Plant Cell Rep (2010) 29:643–650
DOI 10.1007/s00299-010-0850-8

ORIGINAL PAPER

Transgenic hybrid aspen overexpressing the *Atwbc19* gene encoding an ATP-binding cassette transporter confers resistance to four aminoglycoside antibiotics

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Received: 9 September 2009 / Revised: 16 February 2010 / Accepted: 23 March 2010 / Published online: 11 April 2010

Abstract Antibiotic-resistance genes of bacterial origin are invaluable markers for plant genetic engineering. However, these genes are feared to pose possible risk to human health by horizontal gene transfer from transgenic plants to bacteria, potentially resulting in antibiotic-resistant pathogenic bacteria; this is a considerable regulatory concern in some countries. The *Atwbc19* gene, encoding an *Arabidopsis thaliana* ATP-binding cassette transporter, has been reported to confer resistance to kanamycin specifically as an alternative to bacterial antibiotic-resistance genes. In this report, we transformed hybrid aspen (*Populus canescens* × *P. grandidentata*) with the *Atwbc19* gene. Unlike *Atwbc19*-transgenic tobacco that was only resistant to kanamycin, the transgenic *Populus* plants also showed resistance to three other aminoglycoside antibiotics (neomycin, geneticin, and paromomycin) at comparable levels to plants containing a CaMV35S-*npt*II cassette. Although it is unknown why the transgenic *Populus* with the *Atwbc19* gene is resistant to all aminoglycoside antibiotics tested, the broad utility of the *Atwbc19* gene as a reporter gene is confirmed here in a second dicot species. Because the *Atwbc19* gene is plant-ubiquitous, it might serve as an alternative selectable marker to current bacterial antibiotic-resistance marker genes and alleviate the potential risk for horizontal transfer of bacterial-resistance genes in transgenic plants.

Keywords Antibiotic-resistance genes · Aminoglycoside antibiotics · Plant-derived selection marker gene · *Populus* · Transformation

Communicated by S. Merkle.

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Introduction

For plant transformation, it is necessary to include a selectable marker cassette for selecting transgenic cells that will be induced for regeneration into plants for further characterization (Horsch et al. 1985; Ow 2007; Rosellini et al. 2007). Approximately 50 marker genes have been evaluated for their efficiency, biosafety in plant transgenic research, and gene functional validation (Miki and McHugh 2004). Their characteristics, advantages, and disadvantages have been intensively reviewed in recent years (Miki and McHugh 2004; Mentewab and Stewart 2005; Ow 2007; Sundar and Sakthivel 2008). Among these marker genes, antibiotic- and herbicide-resistance genes are the most commonly used because of their broad utility, effectiveness as reliable selection markers, ease of use, and availability (Miki and McHugh 2004; Sundar and Sakthivel 2008).

Kanamycin is an aminoglycoside antibiotic that was isolated from the soil bacterium *Streptomyces kanamyceticus*. It inhibits both prokaryotic and eukaryotic protein synthesis primarily by binding to the ribosomal complex (Bar-Nun et al. 1983; Mingeot-Leclercq et al. 1999; Wright et al. 1998), and has been widely used in plant transformation as a selective agent. When plant cells are transformed with the gene encoding neomycin phosphotransferase (*npt*II), which detoxifies the antibiotic (Wright et al. 1998), resistant cells proliferate and nontransformed cells will be inhibited or

killed, allowing the transgenic cells to regenerate into plantlets (Horsch et al. 1985). Although kanamycin resistance via aminoglycoside-modifying enzymes is widespread among soil bacteria, a remote risk of horizontal gene transfer through food chains from transgenic food crops into bacteria in human and animal guts remains a great public concern (Heinemann and Traavik 2004; Levy-Booth et al. 2007; Nap et al. 1992; Pontiroli et al. 2009).

