Controversy Associated With the Common Component of Most Transgenic Plants – Kanamycin Resistance Marker Gene

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Summary

Plant genetic engineering is a powerful tool for producing crops resistant to pests, diseases and abiotic stress or crops with improved nutritional value or better quality products. Currently over 70 genetically modified (GM) crops have been approved for use in different countries. These cover a wide range of plant species with significant number of different modified traits. However, beside the technology used for their improvement, the common component of most GM crops is the neomycin phosphotransferase II gene (nptII), which confers resistance to the antibiotics kanamycin and neomycin. The nptII gene is present in GM crops as a marker gene to select transformed plant cells during the first steps of the transformation process. The use of antibiotic-resistance genes is subject to controversy and intense debate, because of the likelihood that clinical therapy could be compromised due to inactivation of the oral dose of the antibiotic from consumption of food derived from the transgenic plant, and because of the risk of gene transfer from plants to gut and soil microorganisms or to consumer’s cells. The present article discusses these possibilities in the light of current scientific knowledge.

Key words: plant genetic engineering, nptII, transgenic plants, horizontal gene transfer, antibiotic resistance marker gene, food safety, GM crops

Introduction

Genetically modified (GM) crops are being developed for a variety of reasons, including resistance to herbicides and pests, length of shelf-life in the case of supermarket products, efficiency of processing, improved nutritional value etc. The success of these endeavours is mostly determined by both the ability to deliver foreign genes in host plant cells and by the efficiency with which transgenic plants can be regenerated from transformed cells (1). When the gene of interest is transferred to plant cell, one somehow has to identify the cells that have taken up the additional genetic material. For that purpose, the gene of interest is in most cases linked to a selectable marker gene which confers antibiotic resistance. It allows efficient selection of transformed cells and subsequent regeneration of transgenic plants. Therefore, most GM crops that have been grown on 60 million ha around the world (2) contain antibiotic resistance marker genes. The most commonly used marker gene is neomycin phosphotransferase II gene (nptII), which confers resistance to the antibiotics kanamycin and neomycin. Some transgenic plants also contain antibiotic resistance genes that are under the control of prokaryotic
promoters, and therefore are not expressed in plant cells. These are incorporated into plant genomes because they are present on the same plasmid as the construct used to transform plant cells and have been used for selection in bacteria during gene cloning.

Standard safety evaluation of using any gene including antibiotic resistance marker includes an assessment of the safety of the protein encoded by the gene. In case of antibiotic resistance genes, whether they are expressed or not, experts have to answer to additional questions that have become the subject of intense debate (3): what are the therapeutic uses of the antibiotic that the marker gene products inactivate and how widely are they used? What is the likelihood that clinical therapy could be compromised due to inactivation of the oral dose of the antibiotic by consumption of food derived from the transgenic plants? How prevalent is resistance to these antibiotics among bacteria naturally found in the gut or in the environment? What is the likelihood that the therapeutic use of antibiotic could be compromised from transfer of the antibiotic resistance gene from food to gut epithelium with subsequent expression? What is the likelihood that antibiotic resistance marker genes could be transferred from transgenic plants to soil microorganisms with subsequent expression of the gene? How meaningful is the potential rate of transfer of an antibiotic resistance gene to pathogenic microorganisms thereby rendering them refractory to the antibiotic? The issue is already covered by abundant scientific literature including the opinions of different scientific committees (3–9). The aim of this paper is to summarise the issue in the light of the latest developments of the scientific knowledge.

Neomycin Phosphotransferase II Gene (nptII) and Its Product

The antibiotic resistance marker gene nptII, which encodes aminoglycoside 3'-phosphotransferase II (APH(3')II), is one of the most widely used selectable marker genes in plant genetic engineering. It was originally isolated as a component of transposon Tn5 from the bacterium Escherichia coli (10). The APH(3')II, also called neomycin phosphotransferase II (NPTII) or kanamycin phosphotransferase II, is an enzyme that catalyzes the transfer of a phosphate group from ATP to a hydroxyl group of aminoglycoside antibiotics including neomycin, kanamycin, paromomycin, ribostamycin, gentamicins A and B, as well as butirosins, thereby inactivating the antibiotics (3). Of the antibiotics that are inactivated by APH(3')III, only neomycin and kanamycin are currently in therapeutic use for humans and animals (3).

