Journal of Applied Microbiology 2005, **99,** 7–23 doi:10.1111/j.1365-2672.2005.02595.x

A REVIEW

Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection technologies

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2004/0381: received 5 April 2004, revised 31 January 2005 and accepted 1 February 2005

1. SUMMARY

The first generation of genetically modified plants suitable for agriculture was largely produced using antibiotic resistance markers (ARMs) for the preparation of plant transformation vectors or for the plant transformation process itself. In some instances, the ARM gene remains in the finished commercial plant product. Theoretical concerns regarding the risks posed by such markers have resulted in a demand for the production of commercial plants free of ARMs. This, in turn, has resulted in the development of technologies which avoid the use of ARMs or which allow for the efficient excision of such markers following the initial transformation process, but in advance of the selection of commercial plant lines. We review the current status of ARM safety information relevant to existing genetically modified crops as well as reviewing in some detail the available alternatives to ARM use, the utility of these technologies for commercial production, and the issues that might pertain to the use of these alternative technologies for the production of commercial genetically modified crops. Many of the alternative techniques have the potential to be commercially viable, and one or more will necessarily be used for the future production of genetically modified plants free of ARMs. Nonetheless, existing ARM-containing products will remain in the market for the foreseeable future, and the safety of these products therefore remains a relevant issue. Further, the ease of use, cost, availability, safety profile, regulatory status, and utility of alternative techniques should be kept in proper perspective relative to the long history of safe use of ARMs in plant biotechnology.

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2. INTRODUCTION

Historically, antibiotics are 'chemical substances produced by various species of micro-organisms that suppress the growth of other micro-organisms and may eventually destroy them' (Sande *et al.* 1990). In modern usage, and for the purposes of this document, the term antibiotic has been extended to include both chemically modified natural antibiotics and entirely man-made substances which would

more technically be referred to as semi-synthetic or synthetic antimicrobial agents (Sande *et al.* 1990). Competition among microbial species presumably resulted in the occurrence of natural antibiotics, and micro-organisms responded accordingly by developing multiple mechanisms of antibiotic resistance. Most clinically important antibiotic resistance in bacteria (with the exception of mycobacteria) is the result of the acquisition of antibiotic resistance plasmids or other exchangeable genetic elements, which often carry multiple genes for resistance to different antibiotic classes (Sande *et al.* 1990). Bacteria readily share plasmids among others of their own species and even among other species of bacteria, even if distantly related (Sande *et al.* 1990).

The antibiotic resistance traits useful for plant biotechnology involve the production of proteins that chemically degrade antibiotics (e.g.β-lactamase) or that inactivate antibiotics by addition of a chemical group (e.g. *aad*-3 and *npt*-II), as other mechanisms of antibiotic resistance would not be applicable in living plant cells. Antibiotic resistance markers (ARMs) are used in plant biotechnology for two purposes. ARMs are frequently used in the construction of plasmids or vectors used to accomplish plant transformation, such as the amplification of genetic material in *Escherichia coli* or the production of genetically modified *Agrobacterium tumefaciens* which can directly transform plant cells. In this context, the ARM must follow a bacterial promoter sequence and in some cases the finished plant product may contain an ARM gene that bears a bacterial control sequence (Malik and Saroha 1999) and therefore will inefficiently function in the nuclear genome of plants (Courvalin 1998; European Federation of Biotechnology 2001).

The ARMs are also used in the plant transformation procedure when the resulting phenotype does not allow for direct selection. Plant cell transformation is generally inefficient, and transformed cells must be selected from among a much larger number of nontransformed cells. Antibiotic selection markers are generally not necessary for the insertion of herbicide resistance traits into plant cells, as the herbicide itself can be used to select for transformed cells (Padgette *et al.* 1995; Malik and Saroha 1999). For other traits such as insect resistance a selectable marker is useful, if not essential, for the efficient selection of transformed cells (Flavell *et al.* 1992). Even under these circumstances, the resultant plants do not always carry an ARM gene. In some instances, ARMs have been lost early in the development process as a result of deletion or of segregation between the ARM and the gene of interest, and in a few cases development has proceeded without antibiotic markers through the investment of additional time and resources needed to screen many different cells or plants for the desired trait.

Because ARM genes are not necessary for function of the finished plant product, ARM genes may in principle be removed or become nonfunctional by mutation. However, no efficient mechanism of removal or inactivation of an ARM in an existing product is presently available. New techniques, discussed below, may allow for the selective excision of ARMs or other selectable markers once plant transformation is completed. Other types of marker genes are also discussed at greater length below. In some instances, these markers produce phenotypic modification but do not allow for direct selection using a chemical or physical agent, which greatly decreases the efficiency of the post-transformation selection process.

The most commonly used ARM for plant cell selection is *npt*-II (neomycin phosphotransferase II, also referred to as APH-3′-II or aminoglycoside phosphotransferase 3′-II) (Malik and Saroha 1999; European Federation of Biotechnology 2001). This ARM inactivates the aminoglycoside antibiotics neomycin and kanamycin. Other ARMs are commonly used for bacterial selection during the preparation of DNA for use in plant transformation. These genes include the ampicillin resistance gene (β-lactamase, *bla*), which degrades ampicillin and also the aminoglycoside resistance gene *aad* (aminoglycoside adenyl transferase) that chemically modifies and inactivates the antibiotics spectinomycin and streptomycin. In the past, such genes have occasionally been incorporated into finished plant products (Malik and Saroha 1999), but today such markers are routinely eliminated prior to plant transformation. Markers conferring resistance to hygromycin or other antibiotics (tetracyclines, chloramphenicol) have been used in plant research (Day 2003), but do not presently appear in commercial genetically modified plants (Miki and McHugh 2004).

3. CLINICAL UTILITY OF ANTIBIOTICS USED IN PLANT BIOTECHNOLOGY

3.1. β-Lactamase (*bla*/*tem*-1): ampicillin resistance

Ampicillin use became widespread in the early 1960s because this antibiotic was orally absorbed, well tolerated, and had an extended range of activity that included many of the common community acquired respiratory and urinary tract pathogens. Ampicillin has been largely replaced by amoxicillin, which is better absorbed, produces fewer gastrointestinal (GI) side-effects, and is generally approved for three rather than four times a day dosing. These two agents are, for the most part, interchangeable, with the exception of *Shigella* infection, which is not susceptible to amoxicillin. Clinical indications for ampicillin include common community upper respiratory tract infections (otitis media, sinusitis and pneumonia), acute urinary tract infections, some GI infections, and sexually transmitted diseases. These agents are also used in combination with an

aminoglycoside (frequently gentamicin) to combat unusual infections such as listeriosis or enterococcal endocarditis.

