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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Arabitol dehydrogenase as a selectable marker for rice

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Abstract Arabitol dehydrogenase has been adapted for use as a plant selectable marker. Arabitol is a five-carbon sugar alcohol that can be used by *E. coli* strain C, but not by the laboratory K12 strains. The enzyme converts the non-plant-metabolizable sugar arabitol into xylulose, which is metabolized by plant cells. Rice was transformed with a plant-expression-optimized synthetic gene using Biolistic-mediated transformation. Selection on 2.75% arabitol and 0.25% sucrose yielded a transformation efficiency (9.3%) equal to that obtained with hygromycin (9.2%). Molecular analyses showed that the *atl*D gene was integrated into the rice genome of selected plants and was inherited in a Mendelian manner. This study indicates that arabitol could serve as an effective means of plant selection.

Keywords Transformation · Non-antibiotic selection · Positive selection · *Oryza sativa* L.

Introduction

In developing transgenic plants, selectable markers introduced along with the transgene are used to screen for transgenic cells. An extensive review of selectable marker genes employed in plant research has recently been published (Miki and McHugh 2004). There are currently two main selection systems: positive and negative. A selection system based on the use of D-amino acid oxidase is unique in that both positive and negative selection is possible, depending on the substrate employed (Erikson et al. 2004).

Negative selection kills the cells which do not contain the introduced DNA, and includes antibiotic- and herbicide-based selection. Plant cells dying from antibiotic toxicity release growth inhibitors and toxins which are thought to negatively affect transformed cells and hinder their growth (Haldrup et al. 1998a), thus limiting the transformation efficiency of negative systems. The commonly used antibiotic selective agents, kanamycin and hygromycin, cause genome-wide alterations in DNA methylation (Schmitt et al. 1997). This non-reversible phenomenon leads to gene silencing and is thought to hinder both the selection of transgenic cells and the plant regeneration process. Changes in methylation can be dosage-dependent and lead to sequence mutation (Bardini et al. 2003).

Socio-political issues also affect implementation of selection systems. The European Union has enacted a ban on antibiotic resistance genes for the selection of transgenic plant cells effective at the end of 2004, and thus any future genetically enhanced plants and food products sold in the EU will have to contain alternative selectable markers or have the markers removed (European Parliament 2001). If herbicide resistance is used as a selectable marker, there is always a possibility that resistance might transfer to weedy relatives of crops via outcrossing (Rieger et al. 1999).

In contrast, positive selection gives transformed cells the ability to grow using a specific carbon, nitrogen or growth regulator as the selection agent (Joersbo and Okkels 1996; Bojsen et al. 1998; Haldrup et al. 1998a). Different *E. coli* strains can use a multitude of carbohydrate sources, due to a series of operons that can be located within the *E. coli* genome (Reiner 1975). One of these, in particular, is the ability of *E. coli* strain C, but not the laboratory K12 strains, to grow on D-arabitol (Reiner 1975; Scangos and Reiner 1978). Since most plants cannot metabolize most sugar alcohols, including D-arabitol (Stein et al. 1997), there is an opportunity to develop positive selection systems based on sugar alcohols. The *dal* operon (GenBank AF045245) has been cloned and sequenced from *Klebsiella pneumoniae,* and includes a transporter

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(*dal*T), kinase (*dal*K), dehydrogenase (*dal*D) and a repressor (*dal*R) (Heuel et al. 1997). However, for use as a plant marker, the gene from *E. coli* strain C is preferable, as the use of a gene from a human pathogen (*K. pneumoniae)* can aggravate issues of social acceptance. In *E. coli* strain C, the arabitol genes are located in the *atl* operon and include *atl*T, *atl*D, *atl*K, and *atl*R (Reiner 1975; Heuel et al. 1998). *Atl*D encodes D-arabitol dehydrogenase (EC 1.1.1.11) which converts arabitol into xylulose. Xylulose is an intermediate of the oxidative pentose phosphate pathway (Kruger and von Schaewen 2003). Plants can grow on D-xylulose (Haldrup et al. 1998b), so if a plant cell could express arabitol dehydrogenase, then such a cell would be able to grow in a medium containing D-arabitol, whereas an untransformed plant cell would not proliferate.

Here we describe the potential of using arabitol dehydrogenase from the non-virulent enteric bacterium, *E. coli* strain C, as a plant selectable marker.