Although the marker gene expression is required only during the first steps of the plant transformation process, it is driven by constitutive promoter and therefore the antibiotic inactivating enzyme is produced in all the tissues and throughout the lifetime of the transgenic plant. Since the nptII gene is routinely used as a selectable gene in the production of transgenic plants, the safety of the gene product has been the subject of many investigations and evaluations which concluded that APH(3')II poses no risk to either humans, animals or the environment (3,6,11–17). In these studies the potential toxicity and allergenicity of the APH(3')II have been investigated, as well as the probability that the presence of APH(3')II in food or feed would compromise the therapeutic efficiency of orally administrated antibiotics.

Potential Horizontal Transfer of the Plant Derived nptII Gene to Soil or Intestinal Microorganisms and to Consumer’s Cells

Transfer of plant DNA fragments to microbial or mammalian cells would require the following steps (6,7):

- survival of the released DNA fragments in an aggressive environment
- uptake of the DNA fragments by microbial or mammalian cells
- DNA taken up should be stably established in the recipient cells
- the establishment should at least be neutral otherwise the recipient cells are counter-selected

Fate of the DNA released in soil

Free plant DNA is released into soil by decaying plant material. The persistence of free DNA in soil is affected by different abiotic and biotic factors. However, after cell death plant DNA is protected from degradation by cell wall for some time (18). Additionally, the content and type of clay minerals influence the extent to which free DNA is adsorbed to mineral surface and, thus, protected from degradation by nucleases (19–22). Therefore, large amounts of extracellular DNA are readily found in most soils and persist for months or even years, indicating that the turnover of naturally released extracellular DNA is quite slow (23–27). Since plant DNA can persist adsorbed on soil particles or protected by plant cell wall, competent bacteria colonising in close vicinity could take up this DNA. In addition, for those bacteria that have developed specific symbiotic or pathogenic relationships with plants, conditions for gene transfer could be even more favourable (28–31).

Natural transformation of soil bacteria

Natural genetic transformation is a process by which bacteria are able to take up and integrate exogenous free DNA from their environment. This process enables the recipient organisms to acquire novel genes or heritable traits. Although numerous reports indicated that horizontal gene transfer events would be very rare in the environment it has effectively changed the ecological and pathogenic character of bacterial species (19,32–35).

