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Overexpression of *ScALDH21* gene in cotton improves drought tolerance and growth in greenhouse and field conditions

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Abstract Aldehyde dehydrogenase (ALDH) is essential for scavenging redundant aldehydes when plants are exposed to stress. The aim of the present study was to validate the ectopic expression of the *ScALDH21* gene, which is isolated from *Syntrichia caninervis*, an extremely drought-tolerant moss, to improve drought tolerance in cotton (*Gossypium hirsutum* L.). In our study, the *ScALDH21*-transformed cotton was identified via PCR, RT-PCR, and DNA gel blotting, and the growth and physiological characteristics related to drought tolerance were compared between the transgenic cotton (TC) and non-transgenic cotton (NT) grown in a greenhouse and in field conditions. The results indicated that TC accumulated approximately 11.8–304 % more proline than did NT under drought stress, and produced a lower concentration of lipid peroxidation-derived reactive aldehydes and had a higher peroxidase activity under oxidative stress. Moreover, TC showed reduced loss of the net photosynthetic rate compared with NT. Under field conditions, TC showed greater plant height, larger bolls, and greater cotton fiber yield than NT, but no significant difference in fiber quality between TC and NT following different water-withholding treatments. These results suggest that overexpression of *ScALDH21* can greatly improve the drought tolerance of cotton without reduction in yield and fiber quality.

Keywords *Syntrichia caninervis* · *ScALDH21* · Transgenic cotton · Drought tolerance · Cotton yield

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Introduction

Abiotic stress limits crop productivity (Araus et al. 2002; Tester and Bacic 2005), and various stressors may lead to the disruption of a plant's water status (Verslues et al. 2006), which has become a major environmental problem nowadays (Gale 2002). Cotton (*Gossypium hirsutum* L.) is an important commercial crop grown worldwide, and is considered to be a drought-tolerant crop; however, its sensitivity to water deficiency varies greatly among the different genotypes (Zhang et al. 2011). Therefore, it is imperative to improve its drought tolerance (Light et al. 2005). Compared to conventional selection, genetic transformation technology aims at improving the agronomic traits and economic characteristics of crops by incorporating exogenous genes encoding desired traits. It has become an efficient way to accelerate the breeding process of cotton (Visarada et al. 2009; Sinclair 2011; Deikman et al. 2012). Currently, many efforts are being made to improve the drought tolerance and productivity of crops under water-limited conditions (Zhang et al. 2011). Hundreds of genes that are induced under drought conditions have been identified and used as candidates in genetic engineering (Yue et al. 2012), such as the genes of antioxidative enzymes and enzymes involved in osmolyte biosynthesis (Lv et al. 2007; Zhang et al. 2012), and the regulatory or stress-responsive genes. However, there are few successful studies focusing on genetic modifications to increase the drought tolerance of cotton (but see Pasapula et al. 2011; Lv et al. 2007, 2009).

Drought can cause osmotic stress by reducing the chemical activity of water and affecting the cell turgor (Zhu 2001), which will produce redundant reactive oxygen substances (ROS). Excess ROS can result in membrane lipid peroxidation, protein oxidation, enzyme inhibition, and damage to nucleic acids (Ahmad et al. 2008, 2010), ultimately leading to cell death (Asada 1999; Dat et al. 2000). Antioxidants convert metabolized ROS into less harmful chemicals, thus playing an important role in maximizing cellular function and minimizing undesirable environmental stressors that limit yield and quality (Apel and Hirt 2004). Currently, many antioxidant enzymes have been experimentally proven to be capable of alleviating the damage caused by ROS, making them potential candidate genes for crop improvement via genetic engineering. The overexpression of antioxidant glutathione reductase (GR), ascorbate peroxidase (APX), and catalase has been proven to confer tolerance to various abiotic stressors (Chaves and Oliveira 2004) and to greatly improve the drought tolerance of transgenic crops (Eltayeb et al. 2010; Park et al. 2012; Zhang et al. 2014).

The enzymatic scavenging of ROS may also involve proteins of the aldehyde dehydrogenase (ALDH) superfamily (Wenzel et al. 2008). ROS lead to elevated oxidative stress and damage to the lipid membrane accompanying the accumulation of 200 types of aldehydes, many of which are highly reactive and toxic (Singh et al. 2013). The ALDH superfamily contains NAD(P)-dependent enzymes that metabolize endogenous and exogenous aldehydes to their corresponding carboxylic acids using the coenzyme NAD(P)+ with the production of NAD(P)H (Kirch et al. 2004), and thereby mitigate oxidative/electrophilic stress. The overexpression of *ALDH* in *Arabidopsis thaliana* confers tolerance, and the ROS content of the transgenic plant in detached leaves was significantly lower than that of non-transgenic cotton (NT) (Kotchoni et al. 2006; Xu et al. 2013). These results highlight the role of ALDHs in cell metabolism and stress physiology. In recent years, the enhancement of drought and salt tolerance or the improvement of crop yields via the overexpression of *ALDH* (Zhang et al. 2012) has been reported. However, only a few of the transformed plant ALDHs have reported functions, particularly in crops.

