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## Review article

# Risks from GMOs due to Horizontal Gene Transfer

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**Horizontal gene transfer (HGT) is the stable transfer of genetic material from one organism to another without reproduction or human intervention. Transfer occurs by the passage of donor genetic material across cellular boundaries, followed by heritable incorporation to the genome of the recipient organism. In addition to conjugation, transformation and transduction, other diverse mechanisms of DNA and RNA uptake occur in nature. The genome of almost every organism reveals the footprint of many ancient HGT events. Most commonly, HGT involves the transmission of genes on viruses or mobile genetic elements. HGT first became an issue of public concern in the 1970s through the natural spread of antibiotic resistance genes amongst pathogenic bacteria, and more recently with commercial production of genetically modified (GM) crops. However, the frequency of HGT from plants to other eukaryotes or prokaryotes is extremely low. The frequency of HGT to viruses is potentially greater, but is restricted by stringent selection pressures. In most cases the occurrence of HGT from GM crops to other organisms is expected to be lower than background rates. Therefore, HGT from GM plants poses negligible risks to human health or the environment.**

**Keywords:** horizontal gene transfer / risk assessment / genetically modified plant / lateral gene transfer / antibiotic resistance / risk regulation

## INTRODUCTION

The discovery of horizontal gene transfer (HGT) can be traced to 1928 when Fred Griffith reported the transfer of genetic material from heat-killed virulent *Streptococcus pneumoniae* to an avirulent form of the bacterium by a process he described as transformation (Bushman, 2002). It wasn't until 1946 that other forms of non-reproductive gene transfer between organisms were identified and variously described as conjugation, transduction, recombination, rearrangement, linkage disequilibrium, etc. (Bushman, 2002). Since the 1980s these different examples of gene transfer have become known collectively as either horizontal or lateral gene transfer (Gogarten et al., 2002; Koonin et al., 2001; Ochman et al., 2000; Syvanen, 1994). Both terms are used to describe gene exchange in nature that occurs between organisms without recourse to reproduction.

The rapidly growing library of complete genome sequences reveals that HGT is a major factor in shaping the genomes of all organisms. Comparison of three *Escherichia coli* strains reveals that only 39% of the genome is conserved, and the rest differs, mainly as a consequence of HGT (Welch et al., 2002). About 50% of the human genome is composed of mobile genetic elements, which have largely originated through HGT at different times in our evolutionary past, and undergone varying degrees of expansion through gene duplication (International Human Genome Sequencing Consortium, 2001).

The impact of HGT remains more controversial (Kurland et al., 2003). In the short term, HGT can increase genetic diversity and promote the spread of novel adaptations between organisms (Marri et al., 2007; Thomason and Read, 2006). HGT has been a major contributory factor to the rapid spread of antibiotic resistance amongst pathogenic bacteria in the last 50 years (Mazel and Davies, 1999), and the emergence of increased virulence in bacteria, eukaryotes and viruses (Derbise et al., 2007; Friesen et al., 2006; Mild et al., 2007). In the long term it has been proposed that HGT has contributed to the major transitions in evolution (Koonin, 2007).

More recently, concerns have been raised that HGT from genetically modified organisms (GMOs) could have adverse effects (Pontiroli et al., 2007). HGT of an introduced gene in a GMO may confer a novel trait in another organism, which could be a source of potential harm to the health of people or the environment. For example,

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the transfer of antibiotic resistance genes to a pathogen has the potential to compromise human or animal therapy (Bennett et al., 2004), transfer of a viral gene to a non-homologous virus may result in an emerging disease (Falk and Bruening, 1994) or gene transfer to humans has been controversially proposed as a potential trigger for oncogenesis (Ho et al., 2000).

## WHAT IS HORIZONTAL GENE TRANSFER?

Gene transfer refers to the movement of genes within or between individual organisms. HGT is often used to refer to all forms of gene transfer that do not involve parent-to-offspring transfer (sexual or asexual). It can occur either naturally or by human intervention (*e.g.* gene technology, embryo rescue, *in vitro* fertilization, protoplast fusion, self-cloning). In some cases, HGT may be transient, and not perpetuated in the offspring. Each form of HGT comes with different considerations of risk, which in the case of genetic modification (genetic engineering) is commonly regulated through legislation. In this review, the focus is on HGT that is perpetuated in the offspring.

## DETECTION OF HORIZONTAL GENE TRANSFER

There are several approaches to identify genetic changes due to HGT, including:

(1) *experimental evidence*, whereby a genetic marker is monitored for gene transfer to a recipient organism;
(2) *phylogenetic analysis* of gene sequences to identify topological inconsistencies between different gene families;
(3) *nucleotide compositional analysis* to identify any gene that has a nucleotide pattern that differs significantly from the overall genome; and
(4) *evolutionary scenarios* to explain the patchy appearance of a genetic signature, sequence or function that is not shared by close relatives.

### Experimental evidence

Many laboratory studies of bacterial conjugation, transduction and transformation over the last 80 years have provided valuable insights into some of the mechanisms and frequencies of HGT. More recently, field studies on HGT support many of the laboratory findings (Souza et al., 2002) and reveal the widespread occurrence of HGT amongst bacteria and viruses (Maeda et al., 2006; Sander and Schmieger, 2001; van den Eede et al., 2004; Weinbauer, 2004).

HGT has been recorded in a number of environmental situations such as soil, seawater, freshwater, animal and industrial waste products, plant surfaces, animal intestines, human saliva and food products (Bushman, 2002; Davison, 1999; Lilley et al., 2003; van den Eede et al., 2004; Wolska, 2003). Some settings, such as bacterial biofilms, reveal highly efficient HGT (Molin and Tolker-Nielsen, 2003; van Elsas et al., 2003; Wuertz et al., 2004), whereas the simplified conditions in laboratory studies probably lack many of the appropriate biotic and abiotic signals that facilitate HGT in nature (Mel and Mekalanos, 1996; Nielsen and van Elsas, 2001). For example, the presence of algae stimulates the release of bacterial plasmid DNA that is suitable for HGT (Matsui et al., 2003) and chitin induces natural competence in *Vibrio cholerae* (Meibom et al., 2005).

Nevertheless, the number of HGT events is generally many orders of magnitude lower relative to gene transfer by reproduction (Babić et al., 2008). Consequently, experimental screening for HGT has relied on testing organisms such as bacteria and viruses that can be cultivated in vast numbers and have short generation times. In addition, powerful selection methods such as the use of antibiotics have been used to identify rare transfer events. More recently, other markers like the green fluorescent protein have been shown to allow monitoring of individual occurrences of HGT and provide accurate measures of their frequency (Babić et al., 2008; Perumbakkam et al., 2006; Sørensen et al., 2005). In some cases, the transformation frequencies determined from these studies are much greater than cultivation-based selection systems (Rizzi et al., 2008).

### Phylogenetic analysis

Phylogenetic analysis of gene or protein sequences for the presence of an incongruent phylogenetic signal is considered to represent the most rigorous method for detecting past HGT events (Koonin et al., 2001; Syvanen, 1994). Smith et al. (1992) established the basic requirements for application of the phylogenetic congruency test to identify and support cases of HGT. The test compares the phylogenetic tree constructed from a specific protein or gene sequence with the known phylogeny for the species. Therefore, if a bacterial gene groups with homologs from a particular eukaryotic lineage and only distantly with homologs from other bacteria, then HGT seems likely.

However, the construction of unambiguous trees is time-consuming and susceptible to a number of confounding factors such as gene loss, insufficient phylogenetic signal, rapid nucleotide changes in some lineages, strongly biased nucleotide composition, poor sequence alignments, inadequate algorithms, evolution not

following the most parsimonious path, saturation where multiple changes can lead to revertants, long branch attraction, insufficient gene sampling, inclusion of paralogs and uncertain species tree (Koonin et al., 2001; Lebrun et al., 2006).

### Anomalous nucleotide composition

HGT inferred from anomalous nucleotide composition is based on the premise that genomes evolve to species-specific values due to replication, transcription and translation biases, variations in nucleotide and amino acid pools, and different DNA repair preferences. Thus, if codon usage, GC content or oligonucleotide signatures differ significantly from the mean for a given genome, then HGT may be invoked (Dufraigne et al., 2005; Hooper and Berg, 2002; Kanaya et al., 2001; Karlin et al., 1998; Lawrence and Ochman, 1998; Sandberg et al., 2001; Tsirigos and Rigoutsos, 2005). A significant fraction of many prokaryote genomes, up to 20%, has been classified as recent HGTs according to these criteria (Garcia-Vallve et al., 2003).

The advantage of detecting HGT by anomalous nucleotide composition is that it requires only a single genome to examine and is very rapid to assess. However, it usually detects more recent changes and requires the genome of the donor organism to be distinctive relative to the recipient genome. In addition, some native genes may also show atypical patterns due to chance, gene-specific selection pressure or some higher-order structural constraints on chromosome structure (Koski et al., 2001).

