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A Chimeric Tunicamycin Resistance Gene as a New Selectable Marker for *Arabidopsis thaliana*

Nozomu KOIZUMI*

Research and Education Center for Genetic Information, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0192, Japan
**Corresponding author* E-mail address: nkoizumi@gtc.aist-nara.ac.jp

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Abstract

Cultivation of genetically modified (GM) crops has been banned in many countries because of fears about their effects on human health and the environment. One reason cited by critics of these crops is the use of bacterial antibiotic resistance genes or herbicide resistance genes as selectable markers. To avoid using such genes, I employed the *Arabidopsis thaliana* gene encoding UDP-*N*-acetylglucosamine:dolichol phosphate *N*-acetylglucosamine-1-P transferase (GPT) as a selection marker in transformation of Arabidopsis. GPT catalyzes the initial reaction for the synthesis of asparagine-linked glycans that is inhibited by tunicamycin. Using the GPT gene in combination with tunicamycin functioned efficiently in the selection of transformed Arabidopsis. In addition, this selection was able to identify transformants at a very early stage post germination compared with the selection by kanamaycin. Application of this strategy to produce GM crops may help their acceptance by the public.

Key words: *Arabidopsis thaliana*, transgenic plants, tunicamycin, UDP-*N*-acetylglucosamine: dolichol phosphate *N*-acetylglucosamine-1-P transferase.

Abbreviations

GM, genetically modified; GPT, UDP-*N*-acetylglucosamine:dolichol phosphate *N*-acetylglucosamine-1-P transferase.

Introduction

The use of antibiotic and herbicide resistance genes as selectable markers (Bevan *et al.*, 1983; Bernasconi *et al.*, 1985; van den Elezen *et al.*, 1985; Sathasvian *et al.*, 1991) in the creation of genetically modified (GM) crops is often cited by critics of such crops as a contributing factor to their potential negative impact on human health and the environment. This unfortunate misunderstanding of the danger that the use of these genes poses has been a contributing factor to the negative public reaction to foods derived from GM crops. The negative reaction comes from the supposition that antibiotic resistance genes could be transferred to the human intestinal flora and possibly to human pathogens, and that the herbicide tolerance genes might be transferred to other plants, possibly by cross-pollination (See references for debates on GM crop issues; Poppy, 2000; Beachy *et al.*, 2001; Schubert, 2002; Thompson *et al.*, 2003; Wilkinson *et al.*, 2003). Although the possibility of such transfers is considered to be extremely low, we can nevertheless not rule out that they would never occur (Rieger *et al.*, 2002). In order to avoid such anxiety about GM crops, strategies that make GM crops marker free have been developed. However, these strategies are often time consuming and laborious.

Previously we isolated the gene that encodes UDP-*N*-acetylglucosamine (GlcNAc): dolichol phosphate GlcNAc-1-phosphate transferase (GPT), the enzyme that catalyzes the transfer of GlcNA-1-phosphate from UDP-GlcNAc to dolichol phosphate to form GlcNAC-PP-dolichol from Arabidopsis (Koizumi *et al.*, 1999). In brief, GPT catalyzes the initial step of the synthesis of the glycan core that is transferred to the asparagine residues of nascent protein synthesized in the endoplasmic reticulum. This reaction is specifically inhibited by the nucleoside antibiotic tunicamycin, which is often used experimentally for inhibition of asparagine (N)-linked glycosylation (Hori and Elbein, 1981). Since N-linked glycans play important roles in polypeptide folding, stability and activity of proteins (Helenius and Aebi, 2001),

tunicamycin treated cells are unable to synthesize N-linked glycans and unable to proliferate. If the dose of tunicamycin is high enough, the cells will die and thus lethality caused by tunicamycin is dose-dependent. When the GPT gene was overexpressed in Arabidopsis, the transformants acquired resistance to a concentration of tunicamyicn that killed the wild type seedlings (Koizumi *et al.*, 1999). This result suggested that the combination of GPT gene and tunicamycin could be used as a selection system. The present study was conducted to test this idea and to develop a new method to produce transgenic crops.