It is estimated that an average soil bacterial community contains 10^9 cells/g of soil belonging to 2000–1800 distinct genomes (36–39). Although relatively few species have been shown to carry the genes required to develop a natural state of competence (33), their number could be significantly higher considering that more than 99% of all soil bacteria remain uncultured in vitro (40). Selective screening of soil bacteria from field trials of genetically modified plants containing antibiotic resistance marker genes have shown no bacterial transformants (26,27,41). In addition, several groups have failed
to detect horizontal gene transfer from transgenic plants to bacteria under laboratory conditions (42–44). However, uptake of transgenic plant DNA fragments by bacteria based on recombination of a partially deleted nptII gene after recombination with transgenic plant homologues has been demonstrated in various conditions (45–50). De Vries and Wackernagel (45) studied the ability of the opportunistic soil bacterium Acinetobacter sp. strain BD413 to take up and integrate transgenic plant DNA under the optimised laboratory conditions. The assay was based on the recombinational repair of an nptII gene with an internal 10-bp deletion located on a plasmid downstream of a bacterial promoter. When competent Acinetobacter sp. strain BD413 cells containing the mutant nptII gene on a plasmid were transformed with DNA from various transgenic plants (potato, tomato, sugar beet, oilseed rape and tobacco) carrying nptII as a marker gene the kanamycin-resistant transformants were obtained (45). Similar observations were also made using transgenic sugar beet DNA and sugar beet homogenates for transformation of Acinetobacter strain BD413 carrying nptII gene with 317-bp deletion on a plasmid (46). Both groups showed that the bacterium could access plant DNA under optimised in vitro conditions if homologous stretches of DNA were present, but studies done under optimised in vitro conditions sometimes have little relevance to natural systems such as soil. However, Nielsen et al. (47) showed that horizontal transfer of DNA, extracted from transgenic sugar beets, to bacteria, based on homologous recombination, can occur in soil. They demonstrated that restoration of a 317-bp-deleted nptII gene on plasmid in Acinetobacter sp. strain BD413 cells could be obtained in sterile soil microcosms after addition of nutrients and transgenic plant DNA encoding a functional nptII gene. In a similar assay Tepfer et al. (50) obtained kanamycin-resistant transformants of Acinetobacter sp. strain BD413 in sterile soil without addition of nutrients. Since competent cells prepared in vitro were used in both above studies, it should be considered that the Acinetobacter sp. strain BD413 is unable to develop competence when grown in soil (40), and that competence is lost very rapidly in situ when competent cells are inoculated into soil (51). However, Demanche et al. (40) found that two other bacteria species also present in most soils (Agrobacterium tumefaciens and Pseudomonas fluorescens) were able to take up replicating plasmid in their natural environment, soil microcosms, without any specific physical or chemical treatment. Interestingly, P. fluorescens produced transformants in both sterile and nonsterile soil microcosms but failed to do so in various in vitro conditions. Therefore, the actual status leading to a physical, chemical, physiological, or genetic induction of competence in A. tumefaciens and P. fluorescens in soil remained unknown. Concerning natural conditions that might allow soil bacteria to take up DNA from the environment, it has been shown recently that thunderstorms and lightning discharges might play important role in that process (52). The Escherichia coli strain DH110B has been efficiently transformed with replicating plasmid in soil microcosms only after the soil was subjected to laboratory-scale lightning that had an electrical field gradient and current density similar to those of full-scale lightning. Although E. coli is not a soil bacterium the universality of this process needs to be considered since the cells from nearly all bacterial taxa can be electroporated in the laboratory more or less efficiently and independently of their physiological state and with transforming DNA of prokaryotic or eukaryotic origin (52). In addition, it has been demonstrated that Acinetobacter sp. strain BD413 developed a competence state in planta during the active colonisation of Ralstonia solanacearum-infected plants (30, 53). The study was performed with transplastomic tobacco plants (transgene aadA that confers resistance to spectinomycin and streptomycin was integrated into chloroplast genome). The Acinetobacter sp. cells carrying a plasmid containing tobacco plastid sequences were transformed by the plant's transgene (aadA) during co-infection of transplastomic tobacco plant with R. solanacearum. However, no transformants were observed when the homologous sequences to the chloroplast genome were omitted from the Acinetobacter sp. plasmid (30).

The results of all studies dealing with the transfer of plant transgenes to soil bacteria by natural transformation have shown that the main limitation to such events would be related to the presence of homology between foreign DNA and recipient genome (45–50). For instance, competent cells of the soil bacteria Pseudomonas stutzeri and Acinetobacter sp. BD413 (both harbouring a plasmid with an nptII gene containing a small deletion) were transformed with the same efficiency with an nptII gene whether it was present in the genome of transgenic potato or on plasmid DNA. However, in the absence of homologous sequences in the recipient cells the transformation by nptII dropped by at least about 106-fold in P. stutzeri and 105-fold in Acinetobacter resulting in the latter strain in ≤1·1013 transformants per nptII (48). This indicates a very low probability of non-homologous DNA fragments to be integrated by illegitimate recombination events during transformation (48). Working also with the nptII gene, de Vries and Wackernagel (49) found that the integration of nonhomologous foreign DNA into the genome of the Acinetobacter sp. BD413 during transformation indeed was at least 105-fold lower than that of homologous DNA, but the integration of nonhomologous foreign DNA increased at least 105-fold when it was linked on one side to a piece of DNA homologous to the recipient genome. This homology-facilitated illegitimate recombination decreased with decreasing homology length but was still measurable at 183 bp (49).

