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 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).

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

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 Tn5 neomycin phosphotransferase gene (*nptII*), which confers resistance to several aminoglycoside 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).

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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 10⁶ and 10⁵ 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 10⁴ and 10³ 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-
formation 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 $2 \times 10^{-17}$ 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
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 vacuolar 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 µg ml⁻¹ kanamycin and (B) serially diluted 0 to 100 mg ml⁻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. This material is available as part of the online article from http://www.blackwell-synergy.com