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An *Arabidopsis thaliana* ABC transporter that confers kanamycin resistance in transgenic plants does not endow resistance to *Escherichia coli*

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Summary

Concerns have been raised about potential horizontal gene transfer (HGT) of antibiotic resistance markers (ARMs) from transgenic plants to bacteria of medical and environmental importance. All ARMs used in transgenic plants have been bacterial in origin, but it has been recently shown that an *Arabidopsis thaliana* ABC transporter, *Atwbc19*, confers kanamycin resistance when overexpressed in transgenic plants. *Atwbc19* was evaluated for its ability to transfer kanamycin resistance to *Escherichia coli*, a kanamycin-sensitive model bacterium, under simulated HGT, staged by subcloning *Atwbc19* under the control of a bacterial promoter, genetically transforming to kanamycin-sensitive bacteria, and assessing if resistance was conferred as compared with bacteria harbouring *nptII*, the standard kanamycin resistance gene used to produce transgenic plants. *NptII* provided much greater resistance than *Atwbc19* and was significantly different from the no-plasmid control at low concentrations. *Atwbc19* was not significantly different from the no-plasmid control at higher concentrations. Even though HGT risks are considered low with *nptII*, *Atwbc19* should have even lower risks, as its encoded protein is possibly mistargeted in bacteria.

Introduction

Antibiotic resistant markers (ARMs) derived from bacteria are the most commonly inserted genes in transgenic plants (Miki and McHugh, 2004) and allow for the detection and selection of transformed plants. The entire marker or a portion of the marker may remain in the final commercial product, which has produced concerns about horizontal gene transfer (HGT) of ARMs back to bacteria, thus creating potentially greater antibiotic resistance problems. The most frequently used ARM in plant transformation is the *Escherichia coli* K12 transposon Tn*5* neomycin phosphotransferase gene (*nptII*), which confers resistance to several aminogloycoside antibiotics including kanamycin and geneticin (Garfinkel *et al.*, 1981), and has been engineered with plant-specific promoters to facilitate expression in plant cells (reviewed in Miki and McHugh, 2004).

Even though HGT risks from transgenic plants are considered low by regulatory agencies (Calgene, 1990; USFDA/CFSAN, 1998), there are concerns that HGT between plants and soil microorganisms may be underestimated (Heinemann and Traavik, 2004). However, it has been argued that the likelihood of a gene transfer event from a transgenic plant to a bacterium is so rare as to be negligible (Schluter *et al.*, 1995; Broer *et al.*, 1996; Nielsen *et al.*, 1997; Gebhard and Smalla, 1998; de Vries and Wackernagel, 1998). This argument is based on several factors that have the potential to restrict HGT between plants and soil bacteria: availability of DNA, ability of bacteria to take up DNA, stabilization of DNA in the bacteria and expression of DNA in the bacteria (Nielsen *et al.*, 1998). The main barriers to HGT between species are the ability of the bacteria to take up plant DNA (competence) and establishment or the stabilization of plant DNA (Nielsen *et al.*, 1998). Several bacteria have natural competence, such as *Bacillus subtilis* and *Acinetobacter* sp. strain BD413 (Lorenz and Wackernagel, 1994). However, competence is only one factor in the ability of genetic exchange; once the DNA is taken up by the bacteria, the DNA must be stably maintained (Nielsen *et al.*, 1998).

The most likely mechanism of gene transfer from transgenic plants to bacteria is natural transformation (Nielsen *et al.*, 1998; Bertolla and Simonet, 1999), in which competent bacteria can take up free DNA (Stuart and Carlson, 1986). This DNA can then either be incorporated and stably maintained in the bacterial genome through homologous recombination or can form an autonomous

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replicating element (Smalla *et al.*, 2000). There are more than 70 naturally transformable prokaryotic species including many found in the soil as well as some of medical importance (de Vries and Wackernagel, 2004).

An alternative to bacterial-derived ARMs is a plant-derived ARM: an *Arabidopsis thaliana* ABC transporter, *Atwbc19*, which confers kanamycin resistance, at similar levels as *nptII*, when overexpressed in transgenic plants (Mentewab and Stewart, 2005).