One alternative selectable marker for antibiotic resistance is the *Atwbc19* gene (*Arabidopsis thaliana* white-brown complex homolog 19 gene), a kanamycin-resistance gene encoding an ATP-binding cassette (ABC) transporter, which was discovered when its knockout mutants showed much slower root growth on medium containing kanamycin than those of other T-DNA insertional knockout mutants containing *npt*II (Mentewab and Stewart 2005). Further characterization demonstrated that *Atwbc19* can provide resistance to kanamycin at the level comparable to that conferred by the *npt*II gene when overexpressed in tobacco (Mentewab and Stewart 2005). The *Atwbc19* gene was suggested to be broadly useful for other dicot species, as well as for trees (Mentewab and Stewart 2005), yet there have been no reports in other species so far. In this report, we compared the *Atwbc19* gene with the *npt*II gene, both under control of the CaMV35S promoter in *Populus*, a forest tree which remains relatively undomesticated and has been subject to vigorous regulation and public scrutiny (Farnum et al. 2007; Heinemann and Traavik 2004; Strauss et al. 2009). Our results demonstrated that the *Atwbc19* gene confers a level of kanamycin resistance similar to the *npt*II gene. However, we also found that in *Populus*, the *Atwbc19* gene also confers resistance to three other aminoglycoside antibiotics (neomycin, geneticin, and paromomycin). Therefore, the *Atwbc19* is a useful alternative to the *npt*II gene in *Populus* transformation. This is the first confirmative report that *Atwbc19* is an effective alternative marker gene to the *npt*II gene (Mentewab and Stewart 2005).

Materials and methods

Plants

In vitro aspen shoot cultures were established from actively growing shoots of an aspen hybrid clone [*Populus canescens* × *P. grandidentata*, 36-67-S-1 (pcg)] as described by Dai et al. (2003), and maintained in a culture room at 25°C under a 16-h photoperiod by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 μmol m^{-2} s^{-1} as measured by a LI-COR LI-250 handheld light meter (LI-COR Inc., Lincoln, NE, USA) positioned at the top of the culture vessels. All explants were aseptically grown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.1% (v/v) MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), 100 mg L^{-1} myo-inositol, 3.0% (w/v) sucrose, and 0.3% (w/v) Gelrite™ (Plantmedia, Dublin, OH, USA). All media were adjusted to a pH of 5.7–5.8 prior to autoclaving at 120°C and 103.5 kPa (15 lb in^{-2}) for 20 min. All of the stock cultures in the following experiments were maintained under these conditions.

Vector and Agrobacterium strain

The vectors used in this study were pABC and pNPT, as described in Mentewab and Stewart (2005). Briefly, pABC carried the *Atwbc19* gene driven by a CaMV35S promoter while pNPT carried the *npt*II gene driven by the same promoter. Both vectors also carried a CaMV35S-driven gus gene. Each binary vector was transferred into *Agrobacterium tumefaciens* strain GV3850 by electroporation. Prior to inoculating aspen leaf segments for transformation, the *Agrobacterium* strain harboring each vector was grown overnight at 28°C to a final density of 0.8–1.0 at OD_{600}.

Transformation of aspen using the *Atwbc19* and *npt*II genes

For transformation, previously established in vitro hybrid aspen shoots were transferred from MS medium to woody plant medium (WPM) (Lloyd and McCown 1981), supplemented with 100 mg L^{-1} myo-inositol, 0.1% (v/v) MS vitamins, 3.0% (w/v) sucrose, and 0.3% (w/v) Gelrite™. Leaves harvested from in vitro-grown aspen plants were cut into 0.5 cm^2 segments. One hundred leaf segments were infected by *Agrobacterium* strain GV3850 harboring each vector by immersing them for 30 min in an *Agrobacterium* inoculum solution consisting of 5 ml Lysogeny broth (LB). After infection, the leaf segments were blotted on sterile filter paper to remove the excess bacterial inoculum and then placed on co-cultivation medium [WPM supplemented with 8.9 μM N^6 benzylaminopurine (BA), 5.37 μM α-naphthaleneacetic acid (NAA), and 100 μM acetosyringone]. After 3 days of co-cultivation in the dark, the inoculated leaf segments were washed with WPM liquid medium supplemented with 250 mg L^{-1} cefotaxime, and transferred to a callus/shoot induction medium [(CSIM) WPM supplemented with 8.9 μM BA, 5.37 μM NAA, 100 mg L^{-1} myo-inositol, 0.1% (v/v) vitamins, 3.0% (w/v) sucrose, and 0.3% Gelrite™]. The antibiotics carbenicillin and cefotaxime (each 250 mg L^{-1}) were added to eliminate *Agrobacterium* and 80 mg L^{-1} kanamycin was used for selecting transformed cells (Dai et al. 2003, 2004). The control leaf segments were treated similarly in LB broth except without *Agrobacterium*. After 3 weeks, induced calluses were transferred to fresh CSIM for shoot induction. After