Organisms resistant to ampicillin by virtue of carrying the *bla* gene on a plasmid were first described in the 1970s, and ampicillin use has been progressively limited by the emergence of clinical resistance among common pathogens. Resistance is especially prevalent in *E. coli*, *Salmonella*, and *Branhamella*, which affect the urinary, GI, and respiratory tracts, respectively, and is also found with increasing frequency in *Haemophilus influenzae* and gonococcus. *Bla* is reported from many clinical isolates including *Haemophilus*, *Escherichia*, *Neisseria*, *Citrobacter*, *Salmonella*, *Proteus*, *Klebsiella*, and *Pseudomonas* species (Sykes and Matthew 1976; Neu 1992; Malik and Saroha 1999). As a result, ampicillin and amoxicillin can no longer be recommended as first-line treatment for a number of common infections. They do remain useful in the management of some community-acquired infections and in the treatment of susceptible infections once sensitivity has been demonstrated in the laboratory.

The *bla* gene product, β-lactamase, also hydrolyses other penicillins. Penicillin G was the first injectable penicillin drug to be used clinically, but has a limited role today. Resistance among staphlococci was documented shortly after the introduction of penicillin during the World War II (Sande *et al.* 1990; Courvalin 1998). It is no longer the drug of choice for gonorrhoea due to emerging resistance and to the availability of oral agents like ampicillin/amoxicillin. Penicillin V is an orally available analogue of penicillin G and remains a mainstay for use in children with tonsillitis or pharyngitis and to prevent rheumatic fever. However, resistance is beginning to emerge even among the group A streptococci responsible for these conditions. The anti-pseudomonal penicillins (ticarcillin, piperacillin, azlocillin and mezlocillin) are also degraded by β-lactamase and have been extensively used, usually in combination with an aminoglycoside, to treat serious infections including fevers of unknown origin in immunocompromised patients. As a result of the emergence of β-lactamase in hospital-acquired infections, newer β-lactamase-resistant drugs such as the third-generation cephalosporins and the carbapenems are rapidly replacing the older anti-pseudomonal penicillins.

There are a number of alternative antibiotics available to combat infections caused by β-lactamase-producing organisms, including the combination of amoxicillin with the β-lactamase inhibitor, clavulanic acid, and the oral cephalosporins, all of which are stable to β-lactamase. Over the last 10 years, however, a large number of modified β-lactamases have been described in clinically important bacteria. All of these modified β-lactamases are closely related to the original β-lactamase, referred to as *tem*-1, and are derived from *tem*-1 by mutation. Currently, these extended spectrum β-lactamases are found infrequently (<1% of *E. coli* or *Klebsiella pneumoniae* isolates), but they are expected to become increasingly frequent as a result of antibiotic-mediated selection. These newer β-lactamase genes are not used as markers in plant biotechnology. It is theoretically possible for these newer ARM genes to arise by mutation of older β-lactamases inserted during plant transformation or to arise in bacteria that have acquired an ARM from plant material. However, in the authors' opinion such events would not make a significant contribution to the occurrence of these new markers given exceedingly low gene transfer rates (see below), the widespread presence of the original (*tem*-1) β-lactamase gene in bacteria, the overuse of antibiotics resulting in ongoing selective pressure, and the bacterial dissemination of mutant *tem*-1 genes already in existence.

3.2. *npt*-II: neomycin and kanamycin resistance

The *npt*-II gene confers resistance to neomycin, kanamycin, and a number of aminoglycoside analogues such as paromomycin, geniticin, and butirosin that are not in clinical use. NPT-II does not inactivate clinically used 'gentamicin' (a mixture of gentamicins C1, C1a, and C2, which lack the 3′ hydroxyl necessary for the action *npt*-II) but will inactivate some other nonclinically relevant members of the gentamicin family (Azucena and Mobashery 2001; Smith and Baker 2002). Neomycin is the most toxic of the aminoglycoside antibiotics in current use. The drug is too toxic to be given intravenously, and it can be given orally only because it is poorly absorbed. Neomycin is available for a limited number of topical and oral uses (Sande *et al.* 1990). Various preparations of neomycin with or without polymyxin and/or bacitracin and with or without anti-inflammatory steroids are used for the treatment of otitis externa (swimmer's ear), conjunctivitis, and various bacterial skin conditions. Orally, neomycin is used in combination with other antibiotics to sterilize the gut prior to bowel surgery in order to prevent postoperative infections. However, toxic effects are observed even when neomycin is given by mouth, and a number of publications recommend substitution of tobramycin in preoperative procedures (Donnelly 1993). Although bacterial resistance to neomycin is widespread, this does not necessarily compromise its efficacy as currently employed, as pathogens are exposed to very high local concentrations of drug in the various approved topical uses.

Although kanamycin was one of the first aminoglycosides in clinical use, it has largely been supplanted by other aminoglycosides (gentamicin, tobramycin and amikacin) that have both a broader spectrum of antimicrobial activity and a reduced incidence of severe side-effects. Amikacin remains a second-line treatment for mycobacterial infections, including tuberculosis (Bartlet 1997; Gillespie 2002); but the

existence of HIV/AIDS in tuberculosis-infected populations in the developing world has severely limited the use of parenteral agents. Because kanamycin is no longer in use for tuberculosis, any discussion of *npt*-II is essentially irrelevant to the issue of drug-resistant tuberculosis. Nevertheless, as this issue has been raised repeatedly in the scientific literature, a discussion will be included here.

Antibiotic resistance in *Mycobacterium tuberculosis* results from a mutation of the mycobacterial chromosome, not from acquisition of a new, exogenous ARM gene. Mycobacteria are not known to exchange DNA under natural conditions (Courvalin 1996, 1998; Potera 1998). In the laboratory these organisms can generally be made to exchange genetic material only under the most extreme assistive conditions, such as the combination of homologous sequences (homologous recombination) and electroporation (Kalpana *et al.* 1991). The natural conditions of *M. tuberculosis* during clinical infection further mitigate against the occurrence of recombination events as summarized by Gillespie (2002):

…individual strains of *M. tuberculosis* have little opportunity to interact and exchange genetic information with other strains compared with, for example, organisms that colonize the nasopharynx or the gastrointestinal tract. In these locations, other bacteria may transmit antibiotic resistance determinants through transmissible genetic elements, transposons, integrons, and plasmids, by transduction or transformation. This option is not available for *M. tuberculosis*, so resistance can only occur through chromosomal mutation….

Further, mycobacteria are believed to be intracellular throughout most of the infection process, and have little replicative activity in the gut proper, although they may inhabit lymph nodes of the GI tract. Although Gillespie (2002) goes on to describe the rare occurrence of mycobacterial resistance due to 'mobile genetic elements', this occurs via inactivation of a specific chromosomal gene, does not result in the acquisition of novel enzymatic activity, applies only to the antibiotic pyrazinamide, and is not believed to be readily transferable among tuberculosis strains (S.H. Gillespie, personal communication). This latter mechanism is not at all relevant to the use of ARMs in plant biotechnology.

