Journal of Experimental Botany, Vol. 57, No. 9, pp. 1909–1918, 2006
doi:10.1093/jxb/erj132 Advance Access publication 4 April, 2006

Journal of Experimental Botany

RESEARCH PAPER

Arabidopsis and tobacco plants ectopically expressing the soybean antiquitin-like *ALDH7* gene display enhanced tolerance to drought, salinity, and oxidative stress

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Received 19 September 2005; Accepted 23 January 2006

Abstract

Despite extensive studies in eukaryotic aldehyde dehydrogenases, functional information about the ALDH7 antiquitin-like proteins is lacking. A soybean antiquitin homologue gene, designated *GmTP55*, has been isolated which encodes a dehydrogenase motif-containing 55 kDa protein induced by dehydration and salt stress. GmTP55 is closely related to the stress-induced plant antiquitin-like proteins that belong to the ALDH7 family. Transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* (*Arabidopsis thaliana*) plants constitutively expressing *GmTP55* have been obtained in order to examine the physiological role of this enzyme under a variety of stress conditions. Ectopic expression of *GmTP55* in both *Arabidopsis* and tobacco conferred tolerance to salinity during germination and to water deficit during plant growth. Under salt stress, the germination efficiency of both transgenic tobacco and *Arabidopsis* seeds was significantly higher than that of their control counterparts. Likewise, under progressive drought, the transgenic tobacco lines apparently kept the shoot turgidity to a normal level, which contrasted with the leaf wilt phenotype of control plants. The transgenic plants also exhibited an enhanced tolerance to H_2O_2- and paraquat-induced oxidative stress. Both *GmTP55*-expressing *Arabidopsis* and tobacco seeds germinated efficiently in medium supplemented with H_2O_2, whereas the germination of control seeds was drastically impaired. Similarly, transgenic tobacco leaf discs treated with paraquat displayed a significant reduction in the necrotic lesions as compared with control leaves. These transgenic lines also exhibited a lower concentration of lipid peroxidation-derived reactive aldehydes under oxidative stress. These results suggest that antiquitin may be involved in adaptive responses mediated by a physiologically relevant detoxification pathway in plants.

Key words: Aldehyde dehydrogenase, antiquitin, detoxification pathway, environmental stresses, stress tolerance, turgor-responsive protein.

Introduction

Environmental stress conditions, such as water deficit and salinity, have become major constraints for plant growth, crop productivity, and species distribution. Among other consequences, both drought and high salinity produce osmotic stress by decreasing the water chemical activity and affecting the cell turgor (Zhu, 2001). These environmental stressors also cause a rapid and excessive accumulation of reactive oxygen species (ROS) in plant cells (Bartels, 2001; Zhu, 2001). In addition to reacting directly to proteins, amino acids, and nucleic acids, ROS cause a lipid peroxidation chain reaction resulting in chemically reactive cleavage products, largely represented by aldehydes. ROS accumulation is counteracted by antioxidant systems that include low-molecular-mass molecule and enzyme scavengers (Allen, 1995; Mittler, 2002), whereas the potentially toxic nature of aldehydes is challenged by increased aldehyde dehydrogenase (ALDH) activity as one of the cellular defence strategies in the detoxification

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of these stress-generated chemically reactive compounds (Sunkar *et al*., 2003). While some ALDHs have been described as part of this antioxidant defence system, other drought and salt stress-induced ALDHs seem to play a direct role in cellular osmoregulation by catalysing the synthesis of osmoprotectants (Kirch *et al.*, 2004).

Aldehyde dehydrogenase is a superfamily of enzymes catalysing the conversion of various endogenous and exogenous aldehydes to the corresponding carboxylic acids using the coenzyme NAD^+ or $NADP^+$ (Yoshida *et al.*, 1998). The eukaryotic ALDHs can be organized into 21 families based on sequence identity (Sophos and Vasiliou, 2003). In general, 40% sequence similarity or less places the enzymes in a different family category, whereas 60% sequence identity categorizes members of a subfamily (Kirch *et al.*, 2004). In mammals, different ALDH representatives have been implicated in intermediate metabolism, such as vitamin A biosynthesis and amino acid metabolism, as well as in detoxification of stress-generated aldehydes and osmoprotection (Perozich *et al.*, 1999).

The plant *ALDH* genes are represented in 11 ALDH families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH19, and ALDH21. The ALDH11, ALDH19, and ALDH21 families are unique to plants and, more recently, *ALDH* sequences encoding members of a novel family, ALDH22, have been identified in *Arabidopsis*, maize, and rice genomes (Kirch *et al.*, 2004). Biological function has been assigned to members of the plant ALDH superfamily in development and/or stress adaptation. Among the stress-related ALDHs, the plant ALDH3 and ALDH5 families are involved in detoxification of aldehydes (Bouché *et al.*, 2003; Sunkar *et al.*, 2003), whereas the ALDH10, ALDH11, and ALDH12 families act primarily in cellular osmoregulation by catalysing the synthesis of osmoprotectant (Kirch *et al.*, 2004). The ALDH10 family is represented by the dehydration and salt-inducible betaine aldehyde dehydrogenase (BADH), which catalyses the oxidation of betaine aldehyde to the compatible solute glycine betaine, as an adaptation to osmotic stress (Weretilnyk and Hanson, 1990; Kumar *et al.*, 2004). In *Arabidopsis*, the ALDH11 family consists of a single gene that encodes a non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase, GAPDH (*GAPN*), whereas the ALDH12 family is represented by a mitochondrial Δ^1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*) gene (Kirch *et al.*, 2004). P5CDH is a key enzyme in the degradation of proline to glutamate and its transcript is upregulated by exogenous proline and salinity (Deuschle *et al*., 2001). Among the stress-associated ALDHs, the members of the ALDH7 family, also designated antiquitin, have not been related to any biochemical pathway. The aldehyde-oxidizing activity of the enzyme has been assayed using acetaldehyde and aromatic benzaldehyde as substrates, but the specific physiological substrate for antiquitin remains to be identified (Tang *et al.*, 2002; Chan *et al.*, 2003; Fong *et al.*, 2003).

