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RESEARCH PAPER

Overexpression of the *AtLOS5* gene increased abscisic acid level and drought tolerance in transgenic cotton

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Abstract

Drought is the major environmental stress that limits cotton (*Gossypium hirsutum* L.) production worldwide. LOS5/ABA3 (LOS5) encodes a molybdenum co-factor and is essential for activating aldehyde oxidase, which is involved in abscisic acid (ABA) biosynthesis. In this study, a *LOS5* cDNA of *Arabidopsis thaliana* was overexpressed in cotton cultivar Zhongmiansuo35 (Z35) by *Agrobacterium tumefaciens*-mediated transformation. The transformation and overexpression of *AtLOS5* were assessed by PCR and RT-PCR analysis. Detached shoots of transgenic cotton showed slower transpirational water loss than those of Z35. When pot-grown 6-week-old seedlings were withheld from watering for 3 d, transgenic cotton accumulated 25% more endogenous ABA and about 20% more proline than Z35 plants. The transgenic plants also showed increased expression of some drought-responding genes such as *P5CS* and *RD22*, and enhanced activity of antioxidant enzymes such as superoxide dismutase, peroxidase, and ascorbate peroxidase. Their membrane integrity was considerably improved under water stress, as indicated by reduced malondialdehyde content and electrolyte leakage relative to control plants. When the pot-grown plants were subjected to deficit irrigation for 8 weeks (watering to 50% of field capacity), transgenic plants showed a 13% increase in fresh weight than the wild type under the same drought condition. These results suggest that the *AtLOS5* transgenic cotton plants acquired a better drought tolerance through enhanced ABA production and ABA-induced physiological regulations.

Key words: abscisic acid, *AtLOS5*, antioxidant enzymes, drought tolerance, proline, transgenic cotton.

Introduction

Cotton (*Gossypium hirsutum* L.) is an important commercial crop grown worldwide as a source of fibre and edible oil. As a glycophytic plant, cotton shows higher drought and salt tolerance than other major crops such as wheat and rice, so it is classified as a drought- and salt-tolerant crop. Drought stress greatly impacts on cotton growth and limits fibre yield and lint quality, so plant breeding has been used to improve drought tolerance of cotton. Compared with conventional selection in cotton breeding, genetic transformation technology to improve agronomic traits and economic characteristics of crops by incorporating exogenous genes encoding the desired transgenic traits has become an efficient way to accelerate the breeding process (Zhang *et al.*, 2011).

Drought stress, a major environmental stress that negatively impacts on growth and production of crops worldwide, induces a range of physiological and biochemical responses in plants. It triggers expression of stress-related genes, accumulation of metabolites such as abscisic acid (ABA) or osmotically active compounds, and synthesis of specific proteins (Shinozaki and Yamaguchi-Shinozaki,

Abbreviations: AAO, abscisic aldehyde oxidase; ABA, abscisic acid; APX, ascorbate peroxidase; DW, dry weight; FW, fresh weight; MDA, malondialdehyde; NCED, 9-cis-epoxycarotenoid dioxygenase; POD, peroxidase; RWC, relative water content; SE, standard error; SOD, superoxide dismutase; TW, turgid weight; ZEP, zeaxanthin epoxidase.

2007). In fact, there have been many efforts in crops to improve drought tolerance and productivity under water-limiting conditions.

Hundreds of genes induced under drought conditions have been identified, cloned, and used as candidate genes in genetic engineering. These examples include proteins that function in abiotic stress tolerance, such as late embryogenesis abundant proteins, and key enzymes for osmolyte biosynthesis, and regulatory proteins involved in signal transduction regulation or stress-responsive gene expression, such as transcription factors and protein kinases (Shinozaki and Yamaguchi-Shinozaki, 2007).

ABA, classified as a stress hormone, plays significant roles in the regulation of plant growth and development and in plant responses to environmental stresses (Xiong *et al.*, 2001; Xiong and Zhu, 2003). Genes involved in ABA biosynthesis have been cloned and characterized in *Arabidopsis thaliana* and other plant species (Xiong and Zhu, 2003; Taylor *et al.*, 2005). These genes included zeaxanthin epoxidase (ZEP; Marin *et al.*, 1996), which catalyses the epoxidation of zeaxanthin to produce epoxycarotenoid, 9-*cis*-epoxycarotenoid dioxygenase (NCED; Schwartz *et al.*, 1997), which catalyses the cleavage reaction of epoxycarotenoids to produce xanthoxin, and abscisic aldehyde oxidase (AAO; Seo *et al.*, 2000), which catalyses the final step of ABA biosynthesis whereby ABA aldehyde is converted to ABA. Moreover, LOS5/ABA3 (LOS5) is involved in the regulation of ABA biosynthesis by encoding molybdenum co-factor sulfurase, which is required by aldehyde oxidase in the last step of ABA biosynthesis in plants (Bittner *et al.*, 2001; Xiong *et al.*, 2001).

