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Overexpression of an *Arabidopsis thaliana* ABC transporter confers kanamycin resistance to transgenic plants

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Selectable markers of bacterial origin such as the neomycin phosphotransferase type II gene, which can confer kanamycin resistance to transgenic plants, represent an invaluable tool for plant engineering. However, since all currently used antibiotic-resistance genes are of bacterial origin, there have been concerns about horizontal gene transfer from transgenic plants back to bacteria, which may result in antibiotic resistance. Here we characterize a plant gene, *Atwbc19*, the gene that encodes an *Arabidopsis thaliana* ATP binding cassette (ABC) transporter and confers antibiotic resistance to transgenic plants. The mechanism of resistance is novel, and the levels of resistance achieved are comparable to those attained through expression of bacterial antibiotic-resistance genes in transgenic tobacco using the CaMV 35S promoter. Because ABC transporters are endogenous to plants, the use of *Atwbc19* as a selectable marker in transgenic plants may provide a practical alternative to current bacterial marker genes in terms of the risk for horizontal transfer of resistance genes.

ABC proteins are ubiquitous proteins that share 30–40% identity between family members and are characterized by the presence of an ATP-binding cassette[1]. The vast majority of ABC-transporters are membrane bound and contain transmembrane domains (TMDs). These TMDs are thought to create a path for solute movement across the phospholipid bilayer and appear to determine, or at least contribute to, the substrate selectivity of the transporter. Resistance of human cancer cells to chemotherapeutic agents and multidrug resistance in infectious microorganisms often arises from the overexpression of ABC transporters. Their clinical significance has spurred a number of structural studies to better understand how ATP hydrolysis is coupled with a substrate-specific transport.

Among all multicellular organisms whose genomes have been sequenced so far, plants have the largest number of ABC proteins encoded in their genome. Why plants allocate proportionately more genes to this superfamily relative to the size of their genomes is not clear, but raises the question of its potential adaptive significance[2]. Unlike most animals, plants are sessile and must cope with environmental and biotic stresses and have a central vacuole for sequestration of toxins. Perhaps the plethora of ABC transporters in plants interact with the vacuole to aid in detoxification processes. *A. thaliana* ABC transporters have been classified on the basis of their domain organization and their homology to orthologous genes, but their functions remain largely unknown[3]. The AtWBC family (White-Brown Complex homologs) is the largest group of all *A. thaliana* ABC transporters, with 29 members. One recent publication has proposed that *Atwbc12* encodes a protein that secretes cuticular wax[4]. Here we show that, when overexpressed, *Atwbc19*, a 2,178-bp gene from *A. thaliana*, confers kanamycin resistance to transgenic plants.

Kanamycin is an aminoglycoside antibiotic isolated from the soil bacterium *Streptomyces kanamyceticus*. Aminoglycosides act primarily by binding to the 30S subunit of prokaryotic ribosomes and inhibiting protein synthesis[5]. In eukaryotes they also inhibit protein synthesis by binding to the ribosomal complex[6,7], hence their usefulness as selection agents for plant and mammalian genetic transformation. In bacteria, aminoglycoside resistance is most often associated with the presence of inactivating enzymes. These enzymes, classified as aminoglycoside acetyltransferases, nucleotidyltransferases or phosphotransferases, catalyze the transfer of acetyl, adenosine monophosphate or phosphate groups onto the aminoglycoside antibiotics[8]. Neomycin phosphotransferase type II (*nptII*), originally isolated from the Tn5 transposon of *Escherichia coli*, encodes a phosphotransferase. Since its first use in 1983 (refs. 9–11), it is the most commonly used selectable marker for the production of transgenic plants used in research, in commercial varieties and in US field trials (reviewed in ref. 12). The fact that kanamycin resistance via aminoglycoside-modifying enzymes is widespread among soil bacteria has contributed to the deregulation by many regulatory agencies of transgenic plants containing *nptII*.