The physiological function of antiquitin is believed to be related to the regulation of turgor pressure or to a general stress response. The plant antiquitin homologue gene has been shown to be induced by water deprivation and exposure to high salinity in pea (*Pisum sativum*), in canola (*Brassica napus*), and in *Arabidopsis* (Guerrero *et al.*, 1990; Stroeher *et al.*, 1995; Kirch *et al.*, 2005). In mammals, the antiquitin gene is highly expressed in organs in which the osmotic balance must be maintained for proper function, such as the cochlea and kidney (Skvorak *et al.*, 1997). Despite the stress-induced pattern of antiquitin gene expression, the functional significance of the evolutionary conserved antiquitin family has yet to be elucidated. In this investigation, an antiquitin homologue cDNA was isolated from soybean (*Glycine max*) that was highly represented in a dehydrated-seed cDNA library. To elucidate the function of ALDH7 from soybean, the current study was conducted using transgenic tobacco as a model system, as it can easily be transformed by *A. tumefaciens*. It was also reasoned that the wide distribution of plant ALDH7 homologues in distantly related genera would argue strongly for the conservation of the ALDH7 function as well. To test this prediction further, *Arabidopsis* was also included as a model system for the functional analyses. Both tobacco and *Arabidopsis* transgenic lines producing the soybean enzyme displayed a lower concentration of reactive aldehydes and enhanced tolerance to drought, salinity, and ROS-producing chemical treatments. These results suggest that the antiquitin-like *ALDH7* gene might be involved in the detoxification of reactive aldehyde species generated by oxidative stress-associated lipid peroxidation.

Materials and methods

Plant material and greenhouse experiments

Soybean plants (*Glycine max* cv. Cristalina) were germinated in 5.0 l pots containing a mixture of soil, sand, and dung (3:1:1 by vol.) and grown under standardized greenhouse conditions. The water-stress condition was induced in 40-d-old plants by withholding watering for 9 d before harvesting of tissues (Cascardo *et al.*, 2000). Salt stress was imposed by irrigating the plants with 0.66 M NaCl solution for 9 d. Leaves, roots, and stems were harvested from unstressed and stressed plants, immediately frozen in liquid nitrogen, and stored at −80 °C.

Isolation of an antiquitin homologue cDNA (ALDH7) from soybean

The antiquitin-like cDNA, also designated *GmATQ* or *GmTP55* (GenBank accession number AY250704), was isolated through BLAST searches of a *Glycine max* EST database that was developed from a soybean seed cDNA library prepared in this laboratory. The computer program CLUSTALX was used for sequence alignment and the phylogenetic tree was produced using the Genebee program (http://www.genebee.msu.su/services/phtree_reduced.html).

Construction of plasmids

The GmTP55 (GmATQ) protein was expressed as an N-terminal His-tagged fusion protein. For this purpose, the 1300 bp *Bam*HI fragment

was released from *GmTP55* cDNA (pUFV388) and inserted into pET-16b. The resulting clone, pUFV458, contains a *GmTP55* truncated fragment, which encodes amino acids 97–517, fused in-frame to the His tag.

For plant transformation, the full-length *GmTP55* cDNA fragment was transferred as an *Eco*RI fragment from pUFV388 to pCAMBIA1301Z, generating pUFV408. Alternatively, a binary recombinant plasmid was obtained through the GATEWAY system (Invitrogen Life Technologies Inc.). Briefly, the *GmTP55* full-length cDNA fragment was amplified by PCR with appropriate extensions and introduced by recombination into the entry vector pDONR201, sequenced, and then transferred to the binary vector pK7WG2, yielding pK7-GmATQ. The resulting clones, pUFV408 and pK7-GmATQ, harbour the *GmTP55* cDNA under the control of the CaMV 35S promoter and the polyadenylation signal of the T-DNA nopaline synthase (*nos*) gene.

Antibody production

The recombinant plasmid, pUFV458, was transformed into *E. coli* strain BL21 (DE3) and the synthesis of the recombinant protein was induced by 0.5 mM isopropyl thio-β-D-galactoside for 4 h at 37 °C (IPTG). The His-tagged protein was purified by affinity chromatography using Ni-chelating Sepharose resin (Amersham Pharmacia Biotech.) according to manufacturer's instructions. The recombinant purified protein was used as an antigen to raise polyclonal antisera in rabbits, which were immunized through subcutaneous injections at 2-week intervals.

Protein extraction and immunoblotting analysis

Total protein was extracted from an acetone dry powder, using a protocol adapted from Görg *et al.* (1988). Briefly, plant tissues (roots, leaves, and stems) were crushed in liquid nitrogen, and 2 g of the powder was homogenized with 10% (w/v) TCA in acetone containing 0.07% (v/v) 2-mercaptoethanol. Total protein was precipitated for 40 min at −20 °C, recovered by centrifugation at 16 000 *g* for 15 min, and washed two or three times with acetone containing 0.07% (v/v) 2-mercaptoethanol. The pellet was dried under vacuum, and 100 mg of the acetone dry powder was homogenized in 1 ml of 50 mM TRIS-HCl (pH 7.5), 1% (w/v) SDS, and 25 mM EDTA. Cell debris was removed by centrifugation at 25 000 *g* for 20 min and protein concentration was determined as described by Hill and Straka (1988). Equivalent amounts of total protein (30 mg) were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membranes by electroblotting. The membrane was blocked with 3% (w/v) bovine serum albumin in TBST [100 mm TRIS-HCl (pH 8), 150 mm NaCl, 0.05% (v/v) Tween 20]. GmTP55 (GmALDH7) was detected using a polyclonal antibody raised against the recombinant protein at a 1:1000 dilution, followed by a goat anti rabbit IgG conjugated to alkaline phosphatase (Sigma) at a 1:5000 dilution. The activity of alkaline phosphatase was assayed using 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies do Brasil Ltda, São Paulo, Brazil) and *p*-nitroblue tetrazolium (Life Technologies).

Plant transformation

Leaf discs from *in vitro*-grown tobacco (*Nicotiana tabacum* L. cv. Havana) plants were co-cultivated for 15 min with *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pUFV408. Transformed shoots were selected on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 6-benzylaminopurine (400 μg ml^{-1}), timentin (500 μg ml^{-1}), and hygromycin (50 μg ml^{-1}). Regenerated shoots were rooted on phytohormone-free medium containing hygromycin (50 μg ml^{-1}), transferred into soil, and grown under standardized greenhouse conditions (T_0 plants) to generate seeds. Four independently regenerated hygromycin-resistant plants harbouring the *GmTP55* sense construct were grown for further analyses. Tobacco plants were also transformed with the binary vector pCAMBIA1301Z without any insert. These hygromycin-resistant plants for the pCAMBIA1301Z incorporated binary vector as well as wild-type plants were used as controls.

