine; GI, gastrointestinal; GM, genetically modified; GMO, genetically modified organism; GMOBILITY, EU-project on safety evaluation of horizontal gene transfer from genetically modified organisms to the microflora of the food chain and human gut; GURT, Genetic Use Restriction Technology; HFA, human flora associated; HGT, horizontal gene transfer; Kan^R, kanamycin resistance; *L. curvatus*, *Lactobacillus curvatus*; *L. delbrueckii subps. bulgaricus*, *Lactobacillus delbrueckii subps. bulgaricus*; *L. lactis*, *Lactococcus lactis*; *L. monocytogenes*, *Listeria monocytogenes*; mRNA, messenger RNA; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; NLS, nuclear location signal; *P. stutzeri*, *Pseudomonas stutzeri*; PCR, polymerase chain reaction; PEG, polyethylene glycol; ptDNA, plastid DNA; rDNA, ribosomal DNA; RE, restriction endonucleases; Ri, root inducing; RNA, ribonucleic acid; rRNA, recombinant RNA; *S. gordonii*, *Streptococcus gordonii*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. thermophilus*, *Streptococcus thermophilus*; SAAT, sonication-assisted Agrobacterium-mediated transformation; SCP, Scientific Committee on Plants; ss DNA, single-stranded DNA; T-DNA, transfer DNA; TGMV, tomato golden mosaic virus; Ti, tumour inducing; tRNA, transfer RNA; WDV, wheat dwarf virus; YAC, yeast artificial chromosome; ZYMV, zucchini yellow mosaic virus.

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for obtaining transgenic crops are described. Next, HGT mechanisms and its possible evolutionary role are described. The use of marker genes is presented in detail as a special case for genes that may pose a risk. Furthermore, the exposure to GMOs and in particular to genetically modified (GM) deoxyribonucleic acid (DNA) is discussed as part of the total risk assessment. The review finishes off with a number of conclusions related to GM food and feed safety. The aim of this paper is to provide a comprehensive overview to assist risk assessors as well as regulators and the general public in understanding the safety issues related to these mechanisms.

Keywords: Aantibiotic resistance; Marker genes; Marker gene removal; Biosafety; Transformation; Conjugation; *Agrobacterium tumefaciens*; risk assessment; safety assessment; DNA degradation; DNA integration; Horizontal gene transfer; Marker rescue; GMO; Gene transfer; DNA stability; Transgenic crop plants; Recombinant DNA; Dietary intake; Food safety; Intestinal microflora; Antibiotic resistance markers; DNA in food; DNA in feed

1. Introduction

Horizontal gene transfer (HGT) is the transfer of genetic material directly to a living cell or an organism followed by its expression. It occurs widely among prokaryotes. HGT has been shown to engage members of the same species, of different species, or even of different domains of life. HGT is distinguished from the process of vertical gene transfer, which is the passage of genetic information from parents to offspring as it occurs in reproduction.

HGT plays a major role in the evolution of genomes: it is considered to be more important in the adaptation of bacteria to new environments than the alteration of gene function through point mutations. It is likely that in early evolution, horizontal gene transfer was pervasive and that it was dominating the evolutionary dynamics. Ancient cells were simpler and more modular in design than are modern cells (Woese, 2000). Retrospective research, such as the comparison of the genomes of bacteria and plants, provides more and more evidence for the singular and modular transfer of genes involving different mechanisms between lower and higher organisms. Ravi et al. (1999) suggested that horizontal transfer occurs more frequently for operational genes (those involved in housekeeping) than for informational genes (those involved in transcription, translation, and related processes), because the latter are typically members of large, complex systems thereby making horizontal transfer of such gene products less probable. Garcia-Vallve et al. (2000) collected statistical parameters such as G+C content, codon and amino-acid usage, as well as information about which genes deviate in these parameters from prokaryotic complete genomes in order to compile a database of genes that may have been acquired by HGT. As an example, such analyses showed that at least 234 HGT events have contributed to the genome structure of *Escherichia coli*, as could be inferred from the deviation of this species from the *Salmonella* lineage (Lawrence and Ochman, 1998). These analyses have also shown that the genome of *E. coli* strain MG1655 differs from related *E. coli* strains by 67 events, including 37 gene transfers and 30 gene losses (Ochman and Jones, 2000).

HGT requires a number of steps including uptake of DNA, integration and expression. If the acquired DNA does not provide selective advantage, it is likely to be lost in the population. The probability that a specific gene will be successfully transferred to a new host depends on the specific mechanistic details of the transfer (transformation, transduction, or conjugation), on the relationships of these mechanisms to the types of nucleic acids that are being transferred (single-stranded, double-stranded, linear, circular, etc.), and even on factors such as the distribution of integrases in organisms (Jain et al., 1999). De Vries and Wackernagel (2002) suggest that the integration of non-homologous DNA into the genome of the Gram-negative *Acinetobacter* sp. BD413 during transformation was at least 10^9-fold lower than that of homologous DNA, and that the integration could be increased 10^5 times by linking the non-homologous DNA on one side to a piece of DNA homologous to the recipient genome. This suggests that homologous DNA can serve as a recombinational anchor facilitating illegitimate recombination acting on the same molecule.

HGT as a biosafety issue has been addressed in several studies and a number of potential hazards have received much attention and have been debated intensively in the scientific and popular press (Ho et al., 1999). A lot of attention has been spent on the transfer of antibiotic resistance genes from GM plants to soil- and plant-related micro-organisms. HGT to bacteria in these environments has been shown by marker rescue experiments via homologous recombination. However, HGT may also occur under different conditions, for instance transformation of bacteria in the food chain. Free DNA persists in some materials for weeks, and furthermore, some bacteria develop natural/chemical competence to take up DNA from the environment. In addition, in the gastrointestinal tract of man and husbandry animals, DNA may remain stable for some time, particularly in the colon. However, degradation already begins before the DNA or the material containing the DNA arrives at the critical sites for HGT, which are generally believed to be the lower part of the small intestine, caecum, and the colon. And if DNA reaches

these sites, the DNA is most probably fractionated in fragments smaller than the length of a complete gene. Thus, breakdown of DNA in the gut, combined with the breakdown of the DNA due to food processing, reduces the risk of dissemination.

The purpose of this review is to consider all the elements of HGT that are relevant for evaluation of the safety of transgenic food and feed and to arrive at a number of conclusions.

The EC Scientific Steering Committee defined risk assessment—in line with earlier definitions—as "a process of evaluation including the identification of the attendant uncertainties, of the likelihood and severity of an adverse effect(s)/event(s) occurring to man or the environment following exposure under defined conditions to a risk source(s)" (SSC, 2003). It is generally accepted that risk assessment is composed of four different steps, namely hazard identification (i.e. the identification of the hazard, the danger it presents, the impact in terms of human health, and the circumstances under which it has an impact), hazard characterisation (i.e. the qualitative and/or quantitative evaluation of the adverse effects of the hazard on human health), exposure assessment (i.e. qualitative and quantitative evaluation of the degree of consumption or intake of the hazardous agent that is likely to occur) and finally the risk characterisation (i.e. the integration of the three prior steps into an estimate of the adverse effect likely to occur in the target population). The various elements of the risk assessment approach are discussed by König et al. (2004).

This framework can also serve in the risk assessment when the relevance of HGT for the safety of food and feed derived from GM plants has to be investigated. Strictly spoken, the agent of interest for hazard identification is the DNA as it is the "chemical" which is delivered in the recipient plants for the construction of the genetically modified organism (GMO). However, DNA in food has never been considered to constitute a health risk, except for the—in this case irrelevant—potential to cause gout when consumed in high amounts. It is rather the eventual subsequent transformation of cells by DNA from a GMO that should be the substance of the hazard identification process. Up to now, micro-organisms are known to be the only transformable organisms under natural conditions (i.e. cells taking up free DNA and incorporating it into the genome) and therefore, the acquisition of new heritable information is of concern only for micro-organisms. Evidently, this type of DNA transfer is not restricted to the DNA, that has been newly inserted into plants and thus, the hazard identification has to be focused in a case by case consideration on the unique properties of the microbial transformants that may result from the newly combined plant DNA.

The first GM plant introduced into the market was the Flavr Savr tomato, the safety assessment of which may serve as an example for a case to demonstrate the principle of risk analysis with GM plants. Among numerous hazards considered (Calgene, 1990), the safety of the newly introduced npt II gene encoding resistance against the aminoglycoside antibiotics kanamycin and neomycin was of special concern (Redenbaugh et al., 1991). Nap et al. (1992) summarised seven specific concerns with respect to kanamycin and kanamycin resistance, among which an unacceptable increase in resistant, potentially pathogenic micro-organisms and thus the impaired therapeutic use of the antibiotic has been identified as a defined hazard. The closest contact of the GM-plant material and the human associated microflora takes place in the large bowel, were high microbial counts can be detected. For example, in the faeces ca. 40% of the weight consists of bacterial biomass (equivalent to 4×10^{11} bacteria per gram), and >1500 species can be detected. Once one bacterium acquires the genetic information for antibiotic resistance and selective pressure is exerted (by therapeutic use of the agent), this information will spread via all mechanisms of HGT in this population. It has to be taken into account that kanamycin and neomycin are rather seldomly used in human therapeutics because of their potential side effects. Because of its importance, the authors have addressed a whole part on "marker genes".

To evaluate the exposure—which we address separately in this review—to the biological agent by consumption of the GM tomato, the amount of transformable *nptII* encoding DNA in the tomato at the point of contact with the recipient bacteria has to be known. Of importance is also the knowledge of transformation frequencies in a given habitat, such as the content of the human intestine. Evaluation of the likelihood that a consumer suffers from adverse health effects as a result of gene transfer from GM plants is case-dependent, but available data are still very limited (Jonas et al., 2001).

The entire risk assessment process requires the use of scientific data of the highest quality and the application of established scientific procedures. While research and scientific studies continue to provide answers needed to make informed decisions in risk analysis related to hazards in food, uncertainty and unresolved questions still cause concern to decision-makers. These data enable the individuals responsible for risk management to decide on the measures to handle the risk and to communicate adequately with the public in order to provide the private and public sectors with the information necessary for preventing, reducing, or minimising risks to acceptably safe levels through mandatory or voluntary systems of quality and safety management (see also Frewer et al., 2004).

It is inherent to science that new data are continuously generated and that hence the outcome of a

risk assessment may change over time. The authors of this review have had the privileged position to have insight in the newest data generated within the EU supported shared cost action "GMOBILITY" and have based their views partly on that unpublished information.

2. Mechanisms of plant transformation and the relation to gene transfer issues

Plant transformation has been defined as the stable incorporation and expression of foreign genes into plants. Since the first reports of successful plant transformation in 1983, successful transformation has been described of over 120 species in 35 different families, embracing monocots and dicots (Birch, 1997), as well as algae (Cheney, 2000), fungi (Cantoral et al., 1987), and HeLa cells (Kunik et al., 2001). For a review of patent-protected species transformation, see Roa-Rodríguez and Nottenburg (2003).

We will limit the number of references here, since over the last few years, a number of excellent detailed review papers have been published (Tzifra et al., 2000; Gelvin, 2000; Zhu et al., 2000, Tzfira and Citovsky, 2002).

The process of plant transformation is currently a complex multi-stage procedure involving three discrete phases:

- Phase 1 involves the selection and application of a delivery system, by which the DNA of interest is transferred into a viable host cell.
- Phase 2 involves integration of the DNA in plant cells leading to cells properly expressing the gene of interest.
- Phase 3 involves the recovery of a viable transgenic plant. In many cases, recovery requires tissue culture and plant regeneration, thus representing a rate-limiting step.

2.1. Delivery systems

A delivery system involves the use of specific technologies for the transfer of isolated DNA into a viable host cell. There are two main classes of delivery systems: biological and physical. We will present a comprehensive overview of the different systems and of the various factors that may affect subsequent gene transfer and/or the safety of food and feed derived from the transformed plants.

2.1.1. Biological delivery systems

Only two bacterial species, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* can at present be used as delivery systems for plant transformation. These bacteria, which normally cause crown gall disease, both have the unique ability to transfer portions of their DNA into the genomes of plants.

These strains contain a large megaplasmid (more than 200 kb) which plays a key role in tumour induction, and for this reason was named the Ti plasmid (after tumour-inducing), or Ri (after root-inducing) in the case of *A. rhizogenes*. During bacterial infection of plant tissue the T-DNA (transfer DNA), a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into a plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act in *cis* as a signal for the transfer apparatus. The process of T-DNA transfer is mediated by the co-operative action of proteins encoded by genes of the Ti plasmid virulence region (*vir* genes) and in the bacterial genome. Following transfer and expression of the T-DNA into the plant nucleus, the plant cells proliferate and synthesise certain types of opines, depending upon the T-DNA type that has been integrated. These opines in turn can serve as a sole C and N source for *Agrobacterium* (for a review see Hooykaas and Schilperoort, 1992). The 30 kb *vir* region is a regulon organised of seven operons that are essential for T-DNA transfer (*virA, virB, virD,* and *virG*) and for the increasing of transfer efficiency (*virC* and *virE*). Several genes of the bacterial chromosome have been shown to function in the attachment of *Agrobacterium* to the plant cell and in bacterial colonisation (Ward and Zambryski, 2001).