Aminoglycoside resistance in *M. tuberculosis* is the result of alterations in the antibiotic target molecule within the mycobacterial cell, generally due to an alteration of the 16S ribosomal RNA (Alangaden *et al.* 1998; Courvalin 1998; Ramaswamy and Musser 1998; Silva *et al.* 2001; Gillespie 2002). In regard to other antibiotics used in the treatment of tuberculosis, extensive reviews by Ramaswamy and Musser (1998) and Gillespie (2002) clearly demonstrate that chromosomal resistance, not ARM transfer, is the only identified and characterized mechanism resulting in resistance to rifampin, isoniazide, ethionamide, streptomycin, pyrazinamide, ethambutol, fluoroquinolones, kanamycin, amakacin, viomycin, capreomycin and cycloserine. (Data for individual agents are available in numerous publications including Alangaden *et al.* 1998; Wu *et al.* 1999; Silva *et al.* 2001.) This finding is rather striking in view of the known high prevalence of antibiotic resistance among other (non-mycobacterial) species of bacteria and the frequent presence, therefore, of these antibiotic resistance genes in the environment. That a relatively infrequent (see below) event such as mutation accounts for virtually all resistance in mycobacteria is testimony to the extraordinarily low frequency, if any, with which mycobacteria acquire exogenous genetic material.

Resistance in *M. tuberculosis* emerges through individual point mutations at varying rates for different antibiotics. For example, streptomycin-resistant mutants emerge at a rate of approx. 10^{-6} per cell division. Patients with tuberculosis harbour between 10^{10} and 10^{13} bacteria. This means that a patient with a large mycobacterial load already has bacteria that are streptomycin resistant even though they have never received this drug. It is for this reason that therapy for tuberculosis employs three or four agents: the risk that a bacterium would be resistant to four agents before treatment is very low (10^{-12} or less). Multiple-drug resistance in tuberculosis (MDR-TB) generally indicates a poor therapeutic choice by the physician or poor adherence on the part of the patient. Indeed, the report of the World Health Organization (Pablos-Mendez *et al.* 1998, emphasis added) states that:

Multidrug regimens can prevent the emergence of clinical drug resistance. The problem of resistance results from treatment that is inadequate, often because of irregular drug supply, inappropriate regimens, or poor compliance.

In regard to the emergence of MDR-TB, Ramaswamy and Musser (1998) state (emphasis added):

In principle, MDR-TB strains could arise as a consequence of sequential accumulation of mutations conferring resistance to single therapeutic agents, or by a single-step process such as acquisition of an MDR element, or mutation that alters (for example) cell wall structure. All available evidence indicates that the former mechanism is critical to the emergence of these organisms. Currently, there is no evidence that the processes commonly mediating multidrug resistance in other bacteria, such as conjugal transfer of plasmids encoding combinations of resistance genes or transfer of transposable elements with resistance genes occur in *M. tuberculosis*.

Once one or more mutations have occurred, the existence of antibiotic-selective pressure allows resistant organisms to preferentially survive in spite of apparent competitive

disadvantages conferred by the acquired mutation (Pablos-Mendez *et al.* 1998). There is now evidence that the emergence of antibiotic resistance in *M. tuberculosis* following stress to the organism is substantially accelerated by inducible changes in gene expression, including expression of a variant, low-fidelity DNA polymerase which substantially enhances mutation rate (Boshoff *et al.* 2003; Friedberg and Fischhaber 2003). This mechanism, not dependant in any way upon ARM genes, may well explain the frequent and rapid development of antibiotic resistance in the absence of multi-drug therapy.

3.3. *aad*-3: streptomycin and spectinomycin resistance

The *aad*-3 gene confers resistance to two little-used aminoglycoside antibiotics, streptomycin and spectinomycin (Gilman *et al.* 1996). However, both agents have seen some resurgence recently as a result of bacterial resistance to other antibiotics, especially for the treatment of tuberculosis.

Streptomycin was commonly used during the 1950s and 1960s but has largely gone out of use due to a high frequency of bacterial resistance and the occurrence of ototoxicity (Gilman *et al.* 1996; Reynolds 1996). The re-emergence of tuberculosis in the developing world has, however, caused some resurgence in the use of streptomycin. Infection with *M. tuberculosis* is invariably treated with multiple drugs due to the chronic nature of the infection and the propensity of the organism to rapidly develop resistance to single-agent therapy. Because of low cost, streptomycin is still the most common aminoglycoside used to treat tuberculosis in developing nations (Bartlet 1997). However, as noted above, parenteral agents have largely fallen out of favour in the context of HIV/AIDS. As with kanamycin (see above), resistance in *M. tuberculosis* results from a mutation of the gene for ribosomal RNA, not from acquisition of a new ARM gene. Other indications for streptomycin include the treatment of rare infections such as plague, tularaemia, brucellosis and ratbite fevers. It is also used in combination with β-lactams to treat endocarditis caused by enterococci that are highly resistant to gentamicin but still moderately susceptible to streptomycin (Chiew *et al.* 1998).

Spectinomycin is an aminoglycoside-like antibiotic used for the treatment of anogenital and joint infections caused by gonorrhoea (*Neisseria gonorrhoea)*. It is not effective in the treatment of pharyngeal gonococcal infections. Because of the need for high compliance in treating these infections, therapy has focused on agents having efficacy on a single dose. While the preferred antibiotics had been intramuscular penicillins, these drugs are no longer first-line therapy due to emerging drug resistance (WHO 1991). Currently, the third-generation cephalosporin, ceftriaxone, is the treatment of choice, with the fluoroquinolones and spectinomycin considered as satisfactory alternative drugs. The latter is recommended for pregnant women allergic to β-lactam agents.

3.4. *hph* [*APH*(4)]: hygromycin resistance

Hygromycin is a fungally derived aminoglycoside analogue which has been utilized as a selective agent for the generation of genetically modified organisms, including plants (Day 2003; Miki and McHugh 2004). Resistance to hygromycin is conferred by several different phosphotransferases, including aminoglycoside phosphotransferase (APH) 4-Ia (derived from *E. coli*); APH(4)-Ib (derived from *Pseudomonas pseudomallei*, also confers resistance to glyphosate herbicide); and APH (7″) (derived from *Streptomyces hygroscopicus*) (Wright and Thompson 1999). Only the *E. coli*-derived APH(4)-Ia, also referred to as hygromycin phosphotransferase (*hph*) appears to have been utilized in plant transformation (Day 2003; Miki and McHugh 2004).

Hygromycin does not appear to have seen significant clinical use in either human or veterinary medicine, as the authors have been unable to locate reports of ongoing clinical use (or of proposed future utility for emerging resistant organisms) published since 1966. Further, the *hph* gene does not appear to confer resistance to other clinically relevant aminoglycosides (Wright and Thompson 1999). While laboratory use of *hph* continues (Miki and McHugh 2004), this marker does not appear in commercialized plants available as of 2003 (Miki and McHugh 2004).

Overall, the clinical utility of the antibiotics potentially impacted by the ARMs used in plant biotechnology varies widely. While ampicillin, amoxicillin, and the penicillins are still in general clinical use, the theoretical transfer of ARMs from plants to bacteria would have little impact on clinical antibiotic resistance rates as ARMs for this class of antibiotics are already widespread in bacterial communities. Of the other potentially affected agents, neomycin sees use only as a topical agent and, although resistance is common, resistant bacteria still succumb to the high concentrations achieved with topical therapy. Kanamycin, spectinomycin, and streptomycin have very limited utility in clinical medicine, and hygromycin appears to have no clinical utility at this time or in the foreseeable future. While some of these agents play a limited role in the treatment of tuberculosis, *M. tuberculosis* does not acquire antibiotic resistance by means of plasmid transfer, and the clinical use of these agents has been greatly restricted by concerns related to the risk of disease transmission with intravenous administration.