The enzymes involved in ABA biosynthesis have been investigated transgenically to improve plant stress tolerance. For example, transgenic *Arabidopsis* overexpressing *AtZEP* under drought stress showed increased leaf and lateral root development and longer primary roots compared with control plants, and exhibited much higher expression of the endogenous stress-responsive genes *RD29A* and *Rab18* than wild-type plants under salt stress (Park *et al.*, 2008). When subjected to drought stress, overexpression of *NCED* improved drought tolerance and caused an increase in endogenous ABA or a reduction in transpiration rate from leaves in transgenic *A. thaliana* (Iuchi *et al.*, 2001), transgenic tobacco (*Nicotiana plumbaginifolia*) (Qin and Zeevaart, 2002), transgenic creeping bentgrass (*Agrostis palustris*) (Aswath *et al.*, 2005), and transgenic tomato (*Solanum lycopersicum*) (Thompson *et al.*, 2000, 2007; Tung *et al.*, 2008) with improved tolerance. *AtLOS5*-overexpressing transgenic rice (*Oryza sativa*) (Xiao *et al.*, 2009) and transgenic tobacco (*Nicotiana tabacum*) (Yue *et al.*, 2011) under water-deficit conditions had better drought tolerance than non-transgenic controls.

Based on these studies, *AtLOS5* was introduced into cotton by *Agrobacterium tumefaciens*-mediated transformation under the control of a superpromoter, and several independent transgenic lines were produced in this study. To test the function and potential use of *AtLOS5* in improving drought tolerance of cotton, transformed phenotypes of transgenic cotton were evaluated by determining stress-related physiological and biochemical parameters under water-deficit conditions in a growth chamber.

Materials and methods

Construction of the binary vector

A modified pCAMBIA1300 vector was constructed by adding the superpromoter, which consisted of three copies of the octopine synthase enhancer in front of the manopine synthase promoter, as a *Sal*I–*Xba*I fragment, into the pCAMBIA1300 binary vector containing a hygromycin phosphotransferase gene for resistance to hygromycin as a selectable marker. The *LOS5* cDNA of *A. thaliana* ecotype Columbia (Xiong *et al.*, 2001) was provided by Dr J.K. Zhu (University of California, Riverside, CA) and cloned as an *Xba*I–*Kpn*I fragment downstream of the superpromoter in the modified pCAMBIA1300 (Fig. 1). The recombinant plasmid, pCAMBIA1300-*LOS5*, was introduced into *A. tumefaciens* strain EHA105 by freeze-thawing and used for plant transformation.

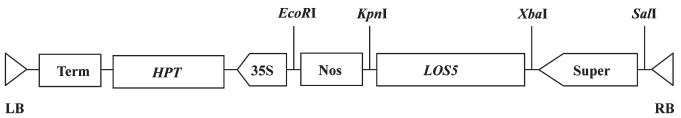

Fig. 1. Schematic representation of the T-DNA region of the binary vector pCAMBIA1300-*LOS5*. LB, left border; RB, right border; *HPT*, hygromycin phosphotransferase gene; Super, super-promoter; 35S, cauliflower mosaic virus (CaMV) 35S promoter; Nos, nopaline synthase terminator; Term, CaMV 35S terminator.

Cotton transformation

Transformation of the cotton cultivar Zhongmiansuo 35 (Z35) by *A. tumefaciens* was performed as described previously (Gould and Magallanes-Cedeno, 1998) with minor modifications. Briefly, *A. tumefaciens* strain EHA105 harbouring pCAMBIA1300-*LOS5* was cultured at 28 °C and 250 r.p.m. overnight in a modified Luria–Bertani medium (Sambrook *et al.*, 1989) supplemented with 50 mg l^{-1} of kanamycin and 50 mg l^{-1} of rifampicin. The bacteria were collected and resuspended in half-strength liquid MS (Murashige and Skoog, 1962) medium at an optical density of 0.4–0.6 at 600 nm.