5–6 weeks regenerated shoots formed, grew, and were transferred to a rooting medium [1/2 MS salts supplemented with 3.0% (w/v) sucrose, 0.3% (w/v) Gelrite™, 250 mg L^{-1} carbenicillin, 250 mg L^{-1} cefotaxime, and 100 mg L^{-1} kanamycin]. In this medium, the non-transgenic shoots were previously shown to fail to root, while the *npt*II-transgenic shoots could root (Dai et al. 2003, 2004). The shoots were later transplanted to sterilized soil after confirmation by PCR.

Plant genomic DNA isolation and PCR analysis

To confirm the presence of the transgene in plants that were kanamycin resistant, genomic DNA was isolated from 0.5 g transgenic and control plant leaf tissues using a DNeasy® Plant Maxi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. To ensure that samples were free from *Agrobacterium* contamination, each sample was taken from shoots growing on rooting medium with 100 mg L^{-1} kanamycin for 4 weeks, and samples were also grown in LB agar medium. To detect the *Atwbc19* and *npt*II genes, 50 ng genomic DNA was used for PCR-amplification by using the *Atwbc19* primers (forward 5′- GTC TTC CCG GAT TCT TCT CC-3′ and reverse 5′- TGT GCT CCT CTT GGA GTG TG -3′), and the *npt*II primers (forward 5′-GAG GCT ATT CTA TGA TG-3′ and reverse 5′-ATC GGG AGC GGC GAT ACC GTA-3′). The expected size for the *Atwbc19* gene was 472 bp and that for the *npt*II gene was 679 bp. Amplified fragments were separated on 1% agarose gels. A DNA 2-kb ladder was used as the size-marker (Fisher Scientific Inc., Pittsburgh, PA, USA).

β-glucuronidase (GUS) histochemical assay

The expression of the GUS gene in the aspen transformants was assayed by histochemical staining. The test was based on the common histochemical GUS assay suggested by Stomp (1992). Tissues were dipped into a GUS assay solution comprised of 50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-GLUC) substrate and incubated for 1 day at 37°C. The chlorophyll in the petioles and leaves was destained by rinsing with 70% ethanol twice over a 24-h period until all of the chlorophyll was removed and the blue GUS stain was clearly visible (Jefferson et al. 1987).

Reverse transcription (RT)-PCR

Total RNA was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and treated with AMPD1 DNase I (Sigma-Aldrich) to remove any traces of DNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the iScript™ select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) with oligo dT primers. Both sets of primers were the same as those used in the original PCR. The "Eleven golden rules of quantitative RT-PCR" was followed (Udvardi et al. 2008). The RT-PCR reaction was carried out for 1 h at 42°C, followed by PCR activation at 95°C for 5 min, 35 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min), and a final extension of 10 min in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Confirmation of resistance to aminoglycoside antibiotics by regeneration of adventitious shoots from leaf tissues of transgenic plants on media with kanamycin, neomycin, geneticin, and paromomycin