During characterization of *Atwbc19* knockout mutants, we observed that root growth on medium containing kanamycin was much slower than that of other T-DNA insertional knockout mutants containing *nptII* (**Supplementary Notes** and **Supplementary Fig. 1** online). We hypothesized that *Atwbc19* could be involved in resistance to kanamycin and thus useful as a selection marker. Thus, we compared tobacco transformation efficiencies among three plasmids containing

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overexpressed *nptII*, *Atwbc19* and both genes together (**Fig. 1a**). Transgenic plants were recovered from each construct using selection of 50, 100 and 200 mg/l kanamycin, but the transformation efficiencies differed. A selection regime of 50 mg/l kanamycin resulted in <8% efficiency for any of the three plasmids, indicating that the selection pressure is not sufficient, at least in tobacco (**Fig. 1b**). When we used *Atwbc19* alone as the selection gene, the majority of plants recovered from medium containing 100 or 200 mg/l kanamycin were transgenic (73% and 69%, respectively). However, selection at 200 mg/l kanamycin was less effective overall because the total number of transgenic plants regenerated on a per explant basis was lower than at 100 mg/l (19% versus 40%). Therefore, it appears that 100 mg/l kanamycin is the most appropriate selection regime for tobacco using the ABC transporter under the control of the 35S promoter. Under these conditions, its efficiency is comparable to that of *nptII* driven by the double 35S promoter with a selection regime of 200 mg/l kanamycin. When both cassettes were present, the transformation efficiency was significantly higher than either of them separately using 200 mg/l kanamycin ($P < 0.05$).

All plants that appeared GUS-positive based on histochemical staining exhibited the expected *Atwbc19* gene fragment in Southern blot analysis (**Fig. 1c**), confirming that *Atwbc19* was stably integrated. Additionally, all transgenic plants appeared morphologically normal. For example, there were no significant differences among 9- week-old transgenic and nontransgenic plants in plant height and number of leaves (**Supplementary Table 1** online). No deleterious or pleiotropic effects of the *Atwbc19* gene were observed (see **Supplementary Fig. 2** online).

Segregation analysis was performed by plating seeds from self-pollinated *Atwbc19*-T_0 plants on medium containing 200 mg/l kanamycin (**Fig. 2a**). Lines segregating 3:1 were selected for northern blot analysis to examine patterns of *Atwbc19* transcription. Expression of *Atwbc19* was confirmed (**Fig. 2b**) and progeny of two transgenic events, nos. 28 and 30 were chosen for root growth assays (**Fig. 2c**). ELISA demonstrated that plants from both events contained no NPTII. As expected, progeny from both events showed a highly significant increase in kanamycin resistance compared to nontransgenic tobacco (*t*-test, $P < 0.001$). The root length was 0.37 ± 0.09 cm for control tobacco; 1.03 ± 0.55 cm for no. 28 and 2.02 ± 1.16 cm for no. 30 on medium with 200 mg/l kanamycin. Because the experiment was conducted on segregating T_1 seeds, we observed a clear difference between transgenic and nontransgenic seedlings. The nontransgenic segregants were bleached and stunted, which is characteristic of kanamycin toxicity. The segregation pattern was also clear when we examined the root length distribution frequency. A bimodal distribution was observed for the transgenic lines (see **Supplementary Fig. 3** online). Plants from these two transgenic events displayed no resistance to other aminoglycosides (amikacin, geneticin, gentamycin, streptomycin, hygromycin) or chloramphenicol, despite some structural similarities among aminoglycosides (**Fig. 3**). Thus, unlike *nptII*, which confers broad tolerance to several antibiotics, *Atwbc19* is apparently very specific to kanamycin resistance. This trait might also have biosafety significance if horizontal gene transfer were to occur.

AtWBC19 apparently possesses a predicted domain organization typical of most members of the WBC family consisting of one ABC domain and one membrane-spanning domain with six spans (**Supplementary Fig. 4** online). From our results it can be inferred that AtWBC19 forms a homodimer since its overexpression is sufficient to confer kanamycin resistance, which would be unlikely in the case of a heteromeric complex. Several mammalian half-molecule ABC transporters form homodimers, including breast cancer