The presented data indicate that gene transfer from transgenic plants to soil bacteria could occur under natural conditions at least when certain homologous sequences are shared between transgene and the DNA of the recipient cell. Studies on transfer of nptII gene and other antibiotic resistance marker genes have revealed that the transfer of a marker gene from a genetically modified plant to soil bacteria that do not already contain the same resistance gene is extremely unlikely. Even in case a kanamycin sensitive soil bacterium acquires plant derived nptII gene the spread of the new trait in the bacterial population would depend on competitive (selective) advantage of any transformed cells. As the occurrence of kanamycin in natural soil has not yet been detected, Nielsen et al. (47) have suggested that...
natural soil conditions would rarely produce the selective pressure required for fixation of possible transfers of the nptII gene from transgenic plants into the recipient bacterium. On the other side, kanamycin-resistant bacteria are abundant in natural soils (29,45,47,54). For instance, de Vries and Wackernagel (45) examined the DNA extracted from four soil samples taken in different areas on farm land and all four samples contained DNA that could be amplified by nptII-specific PCR primers. The concentration of the target nptII sequence varied between $2 \cdot 10^5$ and $3 \cdot 10^8$ molecules/g of soil (dry weight). Therefore, under the worst-case assumptions, kanamycin-resistant transformants resulting from plant DNA left in the fields would represent not more than one in 10 million of the existing kanamycin-resistance soil population (3).

Fate of the DNA released in the intestinal tract of mammals and birds

To address the possibility that bacteria or epithelial cells of gastrointestinal tract may take up and integrate diet-derived DNA fragments, the stability of DNA in all regions of the digestive system must be taken into consideration.

Dietary intakes of nucleic acids in humans depend on the origin of the diet and will vary widely between individuals but are typically in the range of 0.1–1 g/person/day (41). The oral cavity is the site first contact between the incoming food-derived DNA and the resident bacteria and it is one of the most complex and heterogeneous microbial habitats in the human body. Mercer et al. (55) investigated the survival of plasmid DNA in human saliva in vitro. Competitive PCR was used to monitor the survival of a 520-bp DNA target sequence from a plasmid after admixture of the plasmid with fresh saliva indicating that the nucleic acid did not survive the passage through the complete intestine (54). Natural transformation of gastrointestinal bacteria

The data presented above demonstrate that food-derived DNA fragments of various sizes could be detected in different parts of gastrointestinal tract of mammals and birds. Since the concentration of bacteria is high [e.g. $10^{12}$ cells/cm$^3$ in human colon, $10^{11}$ cells/cm$^3$ in rumen (5,59)], the gastrointestinal tract could be very conducive to horizontal gene transfer events if resident bacteria are capable of transformation in that environment.
Mercer et al. (55,60) showed that the oral bacterium *Streptococcus gordonii* DL1 could be transformed in *vitro* by replicating and non-replicating plasmid in the presence of human saliva, implying that naturally transformable bacteria could successfully take up DNA fragments released in human oral cavity. However, the transformation with non-replicating plasmid was obtained only when homology to bacterial chromosomal sequences was provided (60). Additionally, it has been demonstrated that another oral bacterium *Streptococcus mutans* can be transformed in biofilm, its natural environment (61). It has also been found that a small proportion of the indigenous intestinal microflora of ileostomists fed transgenic soybean acquired a food derived transgen, but the authors were unable to isolate those bacteria by colony blot hybridisation or a PCR pooling strategy (54). Duggan et al. (62) successfully transformed competent *E. coli* DH5α cells in filter-sterilised ovine rumen fluid with replicating plasmid containing ampicillin resistance gene. However, the significance of this investigation should be considered under the light of the following facts: bacteria *E. coli* is a minor constituent of the resident microflora of the rumen (representing some 10^9/cm^3 cells in the rumen of healthy adult cattle and sheep) and the population levels of *E. coli* strains carrying transmissible multiple antibiotic resistance can reach 10^11/cm^3 cells in the rumen of sheep maintained under common dietary conditions (62).