Materials and methods

Cloning and characterization of the *E. coli atl*D gene

Genomic DNA of *E. coli* strain C (LaFayette and Parrott 2001) was digested with *Pst*I, ligated to *Pst*I-digested pBluescript and used to transform *E. coli* DH10B. Each transformed culture was placed into 25 ml of D-arabitol-supplemented 2B minimal medium (LaFayette and Parrott 2001). *E. coli* transformed with a plasmid containing the arabitol operon can replicate in the arabitol medium, whereas those not containing the operon do not replicate. After noticeable growth was observed, a 1:100 dilution into fresh arabitol-modified 2B medium was performed and repeated. The bacteria were streaked onto 2B arabitol plates to obtain single colonies containing the arabitol operon insert. Plasmids were isolated using the Quantum Prep® Plasmid Miniprep Kit (www.biorad.com) using the manufacturer's protocol. *Pst*I digestion and subsequent gel electrophoresis revealed three *Pst*I fragments within the clone. These were subcloned individually into *Pst*I -digested pBluescript™ and transformed into *E. coli* DH5α™ (www.invitrogen.com), but none were able to grow in 2B arabitol medium, indicating none had an intact *atl* operon. The largest fragment, approximately 4.5 kb, was sequenced via the EZ::TN™ ⟨KAN-2⟩ Insertion Kit using the manufacturer's suggested protocols (www.epicentre.com). The sequence of the dehydrogenase, *atl*D, was assembled using GeneRunner 3.00 (Hastings Software, Inc., Hastings-on-Hudson, NY) after comparing sequence runs with the sequence of the arabitol operon from *K. pneumoniae* via BLAST analysis (Altschul et al. 1997). Primers AtlD-F 5′-GAGAACGAAACAATGAACG-3′) and AtlD-R (5′-GATACATAACCGCCTCCTG-3′) were constructed from the 5′ and 3′ ends outside of the coding region, and used to amplify *atl*D via PCR using *Pwo* polymerase (www.roche-applied-science.com) according to the manufacturer's suggested protocol. This fragment was cloned into the *Eco*RV site of pBluescript KS+ (www.stratagene.com) forming pAtlD and sequenced. Subsequently, the entire arabitol operon was sequenced using primer walking from the plasmids obtained from *E. coli* DH10B™ cells able to grow on D-arabitol. The sequence was deposited in GenBank as accession number AF378082.

Gene constructs used for transformation

A synthetic *atl*D sequence was designed by GeneArt (www.geneart.com) using their GeneOptimizer™ software to optimize expression of *atl*D in rice and soybean and to remove cis-active DNA motifs and cloned to create pAtlD-S. *Atl*D-S was excised from pAtlD-S by digestion with *Xho*I and *Sma*I and ligated to the *Xho*I and *Stu*I site of pMECA (Thomson and Parrott 1998) creating pMAtlD. *Atl*D-S was excised from pMAtlD by digestion with *Xho*I and *Sal*I and ligated between the *Xho*I sites of pCAMBIA 1305.2 (GenBank AF354046, www.cambia.org), replacing the hygromycin resistance gene to create pAS1305.2 (Fig. 1). Vector pCAMBIA 1305.2 contains an improved version of the GUS gene, GUS*Plus*™, that is fused to a signal sequence for secretion in plant cells, and was used as the plasmid for the hygromycin selection studies.

Digestibility of the ATLD protein

Vector pAtlD was modified by PCR to have a *Nde*I site upstream of the *Atl*D start codon. *Atl*D was inserted as a translational fusion into pET15b (Novagen, www.emdbiosciences.com) using *Nde*I-*Bam*HI sites to create pET15AtlD. BL21(DE3) cells (www.Novagen.com) were transformed with pETAtlD and induced to express the recombinant His-tagged AtlD protein according to

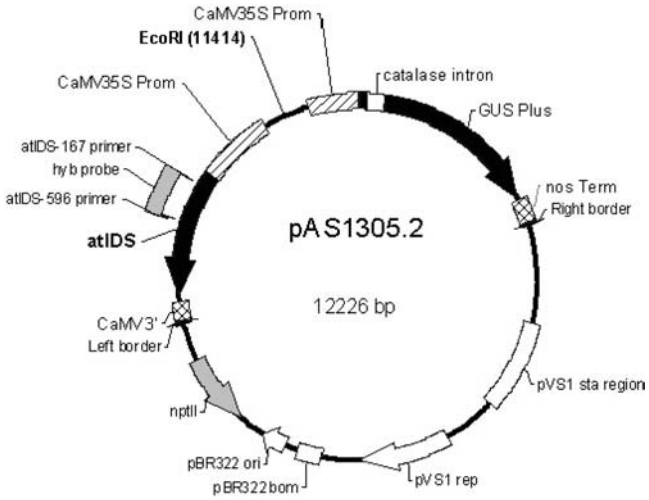

Fig. 1 Schematic diagram of the arabitol dehydrogenase plant transformation vector pAS1305.2. The *B*-glucuronidase (GUS Plus) and the synthetic arabitol dehydrogenase (atlD-S) genes are under the control of the CaMV 35S promoter. The locations of the oligonucleotide primers used for PCR and the unique *Eco*RI restriction site used for Southern analysis are indicated

