Plant Biotechnology 22, 271–276 (2005)

Original Paper

Rice *OASA1D*, a mutant anthranilate synthase α subunit gene, is an effective selectable marker for transformation of *Arabidopsis thaliana*

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Received May 31, 2005; accepted September 1, 2005 (Edited by M. Umeda)

Abstract We have previously developed an antibiotic-free system for the selection of plant transformants that is based on a gene (*OASA1D*) for a mutant α subunit of rice anthranilate synthase. The product of this gene shows a reduced sensitivity to negative feedback regulation by tryptophan. Whereas 5-methyltryptophan (5MT) is lethal for normal plant cells because it causes tryptophan deficiency, expression of *OASA1D* confers resistance to this tryptophan analog. We used this *OASA1D*-5MT system for the transformation of *Arabidopsis thaliana*. An expression vector containing *OASA1D* under the control of the 35S promoter of cauliflower mosaic virus was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. Transgenic plants that harbored *OASA1D* exhibited resistance to 5MT but did not manifest any other differences in growth, morphology, or fertility. The *OASA1D*-5MT selection system performed as well as the *HPT*-hygromycin system for the transformation of *Arabidopsis*. Given the limited number of conventional marker genes currently available, *OASA1D* should prove to be a useful tool in *Arabidopsis* transformation, especially for the generation of plants carrying multiple transgenes.

Key words: Anthranilate synthase, *Arabidopsis thaliana*, 5-methyltryptophan, selectable marker, transgenic plant.

Arabidopsis thaliana is the most widely studied model organism in basic plant research, and an important approach to the analysis of gene function in *Arabidopsis* is the establishment of transgenic plants (Anderson and Wilson 2000; Weigel and Glazebrook 2002). The generation of transgenic plants requires the use of selectable markers that are introduced together with the exogenous gene of interest. Recent progress in plant molecular biology and genome research has led to a desire to introduce several genes into a single transgenic plant line, necessitating the development of various types of selectable marker. Among many selectable marker genes applied to plant transformation (Yoder and Goldsbrough 1994), the most popular markers are bacterial genes that confer resistance to the antibiotics kanamycin and hygromycin or to the herbicide glufosinate ammonium. Although these resistance genes —neomycin phosphotransferase (*NPTII*), hygromycin phosphotransferase (*HPT*), and phosphinotricin acetyl transferase (*PAT*), respectively (De Block et al. 1989; Fraley et al. 1983; Waldron et al. 1985)—are effective, the development of safer systems for environment that do not require the use of antibiotics or herbicides is an important goal in plant biotechnology.

Anthranilate synthase catalyzes the first reaction in the branch from the aromatic amino acid biosynthetic pathway that leads to tryptophan. Given that it is a branch-point enzyme, the regulation of anthranilate synthase is important for the control of metabolic flux in this pathway. The activity of anthranilate synthase is thus subject to feedback inhibition by the end product tryptophan (Belser et al. 1971). We previously showed that the protein encoded by a mutant form of the rice gene for the α subunit of anthranilate synthase (*OASA1*) is insensitive to such feedback inhibition (Tozawa et al. 2001). Rice cells harboring this mutant gene, designated *OASA1D* [previously referred to as the *OASA1*(*D323N*)], thus continue to synthesize tryptophan even under conditions of tryptophan excess.

The tryptophan analog 5-methyltryptophan (5MT)

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Abbreviations: CaMV, cauliflower mosaic virus; Hyg, hygromycin; 5MT, 5-methyltryptophan.

also inhibits the activity of anthranilate synthase and therefore results in tryptophan deprivation (Belser et al. 1971). The introduction of *OASA1D* into rice cells renders them resistant to 5MT (Tozawa et al. 2001), suggesting that *OASA1D* may prove useful as a selectable marker for plant transformation in combination with 5MT. Indeed, we previously showed that rice and potato cells transformed with *OASA1D* binary constructs were selected directly by growth on 5MT-containing medium (Yamada et al. 2004). This selection system does not require the use of antibiotics or herbicides and uses the *OASA1D* gene originated from rice, and thus has potential for widespread agricultural and industrial applications.

We have now demonstrated that the selection system based on rice *OASA1D* and 5MT functions in *Arabidopsis* transformation by an in planta method, suggesting that this approach will prove useful for the generation of transgenic *Arabidopsis* lines, especially for those harboring multiple transgenes.