Arabidopsis thaliana Col-0 was transformed with the binary construct pK7-GmATQ by *Agrobacterium tumefaciens*-mediated transformation using the floral dip method (Clough and Bent, 1998). Transformants (T_1) were selected on MS agar plates containing 50 mg l^{-1} kanamycin.

Analysis of transgenic plants

The presence of *hyg* or *npt*II and *GmTP55* transgenes was analysed by PCR from leaf tissue samples. PCR was carried out on 20 ng of genomic DNA isolated from 4-week-old greenhouse-grown tobacco transgenic plants, using 0.4 μM each of *hyg* primers or *GmTP55* gene-specific primers and one unit of *Taq* polymerase in a final volume of 25 μl. For confirmation of *Arabidopsis* transgenic lines, PCR was conducted on 20 ng of genomic DNA isolated from seedlings, using 0.4 μM each of *npt*II primers or *GmTP55* gene-specific primers and one unit of *Taq* polymerase in a final volume of 25 μl. The *GmTP55*-specific primers were 4076-AntiF501 (coordinates 539–559, upstream) and 4077-AntiR506 (positions 1042–1063, downstream). The primers specific for the *npt*II gene were 5′-TCGACGTTGTCACTGAAGCGCG-3′ (sense) and 5′-GCGGTCAGCCCATTCGCCGCC-3′ (antisense) and for the hygromycin gene were 5′-CGCTTCTGCGGGCGATTTGTGTACG-3′ (sense) and 5′-TCAGCTTCGATGTAGGAGGGCGTGG-3′ (antisense). Transgene copy number was determined by segregation analyses.

For segregation analysis, tobacco seeds were germinated on Murashige and Skoog medium containing 50 μg ml^{-1} hygromycin. Homozygous T_1 lines with respect to the T-DNA loci were selected by determining the frequency of their antibiotic-resistant T_2 seeds after self-pollination.

Analysis of transgene expression

The detection of transgene expression was carried out by gene-specific RT-PCR. Leaves were harvested from 4-week-old tobacco transgenic plants that were grown under standardized greenhouse conditions and immediately frozen in liquid nitrogen. Total RNA was extracted from leaves by the TRIZol method (Invitrogen). First-strand cDNA was synthesized from 2–5 μg of total RNA using the SuperScript III Kit (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions. PCR assays were performed with *GmTP55*-specific primers as described (Cascardo *et al*., 2000). Control reactions were conducted with $polyA^+$ RNA without reverse transcriptase. PCR was also carried out with actin gene-specific primers to assess the quantity and quality of the cDNA. Protein accumulation was monitored by immunoblotting of total protein extracted from leaves of 4-week-old tobacco transgenic plants that were grown under standardized greenhouse conditions.

Stress treatments

To induce salinity stress during germination, both tobacco and *Arabidopsis* seeds were surface-sterilized and sown on agar plates containing MS medium with different concentrations of NaCl (0, 100, 150, and 200 mM). Tobacco seedlings were grown with a day/night cycle of 14/10 h at 28 °C and an irradiance of 200 μmol m^{-2} s^{-1} and *Arabidopsis* seedlings were grown in a growth chamber with a day/night cycle of 16/8 h at 22 °C and an irradiance of 50 μmol m^{-2} s^{-1}.

For H_2O_2 treatment, both surface-sterilized tobacco and *Arabidopsis* seeds were placed on filter paper prewetted with deionized water containing different concentration of H_2O_2 (0, 10, 15, and 20 μM). The conditions of seed germination were as described above.

For paraquat-induced oxidative stress, 15 mm leaf discs from 2-month-old transgenic and untransformed plants were incubated with a series of paraquat (methyl viologen, Sigma) concentrations (0, 1, 2, and 4 μM) and kept for 1 h in the dark, followed by incubation for 18 h with a light intensity of 200 μmol m^{-2} s^{-1} at 24 °C. The extent of the oxidative damage was assessed by determining the necrotic area of the disc leaf using the QUANT 1.0.1-R1 program.

The water-stress treatment was induced in soil-grown tobacco plants, as described previously (Alvim *et al.*, 2001). Briefly, transgenic T_1 seeds were germinated in kanamycin-containing medium for 3 weeks before transplantation. Plants were grown in a mixture of soil, sand, and dung (3:1:1 by vol.) for 2 weeks in the greenhouse under natural conditions of light, relative humidity at 70%, and controlled temperature, 18 °C and 30 °C (night and day). After 30 d of growth with normal water supply, drought stress was imposed by withholding water for 2 weeks from half of the transgenic plants. The remaining transgenic plants received normal water supply continuously. In control experiments, the same conditions of water availability were applied in untransformed control plants at the same developmental stage as transgenic GmTP55 plants. All the experiments were conducted with five clones from the independently transformed lines.

Assay of lipid peroxidation

The malondialdehyde (MDA) content was determined by the reaction of thiobarbituric acid (TBA) as described by Cakmak and Horst (1991).

Results

Isolation of GmTP55, a soybean antiquitin homologue that belongs to the ALDH7 family

A soybean antiquitin-related cDNA (GenBank accession number AY250704) was randomly isolated from a *Glycine max* seed cDNA library constructed in λZAPII. The full-length soybean antiquitin cDNA sequence encodes a protein with an estimated M_r of 55 562 and pI 5.27. The Gm TP55 (*Glycine max* turgor-reponsive 55 kDa protein), also designated GmATQ (*Glycine max* antiquitin), is most closely related to the pea turgor-reponsive protein 26g, an antiquitin-related ALDH (82% sequence identity). Based on ALDH superfamily phylogeny, GmTP55 (ALDH-Gm) segregates more closely with clusters of the ALDH7 family, which contains antiquitin-like (ATQ) proteins found in plants, fish, and mammals (Fig. 1). It retains more than 70% sequence identity with other plant antiquitin-like proteins and shares remarkable conservation of primary structure with the human and mouse antiquitin (about 60% sequence identity). In addition, the soybean antiquitin-like protein possesses a conserved domain characteristic of