the manufacturer's protocol (Novagen). Attempts to purify AtlD from the bacterium host proteins were unsuccessful. Therefore, in vitro digestion of the cleared bacterial lysate was performed using simulated gastric fluid (SGF; 0.32% pepsin, 0.2% (w/v) NaCl, 0.7% (v/v) HCl, pH 1.2 and simulated intestinal fluid (SIG; 1% pancreatin, 0.68% KH_2PO_4, pH 7.5 (US Pharmacopia 1990). Digestion reactions were prepared and the resulting polypeptides visualized with Coomassie blue staining of SDS-PAGE gels.

Plant material

Rice, *Oryza sativa* L. japonica cv TN67, was used for these studies. Embryogenic calli from mature seeds were initiated as described by (Zhang et al. 1996) on medium hardened with 3 g l^{-1} of GelRite (www.sigma-aldrich.com).

Determination of arabitol concentration for selection

Non-transformed embryogenic rice calli were plated on a modified NB medium (Chen et al. 1998) containing different levels of arabitol and sucrose as listed in Fig. 2, and the number of calli clusters showing growth was determined. Twenty four embryo clusters (0.3–0.4 cm in diameter) were placed per plate, and three plates were used for each arabitol/sucrose concentration. Cultures were maintained in the dark at 27°C.

Microprojectile bombardment

Transformation parameters were according to (Chen et al. 1998). Calli were bombarded using the PDS-1000/He system (www.biorad.com) with 2.5 mg gold particles of 0.6 m diameter coated with 10 µl of plasmid DNA (80 ng μl^{-1}) at 7,584 kPa (1,100 psi) helium gas pressure under 91 kPa (27 in) of Hg vacuum, at a shooting distance of 13 cm, as described by Hazel et al. (1998). Each plate was shot once, using a randomized block design with three replicates.

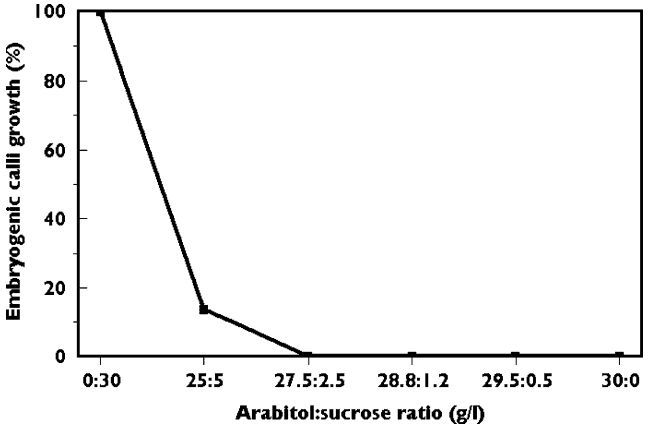

Fig. 2 Growth response of embryogenic rice calli on medium containing various concentrations of arabitol and sucrose. The 27.5 g l^{-1} arabitol with 2.5 g l^{-1} sucrose mix was selected for transformation

Selection and regeneration of transformants

Bombarded calli were cultured for 1 week in the dark at 27°C in the absence of selection and then transferred (approximately 65 colonies of 0.3–0.4 cm diameter per plate) onto selection medium containing either 27.5 g l^{-1} arabitol and 2.5 g l^{-1} sucrose, or 50 mg l^{-1} hygromycin B and 30 g l^{-1} sucrose. After 3–4 weeks, visibly growing calli were moved to fresh selection medium. After approximately 12 weeks, resistant calli (approximately >0.8 cm in diameter) were transferred to regeneration medium as described by Chen et al. (1998). Recovery of transformed callus and plants was also according to Chen et al. (1998). Cultures were maintained under a 23/1 h (light/dark) photoperiod with a photon flux of approximately 66–95 µmol m^{-2} s^{-1} at 26°C. Regenerated plantlets were transferred to medium containing half-strength MS salts and vitamins, 15 g l^{-1} sucrose and 3 g l^{-1} GelRite to allow for root development. Rooted plants were potted into soil and grown to maturity in a greenhouse.