The first protein that is involved in the activation of the T-DNA transfer is VirA, a transmembrane dimeric sensor protein that detects signal molecules, mainly small phenolic compounds such as acetosyringone, released from wounded plants. Activated VirA has the capacity to transfer its phosphate to the cytoplasmic VirG that in turn functions as a transcription factor regulating the expression of the other *vir* genes.

The expression of *vir* genes results in the generation of single-stranded DNA (ss DNA) complementary to the bottom T-DNA strand. Any DNA placed between the T-DNA borders will be transferred to the plant cell, as ss DNA, and integrated into the plant genome as duplex DNA. T-strand synthesis is initiated at the right border and proceeds in the 5′ to 3′ direction.

An ss T-DNA–protein complex is transferred to the plant nucleus. This DNA–protein complex must pass through three membranes (bacterium, plant cell and nucleus). According to the most widely accepted model, the ssT–DNA–VirD2 complex is coated by the 69 kDa VirE2 protein, an ss DNA binding protein. The association occurs co-operatively and prevents the attack of nucleases and, in addition, stretches the ssT–DNA strand reducing the complex diameter to approximately 2 nm. This is thought to make the translocation through membrane channels easier. VirE2 contains two plant nuclear location signals (NLS) and VirD2 one. This suggests that both proteins (and probably also VirF)

presumably play important roles once the complex is in the plant cell, mediating uptake of the complex into the nucleus.

The final step of T-DNA transfer is its integration into the plant genome. The mechanism effecting T-DNA integration is not yet known. It is considered that integration occurs by illegitimate recombination processes (see for a detailed discussion the paper by Cellini et al., 2004). According to this model, pairing of a few bases, known as microhomologies, are required for a pre-annealing step between T-DNA strand coupled with VirD2 and plant DNA. The microhomologies can provide a minimum specificity required for the recombination process.

The Ti system probably inserts only one T-DNA segment per bacterium into the host's nuclear genome, but several bacteria could infect the same host cell, effectively increasing the T-DNA copy number in the genome. Some of the inserts may be located on the same or on different chromosomes. Upon integration, some of them may have undergone rearrangements or may be aberrant.

2.1.1.1. Making the Ti plasmid a vector. Since 1983, many different strains of *Agrobacterium* have been used as delivery systems. Each strain differs in its ability to infect and transform plants. Thus, certain bacteria have proven to be more effective on some plants and less effective on others. Although the composition of the Ti plasmid may be different between *Agrobacterium* strains, the mechanism of integration of the T-strand is the same.

The first method employed to produce a vector to deliver genes of interest to plants by using Ti plasmids relied on cointegration of a plasmid with the gene of interest into the T-region in the Ti plasmid. This method was used to overcome many of the problems associated with the manipulation of large pieces of DNA. However, cointegration vectors have the disadvantage that they contain large areas of DNA that are not required subsequent to the transformation and regeneration of transformed cells. Therefore they are not considered suitable to be used for the production of agronomically important GM crops.

As the technology progressed, the long and cumbersome cointegration method was replaced by the binary system, which is now the standard method for *Agrobacterium* vector production. This system was developed when it was discovered that the genes responsible for integration might act *in trans* independent of the presence of the T-region. The binary vector system consists of two plasmids; one is a broad host vector that replicates in *E. coli* as well as in *A. tumefaciens,* and that contains a bacterial selectable marker, a bacterial origin of replication, and an integration region into which the gene of interest is inserted. The integration region is bordered by the T-region left and right border sequences and contains a multiple cloning site and, for example, an *nptII* gene for Kan^R selection in plants. The gene of interest is fused to a strong eukaryotic promoter for expression in the crop plant and is inserted into the multiple cloning site. Although the binary vector can be introduced directly into *Agrobacterium* via electroporation or transformation, it is often first transformed into *E. coli* and transformants are subsequently mated with a strain of *Agrobacterium* that possesses a *vir* helper plasmid (the second plasmid of the binary system), which is essentially a Ti plasmid that contains the *vir* genes and lacks the T-region. *Agrobacterium* cells containing the *vir* helper plasmid and the binary vector are selected and allowed to infect plant cells/protoplasts. The trans-acting *vir* gene products from the helper plasmid allow the transfer of the binary vector's integration region along the normal *Agrobacterium* integration pathway, as described above.

Studies of natural tumours have shown that foreign DNA is confined to the T-DNA region only, but recent work has shown that, when using binary vectors, transformed cells can sometimes contain sequences from outside of the T-DNA, i.e. from the vector backbone. The structure of the small plasmid carrying the *vir* genes may frequently result in integration of vector backbone sequences. One example is a transgenic potato that has been submitted for marketing approval in the EU and that has been reviewed by the SCP (1998).

There are several advantages to the binary system over the cointegration system, one most important being that it is possible to limit the transferred DNA to its smallest size, which is bordered by the left and right border sequence.

2.1.1.2. Limitations of biological delivery systems. Currently, *Agrobacterium* vectors are used in the successful transformation of many dicots. However, the fundamental limitation of this system is the reduced ability of the system to transfer DNA to cells of monocots.

The *Agrobacterium* system requires physically injured plant tissue that the bacteria can enter. Whether the procedure involves the whole plant or plant culture, such as calli, protoplasts, or leaf disks, the process requires the regeneration of a viable plant (as with plant cultures), or the generation of viable transformed gametes. Thus, the *Agrobacterium* system will not function with plants that cannot be regenerated from culture or do not tolerate physical injury. In addition, if surface sterilisation has not been carried out sufficiently well, the *Agrobacteria* may proliferate further, along with the transformed plant and consequently may be released into the environment where uncontrolled transformation may further occur.

Finally, the *Agrobacterium* binary vector system relies completely on the functioning of the Vir proteins, which

target only to the nuclear genome and not to organelle genomes. For those purposes, alternative delivery systems, such as particle bombardment (see below) must be employed.

2.1.1.3. Current developments and techniques in biological delivery systems. A number of strategies have been employed over the last few years to develop alternative gene delivery systems for higher plants. Whereas most of them have given successful results in only a limited number of cases, they are presented here, because they may acquire a much wider potential over the next few years.

Modified viruses such as derivatives of the tomato golden mosaic virus (TGMV), in which an antibiotic resistance gene replaces the viral coat protein genes, can be used to enhance integration (Elmer and Rogers, 1990). Once inside the genome, the virus has the ability to propagate, whilst the removal of the coat protein genes inhibits the production of new viable virus thus leaving replicated copies of the viral DNA within the nucleus. Similar results have been obtained using other viral vector combinations such as the *Agrobacterium*-geminivirus vectors and *Agrobacterium*-cosmid vectors.

A recent advance in vector construction for high molecular weight DNA involved the generation of a bacterial artificial chromosome (BAC) for use in conjunction with the *Agrobacterium* binary vector strategy (Hamilton, 1997). The BAC can accommodate inserts between 100–180 kb and was chosen over the more commonly used yeast artificial chromosome (YAC), as BAC is much easier to construct, screen, and maintain than YAC. The BAC has shown stability in both *E. coli* and several strains of *Agrobacterium*, making it a practical tool for library construction and positional cloning (Hamilton, 1997).

The wounding of plant material appears to be an important factor in *Agrobacterium*-mediated transformation, as it allows the bacteria to enter the host tissue and to multiply in damaged cells. As pointed out in Section 2.1.1., the wounding also triggers the activation of T-DNA transfer in *Agrobacterium*. From the damaged plant cells, the transfer of T-DNA by the multiplying *Agrobacterium* cells is more effectively carried out to viable plant cells in the vicinity. It is thought that poor tissue penetration is a major factor in *Agrobacterium*'s low ability to transform monocots. Traditionally, leaf disks or cuttings have been used, but new technologies make it possible to transform almost all plant tissues.

A recently developed technique, sonication-assisted *Agrobacterium*-mediated transformation (SAAT), greatly increases the entry of bacteria into the host tissue (Trick and Finer, 1997). The technique involves brief periods in which the target tissue is subjected to ultrasonic treatment in the presence of *Agrobacterium*. The treatment produces small fissures in the tissue and holes in the cells allowing the bacteria to enter the cytosol. The SAAT has the potential for uniform transformation of meristematic tissue, as previous techniques produced scattered transformed cells in a tissue infiltration. Furthermore, the SAAT procedure produced a 100- to 1400-fold increase in the expression of the gene of interest in a variety of tissues and organisms. It is unclear why this is the case, but it may well be that the SAAT technology has a substantial impact on the plant signal transduction and/or translation machinery. Accordingly, a large number of unintended effects may appear as well.

Another wounding technique, vacuum infiltration, has also been shown to improve *Agrobacterium*-mediated transformation (Bechtold et al., 1993). For a while, this method could be successfully applied only to one plant species, the model plant *Arabidopsis thaliana*. However, success has recently been reported for rapeseed, *Brassica campestris*, and radish, *Raphanus sativus*, suggesting that this method may not be limited to *Arabidopsis* (Desfeux et al., 2000; Curtis and Hong, 2001). Concentrated solutions of *Agrobacterium* were applied *in planta* and infiltrated the tissue under a vacuum. It is thought that the negative pressure forces the bacteria into the host cells, thus increasing the susceptibility of the host. Furthermore, the vacuum process stimulates the plants wound response, again increasing the virulence of the bacteria. The method of vacuum infiltration has made it possible to obtain transformed plants without plant tissue culture or regeneration. It is simple and reliable, and transformed plants can be obtained at sufficiently high rates.

Improvements related to this technique have been made, either by simply dipping of developing floral tissues into a solution of *Agrobacterium* (Clough and Bent, 1998) or a floral spray (Chung et al., 2000) or a combination of floral dipping and vacuum infiltration. This efficient plant transformation procedure relies on the gene transfer by *Agrobacterium* to the female reproductive tissues in the flower. Seeds are collected from the transformed plant and transformants can subsequently be selected for either by antibiotic or herbicide resistance markers present in the transferred DNA. Most of the transformed progeny are genetically uniform and the somaclonal variation associated with the tissue culture and regeneration is minimised.

Another improvement to the *Agrobacterium* system involves the stimulation of the *vir* genes by addition of *vir* inducing molecules, such as acetosyringone, to enhance transformation.

2.1.2. Physical delivery systems

In 1987 the microprojectile-mediated transformation (Klein et al., 1987) was published, which involved shooting of DNA-coated metal particles into plant cells.

The "brute force" approach gives the microprojectile technique the unique ability to transform any plant tissue. Mechanical forces, generated by high velocity particles, drive the foreign DNA past all biological barriers, allowing for genomic integration. Furthermore, this approach has the unique ability to target the transforming DNA to the chromosomal DNA of a cell and also to the chloroplast and mitochondrial DNA.

In the initial experiments, DNA was bound to tiny tungsten particles, approximately one micrometer in diameter and accelerated with a gunpowder driven piston, fired at the target cell with a velocity of about 430 meters per second. Host cells in the front line are usually destroyed, but just behind, an area of cells exists where the projectiles penetrate the cell without killing them. Some of the cells that survive the bombardment incorporate the DNA from the projectile into the genome. After successful incorporation, and if the inserted DNA did not knock out any critical gene function, host cells may begin to express the gene product of the foreign DNA.

A variation on the microprojectile transformation is an electric discharge particle acceleration device (DPAD) (Christou et al., 1988). This apparatus uses shock waves to accelerate the particles. The force of the shock wave drives a mylar sheet containing particles and precipitated DNA towards a retaining screen. The mylar sheet is stopped by the screen, but the particles and DNA, now at maximum velocity, continue past the screen and collide with the target tissue. As technology progressed, other improvements, which significantly lowered the cost of these experiments, were developed.

2.1.2.1. DNA preparation and delivery. DNA Construct: Most commonly, plasmids are used in microprojectile treatments. However, the size of the plasmid is limited, as large plasmids will be sheared on impact and gene transfer to the cell is reduced. The plasmids contain a bacterial selectable marker and an *E. coli* origin of replication for propagation in *E. coli*, a multiple cloning site for insertion of foreign DNA, and a marker gene fused to a promoter active in plants for identification of transformants. The constructs are generated in vitro, amplified in *E. coli*, and fused to the delivery particles.

Microprojectiles: Typically, metal particles have been used for microprojectile transformation, as a minimum density (19 g/cm^3) is needed to generate enough force to penetrate plant cells. A further requirement is that the size of the particles is proportional to the target cell size. Tungsten and gold particles are the most common, as they are sufficiently dense and chemically inert.

Binding DNA to microprojectiles: To prepare particles for tissue bombardment the DNA must be precipitated onto the particle. The most commonly used precipitation procedure for plasmids and tungsten uses $CaCl_2$ and spermidine. This $CaCl_2$/spermidine procedure has also been maximised for different tissues and microprojectile transformation systems.