As this marker is endogenous to and presumably ubiquitous in plants, its use in the selection of transgenic plants might be more widely accepted by the public and regulators and inherently less risky than bacteria-derived resistance markers. ABC transporters exist in prokaryotes; however, database searches have not yielded any close matches with *Atwbc19* in regards to plant codon-like usage (Stewart and Mentewab, 2005). Furthermore, this particular ABC transporter is putatively targeted to tonoplasts, which are absent in bacterial cells. *Atwbc19* has been shown to be highly specific for kanamycin resistance in transgenic plants; that is, it does not confer resistance to geneticin, neomycin or other clinically used aminoglycoside antibiotics (Mentewab and Stewart, 2005). In addition, *Atwbc19* is also rather large in size, 2.2 kb, almost 2.75 times larger than *nptII*, making it less likely to be fully incorporated into the bacterial genome. Finally, unlike bacterial markers, *Atwbc19* has plant codon usage, which means that if it were somehow integrated into the bacterial genome and under the control of a bacterial promoter, it might be expressed at a much lower level than that of a bacterial gene such as *nptII* (Stewart and Mentewab, 2005).

We overexpressed *Atwbc19* in *E. coli* to determine whether kanamycin resistance was conferred to bacteria under simulated HGT, e.g. HGT risk assessment using a kanamycin-sensitive model bacterium. We initially screened several soil, plant or human bacteria (*Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Variovorax* sp. UTK 037, *Acidovorax* sp. UTK 052 and *Rahnella* sp. UTK 034) for antibiotic resistance and unlike *E. coli* DH5α these organisms were determined to have resistance to either ampicillin or kanamycin. Therefore, potentially relative bacteria were all found to not be appropriate for this study. Resistance to a similar aminoglycoside, geneticin (G418), was also assessed to determine if there was potential for cross-resistance to occur.

Results and discussion

Escherichia coli DH5α without any of the experimental plasmids was examined for resistance to kanamycin. No growth (i.e. no turbidity) was observed at 25 mg l^{-1}, 50 mg l^{-1} or 100 mg l^{-1}. Turbidity was only observed at the 0 mg l^{-1} (no kanamycin) control.

The *nptII* gene provided greater resistance to kanamycin in *E. coli* than that of the *Atwbc19* gene and was significantly different from the no-plasmid control at low concentrations of kanamycin (e.g. over 10 mg l^{-1}) ($P < 0.05$) (Fig. 1A), approximately $10^{6.8}$ and $10^{7.3}$ colony-forming units (cfu) ml^{-1} respectively (Fig. S1A). The *Atwbc19* gene was not significantly different from the no-plasmid control at higher concentrations of kanamycin (e.g. over 25 mg l^{-1}) ($P < 0.05$), approximately < 0 cfu ml^{-1}. *Escherichia coli* transformed with pKS-*ABC* had little resistance to kanamycin at 100 mg l^{-1}, the level that was most effective in the selection of transgenic tobacco (Fig. 1A) (Mentewab and Stewart, 2005). *NptII*, however, conferred kanamycin resistance at the 100 mg l^{-1} concentration as well as at the 400 mg l^{-1}, the highest concentration tested (Fig. 1A). *Atwbc19* conferred resistance to only lower concentrations of kanamycin to *E. coli* (below 12.5 mg l^{-1}) that was not significantly different from that conferred by *nptII.*

The *nptII* gene provided greater resistance to G418 in *E. coli* than that of the *Atwbc19* gene and was significantly different from the no-plasmid control at concentrations over 1 mg l^{-1} ($P < 0.05$) (Fig. 1B), approximately 10^4 and 10^7 cfu ml^{-1} respectively (Fig. S1B). *NptII* endowed G418 resistance at the 100 mg l^{-1} concentration, the highest tested (Fig. 1B).

There was no significant difference between the no-plasmid control, pKS-*nptII* or pKS-*ABC* at gentamicin concentrations greater than 1 mg l^{-1} (data not shown).

Gene expression levels were found to be similar between pKS-*ABC* and pKS-*nptII* from reverse transcription polymerase chain reaction (RT-PCR) analysis (data not shown). Transcripts of *nptII* and *Atwbc19* were detected by northern blot and similar expression levels between concentrations of the antibiotic kanamycin tested were observed respectively (Fig. 1C).