Expression of the GUS*Plus* gene in transgenic plants

Histochemical assays to assess expression of the gus gene in rice tissues were performed by staining with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide acid as described by Jefferson et al. (1987).

DNA isolation, PCR and Southern blot analyses

Genomic DNA was extracted from leaves of soil-grown plants using a modified CTAB-protocol (Murray and Thompson 1980). PCR was carried out in 25-µl reaction volume using: 1×PCR buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, DNA (50 ng genomic or 1 ng plasmid), 0.25 µM each oligonucleotide primer and 1 U *Taq* polymerase (www.PerkinElmer.com). Primers specific for *atl*D were atlDS-167 (5′-GGTGCAAGCCCTCTCC 3′) and atlDS-596 (5′-CAGGTGGTGTTG GTGGAG -3′). Thermal cycling consisted of one cycle at 94°C for 4 min; 30 cycles at 94°C for 45 s, 60°C for 60 s, 72°C for 60 s; a final extension at 72°C for 7 min.

To check for nuclear integration of atlDS, 5 µg of genomic DNA from transformed and control plants were digested with *Eco*RI (www.invitrogen.com) and used for Southern blotting according to a standard protocol (Sambrook et al. 1989). The probe was obtained by PCR amplification of atlD-S using primers atlDS-167 and atlDS-596 and random-primed with αP^{32} dCTP using the RediPrime Labeling kit (www.amersham.com).

Germination of progeny on arabitol-containing medium

T_1 seeds from self-pollinated transformed and non-transformed rice plants were dehulled and surface-sterilized. These were germinated on half-strength basal MS medium supplemented with 20 g l^{-1} arabitol and 3 g l^{-1} GelRite. Seeds were incubated under conditions as described before for plant regeneration. After 20 days, the number of resistant and sensitive seedlings was determined. In addition, the segregation of the *atl*D-S transgene was determined in T_1 seeds and plants by PCR using the primers atlDS-167 and atlDS-596.

Results and discussion

Gene characterization

Analysis of the putative sequence for *atl*D found two possible start codons, one upstream from the start codon for *dal*D. The most suitable start codon for plant genes was determined using NetStart 1.0 software (Pederson and Nielsen 1997). The program gives a score to each "ATG" site for its potential as a plant gene start codon. Accordingly, the second "ATG" (homologous to the start codon in *dal*D) was determined to be the most suitable start codon for a plant gene, generating a score of 0.764 compared to 0.388 for the upstream methionine. The gene was annotated as such when deposited to GenBank (AF359520).

A BLAST analysis was performed between the genes of the arabitol operon (*atl*) of *E. coli* and the arabinitol operon (*dal*) of *K. pneumoniae*. Overall, the operons are 75% identical at the DNA level. The repressor, *atl*R, is 75% identical at the DNA level to *dal*R and 82% identical at the protein level. The dehydrogenase, *atl*D, is 77% identical to *dal*D at the DNA level and 84% identical at the protein level. The xylulose kinase, *atl*K, is 75% identical to *dal*K at the DNA level and 83% at the protein level. Finally, the transporter gene, *atl*T, is 77% identical to *dal*T at the DNA level and 84% at the protein level.

Putative intron splice sites were discovered within the sequence of atlD using NetGene 2.0 (Hebsgaard et al. 1996). In addition, ATTA motifs, which are associated with mRNA instability (Perlak et al. 1991) were present. The synthetic gene, *atl*D-S, had 353 bases changed to avoid AT-rich or GC-rich sequence stretches, repeat sequences as well as RNA secondary structures, and potential cryptic intron splice sites, without changing the translated protein sequence. The plant-optimized sequence has been deposited into GenBank as accession number AY863020.

Expression of a novel protein in the consumable organs of transgenic plants requires an assessment of the potential allergenicity of that protein. The *atl*D translated protein sequence was compared to amino acid sequences of allergenic proteins at www.allermatch.org (Fiers et al. 2004). AtlD did not have a match above the default setting of 35% identity with allergenic proteins in the database, using a sliding window of 80 amino acid length for comparison.