Integration: The particle/plasmid precipitate suspension is applied to the microprojectile acceleration device. The tissue is bombarded by the particles and the plasmid DNA dissociates from the particle upon entering the cell. Once in the nucleus, plastid or mitochondrion, the plasmid is free to undergo recombination to become incorporated into the genome (Iida et al., 1990).

2.1.2.2. Limitations of physical delivery systems. The most obvious drawback of the microprojectile systems is tissue damage and the relatively low yield of stable transformants. Generally, the outer layers of cells, coined the "zone of death", will be destroyed by the impact of the particles, particle fragmentation, or air blasts. The system is also limited in the size of DNA constructs that can be used.

As was done with the biological delivery systems, viral vectors have been developed to increase transformation efficiency and marker gene expression. It has been reported that both DNA viruses such as geminiviruses and ribonucleic acid (RNA) viruses such as the zucchini yellow mosaic virus (ZYMV) can be successfully integrated in microprojectile systems (Hayes et al., 1988). As noted before, viral vectors can enhance expression through increased copy number by self-replication and integration. Yeast artificial chromosome (YAC) is also being applied to the microprojectile technique. YAC was specially designed for this process and the results suggest it could be used for map-based cloning of plant genes. Furthermore, YAC could also be used to create genetic libraries in transgenic plants (Van Eck et al., 1995).

2.1.3. Alternative delivery systems

Although *Agrobacterium* and microprojectile mediated transformation systems are the most commonly used, they are not the only techniques available. Researchers are continually developing new- and improving established-delivery systems.

2.1.3.1. Polyethylene glycol (PEG) mediated transformation. The most common method of delivering foreign DNA into plant protoplasts involves treatment with PEG (Paszkowski et al., 1984). It is still not clear how PEG causes protoplasts to take up DNA, but it is thought that it induces reversible permeabilisation of the cytoplasmic membrane, which allows passage of macromolecules. The system is only rarely employed due to the low yields of transformants (approximately 1–2%) and the inability of many species to be regenerated into viable plants from protoplasts (approximately 0.1%), making the whole system only about 0.0004% effective.

2.1.3.2. Liposomes. An advance upon PEG-mediated transformation was the liposome-mediated transforma-

tion technique. Foreign DNA is encapsulated in a spherical lipid bilayer termed a liposome (Gad et al., 1990). In the presence of PEG, the host protoplast will bind and envelop the liposome through endocytosis (Fukunaga et al., 1983). After endocytosis, the DNA is free to recombine and integrate into the host genome. As with other transformation systems, a variety of vectors including viral vectors can be employed in this system.

2.1.3.3. Electroporation. An alternative to the method of permeabilising a membrane is electroporation, the exposure of cells to a short-time intense electric field. This technique, which is commonly used in bacterial transformation, is also being applied to plant protoplasts. Transformation by electroporation has been achieved in a variety of species and tissue types, but often species-specific protoplast regeneration problems still necessitate the use of other techniques.

2.1.3.4. Silicon carbide fibres. The size, shape, and chemical composition of silicon carbide fibres make them capable of puncturing the cells without killing them. The technique is quite simple: plasmid DNA (containing a marker gene), silicon carbide fibres, and a suspension of cultured plant cells are added to a tube and mixed vigorously. The hydrodynamic forces drive the fibres and DNA into the cells. The advantages of this system are that it is rapid, inexpensive, and easy to set-up and that it can work on a variety of cell types.

2.1.3.5. Microinjection. This technique uses fine glass needles, which inject the foreign DNA directly into protoplasts (Crossway et al., 1986) or cultured embryonic cells (Nomura and Komamine, 1986). Although it has a fairly high transformation frequency (20–50%), microinjection is a time consuming process that requires specific equipment and considerable training.

2.1.3.6. Electrophoresis. First described in 1989, an electric field is used to force the negatively charged foreign DNA into the host cells (Ahokas, 1989). Meristematic plant tissue is imbedded between two tubes. DNA is mixed with agar, poured into an open-ended tube containing the cathode and just agar is poured into the tube containing the anode. Under an optimised electric field, the DNA passes through the agar, onto the tissue, passes between the cellulose fibres of the cell wall and into the cell. On average, this technique gives about 55% survival rate and of the survivors up to 57% expresses a marker gene (Songstad et al., 1995).

2.1.3.7. Desiccation. Previous research has shown that dried embryos have the unique ability to take up DNA during rehydration (Topfer et al., 1989). This approach takes advantage of the natural changes that occur within the embryonic plant cell wall during rehydration. Dried embryos can be mixed with a nutrient solution containing the foreign DNA. The DNA would be taken up as the embryo rehydrates and seedlings can be germinated in the presence of a selection medium to assess the incorporation of the foreign DNA.

2.2. DNA delivery to different cell compartments

It is also possible that DNA can be integrated in organelle genomes, such as those of mitochondria or chloroplasts.

Plastid/mitochondrial transformation is obtained by (i) introduction of transforming DNA encoding antibiotic resistance by the biolistic process or PEG treatment; (ii) integration of the transforming DNA by two homologous recombination events; and (iii) selective elimination of the wild-type genome copies during repeated cell divisions on a selective medium (Zoubenko et al., 1994).

Apart from a highly regenerable tissue culture system, the generation of plants with transformed plant organelles requires:

- A method to deliver foreign DNA through the cell wall, the plasma membrane, or the double membrane of the plastid.
- A plastid-specific selectable marker gene.
- A mechanism to integrate foreign sequences into the plastid DNA (ptDNA).

Some of the advantages of transplastomic plants over classical transgenic plants (generated by transformation of the nuclear genome) are:

- High levels of transgene expression and foreign protein accumulation due to the higher number of gene copies in multiple organelles.
- Efficient translation of polycistronic mRNAs is possible, in contrast to DNA inserted into the nucleus.
- Absence of epigenetic effects in plastids like gene silencing or co-suppression.
- Transgene containment:
  - Maternal inheritance of chloroplasts in most major crop plants.
  - No transmission of transgenes via pollen.

This transgenic containment is known as cytoplasmic or maternal inheritance. In the majority of plants and animals, chloroplasts and/or mitochondria are passed from generation to generation only by the maternal parent because the egg parent (not the pollen parent) donates all of the chloroplasts and mitochondria. This does not apply to conifers in which chloroplasts are mainly inherited through pollen. In the important crop plant alfalfa, plastids are inherited from both pollen and

egg. In rice there is occasional biparental inheritance of chloroplast genes, while in peas there is cultivar variability for the presence of plastid DNA in pollen. Interestingly in rapeseed, paternal mitochondrial DNA is transferred to the egg but not paternal chloroplast DNA.

Whereas plastid transformation has been achieved in photosynthetic algae such as *Chlamydomonas*, there is little reference to similar experiments in higher plants. Thus far, almost all plastid genome manipulations have been carried out in tobacco (Ruf et al., 2001). Application to other (agriculturally important) species has been largely hampered by technical limitations, in particular due to the insufficiencies of the tissue culture and regeneration methods. In mitochondria, the available selectable markers have the caveat that multiple copies must be present in the cells before the marker is expressed to the selectable level. The cytochrome electron transfer chain inhibitors antimycin A and myxothiazol are potentially useful for the selection of plant mitochondrial genome transformants. These inhibitors bind directly to cytochrome b and block electron flow, while resistance may be achieved by the expression of a modified plant cytochrome b (*cob*) gene (Bennoun and Delosme, 1999).

Mitochondria are unique among the constituents of the eukaryotic cell in that they are semi-autonomous organelles that contain their own genetic machinery. As such, they operate under the dual genetic controls of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). A large degree of the complexity is due to the fact that, among the over 1000 proteins located in the mitochondria, only 13 are encoded by the mtDNA, while the remainder are nucleus-encoded and imported into mitochondria.

Mitochondrial genetics differs markedly from Mendelian genetics, for instance because mitochondria are inherited exclusively from the mother. In addition, mtDNA replication is unrelated to the cell cycle, and mtDNA gene organisation, DNA replication, RNA synthesis, and DNA codon use for translation into proteins all have a prokaryotic "look" about them. The latter stems from the evolutionary history, in which mitochondria evolved from bacteria that were taken up by the proto-eukaryotic cell early in evolution. Consequently, whereas bacterial promoters are unlikely to be expressed when inserted in nuclear plant DNA, they may well be expressed in mtDNA.

2.3. Integration, recombination, and expression

2.3.1. Directed vs. random insertion

The point of insertion of the foreign DNA in the plant genome may have serious implications for its expression as well as on the plant phenotype as a whole. If the DNA is integrated such that it affects the function of important genes, the host cell may be seriously damaged or even die. On the other hand, if the DNA is inserted into a region of the host genome with normally suppressed expression, such as areas of heterochromatin, then the expression of the insert will also be suppressed. Such cases may be eliminated during subsequent characterisation or breeding of the GM plant lines.

As discussed above the transfer of T-DNA from *Agrobacterium* to the host genome is a highly co-ordinated process. The transfer polarity of the T-strand is maintained using pilot and coat proteins, from its excision from Ti plasmid to the integration into the host genome. Visualisation through in situ hybridisation studies has shown that T-DNA can be localised to particular arms of chromosomes in the host genome (Kado, 1991). Host DNA repair enzymes are believed to play a significant role in T-DNA incorporation. However, it is still unclear how the site for incorporation is chosen and what the details of the mechanism are (see Section 2.1.1.). Some believe that the T-DNA border repeats are involved in site selection (Sonti et al., 1995).

Physical delivery systems are less controlled than the *Agrobacterium* system with respect to copy number or molecular integrity of inserts. In most physical delivery systems, where plasmid vectors are used to shuttle the foreign DNA into the host cell the DNA appears to randomly integrate into the host genome. In addition, a bacterial marker gene, together with a bacterial origin of replication, is mostly co-inserted. Therefore, we have to consider the possibility that large DNA fragments derived from the plant genome may be transferred (e.g. via naked DNA) to prokaryotes where they are able to replicate after circularisation. This may lead for instance to the transfer of antibiotic resistance genes, which will be discussed in Section 4.

Plasmids based on viral vectors have the ability to copy and reinsert themselves several times into the plant genome. Furthermore, depending on the viral vector, these insertions are often non-random and can result in tandem arrays.

2.3.2. Promoters and gene expression

2.3.2.1. Viral promoters. The two promoters most commonly used in plant transformation, the 19S CaMV and 35S CaMV promoters, are derived from the cauliflower mosaic virus (CaMV). The CaMV promoters can function in both monocots and dicots and have been successfully applied in a variety of transformation experiments. Once inside the host genome, the CaMV promoter maintains a high and constant non-regulated level of transcription, which is largely independent of the specific tissue of the plant. Ho et al. (1999) suggests that the CaMV promoter may recombine with dormant endogenous viruses, which in turn may result in new infectious viruses. Despite intensive research, CaMV has never been found to generate new viruses by recombi-

nation. In addition, CaMV infects naturally a large range of crucifers that are consumed by man, but there is no record of any adverse effects caused by CaMV recombination.

Other viral promoters were also characterised and cloned for use in transformation experiments. The brome mosaic viruses (BMV) have been engineered as both promoter and self-replicating vectors. Another family of viruses, the Gemini viruses are commonly used as expression vectors. Other DNA viruses, such as the wheat dwarf virus (WDV), are also routinely used. WDV is particularly effective in monocots (Hofer et al., 1992).

2.3.2.2. Tissue-specific and environmentally regulated promoters. In order to have a correct expression and translation, a number of regulatory sequences need to be added to the inserted coding sequences. Examples are the promoter sequences, which bind and direct RNA polymerase to the correct transcriptional start site and thus permit the initiation of transcription, and the terminator, which is a sequence lying beyond the 3′ end of the coding segment of a gene which is recognised by RNA polymerase as a signal to stop synthesising messenger RNA (mRNA). It is also important to have an appropriate leader sequence, which is a polynucleotide region between promoter and structural gene, necessary for the correct transcription of DNA into mRNA, and/or a signal sequence, which is necessary for the transport of the protein into or through the membrane for its secretion into the extracellular medium.

In many instances tissue-specific expression of the gene of interest is very important and thus tissue-specific promoters have been isolated, e.g. tapetum-specific promoters for eliciting male sterility (Daniell, 2002), root-specific promoters for providing pest control (Borisjuk et al., 1999), and tuber-specific promoters to modify starch biosynthesis in potato (Keil et al., 1989). As more plant genes are being identified and sequenced along with their regulatory elements, the knowledge on the specificity of promoters will increase.

In addition to tissue specific promoters, currently promoters responding to light are under intense study. Light-induced genes regulate much of plant development. Many promoters of these genes are controlled by the phytochrome system, a series of membrane-bound photoreceptors. This makes the light-induced response highly regulated and even specific to the wavelength of light from the visual spectrum. The strong activity of some of these promoters makes them an ideal choice for gene expression (Shimizu-Sato et al., 2002). Furthermore, it gives researchers the ability to regulate the expression of the foreign gene along a developmental pathway.