The explants (sterile shoot apices of seedlings) were infected with *Agrobacterium* suspension for 15–20 min and co-cultured on co-cultivation medium (MS salts with 60 μg l^{-1} of acetosyringone, 100 μg l^{-1} of kinetin, 30 g l^{-1} of glucose, and 2.5 g l^{-1} of Phytagel™) with a piece of sterile filter paper on the surface in the dark for 2 d. They were then transferred on to selection medium (MS salts with 100 μg l^{-1} of kinetin, 500 mg l^{-1} of cefotaxime, 15 mg l^{-1} of hygromycin, 30 g l^{-1} of glucose, and 2.5 g l^{-1} of Phytagel) for selection and incubated in a growth chamber at 25 °C (±2 °C) with a light intensity of 50 μmol m^{-2} s^{-1} under a long-day photoperiod (16 h light, 8 h dark) for 4–6 weeks. Hygromycin-resistant shoots were transferred on to recovery medium (MS salts with 250 mg l^{-1} of cefotaxime, 30 g l^{-1} of glucose, and 2.5 g l^{-1} of Phytagel) to restore growth. After 2 weeks, the shoots were grafted onto 6-d-old cotton seedlings of Z35 and placed in a mist chamber at high humidity and 25 °C (±2 °C) for 7 d. The plants were gradually hardened in growth chambers, transferred to large pots with soil, and grown to maturity in the greenhouse.

PCR detection and RT-PCR analysis

Genomic DNA was isolated from the young leaves of control and transgenic cotton according to the cetyltrimethylammonium bromide method (Chaudhary *et al.*, 1999). PCR analysis for detection

of the *LOS5* gene was carried out with specific forward (5′-CTGGGAATGGAACCGTCGTCA-3′) and reverse (5′-GAGCCCGTTTGTAATCCTCGTCTT-3′) primers. PCR amplifications were carried out at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, with a final elongation at 72 °C for 10 min. PCR products were separated by 1% (w/v) agarose gel electrophoresis.

For RT-PCR analysis, total RNA was extracted from the young leaves of control and transgenic cotton under normal and water-deficit conditions using a method described previously (Wan and Wilkins, 1994). Synthesis of cDNA was performed using a Takara RNA PCR kit (AMV) (Takara Biotechnology Co., China). The cDNA samples were used to amplify a *LOS5* segment with forward (5′-GGGAAAGGGTGGAGGAGT-3′) and reverse (5′-GTAGCCAAACCAAGAGCC-3′) primers. The *UBQ7* gene (GenBank accession no. DQ116441) was used as the internal control with specific primers (forward, 5′-GGCCTGATGGGACGAAGA-3′, and reverse, 5′-CAACGTCCAAAGCATCATAGTCA-3′).

Plant growth conditions and drought treatment

Seeds of Z35 and transgenic cotton, the T_3 generation of independent lines L5 and L8, were planted in 3.6-litre pots filled with a mixture of vermiculite and peat (1:1) and grown in a growth chamber with a 14 h photoperiod at a 20/30 °C night/day temperature cycle, with a light intensity of 400 μmol m^{-2} s^{-1} and at 60% relative humidity. Z35 and each transgenic cotton line had seven pots (replicates). Z35 and transgenic cotton seedlings were cultured with a constant supply of Hoagland nutrient solution diluted 1:8.

Drought treatments were performed with 6-week-old cotton seedlings at the seven to eight leaf stage by withholding irrigation, while the control plants were grown normally. Completely expanded leaves at identical positions of Z35 and transgenic cotton seedlings were harvested 3 d after initiation of the stress treatment for biochemical measurements. Photographs were taken 5 d after initiation of the stress treatment.

In another assay of drought tolerance, seedlings of Z35 and transgenic cotton were grown for 3 weeks in 13-litre pots filled with the vermiculite/peat mixture described above. Seedlings of Z35 and transgenic cotton for the control treatments were watered to 75% of the maximum water-holding capacity of the mixture, while for water-deficit treatment the plants were watered to 50% of the maximum water-holding capacity. These cultural conditions were maintained for another 8 weeks and the samples were then harvested for biomass analysis.

Transpirational water loss

Seeds of Z35 and transgenic cotton, the T_3 generation of independent lines L5 and L8, were germinated in sand medium and cultured hydroponically by transferring to pots filled with half-strength Hoagland's nutrient solution in the growth chamber with 40 % relative humidity. Uniform seedlings cultured for 4 weeks were removed from the nutrient solution, detached at the cotyledonary node, and weighed immediately. They were then placed on filter paper to remove water under the growth chamber conditions and weighed at designated time intervals. Each had six replicates. The rate of transpirational water loss was calculated on the basis of the initial fresh weight of the plants.

Relative water content (RWC), electrolyte leakage, and lipid peroxidation

The RWC of leaves and roots was measured according to the method of Parida *et al.* (2007). Fully expanded leaves or roots were cut from plants and the fresh weight (FW) was recorded immediately. The fresh parts were then immersed in distilled water for 4 h and the turgid weight (TW) was recorded. Finally, the dry weight (DW) was recorded after drying for 48 h at 80 °C in an oven. RWC was calculated using the formula: RWC (%)= (FW−DW)/(TW−DW)×100.

