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Horizontal Gene Transfer to Bacteria of an Arabidopsis Thaliana ABC Transporter That Confers Kanamycin Resistance in Transgenic Plants

Kellie Parks Burris
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To the Graduate Council:

I am submitting herewith a thesis written by Kellie Parks Burris entitled "Horizontal Gene Transfer to Bacteria of an Arabidopsis Thaliana ABC Transporter That Confers Kanamycin Resistance in Transgenic Plants." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

C. N. Stewart, Jr., Major Professor

We have read this thesis and recommend its acceptance:

Steve Ripp, Janice Zale

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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<div align="right">

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Major Professor

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We have read this thesis
and recommend its acceptance:

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Horizontal gene transfer to bacteria of an *Arabidopsis thaliana* ABC transporter that confers kanamycin resistance in transgenic plants

**A Thesis presented for the
Masters of Science degree
The University of Tennessee, Knoxville**

**Kellie Parks Burris
December, 2006**

Acknowledgements

Abstract

The use of antibiotic resistance markers is an important tool in the production and selection of transgenic plants. There have been increased concerns about the potential horizontal gene transfer (HGT) from transgenic plants to bacteria of medical and environmental importance. Until recently all antibiotic resistance genes used in transgenic studies have been bacterial in origin. An *Arabidopsis thaliana* ABC transporter, *Atwbc19*, was the first plant gene shown to confer kanamycin resistance when overexpressed in transgenic plants. The *Atwbc19* gene was evaluated for its ability to transfer antibiotic resistance to *Escherichia coli*, which are found in the human gut and environment. Simulated HGT was staged by subcloning *Atwbc19* under the control of a bacterial promoter, genetically transforming the bacteria and assessing if resistance was conferred as compared to the same treatment of *the E. coli nptII* gene. 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 higher concentrations of kanamycin (e.g., over 10 mg L^{-1}) ($p < 0.05$). The *Atwbc19* gene was not significantly different from the no-plasmid control at higher concentrations of kanamycin (e.g., over 25 mg L^{-1}) ($p < 0.05$). *E. coli* transformed with *Atwbc19* conferred little resistance to kanamycin at 100 mg L^{-1}.

Results from Northern gel blot analysis indicated that expression levels for *nptII* were similar to that of *Atwbc19* for the two concentrations of the antibiotic kanamycin tested. However, there was a slightly apparent decrease in the level of expression for *Atwbc19* compared to the *nptII* gene that was most likely the result to the plant codon usage of the *ABC* gene or its large size (over two-fold greater than *nptII*). This research

supports the use of the *Atwbc19* gene in transgenic plants as a selectable marker and

potential replacement of the *nptII* gene for kanamycin selection systems.

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1.   Introduction

Antibiotic resistant markers (ARMs) derived from bacteria are the most

commonly inserted genes in transgenic plants (Miki and McHugh 2004). ARMs allow

for the detection and selection of transformed plants which can then be manipulated to

acquire transgenes. The entire marker or a portion of the marker may remain in the final

commercial product, a concern to those interested in horizontal gene transfer (HGT) of

antibiotic resistance genes to bacteria.   One of the most frequently used antibiotic

resistant marker genes used in plant selection is the *Escherichia coli* K12 transposon Tn5

neomycin phosphotransferase gene (*nptII*), also referred to as aminoglycoside-3'-

phosphotransferase II, which is a selectable marker conferring resistance to several

aminoglycoside antibiotics including kanamycin and neomycin, of bacterial origin

(Garfinkel et al. 1981), and engineered with plant-specific promoters to facilitate

expression in plant cells (reviewed in Miki and McHugh 2004). Although selectable

markers derived from bacteria have been recognized by many regulatory agencies as safe,

there have been concerns raised about the potential risks of creating antibiotic-resistant

pathogens of human health or environmental importance via horizontal gene transfer

from transgenic plants. There is evidence that drug resistance is already widespread in

bacteria as a consequence of overuse in human and veterinary medicine and as animal

growth promoters (IFT 2006). There has been much research in the area of gene transfer

from bacteria to bacteria (Eisen 2000; Beiko et al. 2005; Gogarten and Townsend 2005;

Thomas and Nielsen 2005); from transgenic plants to soil- or plant-bacteria (Broer et al.

1996; de Vries and Wackernagel 1998; de Vries and Wackernagel 2001; de Vries and

Wackernagel 2004; Gebhard and Smalla 1998; Gebhard and Smalla 1999; Kay et al.

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2002; Kowalchuk et al. 2003; Nielsen et al. 1997; Nielsen et al. 2000; Schluter et al. 1995; Smalla et al. 2000; Tepfer et al. 2003;) and from transgenic plants to gut microorganisms (Chambers et al. 2002; Deni et al. 2005; Duggan et al. 2000). Detection transformation frequencies vary widely between studies (Schluter et al. 1995; Broer et al. 1996; Nielsen et al. 1997; Gebhard and Smalla 1998; de Vries and Wackernagel 1998) and are estimated at low frequencies, 2×10^{-17} (Schluter et al. 1995), 6×10^{-12} (Broer et al. 1996), 10^{-2} (Nielsen et al. 1997), 5.4×10^{-9} (Gebhard and Smalla 1998) and 3.5×10^{-8} (de Vries and Wackernagel 1998). With such low detection frequencies, questions still remain on the impact and risks HGT (from transgenic plants to bacteria) may have on human health and the environment.

Recent publications raise 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 rare (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 uptake 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 uptake 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

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ability of genetic exchange; once the DNA is taken up by the bacteria, the DNA must be stably maintained (Nielsen et al. 1998).

There are three mechanisms of gene transfer utilized by bacteria—general transduction, conjugation, and transformation. The most likely mechanism of gene transfer from transgenic plants to bacteria is natural transformation (Nielsen et al. 1998; Bertolla and Simonet 1999). Natural transformation is a process by which competent bacteria can uptake 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 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).

There are alternatives to using bacterial derived antibiotic resistance markers in transgenic research, such as the production of marker-free plants, which can be more difficult and less efficient (Miki and McHugh 2004), the use of reporter genes as visible markers (Miki and McHugh 2004), or the use of selectable markers derived from plants (Mentewab and Stewart 2005). The potential barriers bacteria must surpass in order to obtain antibiotic resistance from transgenic plants are myriad. By adding another "barrier", such as the utilization of a plant-derived antibiotic resistance marker, the frequency of gene transfer from transgenic plants to bacteria should be even less of a risk. Mentewab and Stewart (2005) recently demonstrated that an overexpressed *Arabidopsis thaliana* ABC transporter, *Atwbc19* gene, conferred kanamycin resistance to transgenic tobacco plants. Since this marker is endogenous to and presumably ubiquitous in plants,

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its use in the selection of transgenic plants should be more widely accepted by the public and regulators and inherently less risky than bacteria-derived resistance markers for several reasons. First, ABC transporters are ubiquitously found in plants. There is no evidence that the *Atwbc19* transporter has been transferred to bacteria during evolution (Mentewab and Stewart 2005). ABC transporters exist in prokaryotes and no database searches have yielded any matches with plant-like codon usage patterns (Mentewab and Stewart 2005). Second, this particular ABC transporter is putatively targeted to tonoplasts, which are absent in bacterial cells. Furthermore, *Atwbc19* has been shown to be highly specific for kanamycin resistance in transgenic plants; it does not confer resistance to geneticin, neomycin or other aminoglycoside antibiotics used clinically (Mentewab and Stewart 2005). *Atwbc19* is also rather large in size, 2.2 kb, almost 2.75 times larger than *nptII*, 0.8 kb, 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 somehow did find its way into the bacterial genome and under the control of a bacterial promoter, it would most likely be expressed at a much lower level than that of a bacterial gene such as *nptII*.

The objective of this research was to examine the ability of the *Atwbc19* gene derived from transgenic tobacco to horizontally transfer antibiotic resistance to bacteria that are naturally found in gut and soil environments (*Escherichia coli*). Simulated HGT was staged by subcloning *Atwbc19* under the control of a strong bacterial promoter, transforming bacteria with appropriate plasmids and assessing whether kanamycin resistance was conferred compared to *nptII*. Resistance to a similar aminoglycoside,

geneticin (G418), was also assessed to determine if there was potential for cross-resistance to occur.

2.   Literature Review

2.1   Aminoglycoside antibiotics

2.1.1   Introduction

Aminoglycosides are powerful bactericidal antibiotics that function by either

inhibiting protein synthesis by irreversibly binding to prokaryotic ribosome (Recht et al.

1999) or by creating gaps in the outer membranes of the bacterial cell causing the leakage

of intracellular contents and enhanced uptake of the antibiotic (Montie and Patamasucon

1995). They are among the most common broad-spectrum antibiotics utilized in

medicine. Aminoglycosides are effective against gram-negative, aerobic bacteria, but

also have the ability to act in conjunction against gram-positive bacteria.

Aminoglycosides are structurally similar and include a variety of compounds:

kanamycin, neomycin, gentamicin, streptomycin, amikacin, netilmicin and tobramycinn

(Mingeot-Leclercq et al. 1999). Their basic chemical structure includes one of several

aminated sugars joined to dibasic cyclitol using glycosidic linkages (Mingeot-Leclercq et

al. 1999). They are polycationic with a high binding affinity for nucleic acids, such as

portions of RNA or more specifically prokaryotic rRNA (Mingeot-Leclercq et al. 1999).

Geneticin (G418) has been shown to directly bind to the 80S ribosomal complex of the

eukaryote and its mechanism may be different from other aminoglycosides (Bar-Nun et

al. 1983).   Gentamicin, isolated from *Micromonospora*, is the most commonly used

aminoglycoside because of its low cost and reliability; however, amikacin is the most

effective against resistant bacteria.   Streptomycin, the first aminoglycoside, was isolated

from *Streptomyces griseus*. Neomycin, isolated from *Streptomyces fradiae*, has greater

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potency against gram-negative bacteria; however, because of its high toxicity, it is not to be used systemically. Clinical uses of aminoglycosides include serious gram-negative infections, skin, bone or soft tissue infections, urinary tract infections, septicemia, peritonitis, severe pelvic inflammatory disease, endocarditis, *Mycobacterium* infection, neonatal sepsis, ocular infections and *Otitis externa* (Gonzalez and Spencer 1998).

2.1.2 Prevalence of antibiotic resistance in nature

Antibiotic resistance is relatively common in natural microbial habitats—soil, water and gut. Resistance to ampicillin, penicillin and kanamycin occurs naturally in many soil bacteria. Humans consume ca. 1.2 million kanamycin-resistant bacteria each day, primarily from fresh vegetables, and resistant bacteria are already present in 10-20% of human digestive systems (Calgene 1990). Each human gut naturally contains 10^{12} kanamycin-resistant bacteria, and consumption of the kanamycin-resistance gene would merely increase the frequency of kanamycin- resistant bacteria in the gut by 0.000001% (Calgene 1990).

Many of the antibiotics in use have been derived and produced from naturally occurring microorganisms (i.e. streptomycin from *Streptomyces*; Davies and Wright 1997). These microorganisms, in turn, have a natural resistance to the antibiotic which they produce. Furthermore, bacteria that produce antibiotics themselves contain antibiotic resistance genes as a means of self-protection. This production of antibiotics is believed to be a means of defense against competing bacteria and is a key mechanism of survival in nature.

Since their increased use in the 1960s and 1970s, the appearance of bacterial resistance to aminoglycoside antibiotics has become more prevalent in hospitals and patient-associated environments (Davies and Wright 1997).

2.1.3   Aminoglycosidic antibiotics and their uses

Antibiotics are used as antibacterial compounds, internally and externally, to treat and prevent bacterial infections in both humans and animals. Further, some antibiotics are used in livestock farming animals as antibiotic growth promoters to enhance their growth and feed efficiency and in plants to help maintain their health. Antibiotic growth promoters are used most widely in swine and poultry. Antibiotics have been used in food animal production for more than 50 years to treat, prevent, or control infectious diseases or to improve feed efficiencies and weight gain (IFT 2006). The estimated total annual production of antibiotics between the 1970s and 1990s ranged from 31 million to 50 million pounds, while 18.4 million to 30 million pounds were used in production agriculture (IFT 2006). The amount of antibiotics used by humans in the United States is unknown (IFT 2006), and the distribution of antibiotics among humans, veterinary and plant applications as well as any estimates for the portion introduced annually into the environment are difficult to estimate (IFT 2006). Fruit trees account for the majority of antibiotic use on U.S. plants, with the antibiotics mainly applied to the tree and its blossoms, not the fruit (IFT 2006). The antibiotics most commonly used on plants are streptomycin and oxytetracycline. In the United States, antibiotics applied to plants accounts for less than 0.5% of total antibiotic use (Levy 1998).