4. THEORETICAL CONCERNS SURROUNDING THE USE OF ARMS

There are four basic questions that have been raised regarding ARMs: (i) Is the gene itself harmful to humans

or animals? (ii) Is the protein product produced by the gene harmful to humans or animals? (iii) Can ARM genes transfer into bacteria and adversely impact antimicrobial therapy? (iv) Can ARM genes be deleterious as a result of transfer into human cells? Each of these questions will be addressed below.

4.1. Is the gene itself harmful to humans or animals?

The ARM genes are no different than any other DNA present in plants or animals, are digested and processed in the gut just like the DNA from any other source, and are normally present in gut bacteria (Jonas *et al.* 2001). The quantity of ingested transgenic DNA is actually quite small in comparison with total ingested DNA from plant and animal sources. For example, in dairy cows fed transgenic insect-protected maize, the estimated intake (54 μg) of transgene DNA compared with a total DNA intake of 54 g d^{-1}, is about one one-millionth of total ingested DNA (Aumaitre *et al.* 2002). In transgenic maize, transgenes represent only 0·0001% of total DNA (Society of Toxicology 2002). The FDA regards all DNA, from genetically modified organisms or otherwise, to be Generally Recognized as Safe (GRAS) in food or pharmaceutical use and the Society of Toxicology (2002) and others have concluded that the possibility of adverse effects from the introduced DNA is minimal (Jonas *et al.* 2001). Thus, it appears that ARM genes themselves, as with other genes in food or feed, pose no health risk to humans or animals.

4.2. Is the protein product produced by the gene harmful to humans or animals?

When expressed in plant cells, ARMs produce a protein product that will be ingested along with all of the tens of thousands of proteins that constitute a major portion of the human diet. ARM proteins most commonly used in plant biotechnology are not fundamentally different from other dietary proteins, have no toxic or harmful effects on human beings or experimental animals, and are categorized by FDA as GRAS in food, feed, and pharmaceutical use. These proteins are rapidly digestible with pepsin, are heat labile, and have been subjected to bio-informatic analysis to assure the absence of significant homology to known food allergens. ARMs thus share the characteristics of proteins which are unlikely to produce food allergic reactions. NPT-II, for example, fulfils these criteria and, in addition, has been shown to be nontoxic when consumed in foods in animal studies and in biotechnology pharmaceutical agents given intravenously to humans (Flavell *et al.* 1992; Calgene, Inc. 1993; Fuchs *et al.* 1993). Further, ARM genes regulated by bacterial sequences, if present in plants, will be ineffective in protein production.

The ARM proteins are frequently produced by bacteria in the human intestine (see Section 5). Thus, humans have been exposed to these proteins throughout history. Following antibiotic treatment, antibiotic-resistant bacteria are selected for in the gut, and the frequency of resistant bacteria rises. Adverse effects of ARM proteins produced under these circumstances have not been identified. Taken together, these considerations allow one to conclude reasonably that ARM proteins present no unique hazards above those of other dietary proteins.

4.3. Can ARM genes transfer into bacteria and adversely impact antimicrobial therapy?

In the laboratory, several ARM genes have been shown to be transferred in functional form when a high degree of homology is present between the donor ARM and the recipient bacterial genome and when a suitable selection pressure for the functional ARM is also applied. This has been the subject of extensive investigation *in vitro* and *in vivo*.

deVries and Wackernagel (1998) and Gebhard and Smalla (1998) explored potential horizontal gene transfer from plants to bacteria using a strain of *Acinetobacter* which has known ability to take up foreign DNA. To facilitate the gene transfer, the bacteria were first genetically modified with an *npt*-II gene that had been inactivated by an internal deletion, to allow homologous recombination. The bacteria were then exposed experimentally to plant DNA carrying an intact *npt*-II gene, and activity of the incomplete *npt*-II gene was restored, demonstrating that transformation had occurred. This presumably occurs because of an exchange of DNA between the damaged and intact copy of the *npt*-II gene, and demonstrates both uptake and incorporation of the test DNA. This exchange was dependent on the presence of homologous sequences between the donor plant DNA and reconstitution of the genetic marker in the recipient strain was not observed in the absence of ongoing antibiotic selection.

The homologous recombination of plant DNA into bacterial DNA has also been investigated by Nielsen *et al.* (1997b), who found that the modified *Acinetobacter* strain described above could be transformed in a laboratory mix of soil and that the ability of DNA to transform bacteria was lost after several hours, depending upon soil conditions and other factors. Gebhard and Smalla (1998) demonstrated a similar phenomenon using the homologous DNA system and, in additional experiments, were able to achieve transformation using *npt*-II containing DNA derived from genetically modified sugar beets in otherwise sterile soil, but not in soil containing naturally occurring microbial flora (Nielsen *et al.* 2000a,b).

Tepfer *et al.* (2003) demonstrated the homology-dependent reconstitution of the deletion-mutated *npt-II* in

Acinetobacter using material from six different plant donor species [*Arabidopsis*, oilseed rape, morning glory, carrot and tobacco (two species)]. Purified DNA and macerated plant material were utilized as DNA sources. The addition of sterile soil reduced, but did not eliminate, DNA transfer. Soil having normal bacterial flora was not tested. Kay *et al.* (2002) obtained similar results using plastid-transformed tobacco (which carries a high copy number of genes per cell), the *aad*-3 marker gene, and *Acinetobacter*. While higher transformation rates were observed using transplastomic leaf material, as expected due to the higher number of transgenes, rates observed in root tissues were no higher than in nuclear transformed plants.

The critical role of DNA sequence homology was also noted in experimental studies in *Ralstonia* species by Bertolla *et al.* (1997). The transformation rate was reduced 10-fold (from roughly 10^{-8} to 10^{-9}) by a decrease in the length of identical DNA from very long sequences (200–700 bp) down to 50 bp. In further studies (Bertolla and Simonet 1999), transformation of *Acinetobacter* by the *aad*-3 gene in tobacco was reduced by the use of DNA from divergent species to a level commensurate with the spontaneous mutation rate for acquisition of resistance. Transfer of the *aad* marker from transgenic tobacco to *Acinetobacter* could only be demonstrated in the presence, but not in the absence, of homologous sequences (Kay *et al.* 2002; Tepfer *et al.* 2003). Similarly, deVries *et al.* (2001) demonstrated laboratory transformation of soil bacteria by potato DNA (*npt*-II) and found that the transformation rates in the absence of homology dropped by a factor of 100 million or more in *Pseudomonas* and *Acinetobacter*.