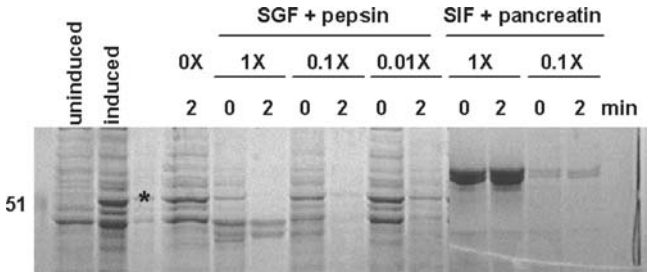

Fig. 3 Digestion of AtlD (approximately 51,000 MW in cleared bacterial lysate) in simulated mammalian gastric fluid (SGF) prepared at various concentrations of pepsin and simulated mammalian intestinal fluid containing pancreatin (SIF). SGF was used at 1x, 0.1x and 0.01x the standard concentration of pepsin. SGF prepared without pepsin was denoted as 0x. SIF was used at 1x and 0.1x the standard concentration of pancreatin. ∗Position of AtlD in the induced bacterial culture

The Allermatch program also searches for short identical stretches of six contiguous amino acids that may be epitopes for IgE antibodies. Although AtlD did show a single six amino acid match to each of two allergens, the presence of a single IgE epitope does not make a protein an allergen, as two IgE binding epitopes are required to induce a mast cell reaction (Kleter and Peijnenburg 2002).

Potential allergenicity of a novel food protein can also be assessed by in vitro digestibility experiments. Resistance to proteolytic degradation is a characteristic of allergenic food proteins. Expression of *atl*D in *E. coli* is shown in induced cultures (Fig. 3). AtlD was completely degraded within 2 min of incubation in 1× SGF and also rapidly degraded in SIF (Fig. 3). These data indicate that AtlD would likely be digested under typical mammalian gastric conditions. Considering the lack of identity with known allergens, together with the rapid digestion, the initial indications is that *atl*D has a very low probability of being an allergen.

Use of AtlD-S for transgenic rice selection

The success of the system hinges on the inability of non-transgenic plant cells to use arabitol. As seen in Fig. 2, callus growth was not supported at a combination of 27.5 g l^{-1} arabitol and 2.5 g l^{-1} sucrose. This ratio of carbon sources was chosen for the selection of transformed rice. After 8 weeks on arabitol selection, resistant calli remained white and increased in size, while non-transformed calli did not grow and eventually died, presumably from carbohydrate deprivation (Fig. 4). A similar time frame was seen using hygromycin as the selective agent.

The transgenic nature of live callus clusters was verified using GUS histochemical staining and PCR. The results are summarized in Table 1 and show that from 259 calli bombarded, arabitol selection yielded a 9.2±1.3% transformation frequency. This frequency was comparable to that obtained from hygromycin selection, which yielded a 9.3±3.8% efficiency from 268 calli bombarded. All calli selected on arabitol regenerated and five lines were further grown for in-depth studies.

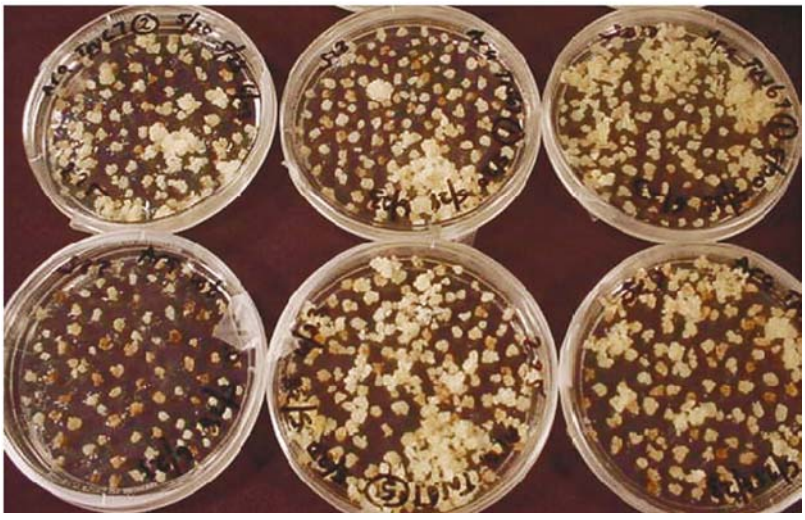

Fig. 4 Selection of embryogenic rice calli after 8 weeks on medium containing 27.5 g l^{-1} arabitol and 2.5 g l^{-1} sucrose. A plate containing non-transformed control tissue is shown at the lower left

Table 1 Comparison of transformation frequency in rice using arabitol and hygromycin as the selective agent. Analysis of variance revealed no significant differences (p=0.05) due to selection agent

Selection agent	Number of calli bombarded	Transformation efficiency[c] (%± SE)
Arabitol[a]	259	9.2 ± 1.3
Hygromycin[b]	268	9.3 ± 3.8