In our previous study, we cloned *ScALDH21* from *Syntrichia caninervis*, a native extremely drought-tolerant moss found in the Gurbantunggut Desert of Xinjiang, China. The overexpression of *ScALDH21* in *E. coli* and tobacco led to a higher drought tolerance compared with the control (Yang et al. 2012). It has been reported that ALDH21A1 is a eukaryotic aldehyde dehydrogenase and is transcriptionally activated by abiotic stress (Chen et al. 2002). However, we wanted to know whether the overexpression of *ScALDH21* (from moss) can confer tolerance to cotton under water deficit. In the present study, several independent transgenic lines were generated via *Agrobacterium*-mediated transformation. To test the function and potential use of *ScALDH21* in improving the drought tolerance of cotton, the phenotypic growth traits of the transgenic cotton were evaluated, and the stress-related physiological and biochemical parameters were measured under water-deficient conditions in both growth

chambers and in field conditions. Furthermore, the seed cotton yield and the fiber quality of the transgenic lines were monitored in field conditions.

Materials and methods

Construction of the plant expression vectors and the plant transformation

The open reading frame of the *ScALDH21* cDNA (GQ245973) was amplified and cloned into the *Sal*I-*Kpn*I site of the pCAMBIA2300 plasmid, such that *ScALDH21* was under the control of the CaMV 35S promoter (Fig. S1a). The recombinant vector contains the neomycin phosphotransferase gene NPTII as the selectable marker and was introduced into the *Agrobacterium tumefaciens* strain EHA105, which was used to transform the cotton according to the protocol from Bayley et al. (1992) as modified by Zhang (2012). The cotton receptor material used in these experiments was the cultivar Xinnongmian 1 (*Gossypium hirsutum*), a newly developed variety with good agronomic traits and economic characteristics developed by the Economic Crop Research Institute, Xinjiang Academy of Agricultural Sciences, China.

PCR, RT-PCR detection, and Southern blot analysis

Genomic DNA was isolated from the cotton seedlings at the five-leaf stage using the cetyltrimethylammonium bromide method (Chaudhary et al. 1999). A PCR detection to identify the *ScALDH21* transgenic plants was performed using specific forward and reverse primers (Table S1). The PCR amplification was carried out as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The PCR products were subjected to 1 % (w/v) agarose gel electrophoresis.

For Southern blot detection, a DNA extraction was performed using the procedure described by Paterson et al. (1993). The plant DNA was extracted from cotton seedlings grown in greenhouses (T5 generation). A total of 90 μg of genomic DNA was resolved via gel electrophoresis and transferred onto a positively charged nylon membrane (Amersham, USA). The digoxygenin dUTP-labeled probe was generated from the PCR production of the *ScALDH21* gene. The hybridization was performed at 42 °C. The hybridization, washing, and chemiluminescence detection were carried out according to the manufacturer's instructions (Roche, Germany).

Total RNA was extracted from the youngest leaves of the cotton plants according to the method from Song and Allen (1997), with the addition of DNase I (TaKaRa, Dalian, China) to remove the genomic DNA. Synthesis of cDNA was performed by using the RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's protocol, and the *ScALDH21* cDNA was used as template in the RT-PCR detection using specific primers (Table S1). In previous experiments, we screened the reference genes in cotton (Yue et al. 2012) and selected *UBQ7* gene (GenBank accession no. DQ116441) as the internal control and amplified it with specific primers (Table S1).

Plant growth conditions and drought treatments

Plant materials

The T3, T4, and T5 generations of the *ScALDH21* transgenic cotton were grown in a greenhouse, outdoors, and in a cotton field respectively, and were analyzed for their drought tolerance performance during 2011, 2012, and 2013. Three *ScALDH21* transgenic lines, 16, 39, and 42, were analyzed in the present study. Young leaves sampled from the cotton plants were immediately frozen in liquid nitrogen, and stored at −80 °C. All of the physiological and biochemical parameters were measured at three different treatment times before drought stress, after withholding water, and after 48 h of sufficient watering for recovery. In addition, to detect the effect of water deficiency on the biomass traits, the plant height, leaf number, root length, lateral root number, stem diameter, fresh weight, leaf diameter, bud number, and total biomass of the transgenic lines and the non-transgenic plants were determined in each experiment.

Cotton growth and drought stress in a greenhouse

The T3 generation of the transgenic lines and the non-transgenic cotton plants were grown in pots (diameter, 31 cm; height, 28 cm) containing homogeneous soil in a greenhouse [approximately 25 % relative humidity (RH), 25 ± 2 °C, natural light]. The pots were fully watered every 5 days. Four pots (replicates) with one plant in each were tested for each line.

The drought treatment was performed using 6-week-old cotton plants by withholding irrigation; the control plants were grown normally. After 18 days of treatment (the control plant was becoming wilted), the plants were fully watered.

Cotton growth and drought stress outdoors (T4 generation)

To analyze the response of the transgenic cotton plants to drought stress at different growth stages, different transgenic lines of the T4 generation were cultivated in larger pots (length, 90 cm; width, 35 cm; height, 45 cm) containing homogeneous soil in outdoor conditions in Urumqi (situated at 43°87′ N, 87°56′ E), Xinjiang, China. The temperature in Urumqi ranges from 15 to 30 °C during the growing season from April to October, and the RH is 15–25 %. Rainfall was blocked out using a plastic shelter during the experimental period. Natural illumination (700–1500 μmol m^{-2} s^{-1}) was used for the plant growth. A total of 15 cotton plants per pot were grown for each transgenic line, and the pots were watered to field capacity every 6 days.