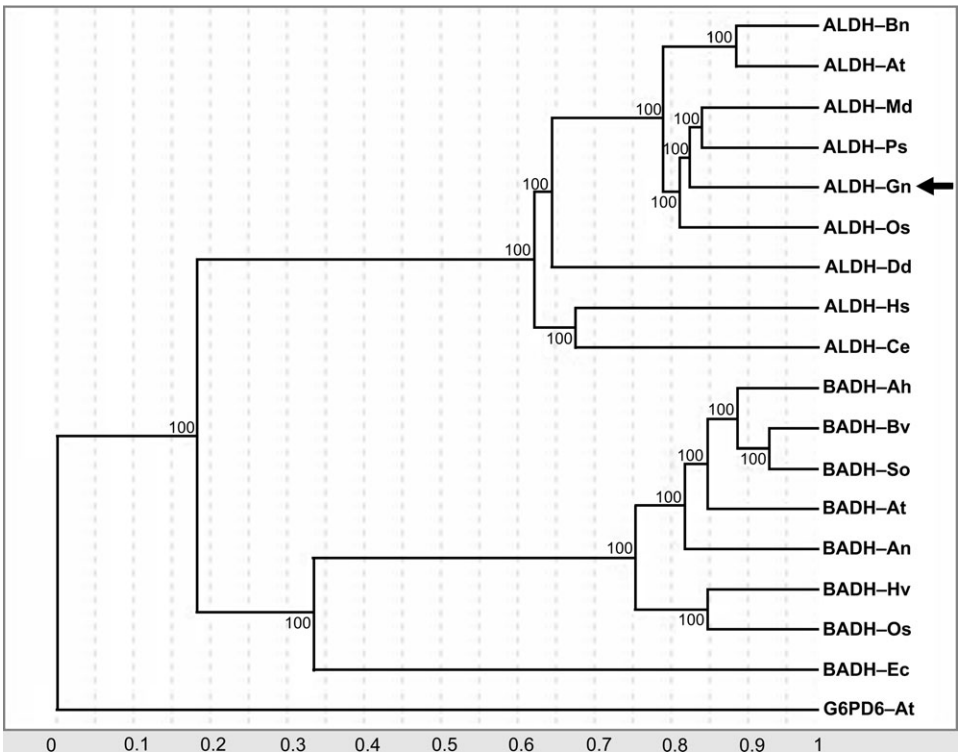

Fig. 1. Phylogenetic tree based on family 7 (ALDH7) and family 10 (BADH) aldehyde dehydrogenase sequences. Amino sequences from ALDHs were aligned using ClustalX, and the dendrogram was generated using Genebee program. The sequences come from members of the ALDH7 family (antiquitin), such as ALDH7 of *Brassica napus* (ALDH-Bn, accession Q41247), *Arabidopsis thaliana* (ALDH-At Q95Y67), *Malus domestica* (ALDH-Md, Q9ZPB7), *Pisum sativum* (ALDH-Ps P25795), *Glycine max* (ALDH-Gm, AY250704), *Oryza sativa* (ALDH-Os, AF323586), *Dictyostelium discoideum* (ALDH-Dd, P83401), human (ALDH-Hs, antiquitin, P49419), *Caenorhabditis elegans* (ALDH-Ce, P46562); members from the betaine aldehyde dehydrogenase, ALDH10 family, such as BADH of *Amaranthus hypochondriacus* (BADH-Ah, O04895), *Beta vulgaris* (BADH-Bv, P28237), *Spinacea oleracea* (BADH-So, P17202), *Arabidopsis thaliana* (BADH-At, Q9S795), *Avicennia marina* (BADH-Am, Prf:2715330A), *Hordeum vulgare* (BADH-Hv, Q40024), *Oryza sativa* (BADH-Os, O24174), *E.coli* (BADH-Ec, P77674), and an *Arabidopsis thaliana* glucose-6-phosphate dehydrogenase (G6PD6-At, Q9FJI5), as an outgroup. Numbers at nodes indicate the percentage bootstrap scores (100 replications) and those shown below the frame indicate the percentage of sequence identity. The arrow indicates the position of the *Glycine max* ALDH7 protein.

members of the ALDH7 family that encompasses the aldehyde dehydrogenase glutamic acid active site ^{270}LELSGNNA^{277} (PROSITE PS 00687; Perozich *et al.*, 1999) and a transmembrane segment ^{158}IVGVISAFNFPCAVLGWNACIAL^{180} (Guerrero *et al.*, 1990; Lee *et al.*, 1994).

GmTP55 is induced by salinity and water deficit

The antiquitin-like protein GmTP55 has been expressed in *E. coli* as N-terminal His-tagged fusion protein and purified to raise antibodies. Immunoblottings of total protein from different tissues of soybean grown under normal conditions demonstrated that the GmTP55 protein accumulates predominantly in the stem (Fig. 2A, lane S) and it is barely detected in leaves (lane L). Several turgor-responsive *ALDH7* genes have been shown to be induced by dehydration and high salinity (Guerrero *et al.*, 1990; Stroeher *et al.*, 1995; Kirch *et al.*, 2005). Accordingly, GmTP55 accumulation is induced by water deficit (Fig. 2B) and salt stress (Fig. 2A) in all organs tested.

Expression of GmTP55 *in transgenic tobacco and* Arabidopsis

Tobacco was transformed via *Agrobacterium tumefaciens* with the *GmTP55* gene, under the control of 35S cauliflower mosaic virus (CaMV) promoter and the *nos* polyadenylation signal. Several independent transgenic lines were established, transferred into soil, and grown in a greenhouse to generate seeds (T_1 seeds). The integration and gene copy number of the construct in the transformed plants were further confirmed by PCR analysis with *GmTP55*-specific primers (Fig. 3A) and segregation analysis of the *hyg* gene in the T_0 progenies (T_1 plants). Four independent transgenic sense lines (TP55-S1, TP55-S2, TP55-S3, and TP55-S5) were selected for further analyses. Under normal, non-stressed conditions, *GmTP55* mRNA was detected in their leaves (Fig. 3B, lanes S1, S2, S3, and S5) but not in the wild type (data not shown) and pCAMBIA1301Z-transformed control (lane pC) tobacco leaves. Apparently, the primers are capable of discriminating between homologous sequences present in the tobacco genome and *GmTP55*-specific sequences, as they also failed to amplify a homologous cDNA from drought untransformed tobacco leaves (data not shown). Under normal, non-stressed conditions, the GmTP55 protein levels were detected in the transgenic leaves (Fig. 3C, lanes S1, S2, S3 and S5) but not in the wild type (data not shown) and pCAMBIA-transformed control (pC) tobacco leaves.