Investigations of DNA transfer have included experiments designed to mimic field conditions as well as studies in the field. Although small fragments of plant DNA can be detected for up to 2 years in soils (Gebhard and Smalla 1999), a number of attempts to transform field samples of bacteria with plant DNA have been unsuccessful (Gebhard and Smalla 1998; Paget *et al.* 1998). Attempts to transform bacteria *in vitro* using DNA (*npt*-II) from transgenic sugar beet and potato and attempts to transform *Erwinia* using DNA (*bla*/*tem*-1) from transgenic potato were also unsuccessful (Schluter *et al.* 1995; Nielsen *et al.* 1997a). Studies of the use of biomass fertilizer derived from genetically modified crops failed to demonstrate transfer of antibiotic resistance to *E. coli* or *B. subtilis* even after 7 years of product use (Andersen *et al.* 2001). Finally, Badosa *et al.* (2004) were unable to demonstrate an increase in ampicillin resistance rates or the transfer of plant-derived ampicillin resistance to culturable bacteria in samples obtained from a commercial site planted with genetically modified corn bearing the TEM-1 marker. While the authors note a number of significant limitations related to culturability and sample size, they also note the high background frequency of ampicillin resistance and suggest that the contribution of gene transfer from plants to bacteria is likely to be negligible.

Turning to mammalian studies, Mercer *et al.* (1999) demonstrated that large quantities of plasmid DNA could survive brief digestion with sterilized human saliva and retain the ability to transform oral bacteria (*Streptococcus gordonii*) previously selected for transformation competence. Additional experiments (Mercer *et al.* 2001) demonstrated transformation by linear DNA as well, but only when homology was present. Survival of the plasmid DNA was brief (half life <6 s), but was sufficient for transformation to occur. Similar findings were recently reported by H.J. Flint, D.K. Mercer, K.P. Scott, C. Melville and A.L. Glover (unpublished data, 2002).

The stability of transgenic DNA in simulated gastric and intestinal fluids has also been investigated (Martin-Orue *et al.* 2002). Naked DNA was largely degraded in simulated gastric fluid, but DNA within intact maize or soya material was stable at 30 min. In simulated intestinal fluid, transgene fragment survival for both naked and whole food DNA was substantial after 30 min of digestion. The authors concluded that some transgenes might survive passage through the small intestine. In chickens, marker gene or gene fragments could be detected in the crop and stomach of some birds following feeding with genetically modified maize. However, no gene survival was detected in the intestinal tract (Chambers *et al.* 2001). Duggan *et al.* (2000) investigated the *in vitro* survival of ARMs (*bla*/*tem*-1) from transgenic maize and the ability of this material to transform *E. coli* following exposure to ovine saliva, ovine rumen fluid, and silage effluent. Plasmid DNA survived 24-h exposure to saliva and retained the ability to transform. Although PCR detection demonstrated some plasmid survival at 30 min in rumen fluid and silage, the ability to transform was lost within 30 s, suggesting rapid gene fragmentation under these conditions.

Recent studies in human volunteers fed a soya-based meal demonstrated that transgenic soya DNA survives passage through the small intestine in patients with ileostomies, but no intact DNA could be detected in the faeces of volunteers with intact digestive tracts (Netherwood *et al.* 2004). Native soya DNA and transgenic DNA did not differ in their degradation in the GI tract. Experimental transformation of gut bacteria with intact transgenic DNA could not be detected using sensitive techniques. While gene fragments could be detected in gut bacterial flora using PCR following expansion in culture, the frequency of such fragments was unchanged by the experiment. Incorporation into bacterial DNA was not directly demonstrated, and no full-length gene transformants were detected. The gene fragments were present in only a very small fraction of overall gut flora, and the organisms carrying these genes could not be amplified or isolated using antibiotic selection. No transformed bacteria

were demonstrated in the faeces of individuals with intact GI tracts.

Based upon available experimental data demonstrating a lack of gene transfer, and considering the sensitivity of the assay system used, Redenbaugh *et al.* (1994) estimated that at the highest achievable gene transfer rate, transfer of the *npt*-II gene from Flavr Savr^{TM} tomato to bacteria, would increase the frequency of kanamycin-resistant bacterial flora in the human gut by only $2{\cdot}6 \times 10^{-13}$, a frequency which is inconsequential in comparison with existing rates of kanamycin resistance (see Section 5).

The extreme rarity of intact, functional gene transfer events in the open environment is not surprising. In order for an ARM to transfer from plants to gut bacteria, the gene would have to be excised from the plant chromosome without being destroyed by cellular enzymes, survive intact in the gut environment, and be acquired in intact form by a transformation-competent bacterial organism (WHO 2000; Jonas *et al.* 2001). Further, the need for a bacterial control sequence means that genes would need to separate precisely from their control sequences and be incorporated in the bacterial DNA in proper relationship to a bacterial control sequence (Courvalin 1998; WHO 2000; Jonas *et al.* 2001). Once incorporated into a recipient microbe, a selection pressure would be required to amplify the transformed population, which otherwise would be diluted and lost due to the existing microflora. The likelihood of such gene transfer events occurring sequentially is exquisitely low, and little-or-no selection pressure exists in either the gut or the soil environments, explaining why plant-bacterial gene transfer, although theoretically possible, has yet to be demonstrated with inserted plant genes in intact animal systems. Even if such rare transfers of genetic material were to occur, other mechanisms of ARM acquisition and the proliferation of antibiotic resistance bacteria caused by overuse of antibiotics are far more important causes of clinical antibiotic resistance.

Overall, there is currently no documentation of intact, functional ARM gene transfer from plants to bacteria occurring in nature. Various aspects of the gene transfer question have been examined in detail and, cumulatively, demonstrate that while transfer of an ARM from plant material to soil microbes is theoretically possible, such events must be exceedingly rare. Based on the published data, the rate of gene transfer to bacteria in the laboratory is not reflective of the rate of spontaneous transfer from plants to bacteria in the absence of other bacterial flora, homologous recombination, and/or appropriate selective pressures in the environment. The available data are thus 'compatible with, but do not prove,' DNA transfer in nature (Tepfer *et al.* 2003).

As noted by Thomson (2001), horizontal gene transfer events 'need to be viewed in the context of evolutionary time.' However, even very rare events may have an ecological impact if the transferred gene alters the fitness of the recipient bacteria or cell. Hence, 'the genes encoded by the transferred DNA in the GM plant should be the focus of biosafety considerations, rather than the transfer process itself' (emphasis added).

Finally, one should note that several options may exist for further minimizing the likelihood of gene transfer from GM plants to bacteria. Transfer rates could be minimized through the selection of DNA sequences lacking in homology with bacterial sequences (Kay *et al.* 2002). Alternatively, the barrier between plant and bacterial species could be effectively restored through the inclusion of introns in the plant sequences, which bacteria are unable to properly excise (Libiakova *et al.* 2001; Tepfer *et al.* 2003).