Electrolyte leakage was determined by relative conductivity as described by Ai *et al.* (2008). Segments (1 cm^2) were obtained from fully unfolded leaves of cotton for the measurements. Electrolyte leakage was calculated by the following formula: electrolyte leakage (%)=L_t/L_0×100, where the conductivity measurements L_t and L_0 corresponded to the plant leaves before and after boiling in water.

Lipid peroxidation was estimated as the content of malondialdehyde (MDA), as described by Ai *et al.* (2008). Leaf segments (0.5 g) were homogenized in 5 ml of 5% (w/v) trichloroacetic acid solution and centrifuged at 10 000*g* for 10 min. The mixture containing 1 ml of sample supernatant, 4 ml f 20% trichloroacetic acid, and 0.5% (w/v) f thiobarbituric acid was heated at 95 °C for 30 min, quickly cooled, and centrifuged at 10 000*g* for 10 min. MDA content was determined in a spectrophotometer at 532 nm (A_{532}) and corrected for non-specific turbidity at A_{600}.

ABA and proline content

ABA was extracted by grinding flesh leaves using a pre-chilled mortar and pestle on ice, homogenizing in 80% (v/v) methanol containing 1 mM butylated hydroxytoluence, and centrifuging at 4000*g* for 20 min. The supernatant liquid was eluted through a Sep-Pak C18 cartridge (Waters Corp.; Milford, MA) to remove polar compounds and then stored at –20 °C for ELISA. Endogenous ABA content was measured by an indirect ELISA technique, as described by Yang *et al.* (2001).

Free proline content was measured using a method described by Bates *et al.* (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenates were centrifuged at 3000*g* for 20 min. The mixture containing 2 ml of sample supernatant, 2 ml of acetic acid and 2 ml of 2.5% acid ninhydrin was boiled for 30 min, and the absorbance was determined at A_{520}.

Antioxidative enzyme activities

The activities of antioxidant enzymes were determined by homogenizing 0.5 g of leaf tissue in 4 ml of extraction buffer [50 mM sodium phosphate buffer (pH 7.0), containing 1% (w/v) polyvinylpyrrolidone] using a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 10 000*g* for 30 min at 4 °C. The resulting supernatant was collected as a crude enzyme extract and assayed for the activity of superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX), as described by Parida *et al.* (2004).

Expression of the genes P5CS, RD22, *and* DREB2B

Expression of the genes *P5CS* (GenBank accession no. EU417651), *RD22* (GenBank accession no. AY464056), and *DREB2B* (At3g11020) in cotton under normal and water-deficit conditions was analysed by RT-PCR as described above. The primers for these genes were: *P5CS* forward (5′-CAAGCGGTCCAATGCTAT-3′) and reverse (5′-TGATGATACAATCTGTGTGTGC-3′); *RD22* forward (5′-AGGAGGTGGTGGTGTAAACGTCAA-3′) and reverse (5′-ATGAAACACGGATCTCCTCCCGAA-3′); and *DREB2B,* forward (5′-GCTGAAATTCGTGAACCCAACCGT-3′) and reverse (5′-AGCTTGGCATCCGAACCATAGAGT-3′).

Statistical analysis

All experimental data are the means of at least three independent replicates, and results were determined using analysis of variance. Variation among treatment means were compared using Duncan's multiple range test (P<0.05).

Results

Generation of transgenic cotton overexpressing AtLOS5

The construct pCAMBIA1300-*LOS5* was introduced into cotton Z35 via *Agrobacterium* mediation, and 24 independent transgenic lines were produced. The primary transformed plants were designated T_0 plants, and the seeds from self-fertilization of T_0 plants were used to raise T_1 progeny. Transgenic cotton harbouring *AtLOS5* was screened on MS medium containing 15 mg l^{-1} of hygromycin. Two dominant lines, L5 and L8, were selected and homozygous T_4 transgenic plants were used for drought-tolerance analysis. The presence and integrity of the transgene were confirmed by PCR analysis of genomic DNA with specific primers for *AtLOS5* (Fig. 2A). Expression of the *AtLOS5* gene was detected by RT-PCR analysis in transgenic cotton under normal and water-deficit conditions, and drought stress increased the expression of *AtLOS5* (Fig. 2B).