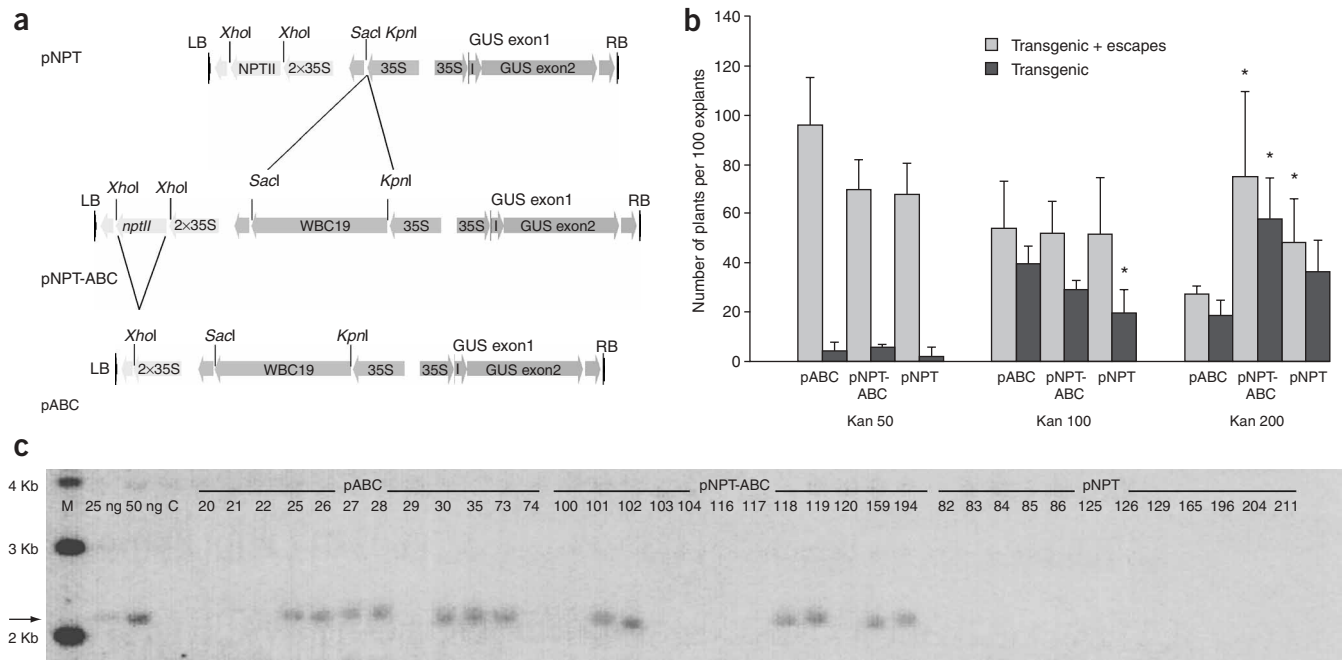

Figure 1 Use of *Atwbc19* as a selection marker. (**a**) T-DNA region of constructs used for transformation. RB, right border; LB, left border; I, castor bean catalase intron. (**b**) Comparison of tobacco transformation efficiency using the three different plasmids and three kanamycin-selection regimes. Asterisks indicate significant differences from pABC plasmid at the corresponding kanamycin-selection regime (*t*-test, $P < 0.05$). (**c**) Southern blot analysis of 12 putative T_0 plants selected with 100 mg/l kanamycin from each construct. DNA was extracted from T_0 plants and digested with *Kpn*I and *Sac*I, such that hybridizations with a ^{32}P-labeled *Atwbc19* probe would produce a 2.2-kb fragment. M, marker; 25 ng, 25 ng pNPT-ABC plasmid; 50 ng, 50 ng pNPT-ABC plasmid; C, nontransgenic tobacco control. Numbers above lanes indicate putative transgenic lines.