### Inconsistent evolutionary scenarios

Deriving a parsimonious evolutionary scenario is based on the premise that over time, genetic change is relentless, such that distant relatives are expected to have fewer features in common than close relatives (Koonin et al., 2001). Therefore, the presence of some distinguishing genetic signal in a distant relative but absent from more closely related organisms contradicts this expectation. The most common causes of these anomalies are loss of the genetic signal in close relatives or gene acquisition in a distant relative.

In practice, genomes provide a wealth of data that can be used as a source of distinctive features that have revealed unexpected relationships indicative of HGT. Some of these features include biochemical/biological properties (Pierce et al., 2003; Wenzl et al., 2005), recombination signals in closely related organisms (Escobar-Páramo et al., 2004; Hakenbeck et al., 2001), presence of mobile genetic elements (MGEs) or viral sequences (Burrus and Waldor, 2004; Paulsen et al., 2003; Welch et al., 2002), gene content pattern (Hall et al., 2005; Hao and Golding, 2004; Homma et al., 2007; Hong et al., 2004; Korbel et al., 2002), presence/absence of genomic features (Gupta and Griffiths, 2002; Kroll et al., 1998; Snyder et al., 2007), database searches for nearest relatives (Aravind et al., 1998; Parkinson and Blaxter, 2003; Ragan and Charlebois, 2002) and unequal rates of genetic divergence (Bromham and Penny, 2003; Novichkov et al., 2004).

Typically, all of these methods for detecting HGT uncover different sets of genes (Ragan, 2001) and may miss true HGT events (false negatives) and incorrectly identify other putative HGT events (false positives) (Canbäck et al., 2004; Daubin and Ochman, 2004; Daubin and Perrière, 2003; Gogarten and Olendzenski, 1999; Guindon and Perrière, 2001; Koski and Golding, 2001; Koski et al., 2001; Mira et al., 2002). Different methods can also detect HGT of different relative ages (Ragan et al., 2006). Therefore, a combination of approaches may be necessary to identify and confirm HGT (Eisen, 2000; Lawrence and Ochman, 2002; Ragan, 2001).

In addition, HGT can be difficult to distinguish from other forms of gene transfer. For example, the large scale intra-genomic transfer of genes from the chloroplast or mitochondrion to the nucleus (Martin, 2003) can confound the detection of bacterium-to-eukaryote transfers by HGT. Also, false diagnosis of gene absence (Zhaxybayeva et al., 2007) or experimental errors, such as contamination of DNA samples, may give misleading indications of HGT (DeMarco et al., 2007).

## PATHWAYS FOR HORIZONTAL GENE TRANSFER

HGT can occur between closely related, but also distantly related organisms such as viruses and animals (Filée et al., 2002; Hughes and Friedman, 2003) or plants and bacteria (Aoki and Syōno, 1999; Intrieri and Buiatti, 2001; Koonin et al., 2001; White et al., 1983).

An overview of the major pathways for HGT between donor and recipient is depicted in Figure 1. The relative impact of each pathway also indicates that MGEs are one of the most important conduits for HGT between organisms (van Elsas and Bailey, 2002; Zaneveld et al., 2008).

### Mobile genetic elements

MGEs are non-essential (accessory) genomic elements composed of genes and structural features that facilitate their spread both within and between organisms (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). They are found in the genomes of all prokaryotes and

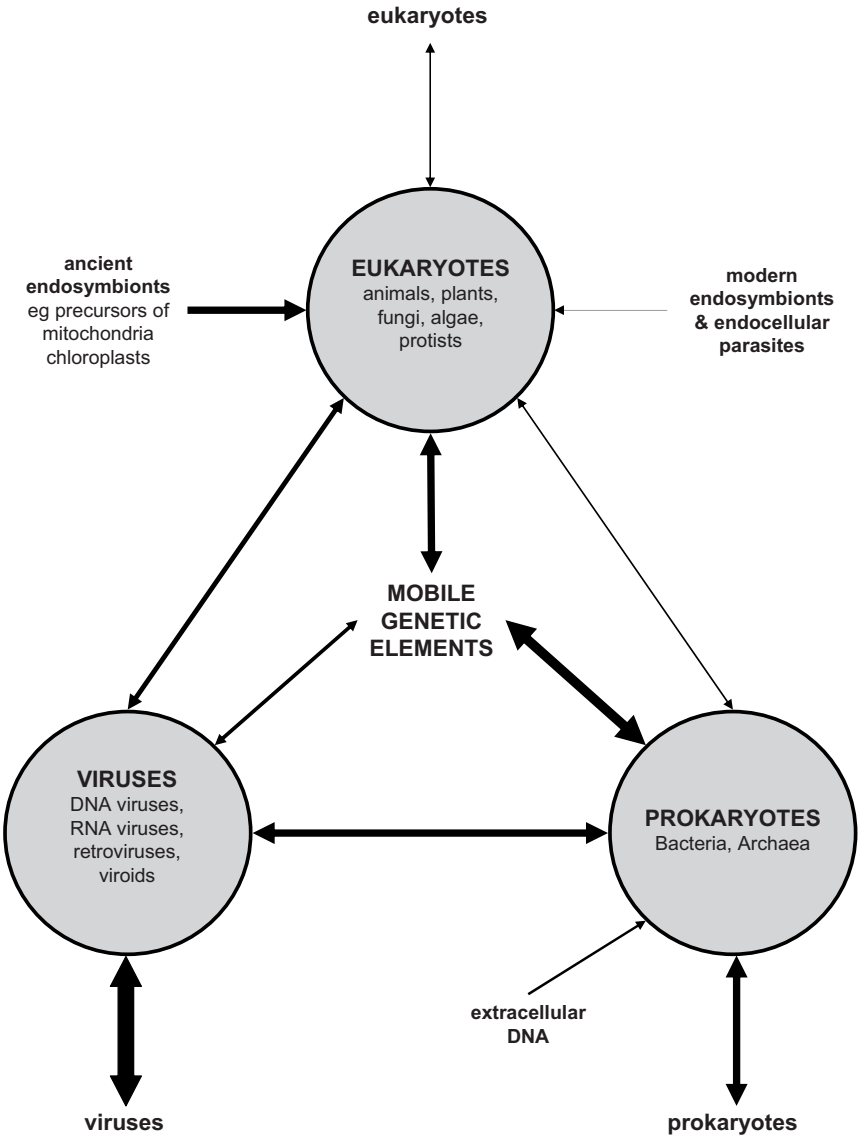


**Figure 1.** Contributions from HGT to the genome composition of different types of organisms. The thickness of each arrow indicates the predicted relative impact of each contribution.

eukaryotes. MGEs share many features and often share a common ancestry with viruses. The main difference is that viruses are usually capable of extracellular persistence.

MGEs, including defective forms, are a dominant feature of most genomes. MGEs constitute 35% of the genome of *E. coli* strain CFT073 (Welch et al., 2002), about 50% of the human genome (International Human Genome Sequencing Consortium, 2001) and about 80% of the maize genome (Whitelaw et al., 2003).

Major types of MGE include DNA transposons, retrotransposons, plasmids, composite mobile elements such as conjugative transposons, genomic islands, pathogenicity islands, integrative conjugative plasmids, mobilisable transposons (Burrus and Waldor, 2004; Osborn and Böltner, 2002), mobile introns/inteins (Lambowitz and Zimmerly, 2004; Poulter et al., 2007; Yamanaka et al., 2002) and non-autonomous mobile elements that lack encoded enzymes or suitable recognition sequences necessary for mobility. For example, the miniature inverted-repeat transposable elements (MITE, Feschotte et al., 2002), short interspersed nuclear elements (SINE, Moran and Gilbert, 2002) and processed pseudogenes (Kazazian, 2004) all lack a recombinase or reverse

transcriptase gene necessary for independent mobility. Other important groups of non-autonomous selfish elements include restriction-modification systems (Kobayashi, 2001) and integrons. The basic integron structure consists of an integrase gene and an outward facing promoter, which together act as an efficient gene capture/gene expression system in bacteria (Michael et al., 2004). When associated with autonomous MGEs, integrons provide an important mechanism for HGT of genes for antibiotic resistance, pathogenicity and other adaptive functions (Boucher et al., 2007; Carattoli, 2001; Ochman et al., 2000; Rowe-Magnus and Mazel, 2001).

MGEs are drivers of genomic and biological diversity (Böhne et al., 2008) and play a significant role in HGT in several ways:

(1) MGEs have evolved mechanisms that enhance the potential for gene transfer between organisms. For example, conjugative elements have evolved highly efficient mechanisms for the passage of genes into a recipient cell.
(2) MGEs can alter the function of genes in the vicinity of the insertion in the host genome. These alterations can include disruption or inactivation of genes at the site of insertion. Conversely, insertional mutagenesis by an MGE can also result in benefits to the host such as provision of regulatory sequences, repair of double-stranded DNA breaks, telomere maintenance in *Drosophila*, foetal implantation in mammals, or genome restructuring and speciation (Jordan et al., 2003; McClure, 2000; Peaston et al., 2004).
(3) MGEs contribute novel structural and functional genetic material that is often further spread throughout the genome (Touchon and Rocha, 2007), and in some cases may promote further horizontal dissemination of genes (Beaber et al., 2004).
(4) HGT of MGEs can result in the transfer of additional genes through genetic piggy-backing. For example, MGEs are the primary vehicle for the spread of antibiotic-resistance genes, pathogenicity determinants and biodegradation pathways amongst bacteria (de la Cruz and Davies, 2000).