Materials and Methods

Plant materials and transformation

Arabidopsis thaliana (ecotype Columbia) was used as plant material. A chimeric construct to overexpress GPT cDNA of Arabidopsis was obtained by replacement of the GPT cDNA with the GUS gene in pBI121 as described previously. This construct was introduced into Arabidopsis with in planta transformation method using *Agrobacterium tumefaciens* (Clough and Bent, 1998). T1 seeds were selected on 1/2 MS plates (half strength of MS salt containing 0.8% agar and 1% sucrose) supplemented with 50 mg l^{-1} cefatoxin and 0.3 mg l^{-1} tunicamycin. Seedlings emerged true leaves were transferred to soil and grown in a greenhouse to harvest T2 seeds.

Sensitivity of transgenic plants against antibiotics

To examine resistance against kanamycin, T2 seeds were sown on 1/2 MS plates supplemented with 25 mg l^{-1} of kanamycin. Plants that died after germination were considered as sensitive to kanamycin and those grew with true leaves were counted as resistant. Sensitivity of seeds against tunicamycin was observed as follows. Seeds were sown on 1/2 MS plates supplemented with various concentration of tunicamycin. Seedlings emerged true leaves were decided as resistant.

Genomic Southern analysis

Genomic DNA was isolated from wild type plants and T4 transgenic plants using plant DNA isolation kit, Nucleon Phytopure (Amersham Life Scinece) according to manufacture's instruction. DNA was digested with restriction enzymes, separated on agarose gel and transferred to nylon membrane (Hybond N^+). The membrane was probed with either ^{32}P-labelled GPT cDNA or neomycin phosphotransferase II (NPTII) gene. After hybridization, membrane was washed with 0.1x SSC containing 0.1% SDS at 68 ℃, and exposed to the X-ray film.

Table 1 Segregation of kanamycin sensitivity of T2 seeds. Seeds of four independent T1 lines rescued from tunicamycin screening were sown on 1/2 MS plate with 25 mg l^{-1} kanamycin.

Line	Resistance	Sensitive	Predicted insertion
1	31	11	1
2	49	13	1
3	55	12	1
4	43	3	2

Results

Selection of transgenic plants with tunicamycin

Arabidopsis was transformed with a binary plasmid harboring the GPT cDNA driven by the CaMV 35S promoter and a neomycin phosphotransferase II (NPT II) gene that catalyzes the detoxification of kanamycin. Approximately 500 T0 seeds were sown on plates containing tunicamycin (0.3 mg l^{-1}). Although growth of most seedlings was arrested just after germination, five plants developed true leaves. These possible T1 plants were transferred to soil, and grown to harvest seeds. Since one plant set only a small amount of seeds, the subsequent experiments were conducted using the other four lines. To examine whether these lines were transformants, T2 seeds were sown on plates containing kanamycin. As indicated in **Table 1**, all four lines (line 1 to 4) showed kanamycin resistance. Judging from the segregation ratio, the transgene was inserted at one locus in the line 1, 2 and 3. Line 4 may have more than one insertion in its genome.

Inheritance of the transgene

Since insertion of the transgene at one locus was predicted, line 1 was used for subsequent analyses. Twelve T2 plants were grown and their seeds were harvested to examine segregation. By sowing T3 seeds on plates containing either tunicamycin or kanamycin, three homozygous lines were identified. The homozygous lines were designated as GPT-OX (for GPT overexpression) and further analyzed. The GPT-OX showed apparent resistance against both tunicamycin and kanamycin, indicating the transgene was inherited in a Mendelian fashion (**Fig. 1**).

