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ORIGINAL PAPER

Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery

Asish Kumar Parida · Vipin S. Dagaonkar · Manoj S. Phalak · Laxman P. Aurangabadkar

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Abstract The relative water content (RWC), free proline levels and the activities of enzymes involved in proline metabolism were studied in drought tolerant (Ca/H 680) and drought sensitive (Ca/H 148) genotypes of cotton (*Gossypium hirsutum* L.) during induction of water stress and posterior recovery. Water stress caused a significant increase in proline levels and P5CS activity in leaves of both tolerant and sensitive genotypes, whereas the activity of P5CR increased minimally and the activity of OAT remains unchanged. The activity of PDH decreased under drought stress in both the genotypes. The leaf of tolerant genotype maintained higher RWC, photosynthetic activity and proline levels, as well as higher P5CS and P5CR activities under water stress than that of drought sensitive genotype. The drought induced proline levels and activities of P5CS and P5CR declined and tend to be equal to their respective controls, during recovery, whereas the PDH activity tends to increase. These results indicate that induction of proline levels by up regulation of P5CS and down regulation of PDH may be involved in the development of drought tolerance in cotton.

Keywords Drought stress · *Gossypium* · Ornithine-δ-aminotransferase · Proline dehydrogenase · Δ^1-Pyrrolline-5-carboxylate reductase · Δ^1-Pyrrolline-5-carboxylate synthetase

Abbreviations

DCIP	2,6-Dichloroindophenol
OAT	Ornithine-δ-aminotransferase
P5CR	Δ^1-Pyrrolline-5-carboxylate reductase
P5CS	Δ^1-Pyrrolline-5-carboxylate synthetase
PDH	Proline dehydrogenase

Introduction

Water deficit or drought stress is a severe environmental factor and the major constraint on plant productivity with an evident effect on plant growth (Rampino et al. 2006). Water deficit occurs in plants whenever water loss through transpiration exceeds supply from the soil. Prolonged water shortages virtually affect all the metabolic processes and often result in severe reductions in plant productivity (Hare et al. 1998). This is especially important for crop species, and a major impetus behind efforts to gain a molecular understanding of plant responses to water deficit is the hope that crop plants can be genetically engineered to maintain productivity while growing in adverse conditions (McCue and Hanson 1990). Well informed manipulation of their responses to water deficit could enable transgenic crop plants to survive productively in conditions that would otherwise be lethal or result in severe reductions in yield. The plant response to drought consists of numerous processes that must function in coordination to alleviate both cellular hyperosmolarity and ion disequilibrium. To cope

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A. K. Parida (✉) · V. S. Dagaonkar · M. S. Phalak · L. P. Aurangabadkar
Biotechnology Division, Ankur Agricultural Research Laboratory, 27, New Cotton Market Layout, Nagpur, Maharashtra 440018, India
e-mail: asishparida2003@yahoo.com

A. K. Parida
Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, G. B. Marg, Bhavnagar, Gujarat 364002, India

with drought stress, plants respond with physiological and biochemical changes. These changes aim at the following, retention of water in spite of the high external osmoticum and the maintenance of photosynthetic activity while stomatal opening is reduced to counter water loss (Parida et al. 2007). One of the most common induced responses in all the organisms undergoing water deficit is the production and/or accumulation of so-called compatible osmolytes. These are osmotically active, neutral organic compounds such as sugars (polyols), certain amino acids, and quaternary ammonium compounds. By lowering water potentials, the accumulation of compatible osmolytes allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortages within the organism. Compatible osmolytes continue to accumulate during prolonged water deficit and it has been proposed that they may help to stabilize protein tertiary structure as cells dehydrate (Kavi Kishor et al. 2005).