## HGT involving prokaryotes

Many studies support the significant role of HGT in the evolution of prokaryotes (Gogarten et al., 2002; Jain et al., 2003; Kunin and Ouzounis, 2003). The principle sources of novel genes are provided by other prokaryotes, viruses and MGEs (Fig. 1). In particular, plasmids and composite mobile elements contribute significantly to HGT between prokaryotes. In some cases, composite elements and megaplasmids are composed of more than 100 genes (Bentley et al., 2002; Hacker et al., 1997), and account for much of the genomic variation between bacterial strains of the same species (Welch et al., 2002). In contrast, DNA transposases, retrotransposons and mobile introns/inteins carry few genes, and are primarily associated with intra-genomic gene movement.

Viruses contribute to prokaryotic genomes, both directly, in the form of viral (prophage) insertions (typically 1–10 copies) that are found in most bacterial genomes, and indirectly, through packaging additional cellular genes during the infection cycle. Prokaryote genomes also contain many ORFs with no dectectable homology to other ORFs in the databases. Some of these are postulated to have been obtained from viruses (Daubin and Ochman, 2004; Yin and Fischer, 2006).

More surprisingly is the apparent rarity of eukaryotic genes in prokaryotic genomes (Andersson, 2005; Guljamow et al., 2007; Pilhofer et al., 2007; Rogers et al., 2007). The abundant opportunities for prokaryotes to encounter eukaryotic genetic material suggest that significant functional (*e.g.* the presence of introns in eukaryotic genes, inefficient gene transfer mechanisms) and selective barriers have restricted the long-term acquisition of eukaryotic genes by bacteria.

One minor pathway for HGT to prokaryotes is the direct uptake of extracellular DNA. Natural genetic transformation is a feature of many bacteria. Although cell lysis of any organism can contribute to this extracellular DNA pool, several studies suggest that secretion of DNA from living bacteria may also be an important source of genetic material (Draghi and Turner, 2006; Vlassov et al., 2007).

## HGT involving viruses

Viruses undergo frequent HGT but this is typically restricted to gene exchange between viral genomes present in the same infection. Many viral genomes have few genes (less than 10), which constrains the number and types of genes that can be expected to increase or even maintain fitness. Consequently, viruses with small genomes have been rarely reported to contain non-virally derived genetic elements (Agranovsky et al., 1991; Becker, 2000; Khatchikian et al., 1989; Masuta et al., 1992; Mayo and Jolly, 1991; Meyers et al., 1991).

The genomes of viruses with large DNA genomes show more examples of host gene acquisitions by HGT (Fu et al., 2008; Hughes and Friedman, 2003; Raoult et al., 2004). However, the repertoire of host genes present in the viral genome is restricted to relatively few gene types. In addition, many viral genes appear to closely follow the evolution of the host, such as the thymidine kinase gene of poxviruses (Boyle et al., 1987). HGT events that result in the acquisition of genes from distantly-related species are relatively infrequent and are

more likely to have occurred over evolutionary time scales that reflect millions of years (Gibbs, 1987).

### HGT involving eukaryotes

The best recognized examples of HGT in eukaryotes include the wholesale absorption of bacterial genomes that once existed as endosymbionts or parasites and now exist as remnant genomes, such as mitochondria and plastids (Gray, 1993). This process of 'you are what you eat' (Doolittle, 1998) has continued throughout evolution and includes gene acquisitions from both prokaryotic (Hotopp et al., 2007) and eukaryotic endosymbionts (Li et al., 2006; Nosenko and Bhattacharya, 2007). For example, the nucleomorph organelle of *Guillardia theta* was originally an absorbed algal cell and is now a vestigal nucleus that contributes 302 genes to the host genome, including genes necessary for photosynthesis (Douglas et al., 2001). Cellular takeover of an endosymbiont genome may proceed over long evolutionary time periods, and some examples may be in the early phase of genetic piracy (Johnson et al., 2007). Bacterial parasites have also contributed to eukaryote evolution (Suzuki et al., 2002).

HGT involving gene transfers to eukaryotes has been more controversial (Doolittle et al., 1990; Kurland et al., 2003; Syvanen, 1994). Nevertheless, fungi and unicellular eukaryotes appear to have participated in many gene exchanges with bacteria (Andersson et al., 2007; Huang et al., 2004; Rosewich and Kistler, 2000). The full sequencing of eukaryotic genomes is likely to considerably add to the knowledge of past HGT events. For example, one group of metazoans, bdelloid rotifers, appear to be subject to far greater HGT than is typical of most other multicellular organisms (Gladyshev et al., 2008).

### Indirect HGT

In addition to direct HGT between organisms as depicted in Figure 1, forms of indirect HGT have been observed, which involve an additional intermediary organism in gene transfer from a host organism to the final recipient organism. The most notable are virus-mediated gene transfer (transduction) between bacteria (Weinbauer, 2004), retrotransfer of a plasmid to a second bacterium, acquiring host genes and returning to the original bacterium (Ronchel et al., 2000), the spread of donor genetic material between several different bacteria coexisting in complex communities or biofilms (Molin and Tolker-Nielsen, 2003; van Elsas et al., 2003; Wuertz et al., 2004); or from virus to virus *via* sequences integrated into a common host organism.

## MECHANISMS OF HORIZONTAL GENE TRANSFER

HGT is a two-step process with mechanistically and biochemically distinct phases. Firstly, there is passage of donor genetic material across the cell membrane(s) of the recipient cell, including other envelope structures such as a cell wall or nuclear membrane. Secondly, there is stable incorporation of the donor genetic material into the genome of the recipient organism such that the new gene may be perpetuated through the offspring. In the case of multicellular organisms this involves gene transfer to germ line cells, which are both fewer and less accessible than somatic cells in most animals and plants. Each HGT event may be accompanied by expression of the donor genetic material or changes to expression of endogenous genes in the recipient organism.

### Translocation of genetic material

The major mechanisms of HGT described in the literature include conjugation, cellular competency (natural transformation) and virus-mediated transduction (Chen et al., 2005; Dreiseikelmann, 1994; Dubnau, 1999). However, these mechanisms only refer to the first step of any successful gene transfer event, namely the passage of genetic material across cellular barriers. Independent mechanisms are involved in integrating the new genes into the genome of the recipient organism.

*Conjugation* – Bacterial conjugation systems belong to a subfamily of type IV secretory pathways that mediate the transport of DNA, proteins and toxins across the cell envelope of bacterial, plant or animal cells (Cascales and Christie, 2004; Ding et al., 2003; Gomis-Rüth et al., 2004). Conjugation is widespread throughout bacteria and is the most important mechanism for translocating DNA between bacteria (Espinosa-Urgel, 2004; Grohmann et al., 2003). In the natural environment conjugation occurs primarily between closely-related strains or species. However, it can occur between distantly-related species, even between members of different domains of the prokaryotes, the Archaea and Bacteria (Koonin et al., 2001).

The conjugation machinery is also used by the bacterial phytopathogen *Agrobacterium* to insert DNA into plant cells as part of the infection process. DNA transfer from *Agrobacterium* to its host has been co-opted by gene technology to introduce genes into plants and has been studied in considerable detail (Gelvin, 2003; Tzfira et al., 2004; Zupan et al., 2000). This mechanism of translocating genetic material across the eukaryotic membrane has been experimentally adapted to introduce genes into yeast (Bundock et al., 1995; Piers et al., 1996), filamentous fungi (de Groot et al., 1998) and mammalian cells

(Kunik et al., 2001). Other types of bacteria have also been modified to transfer genes to yeast or plants by the same mechanism (Broothaerts et al., 2005; Heinemann and Sprague, 1989).

*Cellular competency* (natural transformation) refers to the active uptake of free (extracellular) DNA across the cell wall and cell membrane(s) using cellular machinery designed to facilitate the process (Dubnau, 1999; Lorenz and Wackernagel, 1994). It is a highly regulated physiological state that is often sensitive to environmental cues (Molin and Tolker-Nielsen, 2003). When the DNA is heritably incorporated into the recipient's genome, the process is known as transformation.

Bacterial competency has been closely studied in many systems and is associated with three steps, (1) release of DNA into the environment, (2) binding to specific sites on the bacterial cell surface, and (3) transport across the cell membrane through a specific pore, coupled to degradation of one of the DNA strands. Many of the competency genes are related to those of the type IV secretory pathway used in conjugation (Dubnau, 1999).

Although DNA uptake by competent cells has been observed for many bacteria (Lorenz and Wackernagel, 1994), equivalent processes may occur with eukaryotic cells. There is a growing number of reports of spontaneous DNA uptake by eukaryotes, such as DNA from human erythrocytes by malaria parasites (Deitsch et al., 2001). However, it has not yet been established that eukaryotes have specialized systems equivalent to bacterial competency genes for transporting DNA across the cell membrane.