To confirm that the transgene was inherited by the progeny, the T4 generation of the GPT-OX was subjected to analysis by genomic Southern. As indicated in **Fig. 2**, signals for additional copies of the GPT gene and the NPTII gene were detected in

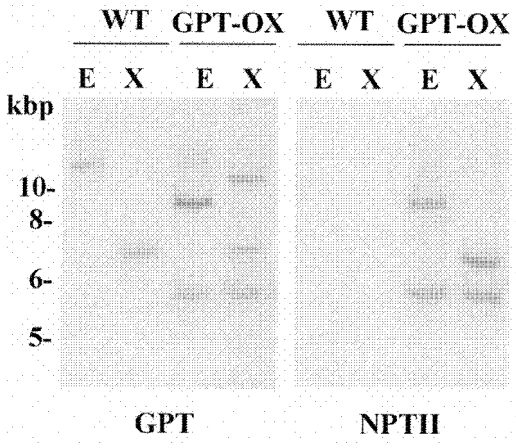

Fig. 2 Genomic Southern analysis of wild type (WT) and GPT-OX plants. Genomic DNA digested with *Eco*RI (E) or *Xba*I (X) was separated on an agarose gel and blotted The same blot was probed with GPT cDNA (left panel) or NPTII gene (right panel).

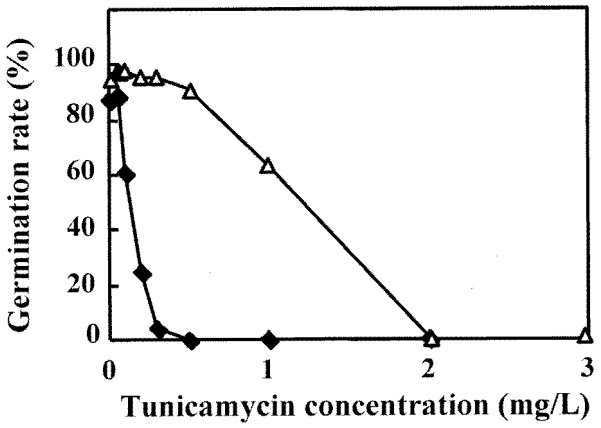

Fig. 3 Germination rate of wild type and GPT-OX plants. Approximately 100 seeds of wild type (closed square) and GPT-OX (open triangle) were sown on 1/2 MS plates supplemented with different concentrations of tunicamycin. Seedlings emerged true leaves were counted as germinated at 10 days after sowing.

the genome of GPT-OX, and these signals were absent from the wild type. In digestion with *Eco*RI, the signals for the additional GPT and NPTII genes were observed at same position on the gel since there is no *Eco*RI site between the NPTII and GPT genes in the vector construct. Two bands for NPTII and additional GPT genes indicated that GPT-OX plants contain tandem copies of transgenes, as is often observed in T-DNA mediated transformation. This result clearly indicated that the transgenes had been inherited in at least the T4 generation.

Response to antibiotics

Sensitivity to tunicamycin was compared for the wild type and the GPT-OX plants. No wild type grew after germination in the presence of 0.5 mg l^{-1} of tunicamycin, while more than 90% of the GPT-OX grew in this condition (**Fig. 3**). I_{50} for tunicamycin was ~0.15 and ~1.3 mg l^{-1} in wild type and in GPT-OX respectively. Namely GPT-OX is approximately ten times more resistant to tunicamycin than wild type.

Subsequently, the growth phenotype for tunicamycin was compared with that for kanamycin which is generally used for the selection of Arabidopsis and many other plants. To mimic selection in the T1 generation, seeds of the GPT-OX (T4) were mixed with wild type (at approximately 5%) and the mixture was sown on plates containing either tunicamycin or kanamycin. When screening was conducted with tunicamycin (0.3 mg l^{-1}), wild type did not develop cotyledons or roots, while the GPT-OX grew in the same way as the plants without antibiotics (**Fig. 4**). The transgenics could be identified a few days after germination. In contrast, on the plates containing kanamycin (25 mg l^{-1}), wild type also developed cotyledons and the difference between wild type and transgenic was not clear at this early stage. Approximately ten days after germination the cotyledons of the wild turned yellow and true leaves did not emerge, while the transgenics had green cotyledons and continued to grow.

Effects of overexpression of GPT

Since GPT-OX showed clear resistance to tunicamycin, the phenotype of GPT-OX is different from the wild type. To examine whether overexpression of GPT affected the growth or morphology of the plants, a dozen plants of wild type and GPT-OX were grown side by side and exposed to the same treatments. No apparent difference in growth or morphology was detected between the two groups. A picture of representative plants is shown in **Fig. 5**. Thus, I concluded that overexpression of GPT did not cause defects in plant growth under normal conditions.