The amino acid proline is perhaps the most widely distributed compatible osmolytes. In organisms from bacteria to maize, there is a strong correlation between the increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity. In plants, the role of proline may not be restricted to that of a compatible osmolyte, it also acts as a protective agent for cytoplasmic enzymes (Paleg et al. 1984), a reservoir of nitrogen and carbon source for post stress growth (Kavi Kishor et al. 2005), a stabilizer of the membranes and the machinery for protein synthesis (Kardpal and Rao 1985), a scavenger of free radicals (Smirnoff and Cumbes 1989) and as a sink for energy to regulate redox potentials (Alia and Saradhi 1993; Lawlor 1995). Among these, the function of proline as an osmoprotectant under drought and salinity stress has been widely advocated (Perez-Alfocea and Larher 1995; Martinez et al. 1996; Petrusa and Winicov 1997; Lutts et al. 1999; Kavi Kishor et al. 2005) despite the fact that accumulation of proline also occurs in response to the factors, which do not lead to any osmotic stress or create low water potential (Saradhi and Saradhi 1991; Alia and Saradhi 1993; Saradhi et al. 1995). Thus, the induction of proline accumulation is not well understood. The major pathway for proline synthesis in plants, which takes place in the cytoplasm, is from glutamate, through γ-glutamyl phosphate and glutamyl-γ-semialdehyde (a two-step reaction that is catalyzed in plants and animals by a single enzyme, Δ^1-pyrrolline-5-carboxylate synthetase; P5CS). The glutamyl-γ-semialdehyde is spontaneously cyclized to Δ^1-pyrrolline-5-carboxylate, which is converted to proline by Δ^1-pyrrolline-5-carboxylate reductase (P5CR). This pathway was first established in *Esherichia coli* and *Saccharomyces cerevisiae* and the corresponding plant genes were identified through the complementation of yeast and bacterial mutants defective in various pathways (Delauney and Verma 1993). The alternative pathway for proline synthesis is from ornithine through ornithine-δ-aminotransferase (Lutts et al. 1999).

It has been reported that drought stress results an increase in free proline levels and OAT activity and decrease in proline oxidase activity in both drought-susceptible and -tolerant cultivars of cassava (Sundaresan and Sudhakaran 1995). In rice proline content and P5CS activity increase during drought stress both in susceptible and tolerant genotypes and the increase was higher in tolerant genotypes (Choudhary et al. 2005). The proline content and γ-glutamyl kinase activity are significantly enhanced and proline oxidase activities are reduced by drought stress in *Abelmoschus esculentus* (Sankar et al. 2007). These results suggest that accumulation of proline by the up regulation of proline biosynthetic enzymes and down regulation of degrading enzymes have significant role in drought tolerance.

Cotton is an important source of fiber, feed, and edible oil. Drought is a major limiting factor affecting the fiber yield and lint quality in cotton. The reports on proline accumulation and drought tolerance in cotton are very scant (De Ronde et al. 2000). In the current study, we have analyzed the levels of proline and the enzymes involved in proline biosynthesis and degradation in two contrasting genotypes of cotton, under drought stress and subsequent recovery conditions. The specific objective of such study was to verify whether the accumulation of proline could be attributed to de novo synthesis and/or catabolism. The observations from such study will provide useful informations for genetic improvement of cotton for drought tolerance to enhance yield and fiber quality.

Materials and methods

Plant materials and growth conditions

Seeds of two cotton genotypes (*Gossypium hirsutum* L.), namely Ca/H 680 (drought tolerant) and Ca/H 148 (drought sensitive) were germinated in pots (size 15″ × 15″) containing soil, peat and sand in the ratio of (1:1:1) and grown under green house conditions. Temperature in the green house were 30 ± 2°C during day and 25 ± 2°C at night with relative humidity ~50% and a photoperiod of 14 h. Metal halide illumination lamps (1,000 W) were used to supplement natural radiation. Light radiation reached a maximum of 1,500 µmole $m^{-2}s^{-1}$ at the top of canopy at midday. Seeds of each genotype were sown in 56 pots. Four seeds were sown per pot. After 2 weeks of emergence seedlings were thinned to one plant per pot. The plants were irrigated at every alternate day with normal tap water. After 45 days of sowing (immediately after flower

initiation) drought was imposed by withholding irrigation for 14 days. A control set was maintained by irrigating the potted plants regularly. After 14 days of drought induction, the drought stressed plants were re-irrigated for 7 days for recovery. The top two expanded leaves from four replications were collected from control and treated plants after 0, 3, 7, 10 and 14 days of drought as well as after 3 and 7 days of recovery for measurements of RWC, photosynthetic activity, proline levels, and activity of P5CS, P5CR, OAT and PDH.