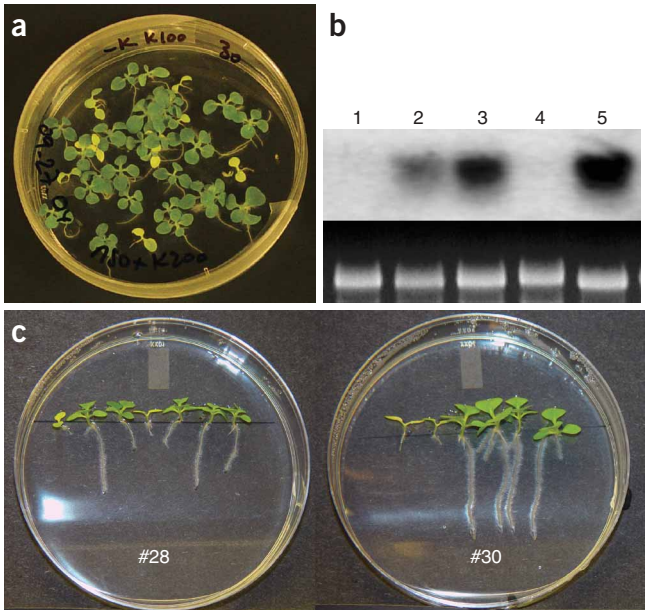

Figure 2 Kanamycin resistance of T_1 transgenic plants transformed with pABC. (**a**) Segregation of T_1 seeds from transgenic event 30 plated on medium with 200 mg/l kanamycin. (**b**) Expression of *Atwbc19* is confirmed by northern blot analysis. Lane 1: nontransgenic tobacco control; lanes 2–5 pABC transgenic events nos. 27, 28, 29, 30. Bottom: 18S ribosomal RNA after ethidium bromide staining is shown for equal loading. (**c**) Root growth of T_1 seeds from transgenic events 28 and 30 plated on medium with 200 mg/l kanamycin.

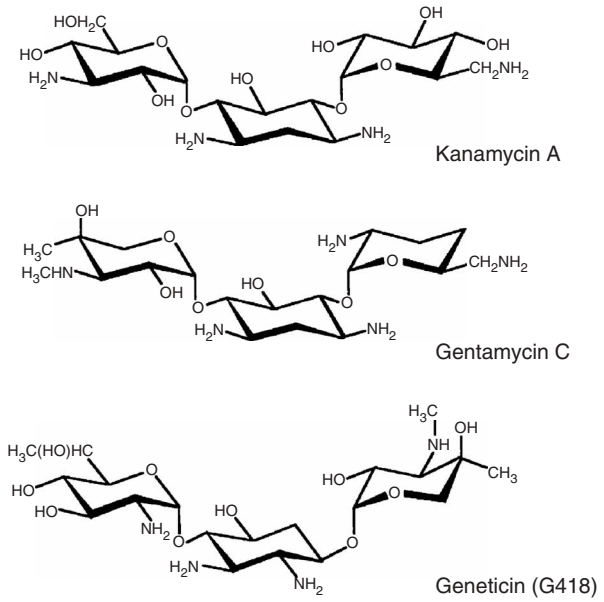

Figure 3 Chemical structures of selected aminoglycoside antibiotics.

resistance protein, the human homolog of AtWBC19, which confers resistance to the anticancer antracyclines and mitoxantrone[13]. Nonetheless it is also possible that AtWBC19 could form a heterodimer with another half-molecule ABC transporter(s) to move other substrates, as is the case with its *Drosophila melanogaster* homologs White, Scarlet and Brown proteins that mediate the transport of pigment precursors into the cells responsible for eye color. White dimerizes with Brown for the transport of guanine and with Scarlet for the transport of tryptophan[14].

To examine the subcellular localization of AtWBC19, we generated transgenic plants containing an *Atwbc19*-N-terminal *gfp* fusion construct (**Supplementary Fig. 5** online). Green fluorescent protein (GFP) accumulated in the vacuolar lumen (**Supplementary Fig. 5** online), which is at least consistent with an association with the vacuolar membrane, although delivery of the fusion protein or its degradation products into this compartment for salvage purposes cannot be excluded. Hence, our hypothesis is that kanamycin is actively sequestered in the vacuole as a substrate of this ABC transporter, where it would not interfere with ribosomal RNA in the cytoplasm, mitochondria and chloroplasts, thereby mitigate its toxicity.

Taken together these results demonstrate that *Atwbc19* is an effective selectable marker candidate as a substitute for *nptII* in the production of transgenic plants. To our knowledge, this is the first identified plant gene that confers antibiotic resistance. The overwhelming majority of selectable markers currently in use are of bacterial origin[12]. Although they are recognized as safe by regulatory agencies, concerns are constantly raised about unpredictable consequences to ecosystems or human health via horizontal gene transfer. Recent publications argue that horizontal gene transfer between transgenic plants and soil microorganisms might be underestimated and should be monitored[15,16] despite contradictory data and a priori biological hurdles for horizontal gene transfer[17]. In this light, there are at least two strategies to improve public acceptance of genetically modified plants[12,18]. The first consists of producing marker-free plants whereby the selectable marker is removed by a variety of methods, including site-specific recombination, transposon-mediated elimination and cotransformation followed by segregation. Such methods are either more difficult to implement or are less efficient than procedures that leave the marker genes in the plant[12,18]. The second, more comprehensive strategy is the use of plant genes, regulatory elements, T-DNA borders-like sequences and selectable markers originating from plants[19,20]. Effective selectable markers derived from plants are lacking apart from the herbicide-resistant forms of the genes encoding EPSP synthase and acetolactate synthase. All other current plant selectable markers are suboptimal in practice or have negative consequences. For example, the gene encoding tryptophan decarboxylase leads to tryptamine accumulation in transformed plants[21] whereas regeneration-promoting genes such as *Atipt*[22] adversely affect plant growth and development when constitutively overexpressed. Therefore they must be used with a tightly controlled inducible promoter or subsequently removed.