Other promoters such as those induced by growth factors and environmental stresses can be employed to control gene expression. Auxins and cytokinins are common growth factors that can induce high levels of transcription at low concentrations. The use of growth factor-controlled promoters allows experimenters to turn on or turn off the expression of the gene of interest through the application or removal of plant hormones. The use of promoters induced by stress allows the foreign gene to be turned on or off, as it is needed. For example, if the inserted gene is designed to confer resistance to cold, then logically one would fuse this gene to a cold-induced promoter, such that the gene is only expressed at low temperature.

2.4. Conclusions

A number of factors such as gene copy number and the point of DNA insertion may have an influence on horizontal transfer. For instance, if a gene becomes intentionally or accidentally inserted in a plant transposable element, then it is likely to spread and multiply readily within the plant population and therefore the probability of subsequent horizontal gene transfer is increased. The number of transgene copies present in a whole plant population and the duration of the propagation of the transgene in the environment are correlated with the probability for horizontal gene transfer. Moreover, multiple identical copies represent multiple sites for homologous recombination and hence, for potential genome rearrangements and genome breakage. One can for instance postulate (in the absence of experimental evidence) the possibility that upon excision of part of a chromosome through homologous recombination between two T-DNA inserts, this piece of DNA can persist due to "illicit" co-integration of a bacterial origin of replication that is present between the two excision sites. Such a molecule may transform a gut bacterium.

Inserted plant and viral promoters may act as regions for homologous recombination, thus bringing together the transformed part with unintended sequences that subsequently replicate in other genomic backgrounds. In addition, strong promoters may affect downstream regions and may lead to unintended effects.

Great care should be spent on the choice of the transformation system. Whereas the Ti system is probably the most controlled, it has the disadvantage that only the dicotyledonous crops are susceptible to it. Most physical delivery systems need careful post-transformation characterisation in order to select the transgenic plant that is safest, particularly in terms of copy numbers. In addition, it must be realised that, for instance during bombardment, pieces of plant chloroplast or mitochondrial DNA may be shot into plant nuclear DNA.

As indicated above, methods such as the SAAT procedure produce a major increase in gene expression in a

variety of tissues and organisms, which has a substantial impact on the plant transduction/translation machinery so that a large number of unintended effects may appear as well.

Great care should be spent also on the purity of the DNA preparation used. Sequences that are not relevant for the genetic modification may be co-inserted in transformation vectors or may be shot into the plant genome, possibly leading to a variety of unintended effects.

3. Marker genes

Marker genes are used to identify and/or select individual organisms, their progeny, or a certain part of a population against a large population. By linking physically the gene of interest to a marker gene, it is possible to recognise/isolate the organism with the gene of interest.

The use of selectable marker genes has been common practice in microbial genetic research for many years. This concept has been extended to genetic engineering of plants including agricultural crops. Besides antibiotic resistance genes, herbicide tolerance genes and reporter genes coding for, for example, metabolic traits are used for selection of transformed plant cells.

Antibiotic resistance genes used as markers for GM plants have reached public awareness. The concern has been raised whether horizontal gene transfer of these genes from the plant material to micro-organisms may lead to an increased level of resistance towards antibiotics in micro-organisms. This may pose a risk to human or animal health by compromising the therapeutic value of antibiotics for treatment of pathogenic micro-organisms. This concern is fuelled by the experience that the extensive use of antibiotics for human and veterinary purposes and as growth promoters for farm animals has led to increased spread of antibiotic resistance genes in the microbial population. The public concern about antibiotic resistance genes is reflected by the provision of the European Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms stating:

> Member States and the Commission shall ensure that GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment, with a view to identifying and phasing out antibiotic resistance marker in GMOs which may have adverse effects on human health and the environment.

Thus, there is the need for science-based assessment of possible adverse effects of antibiotic resistance marker genes in genetically modified plants.

3.1. Classification of antibiotic resistance genes by their distribution and based on the present state of therapeutic importance of the relevant antibiotics

There are two ways in which antibiotic resistance genes can end up in GM plants: (a) antibiotic resistance genes used for the initial molecular cloning in bacteria; and (b) antibiotic resistance genes under control of a plant promoter used for the selection of successfully transformed plant cells.

If the transfer of an antibiotic resistance gene from the genome of a transgenic plant to that of a bacterium should occur at all, this event should be seen against the background of the given distribution of the respective antibiotic resistance gene in soil and enteric bacteria and related to its importance for the therapeutic use of the relevant antibiotics. On the basis of these two criteria for evaluation, the antibiotic resistance genes in Table 1 have been proposed by the authors in accordance with the *Zentrale Kommission für die Biologische Sicherheit*

Table 1
Antibiotic resistance genes used as marker genes

Group	Name of gene	Origin/source	Mode of action	Substrate	References
I	*hpt*	*E. coli*	Hygromycin phosphotransferase	Hygromycin B	Gritz and Davies (1983)
	nptII	*E. coli* Transposon Tn5	APH (3′)II enzyme	Kanamycin, neomycin, geneticin	Garfinkel et al. (1981); Nap et al. (1992)
II	$bla_{(TEM-1)}$	*E. coli*	TEM-1 β-lactamase	Ampicillin, penicillin G, amoxycillin	Sanders and Sanders (1992)
	aadA	*E. coli* plasmid R538-1	Aminoglycoside-3′-adenyl-transferase	Streptomycin, spectinomycin	Davies and Smith (1978); Hollingshead and Vapnek (1985)
	cat	Transposon Tn9	Acetyltransferase	Chloramphenicol	Proctor and Rownd (1982)
III	*nptIII*	*Enterococcus faecalis* R plasmid	APH (3′)III enzyme	Kanamycin, neomycin, geneticin, amikacin	Pietrzak et al. (1986)
	tetA	Transposon Tn10	Efflux mechanism	Tetracycline	Bryan (1984, pp. 191–240)

APH: Aminoglycoside-3′-phosphotransferase

(Central Commission for the Biological Safety, Germany) to be assigned to the following three groups:

3.1.1. Group I

Group I contains antibiotic resistance genes (Table 1) which (a) are already widely distributed among soil and enteric bacteria; and (b) confer resistance to antibiotics that have no or only limited therapeutic relevance in human and veterinary medicine, so it can be assumed that, if at all, the presence of these antibiotic resistance genes in the genome of transgenic plants does not have an effect on the spread of these antibiotic resistance genes in the environment.

The *hpt* and *nptII* genes have selection marker functions in plants and are under the control of a plant promoter, whereas the rest of the genes in Table 1 confer resistance that are not expressed in plants, but are used in the initial cloning process of bacteria.

Hygromycin is not used in human therapy (WHO, 1993) and there is no cross-resistance with other antibiotics used for human therapy.

The *nptII* gene under control of a plant promoter has frequently been used as a marker gene for genetic modification of plants (Nap et al., 1992). Kanamycin is seldom used in human medicine, because more potent aminoglycosides are available. Neomycin is still widely used, but only as a topical drug. Nevertheless, a point mutation in the *nptII* gene can cause resistance against amikacin (Kocabiyik and Perlin, 1992), which is an important drug for human application. However, since the *nptII* gene is under the control of an eukaryotic promoter, an additional step providing a bacterial promoter is needed before expression of the gene. In addition *nptII* genes are widely distributed in the environment (Smalla et al., 1993).

3.1.2. Group II

Group II contains antibiotic resistance genes which (a) are widely distributed in micro-organisms; and (b) confer resistance to antibiotics which are used only for therapy in defined areas of human and veterinary medicine, so it can be assumed that the presence of these antibiotic resistance genes in the genome of transgenic plants has a very low effect on the distribution of these antibiotic resistance genes in the environment.

The $bla_{(TEM\text{-}1)}$ is widely used in molecular biology in a line of cloning vectors (e.g. pBR322 derivatives and pUC series). Of healthy humans, 19% were shown to harbour *E. coli* cells in their intestine conferring resistance to ampicillin (DANMAP, 1997). In human clinical *E. coli* isolates, approximately 35% are resistant to ampicillin (Kresken et al., 1999; DANMAP, 2001), of which 90% in turn are caused by TEM-1 β-lactamases (Livermore, 1995). The corresponding gene is also widespread in other enterobacterial species as *Haemophilus, Neisseria, Salmonella* etc. Data from Denmark from 2001 show that ampicillin resistant *E. coli* in broilers, cattle, and pigs at slaughter were found in 16, 0, and 10%, respectively, of the animals (DANMAP, 2001). In clinical isolates, however, the occurrence of ampicillin resistance can be as high as 80% in cattle (DANMAP, 2001).

In many cases, the use of ampicillin is recommended only when the ampicillin sensitivity has been proven in a test. However, in the case of infections with, for example, enterococci or *Listeria monocytogenes*, ampicillin is still considered to be the drug of choice.

The TEM-1 enzyme has shown only a minor activity against recent cephalosporins and can be inhibited by β-lactamase inhibitors such as clavulanic acid or tazobactam. In the case of *E. coli,* though, a high expression rate may cause resistance to amoxillin/tazobactam and other combinations of β-lactams with β-lactamase inhibitors (Sanders and Sanders, 1992).

Streptomycin and spectinomycin are used in human medicine to a limited degree only (WHO, 1993). However, they are still of importance in human medicine for the treatment of tuberculosis (streptomycin) and gonorrhoea (spectinomycin).

Today, chloramphenicol is used in human therapy only in very rare cases due to the risk of aplastic anaemia caused by it and has not been authorised in the EU for use in food-producing animals. Furthermore, chloramphenicol-resistant micro-organisms are widely found in the environment. However, there are indications that the use on chloramphenicol in human therapy might become more important again due to increase of resistance of micro-organisms to alternative antibiotics.

3.1.3. Group III

Group III contains antibiotic resistance genes, which confer resistance to antibiotics relevant for human therapy and, therefore, should be avoided in the genome of transgenic plants for reason of a high-standard preventive health care.

For use in human therapy, amikacin is an important reserve antibiotic whose therapeutic importance should not, even potentially, be reduced by the use of the *nptIII* gene in the establishment of GM plants.

Tetracyclines are characterised by their wide spectrum of action, and although *tet* genes are widespread in the environment, they continue to be of therapeutic importance in human medicine, and are used to control *Brucella, Chlamydia, Mycoplasma, Rickettsia, Vibrio,* etc.

3.2. Marker genes other than medical antibiotics

GM plant cells can also be selected by growth on media containing herbicides as a selective agent in a concentration inhibitory to unmodified cells not containing the herbicide tolerance gene (Table 2). These genes often have a dual purpose, they are used both for

Table 2
Examples of marker genes other than antibiotic resistance genes

Gene	Gene product	Selective agent or substrate
Genes encoding herbicide resistance		
als (ahas), csr1-1, crs1-2, suRB-S4-hra	Acetolactate synthase	Sulfonylureas, imidazolines, triazolopyrimidines, pyrimidylbenzoate
bxn	Bromoxynil nitrilase	Bromoxynil
epsps, epsps5	5-enolpyruvyl-shikimate-3-phosphate synthase	Glyphosate
gox	Glyphosate oxidoreductase	Glyphosate
Genes encoding metabolic traits		
manA	6-phosphomannose isomerase	Mannose
tdc	Tryptophane decarboxylase	4-methyltryptophane
xylA	Xylose isomerase	Xylose
ipt	Isopentenyl transferase	Shoot development
Reporter genes		
gfp	Green fluorescent protein	Fluorescence, oxygen
luc	Firefly luciferase	Luciferin, ATP, oxygen
luxA, luxB	Bacterial luciferase	Decanal, $FMNH_2$, oxygen
lacZ	β-galactosidase	Galactosides
uidA (gus)	β-glucuronidase	β-glucuronides, blue indicator product

According to Kleter et al. (2000).

selection of the transformed plant cell and to give the plant a selective advantage in the field upon spraying with the herbicide.

Furthermore selection schemes have been developed that are based on the use of special carbon sources allowing transformed plants to metabolise these substrates and permit them to grow on special media, while others are not able to use these substrates. A selection system based on the *E. coli* phosphomannose isomerase gene (*manA*) as selectable gene and mannose as selective agent has been described by Joersbo et al. (1998). Also a bacterial xylose isomerase gene (*xylA*) has been expressed in three plant species and transgenic plants have been selected on xylose-containing medium (Haldrup et al., 1998). A recent strategy is based on the inducible expression of the isopentyltransferase gene (*ipt*) from the Ti plasmid of *A. tumefaciens* under the control of a inducible promoter that increases cytokinin levels, leading to generation of shoots from transformed plant cells under appropriate culture conditions (Kunkel et al., 1999).

Non-selective reporter genes in plants, causing change of colour, fluorescence, or luminescence have also been used.