[a]Average of three replicates, each with approximately 86 embryogenic calli

[b]Average of three replicates, each with approximately 89 embryogenic calli

[c]Percentage of calli positive for GUS expression 10–12 weeks post bombardment

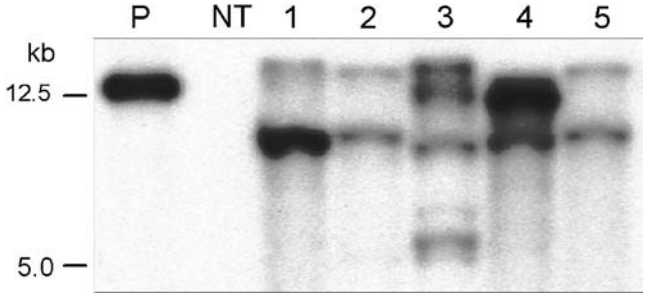

Fig. 5 Southern blot of transgenic rice plants containing the synthetic arabitol dehydrogenase gene. *Lanes*: (P) pAS1305.2 plasmid cut with *Eco*RI; (NT) non-transformed rice; (1–5) transgenic plants 1–5. Plants 1 and 2, 3 and 4, and 5 were obtained from calli on separately bombarded plates. Genomic DNA was digested with *Eco*RI, which has a single recognition site in pAS1305.2, and hybridized with an *atl*D-S probe depicted in Fig. 1

Southern blot analysis of five independent GUS-positive lines indicated that the *atl*D gene was integrated into the rice genome (Fig. 5). The pattern of the hybridizing bands shows integration into at least two to six locations in the transgenic plants, whereas the non-transformed rice shows no hybridization signal.

Transgenic rice plants obtained with the arabitol selection scheme appeared morphologically normal during differentiation and regeneration. GUS expression of the progeny of line 2 (lane 2 in Fig. 5) showed the expected 3:1 segregation ratio for insertion at a single functional locus (Fig. 6). PCR analysis of the progeny of line 5 (lane 5 in Fig. 5) showed that the transgene was inherited at a 3:1 ratio (Fig. 7). The resistance of germinating seeds of transgenic event 3 (lane 3 in Fig. 5) to arabitol was determined and is shown in Fig. 8. The results of the progeny assays are summarized in Table 2 and indicate that the transgenes were inherited in a 3:1 segregation ratio.

Our rate of successful transformation is at least as high as the rate reported for the use of *pmi* (*phosphomannose isomerase*) in rice, which ranges from 6.0% (He et al. 2004) to 41% (Lucca et al. 2001). Lucca et al. (2001) noted that 20 g l^{-1} mannose completely inhibited root development of the transgenic plants, indicating a differential sensitivity to mannose of roots than embryogenic calli. In contrast, T_1 seeds transgenic for *atl*D-S were able to germinate and grow on 20 g l^{-1} arabitol (Fig. 8).

The arabitol selection system resembles other carbohydrate-based systems in the need for optimization of the arabitol/sucrose ratio when used to select transformants in different species. Nevertheless, the use of arabitol selection presents another tool in the growing arsenal of alternative selection agents, and might provide flexibility in special situations. Although mannose

Table 2 Segregation of arabitol resistance, *atlD* gene and GUS activity in progeny of transformed rice selected with arabitol

	Growth on D-Arabitol[a]	atlD gene[b]	GUS activity[c]
Ratio (±)	19:6	32:17	74:21
$\chi 2(3:1)$	0.013	2.46	0.42
p	$0.95<p<0.90$	$0.25<p<0.10$	$0.75<p<0.50$

[a]T_1 seeds of transgenic event 3 were germinated on arabitol-containing medium

[b]T_1 seedlings of transgenic event 5 were analyzed by PCR with *atlD* gene-specific primers