Experimental attempts to establish HGT of transgenic DNA to bacteria under laboratory conditions have been negative. Furthermore, no experiment, to date, has shown evidence of transfer of transgenes or other genes from plants to soil bacteria under non-laboratory conditions. These findings confirm results of previous HGT field test of transgenic crops. There have been no detectable transfer of transgenes to soil bacteria under natural conditions (Paget and Simonet, 1994; Smalla *et al.*, 1994; Badosa *et al.*, 2004); however, HGT from transgenic plants to bacteria under optimized conditions has been demonstrated (Hoffmann *et al.*, 1994; Schluter *et al.*, 1995; Gebhard and Smalla, 1998; de Vries and Wackernagel, 1998; Nielsen *et al.*, 2000; Kay *et al.*, 2002; Meier and Wackernagel, 2003).

Hoffmann and colleagues (1994) used transgenic *Brassica* plants and the filamentous fungi, *Aspergillus niger*, as a model system and demonstrated successful transfor-

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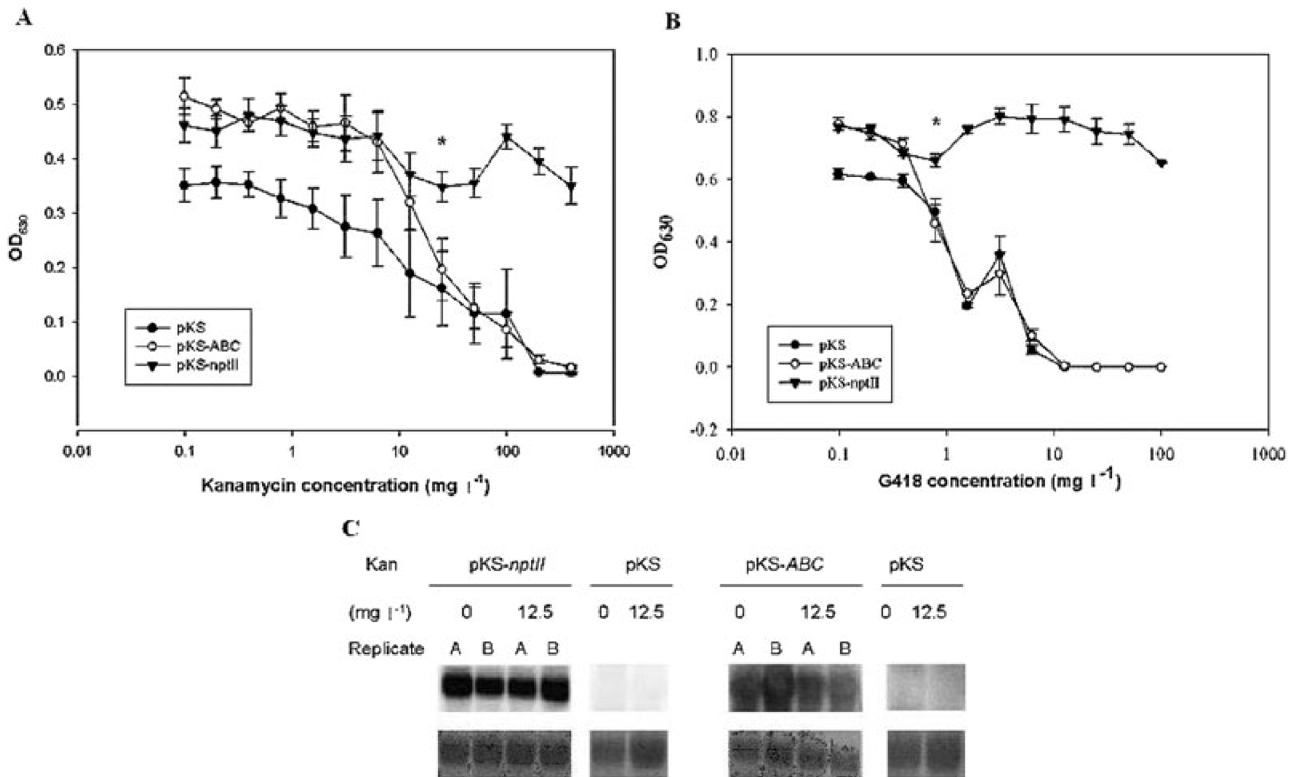