Leaf relative water content

Relative water content (RWC) of leaf was measured according to Barrs and Weatherley (1962). Immediately after sampling, leaves were weighed and then soaked in distilled water for overnight at room temperature. The leaves were then blotted dry and weighed prior to oven drying at 80°C for 48 h. The leaf relative content was calculated using the following formula: RWC = [(FW − DW)/(TW − DW)] × 100, where FW is fresh weight, DW is dry weight, and TW is turgid weight (weight after the leaf was kept in distilled water for overnight).

Isolation of thylakoid membranes and measurement of photosynthetic activity in isolated thylakoids

Thylakoid membranes were isolated from leaves following the procedure of Vani et al. (2001). Fresh leaves (2 g) from control, drought stressed and drought-recovered plants were homogenized in ice-chilled isolation buffer containing 0.4 M sorbitol, 15 mM tricine (pH 7.8) and 5 mM $MgCl_2$ (buffer A), in a tissue homogenizer (Ultra Turaax-T_{25}, Ika, Germany). The homogenates were filtered through four layers of mira-cloth (Calbiochem, Switzerland) and centrifuged at 3,000*g* for 5 min at 4°C. The supernatants and most of the loose pellets were discarded and rest of the pellets was washed in buffer containing 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ (buffer B). The pellets consisting of thylakoid membranes were suspended in buffer consisting of 0.1 M sorbitol, 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ (buffer C) to a final chlorophyll concentration of 1 mg ml^{-1}.

The PS II mediated electron transport activity in terms of DCIP (2,6-dichloroindophenol) photo reduction was measured in thylakoids isolated from control, drought treated and recovered plants following the procedure described earlier (Parida et al. 2003). The assay mixture consisted of (total volume 3 cm^3): 50 mM HEPES-NaOH buffer (pH 7.0), 10 mM NaCl, 2 mM $MgCl_2$, 50 μM DCIP. In each assay thylakoid membranes equivalent to 5 μg Chl ml^{-1} were used. The light induced reduction of DCIP by thylakoid membranes was determined at 25°C by monitoring changes in A_{590} in a spectrophotometer. The rate of loss of absorption was converted to rate of reduction of DCIP by using the extinction coefficient for DCIP as 16 $mM^{-1}cm^{-1}$ at 590 nm (Allen and Holmes 1986).

Estimation of free proline

Free proline content was estimated following the method of Bates et al. (1973). Fresh leaves (0.5 g) were extracted in 4 cm^3 of 3% sulphosalicylic acid and the homogenates were centrifuged at 10,000*g* for 10 min. A total of 2 cm^3 of the supernatant was reacted with 2 cm^3 of acid ninhydrin reagent and 2 cm^3 of glacial acetic acid in a test tube for 1 h at 100°C and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 cm^3 of toluene and mixed vigorously by vortexing for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance measured at 520 nm using a spectrophotometer (Spectramax Plus, Molecular Devices, USA). Proline concentration was calculated from a standard curve using 5–500 μM L-proline (Sigma).

Enzyme extraction for proline metabolism

The leaf tissues stored frozen at −85°C were ground to a fine powder with liquid nitrogen in a chilled mortar and pestle, in the presence of appropriate extraction buffer. Ratios for buffer volume: g fresh weight (FW) was 2:1. All the operations were carried out at 4°C. Extraction buffer used for P5CS, P5CR and PDH was 50 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, 0.6 M KCl, 3 mM EDTA, 1 mM dithiothreitol and 5% (w/v) insoluble polyvinylpolypyrollidine. The homogenates were centrifuged at 4°C for 5 min at 39,000*g*. The supernatants were further clarified by centrifugation at 39,000*g* for 20 min at 4°C. After centrifugation, the supernatants were desalted on a sephadex G-25 column (PD-10, Pharmacia Biotech), eluted with 50 mM Tris-HCl (pH 7.4) containing 10% glycerol and used for the measurement of P5CS, P5CR and PDH activity.