3.3. Techniques used for the removal of markers

Several methods have been developed to remove marker genes from the transformed cell. One system is based on recombination between two homologous DNA sequences, which causes a deletion of the intervening DNA segment. Marker genes located within the intervening regions are removed through site-specific recombination after transformation. Several chemically inducible site-specific recombination systems are reviewed in Hare and Chua (2002). An example is the Cre/*loxP* system (Dale and Ow, 1991). Plasmids carrying the *cre* gene, which encodes a recombinase enzyme, are used for transformation. This plasmid DNA is not incorporated into the host genome, and will be lost in a later stage. The recombinase enzyme removes the DNA sequences inserted between the *lox* sites, including the marker gene (e.g. antibiotic resistance gene). Once the marker gene is deleted, the recombinase gene itself can be segregated away in the next generation through conventional breeding (Gleave et al., 1999).

There are also other tools available to avoid marker genes in GM plants. Using transposable elements (Ac/Ds system) it is possible with the help of transposase either to create loci for precise integration of a gene construct without the need of a selection marker, or alternatively to remove a selection marker after selection has taken place (Goldsbrough et al., 1993; Yoder and Goldsbrough, 1994).

A third method is co-transformation with the gene of interest not covalently linked to a selection marker on a separate DNA, which, in principle, would allow segregating the gene of interest from the selection marker under the assumption that the two DNA molecules have been integrated into different chromosomes of the recipient.

Marker removal systems are applicable for some crop plants. Additional research is still needed before these methods are practical. In future, these methods can solve two major problems. First, consumer concerns may be less when there are no antibiotic resistance genes present in GM plants. Second, removal of marker genes after plant transformation may allow for re-use of the same marker genes in subsequent modifications of the GM plant. For most plant species, only a limited

number of effective marker genes are available and it is not desirable to create GM plants with multiple copies of the same marker gene, which could lead to gene silencing and affect expression of genes.

The Genetic Use Restriction Technology (GURT) is a system for controlling of plant gene expression. It is not a marker removal system, but this controversially discussed so-called "terminator technology" leads to sterile seeds and limits the spread of GM plants and, therefore, limits also the spread of a marker gene that may be present in the GM plant. This system prevents seeds from germinating in the next generation. It consists of a repressor gene, a recombinase gene, and a toxin gene. The whole system is characterised by three steps. Firstly, the addition of terminator genes to a crop, secondly, the initiation of the terminator process before selling the seeds by adding an inducer, and thirdly, the harvest of mature, but sterile seeds (Union of Concerned Scientists, 1998).

There are, however, also some drawbacks. The co-transformation method is very insufficient in order to create GM plants and, therefore the practical value is limited. The Ac/Ds system creates footprints (mutations) at each locus for excision and may jump for more than one time to and from unknown loci. The Cre/*loxP* system will leave a *lox* site after removal of the marker gene. The *lox* site is the target site for the Cre recombinase. If other recombinase enzymes can also target the *lox* site, unpredicted recombination may be possible. As compared to experience of safe use with selectable markers like *nptII*, there is no extended experience with these marker removal systems in commercialised GM plants. Therefore, until safety and stability of the consequences of marker removal systems have been proven, the use of selection markers including certain antibiotic resistance markers, e.g. such as listed in Group 1 of Table 1, may still be a method of interest.

3.4. Conclusions

From the perspective of food safety a possible selective advantage through antibiotic resistance conferred by HGT of antibiotic resistance genes used as markers in plants to the bacterial population is a critical factor. Furthermore, in cases where GM plants are to be placed on the market, the genes introduced by means of genetic engineering should in principle be restricted to those genes that functionally are required, such as the genes of interest, or required as technical sequences or marker genes.

At this stage of the development of marker removal systems, the consequences of applying them might create uncertainties with regard to the induction of point mutations or introduction of sequences that could enhance homologous recombination. Therefore, systems eliminating marker genes are not necessarily safer than the marker genes themselves and further research is required for the increase or assessment of their safety. In particular with the *nptII* marker, there is a 13-year history of safe use in food crops.

4. Natural HGT

Gene transfer plays an essential role in evolution and in assuring diversity in nature. In contrast to vertical gene transfer, where DNA is spread from a parent to an offspring, HGT is the transfer of DNA between cells of the same generation. By HGT it is possible to acquire new genetic information from genetically distinct organisms, and there is a potential that DNA can be spread between all three domains of life: Bacteria, Archaea, and Eukarya (Heinemann, 1991). In Syvanen and Kado (Syvanen and Kado, 1998), some of the proposed transfer events between the domains are reviewed and discussed. Most of the claimed transfer events are based on phylogenetic studies, and the majority of these happened early on when the cells were more primitive (Andersson et al., 2001; Woese, 2000), and are mostly the result of endosymbiotic or symbiotic acquisition of genes (Doolittle, 1998).

In order to evaluate the impact of the transfer of GMO DNA, it is important to consider the gene transfer occurring in nature and the mechanisms behind it. Much of the information we have in relation to bacterial gene transfer in nature is based on the information on dissemination of antibiotic resistance genes where the genes that are nearly identical at the DNA sequence level ($>95\%$) can be found in distantly related bacteria (Salyers et al., 1998).

This part will focus on the evolutionary evidence for HGT and the basic mechanisms used for HGT with special emphasis on transformation, which is the only known mechanism by which bacteria can take up DNA from plants. Once having acquired this DNA, bacteria have efficient mechanisms (e.g. transduction and conjugation) to spread the DNA further to other bacteria. Transfer of DNA from GMO either directly to human cells, or *via* bacteria, is also of concern, and will be discussed.

4.1. Evidence for horizontal transfer between species and genera during evolution

Consideration of the role of horizontal gene transfer in evolution helps to put the possible transfer of GM DNA into context by providing an indication of the likelihood and impact of different types of transfer event. The rapidly increasing availability of sequence data from diverse organisms, including complete genomes, is producing convincing evidence for the role of horizontal gene transfer in evolution (e.g. Ponting et al.,

2000; Lawrence and Ochman, 1998; Nelson et al., 1999; Syvanen and Kado, 1998; Jain et al., 1999; Lower et al., 1996). In the majority of cases, this evidence consists of showing that for a given gene, the sequence-based phylogenetic tree is seriously discordant with that generated from ribosomal DNA (rDNA) or other sequences whose inheritance is assumed to have been largely through vertical transmission (Woese, 2000). Criteria that are independent of phylogenetic tree construction are also reliable indicators of HGT, for example detection of a codon usage pattern or base composition that is anomalous for that phylum. In general, it is important to recognise that the more recent the transfer event, and the more distantly related the partners, the more convincing is the evidence for a horizontal transfer event. Thus antibiotic resistance genes often show almost identical sequences in a range of hosts that are only distantly related in their rDNA sequences and that show quite different % (G+C) contents and codon usage (e.g. Barbosa et al., 1999). On evolutionary timescale the base composition of an acquired gene will approach the base composition of the recipient organism, tending to obscure its origin.

4.1.1. Transfer among Bacteria and Archaea

Evidence is emerging from comparisons of genome sequences that in certain bacterial species (e.g. *E. coli, Neisseria*), gene acquisitions (discounting inserted bacteriophage) may account for a significant proportion, up to 17%, of the genome (Lawrence and Ochman, 1998; Lawrence, 1999; Ochman et al., 2000). There is also clear evidence that exchange has occurred between Archaea and Bacteria (Nelson et al., 1999). Microbial species may differ in their ability to tolerate additional DNA that is of minor or infrequent selective advantage.

4.1.2. Transfer among Bacteria and Eukarya

There are now many convincing examples of the acquisition by bacteria of genes from unicellular eukaryotes and it has been argued that such transfers were a major factor in early evolution (Doolittle, 1998; Woese, 2000). Examples include the presumed replacement of 10 out of 19 tRNA synthetases in spirochaetes by their eukaryotic counterparts (Wolf et al., 1999). There is also persuasive evidence for transfer of genes involved in the degradation of plant cell wall polysaccharides between anaerobic bacteria, fungi, and protozoa from gut habitats (Garcia-Vallve et al., 2000; Devillard et al., 1999).

Transfer of plasmid DNA from *Agrobacterium* spp. to the plants that they associate with is well established (see Section 2) and there is evidence that tobacco plants acquired "hairy root" genes from *A. rhizogenes* (Aoki and Syno, 1999).

Besides the HGT from bacteria to eukaryotes in early time by the evolution of mitochondria, and the following transfer of organellar genomes into the eukaryotic nucleus (Gray, 1999), there is no convincing evidence for bacterial genes transferred into human germ line cells. The initial analysis of the human genome sequence suggested that over 200 bacterial genes were horizontally transferred from bacteria to humans (Lander et al., 2001). Later publications reanalysing the data, however, opposed this conclusion by using data from non-vertebrate genomes (Salzberg et al., 2001; Stanhope et al., 2001). A few examples remain where the evolutionary history is unclear, however the authors state that rather than HGT, other biological explanations are more plausible, e.g. gene loss in non-vertebrates. The rapid evolution in sequencing eukaryotic genomes will probably shed more light on this discussion.

4.2. Horizontal gene transfer: mode and experimental evidence obtained from in situ and in vivo studies

4.2.1. DNA uptake into bacteria

Bacteria propagate by cell division resulting in two identical cells (vertical transfer), so the only way bacteria can receive new DNA is by getting it from other cells by HGT. Bacteria have developed several efficient mechanisms to transfer DNA (transformation, transduction, and conjugation) and the genes of bacteria can be viewed as a huge gene pool, which can be shared with virtually any member of the bacterial world. Besides these inter-cellular gene transfer mechanisms, many bacteria harbour mobile elements in their genome, such as transposons, insertion sequences, and integrons, which contribute to the overall genetic plasticity of bacteria.

4.2.1.1. Transformation. Natural transformation of prokaryotes is the active uptake of free extracellular DNA that becomes heritably incorporated into the genome (Lorenz and Wackernagel, 1994). Eukaryotic cells have no known mechanisms to deliver their DNA to bacteria, so transformation is the only gene transfer mechanism that can be involved in the uptake of GM plant DNA by bacteria. Transformation occurring under natural conditions requires several steps before the plant DNA can be part of the genome of a bacterial cell. First, the plant cell must release DNA into the extracellular space; this may happen during food processing or passage in the gastrointestinal tract. The released DNA must persist in the environment. There is limited knowledge about effects of food components or gut microenvironment upon DNA persistence and integrity in the food chain (see Section 5).

The relevant bacteria must possess or develop competence, which is a physiological state that permits the uptake of exogenous DNA (reviewed in Dubnau, 1991). By now, more than 80 strains among more than 30 species have been found to be naturally transformable—widely distributed among Bacteria and also found in

one example from the Archaea (Lorenz and Wackernagel, 1994; Tonjum et al., 1995). Among these are several that are important for the food chain, e.g. *Bacillus subtilis*, *Acinetobacter* sp., *Lactobacillus lactis*, *Campylobacter* sp., *Helicobacter pylori*, and Streptococci. The increasing list of published sequenced genomes from bacteria shows that DNA sequences homologous to competence genes can be found in many bacteria, e.g. *Lactococcus lactis* that have been regarded as non-competent (Bolotin et al., 2001; Håvarstein, 1998; http://www.tigr.com). Some of these may just be reminiscent of genes or have other functions than competence development. Among the high number of non-cultivable bacteria, there may also be competent bacteria, all this indicating that the capacity to become competent may be more widespread than earlier presumed.

Competence in most naturally transformable bacteria is a transient and inducible physiological property, only *Neisseria gonorrhoeae* is known to be constitutively competent. In *Streptococcus pneumoniae* and *B. subtilis* competence is induced by secretion of a small polypeptide (competence factor) from the cells when a critical cell concentration has been reached (Lorenz and Wackernagel, 1994).

The process of DNA uptake has been extensively reviewed by Dubnau (Dubnau, 1999), and begins with the binding of double-stranded DNA (dsDNA) to the cell surface. Some species, like *Haemophilus influenza*, only take up and integrate DNA from their own or close relatives based on short recognition sequences in the DNA (Danner et al., 1980), others like *Acinetobacter calcoaceticus* and *B. subtilis* take up DNA independent of the sequence (Dubnau and Cirigliano, 1972; Palmen et al., 1993). During translocation, one of the strands is degraded and the remaining strand is stabilised in the cytoplasm by DNA-binding proteins. Natural transformation by chromosomal DNA is strongly affected by restriction endonucleases (RE) in *Pseudomonas stutzeri* (Berndt et al., 2003), whereas in other species, including *B. subtilis* and *S. pneumoniae*, a strong effect was not observed (Majewski, 2001). Natural transformation by plasmid DNA results in duplex DNA molecules formed from single strands taken up and these can be targets for restriction endonucleases (RE) if the methylation pattern differs from that of the new host. The final step requires the heritable incorporation of the DNA either (a) through integration into the chromosome by homologous recombination or (b) under condition that the incoming DNA is carrying an autonomous replicon capable of stable replication in the recipient. The integration can affect directly the expression of genes already present in the recipient organism, either by inactivation or mutation (gene silencing) or by repairing defective genes by marker rescue. The expression of genes present in the recipient can also be affected indirectly due to pleiotropic effects. Functional expression of an acquired gene will require the presence of regulatory elements. These elements may be those of the host or could be constituents of the transforming DNA. Furthermore, for a proper expression of the acquired gene, an adaptation of the expression machinery of the recipient (homing) of the DNA may be needed.