[c]T_1 seeds of transgenic event 2 were assayed for GUS expression

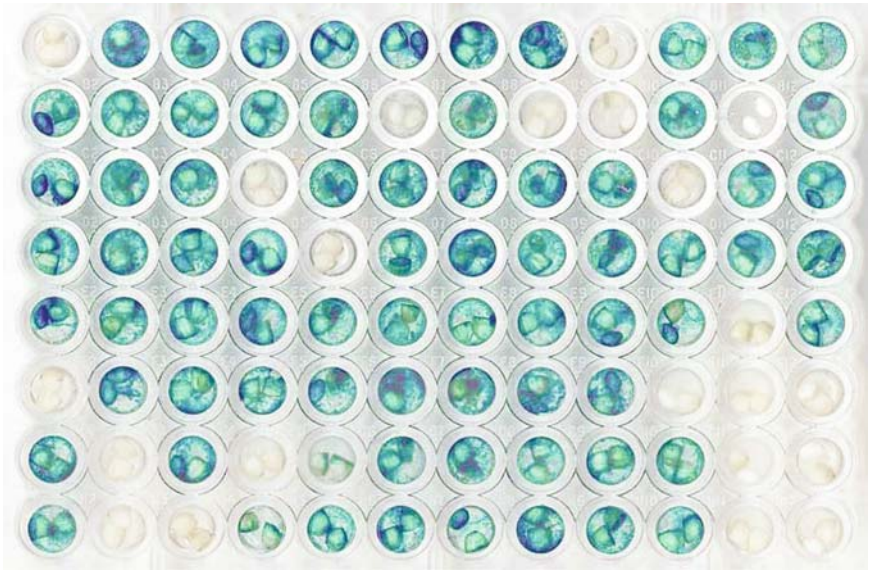

Fig. 6 GUS Plus expression of T_1 rice seeds. Ninety-five seeds of transgenic event 2 were halved and incubated with GUS substrate. A non-transformed seed was placed in the upper left well of the microtitre plate

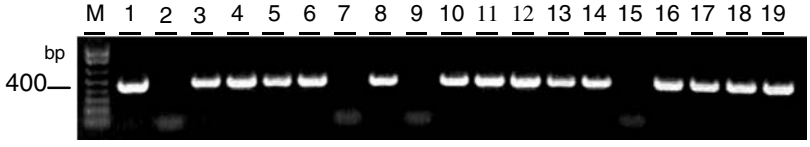

Fig. 7 Molecular analysis of T_1 rice seeds. PCR was performed using primers atlDS-167 and atlDS-596. *Lanes* (1) pAS1305.2 plasmid; (2) non-transformed seedling; (3–19) progeny from transgenic event 5

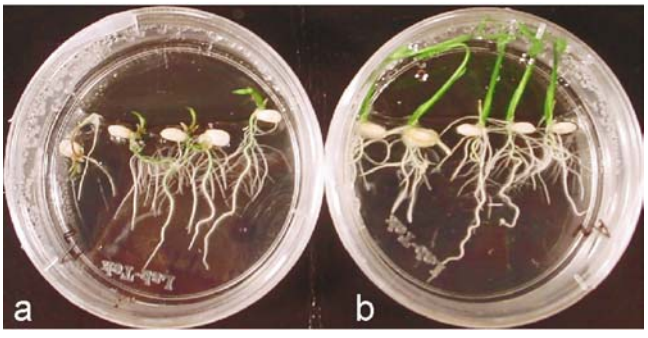

Fig. 8 Resistance of transgenic rice seeds germinated on medium containing 20 g l^{-1} arabitol. (**a**) Non-transgenic seeds; (**b**) progeny from transgenic event 3

selection utilizing *pmi* has been employed for several crop species (Negrotto et al. 2000; Sigareva et al. 2004; Wright et al. 2001), the *pmi* system has not been used in legumes, presumably due to the presence of mannose-6-phosphate isomerase in these plants (Lee and Matheson 1984; Chiang and Kiang 1988). Similarly, mannose and xylose cannot be used as selectable agents for grapevine transformation, since growth and development of embryos occurred on medium containing mannose and xylose as the sole carbohydrate (Kieffer et al. 2004). Hence, the availability of additional alternatives could prove helpful.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acid Res 25:3389–3402

Bardini M, Labra M, Winfield M, Sala F (2003) Antibiotic-induced DNA methylation changes in calluses of *Arabidopsis thaliana*. Plant Cell Tissue Organ Cult 72:157–162

Bojsen K, Donaldson I, Haldrup A, Joersbo M, Kreiberg J, Nielsen J, Okkels FT, Petersen SG (1998) Mannose or xylose based positive selection. US Patent 5767378

Chen L, Zhang S, Beachy RN, Fauquet CM (1998) A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Rep 18:25–31

Chiang YC, Kiang YT (1988) Genetic analysis of mannose-6-phosphate isomerase in soybeans. Genome 30:808–811

Erikson O, Hertzberg M, Nasholm T (2004) A conditional marker gene allowing both positive and negative selection in plants. Nat Biotechnol 22:455–458

European Parliament (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive

Fiers MWEJ, Kleter GA, Nijland H, Peijnenburg AACM, Nap JP, van Ham RCHJ (2004) Allermatch (TM), a webtool for the prediction of potential allergenicity according to current FAO/WHO Codex alimentarius guidelines. BMC Bioinform 5:133