The solution used for extraction of ornithine-δ-aminotransferase (OAT) consisted of 100 mM KPO_4 buffer (pH 7.9) with 1 mM EDTA, 15% (w/v) glycerol and 10 mM β-mercaptoethanol. The extract was centrifuged at 15,000*g* for 15 min at 4°C and 60% $(NH_4)_2SO_4$ was added to the supernatant, which was then allowed to stand for 45 min and used for the enzyme assay (Lutts et al. 1999).

Enzyme assay for proline metabolism

The activity of P5CS (EC 2.7.2.11) was assayed following the method of Zhang et al. (1995). The reaction mixture for

P5CS contained the following in a final volume of 1 cm^3 at pH 7.0: 50 mM L-glutamate, 20 mM $MgCl_2$, 10 mM ATP, 100 mM hydroxamate-HCl and 50 mM Tris. The reaction was started by the addition of 0.1 cm^3 of enzyme extract. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 2 cm^3 of the stop buffer [2.5% (w/v) $FeCl_3$ and 6% (w/v) of trichloroacitic acid dissolved in 100 cm^3 of 2.5 N HCl]. The precipitated proteins were removed by centrifugation and the absorbance was read at 535 nm against a blank identical to the above but lacking ATP. The amount of γ-glutamyl hydroxamate complex produced was estimated from the extinction coefficient of 0.25 mM^{-1} cm^{-1} reported for Fe^{3+} hydroxamate complex of the compound. One unit of P5CS is defined as the amount of enzyme required to produce one nanomole of γ-glutamyl hydroxamate per minute.

The activity of P5CR (EC 1.5.1.2) was assayed following the method described by Lutts et al. (1999). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 1 mM dithiothreitol, 0.25 mM NADH, and 1 mM Δ^1-pyrrolline-5-carboxylic acid. The reaction was started with the addition of 0.1 cm^3 enzyme extract and the decrease in the absorbance of NADH was monitored at 340 nm for 1 min using a spectrophotometer (Spectramax Plus, Molecular Devices, USA). P5CR activity was determined by using an extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADH. One unit of P5CR is defined as the amount of enzyme required to oxidize one nanomole of NADH per minute.

Ornithine-δ-aminotransferase (EC 2.6.1.68) was assayed according to Charest and Phan (1990) in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH in a volume of 2 cm^3. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition of 0.1 cm^3 of the enzyme extract. OAT activity was calculated from the molar extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADPH. One unit of OAT is defined as the amount of enzyme required to oxidize one nanomole of NADH per minute.

Proline dehydrogenase (EC 1.5.99.8) was assayed by measuring the reduction rate of 2,6-dichloroindophenol (DCIP) following the procedure of Sakuraba et al. (2001). The standard reaction mixture was composed of 100 mM L-proline, 0.1 mM DCIP, 200 mM Tris-HCl buffer (pH 8.0), and 0.1 cm^3 enzyme extract in a total volume of 1.0 cm^3. The mixture without the substrate (L-proline) was previously incubated at 50°C for about 3 min in a cuvette and the reaction was started by the addition of L-proline. The initial decrease in absorbance was measured with a spectrophotometer (Spectramax Plus, Molecular Devices, USA). One unit of PDH was defined as the amount of enzyme catalyzing the reduction of one micromole of DCIP per minute at 50°C. The millimolar absorption coefficient of 21.5 $mM^{-1}cm^{-1}$ at 600 nm was used for DCIP.

The protein in the enzyme extract was quantified using the RCDC protein assay kit (Bio-Rad, USA) and the enzyme activity was expressed as unit per milligram protein.