Kanamycin, streptomycin, gentamicin, spectinomycin, butirosin, tobramycin, neomycin, amikacin, netilmicin, and isepamicin are aminoglycosides, or biologically active secondary metabolites, derived from *Streptomyces kanamyceticus*, *Streptomyces griseus*, *Micromonospora purpurea*, *Streptomyces spectabilis*, *Bacillus circulans*, *Streptomyces tenebrarius*, *Streptomyces fradiae*, semisynthetic derivative of kanamycin, semisynthetic derivative of sisomicin, and semisynthetic derivative of gentamicin B, respectively (Davies and Wright 1997). These compounds are used as antibiotics to treat various diseases including but not limited to tuberculosis, septicemia, complicated intraabdominal infections, complicated urinary tract infections, and serious nosocomial respiratory tract infections (Gonzalez and Spencer 1998).

Kanamycin, produced from *Streptomyces kanamyceticus*, is active against most gram-negative bacteria, except *Pseudomonas* species, and strains of *Staphylococcus* and *Mycobacterium*. However, many kanamycin resistant bacteria are widely distributed. A decline in the use of kanamycin has caused a decrease in the prevalence of kanamycin resistant bacteria (Opinion of the Scientific Panel on Genetically Modified Organisms 2004).

Neomycin, produced from *Streptomyces fradiae*, is active against many gram-negative bacteria, except *Pseudomonas* species, and strains of *Staphylococcus aureus*. Neomycin has chemical properties similar to kanamycin. Neomycin is used primarily for the external treatment of skin, eye and ear infections. It is poorly absorbed through the mouth; however, has been given orally to decrease gut microbes. Neomycin is both ototoxic and nephrotoxic. Neomycin has limited use clinically and is primarily used in

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veterinary medicine (Opinion of the Scientific Panel on Genetically Modified Organisms 2004).

Streptomycin, produced by *Streptomyces griseus*, is active against *Mycobacterium tuberculosis* and many other gram-negative bacteria, except *Pseudomonas aeruginosa*. Therefore, it is used in the treatment of tuberculosis and other severe bacterial infections, including gonorrhea. Streptomycin, like neomycin, is poorly absorbed through the mouth and is ototoxic. Streptomycin is also utilized in the treatment of plants and in veterinary medicine (Vidaver 2002; Opinion of the Scientific Panel on Genetically Modified Organisms 2004; Jana and Deb 2006).

Paromomycin is similar to neomycin and is used orally in the treatment of protozoal infections. Gentamicin and tobramycin are used in the treatment of life-threatening gram-negative bacterial infections and oftentimes are used in conjunction with other antibiotics such as beta-lactams. Amikacin and netilimicin are primarily used in infections with bacteria that are resistant to other aminoglycosides and neither is affected by aminoglycoside modifying enzymes (Jana and Deb 2006).

2.1.4 Resistance mechanisms

Antibiotics have the potential for selecting for the growth and spread of resistant bacteria. There are three mechanisms of aminoglycosidic resistance: alteration at the target ribosomal binding site, impaired uptake and decreased cell permeability, and enzymatic modification (Mingeot-Leclercq et al. 1999). Mutations occurring at the site where the aminoglycoside attaches may impede ribosomal binding. This mechanism of resistance is most common with the antibiotic, streptomycin since it binds to a single site

on the 30S subunit of the ribosome. Other aminoglycosides bind to multiple sites on both ribosomal subunits, making their resistance by this mechanism uncommon. Aminoglycoside resistance is also observed due to transport defects or inability of the aminoglycoside to permeate the membrane, most likely mediated by the chromosome (Mingeot-Leclercq et al. 1999).

The most common mode of aminoglycosidic inactivation and one which results in the highest level of resistance is enzymatic modification. Aminoglycosidic antibiotics can be inactivated through the three types of aminoglycoside-modifying enzymes: phosphotransferases, nucleotidyltransferases or acetyltransferases. This reaction requires ATP or acetyl CoA and divalent cations as cofactors (Davies and Smith 1978). O-Phosphotransferases (APH) catalyzes the ATP-dependent phosphorylation of a hydroxyl group, O-Adenyltransferases (ANT) catalyzes the ATP-dependent adenylation of a hydroxyl group, and N-Acetyltransferases (AAC) catalyzes acetyl CoA-dependent acetylation of an amino group (Davies and Wright 1997).

2.1.5 Importance in human health

Aminoglycosides are important antibiotics which are bactericidal against aerobic gram-negative bacilli as well as active against staphylococci and mycobacterium even when inoculum is large. Common uses of aminoglycosides include treatment for serious, life-threatening infections with gram-negative bacteria, infections of the skin, bone or soft tissue, urinary tract infections, septicemia, peritonitis or other severe abdominal infections, pelvic inflammatory disease, endocarditis, mycobacterium infection, neonatal sepsis, ocular infection and otitis externa (Gonzalez and Spencer 1998).

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The development of antibiotic resistant bacteria of human health importance is of greatest concern. Resistant pathogenic bacteria were first discovered soon after the introduction of antibiotics in the 1940s. Resistance is dependent upon presence of antibiotic and resistance gene, and the spread of either the resistant bacteria or resistance gene.

2.2   ABC transporters

2.2.1   Introduction

ATP-binding cassette (ABC) transporters are membrane proteins that function to shuttle nutrient and toxic molecules across the cell's plasma membrane in order to maintain the chemical composition of the cytoplasm. ABC transporters utilize ATP hydrolysis as energy to move both large and small substrates across the membrane. Several ABC transporters in plants have been functionally characterized by both their involvement in detoxification and in plant development; however, many ABC transporters still remain uncharacterized with regards to substrates and functions.

The ATP-binding cassette (ABC) protein superfamily is the largest known protein family with members found in bacteria, fungi, plants and animals (79 members in *Escherichia coli*, 29 members in *Saccharomyces cerevisiae*, 131 in *Arabidopsis thaliana*), and is mostly composed of membrane proteins actively involved in the transport of a variety of substances across phospholipid membranes (Higgins 1992; Henikoff et al. 1997). ABC proteins share 30-40% identity among family members with the majority of members being membrane bound with transmembrane domains (TMDs) (Higgins 1992). TMDs appear to form the pathway for movement of solutes, including

lipids, heavy metal ions, inorganic acids, glutathione conjugates, sugars, amino acids, peptides, secondary metabolites and xenomolecules, across the phospholipid membrane and may contribute to the substrate selectivity of the transporter (Higgins 1992; Rea et al. 1998).

2.2.2 White-Brown Complex subfamily

The White-Brown complex (WBC) subfamily is the largest subfamily of the Arabidopsis ABC protein superfamily and is composed of 29 members (Sanchez-Fernandez et al. 2001). The size of WBC subfamilies sequenced in other species is quite different. To contrast, the yeast genome contains only one WBC homolog—ADP1—of unknown function while the human genome contains only three—ABC5, ABC8, and BCRP1—which are involved in either cholesterol and phospholipids transport or multidrug resistance (Decottignies and Goffeau 1997; Berge et al. 2000; Doyle et al. 1998).

The WBC subfamily has been identified by homology with the White, Scarlet and Brown ABC transporters from *Drosophila* (Sanchez-Fernandez et al. 2001). In *Drosophila*, these transporters mediate the transport of pigment precursors into pigment cells for eye color (Sanchez-Fernandez et al. 2001).

2.2.3 Discovery and functional characterization of *Arabidopsis thaliana* WBC transporters

There have been two genes within the WBC subfamily have been functionally characterized—*Atwbc19* (*At3g21090*), the gene that encodes an *Arabidopsis thaliana*

ABC transporter, was found to confer kanamycin resistance to transgenic plants (Mentewab and Stewart 2005) and *Atwbc12* (*At1g51500*) was found to play a role in the secretion of cuticular wax (Pighin et al. 2004).

Mentewab and Stewart (2005) while investigating responses of Arabidopsis thaliana to explosive 2,4,6-trinitrotoluene (TNT), discovered an endogenous transporter gene that conferred resistance to the aminoglycoside antibiotic kanamycin. Using microarray analysis, Mentewab and Stewart (2005) identified 52 genes that were upregulated in *A. thaliana* grown on TNT. This *Atwbc19* gene was further studied because its product is a member of the ABC protein superfamily with the ability to eliminate and sequester toxins. While characterizing T-DNA insertion mutants, the root growth of *Atwbc19* T-DNA insertion mutants on plates containing kanamycin was reduced as compared to T-DNA mutants with insertions in other genes. Mentewab and Stewart (2005) demonstrated that *Atwbc19* was capable of contributing to kanamycin resistance. When expressed in tobacco, wild-type *Atwbc19* confers kanamycin resistance at levels comparable to other bacterial antibiotic resistance markers, *nptII*.

Pighin et al. (2004) demonstrated that plant ABC transporters were not limited to detoxification, but at least one is required for plant cuticular lipid export. A waxy cuticle surrounds and protects all primary aerial tissues of the plant, and the synthesis of this waxy cuticle requires the transport of lipids out of the epidermal cells to the plant surface. Pighin et al. (2004) identified mutants defective in lipid transport to the cuticle (identified as *cer* mutants). The phenotype of these mutants includes a glossy, bright green stem due to a reduction or alteration in cuticular wax (Koornneef et al. 1989) with cells entirely filled with a central vacuole and a thin layer of cytoplasm around the cell's edge and

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large protrusions of cytoplasm into the vacuole found within the epidermis (Pighin et al. 2004). Stem epidermal cells of cer5 mutants contained reduced wax loads and contained sheet-like inclusions in the cytoplasm of wax-secreting cells (Pighin et al. 2004). These inclusions were abnormal deposits of cuticular wax and were similar in structure to a human disorder caused by a defective peroxisomal ABC transporter. Pighin et al. (2004) isolated the *CER5* gene, localized in the plasma membrane, and determined that its responsible for encoding an ABC transporter and has been designated WBC12.

2.3 Horizontal gene transfer

2.3.1 Introduction

The increased application and usage of biotechnology in the improvement of crops has lead to an increase in field trials with genetically modified crops. A problem with this modern approach to plant breeding is the lack of assurance in its safety on both the environment and consumers. One such concern is the potential development of antibiotic resistance bacteria from the transfer of antibiotic resistance genes from transgenic plants via horizontal gene transfer (HGT). HGT is defined as the movement of genetic material, DNA, to another cell that is not its offspring. HGT is common among prokaryotes, even those not closely related, making it an essential tool in evolution and in maintaining diversity of species in the environment. To contrast, vertical gene transfer is where DNA is transferred from a parent to an offspring while HGT is the exchange of DNA between cells within the same generation.

There are three common mechanisms of HGT: transformation, conjugation and transduction. Of these, transformation is of most interest since it is the only know

method by which plant DNA can become incorporated into bacterial DNA. Natural transformation is defined as a process by which competent bacteria can uptake 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 replicating element (Smalla et al. 2000). There are greater than 70 naturally transformable prokaryotic species including many found in the soil as well as some of medical importance (de Vries and Wackernagel 2004). Recombination frequency in natural populations is dependent upon natural selection and recombination rate. If transformants are at a disadvantage, they will likely be lost or removed from the population. Transformants that are neutral may either be lost from the population or occur at a frequency lower than those that are advantageous. Thus, genes that provide no meaningful function to the bacteria are likely lost from its genome (Ochman et al. 2000). Various methods of detecting HGT from plants to bacteria in both the environment and under laboratory conditions have been studied and have been found to have certain limitations.