Circularisation of linear DNA fragments inside the GM plant containing a bacterial origin of replication, as described in section 2, remains a theoretical possibility that so far has not been found.

Besides the natural transformation system, spontaneous transformation has been recognised in the non-competent bacteria *E. coli* resulting in the uptake of double-stranded (plasmid) DNA in milk and water (Bauer et al., 1999; Baur et al., 1996; Woegerbauer et al., 2002). The uptake of the dsDNA does not require the expression of proteins, but happens by an unknown mechanism.

4.2.1.2. Transduction. Transduction is transfer of DNA from one prokaryotic cell to another by bacterial viruses (bacteriophages). The mechanism results from the erratic packaging of non-viral DNA (bacterial chromosome, plasmid) into phage particles, which transfer the DNA to the next host cell by adsorption and DNA injection. Erratic DNA packaging can occur during normal phage proliferation and may encompass any part of the cellular genome (general transduction). Also during induction of a prophage from its lysogenic state to proliferation, bacterial DNA located next to the prophage insertion site in the host genome may occasionally be incorporated in phage particles (specialised transduction). Most bacteriophages are quite narrow in their host range, restricted to one species; however, some phages have a broad host range (Sayre and Miller, 1991).

Lysogenic conversion is the consequence of gene transfer mediated by bacteriophages where the phage genome is incorporated in the bacterial host. The genes for many bacterial toxins from both Gram-negative and Gram-positive pathogens are carried on these temperate phages (Cheetham and Katz, 1995). Many of these bacteria are natural inhabitants of the human gastrointestinal tract and lysogenic conversion is believed to happen in this environment. Also plasmids can be carried from one bacterial cell to a new host by transduction, because linear plasmid replication intermediates are packaged and form a circular entity in the new host by recombination (Ripp and Miller, 1995).

4.2.1.3. Conjugation. Transfer by conjugation requires cell-to-cell contact between donor and recipient, and is mediated by large conjugative plasmids or conjugative transposons in the donor cell. Besides being self-transmissible, conjugative plasmids and transposons are often capable of mobilising smaller non-conjugative

plasmids and chromosomal DNA. Conjugation is considered to be more promiscuous than transformation and transduction because of less restriction on the similarity between donor and recipient. The *Agrobacterium* conjugation system, where the Ti-plasmid is transferred to plants, is an example of inter-kingdom DNA transfer.

4.2.1.4. In situ and in vivo studies. Table 3 shows selected examples illustrating HGT by different mechanisms in various environments that have relevance for the food chain. Regarding DNA uptake by bacteria, conjugation is best studied and there are numerous examples of inter-species and inter-genus transfer of DNA by conjugation in food and in the intestine. This should therefore be regarded as a common and very efficient way to transfer DNA in these environments. Recent studies indicate that the diet has an effect on conjugation in vivo. Consumption of yoghurt, milk, and lactose lowered plasmid dissemination, presumably by affecting enzyme activities (Maisonneuve et al., 2000, 2001, 2002).

Bacteriophages are quite abundant and ubiquitous in the environment and have been found both in soil and aquatic environments (Jiang and Paul, 1998; Miller, 1998; Paul, 1999). They are also found in great numbers in the intestine of animals and humans (Salyers, 1995). Lysogens of Shiga toxin (Stx)-converting phages were capable of transducing an *E. coli* recipient in the murine gastrointestinal tract (Acheson et al., 1998). This study suggests that the emergence of the many Stx-producing *E. coli* serotypes and the Stx-producing non-*E. coli* genera may have originated from the mammalian gastrointestinal tract. The filamentous phage CTXΦ codes for the cholera toxin of *Vibrio cholerae*, and lysogenic conversion has been shown to be more efficient within the gastrointestinal tract of mice than under laboratory conditions (Waldor and Mekalanos, 1996). These studies may suggest that the gastrointestinal tract (and other mucosal surfaces) may be hot spots for gene transfer by transduction. These sites may provide the environmental signals necessary for expression of important proteins mediating interactions between the phages and their host bacterium.

Uptake of DNA from GM plants and transformation of micro-organisms may occur along the complete chain of events, extending from the field to food processing and storage, to the digestive tract and finally again to the environment. The highest probability of transformation events to occur can be expected when the "concentrations" of the "reactants", i.e. transformable DNA and competent bacteria, are high. Highest microbial counts in a food of plant origin are found in food fermentation processes such as occur in the production of Sauerkraut, fermented olives, tomatoes, cucumbers, egg-plants (Buckenhüskes and Hammes, 1990; Buckenhüskes, 1993), beer, sourdough, and many Asian foods based on soy, beans, peanuts, cereals, or coconut. Fermentation processes are finally also important in post harvest treatment of coffee and cacao. The numbers of bacteria involved in these processes may exceed 10^9 cells per gram, and the species involved comprise bacteria from virtually all genera included in the group of lactic acid bacteria, bacilli (in Asian food), acetic acid bacteria, and enterobacteria. Representative forms of many of these organisms of these groups have been shown to be transformable with DNA (Lorenz and Wackernagel, 1994).

Similar to food fermentation, the production of silage from GM plants may allow HGT by transformation. Transformation at this stage would affect bacteria that enter the intestines of cattle, whereupon the acquired DNA may spread within the intestinal flora by all mechanisms of HGT. Consequently, it can cause a risk for humans, as it may be transferred to the human intestinal flora via consumption of food of animal origin.

It is important to distinguish clearly between transformation events that involve incorporation of sequences into the host chromosome by homologous recombination and those that involve acquisition of self-replicating plasmids. The latter is mainly relevant to GM bacteria that might be used in food or in the environment. Free plasmids are not found in GM plants, although the possibility has not been formally eliminated that plasmid DNA upon insertion into the chromosomes of GM plants might be reconstituted. Bacterial acquisition of plasmids by conjugation will be limited by the plasmid host range for replication, which is quite narrow for most commonly used vectors. HGT from GM crop plants to a recipient bacterium is only believed to take place by homologous recombination. Homologous recombination can potentially result in the acquisition of DNA of any origin provided that it shares homology with the chromosome of a naturally transformable recipient bacterium. Examples of the insertion of GM plant DNA into bacterial chromosomes under simulated environmental conditions have all involved highly homologous regions between an antibiotic resistance marker gene and an identical gene present in the recipient bacterial chromosome, known as marker rescue experiments (Gebhard and Smalla, 1998; Nielsen et al., 2000; de Vries and Wackernagel, 1998). The key question must be whether such events can lead to the capture of adjacent genes flanking the region of homology. Currently, we can state that this possibility does exist where the homologous region is present in a circular molecule, or where multiple regions of homology are present. Based on most recent findings in different bacterial species (de Vries and Wackernagel, 2002; Prudhomme et al., 2002; Meier and Wackernagel, 2003a), a "homology facilitated illegitimate recombination" can

Table 3
Examples of horizontal gene transfer in the food chain

Mechanisms	Recipient	Donor	Environment	Reference
Transformation	*Escherichia coli*	Plasmid DNA isolated from *E. coli*	Milk, vegetable/fruit juices, soy drink, canned cabbage, canned soy beans, canned shrimps, mixed vegetables, spinach potato	Bauer et al. (1999)
	Bacillus subtilis	*B. subtilis* homologous chromosomal DNA	Milk, chocolate milk	Bräutigam et al. (1997)
	B. subtilis	Plasmid DNA isolated from *Lactococcus lactis*	Milk	Zenz et al. (1998)
	Acinetobacter	*E. coli* homologous chromosomal DNA	Surface of raw potatoes, sausage	Schön S. et al., unpublished results
	Acinetobacter	Transgenic plant DNA	Liquefied cooked non-transgenic potatoes	Schön S. et al., unpublished results
	Streptococcus gordonii	Plasmid DNA isolated from *E. coli*. Homologous chromosomal DNA from *L. lactis* and *E. faecalis*	Human saliva	Mercer et al. (1999a); Mercer et al. (2001)
	Campylobacter jejuni	*C. jejuni* homologous chromosomal DNA	Intestinal tract of chicken	Boer et al. (2002)
Transduction	*Streptococcus thermophilus*	Phage a10/J9 (plasmid DNA)	Yoghurt	Heller et al. (1995)
	E. coli	Phage H-19B	Intestinal tract of mice	Acheson et al. (1998)
	Vibrio cholerae	Phage CTXΦ	Intestinal tract of mice	Waldor and Mekalanos (1996)
Conjugation	*E. coli*	*E. coli* (R plasmid)	Minced meat on a cutting board	Kruse and Sørum (1994)
	E. coli	*Aeromonas salmonicida* (R plasmid)	Raw salmon on a cutting board	Kruse and Sørum (1994)
	Lactobacillus curvatus, *Staphylococcus carnosus*	*L. curvatus* (pAMβ1)	Fermentation of sausage	Hertel et al. (1995); Vogel et al. (1992)
	L. lactis	*L. lactis* (pIL205)	Fermentation of cheese	Gabin-Gauthier et al. (1991)
	Enterococcus faecium	*E. faecium* (pAMβ1)	Intestinal tract of chicken	Netherwood et al. (1999)
	Enterococcus faecalis	*E. faecalis* (pCF10)	Intestinal tract of pigs	Licht et al. (2002)
	E. coli	*E. coli* (60 kb plasmid)	Rumen fluid ex vivo	Scott and Flint (1995)
	Bacteroides sp.	*E. coli* (pRK2013, pRRI207)	Intestinal tract of germ-free mice	Garrigues-Jeanjean et al. (1999)
	E. coli	*Serratia liquefaciens* (R plasmid), *E. coli* (pBR plasmids), *Enterococcus faecalis* (pAT191)	Intestinal tract of germ-free and HFA mice	Doucet-Populaire et al. (1992); Duval-Iflah et al. (1980); Duval-Iflah et al. (1994)
	E. faecalis	*L. lactis* (pIL205), *Lactobacillus reuteri* (pAMβ1)	Intestinal tract of germ-free and HFA mice	Gruzza et al. (1994); Morelli et al. (1988)
	Listeria monocytogenes	*E. faecalis* (Tn*1545*)	Intestinal tract of germ-free mice	Doucet-Populaire et al. (1991)
	E. faecalis or human flora	*L. lactis* (genetically modified Tn916 and pIL205)	Intestinal tract of germ-free mice and mice associated with a human flora	C. Alpert, to be published; GMOBILITY (2003)
	L. lactis	*L. lactis* (pAMβ1)	Intestinal tract of germ-free rats	Schlundt et al. (1994)
DNA uptake into mammalian cells	Liver and spleen tissues	Soybean leaves	Mice	Hohlweg and Doerfler (2001)
	Leukocytes, macrophages, B cells, T cells, liver cells, fetal tissues	Phage M13 DNA, plasmid DNA	Mice	Schubbert et al. (1997); Schubbert et al. (1998)
	Cells of the immune system	Attenuated *Salmonella typhimurium*	Mice	Darji et al. (2000)
	HeLa, CHO, COS-1	*E. coli* (inv^+)	Cell culture	Grillot-Courvalin et al. (1998)
	Epithelial cell, macrophages	Wild-type *Shigella flexneri, L. monocytogenes*	Cell culture	Grillot-Courvalin et al. (2002)

HFA, human flora associated.

increase the frequency of a basically illegitimate incorporation of genes when heterologous DNA of up to 2.9 kb is flanked by a short sequence homologous to the integration site. These findings show that the incorporation of a foreign gene and its expression is basically possible, although such an event has not yet been shown to take place in environments in which the DNA is released from the plant genome as it occurs, e.g. in the intestinal tract.

Reported examples of transformation in food matrices are cacao drink, UHT milk, UHT soy drink, tomato juice, carrot juice, and orange juice (Bauer et al., 1999; Bräutigam et al., 1997; Zenz et al., 1998). In all these cases, the DNA was, however, not taken from the GM plant food, but added to the food along with the bacteria and thus the results are indicative that the food matrix has the potential to permit transformation. Furthermore, one result obtained in an EU-supported programme showed that *Acinetobacter* could take up the genetic information by marker rescue from GM plant DNA added to boiled potato (Schön et al., unpublished results in the framework of the GMOBILITY project). *Acinetobacter* is a spoilage organism and has also been detected in the intestines. Thus, a flow of information via food to intestinal bacteria is not unlikely. The EU supported project GMOBILITY showed that transformation of *Streptococcus gordonii* occurred under worst case conditions mimicking the oral cavity, the rumen, and large intestine by homologous recombination (van der Vossen, unpublished results in the framework of the GMOBILITY project). Gene transfer by transformation has also been studied extensively in vivo in the intestinal tract of germ-free animals with different recipients (enterococci, *E. coli*, *Acinetobacter*, *B. subtilis, S. gordonii*) without the detection of any transformants (Midtvedt et al., unpublished results; Schön et al., unpublished results; Wilcks and Jacobsen, unpublished results in the framework of the GMOBILITY project).