The drought treatment was performed in two groups. In group one, drought treatment was performed using 8-week-old cotton plants at the seven-to-nine-leaf stage by withholding irrigation for 10 days (the soil water content was reduced to approximately 5 %). Normal irrigation was then resumed until the cotton began flowering, then another water-withholding period was imposed for 20 days (the soil water content was approximately 5 %, and the NT plants were severely wilted) (Fig. S2). In group two, the drought treatment was applied at the flowering stage by withholding irrigation for 20 days (the NT cotton plants became wilted). After treatment, the plants were watered normally. All of the samples for measurements of the physiological and biochemical parameters were taken at three different time conditions (before the drought stress, after 10 or 20 days of withholding water, and after 48 h of sufficient watering for recovery).

Assay of lipid peroxidation, antioxidative enzyme activities, and proline and soluble sugar content

Lipid peroxidation was estimated by measuring the malondialdehyde (MDA) concentration according to the method from Zhang et al. (2014). The enzyme extraction followed the method from Kochba et al. (1977). The POD activity was determined by monitoring the increase in absorbance of guaiacol at 470 nm using the method of Zhang et al. (2014).

Young leaves were used to determine the free proline and soluble sugar contents. The proline content was measured as previously described by Zhang et al. (2014) with minor modifications. The soluble sugar content was determined according to the method of Khan et al. (2000).

Estimation of the net photosynthetic rate, stomatal conductance, and transpiration rate

Before and after withholding water and recovery, the net photosynthetic rate, stomatal conductance, and transpiration rate were measured, on the third leaf from the top of the plant, by a portable infrared gas analyzer-based photosynthesis system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) (Zhang et al. 2014).

Field test and analysis of the field-grown fiber quality

To analyze the cotton yield of the *ScALDH21* transgenic plants after withholding water, different transgenic lines from the T5 generation were grown in six groups in a field located at Experimental Farm of Economic Crop Research Institute, Xinjiang Academy of Agricultural Sciences, Manasi County (44°18′13.91″N, 86°13′11.03″E), Xinjiang, China, where the light intensity was 700–1500 μmol m^{-2} s^{-1}, the average temperature was 18.8 °C, and the total rainfall was 194.8 mm from April to October in 2013. Six groups were planted with the same transgenic and non-transgenic cotton lines but with different water-withholding treatments at different growing stages (see Fig. 1a), viz.: normal irrigation (A), withholding irrigation once during the seedling stage (B), withholding irrigation once during the bud stage (C), withholding irrigation twice during the bud stage (D), withholding irrigation twice during the flowering stage (E), and withholding irrigation twice during the boll stage (F). Approximately eight irrigations were needed for normal irrigation during the entire cotton growing season. About 9 m^3 water each time was used for full irrigation of each group (total area about 80 m^2). Drip irrigation was employed in the present study (Fig. 1b). To avoid water

uptake by underground crossed root systems between groups, only the middle portions (see the shaded part in Fig. 1b) were considered. The following data were collected and recorded at the time of the cotton's maturity: (1) plant height; (2) number of bolls per plant; (3) seed cotton yield per plant; (4) fiber yield per plant; (5) 100-grain weight of the seeds. This experiment was employed to analysis the cotton yield and quality similar to when the growing cotton encountered drought (no rain) at different growing times.

The following quality traits of cotton fiber were determined after harvesting in October 2013. Seed cotton samples, from each plant, were collected, weighed, and ginned, and the lint percentage was calculated. The ginning out-turn (%) is defined as the lint percentage. The seed cotton was ginned using a small 10-saw experimental gin. The fiber length and uniformity were measured randomly. The micronaire value for testing fineness of fibers was analyzed. The Pressley strength tester was used for testing the fiber strength (cN/tex). Three replicates of the lint sampled from each transgenic line and the non-transgenic plants were analyzed for fiber quality. All these data were measured using a Zellweger Uster model 9000 High Volume Instrument (HVI, Charlotte, NC, USA).

Statistical analysis

All data were means of at least three replicates, and a comparison between the transgenic and non-transgenic plants was performed using the one-way ANOVA method with Duncan's multiple test. $P < 0.05$ was considered to be statistically significant (*) and $P < 0.01$ was considered to be highly statistically significant (**). All statistical analyses were performed using SPSS software (standard release version 17 for Windows, SPSS Inc., IL, USA). Figures were generated by Sigmaplot 10.0.