2.3.2 Mechanisms of HGT

Bacteria replicate by cell division and can only obtain new DNA through HGT from other cells. Bacteria utilize conjugation, transduction and transformation to transfer and obtain new DNA. Conjugation requires cell-to-cell contact for the exchange of DNA between donor and recipient cells, both of which have to be living and metabolically active. Both gram-positive and gram-negative bacteria can transfer DNA via conjugation. Conjugation can occur between related, distantly related, or unrelated

species such as between gram-positive and gram-negative bacteria, from bacteria to yeast, and from bacteria to plants (Droge et al. 1998). Elements involved in conjugation transfer and replicate within a broad host range, making conjugation an important method for gene transfer among bacteria. Transduction is the transfer of DNA from bacteria to bacteria by bacteriophages. Bacteriophages attach to bacteria, inject their DNA, and produce multiple copies of the bacteriophage which are ultimately released to infect other cells. Bacteriophages have the ability to transfer whole plasmids and pieces of chromosomes between hosts. Many bacteriophages have a narrow host range, only infecting one species of bacteria which is usually native to the habitat of the bacteriophage (Nielsen et al. 1998).

Of the bacterial mechanisms to transfer DNA, transformation is the only known method by which bacteria can uptake DNA from plants, and therefore is of most concern for gene transfer from transgenic plants to bacteria (Thomson 2001). Transformation is defined as the uptake and incorporation of free DNA by and into competent bacteria. Transformation requires that the free DNA remains stable and that the bacteria are competent. Currently, there are greater than 70 known bacterial species capable of developing competence for transformation (de Vries and Wackernagel 2004). Natural competence has been observed in several bacterial genera, *Acinetobacter*, *Haemophilus*, *Pneumococcus*, *Streptococcus*, *Bacillus*, *Pseudomonas*, and *Neisseria*. Unlike conjugation, transformation does not require that the donor cell be living since oftentimes during cell death, lysis occurs and provides free DNA. Therefore, transformation can occur with DNA from any source.

2.3.3 Barriers to HGT

There are certain ecological requirements necessary for the transfer of genetic materials from plants to bacteria. The main barriers for exchange between plants and bacteria relate to transfer and establishment (Heinemann 1991). That is, the bacteria must be able to uptake plant DNA and it must have a means to stably maintain it within its genome. Therefore, availability of DNA is the first requirement of HGT. There are four possible fates of free DNA in soil: degradation, inactivation, stabilization or amplification. Degradation can occur when DNA is fragmented chemically by enzymes or mechanically by shearing. DNA can also be inactivated by binding to substances in the soil such as humic acids or clay particles (Stotzky 1989; Tebbe and Vahjen 1993). Furthermore, DNA can bind to clay particles within the soil stabilizing it and allowing it to maintain or enhance its ability to be transformed (Paget et al. 1992; Stewart et al. 1991; Khanna and Stotzky 1992; Gallori et al. 1994; Pietramellara et al. 1997). Finally, DNA can undergo amplification by being taken up, integrated and replicated by transformable bacteria. DNA has been shown to persist in the soil for long periods of time (Lorenz and Wackernagel 1987; Ogram et al. 1988; Ogram et al. 1994; Romanowski et al. 1991; Romanowski et al. 1993; Paget et al. 1992; Recorbet et al. 1993; Smalla et al. 1994; Widmer et al. 1996; Widmer et al. 1997; Gebhard and Smalla 1999).

In order for natural transformation to occur, free DNA must be accessible at the time and place in which competent bacteria inhabit. Thus, the second requirement and potential barrier of HGT is the presence/absence of competent bacteria. The bacteria must be competent to uptake free DNA found in the environment. That is, the bacteria

must have the ability to accept foreign DNA into there genome.   There are currently greater than 70 known bacterial species capable of developing competence for transformation (de Vries and Wackernagel 2004).

Further, the DNA must be stably maintained within the bacteria. This stabilization requires the integration of DNA into the bacterial chromosome or for plant DNA to contain replication functions with a bacterial origin of replication (Andre et al. 1986; Koncz et al. 1990). Integration of DNA into the bacterial chromosome is dependent on sequence homology between the free DNA and recipient bacteria (Baron et al. 1968; Kondorosi et al. 1980; Rayssiguier et al. 1989; Matic et al. 1995; Matic et al. 1997; Vulic et al. 1997). In *E. coli* it has been determined that the minimal length of DNA homology required for homologous recombination is 20 bp (Shen and Huang 1986). Stabilization of DNA in bacteria can also occur if the plant DNA contains replication function and a bacterial origin of replication (Andre et al. 1986; Koncz et al. 1990). If the DNA is fragmented, plasmid rescue-mechanisms may help re-circulate the fragmented DNA (Andre et al. 1986; Koncz et al. 1990).

Finally, another barrier to HGT is the failure to express the genes taken up and stabilized within the bacteria. Although most promoters inserted into transgenic plants demonstrate low activity in bacteria, some promoters may also be active in bacteria (e.g. 35S promoter expresses in *E. coli*; Assad and Signer 1990). Stronger antibiotic resistance in bacteria may occur if a deletion is restored in the resistance gene or if its expression is upregulated following recombination.

2.3.4 HGT detection and its limitations

Many studies have monitored the likelihood of and potential risks associated with HGT between/among bacteria (Eisen 2000; Beiko et al. 2005; Gogarten and Townsend 2005; Thomas and Nielsen 2005), from transgenic plants to soil microbes (Badosa et al. 2004; Broer et al. 1996; de Vries and Wackernagel 1998; de Vries and Wackernagel 2001; de Vries and Wackernagel 2004; Gebhard and Smalla 1998; Gebhard and Smalla 1999; Kay et al. 2002; Kowalchuk et al. 2003; Nielsen et al. 1997; Nielsen et al. 2000; Schluter et al. 1995; Shin et al. 2004; Smalla et al. 2000; Tepfer et al. 2003), from transgenic plants to gut microflora (Chambers et al. 2002; Deni et al. 2005; Duggan et al. 2000) in the environment as well as under laboratory conditions. There have been several methods utilized for the detection of HGT from plants to bacteria: comparison of DNA sequences, screening of bacteria from environmental samples, and experimental studies of HGT from transgenic plants to bacteria under optimized laboratory conditions (Nielsen et al. 1998).

One approach utilized in detecting HGT from plants to bacteria is through the comparison of DNA sequences. This method evaluates similarities between bacterial and plant DNA in nucleotide sequences, G+C content, codon usage and protein sequence and structure and is viewed as controversial (Smith et al. 1992; Shatters and Kahn 1989). Several incidences of HGT from plants to bacteria have been reported using this method of detection. Genes that were examined for HGT include: glutamine synthetase II from a eukaryote to *Bradyrhizobium japonicum* (Carlson and Chelm 1986), glyceraldehydes 3-phosphate dehydrogenase from a eukaryote to *E. coli* (Doolittle et al. 1990), glucose 6-

phosphate isomerase from *Clarkia urgulata* to *E. coli* (Froman et al. 1989), and

leghemoglobin gene to *Vitreoscilla* (Lamour et al. 1994). Problems with this method of

detection include the need for known DNA sequences evaluated in both related and

unrelated organisms (Smith et al. 1992) and the interpretations must be inferred.

A second approach in detecting HGT from plants to bacteria is through the

surveying of bacteria from environmental samples (soil or plants). The persistence of

transgenic plant DNA in the soil has been demonstrated in several studies (Gebhard and

Smalla 1999; Glick 1995; Widmer et al. 1996). A study on the persistence of DNA from

transgenic sugar beet in soil and the horizontal transfer of its DNA to bacteria

demonstrated persistence of its DNA was detectable for up to 2 years following its

release under field conditions; however, there was no evidence of HGT to soil bacteria

(Gebhard and Smalla 1999; Glick 1995). From laboratory experiments, transgenic plant

DNA was detectable by PCR for greater than 130 days (Widmer et al. 1996). Therefore,

the soil can be a potential source of transgenic plant DNA months to years after

transgenic crops are no longer present. The sensitivity of detection for this method is low

due to the differences in cultivation requirements for soil bacteria, the selection agent,

plating efficiency, and background interference.

The final approach used in detecting HGT from plants to bacteria is through

experimental studies. HGT studies have been conducted under the assumption that gene

transfer occurs by natural transformation and have been done in laboratory using gram-

negative soil or plant bacteria (*Agrobacterium*, *Erwinia*, and *Acinetobacter*). The

horizontal transfer of genes from bacteria to plants is most well known in the transfer of

DNA from *Agrobacterium tumefaciens* to plant genomes (Zupan et al. 1998; Smith et al.

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2001). Broer et al. (1996) examined the ability of *A. tumefaciens* to receive T-DNA

integrated by transgenic tobacco. Homologous recombination between this DNA region

and the corresponding region on the T_i plasmid of *A. tumefaciens* was expected to occur.

However, transformants could not be detected (Broer et al. 1996). Transformation

frequencies were determined to be below detection limits of 6×10^{-12}. HGT has been

examined using DNA from transgenic potato and transgenic sugarbeet, both containing

the *nptII* gene, to the bacteria *Acinetobacter calcoaceticus* (Nielsen et al. 1997).

Transformation conditions were optimized with chromosomal DNA and a transformation

frequency of 10^{-2} was determined *in vitro* (Nielsen et al.1997).

Detection transformation frequencies vary widely among studies (Schluter et al.

1995; Broer et al. 1996; Nielsen et al. 1997; Gebhard and Smalla 1998; de Vries and

Wackernagel 1998) and are estimated at low frequencies, 2×10^{-17} (Schluter et al. 1995),

6×10^{-12} (Broer et al. 1996), 10^{-2} (Nielsen et al. 1997), 5.4×10^{-9} (Gebhard and Smalla

1998) and 3.5×10^{-8} (de Vries and Wackernagel 1998). With such low detection

frequencies, questions still remain whether HGT, from transgenic plants to bacteria, may

impact the environment.

Heinemann and Traavik (2004) examined the problems associated with

monitoring HGT from transgenic plants to soil bacteria in the environment and found that

methods of environmental sampling are probably too insensitive for HGT monitoring and

suggested that the sensitivity of current detection techniques for its monitoring HGT may

be a trillion times lower than frequency estimates currently found in the literature.

Recent publications raise concerns that HGT between plants and soil microorganisms

may be underestimated. However, it has been argued that the likelihood of a gene

transfer event from a transgenic plant to a bacterium is rare (Schluter et al. 1995; Broer et al. 1996; Nielsen et al. 1997; Gebhard and Smalla 1998; de Vries and Wackernagel 1998).

2.4   Markers used in plant biotechnology

2.4.1   Introduction

There are many selectable marker genes utilized and expressed in transgenic plants across the United States (Malik and Saroha 1999; Miki and McHugh 2004) (Table 1). The majority of markers used in plant biotechnology are antibiotic resistance markers (ARMs) and were first used either in preparing plant transformation vectors or used within the plant transformation process itself (Goldstein et al. 2005). Although selectable marker genes derived from bacteria have been recognized as safe by regulatory agencies, concerns have been raised on their potential risks to human health. Controversy remains on the potential for human pathogens to develop resistance to antibiotics used in human and animal health. There is evidence that drug resistance is already widespread in bacteria as a consequence of overuse in human and veterinary medicine and as animal growth promoters (IFT 2006).

However, there are alternatives to using bacterial derived antibiotic resistance markers in transgenic research, such as the production of marker-free plants, which can be more difficult and less efficient (Miki and McHugh 2004), the use of reporter genes as visible markers (Miki and McHugh 2004), the use of markers that confer resistance to chemicals other than antibiotics, such as herbicides, (Miki and McHugh 2004), or the use of selectable markers derived from plants (Mentewab and Stewart 2005).

Alternatives include, but are not limited to, the *pat* and *bar* genes encoding phosphinothricin acetyltransferase which detoxify L-phosphinothricin; 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) conferring resistance to glyphosate; acetolactate synthase (ALS) conferring resistance to chlorosulfuron; bromoxinil nitrilase (Bxn) conferring resistance to bromoxinil; and the recently discovered *Atwbc19* gene conferring resistance to kanamycin.

There are numerous safety concerns relating to the use of antibiotic resistance markers in biotechnology, more specifically in the creation of transgenic plants. ARMs that confer resistance to antibiotics of clinical significance, particularly those to which resistance is not widely spread in the environment, should be avoided. The US FDA/CFSAN has published guidance for industry on the use of antibiotic resistance markers in transgenic plants (US FDA/CFSAN 1998).

2.4.2   Antibiotic resistance markers

The main markers used in plant biotechnology confer resistance to some antibiotic. The US FDA/CFSAN (1998) has provided a document on the guidance for industry on the use of antibiotic resistance marker genes in transgenic plants. The most popular antibiotic resistance markers include neomycin phosphotransferase (*nptII*) conferring kanamycin resistance, *aad*-3 conferring resistance to streptomycin and spectinomycin, and *hph* conferring resistance to hygromycin. These antibiotics are toxic to many plant species, especially dicotyledons.