The presented data indicate that naturally competent residents of gastrointestinal tract of mammals and birds could be transformed with food-derived DNA fragments, especially if the homology between exogenous DNA and recipient cell is provided or when exogenous fragments are part of replicating plasmids. The studies on natural transformation of soil bacteria are also relevant to horizontal gene transfer in the gut since many soil organisms are carried into the gut with food especially fresh fruits and vegetables. However, the contribution of plant-derived kanamycin resistance gene is expected to be extremely small since genes for resistance to kanamycin already occur quite commonly in the animal gut microflora (e.g. the flora of the human gut naturally contains about 10^{12} kanamycin resistant bacteria (11)). It has been calculated that consumption of GM tomato containing nptII gene would lead to a maximum projected increase in the number of kanamycin resistant bacteria in the human gut of 2.6 · 10^{-13} % (14). Furthermore, antibiotic resistance genes occurring naturally in gastrointestinal flora are often associated with highly mobile genetic elements like conjugative plasmids and transposons (e.g. nptII gene was originally isolated as a component of transposon Tn5 from the gastrointestinal bacterium *E. coli*) that are readily mobilizable between taxa and represent the most common method of acquiring antibiotic resistance determinants among bacteria. Thus, the practical impact of the transfer of kanamycin resistance gene from GM plants to gastrointestinal bacteria would be negligible.

**Transfer of diet derived DNA to animal cells**

Another concern associated with food derived from GM crops is the possibility that transgenes might become incorporated into the consumer’s genetic make-up. A number of studies have examined the take-up of DNA by mammalian cells from the diet. Schubert et al. (57) demonstrated that phage DNA fragments orally administered to mice were taken up by intestinal wall epithelia and reached the nuclei of leukocytes as well as spleen and liver cells. The isolation of recombinant clones suggested that phage DNA fragments were covalently linked to mouse DNA. Later studies in mice showed that diet derived DNA could be found in the foetuses and newborn mice of pregnant mice consuming large amount of naked DNA fragments (63). This DNA was found in the nuclei but never in all of the cells of the foetus suggesting a transplacental pathway rather than germline transmission (63). In both studies discussed above a large amount of the naked DNA was fed to mice. However, similar results were obtained when the fate of plant specific nucleus encoded gene was followed in mice after feeding them with soybean leaves – a 337-bp fragment was detectable by PCR in DNA from liver and spleen (64). Klotz and Einsparin (65) reported the detection of chloroplast DNA fragment in white blood cells of a cow fed a diet containing GM soybean, but under the same experimental conditions the detection of transgene fragment in white blood cells was unsuccessful. Plant DNA was not found in the milk either (65). Similarly, in a more recent study ten Holstein/Friesian cows receiving up to 26.1% of GM soybean in their diet did not have detectable amounts of transgene DNA in their milk (66). In a study of cattle and chickens fed GM maize under normal feeding conditions, it was found that only short DNA fragments (<200 bp) derived from chloroplasts could be detected in the blood lymphocytes of cows (67). In all other cattle organs investigated (muscle, liver, spleen, kidney) plant DNA was not found although there were faint traces in milk. However, in all chicken tissues (muscle, liver, spleen, kidney) the short maize chloroplast gene fragment was detected, but not in eggs. In contrast, fragments originating from the soybean transgene were not detected in any of the cattle or poultry samples (67). The same strategy was used to study a possible transfer of residual chloroplast specific DNA as well as transgene fragments of GM maize into different pig organs (blood, muscle, liver, spleen and lymph nodes) after feeding pigs with conventional and GM maize (68). Although short chloroplast DNA fragments (199 bp) were successfully amplified from the intestinal juices of pigs up to 12 h after the last feeding, neither chloroplast-specific DNA nor transgene specific fragments were detected in any pig tissue investigated (68). A field study examining supermarket poultry samples (leg, stomach, breast and wing muscle) led to frequent detections of the short (199 bp) chloroplast DNA fragment. Furthermore, faint signals for the maize specific zein gene fragment were detected in these poultry tissues (68). Additional PCR examinations using unhatched chicken embryos showed that neither chloroplast nor maize genes were present endogenously within the wild-type poultry genome indicating that only a transient transfer of short forage DNA into most poultry organs could be suspected (68). The presented studies indicate that depending on the animal species and the type of food, a more or less significant transfer of foreign food DNA into some types
of consumer’s cells can be assumed, especially if the amount of specific DNA in food is large (e.g. organelle derived DNA). Since throughout evolution living beings have been confronted by huge amounts of food derived DNA an uptake of certain amounts of foreign gene material must be accepted as a normal event for man and other animals. Various hypotheses have been proposed on how foreign DNA can reach the mammalian organism through gastrointestinal or placental portals, and which consequences should be considered (41, 64, 67, 69). Today no safe indication for the biological relevance of highly degraded DNA can be provided to prove an interaction with consumers health – although it seems to be very unlikely that functional genes are transferred by this process (67). However, DNA derived from GM crops is equivalent to DNA which has always been consumed with human diets, so any risks from the consumption of any DNA since all DNA is handled by the body in the same way. Furthermore, most of the genes used for the genetic modification of food and feed organisms including nptII gene come from organisms for which there is a long history of human and animal exposure.