Materials and methods

Transformation of Arabidopsis

The binary vector construct p35SASA1D, which contains a 2-kb *Spe*I-*Eco*RI blunt-ended fragment of *OASA1D* downstream of the cauliflower mosaic virus (CaMV) 35S promoter, was described previously (Yamada et al. 2004). This plasmid was introduced into the C58C1Rif^r strain of *Agrobacterium tumefaciens*, and the resulting bacteria were used to transform *Arabidopsis thaliana* ecotype Columbia by vacuum infiltration (Bechtold et al. 1993).

Screening of transformed lines and test of 5MT resistance

Seedlings were grown aseptically under continuous fluorescent illumination at 21°C on MS medium (Murashige and Skoog 1962) supplemented with Gamborg B5 vitamin mix (Gamborg et al. 1968), 2.5% sucrose, and 0.2% Gelrite (Wako, Osaka, Japan). For selection of transgenic plants, hygromycin (20 mg l^{-1}) (Roche Diagnostics, Mannheim, Germany) or 100 μM 5MT (Sigma-Aldrich, St. Louis, MO) was added to the medium.

Southern analysis

Genomic DNA was purified from seedlings of transgenic lines as described previously (Yabe et al. 1994) and then digested with *Eco*RI. The resulting DNA fragments were fractionated by agarose gel electrophoresis, transferred to a nylon membrane (Hybond-N; Amersham Biosciences, Piscataway, NJ), and probed with a 710-bp fragment of rice *OASA1* cDNA that had been amplified by PCR with the primers 5MT-1 (5′-ACCGCTGCCTCGTCAGGGAGGACG-3′) and RAS-4 (5′-CTCAAAACGCTGGCTTAAGAC-3′) and then coupled to alkaline phosphatase. Hybridization was performed with an AlkPhos Direct Labeling and Detection System (Amersham Biosciences).

Segregation analysis

In crosses, we used Landsberg *erecta* or the *ap1* mutant on the Landsberg *erecta* background as the female parent and a *35S::OASA1D* transgenic line as the male parent. Several flowers of the female parent plants were emasculated, and pollen from the male parent plants was dabbed on pistils of the female plants. Seeds were harvested and planted on solid medium containing 100 μM 5MT.

Results

Expression of OASA1D in Arabidopsis confers 5MT resistance

To investigate the effects of expression of rice *OASA1D* in *Arabidopsis*, we developed transgenic *Arabidopsis* lines that express *OASA1D* under the control of the CaMV 35S promoter. The binary vector construct p35SASA1D (Figure 1A), which also contains *HPT*, was introduced into *A. tumefaciens*, and the bacteria were then used to transform wild-type *Arabidopsis* by in planta vacuum infiltration (Bechtold et al. 1993). Seeds harvested from the *Agrobacterium*-treated plants were subjected to surface sterilization and spread on solid medium containing hygromycin (20 mg l^{-1}).

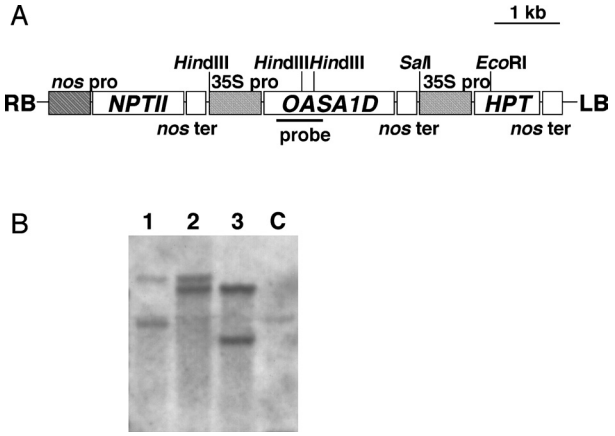

Figure 1. Structure of the expression plasmid and genomic Southern analysis of transgenic lines. (A) Structure of p35SASA1D. RB and LB indicate right and left borders, respectively; *nos* pro and ter indicate the promoter and terminator of nopaline synthase gene, respectively; 35S pro denotes the 35S promoter of cauliflower mosaic virus; NPTII and *HPT* indicate neomycin phosphotransferase gene and hygromycin phosphotransferase gene, respectively. (B) Genomic DNA (2 μg) isolated from seedlings of independent *35S::OASA1D* transgenic lines (lanes 1 to 3) or from wild-type Columbia (lane C) was digested with *Eco*RI and subjected to Southern hybridization with an *OASA1* cDNA fragment as the probe (indicated in A).

Hygromycin-resistant seedlings were cultivated and their seeds were harvested. Genomic DNA was prepared from the seedlings of transgenic lines and nontransgenic controls, digested with *Eco*RI, and subjected to Southern hybridization with a 710-bp rice *OASA1* cDNA fragment. The results confirmed that several independent transgenic lines were obtained (Figure 1B).