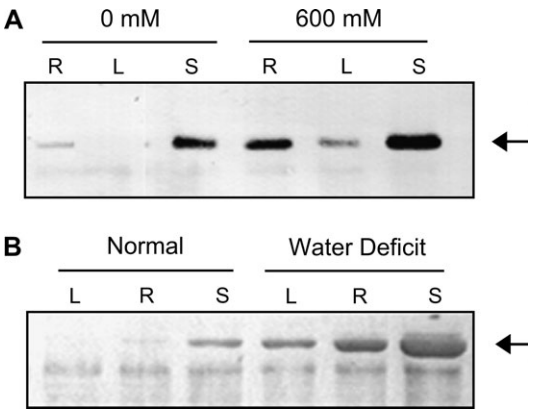

Fig. 2. GmTP55 is induced by salinity and water deficit. (A) GmTP55 accumulation in response to NaCl. Equivalent amounts of total protein from stems (S), roots (R), and leaves (L) from soybean plants grown under normal conditions (0 mM) and from NaCl-treated soybean plants (600 mM) were fractionated by SDS-PAGE and immunoblotted using an anti-GmTP55 serum. (B) Induction of GmTP55 accumulation by drought. Immunoblotting of total protein extracted from soybean plants grown under water deficit for 9 d. Normal shows the GmTP55 levels in plants before withholding watering.

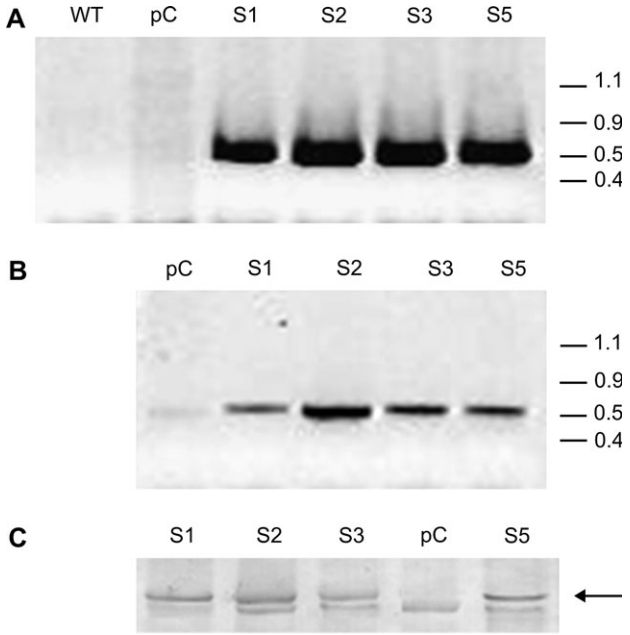

Fig. 3. Analysis of transgene expression in tobacco plants transformed with the *GmTP55* cDNA. (A) Diagnostic of transgene incorporation. Total DNA was isolated from greenhouse-grown transgenic plants and provided the template in PCR reactions using *GmTP55* gene-specific primers. S refers to the plants transformed with the *GmTP55* cDNA. Different numbers following the S symbol indicate that the transgenic plants were originated from independent events of transformation. WT corresponds to the result of a PCR reaction performed with DNA from control plants and pC from pCAMBIA-transformed control plants. (B) Accumulation of *GmTP55* transcripts in tobacco transgenic plants. RT-PCR assays were performed using cDNA prepared from polyA$^+$ RNA of pCAMBIA-transformed control plants (pC), *GmTP55*-transformed plants (S), and the *GmTP55* gene-specific primers. The positions of DNA standard markers are indicated on the left in kb. (C) Enhanced levels of GmTP55 (ALDH7) protein in tobacco transgenic plants. Equivalent amounts of total protein (30 mg per lane) extracted from the fully expanded third leaf of four independent transgenic 35S-TP55-S (sense) tobacco plants (lanes S1, S2, S3, and S5) and pCAMBIA-transformed control plants (pC) were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-TP55 antibody. The arrow indicates the position of the GmTP55 protein.

Segregation analysis suggested that the lines TP55-S2 and TP55-S3 plants appeared to have an integrated T-DNA locus on a single chromosome, since 75% of their T_1 segregating seedlings were resistant to hygromycin (Table 1). In addition, these lines were phenotypically normal and, therefore, they were selected for further studies. The selected transgenic lines also exhibit similar developmental performance as the wild type and pCAMBIA-transformed control lines (Table 2).

Salt tolerance during germination and early seedling development

As a first step towards understanding the function of antiquitin, the effect of the *GmTP55* gene expression on salt tolerance was evaluated. Tobacco transgenic T_1 seeds expressing the soybean antiquitin gene as well as wild-type seeds were allowed to germinate in MS media (Murashige and Skoog, 1962) containing different concentrations of NaCl, as indicated in Fig. 4A. Under normal conditions (0 mM NaCl) both wild-type and T_1 transgenic seeds germinated into seedlings with similar frequency and no phenotypic difference on seedling development was observed. Between wild-type and tobacco transgenic lines, differences of germination efficiency and seedling development became apparent at 100 mM NaCl and were statistically significant at 150 mM NaCl (Fig. 4B). While the germination efficiency of wild-type seeds was 30% reduced at 150 mM, TP55-S2 and TP55-S3 transgenic lines kept the germination frequency to normal levels. Both tobacco transgenic lines exhibited enhanced tolerance to salinity, although to different extents, as the TP55-S2 transgenic seeds exhibited a better performance at high salt concentrations. In fact, TP55-S2 transgenic seeds germinated with high efficiency (>90%) under 200 mM NaCl, whereas only 58% of TP55-S3 seeds germinated into seedlings under this condition. For the TP55-S2 and TP55-S3 independent transgenic lines analysed, the salt-tolerant germination phenotype at 200 mM NaCl was found to be linked to the *hyg* gene because it segregated with the same ratio as the hygromycin-resistant phenotype (Table 1). Since transformation of tobacco with the *hyg* gene alone did not confer tolerance to salinity during seed germination, it was concluded that the salt-tolerant germination phenotype was caused by ectopic expression of the antiquitin-like gene. In fact, PCR analysis of the segregating population with *GmTP55*-specific primers further confirmed the results (data not shown).