The neomycin phosphotransferase (*nptII*) gene encodes aminoglycoside 3'-phosphotransferase II and was first isolated as a component of transposon Tn5 from

Escherichia coli (Garfinkel et al. 1981; Miki and McHugh 2004). The *nptII* gene confers

resistance to the antibiotics neomycin and kanamycin, and it is a popular selectable

marker in plant biotechnology. Neomycin and kanamycin are not of importance in

human/animal medical treatment, and on average, 20 to 40% of naturally occurring gut

bacteria are already resistant to kanamycin (Braun 2001). Further, kanamycin resistant

bacteria are ubiquitous in nature. Humans consume 1.2 million kanamycin-resistant

bacteria each day, primarily from fresh vegetables, and resistant bacteria are already

present in 10-20% of human digestive systems (Calgene 1990). Each human gut

naturally contains 10^{12} kanamycin-resistant bacteria.

The *aad-3* gene confers resistance to both streptomycin and spectinomycin

(Gilman et al. 1996). Streptomycin was used commonly in the 1950s and 1960s;

however, due to a high frequency in bacterial resistance and large occurrences of

ototoxicity, it no longer is highly used in the treatment of tuberculosis (Gilman et al.

1996). Spectinomycin is an aminoglycoside-like antibiotic used in the treatment of joint

infections caused by gonorrhea (WHO 1991).

The *hph* gene, derived from a fungus, confers resistance to hygromycin which is

an aminoglycoside analogue, and is used in the selection of transgenic plants and

organisms (Day 2003; Miki and McHugh 2004). Hygromycin is not used significantly in

human or veterinary medicine and has not conferred resistance to other clinically relevant

aminoglycosides (Wright and Thompson 1999).

2.4.3 Alternative markers to antibiotic resistance genes

There are several alternatives to using bacterial derived antibiotic resistance markers in transgenic research, such as the use of chemical markers, production of marker-free plants, which can be more difficult and less efficient (Miki and McHugh 2004), the use of reporter genes as visible markers (Miki and McHugh 2004), or the use of selectable markers derived from plants (Mentewab and Stewart 2005).

Some markers confer resistance to chemicals other than antibiotics, such as herbicides or lethal concentrations of the amino acids, lysine and threonine, while others rely on the growth of plant cells in the presence of atypical nutrients, such as cytokinin, glucuronides, xylose or mannose (reviewed by Miki and McHugh 2004). Herbicide-resistance selectable markers include, but are not limited to, phosphinothricin acetyltransferase (*pat* and *bar* genes), 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), (resistance to glyphosate), acetolactate synthase (ALS) (resistance to chlorosulfuron), and bromoxinil nitrilase (*bxn*) (resistance to bromoxinil).

Two genes, *pat* and *bar,* encode the enzyme, phosphinothricin acetyltransferase, which confers tolerance to PPT in transgenic plants. PPT is an inhibitor of glutamine synthetase, which catalyzes ammonia into glutamic acid in plants. Inhibition of this enzyme results in the accumulation of toxic levels of ammonia within the plant, causing plant death (OECD 1999). Glyphosate inhibits EPSPS, an essential enzyme in the shikimate pathway for aromatic amino acid biosynthesis. ALS is the target for several herbicides including sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl thiobenzoates (Singh and Shaner 1995). ALS is a regulatory enzyme in the biosynthetic

pathway to branched-chain amino acids in chloroplasts. Bromoxynil nitrilase

hydrolyzes bromoxynil into 3,5-dibromo-4-dihydroxybenzoic acid and ammonia.

Non-selectable marker genes or reporter genes can be used in conjunction with

selectable marker systems. Reporter genes can visible detect transformed cells or tissues.

Popular reporter genes include green fluorescent protein (GFP) (reviewed by Stewart

2001), luciferase (Ow et al. 1986), β-galactosidase (Helmer et al. 1984), and β-

glucuronidase (GUS) (Jefferson et al. 1987; Kilian et al. 1999; Daniell et al. 1991).

A final alternative is the use of markers derived from plant sources instead of

bacterial sources. The plant gene, *Atwbc19*, encodes an *Arabidopsis thaliana* ATP

binding cassette (ABC) transporter has been shown to confer kanamycin resistance to

transgenic plants (Mentewab and Stewart 2005).

2.4.4   Implications of ARMs on human and animal health

There are numerous safety concerns relating to the use of antibiotic resistance

markers in biotechnology, more specifically in the creation of transgenic plants; the

safety of the gene and the gene product and the potential for the gene to be transferred to

bacteria and create a risk to human health (Gay and Gillespie 2005). The latter has been

discussed in detail in section 2.3 Horizontal Gene Transfer.

Antibiotic resistance occurs when an antibiotic is no longer effective in killing or

inhibiting bacterial growth. The major cause of resistance is believed to be the overuse or

inappropriate use of antibiotics in human and animal health (Braun 2001). Gene flow

among bacteria is a well-established process of microbial evolution, as observed in the

development of multi-drug resistant bacteria (Tschape 1994; Salyers and Shoemaker

1994; Witte 1998). The implications of ARMs on human and animal health are related more to the persistence of transgenic plant DNA in the environment from processing, ingestion and digesting (Braun 2001). Further, most ARMs utilized in the production of transgenic plants do not use antibiotics that are used clinically in human and animal health (Braun 2001). Thus, the likelihood of developing resistance is of little concern in human and animal health (Braun 2001).

However, of more concern in human and animal health, is the potential for cross-resistance. Antibiotic cross-resistance is the tolerance to other antibiotics when exposed to a similar substance (Mikkelsen et al. 1999). That is, the development of G418 resistance when exposed to kanamycin resistance. Cross-resistance is of concern with aminoglycosidic antibiotics since many of the aminoglycosides are similar in structure. In general, for human and animal health, ARMs that confer resistance to antibiotics of clinical significance, particularly those to which resistance is not widely spread in the environment, should be avoided.

2.4.5   Regulations regarding the use of ARMs in transgenic plants

The US FDA/CFSAN has published guidance for industry on the use of antibiotic resistance markers in transgenic plants (US FDA/CFSAN 1998. There are five topics discussed relating to the transfer of antibiotic marker genes : direct effects of the ingestion of enzyme encoded by antibiotic resistance marker genes, potential transfer of antibiotic marker genes to gut epithelial cells, gut microorganisms and microorganisms in the environment and approaches to and surveillance for the potential transfer of antibiotic

resistance marker genes and their resistance. These topics have been covered in detail in previous sections.

3. Objectives

The objective of this study was to determine the ability of bacteria to confer aminoglycoside resistance to a newly discovered plant ABC transporter that confers kanamycin resistance when overexpressed in transgenic plants. This study examined the ability of *Escherichia coli* to confer kanamycin, gentamicin and geneticin resistance when transformed with either *nptII* or *Atwbc19*.

Simulated HGT was staged by subcloning *Atwbc19* under the control of a strong bacterial promoter, transforming bacteria with appropriate plasmids and assessing whether kanamycin resistance was conferred compared to *nptII*. Resistance to a similar aminoglycosides, geneticin (G418) and gentamycin, were also assessed to determine if there was potential for cross-resistance to occur.

4. Materials and Methods

4.1 Bacterial strains and plasmids

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). Three plasmids

were used in this study. Plasmids were obtained from Mentewab Ayalew, a former

postdoctoral associate at the University of Tennessee. In all cases HGT is mimicked by

the subcloning of the gene that would be in T-DNA under the control of a plant promoter

so that it is now under the control of a strong inducible bacterial promoter; modeling a

worst-case HGT scenario.   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 (Fig. 1) (all figures and tables located within appendix). The microorganisms

were maintained in glycerol stocks and stored at -80°C. Working cultures were obtained

by inoculating a loopful of culture into 50 ml LB broth supplemented with ampicillin

(100mg L^{-1}) and incubated at 37°C for 24 hr. Inserts were sequenced at 5' and 3' ends as

confirmation.

4.2 Determining susceptibility of *Escherichia coli* to kanamycin

E. coli DH5α without any of the experimental plasmids was examined for

resistance to varying levels of kanamycin prior to transformation experiments. Briefly,

bacteria were grown overnight in 50 ml cultures to approximately 1×10^9 CFU ml^{-1},

serially diluted and placed in LB with kanamycin (0, 25, 50 and 100 mg L^{-1}). Tubes were

incubated overnight at 37°C and turbidity observed after 24 hr incubation. Only those

strains that were susceptible (i.e. no visible turbidity) at the lowest level (25 mg L^{-1}) of kanamycin were used for the horizontal gene transfer experiments.

4.3   Determining potential usage of other bacteria in HGT studies

Other soilborne bacteria were examined for potential use in these experiments: *Variovorax* sp. UTK 037, *Acidovorax* sp. UTK 052 and *Rahnella* sp. UTK 034 were examined for their natural resistance to ampicillin and kanamycin (Fig. 2). Ampicillin was utilized since it was the ARM utilized in initial experiments for plasmid selection in bacteria.

4.4   Transformation

E. coli DH5α was transformed with pKS, pKS-*ABC*, and pKS-*nptII* by a freeze/thaw method (Hanahan 1983). 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 hr.

4.5   Assessment of new antibiotic resistance through natural transformation

Transformed bacteria were observed for their ability to acquire kanamycin, geneticin (G418), and gentamycin resistance using growth assays 96-well microtiter plates. Isopropyl-b-D-thiogalctopyranoside (IPTG) at 1 mM was used to induce the lacZ promoter. Each well was filled with 199 μl LB, IPTG and varying concentrations of antibiotic (kanamycin, geneticin (G418), and gentamycin) and 50 μl bacterial treatments. Each treatment was replicated in triplicate in sterile 96-well microtiter plates and each

experiment was duplicated in time. Optical densities were measured at 630 nm after

incubating microtiter plates at 37°C for 24 hr.

Antibiotics with cationic structures may bind electrostatically to acid or sulfate

groups in LB media and thus, decrease the effectiveness of the aminoglycoside

(Amsterdam 1996). Thus, Mueller-Hinton (Mueller and Hinton 1941) broth was tested

instead of LB to examine if this was the case in this system.

4.6   Statistical analysis

Data were analyzed 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. All analysis was

performed using the SAS program (Statistical Analysis Systems 9.1, SAS Institute, Inc.,

Cary, NC).

4.7   Reverse transcription polymerase chain reaction

Initial expression studies for the antibiotic resistance genes were performed using

reverse transcription PCR. RNA was extracted using the RNeasy Mini Kit following the

manufacturer's protocol (Qiagen, Valencia, CA, USA). Two micrograms of template

was used in the iScript cDNA synthesis reaction following the manufacturer's

instructions using half reactions (Bio-Rad, Hercules, CA, USA). Quality of cDNA

synthesis was examined using 1% agarose gel electrophoresis.   PCR was performed

using the following primers: ABC forward (5'-GAG GCA GGG ACA CAA GAG AG-

3'), ABC reverse (5'-TGT CCC CGT TTT TAT CCA AG-3'), neomycin forward (5'-

CTT GGG TGG AGA GGC TAT TC-3'), neomycin reverse (5'-AGG TGA GAT GAC

AGG AGA TC-3') (Integrated DNA Technologies, Inc., Coralville, IA, USA) and

following protocol Eppendorf MasterMix. Expression was observed using a 1% agarose

gel.

4.8   Northern blot analysis

Northern gel blot analysis was performed to assess gene expression of *Atwbc19*

and *nptII* under the experimental conditions. Fifteen-hundred microliter samples were

taken from triplicate wells for each plasmid (pKS, pKS-*ABC* and pKS-*nptII*) at 0 and

either 6.5 or12.5 mg L^{-1} G418 or kanamycin respectively at 24 h for northern blot

analysis. RNA was extracted using the RNeasy Mini Kit and following manufacturer's

protocol (Qiagen, Valencia, CA, USA). RNA was examined for quality and quantity by

1% agarose gel electrophoresis (Fig. 3). 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 Labeling Kit (Stratagene, LaJolla, CA, USA) and purified using mini Quick Spin

Columns (Roche Applied Science, Indianapolis, IN, USA). Prior to probing, *Atwbc19*

and *nptII* were excised from a 1% gel (Fig. 4), purified using QiaQuick spin columns

(Qiagen, Valencia, CA, USA), and checked for quality (Fig. 5). Hybridization was

performed using ULTRAhyb™ hybridization buffer following the manufacturer's

instructions (Ambion, Austin, TX, USA).