To confirm whether the *Atwbc19*-transgenic aspen plants were resistant to kanamycin and other aminoglycoside antibiotics, in vitro leaf explants from control plants and plants transformed with the *Atwbc19* and *npt*II genes were cultured on shoot induction medium that contained various aminoglycoside antibiotics, similar to that described by Kapaun and Cheng (1999). Several concentrations of kanamycin (0, 50, 100, 150, and 200 mg L^{-1}), neomycin (0, 50, 100, 200, and 400 mg L^{-1}), geneticin (0, 1.25, 2.5, 5.0, and 10.0 mg L^{-1}) and paromomycin (0, 50, 100, 200, and 400 mg L^{-1}) were added to the media to compare the effects of each specific antibiotic on regeneration of adventitious shoots. For each antibiotic, three replicate plates were used, each with five 0.5 cm^2 leaf segments. Each experiment was repeated three times. Regeneration frequency was defined as the percentage of leaf segments that produced shoots in each antibiotic-containing medium. The experimental design was completely randomized and the regeneration frequency was evaluated by analysis of variance using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Means separation was carried out using Duncan's multiple range test.

Results

Transformation of aspen with the *Atwbc19* and *npt*II genes

Among the 100 leaf segments that were infected with *Agrobacterium* that contained either the *Atwbc19* or the *npt*II *genes*, 60 and 63% of the leaf segments, respectively, formed calluses. Of those kanamycin-resistant calluses, 58 and 60%, respectively, regenerated adventitious shoots on

medium containing 80 mg L^{-1} kanamycin (Fig. 1a, b, respectively, and Table 1). While non-infected control leaf segments formed few calluses, when placed on medium with 80 mg L^{-1} kanamycin they did not produce shoots (data not shown). The seven *Atwbc19*- and nine *npt*II-transgenic plants (Table 1) were transferred into pots after confirmation by PCR and all transgenic plants appeared morphologically normal (Fig. 1d). There were no significant differences among 9-week-old transgenic and non-transgenic plants in plant height and number of leaves (data not shown).

Confirmation of transgenic plants by PCR, β-glucuronidase (GUS) histochemical assay, and RT-PCR analysis of transcription level of the *Atwbc19* and *npt*II genes

Both the *Atwbc19* (Fig. 2, left panel) and the *npt*II (Fig. 2, right panel) genes were PCR-amplified using their specific primer pairs, yielding the expected band sizes of 0.47 and 0.67 kb, respectively. GUS gene expression, indicated by blue staining, was detected in the aspen transformants containing the *Atwbc19* gene and the *npt*II gene. GUS expression was not detected in non-transgenic control plant tissues (Fig. 3). RT-PCR was performed on two *Atwbc19*-transgenic lines to confirm the expression of the *Atwbc19* transgene. As shown in Fig. 4, the expected 472-bp bands for the *Atwbc19* gene (left panel, lanes 3 and 4) and 679-bp bands for the *npt*II gene (right panel, lanes 5 and 6) were clearly visible. A non-transformed aspen plant sample was used as the control, which showed no amplification (both panels, lane 2). Total RNA was also screened by PCR without reverse transcriptase to ensure preparations were free of DNA contamination.

Confirmation of transgenic plants by retesting regeneration ability in media containing aminoglycoside antibiotics

Transgenic plants were retested for resistance to kanamycin and other aminoglycoside antibiotics by regeneration of adventitious shoots from leaf segments placed on aminoglycoside antibiotic-containing media. Leaf segments from *Atwbc19*- and *npt*II-transgenic plants regenerated into shoots on medium containing 100 and/or 150 mg L^{-1} kanamycin, respectively, while leaf segments of a non-transformed control plant regenerated into shoots on medium with only 50 mg L^{-1} kanamycin. Leaf segments of *Atwbc19*- and *npt*II-transgenic plants regenerated on medium that contained 200 mg L^{-1} neomycin, 5 mg L^{-1} geneticin, and 100 mg L^{-1} paromomycin, respectively, while those from a non-transformed plant regenerated into shoots on medium containing 50 mg L^{-1} neomycin, 1.25 mg L^{-1} geneticin, and 50 mg L^{-1} paromomycin, respectively. Transgenic plants with either the *Atwbc19* gene or the *npt*II gene showed comparable regeneration ability of adventitious shoots from leaf segments at similar levels of antibiotics, which was about 2–3 times the tolerable antibiotic level used for the non-transformed control plants (Fig. 5; Table 2).