We next evaluated the resistance of the transgenic plants harboring *35S::OASA1D* to 5MT. Progeny of each transformant were grown on solid medium containing 5MT at various concentrations, and their viability was examined. Some of the progeny grew normally on 5MT-containing medium but others did not (data not shown). Genotype analysis of the progeny by PCR revealed that resistance to 5MT cosegregated with *35S::OASA1D* (data not shown). Transgenic plants that were homozygous for the *35S::OASA1D* transgene were grown further and their seeds were also found to be resistant to 5MT (Figure 2). No marked difference in plant growth, morphology, or fertility was detected between *OASA1D*-expressing transgenic lines and nontransformed controls. These results indicated that the introduction of *OASA1D* into *Arabidopsis* confers 5MT resistance without causing plant damage.

Selection of transformed Arabidopsis plants with 5MT

We previously showed that *OASA1D* was functional as a selectable marker in the transformation of rice or potato (Yamada et al. 2004). Calli or tissues were used for *Agrobacterium* infection in rice or potato transformation, and transformed cells were selected by cultivation on medium containing 5MT. Transgenic plants were then regenerated from the selected transformed cells. For *Arabidopsis* transformation, *Agrobacterium* infection is usually performed by an in planta method that does not require tissue culture or plant regeneration. After transformation of *Arabidopsis* by the vacuum infiltration method, transformed plants are usually selected on the basis of antibiotic resistance of seedlings. We therefore designed an experiment to investigate whether *OASA1D* is effective for selection of transformed *Arabidopsis* plants.

Given that p35SASA1D contains *HPT* in addition to *OASA1D* (Figure 1A), transgenic plants would be expected to be resistant to both hygromycin and 5MT. Ten seeds of a *35S::OASA1D* homozygous transgenic line were mixed with 120 mg (~6000 seeds) of nontransgenic Columbia seeds, the surface of the seeds was sterilized, and they were then spread on a 150-mm dish containing solid medium supplemented with either hygromycin (20 mg l^{-1}) or 100 μM 5MT. Ten seedlings grew normally on both media and all of them harbored *OASA1D* DNA (data not shown). The difference in growth between transgenic and nontransgenic plants was more obvious on the medium containing 5MT than on that containing hygromycin (Figure 3). After incubation for 2 weeks on solid medium, transgenic plants were transferred to soil. Those selected with either 5MT or hygromycin grew normally and mature seeds were harvested. These results thus showed that *OASA1D* expressed under the control of the CaMV 35S promoter is an effective selectable marker for *Arabidopsis* transformation and that selection on medium containing 5MT does not have a marked adverse effect on plant growth, morphology, or fertility.

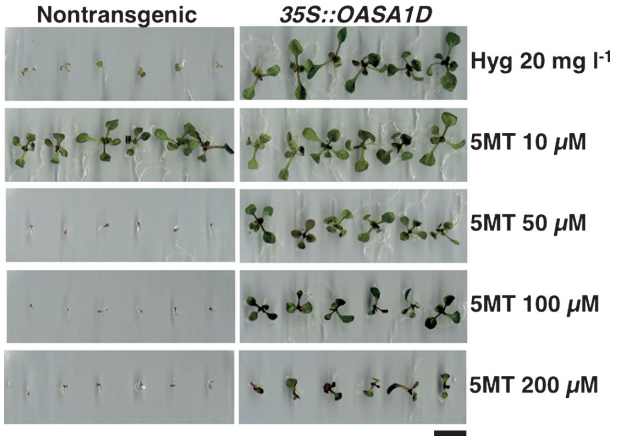

Figure 2. 5MT resistance of transgenic plants harboring *OASA1D*. Seeds of transgenic (right) or nontransgenic (left) plants were grown on solid medium containing 5MT or hygromycin (Hyg) at the indicated concentrations. Scale bar, 10 mm.