5. Results

E. 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.

When testing broth differences, observations indicated a decreased growth overall resulting in OD readings reaching only 0.3 after 24 hr when Mueller-Hinton broth was used in place of LB (Fig. 6). Furthermore, it appeared that the minimum inhibitory concentrations (MICs) were lower when using the alternative media. However, growth of bacteria in Mueller-Hinton broth containing no antibiotic was decreased as well, indicating our bacteria grew better in LB. However, further tests would need to be conducted to fully observe the influence of media type on MIC.

When soilborne bacteria were tested for their ability to be used in subsequent experiments, *Acidovorax* was slow-growing and could not be compared effectively in these experiments (Fig. 2). *Rahnella* and *Variovorax* appeared to demonstrate resistance to ampicillin, making it difficult to screen in cloning experiments (Fig. 2). *Rahnella* appeared less resistant to kanamycin than *Variovorax* (Fig. 2). Thus, these bacteria were not utilized in any further experiments.

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 higher concentrations of kanamycin (e.g., over 10 mg L^{-1}) ($p<0.05$) (Fig. 7). The *Atwbc19* gene was not significantly different from the no-plasmid control at higher concentrations of kanamycin (e.g., over 25 mg L^{-1}) ($p<0.05$). *E. coli* transformed with pKS-*ABC* showed little resistance to kanamycin at 100 mg L^{-1}, the level that was most effective in the

selection of transgenic tobacco (Mentewab and Stewart 2005; Fig. 7). *NptII*, however, conferred kanamycin resistance at the 100 mg L^{-1} concentration as well at the 400 mg L^{-1}, the highest concentration tested (Fig. 7). *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. 8). *NptII* endowed G418 resistance at the 100 mg L^{-1} concentration, the highest tested (Fig. 8).

There was no significant difference between the no-plasmid control, pKS-*nptII* or pKS-*ABC* at gentamicin concentrations greater than 1 mg L^{-1} (Fig. 9). Each plasmid followed a similar growth pattern with none displaying resistance at any level greater than 1 mg L^{-1} (Fig. 9). Gentamicin, used in human therapeutics, is composed of a mixture of antibiotic substances, C1 (25-50%), C1a (10-35%), and gentamicins C2a and C2 (25-55%) (McAllan and Smith 1973). Gentamicins A and B are only minor components of the commercial drug and APH (3') II, and thus, does not confer resistance (Davies 1986). This minor composition may explain the lack of resistance observed in pKS-*nptII* (Fig. 9).

Gene expression levels were found to be similar between pKS-*ABC* and pKS-*nptII* from RT-PCR analysis (Fig. 10). Expression levels for *nptII* were similar to that of *Atwbc19* for the two concentrations of the antibiotic kanamycin tested (Fig. 11). Equal loading of RNA was observed with methylene blue staining of RNA adhered to the nylon membrane (Fig. 11). There was a slightly apparent decrease in the level of expression for

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Atwbc19 compared to the *nptII* gene that was most likely the result to the plant codon usage of the *ABC* gene or its large size (over two-fold greater size than *nptII*) (Fig. 11). All other samples of pKS-nptII were similarly expressed (Fig. 11B).

6.   Discussion

Previous research has addressed the potential for horizontal gene transfer to occur from transgenic plants to environmental bacteria (Gebhard and Smalla 1998; de Vries and Wackernagel 1998). Gebhard and Smalla (1998) demonstrated the ability of *Acinetobacter* sp. BD413 to utilize and integrate transgenic plant DNA under laboratory conditions. Furthermore, de Vries and Wackernagel (1998) reported the restoration of *Acinetobacter* sp. BD413 cells containing an *nptII* gene with a 10 bp deletion when transformed with DNA from transgenic *Solanum tubersosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, and *Lycopersicon esculentum* carrying an *nptII* marker gene.

Mentewab and Stewart (2005) recently described a plant gene that confers kanamycin resistance to transgenic plants—the ABC transporter *Atwbc19*. Transgenic plants with the *Atwbc19* gene, with and without an *nptII* cassette confer resistance to kanamycin similar to that of *nptII* in transgenic plants. Here we have demonstrated that when HGT is staged by subcloning *Atwbc19* under the control of a strong bacterial promoter, it did not confer kanamycin resistance comparable to that of the *nptII* gene in *E. coli*. Many researchers have failed to detect HGT from transgenic plants to bacteria, most likely due to the absence of homologous sequences in the bacteria (Nielsen et al. 1997) or the use of bacteria with decreased transformation efficiency (Schluter et al. 1995; Broer et al. 1996). Here we utilize *E. coli* DH5α, a highly transformable bacteria and optimize transformation using a strong bacterial promoter and demonstrated the inability of the *Atwbc19* gene to confer resistance to the aminoglycoside antibiotic kanamycin to that of the *nptII* in the soil bacterium *E. coli* at high concentrations (e.g., over 10 mg L^{-1}).

Another explanation for the inability to detect HGT may be due to only monitoring the transfer of the complete gene, not merely segments of the DNA (Smalla 2000). As with previous laboratory studies with *nptII* and its transfer to bacteria under laboratory conditions, we found that the *nptII* gene provided resistance to kanamycin in bacteria (Gebhard and Smalla 1998).

Nielsen et al. (1998, 2001), Bertolla and Simonet (1999), Droge et al. (1998) and Thomson (2001) have published reviews of the work on horizontal gene transfer from transgenic plants to bacteria or mammalian cells. Many researchers have conducted experiments to mimic the movement of DNA from transgenic plants to environmental bacteria and fungi under field conditions (Paget et al. 1998; Gebhard and Smalla 1999). 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 (Smalla et al. 1994; Paget and Simonet 1994; Badosa et al. 2004); however, HGT from transgenic plants to bacteria under optimized conditions has been demonstrated (Hoffman et al. 1994; Schluter et al. 1995; Gebhard and Smalla 1998; de Vries and Wackernagel 1998; Nielsen et al. 2000; Meier and Wackernagel 2003).

Hoffman et al. (1994) used transgenic *Brassica* plants and the filamentous fungi, *Aspergillus niger*, as a model system and demonstrated successful transformation of *A. niger* with the hygromycin B antibiotic resistance marker gene; however, the transfer

frequency was too low to calculate. Schluter et al. (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}, a rate far below

detection limits.

Other studies were conducted utilizing the bacterial-derived antibiotic resistance

gene (*nptII*) incubated with natural transformable bacteria with a non-functioning copy of

nptII (Gebhard and Smalla 1998; De Vries and Wackernagel 1998; Nielson et al. 2000;

Meier and Wackernagel 2003). HGT was determined based on the return of antibiotic

resistance of the non-functioning *nptII*. Low transfer frequencies of 3×10^{-8} (de Vries

and Wackernagel 2003) and 5.4×10^{-9} (Gebhard and Smalla 1998) were detected. Broer

et al. (1996) infected *Agrobacterium tumefaciens* with the T-DNA from transgenic

tobacco, *Nicotiana tabacum*, but were unable to detect HGT from plant to bacteria due to

the inability of the bacterium to develop competence and uptake DNA. Nielson et al.

(1997) found no HGT between transgenic plants and *Acinetobacter* under laboratory

conditions.

There is no evidence that *Atwbc19* has been transferred to bacteria during

evolution (Mentewab and Stewart 2005). Furthermore, there are ABC transporters found

in prokaryotes; however, database searches have not disclosed bacterial ABC transporter

orthologues with plant-like codon use patterns (Mentewab and Stewart 2005).

Previous observations have indicated that *Atwbc19* is very specific for kanamycin

and resistance to no other aminoglycoside antibiotics was displayed (Mentewab and

Stewart 2005). In addition *Atwbc19* is 2.75 times larger than *nptII* and has plant codon

usage. *E. coli* transformed with pKS-*ABC* showed little resistance to kanamycin at 100

mg L^{-1}, 10 times less than the level of resistance found in enterococci isolated from

coastal bathing waters (Arvanitidou et al. 2001). This lack of resistance demonstrated by

bacteria transformed with the *Atwbc19* gene along with the *Atwbc19* gene's specificity to

kanamycin, larger size, and its usage of plant codon, demonstrates a decrease in the

potential threat to transfer its resistance to bacterial pathogens. At least if it were

transferred to bacteria it would not confer kanamycin resistance. Mentewab and Stewart

(2005) inferred that *Atwbc19* was targeted to the tonoplast when overexpressed in plants.

Since the tonoplast and central vacuole are absent in bacteria, it is likely mis-targeted to

some degree to the cell membrane (Fig. 12). In plants there is active transport of

kanamycin into the vacuole where it is sequestered. Since bacteria lack a central vacuole

this mechanism cannot occur for toxin sequestration (Fig. 12). The decrease of *Atwbc19*

in bacteria can be largely be explained by differences of transgene subcellular targeting

between plants and bacteria. The low level of *Atwbc19*-conferred kanamycin resistance in

bacteria is likely caused by an inefficient cell membrane targeting of the overproduced

protein that attempts to pump kanamycin out of cells.

  The neomycin phosphotransferase II gene was originally isolated as a component

of the transposon Tn5 from *E. coli* (Beck et al. 1982) and is an enzyme that catalyzes the

transfer of acetyl, adenosine monophosphate or phosphate groups onto the

aminoglycoside antibiotics, including kanamycin, neomycin, paromomycin,

ribostamycin, gentamicins A and B and butirosins (Wright et al. 1998; FDA/CFSAN

1998). This transfer, in turn, contributes to the *nptII* gene's greater ability to resist higher

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kanamycin concentrations in bacteria. Bacteria have membrane transport systems that function to deliver essential nutrients into the cell, maintain homeostasis, export proteins; and control efflux of xenobiotic/antibiotic compounds (Paulsen et al. 1998). Of the above mentioned aminoglycosides, kanamycin and neomycin are not currently used medically for either animals or humans (FDA/CFSAN 1998). Geneticin (G418) is an aminoglycoside antibiotic similar in structure to gentamicin, neomycin and kanamycin. G418 is an analog of neomycin sulfate and interferes with the function of the 80S ribosome and protein synthesis in eukaryotic and prokaryotic cells. And thus, the aminoglycoside transferase gene confers resistance.

7.   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. Currently, the antibiotic resistance genes used in transgenic studies have been bacterial in origin, with the exception of the ABC transporter, *Atwbc19,* the first plant gene shown to confer kanamycin resistance in transgenic plants. The *Atwbc19* gene was evaluated for its ability to transfer antibiotic resistance to bacteria found in the environment (*E. coli* DH5α). 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, that low levels of kanamycin resistance would be conferred as a result.

Further research needs to be conducted to assess the safety of the *Atwbc19* gene as an alternative antibiotic resistance marker in the creation of transgenic plants (i.e. examine bacteria of medical importance). This gene is an effective alternative to the bacterial *nptII* gene in transgenic plants.