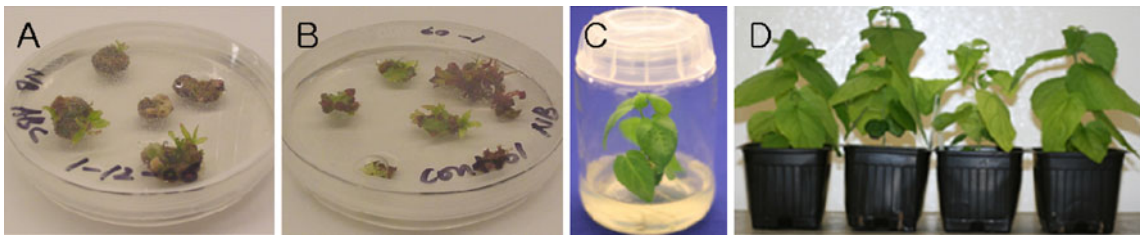

Fig. 1 Transformation of aspen with the *Atwbc19* and *npt*II genes. **a**, **b** Adventitious shoots formed from calluses that were infected with *Agrobacterium* strain GV3850 containing the *Atwbc19* and *npt*II genes, respectively, on 80 mg L^{-1} kanamycin-containing medium. **c** The *Atwbc19* transgenic plant was cultured on rooting medium (1/2 MS plus antibiotics). **d** *Atwbc19*- (*left two*) and *npt*II- (*right two*) transgenic plants in pots

Table 1 Confirmation of aspen transformation with the *Atwbc19* and *npt*II genes by antibiotic selection medium and PCR

Gene	Leaf segments with kanamycin-resistant calluses	Shoots from kanamycin-resistant calluses	Kanamycin-resistant shoots confirmed with PCR
Atwbc19	60/100 (60.0%) a	35/60 (58.3%) a	7/35 (20.0%) a
*npt*II	63/100 (63.0%) a	38/63 (60.3%) a	9/38 (23.7%) a

Control 20/100 (20.0%) b 0/20 (0.0%) b not applicable

Explants were grown on WPM supplemented with 250 mg L^{-1} carbenicillin and cefotaxime, and kanamycin (80 mg L^{-1} for callus and 100 mg L^{-1} for shoots). The same letters in different rows indicate that there is no significant difference ($p \leq 0.05$)

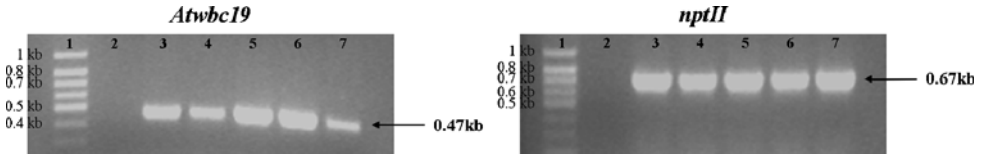

Fig. 2 PCR-amplification with the *Atwbc19*- (*left panel*) and the *npt*II- (*right panel*) specific primers (*Atwbc19*-specific primers: forward 5′-ACTGCAGGTACCATGAATC TATCACTCAGCGG-3′ and reverse 5′-TGTCCCCGTTTTTATCCAG-3′; the *npt*II-specific primers: forward 5′-GAGGCTATTCTATGATG-3′ and reverse 5′-ATCGGGAG CGGCGATACCGTA-3′ primer). Left panel: *lane 1* 2-kb marker, *lane 2* non-transgenic control plant, *lanes 3–7* five independent transgenic lines with the *Atwbc19* gene. Right panel: *lane 1* 2-kb marker, *lane 2* non-transgenic control plant, *lanes 3–7* five independent transgenic lines with the *npt*II gene