Fig. 1. Growth and Northern blot of transformed *E. coli* in kanamycin and G418. *Escherichia coli* DH5α was used in all experiments and cultured on Luria–Bertani (LB) media (Luria and Burrous, 1955; Luria *et al*., 1960; Miller, 1972). *Escherichia coli* DH5α was transformed with pKS, pKS-*ABC* and pKS-*nptII* by a freeze/thaw method (Hanahan, 1983). pKS-*ABC* contains the *AtWBC19* gene under the control of the *lacZ* promoter in pBluescript II SK+; pKS-*nptII* contains the *nptII* gene with the *lacZ* promoter in pBluescript II SK+; and pKS is pBluescript II SK+ with no inserted genes as a control. For selection of transformants, aliquots of bacteria were spread onto solidified LB medium supplemented with ampicillin (100 mg l^{-1}) and incubated at 37°C for 24 h. Transformed bacteria were observed for their ability to obtain kanamycin, geneticin (G418) and gentamicin resistance using growth assays 96-well microtitre plates. Isopropyl-b-D-thiogalctopyranoside (IPTG) at 1 mM was used to induce the *lacZ* promoter. Each well was filled with 199 μl of LB, IPTG and varying concentrations of antibiotic [kanamycin, geneticin (G418) and gentamicin] and 50 μl of bacterial treatments. Each treatment was replicated in triplicate in sterile 96-well microtitre plates and each experiment was duplicated in time. Optical densities (measured at 630 nm) (A and B) of *E. coli* DH5α transformed [pKS-*ABC* ($n = 12$), pKS-*nptII* ($n = 12$) or pKS ($n = 6$)] and serially diluted 0–400 mg l^{-1} kanamycin (A) or 0–100 mg l^{-1} geneticin (G418) (B) and incubated at 37°C for 24 h. Data were analysed by analysis of variance (ANOVA) using the general linear model. Duncan's Multiple Range test was used to compare treatment mean values when significant differences (at the 0.05 probability level) were found. Minimal concentration in which significant difference between pKS-*nptII* and pKS-*ABC* occurs is symbolized with an asterisk. Error bars indicate standard error. Northern blot analysis was performed to assess gene expression of *AtWBC19* and *nptII* under the experimental conditions (C). Fifteen-hundred microlitre of samples were taken from triplicate wells for each plasmid (pKS, pKS-*ABC* and pKS-*nptII*) at 0 or 12.5 mg l^{-1} kanamycin at 24 h for Northern blot analysis. RNA was extracted using the RNeasy Mini Kit and following manufacturer's protocol (Qiagen, Valencia, CA, USA). Five micrograms of RNA was loaded in each well. After electrophoretic separation, bacterial RNA was transferred to a nylon membrane by capillary action and probed with either *AtWBC19*, *nptII* or 16S rRNA radiolabelled with [^{32}P-dCTP] using random primers created using Prime-It® II Random Primer Labelling Kit (Stratagene, LaJolla, CA, USA) and purified using mini Quick Spin Columns (Roche Applied Science, Indianapolis, IN, USA). Hybridization was performed using ULTRAhyb™ hybridization buffer and manufacturer's instructions (Ambion, Austin, TX, USA). Each lane (C) represents 2 μg of total RNA obtained from *E. coli* transformed with either pKS-*nptII* (left panel), pKS-*ABC* (right panel) or pKS (negative control; both panels) and incubated in varying concentrations (0 or 12.5 mg l^{-1}) of kanamycin in duplicate. Expected band sizes were 0.8 kb and 2.2 kb for pKS-*nptII* and pKS-*ABC* respectively.

mation of *A. niger* with the hygromycin B antibiotic resistance marker gene; however, the transfer frequency was too low to calculate. Schluter and colleagues (1995) simulated HGT between transgenic potatoes, *Solanum tuberosum*, and the plant pathogenic bacterium, *Erwinia chrysanthemum*, in an effort to calculate HGT under various conditions and found that even under 'idealized' natural conditions (using a bacterial marker gene linked to a functional origin of replication), a calculated HGT frequency of 2×10^{-17} was obtained, a rate far below detection limits.