A change in the genetic background of a transgene is usually achieved by crossing transgenic lines with different strains or mutants. If transgenic plants that harbor *HPT* or other antibiotic resistance genes are used as the male parent, F_1 progeny can be readily separated from the self-progeny of the female parent because the latter is not able to grow in the presence of antibiotic. We therefore examined whether *OASA1D* is also effective as a dominant marker that allows the isolation of F_1 progeny after cross-fertilization. We used a *35S::OASA1D* transgenic line on the Columbia background as the male parent and Landsberg *erecta* as the female parent. Landsberg *erecta* is an ecotype of *Arabidopsis* that, like Columbia, has been widely used for both molecular and genetic studies (Anderson and Mulligan 1992; Putterill and Coupland 2000; Redei 1992). It harbors the recessive *erecta*(*er*) mutation and manifests an altered organ shape (Torii et al. 1996). We used Landsberg *erecta* to assess selection efficiency because its self-progeny exhibits a phenotype caused by the *er* mutation, whereas this phenotype is complemented in F_1 progeny. After crossing, seeds were collected and planted on solid medium containing 100 μM 5MT. All seedlings that grew on 5MT-containing medium showed a complemented phenotype, suggesting that they were F_1 progeny (Table 1). These

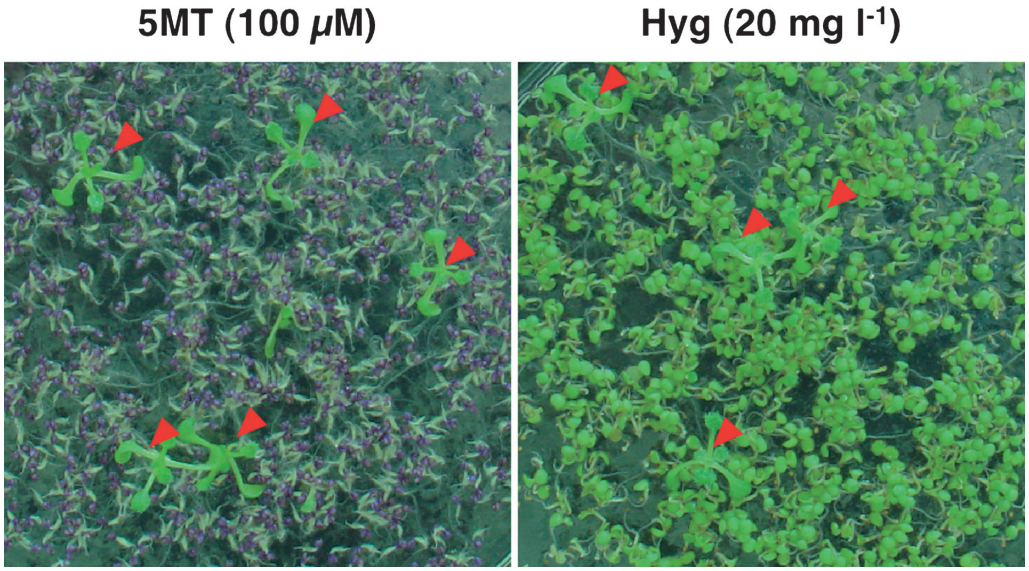

Figure 3. Screening for *OASA1D* transgenic plants on solid medium containing hygromycin or 5MT. Ten seeds of transgenic plants were mixed with 120 mg of wild-type Columbia seeds (~6000 seeds). After surface sterilization, the seeds were spread on solid medium containing either 100 μM 5MT or hygromycin (20 mg l^{-1}). Arrowheads indicate 5MT-resistant transformants (left) and hygromycin-resistant transformants (right).

Table 1. Segregation analysis of the F_1 progeny of crosses between a *35S::OASA1D* transgenic line on the Columbia (Col) background and either wild-type or *ap1* lines on the Landsberg *erecta* (L*er*) background.

	No. of progeny	$5MT^r$	*er*	*ER*	*ap1*	*AP1*
Wild type (*Ler*)×*35S::OASA1D* (Col)						
Experiment 1	26	3	0	3		
Experiment 2	80	7	0	7		
Experiment 3	29	6	0	6		
Experiment 4	57	34	0	34		
ap1 (*Ler*)×*35S::OASA1D* (Col)						
Experiment 1	>200	50	0	50	0	50
Experiment 2	62	3	0	3	0	3
Experiment 3	>200	87	0	87	0	87

$5MT^r$, 5MT resistant

results thus showed that the *OASA1D*-5MT system is effective for the selection of F_1 progeny.

We also crossed a *35S::OASA1D* transgenic line with the mutant line *apetala1* (*ap1*) on the Landsberg *erecta* background. The *ap1* mutant is characterized by the conversion of sepals in the first whorl into leaf-like organs, which often subtend secondary flowers in their axils (Irish and Sussex 1990; Mandel et al. 1992). None of the resulting 5MT-resistant seedlings exhibited the *ap1* mutant phenotype (Table 1), indicating that we had completely eliminated contamination with self-progeny. Together, these results thus indicate that *OASA1D* will likely prove a useful marker for selection of F_1 progeny in *Arabidopsis*.