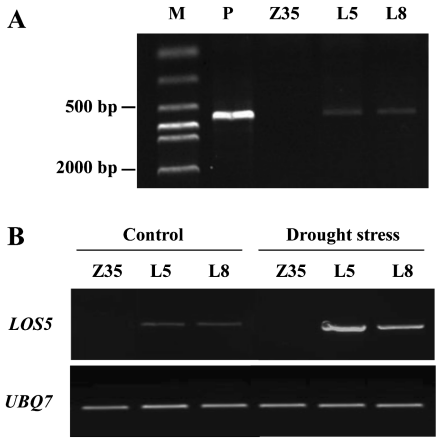

Fig. 2. PCR and RT-PCR analyses of *LOS5* gene in transgenic cotton. (A) PCR analysis using specific primers for the *LOS5* gene to identify two independent transgenic lines. Lane M, DNA marker; lane P, positive control (plasmid pCAMBIA1300-*LOS5*); Z35, non-transgenic control; lanes L5 and L8, transgenic lines. (B) RT-PCR analysis of *LOS5* gene expression in the two transgenic lines under normal and water-deficit conditions. The *UBQ7* gene was used as an internal control.

Drought tolerance in AtLOS5-*overexpressing cotton*

Measurement of transpirational water loss by detached plants showed that transgenic seedlings overexpressing *AtLOS5* lost water more slowly than Z35 cotton in the same period under normal conditions (Fig. 3). The visible growth and morphology of non-transgenic Z35 and transgenic cotton were similar under natural (non-stressed) conditions over a 6-week period (Fig. 4). When subjected to drought stress for 5 d, Z35 and transgenic cotton showed differing degrees of wilting – Z35 plants wilted severely, while transgenic cotton wilted only partially. Non-transgenic Z35 and transgenic cotton had similar FWs of shoots and roots under control conditions (Fig. 5). However, the transgenic cotton plants maintained a 13% higher FW of shoots and a 30% higher FW of roots than that Z35 cotton after 8 weeks of water deficit.

Leaf water status and membrane integrity in AtLOS5-*overexpressing cotton*

The RWC in leaves of Z35 and transgenic cotton under normal conditions were similar (Fig. 6). However, transgenic cotton maintained a higher RWC of 79% compared with Z35 plants with a RWC of 70%.

Drought stress increased oxidative damage in the cotton leaves, as indicated by membrane lipid peroxidation, measured as MDA content (Fig. 6B). Drought stress enhanced MDA production in the leaves of both Z35 and transgenic cotton compared with the control plants. MDA production without drought stress was similar in transgenic and Z35 cotton, whereas the MDA content in drought-stressed plants was 14% lower in the leaves of transgenic cotton compared with Z35 plants.

The background level of electrolyte leakage without drought stress was similar for Z35 and transgenic cotton seedlings (Fig. 6C). Drought stress greatly increased

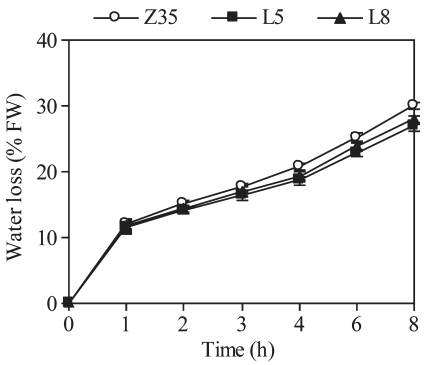

Fig. 3. Comparison of rates of transpirational water loss from detached shoots of non-transgenic Z35 and the transgenic cotton lines L5 and L8. Water loss is expressed as a proportion of the initial FW. Values are means ±standard error (SE) for six plants.

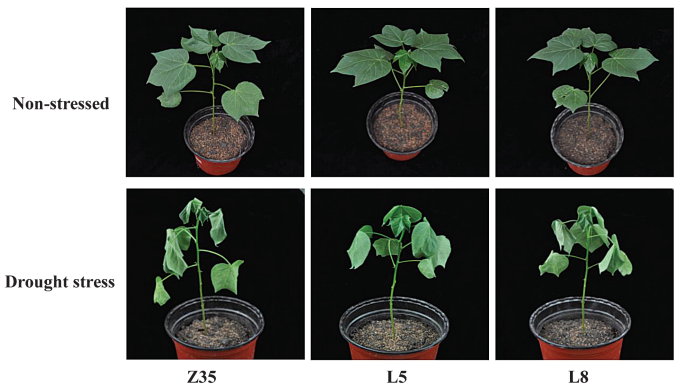

Fig. 4. Six-week-old seedlings of non-transgenic Z35 and the transgenic cotton lines L5 and L8 subjected to drought stress for 5 d. *LOS5*-overexpressing cotton showed less wilting compared with Z35 plants.