Table 1. *Expression of hygromycin resistance and salt stress tolerance in the T_1 generation of transgenic tobacco plants*

χ^2 tests indicate good agreement with segregation ratio indicated.

Plant lines tested	Hygromycin-resistant seedlings	Ratio	χ^2	200 mM NaCl-tolerant seedlings	Ratio	χ^2
TP55-S2	$1377^+/486^-$	3:1	1.23	$316^+/118^-$	3:1	1.10
TP55-S3	$1244^+/455^-$	3:1	2.87	$208^+/68^-$	3:1	0.02
Control[a]	$0003^+/997^-$	–	–	ND[b]	ND	ND

[a] Untransformed, wild-type plants.
[b] Not determined.

Table 2. *Growth measurements of transgenic lines*

Data are given as mean ±SD from three replicates. No significant differences at $P \leq 0.05$ were observed.

Plant lines tested	Height (cm)	Flowering[a]	Aerial part (DW)[b]	Roots (DW)[b]
Wild type	98.7±5.8	62.3±3.2	51.03±21.50	7.37±2.1
TP55-S2	95.4±12.2	61.7±5.1	53.50±15.47	7.32±1.95
TP55-S3	87.2±14.1	58.2±2.3	47.20±7.37	6.83±0.84
Control[c]	85.5±9.8	55.7±5.1	49.2±12.25	6.91±1.12

[a] Days in greenhouse.
[b] Dry weight (g).
[c] Plants transformed with the binary vector alone.

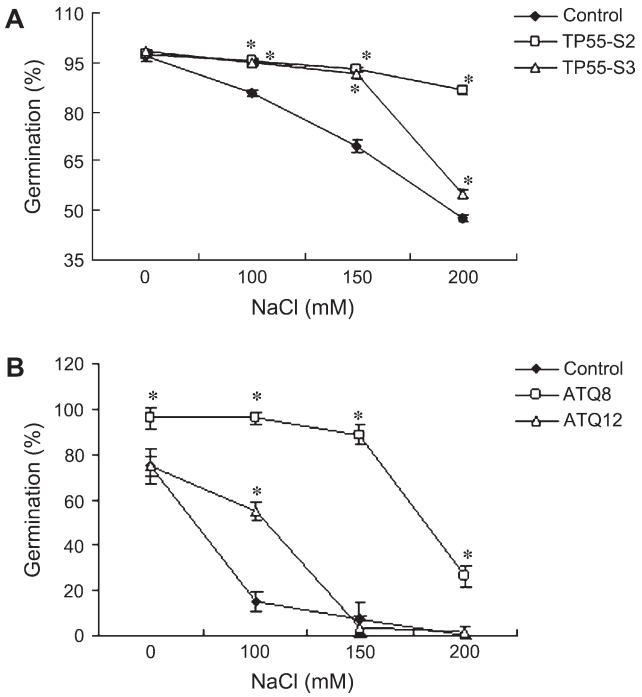

Fig. 4. GmTP55 transgenic lines exhibit increased tolerance to salinity during germination and early seedling development. (A) Germination efficiency of transgenic tobacco seeds under salt stress. Transgenic (TP55-S2 and TP55-S3) and control (WT) seeds were germinated on MS agar plates containing the indicated concentrations of NaCl. The data are expressed as a percentage of 100 seeds germinated from each indicated line and the values are given as mean ±SD of five determinations from each independent transformant line. The asterisks indicate significant differences at P ±0.05. (B) Germination and seedling development of *Arabidopsis* transgenic seeds exposed to NaCl. Transgenic (ATQ8 and ATQ12) and control (WT) *Arabidopsis* seeds were germinated on MS agar plants containing the indicated concentrations of NaCl for 15 d. The data are expressed as a percentage of 200 seeds germinated from each indicated line and the values are given as mean ±SD of three independent experiments. The asterisks indicate significant differences at $P \leq 0.05$.

Similar results were observed for the *Arabidopsis* transgenic seeds, ATQ8 and ATQ12, which displayed an enhanced tolerance to salinity during germination and seedling development (Fig. 4C). At 100 mM NaCl, the *GMTP55*-expressing seeds germinated with high efficiency (100% for ATQ8 transgenic line and >60% for ATQ12 transgenic line), whereas less than 10% of the control, untransformed seeds germinated into seedlings under similar conditions.

Water stress tolerance of soil-grown transgenic plants

Because *GmTP55* (Fig. 2B) and its plant homologues have also been shown to be induced by water deficit (Guerrero *et al.*, 1990; Stroeher *et al.*, 1995; Kirch *et al.*, 2005), the response of the tobacco transgenic lines to drought stress was examined next. For this experiment, young seedlings were transferred to a greenhouse where half of the plants received no irrigation and the remaining ones were irrigated throughout the period of the experiment. After 2 weeks under progressive dehydration, a drought stress-tolerant phenotype was clearly developed by the transgenic plants expressing the *GmTP55* gene (Fig. 5). Whereas the untransformed, wild-type control leaves were completely wilted (relative water content 39.5±1.5%), the transgenic leaves apparently kept the turgidity to a higher level (relative water content of TP55-S2, 61.2±0.3% and TP55-S3, 52.2±0.9%). These results were not due to differences in the developmental performance of the transgenic lines, because, under irrigation, the overall development of wild-type and transgenic lines was phenotypically undistinguishable (bottom panel). The likely maintenance of turgidity in stressed sense plants (Fig. 5) might suggest that some degree of osmotic adjustment in these plants helped to some extent to prevent cellular dehydration.

Fig. 5. *GmTP55* overexpression confers drought stress tolerance to transgenic plants. Drought condition was induced in 6-week-old control (WT) and transformed seedlings grown in a greenhouse by withholding irrigation for 2 weeks. At the bottom, wild-type and transgenic plants received normal water supply continuously.