*Transduction* – Viruses are able to package genes from the genome of one bacterial host within their capsids and transfer these donor genes to a second bacterial cell. Generalized transduction involves the transport of any bacterial gene, whereas specialized transduction transports only selected genes that are close to the attachment site where the virus was integrated in the host bacterial genome.

Marine environments are a major setting for virus-mediated gene transfer between bacteria, where there is an estimated abundance of greater than $10^{29}$ virus particles (Hendrix et al., 1999; Weinbauer and Rassoulzadegan, 2004). For example, virus-mediated gene transfer frequencies of around $10^{-8}$ have been reported from the Tampa Bay estuary, corresponding to around $3.6 \times 10^{11}$ HGT events each day in the estuary (Jiang and Paul, 1998).

In addition to conjugation, transformation and transduction, other less well recognised mechanisms of DNA uptake occur in nature, while other mechanisms of HGT are probably yet to be elucidated, in particular, DNA uptake by eukaryotes:

- *Vesicle-mediated translocation* by a range of Gram-negative bacteria such as *Neisseria gonorrheae*, *E. coli* and *Pseudomonas aeruginosa*, which can bud off vesicle structures that contain genetic material (*e.g.* antibiotic resistance and virulence genes) and then fuse with another bacterium (Dorward et al., 1989; Kadurugamuwa and Beveridge, 1997; Yaron et al., 2000).
- *Virus-like particles* (gene transfer agent) formed from proteins encoded by some bacterial genomes, which can trap random fragments of the genome (about 4400–13 600 base pairs) and transmit them to a second bacterium (Dykhuizen and Baranton, 2001; Lang and Beatty, 2001; Marrs, 1974).
- *Cellular fusion* (fusion between cell membranes) that allows mixing of entire genomes, can occur with some fungi, multicellular bacteria and between membrane-bound viruses and their host (Hijri and Sanders, 2005; Knipe and Howley, 2001).
- *Phagocytosis/endocytosis*, by which certain unicellular eukaryotes (*e.g.* amoeba) engulf entire cells that may be prokaryotic or eukaryotic (Doolittle, 1998).
- *Lysis* of intracellular pathogens/endosymbionts has been shown to deliver DNA into mammalian cells (Grillot-Courvalin et al., 2002).
- *Cellular channels* may account for some HGT events between parasitic plants and their host (Haupt et al., 2001; Mower et al., 2004); this is commonly used by plant viruses, which encode a movement protein that modifies the plasmodesmata, allowing spread of virus between cells in a plant (Hofmann et al., 2007).
- *Vector-mediated translocation* has been postulated as a mechanism for the indirect transport of genes between eukaryotes, such as gene transfer between *Drosophila* species by mites that feed on the eggs (Houck et al., 1991) or *via* other biological vectors such as pollen, fungi, bacteria and nematodes.
- *Adventitious* uptake of genetic material can occur when the cell membrane is accidentally breached, whether mechanically, chemically or electrically, such as in the entry by viruses after physical damage of a plant or animal cell (Bos, 1999), by lightning in a manner analogous to genetic modification by electroporation (Demanèche et al., 2001) or desiccation (Gladyshev et al., 2008).

### Incorporation of donor genetic material

Once the donor genetic material enters the cell or nucleus, there are three basic mechanisms for integrating the donor DNA or RNA into the genome of the recipient organism.

(1) *Break and join*, which involves cleavage of the recipient genome, usually at some specific site, followed

by ligation to the termini of the donor genetic material. Many integrative plasmids, viruses, mobile introns and transposons encode a specific DNA endonuclease/recombinase/integrase that fulfils this function. More recently, some RNA molecules (*e.g.* some mobile introns) have been discovered that possess enzymatic self-cleaving properties. In the case of some mobile introns, the RNA can reverse splice into the RNA of a recipient molecule (Belfort et al., 2002).

(2) *Template strand switching*, which involves a DNA or RNA polymerase jumping from one template molecule to a second molecule during the synthesis process to form an integrated, chimaeric (recombinant) molecule between the donor genetic material and the genome of the recipient organism.

(3) *Autonomous replication*, whereby the donor genetic material (a plasmid or accessory chromosome) is capable of independent replication without physical linkage to the genome of the recipient organism. Typically, plasmids or accessory chromosomes coordinate their multiplication and transmission to daughter cells with replication and cell division of the host genome.

Recombination is often linked to DNA repair mechanisms present in cellular organisms. DNA damage, including double-stranded breakage of the chromosome, is an ongoing facet of cellular life. The machinery used to repair this type of DNA damage is commonly co-opted to integrate foreign DNA into the host genome. Genetic modification of plants using *Agrobacterium*-mediated gene transfer or biolistics is assumed to involve integration of the donor DNA at random double-stranded breaks in the chromosome (van den Eede et al., 2004).

The outcomes of chromosomal integration events are usually described as homologous, site-specific or non-homologous recombinations, depending on the degree of sequence similarity at the site of recombination.

*Homologous recombination.* All cellular organisms have molecular functions dedicated to recognizing and recombining DNA molecules that have extensive sequence similarity at the region of cross-over, usually greater than 200 base pairs. The principle molecule involved in homologous recombination belongs to the family of RecA/Rad51 DNA binding enzymes that catalyse post-replicative strand exchange during meiotic/mitotic recombination or repair of DNA double-strand breaks. These enzymes show some sequence preferences (Raja et al., 2006) and require minimally efficient processing segments of 20–30 base pairs to initiate strand exchange (Majewski and Cohan, 1999).

*Site-specific recombination.* Many integrative plasmids and bacterial viruses recognise a specific sequence (attachment site), usually less than 30 base pairs in the recipient genome, where integration is targeted (Grindley et al., 2006). Nearly all site-specific recombinases belong to either the tyrosine or serine families of recombinases, which use the break and join mechanism of recombination. These recombinases are named after the amino acid that forms a covalent protein-DNA linkage in the reaction intermediate. Tyrosine recombinases proceed by cleavage and ligation of single strands in pairs to form a Holliday junction intermediate, whereas serine recombinases cut all strands in advance prior to strand exchange and religation.

*Non-homologous (illegitimate) recombination.* Double-strand breaks in DNA can be repaired by homologous recombination, single-strand annealing or non-homologous end-joining. Non-homologous end-joining occurs at sites that show weak or no sequence specificity. The crucial step involves binding of DNA Ku protein dimers to the broken ends, which then catalyses the recruitment of other cellular components that complete the ligation step (Pastwa and Błasiak, 2003). Although best characterised in eukaryotes, genes homologous to *Ku* are present in bacteria (Weller et al., 2002) and viruses (d'Adda di Fagagna et al., 2003).

Some cases of recombination include both homologous and non-homologous pathways. For example, one terminus of the donor genetic material may integrate at a homologous region, which assists the random insertion of the other terminus at a non-homologous site of the recipient genome (de Vries and Wackernagel, 2002). Except for higher plants, homologous recombination appears to be more readily observed than non-homologous recombination. This may partly reflect the selection process in which the majority of products of homologous recombination are likely to be related and therefore able to maintain the functional integrity of the recipient genome.

Both homologous and non-homologous recombination can be detected in viruses with RNA genomes. However, any regions of homology are usually short and may be associated with secondary structures that facilitate the recombination process (Bujarski and Nagy, 1996). Some of the typical properties of sites involved in recombination between viral RNAs include an AU-rich sequence adjacent to a 5' GC-rich region (Nagy and Bujarski, 1996; Ohshima et al., 2007; Vives et al., 2005).

## Fate of donor genetic material

Once the donor DNA has been incorporated into the genome of the recipient organism, it may alter the phenotype of the recipient, either by disrupting endogenous genes, supplanting a homologous gene product but with altered expression/enzymatic properties or introducing a novel trait. However, expression of a novel trait requires a combination of several processes including appropriate transcription with regard to timing, amount and sites

of initiation and termination, correct processing of transcripts, including splicing and polyadenylation, efficient translation and maintenance of a functional protein product, correct folding and secondary modification of the protein product (*e.g.* phosphorylated, glycosylated, acetylated, farnesylated, ubiquinated or sulfated) and appropriate interactions with other proteins and substrates.

The mechanisms and regulatory signals for appropriate gene expression differ between organisms. The extent of incompatibility often correlates with the degree of evolutionary distance. Consequently, eukaryotic genes may be inefficiently expressed if transferred to a prokaryote, and *vice versa*. For example, introns in eukaryotic genes would prevent appropriate expression in a prokaryotic recipient (Andersson, 2005).

The short- and long-term impact of the donor genetic material is also dependent on the dynamic interplay between the recipient organism and the environment, honed by selection and historical contingencies. This includes any selective advantage provided by the donor genetic material that facilitates its spread throughout the population of the recipient organism.