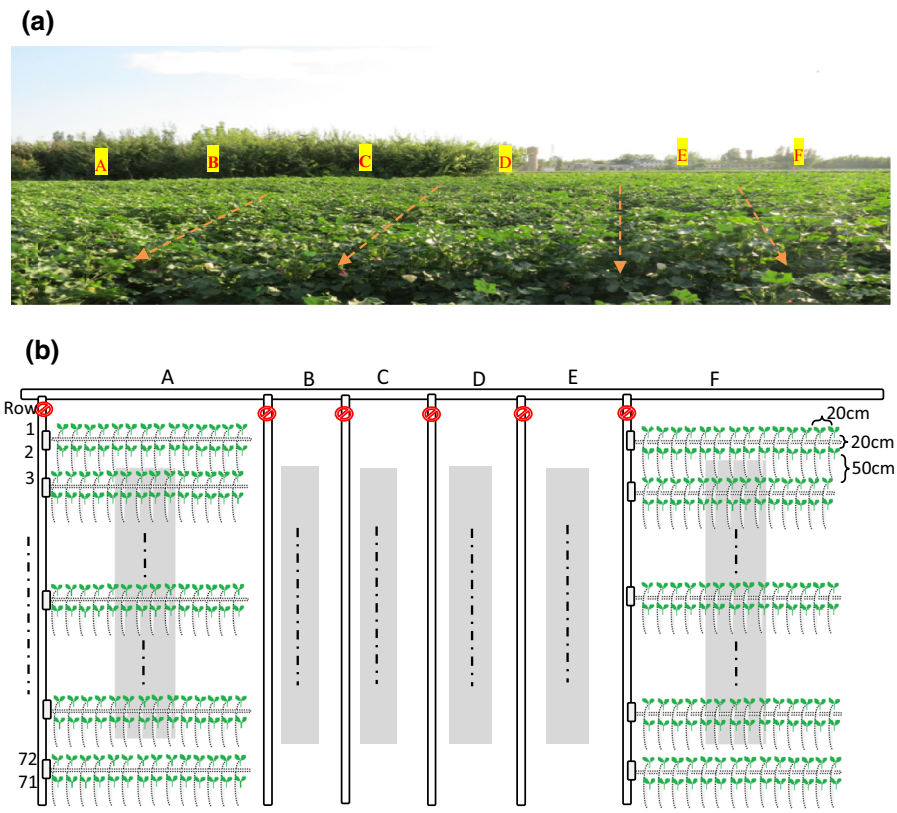

Fig. 1 A picture of the field cotton (**a**) and the mimic growing mode in the field (**b**). *A* to *F* show the different treatment groups: normal irrigation (*A*), withholding irrigation once during the seedling stage (*B*), withholding irrigation once during the bud stage (*C*), withholding irrigation twice during the bud stage (*D*), withholding irrigation twice during the flowering stage (*E*), and withholding irrigation twice during the boll stage (*F*). The *red circles* show the water control valves and the *hollow bars* show the drip irrigation pipes. The *shaded portion* shows the sampling area. Six rows of non-transgenic cotton were arranged in each treatment group. (Color figure online)

Results

Generation of the transgenic cotton lines via *ScALDH21* overexpression

The cotton material (cv. Xinnongmian 1) was transformed using the plant expression vector construct pCAMBIA2300-*ScALDH21*, as shown in Fig. S1. The transgenic plants with *ScALDH21* overexpression were identified. The presence of the target gene was confirmed via PCR analysis (Fig. S1b) in different generations, which showed that 48 of the 50 randomly sampled plants were positive for the target gene in the T5 generation. Transcriptional expression of the *ScALDH21* gene in the transformed cotton plants was determined via RT-PCR analysis (Fig. S1c). The integration of the *ScALDH21* gene in the genome of the transgenic cotton plant was demonstrated by Southern blot analysis (Fig. S1d).

Overexpression of *ScALDH21* in cotton improved growth performance under drought stress

After the transgenic cotton was identified, the growth performance between the non-transgenic cotton (NT) and transgenic cotton (TC) was investigated and compared under drought stress in 2011, 2012, and 2013. The results of the plant growth and morphological analysis showed that the plant height, leaf number, and root length of the TC plants were greater than NT (Fig. S3). Similarly, the stem diameter, fresh weight, and dry weight of TC were greater than NT. Interestingly, the drought stress led to a lateral root number and leaf area of TC significantly larger than those of NT (Fig. S3a, S3b). This indicates that *ScALDH21* overexpression enhanced the performance of plant growth in TC under the drought stress conditions.

Lipid peroxidation in transgenic cotton was reduced under drought stress conditions

Drought stress was applied during the seedling (Fig. 2a, c) and flower stages (Fig. 2b, d). The results indicated that water deficit promoted MDA production in the leaves of both NT and TC compared with the control (Fig. 2), and a high level of MDA was detected in NT and TC when compared with the normal conditions. Although the MDA content varied between the different transgenic lines, it was significantly lower in TC than in NT under the drought stress. After two continuous water-withholding treatments during the flowering stage, the MDA level was found to be much higher [>50 mmol g^{-1} fresh weight (FW)] (Fig. 2d; SF2) than that following only a single drought treatment during the other stages (<30 mmol g^{-1} FW) (Fig. 2; S, SF1, F). Under severe drought stress, the plants failed to recover after 48 h of watering (Fig. 2d).

Overexpression of *ScALDH21* in transgenic cotton improved proline and soluble sugar levels

We measured the free proline (Fig. 3) and soluble sugar (Fig. S4) content in the leaves during the seedling stage (S, SF1) and flower stage (F, SF2) following one or two continuous water-withholding treatments (Fig. 3; Fig. S4; SF1, SF2). The results indicated that they increased significantly in response to the drought stress in both TC and NT, and the proline content in TC was 11.8–304 % greater than in NT. The proline content accumulation was enhanced by 0.35-fold (Fig. 3; SF2) following the second continuous water withholding when compared with the other treatments (Fig. 3; S, SF1, F).