Discussion

Marker genes of bacterial origin that confer antibiotic resistance, such as *npt*II, are commonly used for plant transformation, especially for dicotyledonous species (Bevan et al. 1983; Horsch et al. 1985; Miki and McHugh 2004; Sundar and Sakthivel 2008). Although these bacterial antibiotic resistance marker genes are regarded by many regulatory agencies to be safe (Miki and McHugh 2004), there is a remote probability of horizontal gene transfer between transgenic plants and soil microorganisms or bacteria in human and animal guts that might cause unpredictable consequences to ecosystems or human health (Heinemann and Traavik 2004; Nielsen and Townsend 2004). These strong public concerns of antibiotic-resistance genes of bacterial origin in transgenic plants has prompted development of alternative means to remove them from commercial products, such as deletion of the marker gene after selection (Darbani et al. 2007; Luo et al. 2007; Ow 2007), using plant-native genes as selective marker genes (Mentewab and Stewart 2005; Rommens et al. 2004), and use of non-bacterial antibiotic-resistance genes as marker genes (Mentewab and Stewart 2005).

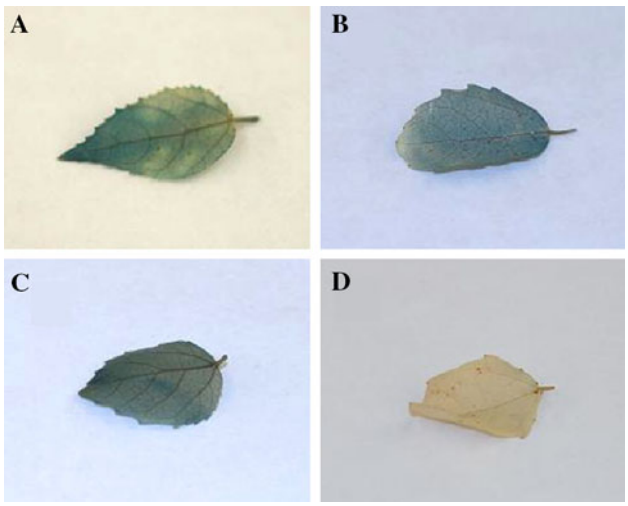

Fig. 3 Histochemical analysis of *β*-Glucuronidase (GUS) gene expression in transgenic plants. Leaves were stained with X-GLUC (**a**) and **b** transformed with *Atwbc19,* **c** transformed with *npt*II, **d** non-transgenic control plant showing absence of *blue* color

The *Atwbc19* gene is a plant-indigenous gene that confers resistance only to kanamycin in transgenic tobacco at levels similar to the bacterial *npt*II gene, but does not confer resistance to other aminoglycoside antibiotics (Mentewab and Stewart 2005). However, this gene's potential as a plant-derived antibiotic-resistance marker gene has not been explored in other plant species. Using a well-established protocol for aspen transformation (Dai et al. 2003, 2004) with *npt*II as a selective marker gene which confers resistance to aminoglycoside antibiotics, we compared the *Atwbc19* and *npt*II genes in aspen transformation. PCR-amplification, RT-PCR evaluation on gene expression, GUS staining, and shoot regeneration from leaves of transgenic plants on antibiotic-containing media together suggest that the transgenes are stably integrated into the aspen genome, expressed, and functionally conferring resistance to four aminoglycoside antibiotics (kanamycin, neomycin, geneticin, and paromomycin). The results confirmed that transformation efficiencies with the

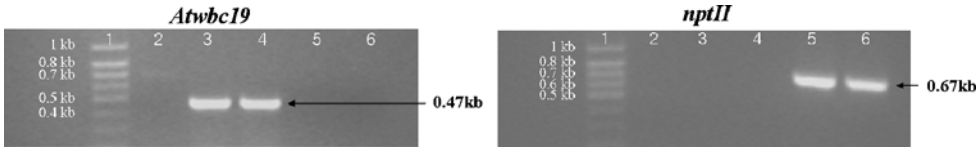

Fig. 4 Expression of the *Atwbc19* (*left panel*) gene and the *npt*II (*right panel*) gene analyzed by RT-PCR. RT-PCR analysis of selected transcripts in leaf tissues of aspen. Left panel: *lane 1* 2-kb marker, *lane 2* non-transgenic control plant, *lanes 3, 4* two independent transgenic lines with the *Atwbc19* gene, *lanes 5, 6* two independent transgenic lines with the *npt*II gene. Right panel: *lane 1* 2-kb marker, *lane 2* non-transgenic control plant, *lanes 3, 4* two independent transgenic lines with the *Atwbc19* gene, *lanes 5, 6* two independent transgenic lines with the *npt*II gene