Discussion

This study clearly indicated that the combination of GPT and tunicamycin could be used as selectable marker system in Arabidopsis. As shown in **Fig. 3**, selection at a tunicamycin concentration of 0.3-0.5 mg l^{-1} can discriminate transformants from wild type plants. The GPT-OX was approximately ten times more resistant to tunicamycin than the wild type. This difference of sensitivity against tunicamycin probably reflects the difference of GPT activity, since we previously observed approximately ten times higher GPT activity in plants that had the same construct as the one used here

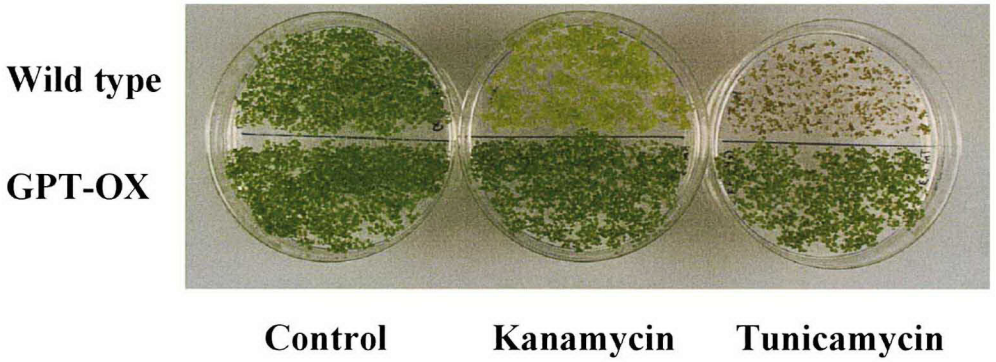

Fig. 1 Sensitivity of wild type and GPT-OX plants to kanamycin and tunicamycin. Seeds were sown on 1/2 MS plates without antibiotics (control), with 25 mg l^{-1} of kanamaycin or 0.3 mg l^{-1} of tunicaymcin. Photo was taken two weeks after sowing.

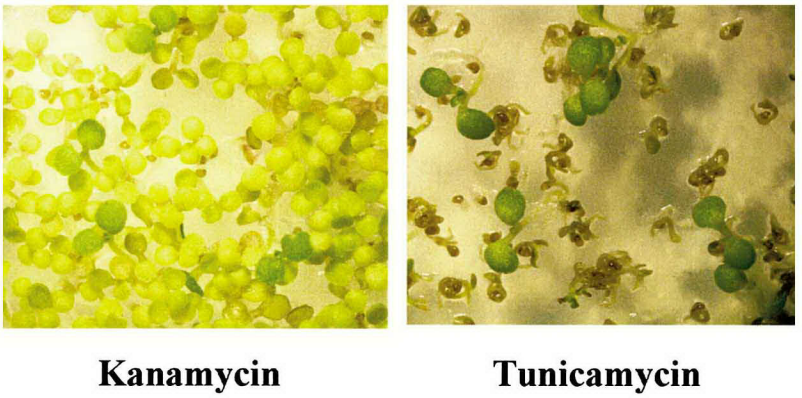

Fig. 4 Screening of a transformants. A mixture of GPT-OX (approximately 5%) and wild type seeds were sown on 1/2 MS plates with 25 mg l^{-1} of kanamycin or 0.3 mg l^{-1} of tunicamycin. Photo was taken at 10 days after sowing.