However, the transfer of genetic material by HGT would be expected to impose a fitness cost in most cases, both in terms of metabolic burden and interference with normal cellular function. In the case of plasmids and composite mobile elements, where many genes may be transferred, one solution appears to be the presence of a gene that encodes a histone-like nucleoid structuring protein (Doyle et al., 2007). This protein is found in many Gram-negative bacteria and binds to regions of curvature in the A+T-rich DNA typical of many plasmids and pathogenicity islands (complex composite MGEs). As a result of binding to the DNA, the histone-like nucleoid structuring protein represses transcription, minimising impacts on global expression patterns and fitness costs.

Over time, further adaptations of the donor genetic material may occur that promote the long-term interests of both the genes and future generations of their newly-acquired host. These changes include altered nucleotide composition, modified regulation or function of the gene, or changes at the genome level, including recombination, duplication and transposition. In some cases, the foreign gene becomes so fully integrated that it fully replaces the endogenous gene homologue (Koonin et al., 2001).

### Barriers to HGT

HGT can increase genetic diversity and promote the spread of novel adaptations, but it can also result in excess genetic baggage and the import of deleterious genes. Therefore, organisms possess a number of physical, biochemical and genetic barriers to restrict the frequency of HGT (Kurland, 2005; Matic et al., 1996; Nielsen, 1998; Nielsen et al., 1998; Thomas and Nielsen, 2005).

The barriers to HGT include the physical integrity of the cell and nucleus, limited physical access to germline cells in plants and animals, restriction-modification systems in bacteria and algae that recognize and hydrolyse foreign gene sequences, requirements for self-recognition sequences (Ambur et al., 2007), sequence specificity for integration into the recipient genome by homologous recombination, presence of inappropriate regulatory signals (*e.g.* presence of an intron, host or tissue specific promoter, cryptic splice site), nucleotide composition adaptations for optimised gene expression, mismatch repair systems and natural selection (Matic et al., 1996; Nielsen, 1998; Thomas and Nielsen, 2005).

In general, the stringency of the barriers to HGT increases proportionally with genetic distance. Consequently, the frequency of HGT is much greater within species than between unrelated or distantly-related species (Fraser et al., 2007).

## RISKS DUE TO HORIZONTAL GENE TRANSFER

Genetic modification has many potential applications in agriculture, therapeutics and industrial chemical production. With these new opportunities has come greater public scrutiny and government regulation. Risk assessment is a common regulatory tool used in the decision-making process for a proposed commercial release of a GMO into the environment (Hill, 2005). A critical step in risk assessment is identification of those sets of circumstances that may give rise to an adverse effect(s) (risk identification, or "what could go wrong"). The level of risk is then estimated from both the likelihood and severity associated with those circumstances of concern. In some jurisdictions, risk assessments also consider potential benefits as part of the decision-making process.

HGT of the introduced gene(s) from a GMO to other organisms has been commonly cited as one potential risk. However, it is important to note that HGT is not an adverse effect as such, but an event that may or may not lead to harm. HGT is widespread in nature and in some cases occurs frequently. All organisms have a history of HGT and every gene, including those introduced by gene technology, is capable of being transferred between organisms by HGT. The transferred gene could confer a novel trait to the recipient organism, which may result in negative, neutral or positive effects. For example, bioremediation of contaminated groundwater or wastewater may be enhanced when catabolic genes used for genetic modification reside on MGEs and can be readily transferred to endogenous bacteria (Bathe et al., 2004; Taghavi et al., 2005).

## Impact of HGT from GMOs

Some of the considerations on potential impacts of HGT from GMOs include the following.

- *Adverse effects on the health of people or the environment*. The criteria for harm to human health or the environment that are commonly considered in risk assessments of GMOs include: enhanced pathogenicity or virulence in people or animals (Kleter et al., 2005), emergence of a new disease, pest or weed, increased disease burden if the recipient organism is a pathogenic microorganism or virus, increased weed or pest burden if the recipient organism is a plant or invertebrate and adverse effects on species, communities or ecosystems.
- *Unpredictable and unintended effects*. HGT has the potential to transfer introduced genes from a GMO to a multitude of other species, some of which are potential pests or pathogens, and many organisms are yet to be identified and characterized. The genes introduced to bacteria could be transferred to indigenous bacteria, altering the ecological niche or ecological potential of the recipient organism (Heuer and Smalla, 2007) or through unexpected changes in structure or function (Prescott et al., 2005). The wide diversity of recipient genomes makes it difficult to predict the outcome from the introduction of a particular gene. Furthermore, the gene transferred may insert at variable sites of the recipient gene, not only introducing a novel gene, but also disrupting an endogenous gene, with unpredictable and unintended effects.
- *Genomic disruption*. It has been proposed that more complex cells, such as eukaryotic cells, are more intolerant of change (Woese, 2004) such that the gene technology could lead to genome instability and an increase in horizontal gene transfer (Ho et al., 2000).
- *Loss of management control measures*. Regulatory approvals for field trials of GMOs often require measures to limit and control the release in space and time. With the spread of the introduced gene(s) to another species by HGT, a new GMO is created. This new GMO may give rise to adverse effects not controlled by management measures imposed by the original licence or permit.
- *Long-term effects*. On some occasions the impact of HGT may be more severe in the long term. Even under relatively strong selection pressure, it may take thousands of generations for a recipient organism to become the dominant form in the population (Nielsen and Townsend, 2004). In addition, there are many other factors that could delay significant impact, such as timing of appropriate biotic or abiotic environmental conditions for an adverse effect to be realized, additional changes in the recipient organism that may be necessary before an adverse effect is realized by complementing the function of the donor genetic material, secondary transfers of the donor genetic material to another species or delay in the uptake of donor DNA, which can persist, on rare occasions, for more than 400 000 years (Pääbo et al., 2004).
- *Ethical concerns*. A number of ethical issues associated with HGT from GMOs have been raised, including perceived threats to the integrity and the intrinsic value of the organisms involved, to the concept of natural order and the integrity of species, to the integrity of the ecosystems in which the genetically modified organism occurs and to the different ethical values to be attributed to different species and kingdoms, especially as this affects specific people (GTEC, 2006).

Most commonly, the risk assessment focuses on the potential to cause harm to human health or the environment, but depends on value judgements as to what constitutes harm and its severity. The criteria for harm should be made explicit in legislation or guidance documents (OGTR, 2007; USEPA, 1998).

## Frequency of HGT from GMOs

The risk assessment considers not only the severity, but also the likelihood of adverse effects. The likelihood of harm involving HGT relies on several links in a causal chain that includes the opportunity for the recipient organism to encounter genetic material from the donor organism, the occurrence of HGT, the expression of a novel trait in the recipient organism, the persistence of the recipient organism such that it passes the novel genetic material to its offspring, and a selective advantage that allows the spread and maintenance of the genetic material in the population and species (van Elsas and Bailey, 2002). The final links in the causal pathway require exposure of people or the environment to the recipient organism or its offspring that results in harm due to the novel trait acquired by the recipient organism.

The occurrence of HGT from donor to recipient organism is only one of those links considered in the overall likelihood calculation. Even when the frequency of HGT may be high, a close-to-zero probability of any of the other steps will reduce the overall likelihood that harm is realized to also near zero.

Features that have an effect on the frequency of HGT are listed in Table 1. These include the nature of the donor and recipient organisms, their genetic and ecological relationship and the type and function of the genetic material that is transferred. However, it is important to note that the frequency of gene transfer by HGT for all organisms (including viruses and prokaryotes) is orders of magnitude lower than vertical gene transfer by sexual or asexual reproduction.

**Table 1.** Characteristics that correlate with different frequencies of HGT.

| Property | Relative frequency of HGT | | |
|---|---|---|---|
| | Low | Moderate | High |
| Type of organism | multicellular eukaryote | prokaryote/single-celled eukaryote | virus |
| Genetic relationship between donor and recipient | distantly related species | closely related species | same species or closely related strains |
| Ecological relationship between donor and recipient | separated in space or time | parasitic/symbiotic | same ecological niche |
| Function of gene transferred | toxic/informational | structural/metabolic | MGE associated/ pathogenic/defence/ ecologically opportunistic |

*Type of organism*

The frequency of HGT is strongly influenced by whether or not the organism is multicellular. The vast majority of cells in multicellular organisms are somatic (*e.g.* plant leaves, stems, roots, most flower parts or most animal organs and body parts). A minor portion of cells in multicellular eukaryotes constitute the germ line. Cells in the germ line are destined to become sex cells, thereby contributing their genome to the next generation through the egg cell and sperm or pollen. Therefore, only gene transfers that stably integrate into the genome of the germ line will perpetuate the change in the offspring.

One exception to low frequency of HGT in multicellular organisms includes bdelloid rotifers, which apparently lack sexual reproduction (Gladyshev et al., 2008).

In contrast, every prokaryote or unicellular eukaryote is potentially competent for reproduction and thus capable of transmitting any genetic novelty to its descendents. Consequently, prokaryotes and unicellular eukaryotes may be inherently more prone to HGT events than multicellular eukaryotes, whose germ line cells often have limited exposure to the environment and other organisms (Kurland, 2005).