Following the drought treatment, more soluble sugar accumulated in both the NT and TC plants. However, the sugar level in the transgenic lines was greater (24.9–83.6 %) than that observed in the NT plants (Fig. S4). After the stress was relieved, the soluble sugar content reduced back to levels similar to the control (Fig. S4).

POD activity improved in *ScALDH21* transgenic cotton under drought stress conditions

In the seedling (Fig. 4; S, SF1) and flower stages (Fig. 4; SF2, F), the activity of the antioxidant enzyme POD was detected following one or two continuous water-withholding treatments (Fig. 4; SF1, SF2). The results indicated that the drought stress markedly increased the activity of the POD enzyme in the leaves of both NT and TC, and the TC plants exhibited significantly higher POD activity compared with the NT plants (17.6–243 % higher).

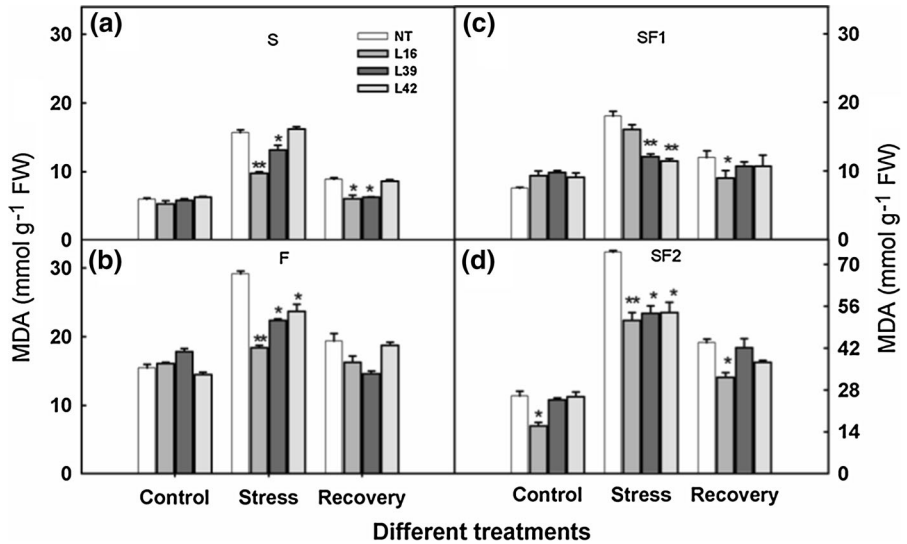

Fig. 2 MDA content during drought stress and after 48 h recovery at different growth times. *Control*, before the water-withholding treatment; *Stress*, water-withholding treatment; *Recovery*, 48 h after re-watering. *S*, withholding water during the seedling stage for 18 days in a greenhouse in 2011; *SF1*, withholding water during the seedling stage for 10 days outdoors in 2012; *SF2*, the SF1 samples that underwent an additional water-withholding period of 20 days during the flower stage outdoors in 2012; *F*, withholding water during the flower stage for 20 days outdoors. *L16*, *39* and *42* are the different transgenic lines

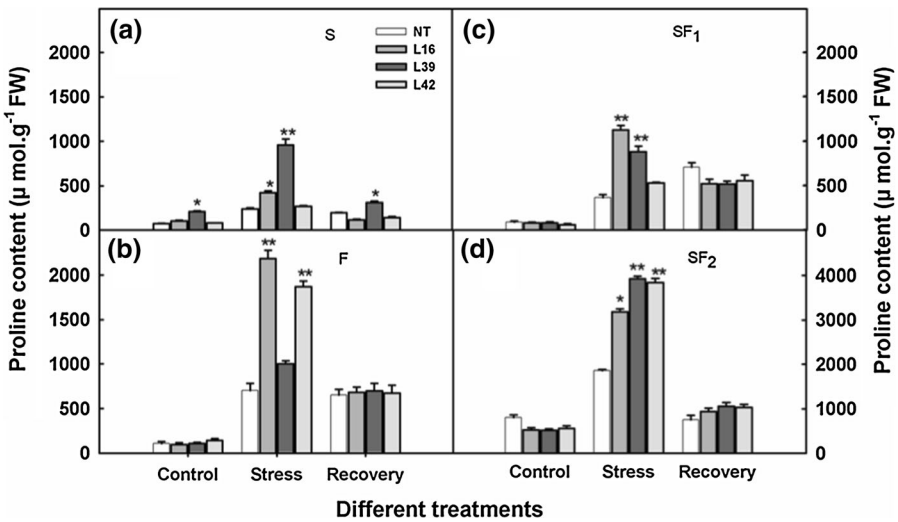

Fig. 3 Proline content during drought stress and after 48 h recovery at different growth times. For the graph annotations, see Fig. S4

Photosynthesis indices improved in *ScALDH21* transgenic cotton under drought stress conditions