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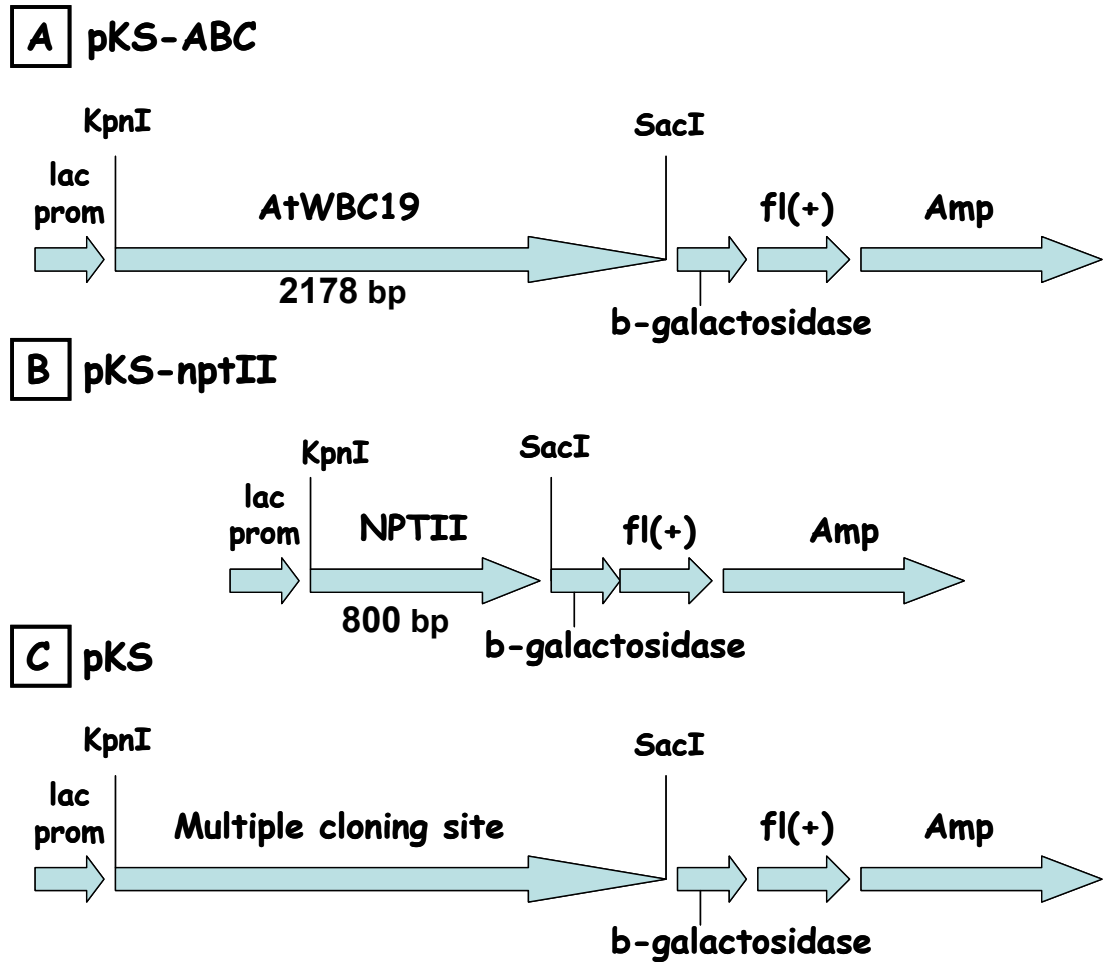
Appendix