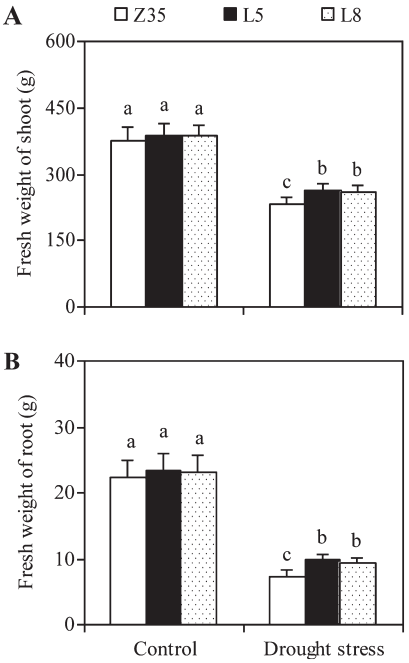

Fig. 5. Biomass of non-transgenic Z35 and the transgenic cotton lines L5 and L8 grown in the pots under normal and water-deficit conditions. Three-week-old seedlings were watered to 50 % of the maximum water-holding capacity of the pots for 8 weeks as water-deficit treatment. For the control, seedlings were watered to 75 % of this value. Values are means ±SE, Values with the same letter were not significantly different according to Duncan's multiple range tests (P<0.05).

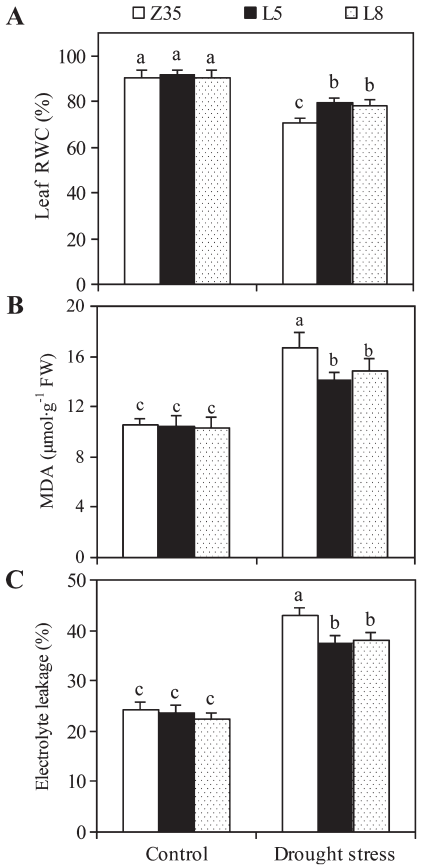

Fig. 6. Leaf RWC (A), MDA content (B), and electrolyte leakage (C) in leaves of non-transgenic Z35 and the transgenic cotton lines L5 and L8 under normal and water-deficit conditions. Values are means ±SE. Values with the same letter were not significantly different according to Duncan's multiple range tests (P<0.05).

electrolyte leakage in Z35 leaves by 76% and in transgenic cotton by 64%.

Antioxidant enzymes in AtLOS5-*overexpressing cotton*

Baseline levels of the antioxidant enzymes SOD, POD, and APX under non-stressed conditions were similar for transgenic and Z35 cotton (Fig. 7). However, drought stress markedly increased the activities of the antioxidant enzymes in the leaves of both Z35 and transgenic cotton. Transgenic cotton under drought stress exhibited higher activities of SOD by 17%, POD by 21%, and APX by 15% compared with Z35 plants.

ABA and proline in AtLOS5-*overexpressing cottons*

Accumulation of endogenous ABA and proline under normal conditions was similar for both Z35 and transgenic cotton (Fig. 8). Drought stress greatly increased the endogenous ABA and proline contents in the leaves of both Z35 and transgenic cotton, but transgenic cotton accumulated 25% more endogenous ABA and 26% more proline than Z35 cotton.

Drought-related gene expression in AtLOS5-*overexpressing cotton*

The drought-related genes *P5CS*, *RD22*, and *DREB2B* had low expression under normal conditions, but their expression was greatly promoted by water-deficit stress (Fig. 9). Although expression of *RD22* and *DREB2B* under water-deficit conditions was improved to varying degrees in Z35 and transgenic cotton, the expression of *P5CS* in *AtLOS5*-overexpressing cotton, especially in line L5, was higher than in Z35 plants.

Discussion

Previous studies have demonstrated that transgenic rice (Xiao *et al.*, 2009) and transgenic tobacco (Yue *et al.*, 2011) showed higher expression levels of *AtLOS5* under normal conditions compared with non-transgenic plants. Consistent with these studies, the *AtLOS5* gene driven by the super-promoter had higher expression levels in transgenic cotton than in non-transgenic plants under both normal and water-deficit conditions (Fig. 2B). As reported previously in *Arabidopsis* (Xiong *et al.*, 2001), the transcript levels of *AtLOS5* in transgenic cotton were increased significantly in response to drought stress.