4.4. Can ARM genes be deleterious as a result of transfer into human cells?

Plant gene fragments have been demonstrated in the cells that line the GI tract and in animal immune system cells (white blood cells). However, these gene fragments have never been shown to incorporate into animal DNA, and no expression of an intact, functional plant-derived gene in humans or animals has been reported. Because all whole foods contain DNA, humans have been exposed to plant DNA as well as DNA from gut bacteria throughout evolutionary history. In spite of this, there is no evidence for the regular incorporation of intact plant or bacterial genes into human cells. It is interesting to note that while ARM genes would have no utility for human cells as they are relatively resistant to antibiotics, even genes which would seem to be of extremely high utility, such as the genes allowing for production of essential dietary nutrients, have not been acquired from plants in the course of human evolution.

Regarding other investigations of DNA transfer to humans, the transfer of SV40 virus DNA from bacteria (*E. coli*) to human cells has been demonstrated in the laboratory (Schaffner 1980). However, this virus is known to be adapted for function in mammalian systems. Schubbert *et al.* (1994, 1997) demonstrated that when large doses of purified bacterial or bacteriophage (a virus that infects bacteria) DNA were given orally to rodents, fragments of that DNA could be detected in various body cells. These cells consisted primarily of phagocytic cells, which normally ingest fragments of DNA and other material. In one case, partial integration of DNA into a single cell may have occurred, but there was no evidence that mammalian cells were transformed with an intact, functional gene. Methylation patterns in DNA differ between plant and mammalian species. Bacterial ARMs inserted into plants acquire plant DNA modification patterns which are believed to trigger phagocytosis and which may be responsible for this observation (Beever and Kemp 2000).

Overall, these data indicate that when large quantities of specific DNA are given to animals and when sensitive detection methods for gene fragments are employed, transfer of some genetic material into phagocytic cells can be demonstrated. However, the incorporation of a functional gene has not yet been demonstrated. If transfer of DNA to human cells were to occur, proliferation of such genes would be unlikely, as they would offer no selective advantage (Society of Toxicology 2002). Transfer to gut cells, in particular, is not likely to be a concern as these cells are routinely shed, and transfer to isolated immune cells is unlikely to affect clinical immune function (Society of Toxicology 2002).

5. ALTERNATIVE SOURCES OF MICROBIAL ANTIBIOTIC RESISTANCE

Bacteria can easily acquire antibiotic resistance from other bacteria. Bacteria in the intestinal tract of animals and humans, as well as in the environment, frequently carry ARM genes. These genes may serve as a source of antibiotic resistance in gut bacteria, as soil bacteria are frequently ingested (Nwosu 2001) and interchange of genetic material among bacteria occurs readily in the environment (Jiang and Paul 1998; Davison 1999). Such 'horizontal gene transfer' is the major cause of resistance among bacteria (Courvalin 1998; Smalla *et al.* 2000; Nwosu 2001).

β-Lactamase genes exist on hundreds of different naturally existing plasmids (Sykes and Matthew 1976). Most of these plasmids carrying the original *bla* (*tem*-l) gene, are capable of transferring other bacteria (conjugative plasmids), are found commonly in soil organisms (Nwosu and Ladapo 1999), and transfer readily in soil from one bacterial species to another (Hefron *et al.* 1975; Yamamoto *et al.* 1982; Bunny *et al.* 1995). Consequently, these genes are widespread in Gram-negative bacterial species of clinical importance, such as *Haemophilus*, *Escherichia*, *Neisseria*, *Citrobacter*, *Salmonella*, *Proteus*, *Klebsiella* and *Pseudomonas*. β-Lactamase (*tem*-l) has also been demonstrated in isolates from patients infected by these same organisms (Sykes and Matthew 1976) and is widely distributed geographically (Neu 1992). This commonly occurring gene accounts for approx. 50% of the cases of clinical resistance to penicillins in Gram-negative bacteria (Malik and Saroha 1999; Medeiros and Crellen 2000).

Resistance to aminoglycosides was extensively reviewed by Shaw *et al.* (1993), well before the introduction of plant biotechnology products into the environment. At that time, resistance to neomycin and kanamycin was frequent in bacterial populations. Resistance to both antibiotics almost invariably occurs together as a result of a single antibiotic resistance gene. A survey of 19 European hospitals published in 1999 reported that of the nine known resistance genes, three were responsible for resistance to kanamycin in 211 strains among 699 staphylococcus isolates (Schmitz *et al.* 1999). Among *M. tuberculosis* isolates ($n = 68$) and reference strains ($n = 4$), kanamycin resistance was found in 35 strains (Bastian *et al.* 2001). Similarly, kanamycin resistance has become widespread in *N. gonorrhoeae* in Indonesia (Lesmana *et al.* 2001). In food animals, the level of faecal kanamycin resistance in *Enterococcus faecium* and *Ent. faecalis* ranges from 5 to 35%, and from 2 to 18% respectively (DANMAP Report 1997).

Resistance to streptomycin/spectinomycin is among the most widespread in nature (Kelch and Lee 1978; Atkinson 1986; Levy *et al.* 1988). Resistant bacteria are widespread (Levy 1978) and are found in food (Corpet 1988), landfills (Nwosu and Ladapo 1999), drinking water (Kelch and Lee 1978) and faeces (Levy 1978). Levels of resistant organisms are higher in purified water than unpurified water (Armstrong *et al.* 1981) and can represent up to 50% of the isolated bacteria (Calomiris *et al.* 1984). In a human study, about one-third of individuals not taking antibiotics have >10% of gut bacteria resistant to streptomycin, and in roughly half of these individuals resistance is 50% or greater (Levy *et al.* 1988). Gilbert *et al.* (1993) demonstrated that a large percentage of bacteria in the root zone of field-grown soyabeans were resistant to streptomycin (18–52%) or spectinomycin (44–84%).

Resistance to hygromycin does not appear to have been subjected to systematic study, as no systematic investigation of the prevalence of this gene in general bacterial populations was located by the authors. This presumably reflects the fact that hygromycin has no clinical utility in human or veterinary medicine (see Section 3.4).

Bacteria can also acquire antibiotic resistance when spontaneous mutation occurs in the context of antibiotic-selective pressure. In this context, it is informative to look specifically at the issue of tuberculosis (*M. tuberculosis*) in developing nations. Extensive data are available for India, where antibiotic resistance was noted in the 1980s, became common in the 1990s and has continued to increase (Prakash 2002). In a 1989 study in Madras of TB patients who survived following 6 months of treatment, 31% still had active infections, with 65% being resistant to isoniazid, 12% to rifampicin and 19% to streptomycin. A more recent study in Mumbai, found that 53% of isolates recovered from sputum were resistant to streptomycin and 25% resistant to kanamycin as well as demonstrating variable resistance to other antibiotics (Chowgule and Deodhar 1998). Similar results have been published by other authors (Varaiya and Gogate 1998; Mathur *et al.* 2000). Clearly, streptomycin and kanamycin resistance in *M. tuberculosis* is quite common and has been so since long before genetically engineered crops were introduced. Further, it should be remembered that resistance to aminoglycosides among

clinical isolates of *M. tuberculosis* is invariably due to mutation of ribosomal RNA rather than the acquisition of antibiotic resistance plasmids in spite of the fact that *aad* and *npt*-II like genes exist in natural mycobacterial populations, and that mycobacteria do not generally exchange DNA under natural conditions.