Statistical analysis

Standard errors were computed from the values of two independent experiments with four replicates. Statistical analysis of the results from two different experiments was carried out according to Duncan's multiple range tests. Data were subjected to a two-way analysis of variance (ANOVA) and the LSD at $P \leq 0.01$ was determined (Sokal and Rohlf 1995). The correlation coefficient (r) significant at 5% level ($P < 0.05$) was calculated between various enzymes of proline metabolism and proline accumulation in both the genotypes of cotton under drought stress and recovery (Gomez and Gomez 1984).

Results

With the progression of drought, leaf of drought sensitive genotype (Ca/H 148) wilted within 7 days of stress and the wilting became more pronounced after 14 days of stress under pot culture. There was no visible symptom of leaf wilting in drought tolerant genotype (Ca/H 680) up to 10 days of drought induction and after 14 days the lower leaves slightly lost their turgidity. As the water stressed plants were recovered from drought, the wilted plants became normal in terms of morphological appearance within 7 days of recovery.

We observed that the differences in each parameter between the control plants (normally irrigated) of each genotype was statistically insignificant during entire experimentation period (from the day of induction of stress to 7 days posterior recovery). Therefore, we choose one control data point (0 days of stress) for each measurement for each genotype.

Relative water content of leaf

Relative water content of leaves were 73 and 66% respectively in Ca/H 680 and Ca/H 148 genotypes under control conditions (Fig. 1), while after 14 days of drought stress RWC declined to 68 and 41% respectively in Ca/H 680 and Ca/H 148 genotypes. However, on re-watering, the genotype Ca/H 680 recovered fully in terms of RWC; whereas there is still a 7% decline in RWC in the genotype Ca/H 148 after 7 days of recovery (Fig. 1).

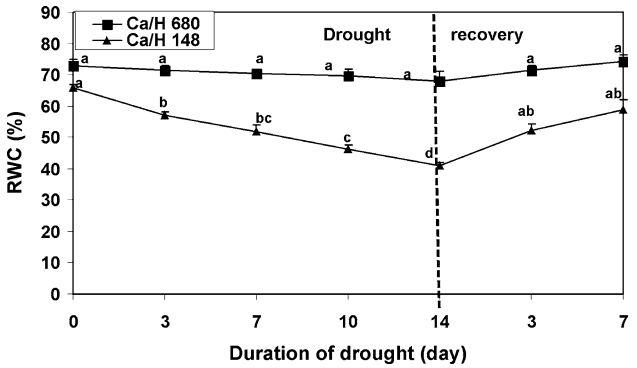

Fig. 1 Changes in relative water content (RWC) of leaves in drought tolerant (Ca/H 680) and drought sensitive (Ca/H 148) genotypes of cotton with progression of drought stress and recovery. The values are mean ± SE ($n = 8$). *Different letters* besides the *error bars* indicate statistically different means at $P \leq 0.01$

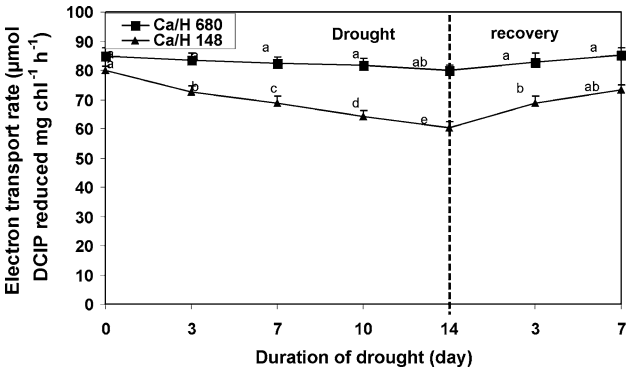

Fig. 2 Effects of drought on PS II mediated photosynthetic activity in thylakoids isolated from leaves of cotton genotypes as monitored in terms of DCIP photoreduction (µmoles mg chl^{-1} h^{-1}). The rate was measured at different days after drought treatment and recovery in a reaction mixture at pH 7.0 as $H_2O \rightarrow$ DCIP without any uncoupler and inhibitors. The values are mean ± SE ($n = 8$). *Different letters* besides the *error bars* indicate statistically different means at $P \leq 0.01$