However, all these studies used homologous recombination as the mechanisms of DNA incorporation, and therefore no "new" genetic information was introduced by transformation.

There are also examples of extensive research that could not demonstrate gene transfer in matrices that are very relevant for the food chain. Heller et al. (1995) studied the ability of two yoghurt cultures, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subps. *bulgaricus*, to participate in gene transfer by conjugation, transduction, and transformation. While *S. thermophilus* was able to participate in gene transfer by all three mechanism *in vitro*, no transfer at all into *L. delbrueckii* subps. *bulgaricus* was observed. When performing *in situ* studies with *S. thermophilus* in yoghurt, no transfer by conjugation could be observed (Heller et al., 1995; Kleinschmidt et al., 1993), maybe because yoghurt does not allow intimate cell-to-cell contact.

4.2.2. DNA uptake into mammalian tissues

The work of Schubbert et al. (1997, 1998) showed that following feeding of naked M13 bacteriophage DNA replicative form (dsDNA circular) to mice, small DNA fragments could be detected not only in gut contents but also in cells of the intestinal wall, liver and B and T cells (see Table 3). Evidence was produced for covalent linkage of M13 DNA to mammalian chromosomal DNA (Schubbert et al., 1997) and for transplacental transfer of the ingested DNA in pregnant mice. This suggests that DNA from the gut can enter mammalian cells. Gut cells are exposed constantly to DNA fragments derived from food and DNA may enter various tissues as shown for maize chloroplast DNA in cattle and chicken after feeding with maize (Einspanier et al., 2001).

Recent interest in gene therapy has led to new insights into the fate of foreign DNA in mammalian tissues. Invasive bacterial strains have been used to deliver stably inherited genes into mammalian cells (Courvalin et al., 1995; Grillot-Courvalin et al., 1998). Naked DNA is extensively broken down following injection into tissues (Barry et al., 1999). Naked DNA of bacterial origin can be distinguished from DNA of eukaryotic origin through the presence of unmethylated CpG dinucleotides in particular base contexts (Sato et al., 1996; Krieg, 1999; Beever and Kemp, 2000). Clearly, the behaviour of foreign DNA of plant and bacterial origin within mammalian tissues may differ markedly.

4.3. Conclusions

HGT is a natural process and an integral part of microbial life with a high evolutionary potential. The transfer of genes, along with mutations, contributes to the genetic adaptation of bacteria to a changing environment by expressing genetic information, which has evolved in other cells. The spread of antibiotic resistance genes is an example of transfer of genes emerging with the introduction of antimicrobial agents providing the selective pressure. It is important to keep in mind that the transfer event by itself, the integration of a gene and its expression, is not responsible for the spread of genes, but that the selective pressure drives the spread of genes in the particular environment. The possible co-existence and co-transfer of genes neighbouring the gene encoding antibiotic resistance that are susceptible to the environmental pressure further complicate this.

Genes of bacterial origin in GM plants may theoretically be capable of being taken up by bacteria in the food chain. However, the chance of acquiring the same genes from another bacterial species in the environment is much greater. The probability of a gene transfer from transgenic plants to bacteria has been estimated to be 2×10^{-11} to 1.3×10^{-21} per bacterium and that of a gene transfer by conjugation between soil and enterobacteria is, however, 10^{-1} to 10^{-8} per donor cell (Dröge et al., 1998).

Since the integration of foreign DNA by bacteria after transfer is much more likely when there are homologous sequences between the foreign DNA and the bacterium, the probability of expression of DNA of non-bacterial origin in gut bacteria following transfer will be even lower than the low transfer probability. Since any bacterial DNA that has been used in the construction of the transgenic plant may serve as a site for homologous recombination, increasing the probability of gene transfer from GM plant DNA to bacteria in the gut and elsewhere, such sequences should be kept to a minimum.

If we accept the possibility of rare uptake of DNA from food by mammalian cells, are there serious consequences for safety? Two points are of paramount importance here. First, DNA sequences of various origins (plant, animal, microbia, virus) have been present in human food and farm animal feed throughout history. Therefore, most sequences to be found in GM crop plants will have entered the mammalian gut before present time. Second, it is clear that uptake is very much more probable for somatic cells (particularly those of the gut and immune systems) than for germ line cells. This may account for the almost complete lack of evidence for sequences of plant origin in mammalian genomes. Somatic cells of the gut lining have a rapid turnover, such that the most likely fate of most modified cells is to be lost in the faeces. These considerations make deleterious consequences improbable.

5. Availability of DNA from food and feed for gene transfer

The dietary exposure to GMO DNA is a crucial parameter in the overall risk assessment of the consumption of GMO products in relation to HGT of DNA from GMOs, present in the food and feed to the intestinal microflora. This exposure depends on the amount of consumed GMO material; the quantity of recombinant DNA present in the specific transgenic plants; the food and feed processing, which may reduce the amount of DNA and thus recombinant DNA prior to consumption; whether or not the DNA is released from the plant material and therefore would be available to intestinal microflora; and finally, how stable DNA would be along the passage through the intestinal tract.

5.1. Dietary exposure

A first step in the exposure assessment is the identification of those food products that contribute most to the daily intake of GMOs. This identification relies heavily on the availability of food consumption data. Fortunately, these data are available from nation-wide food consumption surveys performed in several countries (Jonas et al., 2001). After identifying the food products that contribute to the exposure to GM DNA, it is necessary to determine the amount and integrity of the DNA present. Food processing (e.g. heating steps) and the characteristics (acidic pH conditions) of the food matrix may promote DNA degradation (Jonas et al., 2001) and may strongly affect the quantity and integrity of the DNA. Both the daily intake of GMO DNA and data on the quantity and integrity of the DNA are necessary to determine the exposure to GM DNA via the food. However, when assessing the risk of HGT in the human intestinal tract we also need to consider the stability of the DNA within the gastrointestinal (GI) tract. Within the GI tract, DNA is exposed to various conditions (e.g. different pH values) and enzymes (e.g. pancreatic DNases) that have a degradative effect. However, DNA that remains after exposure to these degradative influences may reach the colon and become available to bacteria being present there in high concentrations. Thus, transformation may occur along the GI tract with a particularly high chance in the colon, as this part of the GI tract contains the largest amounts of bacteria and the lowest DNA degrading activity.

Dietary assessment studies can be performed using the point estimate approach or the probabilistic approach. Of these two, probabilistic approaches allow, in the case of dietary exposure to GM DNA, the inclusion of variability in GM DNA levels present in food products as well as variability in food consumption patterns within a population. Probabilistic modelling using this information will result in a distribution of possible daily exposure levels for a certain population. An example of a probabilistic method is the Monte Carlo technique (Petersen, 2000). Compared to the point estimate approach, which results in single "worst case" extreme estimates of exposures, probabilistic methods better simulate what happens in real life. When using probabilistic modelling to assess the risk of HGT in the GI tract, information is needed on the consumption of the GMO ingredient via the diet, DNA quantity and integrity per food product, DNA survival in the GI tract and transfer efficiency within the GI tract. Eventually, the outcome of these studies will be an estimation of the risk of HGT of GM DNA present in the food to the intestinal flora of humans.

5.2. Persistence of DNA in food and feed

A prerequisite for transformation is the availability of DNA. Transforming DNA must obey some minimal specifications related to structure, sequence, and length. The length of the fragments has different implications in relation to their transforming potential:

- To transfer a complete gene or a functional regulatory element, the minimal length ranges

between about 150 and 6000 bp, and 100 and 500 bp, respectively.

- To achieve the insertion of a sequence via homologous recombination, the minimal length ranges between 2040 bp in *E. coli* and 280 bp in *Campylobacter coli* (Richardson and Park, 1997). In addition, the shorter the fragments are, the lower is the uptake efficiency. For example, a decrease from 10,000 to 1000 and 300 bp reduces the efficiency by factors of 10–2 and 10−6 respectively in transformation (Palmen and Hellingwerf, 1997; Meier and Wackernagel, 2003b).

The integrity of DNA is affected by various parameters (see Table 4) such as temperature, enzymatic activities, a_w, pH, pressure, shear forces, and reactive chemicals (radicals). Enzymatic activities, such as exonuclease and endonucleases including DNaseI and DNaseII, have their optimal pH range for activity. DNaseI is optimally active at neutral pH and DNaseII is optimally active around pH 5.0 (Baker et al., 1998). Low pH conditions also affect DNA integrity via depurination reactions, which affect the adenine and guanine bases from the naked DNA fragments (Klinedinst and Drinkwater, 1992). The review of Jonas et al. (2001) discusses the DNA degrading activities in relation to food safety. As far as is relevant to food processing and storage, and to the gastrointestinal tract of mammalian species, these parameters will be extensively discussed in the next paragraphs in view of the transforming potential of DNA.

The stability of DNA is relevant, because it is a prerequisite for its potential for natural transformation of native bacteria. From a chemical point of view, donor DNA and the recipient organism are the reaction partners in a transformation event. To assess the probability of an event, the concentrations of the two "reactants" as well as all chemical (biochemical), physical, and biological properties of the "reacting system" have to be taken into consideration. In a natural system, double-stranded plasmid DNA and linear duplex DNA are the transforming nucleic acid species. Their availability in diverse environments after DNA release from a GMO is of key importance for the safety assessment. The following factors make the estimation of the exposure of a bacterium or an enterocyte to transforming recombinant DNA difficult:

- Competition with a large surplus of non-recombinant DNA in the diet as well as from endogenous sources, such as the intestinal microbial population or shed epithelial cells.
- The effect of the diet on DNA degradation by binding to or inclusion into compartments of particles or compounds that protect DNA from enzymatic attack.
- The effect of food processing and food storage on the amounts of DNA present and DNA integrity.

5.2.1. Degradation of DNA during food processing and food storage

The structure of DNA and the degradation of the macromolecule have been reviewed extensively by

Table 4
Factors affecting the integrity of DNA molecules in the environment and food

Factor (effect)	Importance in food	Site of action on DNA
Shear forces	variable between minimal to high during processing of foods	sugar-phosphate backbone (double-strand breaks)
Enzymes	e.g. DNases; potentially high in foods originating from tissues of plant or animal origin or subjected to microbial activities	sugar-phosphate backbone, nucleobases (modification, hydrolysis)
pH	most foods are in the neutral pH or weak acid range, then weak effects; during processing pH may vary between 1 to ~12, then strong effects	base-sugar bonds at low pH (hydrolysis) with secondary effects on sugar-phosphate backbone (strand breaks), bases (deamination)
Temperature	when foods are subjected to thermal processes (> 80 °C), then strong effects	denaturation of DNA, various hydrolysis and deamination reactions, $Q_{10} = 2–3$
Water activity	low in dried foods; high at high water activity	all reactions leading to damage
Chemical agents (e.g. reactive oxygen species, bisulfite, nitrite, alkylating agents)	partially high when SO_2 is used as additive, curing agents are employed or when stored under air	nucleobases (chemical modification, loss of bases)
Protective interactions	in certain food matrices highly important; variable influences by adsorption of DNA to particles, inclusion into compartments, or interaction with polyamines, cations etc.	protection against various damaging factors including shear forces, enzymes, heat etc.

Lindahl (1993) and have also been discussed by Jonas et al. (2001). Native DNA is present virtually exclusively in living cells or resting forms. As soon as a cell dies, any repair of the inevitably ongoing damage stops and enzymatic degradation starts. Factors of the food chain listed in Table 4 affect the degradation.

As a result of degradation, DNA fragments of variable size will be produced, which can be detected. Information can be obtained on the length of DNA fragments, on the integrity as determined by using the DNA as template for in vitro amplification by polymerase chain reaction (PCR), or on the "biological activity", i.e. whether or not the DNA can serve as transforming molecules.

DNA stability in food can be addressed indirectly by analysing the DNA content of processed food (see section 5.2.1.3.) and directly by monitoring DNA degradation in food processing (see Section 5.2.1.1.). In addition, the protective effect of food ingredients on DNA stability has been studied (see Section 5.2.1.2.).

5.2.1.1. DNA degradation during food processing. The degradation of DNA during the production process of sugar was investigated by Klein et al. (1988). It was concluded that the sugar beet chromosomal DNA was strongly degraded during processing by nucleases. By spiking of intermediate products with pUC18 DNA and analysis by competitive PCR, an overall reduction of DNA by a factor $>10^{14}$ was determined. Gawienowski et al. (1999) observed also a strong reduction in DNA concentration during sugar production from maize. At the other extreme, GM sequences were detectable in maize silage after 7 months (Hupfer et al., 1999). There could therefore be many circumstances where GM plant material in animal feed, or forming part of human foodstuffs, would contain incompletely degraded DNA. DNA survival in foods, as in the wider environment, can be expected to depend on a variety of factors including nucleases, reducing agents, metal ion concentrations, and the presence of binding agents (e.g. Khan et al., 2000; Jain et al., 1996).