To gain insights into the possible reason for the increased biomass in the *ScALDH21* transgenic cotton line, the photosynthetic performance of the plants under drought stress was investigated at different developmental stages (seedling and flowering). Under non-stressed conditions, the TC plants displayed a greater photosynthetic rate than the NT plants (Fig. 5). The net photosynthetic rate of both the NT and TC plants was significantly reduced under water deficiency, and were both lower than 2 μmol CO_2 $m^{-2}s^{-1}$ in SF1 and SF2 (Fig. 5c, d) under severe drought stress conditions,

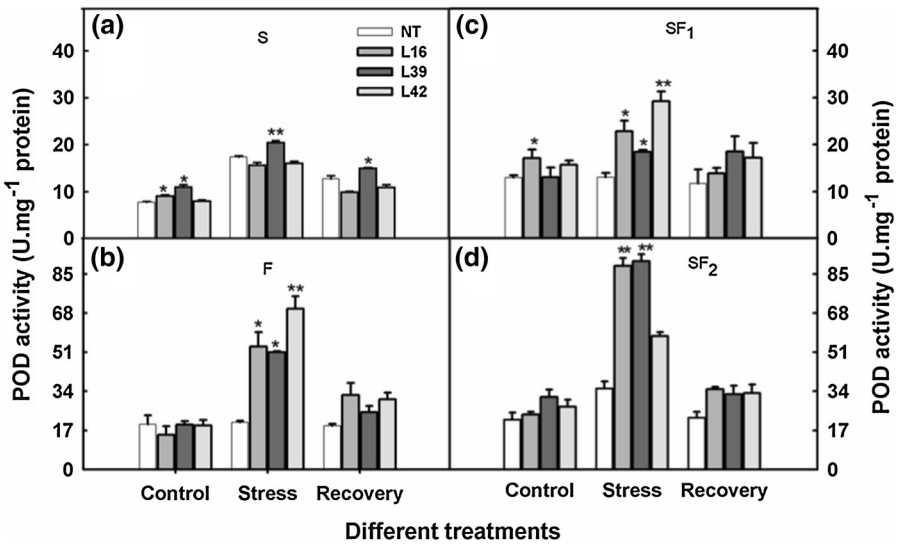

Fig. 4 Changes in POD activity during drought stress and after recovery at different growth times. Control, before the water-withholding treatment; *Stress*, water-withholding treatment; *Recovery*, 48 h after re-watering. *S*, withholding water during the seedling stage for 18 days in a greenhouse in 2011; *SF1*, withholding water during the seedling stage for 10 days outdoors in 2012; *SF2*, samples that underwent an additional water withholding period for 20 days during the flower stage outdoors in 2012; *F*, withholding water during the flower stage for 20 days outdoors. *L16*, *39* and *42* are the different transgenic lines

although the TC cotton still maintained a significantly higher photosynthetic rate than NT. TC also showed higher stomatal conductance and transpiration rates compared with the NT cotton (Fig. 5e–h; i–l). Overall, TC cotton showed reduced inhibition of the photosynthetic performance under stress compared with the NT plants.

Cotton yield and fiber quality in the field improved in *ScALDH21* transgenic cotton under drought stress conditions

To investigate the performance of the *ScALDH21* transgenic cotton under field conditions, the transgenic lines were grown in a trial farm in 2013. After applying six different water-withholding treatments, TC lines were found to perform better than the NT in plant height, boll number per plant, seed cotton yield, fiber yield per plant, and 100-grain weight of the seeds to some extent (Fig. S5). Drought stress during the bud stage reduced the plant height (Fig. S5a) and boll numbers (Fig. S5b), whereas a double drought treatment during the bud and flower stages resulted in reduction in the seed cotton yield (Fig. S5c), fiber weight, and seed weight (Fig. S5d, e). The order of the impact of the water deficiency can be ranked as follows: bud stage > flower stage > boll stage. The transgenic cotton showed significantly higher yields than the NT plants during the bud stage following the single (C), and double drought stress (D) treatments and during the flower stage following the double drought stress (E) treatment. Clearly, the bud stage is very important for cotton growth and yield. Furthermore, the fiber strength (Fig. S6a), ginning out-turn of the fibers (Fig. S6b), fiber length (Fig. S6c), and length uniformity (Fig. S6d) of the transgenic plants were not significantly different from the control (A; normal irrigation), but the TC lines generally showed more uniform, stronger, and longer fibers compared with those from NT (Fig. S6; Fig. S7). The micronaire value of TC was similar to or slightly lower than to NT (Table S2).

Discussion

Plant ALDH proteins belong to a large family and participate in normal cell metabolism. Currently, more and more ALDH subfamilies have been shown to play important roles in plant resistance to biotic and abiotic