Figure 1. Constructs utilized in all transformation experiments. pKS-*ABC* (A) contains the *Atwbc19* gene under the control of the lacZ bacterial promoter in pBluescript II SK+; pKS-*nptII* (B) contains the *nptII* gene with the lacZ bacterial promoter in pBluescript II SK+; and pKS (C) is pBluescript II SK+ with no inserted genes as a control. Genes were inserted into pKS using KpnI and SacI restriction sites. Initial transformants were selected using ampicillin resistance.
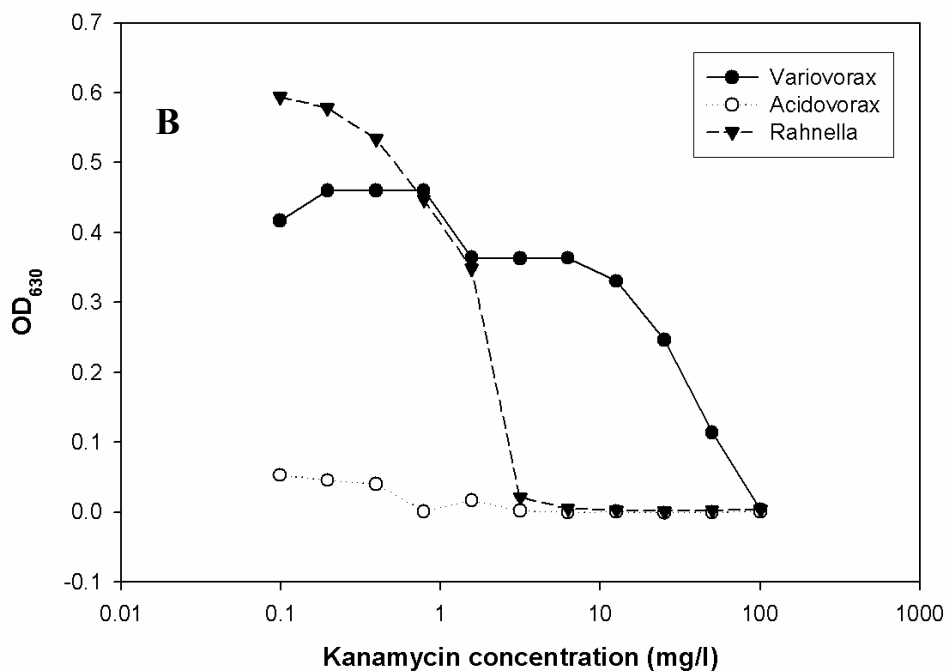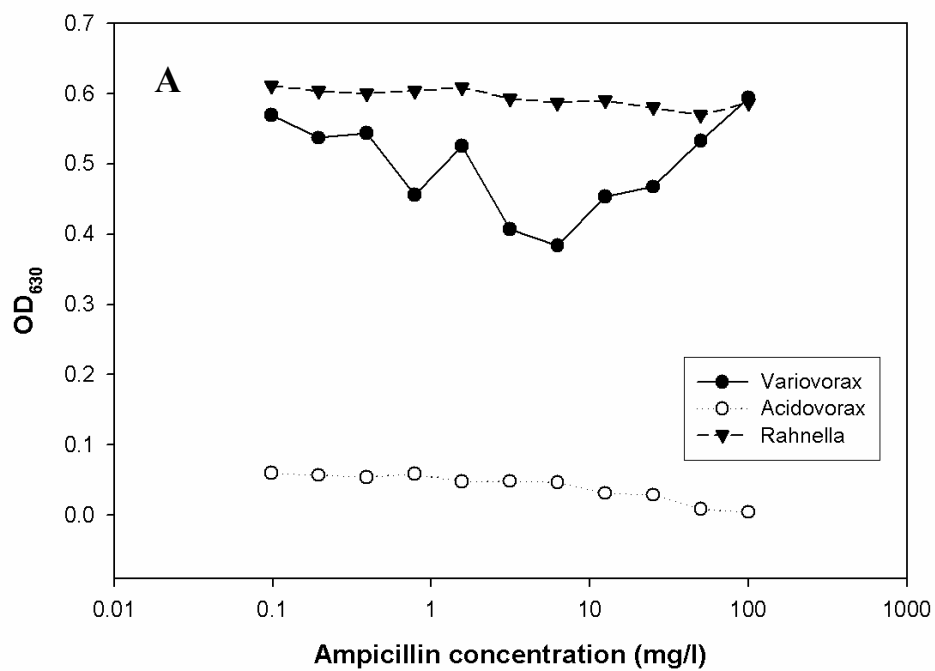
Figure 2. Effect of ampicillin (A) and kanamycin (B) on the growth of the soilborne bacteria, *Variovorax*, *Acidovorax* and *Rahnella*. Optical densities were measured at 630 nm to determine the growth of *Variovorax*, *Acidovorax* and *Rahnella* incubated at 30°C in 96 well microtiter plates containing 199 μl LB and serially diluted 0 to 100 mg L^{-1} kanamycin.
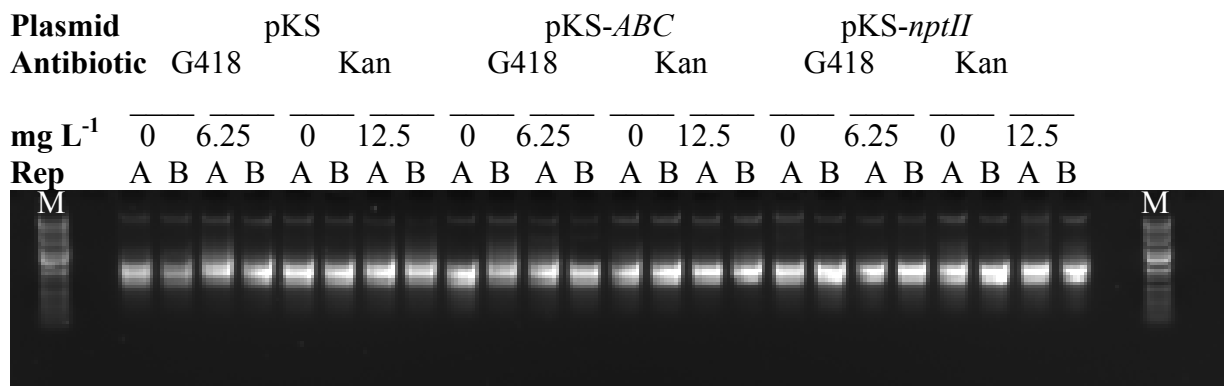
Figure 3. A check of quality and quantity of RNA using a 1% agarose gel prior to running the formaldehyde gel. Each lane represents 2 μg total RNA obtained from *E. coli* transformed with either pKS, pKS-*nptII* or pKS-*ABC* and incubated in varying concentrations (0, 6.25 or 12.5 mg L^{-1}) of antibiotics (G418 or kanamycin) in duplicate (A, B). The name of each sample is labeled above the panel. M=marker.
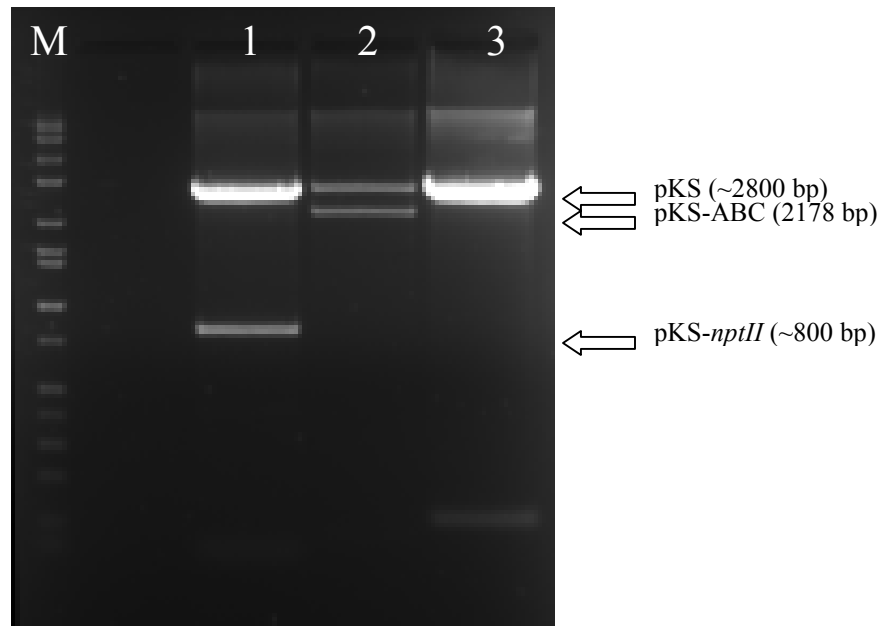
Figure 4. Plasmids (1=pKS-*nptII*, 2=pKS-*ABC*, and 3=pKS) were digested with KpnI and SacI and ran on a 1% agarose gel. Bands were excised, gel purified and were used in the creation of probes for the northern blotting. M=marker.
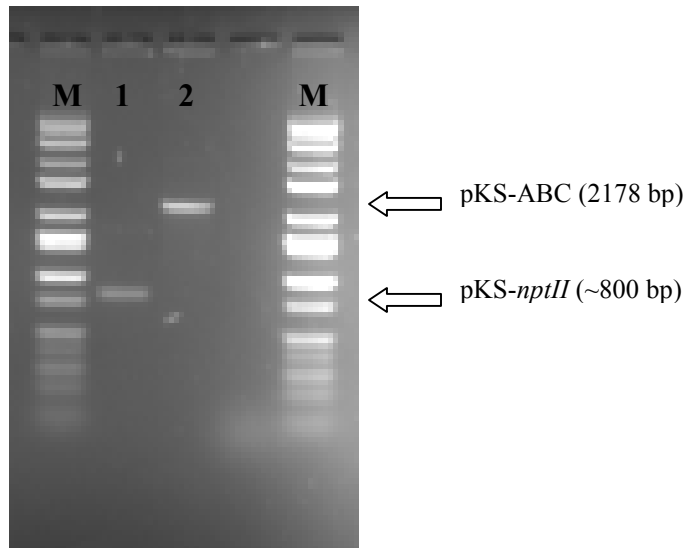
Figure 5. Quality control check of probes used in the northern blot. Probes, 1=*nptII* and 2=*ABC*, at 200 ng were ran on a 1% agarose gel. M=marker.
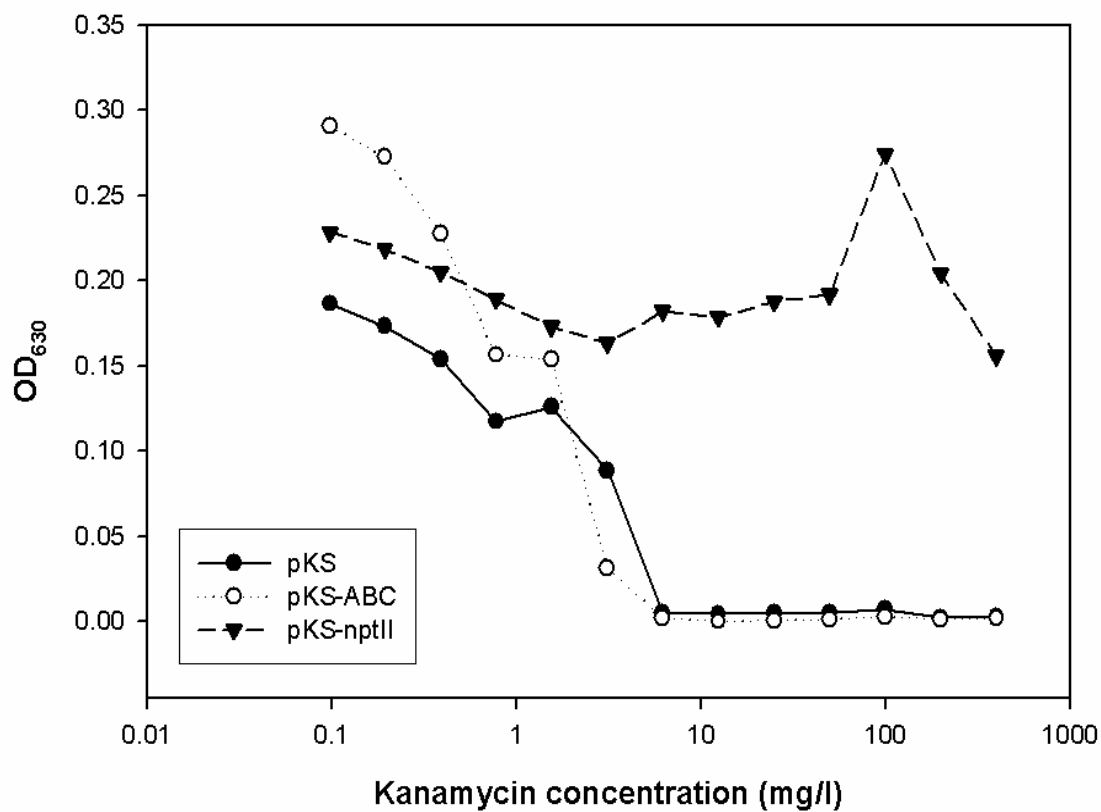
Figure 6. Effect of media type (Mueller-Hinton broth) on the growth of *E. coli* DH5α. Optical densities (measured at 630 nm) of *E. coli* DH5α transformed (pKS-*ABC*, pKS-*nptII* or pKS) incubated at 37°C in 96 well microtiter plates containing 199 μl Mueller-Hinton broth, induced using 1 mM IPTG and serially diluted 0 to 400 mg L^{-1} kanamycin.
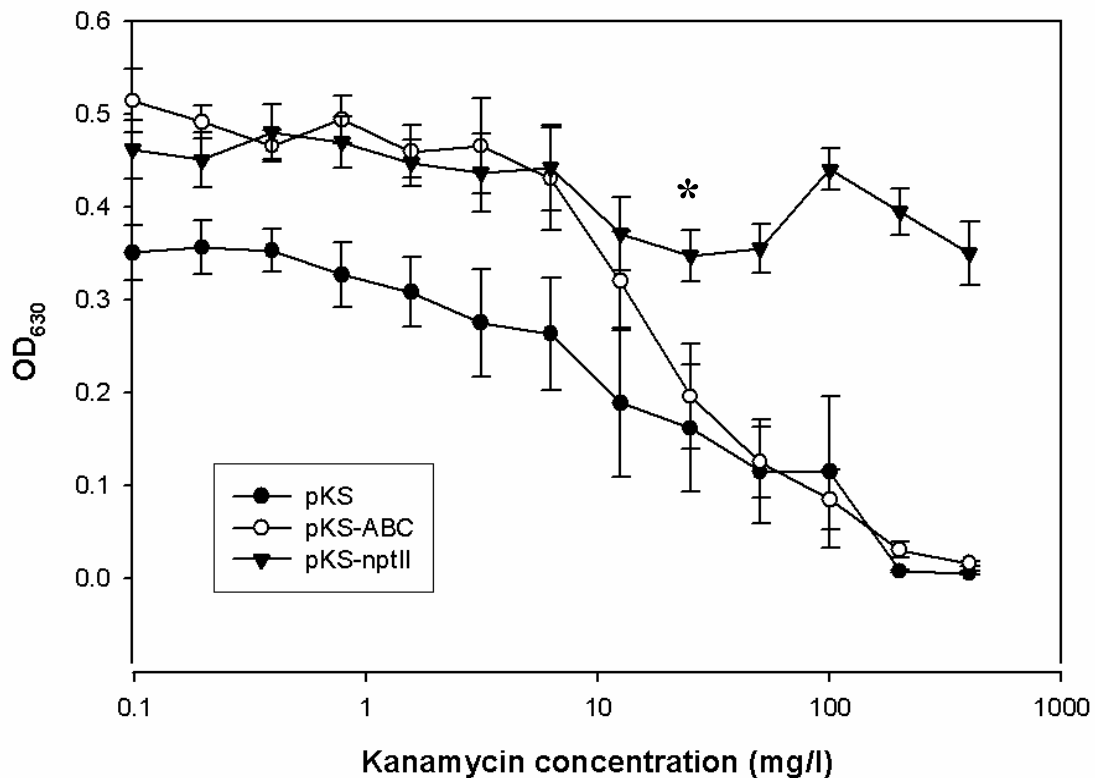
Figure 7. Optical densities (measured at 630 nm) of *E. coli* DH5α transformed (pKS-*ABC* (n=12), pKS-*nptII* (n=12) or pKS (n=6)) incubated at 37°C in 96 well microtiter plates containing 199 μl LB, induced using 1 mM IPTG and serially diluted 0 to 400 mg L^{-1} kanamycin. Data were analyzed by ANOVA using the general linear model, and 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.
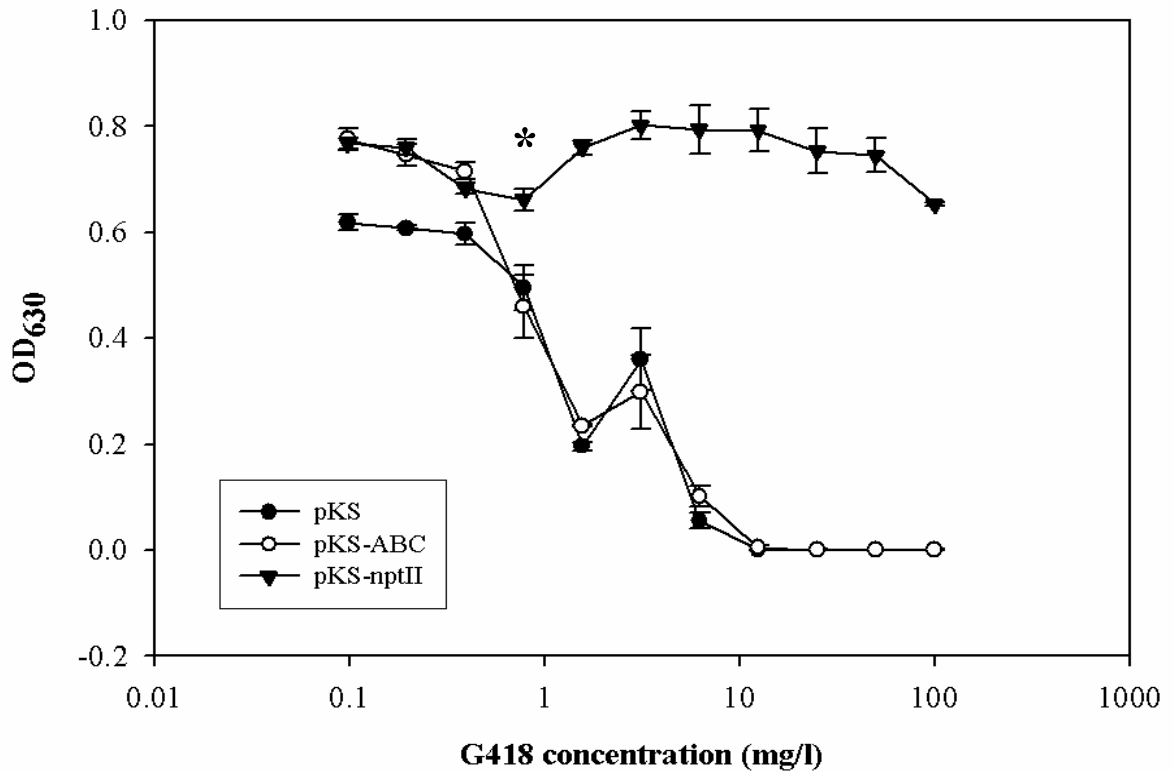
Figure 8. Optical densities (measured at 630 nm) of *E. coli* DH5α transformed (pKS-ABC (n=12), pKS-nptII (n=12) or pKS (n=6)) incubated at 37°C in 96 well microtiter plates containing 199 μl LB, induced using 1 mM IPTG and serially diluted 0 to 100 mg L^{-1} geneticin (G418). Data were analyzed by ANOVA using the general linear model, and 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.
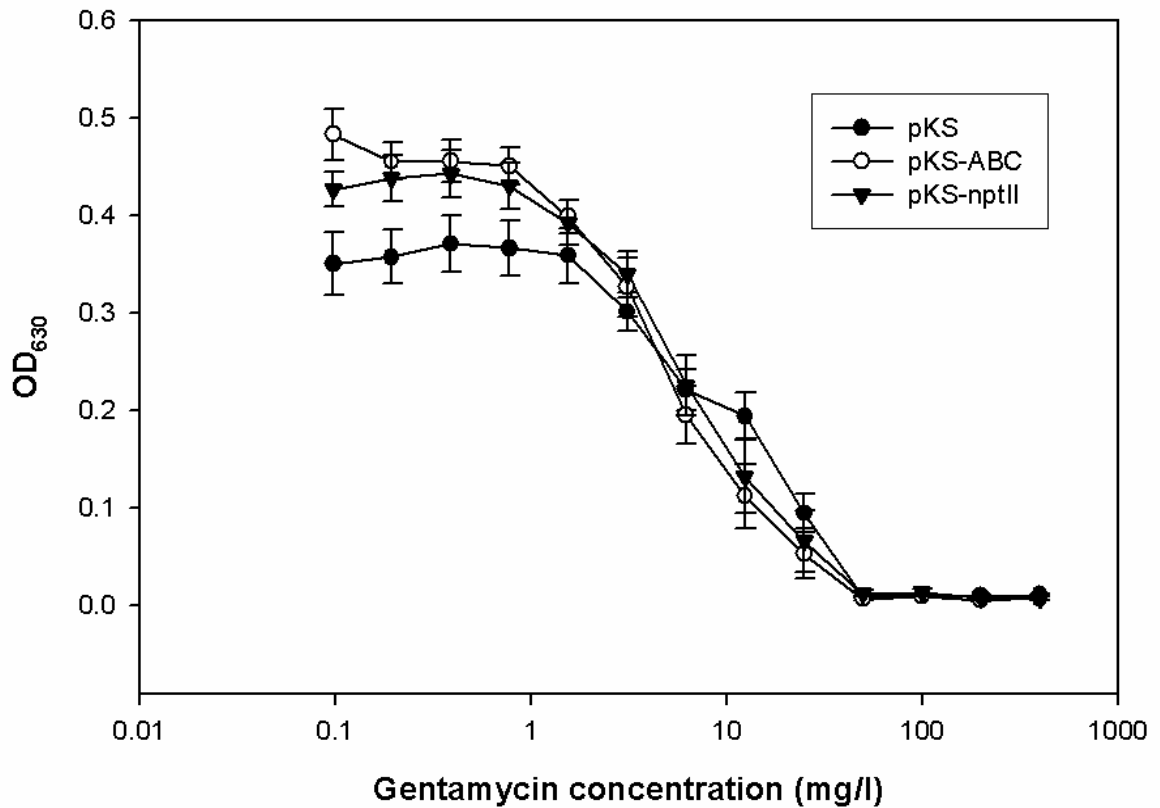
Figure 9. Optical densities (measured at 630 nm) of *E. coli* DH5α transformed (pKS-*ABC* (n=12), pKS-*nptII* (n=12) or pKS (n=6)) incubated at 37°C in 96 well microtiter plates containing 199 µl LB, induced using 1 mM IPTG and serially diluted 0 to 400 mg L^{-1} gentamicin. Data were analyzed by ANOVA using the general linear model. No significant differences (at the 0.05 probability level) were found between treatments. Error bars indicate standard error.
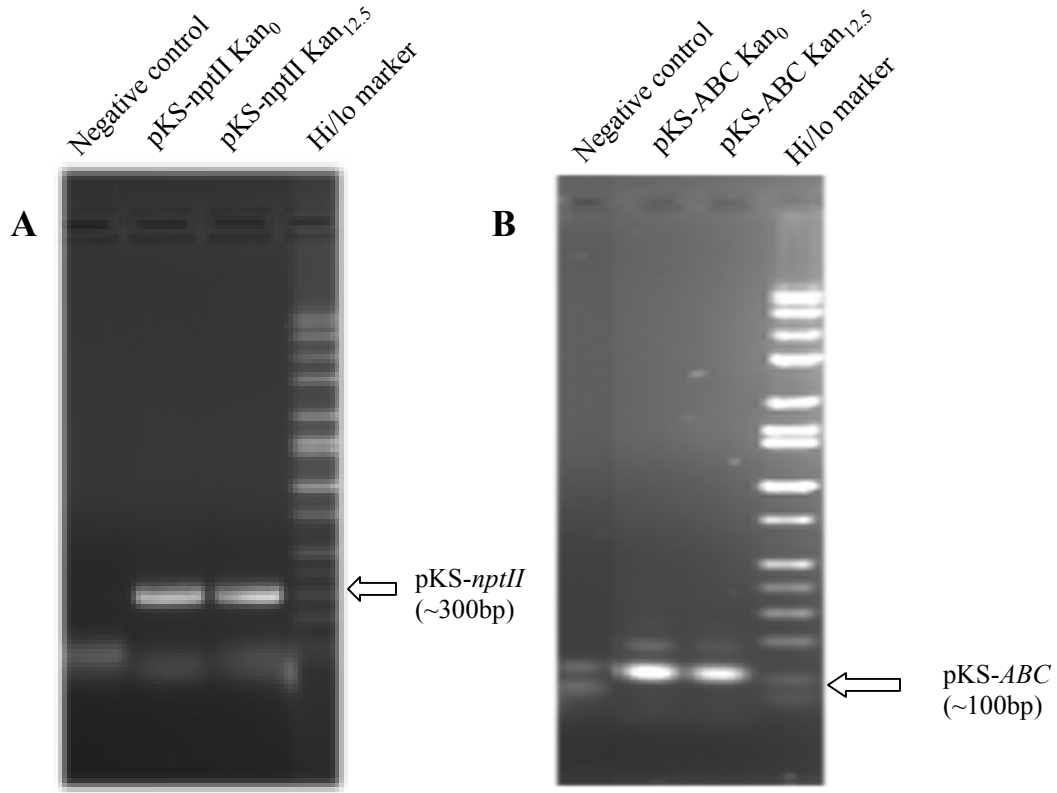
Figure 10. Products following reverse transcription PCR of pKS-*nptII* (A) and pKS-*ABC* (B) grown at 0 and 12.5 mg L^{-1} kanamycin and ran on a 1% agarose gel.
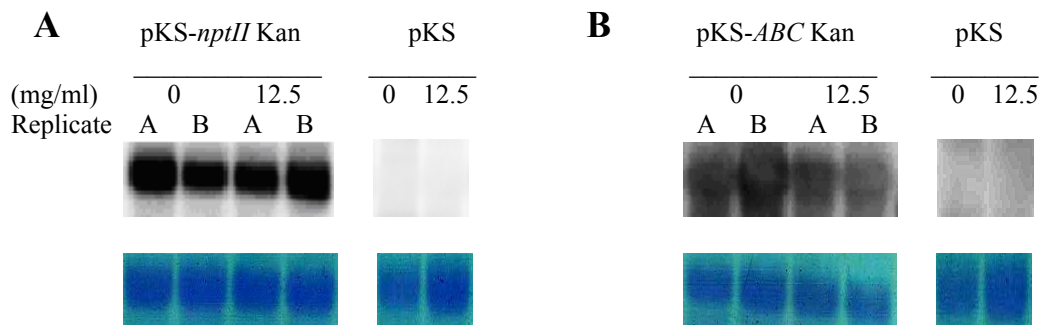
Figure 11. Each lane represents 2 μg total RNA obtained from *E. coli* transformed with either pKS-*nptII* (panel A), pKS-*ABC* (panel B) or pKS (negative control) and incubated in varying concentrations (0 or 12.5 mg L^{-1}) of kanamycin in duplicate (A, B). The blots were hybridized with a [32-P] radiolabelled *nptII* or *ABC* probe respectively. Expected band sizes were 0.8 kb and 2.2 kb for pKS-*nptII* and pKS-*ABC* respectively. The name of each sample is labeled above the panel**.**
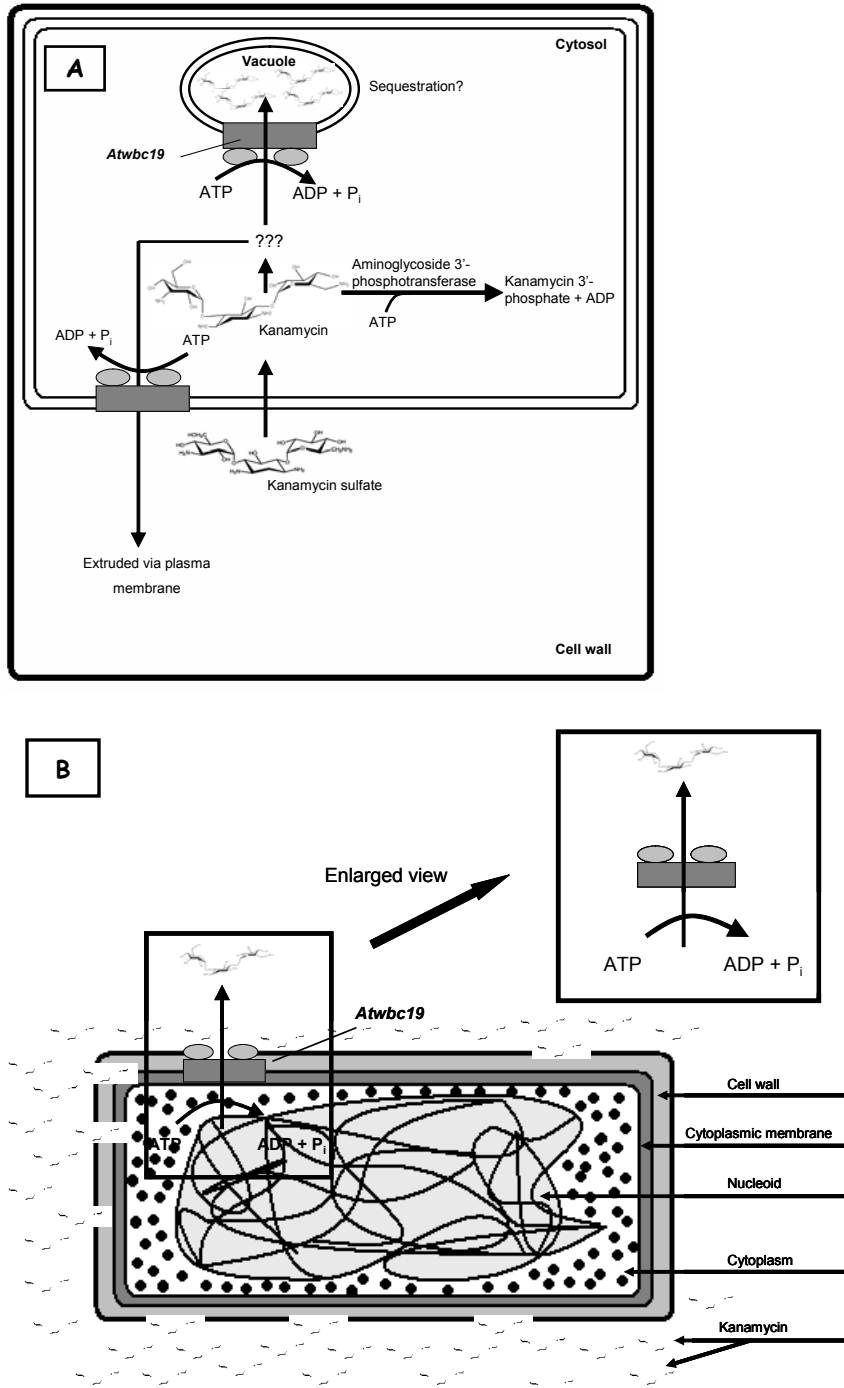
Figure 12. Possible modes-of-action of *Atwbc19*, an *Arabidopsis thaliana* gene shown to confer kanamycin resistance, in plants(A) (Adapted from Rea 2005) and not in bacteria (B). Bacteria have no mechanism of eliminating the kanamycin; therefore, the bacterial membranes become overwhelmed and the cells become lysed, leading to the death of the bacteria. *Atwbc19* may be targeted to the bacterial membrane and aid in some removal of kanamycin.