Viruses appear to have the potential for greater rates of HGT than bacteria. Most types of DNA and RNA viruses are prone to high rates of recombination, except for viruses with negative strand RNA genomes (Chare et al., 2003). Major innovations in virus evolution are often the result of extensive exchange of a common pool of genetic modules (Botstein, 1980; Gibbs, 1987; Hendrix et al., 1999; Pedulla et al., 2003). Viruses also provide a substantial conduit for HGT between other organisms such as bacteria (Breitbart and Rohwer, 2005).

One study that measured the rate of recombination demonstrated frequent HGT between viruses during every infection, higher than the rate of mutations per base (Froissart et al., 2005). Using Cauliflower mosaic virus as a model, over 50% of viral genomes recovered after a single host infection were recombinant with an estimated baseline recombination frequency of $2{-}4 \times 10^{-5}$ per base each replication cycle (Froissart et al., 2005). Other studies support these findings of recombination amongst many groups of viruses (Aaziz and Tepfer, 1999; Banner and Lai, 1991; Bruyère et al., 2000; Revers et al., 1996; Rokyta et al., 2006) and viroids (Keese and Symons, 1985; Rezaian, 1990).

Nevertheless, selection amongst viruses provides a significant bottleneck to the propagation and persistence of offspring from HGT. All viruses that infect higher plants have small RNA or DNA genomes, usually with less than 20 encoded proteins. These viruses are therefore highly constrained as to the type and size of novel genetic material that can be acquired by HGT. Although most plant viruses routinely incorporate other genes by template switching during the replication process, nearly all gene transfers that survive selective pressures are homologous genes from the same strain of virus (Tan et al., 2004; Worobey and Holmes, 1999).

*Genetic relationship between donor and recipient organism*

There is a close correlation between the genetic similarity of the donor and recipient organisms, and the frequency of HGT between them (Beiko et al., 2005). Experimental studies of HGT from widely different genera of bacteria reveal a consistent decline in gene transfer rates as a function of genetic distance (Majewski, 2001). Fraser et al. (2007) report that 5% genetic divergence correlates with a 10-fold decrease in the relative rate of recombination and 15% genetic divergence correlates with a 1000-fold decline. In some cases, the rate of intraspecific HGT amongst some bacteria can far exceed the point mutation rate (Vergin et al., 2007).

Similarly, the highest frequency of HGT involving viruses is between closely-related strains. Bonnet et al.

(2005) reported a level of 17% recombinants in natural populations of Cucumber mosaic virus, all attributable to HGT exchange between strains of the one virus species.

The greater frequency of HGT between closely-related organisms is probably due to fewer genetic barriers to gene exchange, having shared sequences that facilitate DNA uptake and/or homologous recombination and also greater chance that the recipient genetic material is functionally compatible and useful. A notable exception is the transfer of genes whose expression is toxic in the recipient cell (Sorek et al., 2007). In these cases, similar expression control signals expected in closely-related organisms would facilitate the production of toxic effects.

HGT occurs less frequently between distantly-related organisms, except for the exchange of broad host range plasmids between bacteria and integration of certain viruses in the genome of their host. The most infrequent HGT events that have been detected involve gene transfers from eukaryotes to prokaryotes.

### *Ecological relationship between donor and recipient organism*

HGT usually occurs where donor and recipient share a common environment at the same time. Rarely, HGT occurs indirectly, such as the proposed gene transfer of retrotransposons between reptiles and mammals *via* poxviruses (Piskurek and Okada, 2007) or photosynthetically-important genes exchanged between cyanobacteria *via* viral intermediates (Zeidner et al., 2005).

Of intermediate frequency is HGT between symbionts or parasites and their hosts. In particular, eukaryotic genomes often reveal the footprints of past gene acquisitions from resident intracellular invaders. These HGT events include genetic material obtained from many types of organism, including other eukaryotes, such as algal symbionts (Douglas et al., 2001), prokaryotic genes, such as *Wolbachia* genes in insect and nematode genomes (Hotopp et al., 2007), the ancestors of mitochondria and chloroplasts and viral genes, from both integrating viruses and, more surprisingly, non-integrating viruses (Harper et al., 2002).

HGT occurs most frequently between organisms that occupy the same ecological niche (Beiko et al., 2005). In particular, environments that allow frequent, multiple interactions between donor and recipient organisms favor high levels of HGT. Some examples include aquatic environments (Audic et al., 2007; Jiang and Paul, 1998), biofilms (Hendrickx et al., 2003; Sørensen et al., 2005), the human gut (Kurokawa et al., 2007; Xu et al., 2007) and cells co-infected by viruses or bacteria (Abbot et al., 2007; Rekab et al., 1999; Worobey and Holmes, 1999). Consequently, prokaryotes that inhabit extreme environments reveal multiple transfers between genetically-distant archaea and bacteria that co-inhabit these environments (Nesbø et al., 2001).

In addition, organisms that share other external and internal determinants correlate with higher rates of HGT in prokaryotes. These determinants include oxygen tolerance, temperature parameters, carbon usage, G/C content and genome size (Jain et al., 2003).

### *Function of gene transferred between organisms*

Every type of gene appears to be capable of HGT (Sorek et al., 2007). This includes HGT of highly-conserved genes such as ribosomal genes (Acinas et al., 2004; Ueno et al., 2007; Yap et al., 1999), even though ribosomal RNAs are functionally constrained by interactions with dozens of proteins, RNAs and other molecules. Nevertheless, there is considerable bias in the biological functions of horizontally transferred genes.

Genome comparisons reveal that informational genes involved in transcription and translation are the least likely to be transferred between organisms (Jain et al., 2003). This finding is supported by experimental data that studied the attempted transfer of 246 045 genes from 79 prokaryotic genomes into *E. coli*. Amongst the genes that were unclonable most were certain highly conserved, single-copy informational genes, whose expression was toxic to *E. coli* (Sorek et al., 2007).

The most commonly transferred genes amongst cellular organisms are selfish genes associated with replication, translocation and integration of MGEs and viruses (Bushman, 2002). Other frequently transferred genes include those associated with pathogenicity, defence or host-pathogen interaction through cell surface proteins (Hughes and Friedman, 2005; Nakamura et al., 2004; Pallen and Wren, 2007). In addition, many of the genes transferred between prokaryotes on a variety of MGE backbones include ecologically opportunistic genes that allow opportunities for innovation. For example, genes acquired by strains of *E. coli* have facilitated infections outside its typical environment of the gastrointestinal tract (Welch et al., 2002), or HGT of genes that degrade aromatic pollutants (Phale et al., 2007).

Of intermediate frequency is HGT of genes associated with core structural or metabolic functions (Koonin et al., 2001). However, even in the case of prokaryotes, these HGT events are more commonly detected during phylogenetic reconstructions of organisms that diverged many millions of years ago.

In conclusion, a number of factors influence the frequency of HGT. The highest rates of HGT can be expected for the exchange of pathogenic markers between closely-related strains of viruses that infect the same host.

In contrast, low rates of HGT occur for multicellular eukaryotes, between distantly related species, between donor and recipient organisms that are separated in space or time or involve a gene toxic in the recipient organism. In all of these later cases, the frequency of HGT is expected to be too low to give rise to a significant risk.

## Risks from GM plants due to HGT

Assessing the risk posed by HGT from dealing with GMOs intentionally released into the environment requires consideration on the introduced genes increasing either the severity or likelihood of an adverse outcome. Globally, GM plants constitute the great majority of GMOs released into the environment, including cotton, soybean, oilseed rape and maize. The majority of genes introduced into these crops confer herbicide tolerance or insect resistance together with a range of accessory genetic material, such as the antibiotic resistance genes used in the process of genetic modification. Other types of genes that have been inserted into crop genomes include genes that confer disease resistance or stress tolerance. Future GM crop plants may include genes for therapeutic uses such as the production of vaccines or nutritionally enhanced foods such as modified oils.

Therefore, consideration of HGT in case-by-case risk assessments of these GM plants examines the nature of the potential recipient organism (bacterium, virus or eukaryote), as well as the properties of the introduced gene(s) and all steps in the causal pathway leading to harm.

### *HGT from GM plants to bacteria*

There are many opportunities for bacteria to encounter DNA from GM and non-GM plants. This includes bacteria that directly interact with plants as commensals, symbionts or parasites, bacteria that inhabit soil or water environments that are exposed to plant tissue decomposition products, as well as in the guts of herbivores. However, the role of HGT in the adaptation of bacteria to an environmental niche involving interactions with plants is not well understood (van Elsas et al., 2003). There are almost no evolutionary examples of HGT to bacteria from eukaryotes (Andersson, 2005). Although cultivation-based selection systems underestimate the HGT frequencies (Rizzi et al., 2008), experimental and laboratory studies also suggest limited circumstances that support the transfer and persistence of DNA from plants to bacteria (Nielsen et al., 1998). The only genes from GM plants that are likely to be successfully transferred to bacteria are other bacterial genes, including antibiotic resistance genes used in the transformation process (Pontiroli et al., 2007).