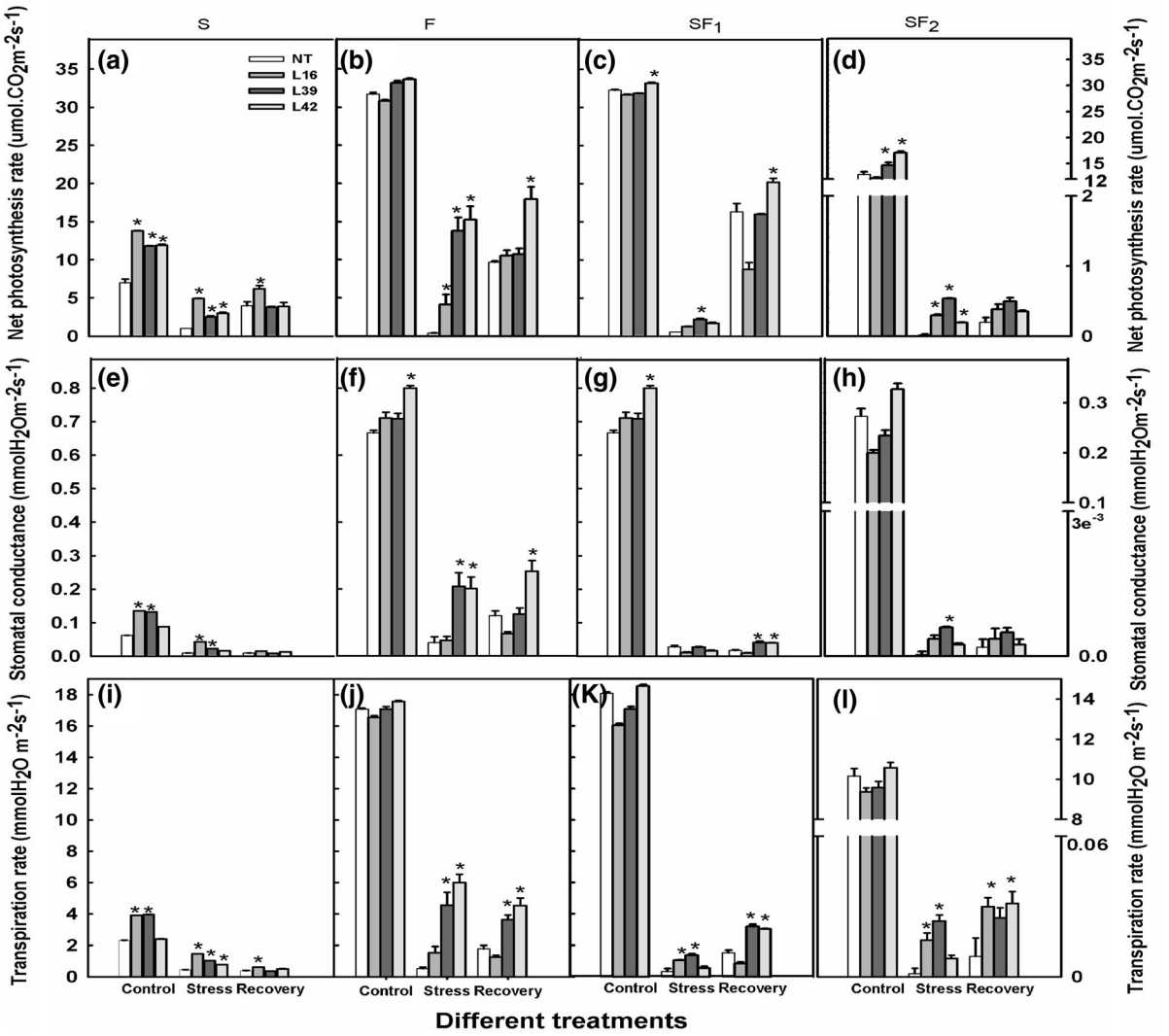

Fig. 5 The effect of drought stress on photosynthetic rate, stomatal conductance, and transpiration rate. For the graph annotations, see Fig. S5

stressors (Sophos et al. 2001; Kotchoni et al. 2006;Xu et al. 2013). The unique *ALDH21* subfamily genes exist only in moss and respond to the plant stress pathway at the transcription level (Chen et al. 2002; Yang et al. 2012). In the present study, experiments in a greenhouse, outdoors, and in the field were performed, and the *ScALDH21* transgenic plants exhibited a significant improvement in drought tolerance and fiber yield compared with non-transgenic cotton (NT) under drought stress (especially at the more sensitive stages, e.g., the bud and flower stages). It is imperative for scientists to develop crop varieties with high productivity under stressful conditions (He et al. 2005). Currently, only a few reports focusing on improving the drought tolerance of cotton using transgenic technology have been published. *Arabidopsis GF14λ* (Yan et al. 2004), the pyrophosphatase gene (*AVP1*) (Zhang et al. 2011), and the molybdenum cofactor gene *AtLOS5* (Yue et al. 2012) have been introduced into cotton to enhance drought tolerance. It is even more difficult in transgenic cotton lines (TC) to achieve improvements in yield and fiber quality at the same time. Excitingly, the results of the present study are the first to show that transgenic cotton lines can

survive drought stress with a minimum loss of yield, which is encouraging for cotton breeding studies.