ABA accumulation is a common trait of transgenic plants that overexpress genes involved in ABA biosynthesis, such as *AtZEP* in transgenic *Arabidopsis* (Park *et al.*, 2008), *NCED* in transgenic lines of tomato (Thompson *et al.*, 2000), *Arabidopsis* (Iuchi *et al.*, 2001), tobacco (Qin and

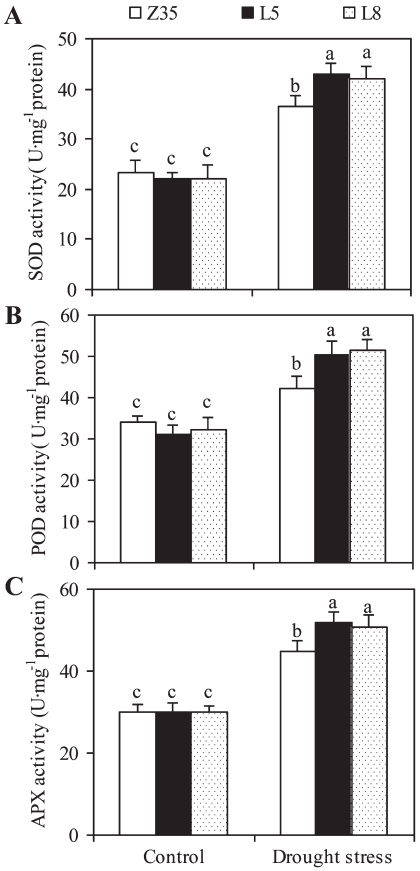

Fig. 7. Activities of SOD (A), POD (B), and APX (C) in leaves of non-transgeneic Z35 and the transgenic cotton lines L5 and L8 under normal and water-deficit conditions. Values are means ±SE. Values with the same letter were not significantly different according to Duncan's multiple range tests (P<0.05).

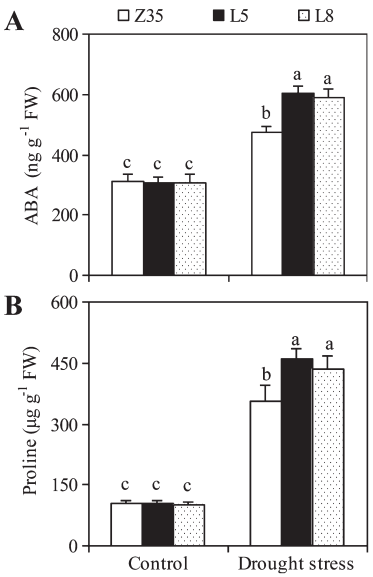

Fig. 8. Levels of endogenous ABA (A) and proline (B) in the leaves of non-transgenic Z35 and the transgenic cotton lines L5 and L8 under normal and water-deficit conditions. Values are means ±SE. Values with the same letter were not significantly different according to Duncan's multiple range tests (P<0.05).

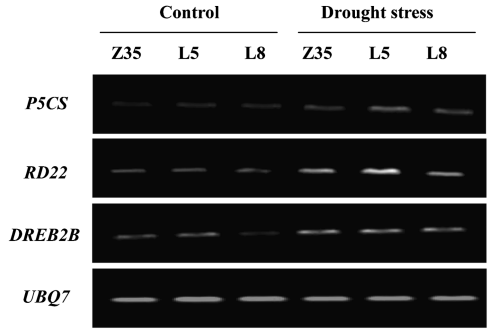

Fig. 9. RT-PCR expression analysis of genes *P5CS*, *RD22*, and *DREB2B* in both non-transgenic Z35 and the transgenic cotton lines L5 and L8 under normal and water-deficit conditions. The gene *UBQ7* was used as an internal control.

Zeevaart, 2002), and bentgrass (Aswath *et al.*, 2005), or *AtLOS5* in transgenic tobacco (Yue *et al.*, 2011). The accumulation of ABA can be explained by a model for stress induction of ABA biosynthesis in *Arabidopsis* (Xiong *et al.*, 2002). Osmotic stress first induces expression of *NCED*, an early limiting step in controlling drought stress-induced ABA biosynthesis. Initially, accumulation of ABA potentiates expression of other ABA biosynthesis genes such as *AAO*, *LOS5*, and *ZEP*, which leads to more ABA biosynthesis. This coordinated increase in the transcription of all ABA biosynthesis genes would result in a more rapid and sustained increase in ABA biosynthesis. Therefore, overexpression of *AtLOS5* increased ABA accumulation greatly in transgenic cotton under water-deficit conditions (Fig. 8A).