The widespread use of antibiotics for both human and animal use has imposed strong selection pressures for bacterial resistance. The rapid rise in antibiotic resistance over the last 50 years has compromised therapeutic treatments, adding significant costs to medical and veterinarian care. The most common mechanism for the development of antibiotic resistance in disparate groups of pathogenic bacteria has been through HGT, *via* plasmids and other MGEs.

Antibiotic resistance genes have been introduced to GM plants either as part of the bacterial cloning vectors used for the initial gene constructions or under the control of plant promoters to select for successfully modified cells. The concern is that the presence of antibiotic resistance genes in GM plants could provide a reservoir for the appearance of new drug resistant bacteria through HGT from plants to pathogenic bacteria.

Van den Eede et al. (2004) distinguish three groups of antibiotic resistance genes according to therapeutic use in humans and animals. Two of the most common antibiotic resistance genes present in GM plants released are *nptII* (resistance to kanamycin) and *hpt* (resistance to hygromycin B). These genes are assigned to group 1, which have no or limited therapeutic relevance. Other antibiotic resistance genes commonly used in GM plants are *aad* (resistance to streptomycin and spectinomycin) and *bla* (resistance to ampicillin). These belong to group 2, which include resistance to antibiotics restricted to defined areas of human and veterinary medicine.

Potential recipient bacteria for antibiotic resistance genes present in GM plants include bacteria that infect plants or reside on plant surfaces, endosymbionts, soil bacteria and gastrointestinal bacteria of people and animals that eat plant products. However, there are major barriers that restrict the likelihood of gene transfer including the persistence of intact DNA in complete genome segments, its integration into the recipient bacterium and sufficient selective advantage to promote proliferation and spread of the recipient bacterium.

One study has reported evidence of low-frequency transfer of a small fragment (180 bp) of an introduced gene derived from GM soybean to microorganisms within the small intestine of human ileostomists (individuals in which the terminal ileum is resected and digested material is diverted from the body to a colostomy bag) (Netherwood et al., 2004). However, only very low concentrations (1–3 copies per $10^6$ bacteria) of the small fragment were detected in samples of microorganisms taken from the small bowel of three of seven ileostomists. Furthermore, the small fragment was only detected after two steps of amplification: (1) extensive culturing of the samples, and (2) Polymerase Chain Reaction

(PCR) analysis. The introduced gene could not be detected in faeces from human volunteers with intact digestive tracts following the consumption of a meal containing GM soya, indicating that the introduced gene is normally completely degraded in the large intestine.

Transfer of plant DNA to bacteria has been demonstrated under highly artificial laboratory and glasshouse conditions, between homologous sequences and under conditions of selective pressure (De Vries and Wackernagel, 1998; De Vries et al., 2001; Gebhard and Smalla, 1998; Mercer et al., 1999; Nielsen, 1998; Nielsen et al., 2000b) and even then only at a very low frequency.

One report has demonstrated transformation of *Acinetobacter baylyi* by pure plant DNA at low rates of $5.5 \times 10^{-11}$ transformants per recipient (Simpson et al., 2007). However, this rate of transformation is likely to be much greater than what would be possible in natural environments where the persistence of DNA in a transformable state and availability of suitable competent bacteria is expected to be much lower (Nielsen et al., 2007; Simpson et al., 2007).

Using antibiotic selection to detect these extremely rare events, *Acinobacter* sp. cells containing a defective copy of the neomycin resistance *(nptII)* gene (with 10 bp or 317 bp of DNA deleted) were observed to incorporate DNA from GM plants (sugarbeet, tomato, potato or oilseed rape) carrying the intact *nptII gene,* leading to restoration of neomycin resistance (Nielsen et al., 2000a, 2000b). However, without the artificially introduced homology in the recipient strain, no uptake of DNA could be detected in *Acinobacter* sp. (De Vries et al., 2001; Nielsen et al., 2000a, 2000b) or in *Pseudomonas stutzeri* (De Vries et al., 2001).

In conclusion, the most likely candidates for HGT from GM plants to bacteria are bacterial genes. However, these genes are often abundant in the environment and more readily transferable by conjugation and transduction. For example, one study of HGT to gut bacteria in bees that pollinated GM *Brassica* with the herbicide tolerance gene, *pat-1*, was confounded by the high background of glufosinate resistance already present in the bacterial flora (Mohr and Tebbe, 2007). Bacteria in the natural environment also remain the best sources for diversity and abundance of genes that may give rise to adverse effects through HGT, such as antibiotic resistance genes (D'Costa et al., 2006; Jones et al., 1986).

### *HGT from GM plants to animals*

The most feasible route of HGT from GM plants to animals is DNA entry across the gastrointestinal tract. This includes both vertebrates and invertebrates that feed on plants above or below ground, animals that feed on herbivores, and pollinators.

In the case of vertebrates, the fate of DNA from GM corn and soybean has been extensively monitored in cattle, sheep, pigs and poultry (Aeschbacher et al., 2005; Beagle et al., 2006; Chowdhury et al., 2004; Deaville and Maddison, 2005; Duggan et al., 2003; Einspanier et al., 2001; Jennings et al., 2003a, 2003b; Mazza et al., 2005; Nemeth et al., 2004; Sharma et al., 2006; Tony et al., 2003). DNA segments can survive intestinal juices and multicopy forms of DNA could be detected in some tissues examined in chickens (muscle, liver, spleen or kidney), but not in eggs (Einspanier et al., 2001). Except for blood or milk, such DNA has been rarely detected in tissues or organs of cattle, sheep or pigs. One report of transgenic DNA in organs of pigs could not demonstrate the presence of an intact gene or integration into the genome of somatic cells (Sharma et al., 2006). In all cases, there was extensive degradation of DNA in the gastrointestinal tract, reducing the size and frequency of DNA transfers (Beagle et al., 2006; Chambers et al., 2002; Chowdhury et al., 2003; Deaville and Maddison, 2005; Phipps et al., 2003; Rossi et al., 2005; Wiedemann et al., 2006).

Therefore, the GM feeding studies show the potential for transient gene transfer to animal somatic cells but not germ line cells (van den Eede et al., 2004). Although uptake of bacterial and viral DNA by mammalian tissues has also been reported, there has been no demonstration of DNA transfer to the germ line cells. This is consistent with genomic sequences that reveal only rare examples of HGT from plants to animals (Bird and Koltai, 2000; Lambert et al., 1999), despite co-evolution for hundreds of millions of years.

Another theoretical pathway proposed is the HGT of viral regulatory sequences found in most GM plants to the human genome, which may disrupt normal gene function and cause disease. On the basis of a report by Kohli et al. (1999) on a recombination hotspot in the Cauliflower mosaic virus 35S promoter, Ho and colleagues have proposed (Cummins et al., 2000; Ho et al., 2000) that the instability of the 35S promoter can lead to high recombination frequencies, facilitating horizontal transfer to the human genome and resulting in overexpression of human genes responsible for cancer, activation of dormant viruses or toxic metabolites. However, several links in this uncertain chain are scientifically inaccurate, mechanistically doubtful or highly unlikely (Hull et al., 2000).

### *HGT from GM plants to viruses*

One additional route for HGT from GM plants involves viruses as the recipient organisms. Viruses that infect a plant have the potential to recombine with endogenous plant genes or other pathogens that co-infect the plant. However, virtually all viral recombination events

are restricted to gene exchanges with other viral sequences. In contrast to certain viruses of animals and bacteria that have large DNA genomes with many host-acquired genes, plant viruses have small genomes and only rare examples of host sequences in the viral genome (Agranovsky et al., 1991; Khatchikian et al., 1989; Masuta et al., 1992; Mayo and Jolly, 1991; Meyers et al., 1991). Therefore, HGT from a GM plant to an infecting virus is likely to be restricted to crops transformed with viral sequences.

Most GM plants carry regulatory sequences from viruses (*e.g.* the Cauliflower mosaic virus 35S promoter). In other cases, viral sequences are introduced to protect the GM plant from infection by the corresponding virus through some form of cross-protection or to express genetic fragments from animal viruses as the basis of viral vaccine production.

When a GM plant with a viral genetic element is growing in the field it will be subjected to invasion by a wide range of viruses. These viruses may include the same species from which the viral transgene was derived (homologous virus). For example, aphids carrying the fully infectious form of Potato leafroll virus may feed on GM potatoes with the coat protein gene of the same virus. Alternatively, those same aphids may harbor other viruses that are capable of infecting the same GM potatoes (*e.g.* Potato virus Y, a heterologous virus). In yet other cases, aphids could inject a virus that may or may not multiply in the injected cell, but is unable to spread and initiate a general infection (non-host virus).

All three classes of virus (homologous, heterologous, non-host) are then able to recombine with the viral transgene. In the case of RNA viruses, this will be with the transgene RNA in the cytoplasm. In the case of an invading DNA virus, it may also interact with the transgene DNA integrated into the plant genome in the nucleus. One exception is the Nucleorhabdovirus group of single-stranded RNA viruses, which replicates in the nucleus, and could therefore also interact with the transgene in both the nucleus and cytoplasm.