Discussion

We have generated *Arabidopsis* plants that harbor the *35S::OASA1D* transgene and demonstrated that rice *OASA1D* is effective as a selectable marker for *Arabidopsis*. We compared the efficiency of transformant selection based on either *OASA1D* or *HPT*. Resistant seedlings were selected successfully with both systems, and we did not detect any difference in efficiency between the two markers. Our results thus indicate that the combination of *OASA1D* and 5MT is just as effective as is that of *HPT* and hygromycin. The *OASA1D*-5MT system also has a practical advantage over other systems based on the use of expensive chemicals in that 5MT is available from various suppliers at a cost similar to that of hygromycin. In addition, we found that transformants were more readily distinguishable from nontransformants during selection with *OASA1D* than they were during selection with *HPT*. Nontransformants grew normally until the cotyledons opened during selection with *HPT*, whereas they did not reach this stage of development during selection with *OASA1D* on medium containing 100 μM 5MT. The ability to detect the difference between transformed and nontransformed plants at an earlier stage of germination will likely prove an

important advantage of the *OASA1D*-5MT system in that it should allow selection at high seed density within a short period.

Expression of rice *OASA1D* in *Arabidopsis* conferred 5MT resistance without affecting plant growth. This observation suggests that the product of the exogenous gene functions in *Arabidopsis* as an α subunit of anthranilate synthase in the presence of 5MT, whereas 5MT is toxic to wild-type *Arabidopsis*. *Arabidopsis* possesses two endogenous genes, *ASA1* and *ASA2*, that encode α subunits of anthranilate synthase (Niyogi and Fink 1992). The activity of these endogenous proteins is inhibited in the presence of 5MT, which prevents the biosynthesis of sufficient tryptophan for growth. The *trp5* mutant of *Arabidopsis* was isolated by selection for resistance to the herbicide 6-methylanthranilate and is also resistant to 5MT (Li and Last 1996). The *trp5-1* mutant harbors a single amino acid substitution in ASA1 that is identical to that present in rice *OASA1D*. Although the amino acid sequences of rice and *Arabidopsis* ASA1 proteins are highly conserved (74% identity), rice OASA1 is actually more similar to *Arabidopsis* ASA2 in terms of its pattern of gene expression (Tozawa et al. 2001).

We have demonstrated that *OASA1D* is an effective selectable marker for *Arabidopsis* as well as for rice and potato (Yamada et al. 2004), indicating that the *OASA1D*-5MT system is applicable to wide variety of plant species and for selection procedures with various plant materials. Recently, it was also reported that the feedback-insensitive tobacco *ASA2* gene was available as a selectable marker for legume hairy root transformation (Cho et al. 2004). Although our present system will likely prove useful for transformation with various types of genes in *Arabidopsis*, it is possible that it may not be suitable for some genes, such as those associated with amino acid metabolism, given that expression of *OASA1D* may influence the concentrations of tryptophan and related compounds. Expression of *OASA1D* in rice results in accumulation of tryptophan as a result of the fact that the encoded protein is insensitive to feedback inhibition by this amino acid (Tozawa et al. 2001; Yamada et al. 2004). We also detected an increase in the tryptophan content in *35S::OASA1D* transformants of *Arabidopsis*, but the level of tryptophan accumulation was lower than that observed in rice transformants (data not shown). Tryptophan content also varied among transgenic *Arabidopsis* lines, but all lines exhibited normal growth and fertility. The accumulation of tryptophan induced by *OASA1D* in some *Arabidopsis* lines did not affect the effectiveness of the gene as a selectable marker. It is feasible that replacement of the 35S promoter used to control the expression of *OASA1D* in the present study by a promoter that is active only under specific conditions might reduce the extent of tryptophan accumulation.

The analysis of gene function *in vivo* is likely to be facilitated by the ability to generate transgenic plants that harbor two or more transgenes. The availability of several practical markers for the selection of transgenic plants is therefore desirable. For example, it will be easier to generate transgenic plants that carry a test gene and a reporter gene with the use of two marker genes than it will with a single marker. Given that the number of conventional marker genes is limited at present, rice *OASA1D* represents an attractive additional choice.

Acknowledgments

We thank J. G. Dubouzet, A. Komatsu, and K. Toda for helpful discussions. This work was supported by a project grant from the Ministry of Agriculture, Forestry and Fisheries of Japan for the Development of the Next Generation Recombinant DNA Techniques as well as by Core Research for Evolutional Science and Technology/Japan Science and Technology Agency.

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