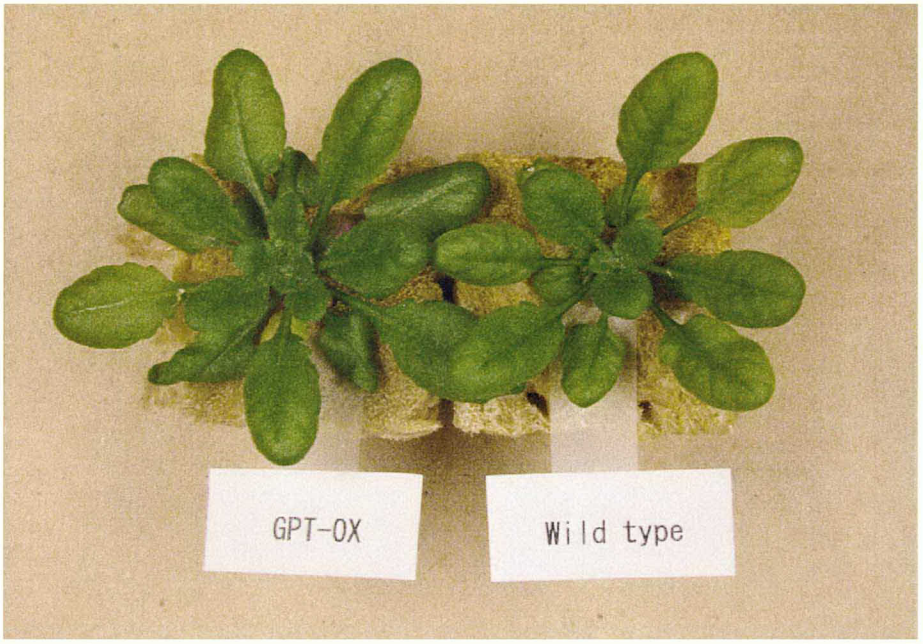

Fig. 5 Growth of wild type and GPT-OX plants. Seeds of wild type and GPT-OX were directly sown on rockwool. Plants were fertilized with Hyponex and grown in 16 h light/8 h dark condition at 21°C for 40 days.

(Koizumi *et al.*, 1999). The response of plants to antibiotics was different between tunicamycin and kanamycin. With an excess of tunicamycin, the wild type seedlings died just after germination while the transformants continued to grow. On the other hand, with kanamycin, both wild type and transformants grew similarly at first, and the difference between wild type and transformants became clear approximately ten days after germination. Thus, quicker selection was possible with tunicamycin. These results indicated that this system is useful as an additional transformation system for Arabidopsis researchers wishing to introduce multiple genes.

In addition, I would like to emphasize the potential application of this system to generate GM crops. To circumvent the negative reaction against GM crops caused by the use of selectable marker genes, there are several selection methods that do not use antibiotic resistance genes. For example, phosphomannose isomerase from *E. coli* was successfully used as a selection marker for transgenic plants (Schiermeier, 2000; Privalle, 2002). However, this method still uses a bacterial gene, which is unacceptable to some people. Technologies that remove selectable marker genes have been also developed. They use a combination of the *cre* recombinase and the *lox* sequence (Gleave *et al.*, 1999), or use intrachoromosomal recombination between attp regions (Zubko *et al.*, 2000). In addition, a system that removes a marker gene in combination with a positive marker, called MAT vectors, has been developed (Ebinuma *et al.*, 1997; Endo *et al.*, 2002). While these are useful tools to produce maker free GM crops, they all involve extra steps. Moreover, aberrations of plants with *cre* recombinase expression have been reported recently (Coppoolse *et al.*, 2003). A method that uses two separate T-DNAs to bring about co-transformation has also been developed. The selective marker genes can then be segregated out resulting in progeny will become marker free (Komari *et al.*, 1996). A strategy that does not use selection marker has been also reported. This system surveys all cells or organs by PCR (de Vetten *et al.*, 2003). All these methods are laborious and some do not answer all the critics.

In the present study, the GPT gene we used originated from the same plant species (Arabidopsis). The rapid increase in sequence information for other crops should make it possible to identify the GPT genes of other plants. Promoters suitable for use of selectable marker are also available from the homologous plants. Thus if the GPT gene is used in combination with such a promoter, one can generate GM crops that harbor only genes of the same species. Overexpression of a plant gene as a selection marker was already reported, however in this case the plants acquired herbicide tolerance and the possibility of spreading the mutant gene to wild relatives of the crop by cross-pollination is feared. Since tunicamycin is not a herbicide, this is not an 1issue here. Moreover, this system is considered to be applicable to animals, since animals also contain GPT that is inhibited by tunicamycin (Lehrman *et al.*, 1988).

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