Haldrup A, Petersen SG, Okkels FT (1998a) Positive selection: a plant selection principle based on xylose isomerase, an enzyme used in the food industry. Plant Cell Rep 18:76–81

Haldrup A, Petersen SG, Okkels FT (1998b) The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. Plant Mol Biol 37:287–296

Hazel CB, Klein TM, Anis M, Wilde HD, Parrott WA (1998) Growth characteristics and transformability of soybean embryogenic cultures. Plant Cell Rep 17:765–772

He ZQ, Fu YP, Si HM, Hu GC, Zhang SH, Yu YH, Sun ZX (2004) Phosphomannose−isomerase (*pmi*) gene as a selectable marker for rice transformation via *Agrobacterium*. Plant Sci 166:17–22

Hebsgaard SM, Korning PG, Tolstrup N, Engelbrech J, Rouze P, Brunak S (1996) Splice site prediction in Arabidopsis thaliana DNA by combining local and global sequence information. Nucl Acid Res 24:3439–3452

Heuel H, Shakeri-Garakani A, Turgut S, Lengeler JW (1998) Genes for D-arabitol and ribitol catabolism from *Klebsiella pneumoniae*. Microbiology 144:1631–1639

Heuel H, Turgut S, Schmid K, Lengeler JW (1997) Substrate recognition domains as revealed by active hybrids between the D-arabitol and ribitol transporters from *Klebsiella pneumoniae*. J Bacteriol 179:6014–6019

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Joersbo M, Okkels FT (1996) A novel principle for selection of transgenic plant cells: positive selection. Plant Cell Rep 16:219–221

Kieffer F, Triouleyre C, Bertsch C, Farine S, Leva Y, Walter B (2004) Mannose and xylose cannot be used as selectable agents for *Vitis vinifera* L. transformation. Vitis 43:35–39

Kleter GA, Peijnenburg AA (2002) Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens. BMC Struct Biol 2:8

Kruger NJ, von Schaewen A (2003) The oxidative pentose phosphate pathway: structure and organisation. Curr Opin Plant Biol 6:236–246

LaFayette P, Parrott WA (2001) A non-antibiotic marker for amplification of plant transformation vectors in *E. coli*. Plant Cell Rep 20:338–342

Lee BT, Matheson NK (1984) Phosphomannoisomerase and phosphoglucoisomerase in seeds of *Cassia coluteoides* and some other legumes that synthesize galactomannan. Phytochemistry 23:983–987

Lucca P, Ye XD, Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. Mol Breed 7:43–49

Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol 107:193–232

Murray HG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8:4321–4326

Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Rep 19:798–803

Pederson A, Nielsen H (1997) Neural network prediction of translation initiation sites in eukaryotes: perspectives for EST and genome analysis. ISMB 5:226–233

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88:3324–3328

Reiner AM (1975) Genes for ribitol and D-arabitol catabolism in *Escherichia coli*: their loci in C strains and absence in K-12 and B strains. J Bacteriol 123:530–536

Rieger M, Preston C, Powles S (1999) Risks of gene flow from transgenic herbicide-resistant canola (*Brassica napus*) to weedy relatives in southern Australian cropping systems. Aust J Agr Res 50:115–128

Sambrook T, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Scangos GA, Reiner AM (1978) Ribitol and D-arabitol catabolism in *Escherichia coli*. J Bacteriol 134:492–500

Schmitt F, Oakeley EJ, Jost JP (1997) Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tabacum* plants. J Biol Chem 272:1534–1540

Sigareva M, Spivey R, Willits M, Kramer C, Chang YF (2004) An efficient mannose selection protocol for tomato that has no adverse effect on the ploidy level of transgenic plants. Plant Cell Rep 23:236–243

Stein R, Gross W, Schnarrenberger C (1997) Characterization of a xylitol dehydrogenase and a D-arabitol dehydrogenase from the thermo- and acidophilic red alga *Galdieria sulphuraria*. Planta 202:487–493

Thomson JM, Parrott WA (1998) pMECA: A cloning plasmid with 44 unique restriction sites that allows selection of recombinants based on colony size. Biotechniques 24:922–924

US Pharmacopia (1990) The National Formulary. USPXXII, NF XVII, US Pharmacopoeial Convention, Inc. Easton PA 1990:1788

Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, Artim-Moore L (2001) Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, pmi, as the selectable marker. Plant Cell Rep 20:429–436

Zhang S, Chen L, Qu R, Marmey P, Beachy R, Fauquet C (1996) Regeneration of fertile transgenic indica (group1) rice plants following microprojectile transformation of embryogenic suspension culture cells. Plant Cell Rep 15:465–469