Photosynthetic activity

Photo system II mediated electron transport activity was monitored in thylakoids isolated from drought tolerant (Ca/H 680) and drought sensitive (Ca/H 148) genotypes in terms of 2,6-dichloroindophenol (DCIP) photoreduction under control, drought stressed and drought-recovered conditions. There were no significant changes in rate of DCIP photoreduction in thylakoids isolated from drought stressed plants of tolerant genotype up to 10 days of treatment and a marginal decrease (6%) was observed after 14 days of stress (Fig. 2). In contrast to tolerant genotypes rate of DCIP photoreduction decrease gradually in thylakoids isolated from sensitive genotype with progression of drought stress and a significant decrease (24%) was noticed after 14 days of stress (Fig. 2). The drought stressed plants of tolerant genotype recovered fully in terms of photosynthetic activity within 7 days, when the stressed plants were re-irrigated, however in sensitive genotype an 8% decline in photosynthetic activity was observed after 7 days of recovery (Fig. 2).

Changes in proline level

As shown in Fig. 3a, the proline contents of leaves increased significantly with progression of drought stress in both the genotypes. It was observed that the proline level increased slowly in early stages of drought induction (3–7 days), whereas it increased steadily after 7 days of stress (Fig. 3a). After 14 days of drought stress, proline level increased by 22-fold in the genotype Ca/H 680, and 14 fold in Ca/H 148. After recovery from drought, the proline contents of both the genotypes decreased significantly and tend to be equal to their respective control (Fig. 3a).

Activity of P5CS and P5CR

Drought caused a significant increase in P5CS activity in both the genotypes. After 14 days of drought stress, P5CS activity increased by 2.1- and 1.6-fold respectively in Ca/H 680 and Ca/H 148 (Fig. 3b). As shown in Fig. 3c, the activity of P5CR also increased in both the genotypes (21 in Ca/H 680 and 14% in Ca/H 148, after 14 days of drought). It was also noteworthy that the activity of P5CS was far greater than that of P5CR in both tolerant and sensitive genotypes. After 7 days of recovery from stress both P5CS and P5CR activity declined and tends to be equal to their respective control (Fig. 3b, c). The correlation analysis revealed that P5CS and P5CR activities were positively correlated with proline accumulation in both the genotypes (Table 1).

Activity of OAT

In contrast to P5CS and P5CR, OAT activity remains unchanged in both the genotypes with progression of drought stress (Fig. 3d). The correlation analysis showed that there was no correlation between OAT activity and proline accumulation in both the genotypes (Table 1).

Activity of PDH

Our results showed that PDH activity declined significantly in both the genotypes with progression of drought stress and increased significantly with subsequent recovery from stress. As shown in Fig. 3e, PDH activity decreased by 36 in Ca/H 680 and 25% in Ca/H 148 after 14 days of drought stress. The correlation analysis