As a major physiological signal, ABA affects many stress-adaptation responses, such as regulation of shoot and root growth and limiting transpiration rate, thereby reducing wilting of plants (Lata and Prasad, 2011). Moreover, ABA modifies gene expression, and a large group of stress-responsive genes are regulated by stress-induced ABA. Overexpression of these genes involved in ABA biosynthesis leads to increased ABA production and reduced leaf transpiration under drought conditions, which consequently increases drought tolerance in transgenic plants.

Transgenic plants under drought conditions showed reduced leaf transpiration in tomato overexpressing *LeNCED1* (Thompson *et al.*, 2000), in *Arabidopsis* overexpressing *AtNCED3* (Iuchi *et al.*, 2001) and in tobacco overexpressing *PvNCED1* (Qin and Zeevaart, 2002). Detached transgenic tobacco overexpressing *AtLOS5* had lower transpirational water loss than control plants under normal conditions (Yue *et al.*, 2011). Moreover, transgenic *Arabidopsis* overexpressing *AtZEP* in response to drought had higher FW compared with control plants (Park *et al.*, 2008). Overexpression of *AtLOS5* in rice under water-deficit conditions increased relative yield production and spikelet fertility of transgenic plants (Xiao *et al.*, 2009). Consistent with these reports, transgenic cotton overexpressing *AtLOS5* showed reduced transpirational water loss (Fig. 3) and enhanced drought tolerance (Fig. 4). Furthermore, transgenic

cotton overexpressing *AtLOS5* under water-deficit conditions maintained a higher RWC (Fig. 6A) and retained more shoot or root FW than non-transgenic controls (Fig. 5).

Reactive oxygen species triggered by drought stress can attack cellular macromolecules, thereby causing membrane damage or MDA accumulation, and affect protein synthesis and stability in plants. Meanwhile, water-deficit stress-induced ABA triggers induction of the antioxidant defence system and upregulates the activities of antioxidant enzymes such as SOD, CAT, and APX to protect plants from oxidative stress (Jiang and Zhang, 2002). Both transgenic cotton overexpressing *AtLOS5* and non-transgenic Z35 plants under water-stress conditions had greatly increased electrolyte leakage and MDA production (Fig. 6B, C) and increased activities of the antioxidant enzymes SOD, POD, and APX (Fig. 7). However, efficient scavenging of reactive oxygen species in transgenic cotton protected membranes and macromolecules better and maintained less electrolyte leakage and MDA content than Z35 plants during drought stress, which contributed to enhanced drought tolerance of transgenic cotton overexpressing *AtLOS5*.

To further define which stress-responsive genes are regulated by stress-induced ABA, the drought-related genes *DREB2B*, *RD22*, and *P5CS* were analysed in transgenic cotton and Z35 plants under normal or water-deficit conditions. *DREB2B*, which is involved in osmotic-responsive gene expression in the ABA-independent stress-tolerance pathway, is induced by drought or high-salt stress (Liu *et al.*, 1998). In our studies, water-deficit stress increased the expression of *DREB2B* in transgenic cotton overexpressing *AtLOS5* and in non-transgenic Z35 plants, but there were no obvious differences in *DREB2B* levels (Fig. 9). Conversely, one ABA-dependent signal transduction pathway in drought and high salinity stress responses, MYC and MYB transcription factors, synthesized following accumulation of endogenous ABA, bound *cis* elements in the promoter and cooperatively activated the dehydration-responsive gene *RD22* (Abe *et al.*, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007). We found that the *RD22* gene could be induced by drought or ABA (Fig. 9). Under water-deficit conditions, *RD22* was expressed more in *AtLOS5*-overexpressing cotton, especially in the line L5, than in non-transgenic Z35 plants.

P5CS, which catalyses the first two steps in proline biosynthesis, plays a key role in biosynthesis under osmotic stress (Savoure *et al.*, 1995; Yoshiba *et al.*, 1995). Expression of the *P5CS* gene was induced by both water-deficit in non-transgenic Z35 cotton and improved endogenous ABA in transgenic cotton overexpressing *AtLOS5* (Fig. 9). The increased expression of *P5CS* further increased proline production in transgenic cotton. Drought stress notably increased proline content in both transgenic cotton overexpressing *AtLOS5* and in non-transgenic Z35 plants, but *AtLOS5*-overexpressing cotton accumulated more proline than Z35 plants (Fig. 8B). Proline accumulates in many plant species in response to environmental stress, such as drought and high salinity, and its accumulation frequently correlates with tolerance to drought or salt stress in plants (Ben *et al.*, 2008; Parida *et al.*, 2008).

In conclusion, overexpression of the *AtLOS5* gene in transgenic cotton seedlings improved drought tolerance, as indicated by increased FW in the growth chamber. The improved drought tolerance is probably attributed to enhanced expression of ABA-responsive genes as a result of increased ABA levels under the drought stress. Further study is required to determine field performance of the transgenic cotton.

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