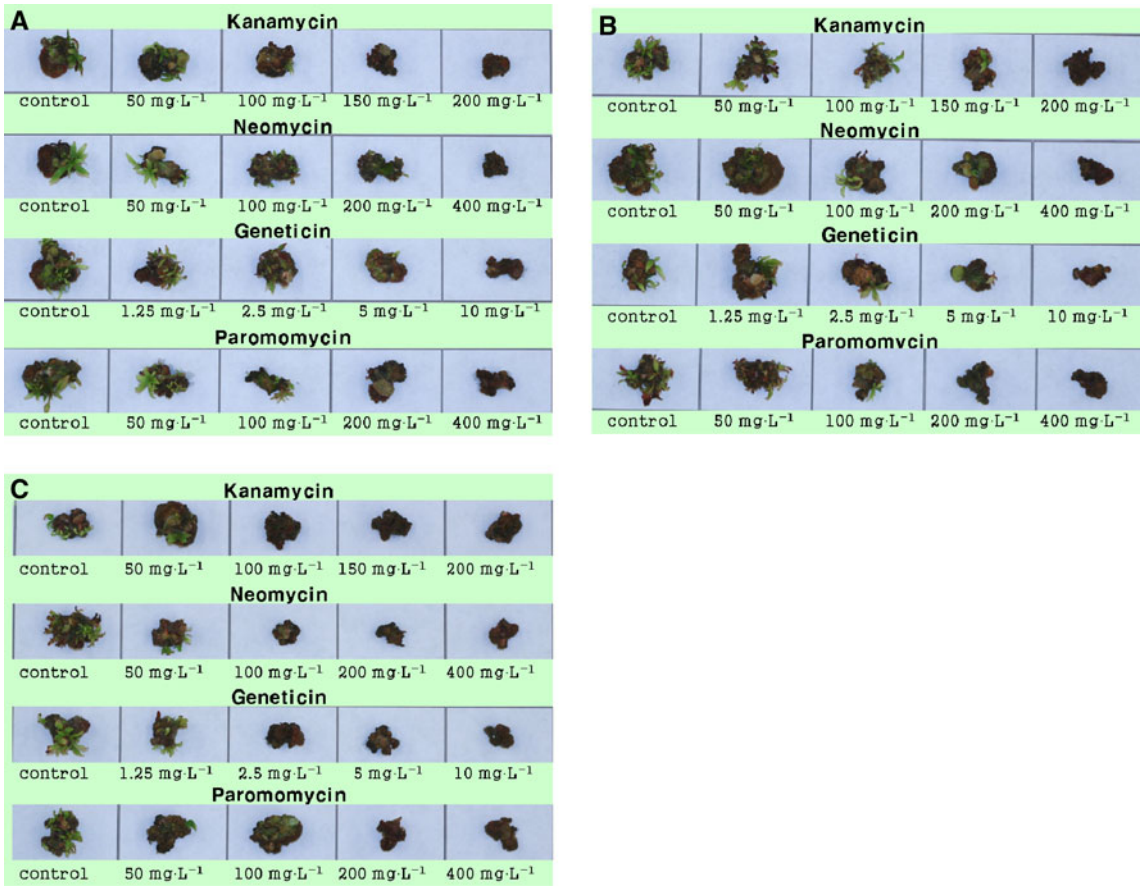

Fig. 5 Regeneration of leaf tissues of *Atwbc19*- (**a**) and *npt*II- (**b**) transgenic plants and non-transformed plants (**c**) on media containing various concentrations of four different aminoglycoside antibiotics

Atwbc19 gene were, as shown by time for callus formation, shoot formation, level of resistance to kanamycin, and normal morphologies of transgenic plants, similar to transformation efficiencies when the *npt*II gene was employed. Since *Populus* species are generally less domesticated, and gene flow is common via natural hybridization between planted and natural *Populus* stands, the release of transgenic *Populus* into the environment has been subject to vigorous regulatory review, public scrutiny, and opposition (Farnum et al. 2007; Heinemann and Traavik 2004; Strauss et al. 2009). Therefore, use of the plant-native *Atwbc19* gene as a selective marker might, at least, partially alleviate the public concerns of field deployment of transgenic *Populus*.