Performance of GmTP55-overexpressing plants under oxidative stresses

Both water deficit and salinity often promote the formation of reactive oxygen species (ROS) that interact directly with different macromolecules. In particular, the destruction of the lipid membranes leads to the formation of lipid hydroperoxides and their toxic aldehyde degradation products. To determine whether the stress protective role of GmTP55 is associated with an aldehyde detoxification pathway, oxidative stress was induced by treating transgenic lines with H_2O_2 and paraquat. While 10 μM H_2O_2 did not affect the germination efficiency and seedling development of tobacco transgenic seeds, it promoted a 50% inhibition of wild-type, control seed germination (Fig. 6A). This significantly enhanced tolerance to H_2O_2 of transgenic seeds persisted with the progressive increase of H_2O_2 concentration (up to 20 μM). Likewise, *Arabidopsis* transgenic seeds germinated more efficiently in the presence of H_2O_2 than wild-type, untransformed seeds (Fig. 6B).

The TP55-S2 and TP55-S3 tobacco transgenic lines were also included in a resistance test against paraquat, as an alternative oxidative stress-inducing agent. The visible injuries, resulted from oxidative damages, were recorded

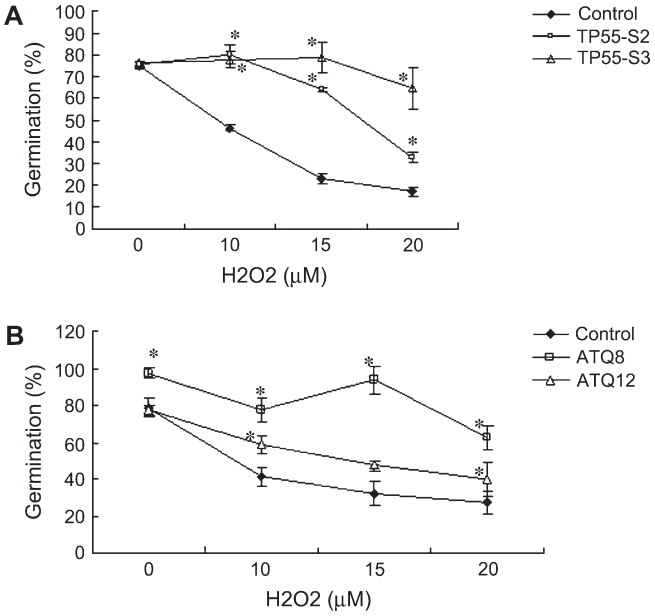

Fig. 6. H_2O_2 tolerance of transgenic lines during germination. Seeds from tobacco transgenic (A) and *Arabidopsis* transgenic (B) lines as well as from their wild-type counterparts (control) were germinated under various concentrations of H_2O_2 (as indicated). Data are the mean ±SD of three independent experiments. The asterisks indicate significant differences at $P \leqslant 0.05$.

on leaf discs from transgenic and untransformed plants (Fig. 7) and the extent of leaf necrotic area was measured with the program Quant 1.0.1-R1 (Table 3). Leaf discs from both TP55-S2 and TP55-S3 transgenic tobacco were able to tolerate high concentrations of paraquat up to 4 μM and 2 μM, respectively, whereas control leaf discs were bleached in concentrations as low as 1 μM of paraquat (Fig. 7A). As indicated by the area of leaf bleaching, while 4 μM paraquat treatment caused almost total chlorophyll loss in untransformed leaves (98% necrotic area), the tobacco transgenic lines were more resistant to paraquat, as their necrotic areas were as low as 29.5% (TP55-S2) and 58.8% (TP55-S3) under the same conditions.

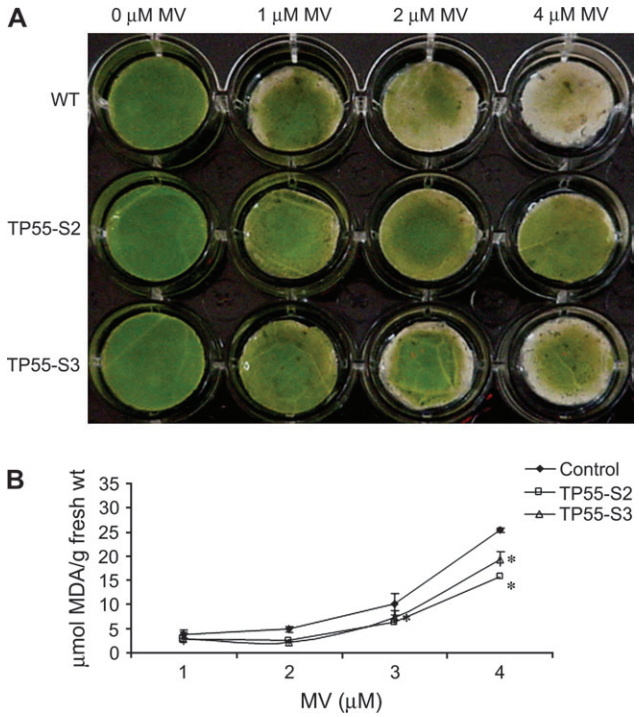

Fig. 7. *GmTP55* reduces paraquat-induced lipid peroxidation in transgenic lines. (A) Paraquat visual injury. Leaf discs of 1.5 cm of diameter from wild type (WT) and tobacco transgenic lines (TP55-S2 and TP55-S3) were incubated with the indicated concentrations of paraquat (MV) for 1 h dark period followed by 18 h under light. (B) Lipid peroxidation expressed as MDA contents of paraquat-treated leaf discs. Leaf discs from control (WT) and tobacco transgenic lines (TP55-S2 and TP55-S3) were treated with paraquat and the malondialdehyde (MDA) content was determined. Values are given as mean ±SD from three replicates. The asterisks indicate significant differences at $P \leq 0.05$.

Table 3. *Leaf necrotic area measured by Quant 1.0.1-R1 software*

Values for leaf necrotic area are the mean ±standard deviation from three replicates.

Plant lines tested	Paraquat concentration			
	0 μM	1 μM	2 μM	4 μM
Control[a]	0%	36.0±0.6%	61.8±0.8%	97.0±0.3%
TP55-S2	0%	13.6±0.2%	21.7±0.1%	29.5±0.7%
TP55-S3	0%	14.1±0.7%	46.2±0.6%	56.9±0.3%

[a] Untransformed, wild-type plants.

Oxidative stress promotes increased levels of ROS and subsequent lipid peroxidation. The levels of lipid peroxidation were measured in paraquat-treated leaves on the basis of the accumulation of malondialdehyde (MDA), a major product of lipid peroxidation (Esterbauer *et al.*, 1991). As shown in Fig. 7, significant differences in the levels of MDA were detected among the paraquat-treated lines. A significant reduced accumulation of lipid peroxidation-derived reactive aldehydes was observed in both TP55-S2 and TP55-S3 transgenic tobacco as compared to control, untransformed lines. These results indicate that antiquitin-like ALDH7 may be involved in the detoxification of aldehydes generated by lipid peroxidation after the formation of ROS.