Conclusions

The nptII gene, which confers resistance to the antibiotics kanamycin and neomycin, is the most widely used antibiotic resistance marker gene in plant genetic engineering. Although the transfer of nptII gene from GM plants to soil and gastrointestinal bacteria or to consumer’s cells has to overcome a series of hurdles, the experimental approaches have demonstrated that each of the steps required can be achieved in nature. At the same time the data show that when both a homology between transgene and recipient genome and selection pressure are not present the chances of transfer to bacteria are extremely low. Furthermore, the nptII gene is already present in very large amounts among the soil and gastrointestinal bacteria (carried by easily exchangeable genetic elements), meaning that humans and animals are permanently exposed to the nptII gene whether they consume GM or non-GM plants. Therefore, the practical impact of both the consumption of GM plants containing nptII gene by humans or animals and of the transfer of nptII gene from GM plants to gastrointestinal or soil bacteria would be negligible.

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References

Rasprave o genu za otpornost na antibiotik kanamycin, zajedničko značajki većine transgenih biljaka

Sažetak

Primjenom genetičkog inženjerskog u oplemenjivanju biljaka dobivene su kulture otporne na nametnike, bolesti i druge nepovoljne činile okoliša, kao i ono pobožljivih prehrambenih i drugih svojstava. Trenutačno se u svijetu uzgaja više od 70 sorata biljaka porne na nametnike, bolesti i druge nepovoljne činile okoliša, kao i one poboljšanih transgeničkim inženjerskim metoda (genetički modificirane kulture – GM kulture), koje obuhvaćaju brojne vrste s različitim značajkama. Međutim, osim tehnologije primijenjen je u njihovom oplemenjivanju, većinu GM biljaka zajednici je gen za neomycin-fosforotransferazu II (nptII), koji određuje otpornost na antibiotike kanamicin i neomicin. Gen nptII

koristi se u biljnom genetičkom inženjerstvu radi olakšanog izdvajanja transformiranih stanica u početnoj fazi postupka. Primjena gena za otpornost na antibiotike u biljnom genetičkom inženjerstvu tema je mnogobrojnih rasprava zbog mogućnosti inaktivacije oralno apliciranih antibiotika u potrošača genetički modificiranih biljaka, te zbog rizika horizontalnog prijenosa gena za otpornost na antibiotike iz GM biljaka u mikroorganizme tla i probavnoga sustava, te prijenosa u stanice potrošača. U radu se raspravlja o tim rizicima u vezi s najnovijim znanstvenim spoznajama.