Unlike the *Atwbc19*-transgenic tobacco which is resistant only to kanamycin, the *Atwbc19*-transgenic *Populus* was resistant to kanamycin and three other aminoglycoside antibiotics: neomycin, geneticin, and paromomycin. Mentewab and Stewart (2005) suggested that the *Atwbc19* gene forms a homodimer, but it might also form a heteromeric complex with another half-molecule ABC transporter. Several mammalian half-molecule ABC transporters form homodimers, including the breast cancer resistance protein, the human homolog of *Atwbc19*, which confers resistance to the anticancer antracyclines and mitoxantrone (Kumie et al. 2002), but there is precedence for heterodimer formation. In *Drosophila melanogaster*, the white, scarlet, and brown proteins can form heterodimers that mediate the transport of pigment precursors into the cells responsible for eye color (Dreesen et al. 1988). Perhaps, *Atwbc19* in transgenic *Populus* plants forms a heterodimer with an endogenous half-transporter, which might account for the resistance to multiple aminoglycoside antibiotics. A priori, heterodimer formation is one mechanism to enlarge and evolve substrate specificity.

Regardless of narrow- or broad-resistance to aminoglycoside antibiotics, our research confirms that the *Atwbc19* gene is an alternative to the bacterial *npt*II gene as a plant transformation selective marker gene. The broad utility of this plant-derived antibiotic-resistance gene as a selective marker gene might be applicable in other dicotyledonous plants and coniferous trees.

Table 2 Regeneration frequency (%) from leaf tissues of *Atwbc19*- and *npt*II-transgenic plants and non-transformed control plants on media containing a range of concentrations of aminoglycoside antibiotics

	Kanamycin (mg L^{-1})				
	0	50	100	150	200
Atwbc19	84 b	65 a	36 a	0 b	0
*npt*II	91 a	67 a	40 a	18 a	0
Control	89 ab	33 b	0 b	0 b	0
	Neomycin (mg L^{-1})				
	0	50	100	200	400
Atwbc19	89 a	69 a	44 a	13 b	0
*npt*II	87 a	73 a	49 a	24 a	0
Control	91 a	27 b	0 b	0 c	0
	Geneticin (mg L^{-1})				
	0	1.25	2.5	5.0	10.0
Atwbc19	91 a	62 a	29 a	11 a	0
*npt*II	89 a	73 a	36 a	20 a	0
Control	89 a	18 b	0 b	0 b	0
	Paromomycin (mg L^{-1})				
	0	50	100	200	400
Atwbc19	89 ab	62 a	33 a	0	0
*npt*II	87 b	56 a	29 a	0	0
Control	96 a	36 b	0 b	0	0

Plants were grown on WPM supplemented with kanamycin, neomycin, geneticin or paromomycin. Cultures were maintained in a growth room at 25°C and a 16-h photoperiod where fluorescent light intensity was 125 μmol m^{-2} s^{-1}. The same letters in different rows indicate that there is no significant difference ($p \leq 0.05$)

Acknowledgments This research is supported in part by the DOE-Bioenergy Science Center grant to ZMC and The Consortium for Plant Biotechnology Research, Inc. by the Department of Energy Cooperative Agreement No. GO12026 to ZMC, and by the Tennessee Agricultural Experiment Station. The BioEnergy Science Center is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. This support does not constitute an endorsement by the DOE or by The Consortium for Plant Biotechnology Research, Inc. of the views expressed in this publication.

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