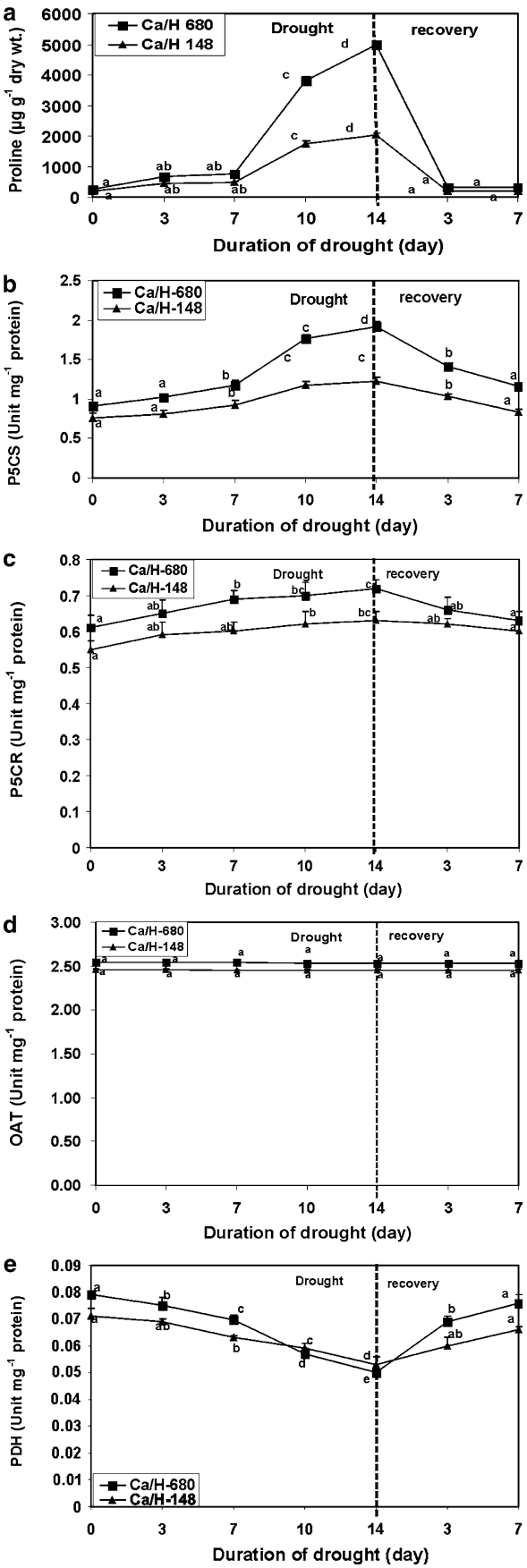

Fig. 3 Changes in **a** proline levels, **b** activity of Δ^1-pyrrolline-5-carboxylate synthetase (P5CS), **c** activity of Δ^1-pyrrolline-5-carboxylate reductase (P5CR), **d** activity of ornithine-δ-aminotransferase (OAT) and **e** activity of proline dehydrogenase (PDH) of leaves in drought tolerant (Ca/H 680) and drought sensitive (Ca/H 148) genotypes of cotton with progression of drought stress and recovery. The values are mean ± SE ($n = 8$). *Different letters* besides the *error bars* indicate statistically different means at $P \leq 0.01$

revealed negative correlation of PDH with proline accumulation in both the genotypes (Table 1).

Discussion

It is well known that the proline contents in leaves of many plants get enhanced by several stresses including drought stress (Lopez et al. 1994; Lee and Liu 1999; De Ronde et al. 2000; Hernandez et al. 2000; Abdel-Nasser and Abdel-Aal 2002; Parida et al. 2004, 2007; Ruiz et al. 2005). Thus, we monitored the proline levels in leaves of cotton genotypes during drought and recovery periods. Our results of drought induced dramatic increase in proline contents in leaves of cotton agree with earlier reports of accumulation of proline during drought exposure (Lopez et al. 1994; Abdel-Nasser and Abdel-Aal 2002). The drought induced foliar accumulation of proline in cotton varies between tolerant and sensitive genotypes. The higher efficiency of the proline accumulation in the genotype Ca/H 680 can be considered as one of the factors responsible for its tolerance to drought, thereby maintaining higher relative water content (RWC) and photosynthetic activity under water stress conditions.

In higher plants under osmotic stress, proline accumulates through stimulation of its de novo synthesis together with repression of its catabolism (Delauney and Verma 1993). Proline can be synthesized using either glutamate or ornithine precursors. The first two steps of the proline biosynthesis from glutamate are catalyzed by a single bifunctional enzyme P5CS (Zhang et al. 1995). Although several studies have indicated that P5CS is the critical enzyme in proline biosynthesis under salt and water stress (Kavi Kishor et al. 1995; Yamada et al. 2005), the stimulation of proline biosynthesis has been associated with increased P5CR and P5CS mRNA levels (Savoure et al. 1995). Recently, Roosens et al. (1999) had demonstrated that the glutamate pathway is the first in the time course to contribute to the proline accumulation in response to salt or drought stress. Our results showed that P5CS activity increased significantly with the duration of drought stress; the highest activity occurring after14 days of stress (Fig. 3b). As compared to P5CS, P5CR activity increase marginally in cotton under drought stress (Fig. 3c).