Forbes et al. (1998) analysed the effect of grinding and milling, heat treatment, and steam pressure on feed sources. Grinding and milling did not cause significant disruption of the plant DNA. DNA fragments smaller then 21 kb in size were not detected. Heat treatment at around 94 °C of wheat grains for 30 min resulted in a much stronger disruption of the DNA (50–250 bp). The application of temperatures between 100 and 150° C during 30 min caused the total disruption of the DNA (<100 bp). Also studies with heat above 90 °C in combination with high pressure (>1.6 kg/cm^2) showed extensive disruption of plant DNA.

The fate of DNA in the course of production of wheat bread was studied by Straub et al. (1999a) and it was observed that DNA was degraded during fermentation to <10 kbp and at the stage of baking to <500 bp. Roundup Ready® specific sequences of 172 bp remained detectable throughout. The baking process of Mehrkornbrot and Mohnschnitte (both containing maize) and wheat bread (containing soy flour) was investigated by Moser et al. (1999). PCR fragments <200 bp from maize and soy were detectable throughout the process and the extent of degradation was affected by the food matrix and the conditions prevailing during baking, e.g. heat and pH. The degradation of DNA during the production of soymilk was monitored by Kharazmi et al. (unpublished results obtained in the framework of the GMOBILITY project). Remarkably, fragments of up to 1.7 kbp were still detectable in the final product. During the production of instant "masa mahrina", maize DNA is efficiently degraded by a step of alkaline cooking to fragments <500 bp (Kharazmi, Hammes and Hertel, unpublished results obtained in the framework of the GMOBILITY project).

5.2.1.2. Protective effects of food ingredients. Investigations of the persistence of recombinant plasmid DNA in summer sausages revealed that the complex food matrix meat can exert a protective effect against DNA degradation (Straub et al., 1999b). Furthermore, in vitro experiments showed that ingredients such as arginine, polyamines, and biogenic amines (positively charged molecules) exerted protective effects against an enzymatic attack (DNaseI) detected by PCR and transformation experiments with *B. subtilis* (Bräutigam and Bauer, Hammes, and Hertel, unpublished results).

5.2.1.3. Studies on DNA persistence in food. In complex food matrices, especially when subjected to multiple processing combinations, a prediction of DNA persistence is difficult and therefore needs experimental verification.

Studies of DNA persistence in GMO-derived foods have been performed in numerous food matrices, and the focus was mainly directed to the traceability of recombinant DNA. In this case the minimum length of the "template"-sequence is about 100 bp. It is conceivable that the smaller the DNA-pieces the longer "DNA" persist. With regard to evaluation of a possible HGT by means of transformation, the length of the fragments has different implications.

The study of the traceability of DNA from GMOs is commonly performed by PCR methods. Data are available for processed food, such as shown in the following. Lipp et al. (2001) detected DNA fragments of 118 bp and 195 bp from the *p35S* promoter and *nos* terminator in biscuit, polenta, or infant formula containing GM maize and/or soybeans in a range of 2 to 100%. Eukaryotic DNA of the 18S recombinant RNA (rRNA) (137 bp) was amplified by Pauli et al. (2000) from various foodstuffs subjected to different degrees of

processing, whereas species-specific genes (*zein* gene, 277 bp; *lectin* gene, 118 bp; *patatin* gene, 146 and 272 bp) could not be detected in highly refined foods such as corn starch or refined corn oil. On the other hand, in rapeseed oil PCR fragments up to 350 bp of the plant specific PE3-PEPCase gene of *Brassica napus* were detected by Hellebrand et al. (1998). It should be noted that this concerned cold-pressed oil, which is a product that as such is not available to the consumer. In soy protein concentrates and processed foods containing this concentrate, soy-specific DNA of *lectin* gene (414 bp) was detected by Meyer et al. (1996). In tofu, soy protein isolates, and soy milk, PCR fragments of the *lectin* gene at a length of up to 714 bp were detectable, and during long-time storage of soy milk over 14 days at 8 °C, they remained stable (Bauer and Kharazmi, Hammes and Hertel, unpublished results obtained in the framework of the GMOBILITY project). PCR products of 145 bp of the *lectin* gene were demonstrated in soy lecithin by Wurz et al. (1999) and in numerous soybean or maize products, GM-specific sequences of the Roundup Ready® soybean, maize Bt176, Bt11, or Mon810 were detected by Busch et al. (2001).

Apart from studies taking advantage of the PCR technology for analysing the stability of DNA in the food environment, such can also be studied by transformation of natural competent bacteria. Transformation has been reported for *A. calcoaceticus* (Lorenz et al., 1992; Chamier et al., 1993; de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Nielsen et al., 2000) and for *P. stutzeri* (Sikorski et al., 1998) by plasmid and chromosomal DNA surviving the addition to soils. *E. coli* has been shown to be capable of transformation in the presence of Ca^{2+} concentrations found in ground waters (Baur et al., 1996). Genetic transformation of *E. coli* and *B. subtilis* by free DNA added to a variety of foodstuffs has been demonstrated (Hertel et al., 1995; Bräutigam et al., 1997; Bauer et al., 1999).

5.2.2. Fate of DNA in the gastrointestinal tract of mammalian species

The gastrointestinal tract from oral cavity down to the colon of mammalians contains various numbers and species of micro-organisms. These micro-organisms are believed to be potential recipients for transformation by DNA ingested and released from the food by physical, chemical, and enzymatic activities of the digestive tract. This DNA is concomitantly subject to degradation by the action of pancreatic nucleases in the small intestine and other enzymatic activities (reviewed by Jonas et al., 2001) as well as chemical degradation by low pH conditions in the stomach. Although the pancreas is a well known source of nuclease activity in mammalian species as reviewed by Jonas et al. (2001), other exocrine cells such as found in the parotid gland (oral cavity) have been described to produce DNase activity (Takeshita et al., 2000). Reports from two decades ago described DNA degrading activities extracted from the intestinal mucosa (Anai et al., 1983; Nagae et al., 1982; Keys and Zbarsky, 1980). Shimada et al. (1998) supported evidence for the presence of DNase1 secreting exocrine cells known as Paneth cells in the crypts of the human small intestine. This DNase1 is hypothesised to be an important component of the host intestinal immune defence system in addition to antibacterial proteins, immunoglobulins, and tumour necrosis factor, which are released from the Paneth cells. In addition to the DNA degrading activities of the host, the microflora of the gastrointestinal tract is also involved in DNA degradation. Some ruminal *Prevotella* species produce abundant extracellular DNase activity (Avgustin et al., 1997; Accetto and Avgustin, 2001). Also other ruminal bacteria such as *Selenomonas ruminantium*, *Streptococcus bovis* and *Bacteroides ovatus* release nuclease activity in culture (Al-Khaldi et al., 2000).

Despite these excreted DNA degrading activities, plasmid DNA added to human saliva showed a half-life of approximately 50 seconds in in vitro studies (Mercer et al., 1999a), although degradation is probably more rapid in vivo (Mercer et al., 2001). DNA exposed to partial degradation by saliva was able to transform naturally competent cells of *S. gordonii* (Mercer et al., 1999b). Free plasmid DNA degradation in a human colon flora was shown to proceed under in vitro conditions at a rate in which half of the linearised plasmid DNA could be detected after approximately six minutes (van der Vossen et al., 1998). From research in the European 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 below detection level. Ex vivo analysis of DNA persistence in the chyme of the small intestines of rats and pigs showed that degradation of DNA proceeds faster in the latter (Wilcks and van der Vossen, unpublished results from the GMOBILITY project). In other parts of the GI tract of these animals, DNA degradation proceeds less fast. Despite rapid DNA degradation in the chyme of the small intestine, DNA was shown to be transiently present in the GI tract of rats and could reach the colon at detectable levels (Aarts and Wilcks, unpublished data from the GMOBILITY project). High molecular weight DNA has also a short half-life in ruminal fluid (Ruiz et al., 2000; van der Vossen, unpublished results from the GMOBILITY project).

Although DNA and RNA are generally held to be rapidly degraded in the mammalian gut (e.g. McAllan, 1980), this degradation is not complete and is not instantaneous. Incompletely degraded fragments of double-stranded M13 bacteriophage DNA were detectable in the faeces of mice for many hours after feeding the free DNA (Schubbert et al., 1994). Fragments of

M13 DNA reached the blood stream and various mouse tissues and evidence was provided that it was covalently linked to mouse DNA (Schubbert et al., 1997). The latter observation was questioned (Beever and Kemp, 2000). Hohlweg and Doerfler (2001) showed that plant-associated naturally fed DNA is more stable in the intestinal tract of mice than naked DNA. These authors could amplify a soybean nuclear gene isolated from spleen and liver of soybean leaves-fed mice. Negative data from additional research argued against the germline transfer of orally administered DNA. Data of Klotz et al. (2002) indicated PCR detectable short maize chloroplast DNA fragments (199 bp) in porcine intestinal juices up to 12 h after feeding of maize. In contrast to the data of the previous authors, maize DNA was not found in any pig organ investigated. This suggests that transient transfer of short forage DNA fragments into the organs is species dependent. Studies in an in vitro gastrointestinal tract model (TIM) showed that DNA of ingested GM tomatoes was recovered at approximately 6% at the end of the ileal compartment (van der Vossen et al., 1998). This recovery was in agreement with the recovery rate of glucose and fructose from the tomato fruits suggesting that these molecules were not released from the cells by the digestive action. Free DNA seemed to be digested rapidly in the small intestine. In the colon, however, DNA degradation proceeded less rapidly. The studies on DNA degradation in the gastrointestinal environment suggest that natural transformation of oral- and gut-bacteria cannot be excluded. However, various attempts to demonstrate such a transfer event under conditions optimised for a transfer to occur in vivo such as gnotobiotic mice have met with failure.

5.3. Conclusions

The DNA from heat-processed crops is often small in size and therefore the likelihood to be transferred to new prokaryotic hosts via the process of transformation is small. Homologous recombination is the main mechanism by which DNA sequences can become incorporated into new bacterial hosts during transformation (de Vries et al., 2001), but this requires regions of homology between the transforming DNA and the host genome which is effective only when at least several hundred nucleotides in length (de Vries et al., 2001; Meier and Wackernagel, 2003b; Palmen et al., 1993). Significant phenotypic alterations may only occur if complete genes are acquired, which assumes a size for the transforming fragments of again at least several hundred base pairs.

Free DNA which is likely to be present in various processed food crops, is rapidly degraded in the upper part of the intestinal tract of mammalians and therefore is not a relevant source for transformation. However, unprocessed food crops eaten as such may release their DNA as rather large molecules. These may transform cells of the microflora in the oral cavity or, with lower chance due to the DNA degrading environment, the GI tract, where it may become available for transformation to the intestinal microflora. A compartment where DNA from plant cells is less rapidly degraded is the distal part of the intestinal tract, where also the bacterial cell density is the highest in the GI tract.

The probability of transfer of antibiotic resistance genes present in plant GM varieties to the indigenous gut micro-flora of humans is one of the major concerns of the public. As shown above, the amount and integrity of the DNA in food depends highly on the stringency of processing conditions and the degrading effect of the digestion process in the gastrointestinal tract. Therefore, it can be concluded that the calculated amount of recombinant DNA consumed per capita (0.049 μg/day for maize and 0.011 μg/day for soy), such as published by Jonas et al. (2001) based on the data of Herbel and Montag (1987) and Lassek and Montag (1990) and on the amount of maize and potato products consumed per capita in Austria (Jonas et al., 2001), is probably overestimated with respect to the availability for gene transfer, because most of these plant food materials are processed before eating. Furthermore, due to the DNA degradative activities in the intestinal tract, only a small percentage of the calculated amounts will finally reach the colon, which contains high numbers of bacteria. Since only linear DNA fragments are available from the food, genes can only be acquired by transformation via homologous recombination to resident prokaryotic genome. This implies that the acquisition of new genes, such as antibiotic resistance genes from food, is—most probably—a rare event. However, the mere fact that it might occur stresses the importance to avoid certain antibiotic resistance marker genes of group II or III in GM plants.

6. General conclusions

The following general conclusions and recommendations are based on published scientific knowledge and on results from ongoing EU-sponsored research projects (GMOBILITY) related to the safe use of GM food and GM feed:

- DNA, once it has been introduced into the recipient organism, is indistinguishable from the host DNA in its physical and chemical properties and behaves identically.
- In order to assess the impact of the transfer of GMO-DNA on food safety, it is necessary to understand the gene transfer processes occurring in nature and the mechanisms behind them,

including their occurrence at different stages along the food chain.

- HGT is at the origin of the variety of life itself and there is very little reason to assume that consumption of transgenic food or feed adds any particular generalised risk.
- The impact of HGT events will depend upon a number of environmental factors, the selective advantage for the bacterial population being the most important. Antibiotic resistance markers are a particular example of this.
- At present, it is not necessarily the case that the use of systems that eliminate marker genes or the use of markers alternative to antibiotic resistance markers is safer than the use of certain antibiotic resistance marker genes themselves.
- Whereas uptake of ingested DNA by mammalian somatic cells has been demonstrated, there is so far no evidence that such DNA may end up in germ line cells as a consequence of the consumption of food.

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