The transfer of antibiotic resistance genes from plants to bacteria, if it were to occur at all, cannot be considered as an important mechanism of bacterial resistance when compared with the ready availability of such genes from neighbouring bacteria and even the lesser frequency of spontaneous mutation. Jelenic (2003) reviewed this issue relative to *npt*-II and concluded that 'the practical impact of both the consumption of GM plants containing *npt*-II by humans or animals and the transfer of *npt*-II gene from GM plants to GI or soil bacteria would be negligible.' More importantly, there is agreement that the problem of clinical antibiotic resistance is aggravated by two common practices: the overtreatment of minor bacterial or viral infections in humans and the use of antibiotics in agriculture. Antibiotics are used in agriculture to treat animal illness, as growth promoters for poultry and livestock and as pesticide on plants (streptomycin). Furthermore, antibiotics enter into the general environment through manufacture, disposal, and therapeutic use in humans and animals. All of these sources of antibiotics apply selective pressure to bacterial populations and encourage the presence of antibiotic resistance genes in the bacterial population (Smalla *et al.* 2000; Nwosu 2001).

6. ALTERNATIVES TO ARM GENES

6.1. Options for selective technologies

Multiple alternatives to the use of ARMs as selectable markers do exist. However, most have not been subjected to regulatory scrutiny for national or international approvals at this time. Because the use of ARM marker replacement or excision systems is new, regulatory agencies will need to consider whether any new issues are involved in approving the use of these technologies.

As noted above, herbicide tolerance is often a desired trait and, when present, can also be used as the selectable marker. Such systems include *bar* (phosphinothricin acetyl transferase/resistance to glufosinate), EPSPS (5-enolpyruvylshikimate-3-phosphate synthase, resistance to glyphosate), ALS (acetolactate synthase, resistance to chlorosulfuron), and *Bxn* (bromoxinil nitrilase, resistance to bromoxinil) (Day 2003). While it is tempting to use this marker even when the trait is not essential in the finished plant product, the presence of herbicide-tolerant selectable markers may be undesirable when the trait is not necessary for product function, perhaps limiting control options for 'volunteer' re-emergence of plants during a following crop rotation or providing a source of herbicide resistance to sexually compatible weed species.

Other new selectable marker systems rely on the growth of plant cells in the presence of unusual nutrients or other factors, including cytokinins, glucuronides, xylose, or mannose, that either will not support the growth of nontransformed plant cells (negative selection) or provide a growth advantage (positive selection) (Joersbo and Okkels 1996; Okkels *et al.* 1997; Haldrup *et al.* 1998; Joersbo *et al.* 1998). These systems have been recently reviewed at length (Day 2003; Miki and McHugh 2004), and share a number of issues. Selectable markers of this type have not yet appeared in approved food plants. These markers invariably entail the addition of new metabolic pathways to an existing plant species, with the possibility of novel products and/or pleiotropic effects. A complete risk assessment will need to be conducted to assess the impact on the overall plant metabolism, nutritional, and compositional characteristics, the safety of the introduced protein, the potential ecological impacts of plants that can grow on a new substrate, and the potential, if any, for such genes to migrate to, and persist in, naturally occurring related species (European Federation of Biotechnology 2001). A unique marker, pepper ferridoxin-like protein (*pflp*), is a plant-derived gene conferring resistance to a bacterial plant pathogen, and represents a unique approach to avoiding the use of genetic material from nonfood species while conferring a potentially useful secondary plant characteristic (Hood 2003).

It is apparent that difficulties with selectable markers – ARM or otherwise – could be avoided with the use of technology to eliminate the marker from the finished plant product once the desired traits have been successfully introduced. The precise and controlled removal of selectable markers after plant transformation is only possible if a mechanism for removal was foreseen at the time of transformation. The perceived need to eliminate selectable markers from finished products has resulted in the development of methods that allow the more efficient elimination of selectable marker genes from the genetically modified crop after transformation but before commercialization.

Three different methods have been identified that hold the potential for removal of marker genes. Co-transformation is an approach that allows separate integration of the marker gene and the desired trait at two different locations in the DNA. If these locations are on separate chromosomes (or sufficiently separated on a single chromosome) traditional breeding can be used to separate the desired trait genes(s) from the selectable marker. Homologous recombination and recombinase-mediated excision are approaches in which specific, enzyme-mediated processes result in excision of the marker gene.

6.2. Co-transformation

In co-transformation, the plant is transformed with a marker gene and a desired trait gene on two different segments of DNA. When this is made, the marker gene will sometimes integrate on a different chromosome than the desired trait and can be removed by traditional breeding (Miki and McHugh 2004). Three strategies using *Agrobacterium* transformation were devised to provide an opportunity for obtaining transformants in which the gene of interest and the marker gene integrate into separate genomic locations, allowing segregation of the two insertions by breeding. Two *Agrobacterium* strains can be used with separate plasmids, each with a single T-DNA (transfer DNA) region, separately encoding the desired trait and the marker (Depicker *et al.* 1985; McKnight *et al.* 1987; DeBlock and Debrouwer 1991; De Neve *et al.* 1997). Alternatively, a single *Agrobacterium* strain containing two plasmids, each with a single T-DNA region, may be used (DeFramond *et al.* 1986; Daley *et al.* 1998). Finally, a single plasmid carrying two independent T-DNA regions can be constructed (Komari *et al.* 1996). The overall frequency of co-transformed events in which it is possible to separate marker and trait-conferring genes during breeding is broadly similar for all three approaches. Although better than attempting transformation without a selectable marker, one must still produce many more plants to obtain the same number of potential commercial genetically modified events one would achieve with current transformation methods (Miki and McHugh 2004).

The use of co-transformation in association with particle gun transformation methods was explored in 10 different species, including tobacco, petunia, *Arabidopsis*, maize, soyabean, rice, oilseed rape, barley, bean and wheat (Peerbolte *et al.* 1985; Christou *et al.* 1989; Damm *et al.* 1989; Lyznik *et al.* 1989; Christou and Swain 1990; Spencer *et al.* 1990; Goto *et al.* 1993; Herve *et al.* 1993; Wan and Lemaux 1994; Aragao *et al.* 1996; Zhong *et al.* 1996; Barro *et al.* 1997). Independent integration of the fragments at two different positions followed by independent breeding segregation was observed only in one report by Herve *et al.* (1993). Thus, it does not appear that co-transformation will be commercially practical for species that require particle gun transformation.

6.3. Homologous recombination

Repeated DNA sequences, particularly when adjacent in a chromosome, can recombine in a way that leads to the excision of DNA sequences that are located between the two repeated DNA sequences (Lichtenstein *et al.* 1994). At least two laboratories have reported successful use of this technique (Ebinuma *et al.* 1997; Zubko *et al.* 2000).