Table 1 Correlation coefficient (r) of various proline metabolic enzymes studied in two genotypes of cotton (Ca/H 680 and Ca/H 148) with proline content under drought stress and recovery conditions

Parameters	Genotypes	
	Ca/H 680	Ca/H 148
P5CS	0.906*	0.862*
P5CR	0.822*	0.617*
OAT	−0.268	0.032
PDH	−0.952*	−0.780*

P5CS Δ1-Pyrrolline-5-carboxylate synthetase, *P5CR* Δ1-pyrrolline-5-carboxylate reductase, *OAT* ornithine-δ-aminotransferase, *PDH* proline dehydrogenase

* Significant at $P < 0.05$

Plants also synthesize proline from ornithine by ornithine amino transferase (OAT, Delauney and Verma 1993). Although some studies showed increased OAT activity under salt or drought stress conditions (Hervieu et al. 1995; Roosens et al. 2002); most studies indicated that under these conditions the free proline increase appears to be mainly due to the activity of the enzymes of the glutamate pathway (Roosens et al. 1999). Our results agree with these later conclusions, given that in our experiment the OAT activity did not vary in its activity during entire period of stress and recovery (Fig. 3d). Therefore, given the results for the enzymes involved in proline biosynthesis (P5CS and OAT), we can define proline synthesis under drought stress in cotton is predominantly due to increase P5CS activity.

Proline metabolism and accumulation also depend on its degradation, which is catalyzed by the mitochondrial enzyme PDH activity (Hare et al. 1999). In general, certain types of stress (mainly drought and salinity) seriously inhibit PDH activity (Hare et al. 1999). Our results indicated that PDH activity was declined by drought stress in both the genotypes of cotton (Fig. 3e), the lowest activity being recorded after 14 days of stress. According to some authors, PDH activity determines proline accumulation (Hare et al. 1999; Ruiz et al. 2005). The relationships found in our experiment between PDH activity and proline concentrations confirm that proline accumulation depends largely on the depression or inhibition of PDH activity (Fig. 3e). These results in response to drought stress in cotton indicate the importance of the enzymes P5CS and PDH in drought tolerance in cotton. Under dehydration conditions, when the activity of P5CS is strongly induced, the activity of PDH is inhibited. By contrast, under rehydration conditions, when the activity of PDH is strongly induced, the activity of P5CS is inhibited. Thus, P5CS, which acts during the biosynthesis of proline, and PDH, which acts during the catabolism of proline, appear to be the rate-limiting factors under water stress (Yoshiba et al. 1997). Several repots have demonstrated that overexpression of proline biosynthetic enzymes such as P5CS, P5CR and OAT increases proline production and confers salt and drought tolerance in transgenic plants (Wu et al. 2003; De Ronde et al. 2004; Hmida-Sayari et al.2005; Simon-Sarkadi et al. 2006; Gruszka Vendruscolo et al. 2007; Yamchi et al. 2007). Transgenic *Arabidopsis* plants with antisense-proline dehydrogenase (antisense-AtPoDH) were more tolerant to freezing and high salinity conditions than wild-type plants (Nanjo et al. 1999). Therefore, it is suggested that genetically engineered cotton plants that overproduce proline might, thus, acquire osmotolerance, namely, the ability to tolerate environmental stresses such as drought.

In short, considering our overall results, we conclude that drought induced foliar accumulation of proline in cotton is predominantly due to up-regulation of proline biosynthetic enzyme P5CS and down regulation of proline degrading enzyme PDH. These results also indicate that during water stress there are alterations in the metabolism of proline in cotton genotypes, and the extent of alteration varies between drought-tolerant and drought-sensitive genotypes. The results also demonstrated that the removal of free proline during the recovery from dehydration stress involves an induction of the PDH while the activity of P5CS declines. The reciprocal regulation of P5CS and PDH appears to be a key mechanism in the control of proline levels during and after drought stress in cotton. Finally, under our experimental conditions, proline can be defined as a drought tolerance indicator in cotton.

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