There are no such undesirable effects or limitations with use of *Atwbc19* as a selection marker, since it amounts to harnessing a plant's own genome for established kanamycin-resistance procedures common for many species. Here we show its usefulness as a selection marker in tobacco, but it may prove to be a valuable substitute for *nptII* in the transformation of agriculturally important dicot species such as soybean[23], cotton[24], *Brassica* crops[25] (**Supplementary Table 2** online), tomato and other *Solanaceae*[26] as well as forest tree species including angiosperms (e.g., poplar and elms) and gymnosperms (e.g., pines and spruce)[27].

METHODS

Plant transformation, segregation and growth analysis. Constructs were made in the binary vector pCambia2301 (CAMBIA). The CaMV 35S promoter and the *nos* terminator were introduced at the *Hin*dIII-*Xba*I and *Sac*I-*Eco*RI

sites to generate the empty vector pNPT. *Atwbc19* was PCR amplified from BAC clone T26I12 obtained from the *Arabidopsis* Biological Resource Center (Ohio State University). The forward (5′-ACTGCAGGTACCATGAATCTAT CACTCAGCGG-3′) and reverse (5′-TGTCCCCGTTTTTATCCAAG-3′) primers used were designed to introduce *Kpn*I and *Sac*I sites. *Atwbc19* was ligated into the appropriate location in pNPT resulting in pNPT-ABC. Plasmid pABC was generated from pNPT-ABC by removing *nptII* through vector digestion with *Xho*I and religation (**Fig. 1a**).

Agrobacterium tumefaciens strain GV3850 was used for leaf disc transformation[28] of *Nicotiana tabacum* cv Xanthi using either 50, 100 or 200 mg/l kanamycin monosulfate (Kanamycin A from Sigma) in addition to 200 mg/L timentin in the media (GlaxoSmithKline). For each selection regime and each plasmid a total of 48–56 explants were plated in three replicate experiments. The number of putative transgenic plants was recorded after transfer of rooted shoots to soil. GUS assays[29] were carried out to test for transgene integration and expression.

Segregation analysis was performed on Murashige & Skoog medium with B5 vitamins and 3% sucrose containing 200 mg/l kanamycin[30]. T_1 seeds from plants transformed with pABC were surface sterilized and germinated on the medium. Root growth analysis was performed as above on vertically positioned plates where six seeds per plate and six replicate plates for each event were germinated with and without 100 and 200 mg/l kanamycin. Root length was measured after 3 weeks. All experiments were carried out in a 25 °C growth room under constant 40 μE irradiance. Similarly, other antibiotics (all from Sigma) were tested at the following concentrations: amikacin at 100 mg/l, geneticin at 100 and 25 mg/l, gentamycin at 200 mg/l, streptomycin at 100 mg/l, hygromycin at 50 and 25 mg/l and chloramphenical at 25 and 10 mg/l.

Southern blot analysis. Genomic DNA was extracted from leaf tissue of T_0 plants and 10 μg was digested with *Kpn*I and *Sac*I to produce an expected 2.2-kb fragment in transgenic events corresponding with the coding region of *Atwbc19*. The digested DNA was separated on a 0.7% (wt/vol) agarose gel and blotted onto Hybond-Ny^+ (Amersham Pharmacia Biotech). The *Atwbc19* fragment was released from the plasmid pNPT-ABC by *Kpn*I and *Sac*I digestion, eluted from the gel and radiolabeled with [^{32}P] using random primers and RadPrime DNA labeling system under manufacturer's instructions (Invitrogen). Autoradiographic exposures were captured using a Personal FX phosphoimager (Bio-Rad).

Northern blot analysis. Transgenic events with single locus inserts (3:1 segregation; X^2 test, $P < 0.05$) were selected for northern blot analysis. RNA was extracted using the Rneasy Mini Kit and manufacturer's protocol (Qiagen). Ten micrograms of RNA was loaded in each well. After electrophoretic separation, plant RNA was transferred to a nylon membrane by capillary action and probed with *Atwbc19* as above.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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