The frequency at which homologous recombination occurs in plants is low, varies according to crop species, and is proportional to the length of the direct repeated DNA sequence. Recombination between closely linked repeated sequences in maize occurs at frequencies of about 0·5% (Sudupak *et al.* 1993). Research is underway to identify conditions which would increase the recombination frequency by several orders of magnitude and thus provide a practical means of targeted gene insertion, perhaps without a need for active selection (Reiss *et al.* 1996).

6.4. Recombinase-mediated excision

An alternative strategy for marker elimination is the use of site-specific recombinase-mediated marker excision systems. At least four different site-specific recombination systems have been shown to function in plant cells: the bacteriophage P1 Cre/*lox* system (Dale and Ow 1990; Odell *et al.* 1990; Russell *et al.* 1992); the *Saccharomyces cereviseae* 2 mm circle FLP/*FRT* system (Lyznik *et al.* 1993; Lloyd and Davis 1994; Kilby *et al.* 1995; Sonti *et al.* 1995); the pSR1 system of *Zygosaccharomyces rouxii* (Onouchi *et al.* 1991; Sugita *et al.* 2000) and the Gin recombinase system of phage Mu (Maeser and Kahmann 1991). These have been reviewed by Gilbertson (2003) and by Miki and McHugh (2004). All are two-component systems involving a recombinase enzyme, which acts to catalyse recombination between two short, specific DNA sequences.

The Cre/lox system of marker excision is perhaps best studied, and will be used as an example of excision systems. The Cre/*lox* system consists of the CRE recombinase enzyme, which specifically recognizes and catalyses recombination between two specific DNA sequences (*lox* sequences). DNA sequences, including selectable markers, which are flanked by two *lox* sites, can be precisely excised in the presence of the Cre recombinase (Gilbertson 2003; Zhang *et al.* 2003). The strategy for the elimination of selectable marker genes is thus to produce transformants in which the selectable marker gene is flanked by the specific recombination target sequences and subsequently introduce the recombinase protein.

There are three strategies for introduction of the Cre protein. In the first strategy, the inserted DNA can contain the marker gene with an adjacent *cre* gene, both flanked by a single pair of lox sites. The expression of the *cre* gene is controlled by an inducible promoter, selected so that the gene remains turned off until particular conditions (for example, the introduction of a hormone or chemical to the media) are met. Secondly, transformants can be constructed which contain the desired trait and a *lox*-site flanked marker gene. The Cre protein is introduced into selected lead lines by crossbreeding with a line already containing the *cre* recombinase gene. The marker gene will be excised and, in

the following generation, the *cre* gene and the desired trait can be separated by conventional breeding (Dale and Ow 1991). This method is not suitable for vegetatively propagated crops. Thirdly, the *cre* gene can be introduced transiently into lines that contain *lox*-flanked markers by direct transformation (Gleave *et al.* 1999).

The Cre/*lox* system appears to have many advantages. There is no reason to believe that the *lox* sequence itself presents any hazard (it is DNA and produces no protein), and the system is present in natural bacterial populations, including gut flora, and is therefore part of normal human exposure (European Federation of Biotechnology 2001). While widespread use of this system will require completion of a full safety assessment ongoing work indicates that stable transformation is obtained in maize and other plant species (Gilbertson 2003; Miki and McHugh 2004).

Each of these systems has advantages and disadvantages. The co-transformation techniques lose the tight connection between the selectable marker and the desired trait, increasing the risk of selecting a plant that has not actually been transformed with the desired trait and thus reducing the efficiency of the overall transformation process. Presently, most companies work with several hundred to several thousand transformed lines to allow selection of, ultimately, a single commercial lead event. An increase in this number would have significant cost and time repercussions. Further, co-transformation for selectable marker gene elimination cannot be used for vegetatively propagated species like potato and sugarcane, as their propagation from cuttings or shoots does not offer the opportunity to separate the trait and marker genes.

Cre/*lox* and other recombinase-mediated systems require the use of an exogenous recombinase gene and the application of traditional breeding or other techniques to express the recombinase activity at an appropriate time in the transformation process. Homologous recombination, in principle, can be used for perfect integration and excision of transgenes without the need for recombinase recognition sites, but this technique appears to be relatively inefficient at producing transformants, and has not been successfully implemented in crop plants.

7. CONCLUSION

Antibiotic resistance genes have played a critical role in the development of plant biotechnology. In contrast to many of the alternatives, there is a long history of safe use of the ARMs in food and nonfood applications in biotechnology. Further, the use of ARMs provides an inexpensive, readily available technology and therefore may be of extreme importance to developing countries or to smaller institutions.

The ARMs chosen for food plant applications affect antibiotics for which little application exists and/or for which resistance is already widespread within bacterial populations. Hazards of the genes themselves and of the direct gene products appear to be negligible, as both DNA and the ARMs are GRAS. Transfer to mammalian cells does not appear to be of biological relevance for plant genes, including the ARMs. Transfer of genetic material between plants and bacteria can be demonstrated at low frequencies under ideal laboratory conditions using high degrees of DNA homology. Gene transfer in the open environment remains so infrequent as to be theoretical. While the limits of science are such that the possibility of such transfer can never be absolutely excluded, it can be stated with great certainty that the transfer of ARMs from plants to bacteria could never become an important cause of bacterial antibiotic resistance when contrasted against the ubiquitous nature of resistance plasmids, the far higher frequency of resistance by spontaneous mutation, and the selective effects of antibiotic use in the environment. Further, risk of transfer could be reduced through the use of nonhomologous sequences (Kay *et al.* 2002) or through the inclusion of introns in plant-expressed ARM genes (Libiakova *et al.* 2001; Tepfer *et al.* 2003).

No expert panel has ever identified significant risk, associated with the use of ARMs in plant biotechnology. Expert groups have, however, correctly pointed out that the ARMs are not necessary for the function of the final plant product, but are needed only for the development process (WHO 2000). Further, they are of public concern even in the absence of scientific evidence (The Royal Society of London 2000). For this reason, efforts are underway to produce the next generation of genetically modified food crops using various techniques to avoid the presence of ARMs in the finished plant product.

Although alternative marker genes are available in theory, all involve the alteration of plant metabolism in some manner, and all (except herbicide tolerance) have yet to navigate the safety assessment and regulatory processes. Excision technologies are highly promising and will allow the removal of ARMs or other markers as desired, but also have yet to undergo full safety assessment and regulatory evaluation.

The ARMs will continue to be present in the current generation of genetically modified crops, and thus will be present in the environment for some time to come. The available data, in the opinion of the authors, support the safety of these existing markers and does not appear to justify removal of these existing products from the market. Alternative markers and new gene removal techniques will necessarily be used for future genetically modified crops, but still require formal safety assessment and do not have the proven track record, research base, and regulatory acceptance of the present ARM systems, and thus may increase

costs and development time for much-needed products. This impact will likely be disproportionately burdensome for individual academic investigators and for laboratories developing products which may bring great benefits, but little commercial return. Given this, one may question the wisdom of replacing a widely accepted, fully evaluated system that has produced no documented harm to date with a novel system whose costs are greater and whose impacts are less fully understood.

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