Drought-tolerant plants possess many advantageous phenotypes. Enhancing water absorption by the roots is one of the main mechanisms by which plants can maintain their water content under stressful conditions (Martinez-Ballesta and Carvajal 2014). In this study, ectopic *ScALDH21* overexpression in cotton improved drought tolerance by changing the plant phenotype characteristics (e.g., plant height, leaf number, leaf area, and lateral root number). Overexpressing a vacuolar pyrophosphatase gene in cotton was shown to increase root length and lateral root number and ultimately improve water-absorbing ability (Zhang et al. 2011; Pasapula et al. 2011). Expression of the isopentenyltransferase gene *IPT* in cotton after a 90-day water deficit led to increased cotton height and more roots (Kuppu et al. 2013). It has been reported that overexpression of the rice *NAC* gene improves the root system under normal and drought conditions (Liu et al. 2014). In our study, overexpression of *ScALDH21* in cotton significantly improved the lateral root number, which consequently accelerated leaf growth compared with NT following drought stress. These phenotypic results were consistent with the performance of other transgenic cottons (Zhang et al. 2011; Liu et al. 2014). In addition to the root system, the plant height, leaf color, and leaf area can also be altered in transgenic cotton. We observed that the height, leaf area, and leaf color of the transgenic *ScALDH21* cotton was improved under normal conditions. Furthermore, overexpression of the *Arabidopsis* 14-3-3 protein gene *GF14λ*in cotton displays a "stay-green" phenotype (Yan et al. 2004). Ectopic expression of a potato sucrose synthase gene in cotton accelerates leaf expansion and vegetative growth (Xu et al. 2012). Besides demonstrating the drought-resistant phenotypic characteristic in transgenic *ScALDH21* cotton, our data also explained the stress memory of cotton in regards to phenotype and physiology via the two continuous water-withholding experiments, which showed that the double water deficit was worse than a single water deficit.

As abiotic or biotic resistance genes, the ALDHs superfamily has been shown to be involved with two classes of downstream genes. First, ALDHs act as 'aldehyde scavengers' (Singh et al. 2013). It was reported that increased activity of the *Arabidopsis* aldehyde dehydrogenase Ath-ALDH3 and soybean ALDH7 appears to constitute a detoxification mechanism that limits aldehyde accumulation and oxidative stress in *Arabidopsis* (Sunkar et al. 2003; Rodrigues et al. 2006). Second, the metabolized products of ALDH enzymes also play a direct role in cellular osmotic homeostasis by catalyzing the synthesis of osmoprotectants (Fu et al. 2011; Li et al. 2014). In our research, the results of the osmoregulation and antioxidant enzyme indices showed that *ScALDH21* transgenic cotton exhibited higher POD activity, lower MDA production, and higher proline and soluble sugar content compared with the non-transgenic plants under stress, which may mean that the *ScALDH21* gene plays an important role in tolerating drought stress. When compared with SOD, APX, and CAT, the enhanced POD activity was the primary reason for the reduced peroxide levels in transgenic *BADH* tomatoes (Li et al. 2014). Our data demonstrated the potential of *ALDH21* to confer tolerance to osmotic and oxidative stress in cotton.

Excessive ROS lead to inhibition of the repair of photodamaged PSII due to suppression of the synthesis of the PSII protein during stresses (Hozain et al. 2012). Under abiotic stress, e.g., water deficit or salinity, stomata closure is considered to be the first mechanism that a plant employs to preserve water, and this involves decreasing the CO_2 availability (Ashraf and Harris 2013). However, crop productivity is associated with a higher rate of transpiration and leaf growth (Upadhyay et al. 2014). Therefore, an optimal balance between water status, nutrient uptake, photosynthesis, and transpiration rate is needed in plants. In the present work, the transgenic cotton showed a higher rate of photosynthesis and less inhibition of the stomatal conductance and transpiration rate under water deficiency (Fig. 5), which means that the overexpression of *ScALDH21* can reduce damage to photosynthesis. More importantly, the height and young leaf area of the transgenic cotton plants was greater than the NT plants following the recovery period after drought stress (Figs. 1, S3). The photosynthetic capacity has a positive association with biomass production or seed yield under drought stress in cotton plants (Ashraf and Harris 2013). There is a persistent hope amongst plant breeders that improved photosynthetic efficiency of crops will improve energy capture, which can be translated into a greater harvestable yield (Dunwell 2000). The improvement in the yield and relevant traits has been observed in transgenic cotton under greenhouse conditions. Overexpression of the rice *NAC* gene (stress-

related transcription factor) increased boll number in transgenic cotton under drought stress (Liu et al. 2014) in the greenhouse. The T3 generation of *AtLOS5* transgenic cotton increased its fresh weight by 13 % compared with NT under drought stress (Yue et al. 2012) in the greenhouse. *AVP1* (vacuolar pyrophosphatase gene) overexpression in cotton leads to a 20 % greater fiber yield compared with NT in dry land conditions (Zhang et al. 2011; Pasapula et al. 2011). Other genes, such as *GHSP26* (heat shock protein gene), *GUSP1* (universal stress protein gene), or *Phyto-B* (phytochrome-B gene) can increase the boll number, boll weight, or seed cotton yield, respectively, in T1 cotton (Shamim et al. 2013). In our research, the transgenic cotton lines also showed a significant increase in their biomass and seed fiber yield (Figs. S5, S6, S7), which is likely due to decreased impact on their photosynthetic capacity under stressful conditions.

In conclusion, the results of the present study suggest that the ectopic expression of the *ScALDH21* gene in cotton enhances drought tolerance and reduces the yield penalty. To our knowledge, this is the first report to show that overexpression of *ALDH21* improves drought tolerance in cotton in several growing seasons and under multi-site field experiments. The work demonstrated that *ScALDH21* can be a candidate gene for the improvement of cotton production in arid and semi-arid regions.

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