The most common type of HGT in viruses arises from homologous recombination, relying on sequence similarity at the point of crossover and results in hybrids with essentially the same properties as the parental virus. Several examples of recombination between a virus and its homologous transgene have been reported (Adair and Kearney, 2000; Borja et al., 1999; Frischmuth and Stanley, 1998; Gal et al., 1992, 1996; Greene and Allison, 1994, 1996; Schoelz and Wintermantel, 1993; Turturo et al., 2008; Wintermantel and Schoelz, 1996). In most cases, a defective virus was used such that recombination would restore full infectivity and thus confer a significant selective advantage on the recombinant. In one case (Wintermantel and Schoelz, 1996), a poorly competitive strain of Cauliflower mosaic virus was used as the infecting virus, whereas the transgene was from a highly competitive strain of the virus. In another case, recombination was obtained under conditions of low selection pressure, but the populations of recombinant viruses was equivalent to that produced from non-transgenic plants (Turturo et al., 2008).

Heterologous recombination may occur at a significantly lower rate and produce less competitive recombinants than homologous recombination. In the case of Brome mosaic virus, homologous recombination occurs experimentally at 5–10 times the frequency as heterologous recombination (Bujarski and Nagy, 1996). However, viral transgenes may create a favorable environment for recombination by complementing some defects that may be associated with an initial recombinant (Jakab et al., 1997).

Recombination between viruses has been associated with major adaptations by increasing virulence, expanding the host range or changing the mode or vector for virus transmission (Gibbs and Weiller, 1999; Rest and Mindell, 2003). For example, genes from influenza strains that infect birds or pigs have recombined by natural means with strains that infect humans (Gibbs et al., 2001). On occasions, these exchanges have resulted in devastating epidemics such as the 40 million deaths from the 1918 "Spanish flu" (Taubenberger, 2006).

Most plant viral genes seem to contribute to pathogenicity (Brigneti et al., 1998; Takeshita et al., 2001). Consequently, recombination with a viral transgene could potentially lead to increased virulence. Many plant viral strains can be distinguished by differences in symptom development. New, highly virulent, strains of African cassava mosaic virus ravage a critical African staple in an epidemic that is still expanding (Legg and Thresh, 2000). These new strains, which can overcome previously resistant lines of cassava, have emerged by recombination between viruses naturally co-existing in cassava (Pita et al., 2001; Zhou et al., 1997). In addition, mutation and recombination of viruses in the laboratory have generated offspring viruses with more severe pathology (Ding et al., 1996). However, these novel recombinant viruses have rarely been tested for fitness or competitiveness (Fernandez-Cuartero et al., 1994).

Like in virus pathology, many viral genes play a part in determining host range (Carrère et al., 1999). Closely-related viruses are often distinguished by differences in host range. Viruses such as those belonging to carlaviruses or sobemoviruses may have a narrow host range as individual members, but the two groups as a whole have very wide host ranges. Therefore, relatively few sequence changes in these viruses have the potential for a change in host range. Accompanying a shift in host, significant new diseases may emerge. For example,

mutations to a potyvirus of cucurbits can give rise to a devastating pathogen of papaya (Bateson et al., 2002).

Although mutation and recombination are commonly attributed to changes in hosts, specific sequence differences that account for the disparity in host range have rarely been identified. Nevertheless, recombination between a virus and any viral transgene can lead to a change in host range (Schoelz and Wintermantel, 1993). Some viruses have exceptionally broad host ranges, such as Cucumber mosaic virus, Tomato spotted wilt virus and Beet curly top virus. All three viruses cause significant losses in many crops. Viral sequences from these viruses have the potential to greatly extend the host range of other viruses through recombination.

A more remote possibility is a large shift in host range, such as between plants and animals. Serious plant pathogens include certain bunyaviruses, rhabdoviruses and reoviruses. Their respective closest relatives are viruses of animals and they can replicate inside their insect vector. The main difference is that the plant viruses possess a movement protein to allow spread between plant cells. The acquisition of a movement protein by certain ancestral animal viruses may have allowed these viruses to multiply in plants and diversify into major groups of plant viruses as seen today.

The presence of a viral movement protein gene in some GM plants may facilitate HGT with an animal virus that results in a host range extended to plants. However, as these HGT events are exceedingly rare, any increase is likely to be insignificant.

In addition, the frequency at which viruses will infect a GM plant and recombine with a viral transgene is dependent on a wide range of factors, including:

- *Nature of the GM plant.* Plant species vary in virus susceptibility. In general, fewer viruses have been isolated from tree species, whilst annual and other short-lived species may be infected concurrently by several different viruses. For example, over 200 viruses belonging to 23 different groups can infect *Nicotiana benthamiana*. In general, however, most crops are usually host to no more than 20 to 30 viruses in nature, of which less than 10 may be of agricultural significance.
- *Availability of virus.* A viral source is required to initiate infection. The abundance of virus and the presence of suitable vectors will affect infection rates.
- *Inherent rate of viral recombination.* Some types of virus may be less prone to HGT than others (Chare et al., 2003).
- *Scale of release.* The number of GM plants, the shape, size and disposition of the plot(s) and the number of release sites will affect the opportunity for infection.
- *Environmental conditions.* These include growing conditions and age of the plants, and climatic factors, which affect the efficiency of virus infectivity or vector availability.
- *Management practices.* People play a critical role in the infectivity and spread of viruses or their vectors, either through cropping systems, choice of crop genetic make-up, farming practices, or movement of infected material.
- *Transgene promoter.* Although the transgene DNA is usually in every diploid cell, transgene RNA may be expressed in only some cells. Nevertheless, the most commonly used promoter is the 35S promoter from CaMV. This promoter gives high expression in the plants vascular system (a common site of viral replication, *e.g.* most aphid-borne viruses) and moderate to high expression in most other cells.
- *Nature and quantity of transgene RNA or product.* If a viral transgene is designed to provide protection based on pathogen derived resistance (Sanford and Johnston, 1985) then high levels of transgene RNA may be expected. However, if resistance is based on induction of gene silencing (Lindbo and Dougherty, 1992) then almost no transgene RNA should be present in the cytoplasm.
- *Selection of offspring from an HGT event.* Spread and persistence of the recombinant virus is dependent on its survival in the cell/plant, that it is reproductively competent, able to be transmissible from cell to cell, capable of transmission by a vector where necessary, is competitive with other viruses, including revertants, target and non-target viruses and can overcome host resistance and agronomic management practices.

In most cases, selection is the major barrier to viruses acquiring heterologous sequences by HGT. Furthermore, to give rise to increased risk, the recombinant virus that acquires a viral transgene should also be more harmful than the parental virus and arise more frequently than background levels of viral HGT. Although recombination events in viruses have sometimes contributed to greater disease burden (Legg and Thresh, 2000), these cases represent only a minute fraction of all HGT events (Froissart et al., 2005).

The main sources for background levels of HGT to plant viruses would be co-infections by two or more viruses and from a broad range of viral sequences that naturally occur in plant genomes. These include multiple direct repeats of geminivirus related gene sequences in the genome of four species of *Nicotiana* (Ashby et al., 1997; Bejarano et al., 1996), multiple copies (mostly defective) of Banana streak virus reside in banana plants (Harper et al., 1999) and a variety of other pararetroviral gene sequences (Caulimoviridae) in tobacco and petunia (Harper et al., 2002). In one case, a caulimovirus sequence is present in the genome of carnation linked to tandem repeats of sequences related to carnation small

viroid (small RNA pathogen) (Vera et al., 2000). Plants, like most other organisms, also have sequences related to retroviruses inserted throughout their genomes. For example, *Arabidopsis thaliana* has 276 retrovirus-related elements related to the Pseudoviridae (Peterson-Burch and Voytas, 2002). The risks from viral transgenes due to HGT are not expected to be greater than risks posed by these endogenous viral sequences.

## GENERAL CONCLUSIONS

HGT is defined as the transfer of genetic material from one organism to another, independent of reproduction. HGT results in unidirectional gene flow, usually of one to several genes, from a donor organism to the genome of a recipient organism. The recipient organism may be closely related to the donor organism or may be an unrelated species.

Sequencing of large numbers of eukaryotic, prokaryotic and viral genomes has shown that HGT is a significant component in the evolution of virtually every organism. Nevertheless, most gene transfers between multicellular eukaryotes and other organisms are detected over time scales of millions of years. This is despite the abundant availability of genetic material in living organisms, and externally, such as in soil, water, feces or even processed foods (Brinkmann and Tebbe, 2007; Douville et al., 2007; Kharazmi et al., 2003; Nielsen et al., 2007). Only a few types of HGT occur sufficiently often to be observed. These frequent HGT events typically involve MGEs such as plasmids and viruses.

From the current scientific evidence, HGT from GM plants to other organisms presents negligible risks to human health and safety or the environment due to the rarity of such events relative to those HGT events that occur in nature and the limited chance of providing a selective advantage to the recipient organism.

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