Table 1. Selectable marker genes (ARMs and herbicides) utilized in transgenic plants in the United States (adapted from Miki and McHugh 2004).

Enzymes	Gene	Selection
ARMs		
Acetyl transferase	*sat3*	Streptothricin
Aminoglycoside-3"-adenyl transferase	*aadA*	Spectinomycin
β-lactamase	*bla*	Ampicillin
Bleomycin resistance	*Ble*	Bleomycin, phleomycin
Chloramphenicol acetyltransferase	*cat*	Chloramphenicol
Dihydropteroate synthase	*sul1*	Sulfonamides
Hygromycin phosphotransferase	*hph*	Hygromycin B
Neomycin phosphotransferase	*neo,nptII*	Neomycin, kanamycin
Streptomycin phosphotransferase	*SPT*	Spectomycin, spectinomycin
Chemical		
Acetolactate synthase	*csr1-1*	Sulfonylureas
Acetolactate synthase	*csr1-2*	Imidazolinones
β-glucuronidase		
Bromoxinil nitrilase	*Bnx*	Oxynils
Cyanamide hydratase	*cah*	Cyanamide
5-enolpyruvylshikimate-3-phosphate synthase	*EPSP synthase, aroA*	Glyphosate
Glutamate-1-semialdehyde aminotransferase	*hemL*	Gabaculine
Glyphosate oxidoreductase	*cp4 epsps, gox*	Glyphosate
Phosphinothricin acetyl transferase	*pat, bar*	Bialaphos, glufosinate, PPT

Vita

Kellie Parks Burris was born on September 5, 1978 in Morganton, NC to Tom and Gail Parks. She attended Burke County Public Schools and graduated with honors from Freedom High School in 1996. Kellie attended North Carolina State University and graduated *Cum Laude* with two Bachelors' of Science Degrees in Animal Science and Poultry Science in May 2000 and *Magna Cum Laude* with a Bachelor's of Science Degree in Food Science with a minor in nutrition in May 2001. In August 2002, Kellie attended graduate school at the University of Tennessee, and graduated with her Masters of Science degree in Food Science and Technology in December 2004. She continued her education by pursuing another Master's degree at the University of Tennessee in Plant Sciences to gain skills and knowledge in plant molecular biology. Her research was focused on examining the risks of a novel kanamycin resistance marker, *Atwbc19*, on the ability of bacteria to develop antibiotic resistance under the direction of Dr. C.N. Stewart, Jr. Kellie graduated with her Masters of Science degree in Plant Sciences December 2006. She is currently a partner and Research Scientist for the start-up biotechnology company, MycoGenomix, LLC.