Discussion

*ALDH*s make a large gene family in plants comprising at least 11 subfamilies whose members are involved in development and/or stress adaptation. Among the stress-related ALDHs, the plant ALDH3 and ALDH5 families are involved in detoxification of aldehydes, whereas the ALDH10 and ALDH11 families act primarily in cellular osmoregulation by catalysing the synthesis of osmoprotectants. Members of the ALDH7 family of aldehyde dehydrogenase superfamily have been identified in humans, fishes and plants, but functional studies are lacking. The characterization of an *ALDH7* gene is described here and it is shown that it can provide a protective function for the plants under a wide range of stresses such as dehydration, salinity, and oxidative stress.

Despite the fact that no biochemical pathway has been assigned to the ALDH7 proteins, it has been hypothesized that this family of ALDHs, that includes the human antiquitin, plays a direct role in the maintenance of osmotic homeostasis. This hypothesis has been raised based on three observations: (i) all the plant ALDH7 proteins already described have been shown to be induced by dehydration and salinity (Guerrero *et al.*, 1990; Stroeher *et al.*, 1995; Kirch *et al.*, 2005), (ii) the expression of the human antiquitin predominated in organs that depend on a tight osmotic balance for proper function (Skvorak *et al.*, 1997), and (iii) representatives of several ALDH families have been demonstrated to be involved in osmoregulation (Kirch *et al.*, 2004). However, two lines of evidence that ALDH7 may function in aldehyde detoxification have been provided. First, the ectopic expression of *GmTP55* in both *Arabidopsis* and tobacco resulted in cross-tolerance to multiple abiotic stresses that share the oxidative stress, as a common component. Oxidative stress is associated with increased levels of ROS that cause peroxidation of

membrane lipids and consequent accumulation of toxic degradation products, largely represented by aldehydes. The *GmTP55*-mediated cross-tolerance mechanism may underscore a potential role of ALDH7 in detoxification of reactive aldehydes derived from lipid peroxidation. Second, the broad stress tolerance of the *GmTP55*-overexpressing transgenic lines was correlated with a decrease in lipid peroxidation, as detected by a significant decrease in the accumulation of MDA in transgenic lines. Similar results were obtained by overexpression of *At-ALDH3* in *Arabidopsis* and *MsALR* from alfafa (*Medicago sativa*) in tobacco (Oberschall *et al.*, 2000; Sunkar *et al.*, 2003). The analyses of both *Arabidopsis* and tobacco transgenic lines indicate that overexpression of both the aldehyde dehydrogenase (*At-ALDH3*) and aldehyde reductase (*MsALR*) improves stress-tolerance most likely by scavenging toxic aldehydes and thus reducing lipid peroxidation. Although further studies will be necessary to discern the precise mechanism of *GmTP55*-mediated stress tolerance, its potential aldehyde detoxifying activity may provide a link for the apparent effectiveness of GmALDH7 protective properties against several abiotic stresses. Nevertheless, these data did not rule out the possibility that lipid peroxidation was reduced in transgenic leaves by activation of alternative osmolyte- producing pathways. Although several functions have been attributed to osmolytes, such as stabilization of cytoplasmic components, water retention, and ion sequestration, the underlying mechanism of osmolyte action and their physiological role under dehydration remain largely unknown (Hare *et al.*, 1998).

Different approaches to increase stress tolerance have been undertaken by manipulating and reprogramming the expression of endogenous stress-related genes. In general, strategies targeting transcription factor expression have been shown to be effective due to the consequent up-regulation of many downstream genes (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Hsieh *et al*., 2002). However, enhanced stress tolerance has also been achieved by changing the expression of a single downstream gene (Zhu, 2001; Kumar *et al.*, 2004). In this case, two distinct functional classes of genes have been demonstrated to be effective targets for engineering stress tolerance. The first class encompasses genes involved in mechanisms that prevent intracellular stress build-up. An example in this class includes the Na^+/H^+-antiporter gene whose overexpresssion in tomato and *Arabidopsis* promoted a significant increase in NaCl resistance (Apse *et al.*, 1999). The second class acts functionally in the detoxification of toxic by-products and in cellular repair, such as the antioxidant system genes (Gupta *et al.*, 1993; Badawi *et al.*, 2004). The aldehyde dehydrogenase superfamily has been shown to encompass both classes of downstream genes, as the ALDH10 gene family is involved in the maintenance of osmotic homeostasis (Kirch *et al.*, 2004), whereas genes from the ALDH3 family are clearly involved in aldehyde detoxification-mediated stress tolerance mechanisms in plants (Sunkar *et al.*, 2003). However, the majority of these *ALDH* genes are involved in some aspects of intermediary metabolism in plants and, as a consequence, their overexpression, while it has been shown to be effective on enhancing stress tolerance, also adversely impacts plant growth under optimal growth conditions. Thus, the pleiotropic effects resulted from up-regulation of *ALDH* genes under optimal conditions may counteract advantages of transgene-mediated improvements in the physiological performance of the transgenic plants under stress conditions. In this context, these results on *ALDH7* ectopic expression in transgenic plants incorporate relevant implications for agriculture as the *ALDH7*-overexpressing lines showed enhanced resistance to water deficit, salinity and oxidative stress, but did not exhibit growth-related phenotypes under normal conditions. In fact, the transgenic plants were phenotypically indistinguishable from the control plants under favourable growth conditions. Collectively, these results indicate that *GmALDH7* may be an effective engineering target to improve environmental stress resistance in agriculturally important crops without affecting the overall field performance of transgenic crops under optimal conditions.

Acknowledgements

This research was supported by the Brazilian Government Agency, CNPq Grants 50.6119/2004-1, 501189/2004-1, 500605/2004-1, and 470787/2004-0 (to EPBF). SMR was supported by a FAPEMIG graduate fellowship from the Minas Gerais State, Brazil. MOA and APSG were supported by a CNPq undergraduate scholarship from the Brazilian Government. The authors thank Dr Andréa Miyasaka and Dr Luiz Orlando de Oliveira for helpful discussions and Dr Alessandro J Waclawovsky for technical assistance.

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