Conclusions

Antibiotic resistance genes are naturally present in soils, water and bacteria. Transgenic research utilizes these markers to select for transformed plant cells. A series of articles has debated the safety of ARMs in transgenic

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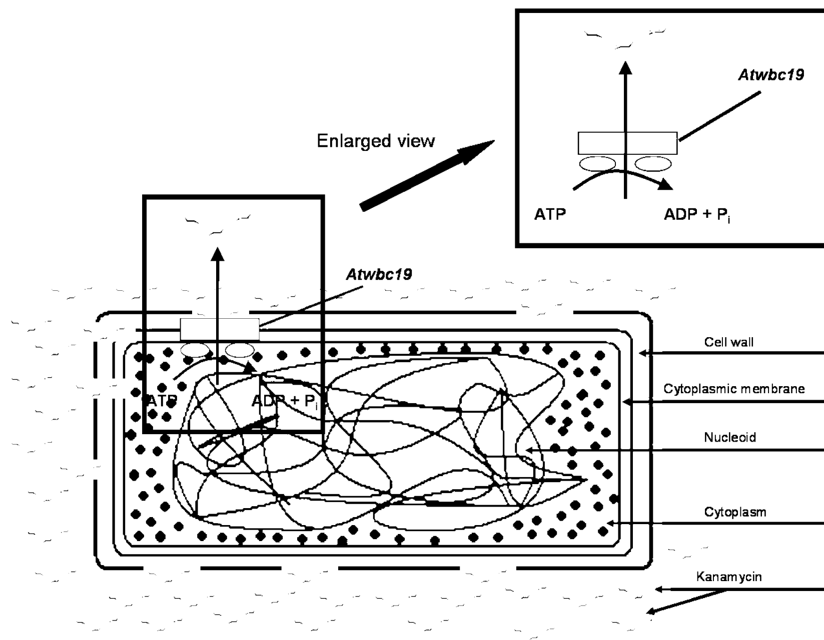

Fig. 2. Possible modes-of-action of *AtWBC19*, an *Arabidopsis thaliana* gene shown to confer kanamycin resistance, in plants but not in bacteria. Bacteria have no mechanism of sequestering kanamycin in a vacuole, but *AtWBC19* may be mistargeted to the bacterial membrane which might cause some kanamycin transport out of cells, conferring low resistance.

plants, and controversy still remains on their impact to environment and human health (Nielsen *et al.*, 1998; Smalla *et al.*, 2000; Kowalchuk *et al.*, 2003; Heinemann and Traavik, 2004; Gay and Gillespie, 2005). One concern that researchers wish to resolve is the potential of horizontal transfer of resistance genes from transgenic plants to bacteria of medical importance. The *Atwbc19* gene was evaluated for its ability to transfer antibiotic resistance to kanamycin-sensitive bacteria found in the environment (*E. coli* DH5α).

When overexpressed in plants, *Atwbc19* is very specific for kanamycin (e.g. resistance to other aminoglycoside antibiotics was not found) (Mentewab and Stewart, 2005). It is unknown exactly why a plant ABC transporter might transport a compound such as kanamycin, and why it would not transport related molecules, but there is evidence that each ABC transporter might have a unique range of substrates (Higgins, 1992). GFP fusion experiments indicated that when overexpressed in plants, *Atwbc19* is likely targeted to the tonoplast, where kanamycin would be sequestered in the central vacuole (Mentewab and Stewart, 2005). As bacteria have no central vacuole, *Atwbc19* targeting is necessarily different than in plants and no vacuoler kanamycin sequestration can occur. *Atwbc19* is most likely mistargeted to the cell membrane in bacteria where it would provide some efflux of kanamycin outside of cells, but it is apparently not sufficient to confer efficient kanamycin resistance (Fig. 2). In addition to the other advantages of *Atwbc19* compared with *nptII* relative to HGT (i.e. greater size and codon usage), the absence of the appropriate membrane target in bacteria would result in low resistance if it were horizontally transferred to bacteria and expressed.

In this research, it was determined that if the *Atwbc19* gene were to be transferred to *E. coli* and it were to land under the control of a strong inducible bacterial promoter, low levels of kanamycin resistance would be conferred as a result. This gene is an acceptable alternative to the bacterial *nptII* gene in transgenic plants.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Standard growth curve of *E. coli* DH5α transformed (pKS-*ABC* (n = 12), pKS-*nptII* (n = 12) or pKS (n = 6)) incubated at 37°C in 96 well microtiter plates containing 199 μl LB, induced using 1 mM IPTG and (A) serially diluted 0 to 400 mg l^{-1} kanamycin and (B) serially diluted 0 to 100 mg l^{-1} geneticin (G418). Growth curves were performed by plating 100 μl from the 96-well microtiter plates, performing serial dilutions, and determining bacterial counts in duplicate.

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