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BRIEF COMMUNICATION

Proline accumulation and the expression of Δ^1-pyrroline-5-carboxylate synthetase in two safflower cultivars

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Abstract

Proline (Pro) accumulation under water stress was measured in safflower (*Carthamus tinctorius* L.) drought tolerant cv. A1 and sensitive cv. Nira. Activities of pyrroline-5-carboxylate reductase (P5C reductase) and pyrroline-5-carboxylate synthetase (P5C synthetase), two enzymes involved in the Pro biosynthetic pathway were also estimated. Water stress resulted in a reduction in the leaf dry mass and chlorophyll content along with a gradual accumulation of Pro. RT-PCR results show higher expression of Δ^1-pyrroline-5-carboxylate synthetase (*p5cs*) gene in correlation with up-regulated Pro accumulation in cv. A1. P5C reductase was found to be the Pro synthesis rate limiting whereas P5C synthetase did not show any specific response to the drought stress in both cultivars.

Additional key words: *Carthamus tinctorius*, safflower, water stress.

Drought stress is one of the main abiotic factors affecting plant growth and productivity. Osmotic adjustment is a part of the drought avoidance mechanisms to counteract the decrease in pressure potential by increasing and maintaining higher amount of intracellular compatible solutes in cytosol (Cushman 2001). Proline (Pro) is one among them (Delauney and Verma 1993, Verbruggen and Hermans 2008) and a dramatic increase in the intracellular Pro content has been observed in a variety of osmotically stressed plants (Yamada *et al.* 2005, Veeranagamallaiah *et al.* 2007, Reddy *et al.* 2008, Silva-Ortega *et al.* 2008, Verbruggen and Hermans 2008). In plants, Pro biosynthesis occurs *via* two pathways, either from glutamate or ornithine. In the first one, glutamate is converted to Pro by two successive reductions, catalyzed by pyrroline-5-carboxylate (P5C) synthetase and P5C reductase, respectively. P5C synthetase is a bifunctional enzyme which initially catalyzes the activation of glutamate by phosphorylation followed by reduction of the labile intermediate γ-glutamyl phosphate into glutamate semialdehyde (GSA), which is in equilibrium with the P5C form (Hu *et al.* 1992). A strong correlation between *p5cs* expression and the accumulation of Pro has been shown in rice and *Arabidopsis thaliana* (Hien *et al.* 2003, Szekely *et al.* 2008). Ornithine (Orn), the alternate precursor can be transaminated to P5C by ornithine-γ-aminotransferase (OAT), a mitochondrial enzyme (Roosens *et al.* 1998). Pro biosynthesis from glutamate appears to be the predominant pathway, especially under abiotic stress (Delauney and Verma 1993, Bhatia *et al.* 1994). There are reports of higher Pro accumulation inducing stress tolerance in many plant species (Sawahel and Hassan 2002).

Safflower (*Carthamus tinctorius* L.) is an oil yielding crop grown throughout the semiarid regions. It is a deep-rooted annual which has the ability to meet its water requirements by exploring a larger volume of soil than the other oil-yielding crops (Dordas and Sioulas 2008). Different osmolytes have also been reported in safflower under stress (Bhatia *et al.* 1994). However, it is not known whether the Pro pathway is activated during stress in safflower. Hence, the objective of our investigation

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Abbrevations: *p5cs* - pyrroline-5-carboxylate synthetase gene; P5C reductase - pyrroline-5-carboxylate reductase; P5C synthetase - pyrroline-5-carboxylate synthetase; Pro - proline; RT-PCR - reverse transcriptase polymerase chain reaction.

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was to determine the content of Pro under drought stress in safflower, and to establish the relationship between the Pro accumulation, the activities of P5C reductase and P5C synthetase and also the expression of *p5cs* gene. Based on the previous reports in rice and *Arabidopsis thaliana* where the drought stress treatments increased *p5cs* transcript level (Hien *et al.* 2003, Szekely *et al.* 2008), we considered the possibility of the *p5cs* being regulated by drought and hence a detailed analysis of the expression of *p5cs* was also carried out. In order to find out the significance of Pro-mediated mechanisms in drought tolerance, the observations in drought tolerant cv. A1 and drought sensitive cv. Nira were compared.

Seeds of safflower (*Carthamus tinctorius* L.) cultivars A1 and Nira (procured from Agricultural Department, Anantapur) were surface sterilized with 0.1 % (m/v) sodium hypochlorite solution for 5 min, thoroughly rinsed with distilled water and sowed in plastic pots containing soil:sand (2:1) mixture allowed to grow at 28 ± 4 ºC with regular water supply and natural irradiance (photoperiod 10 - 12 h). Three weeks old plants of both cultivars were separated into four sets. One set of plants were maintained at soil moisture level (SML) 100 % served as control. Remaining three sets of the plants were subjected to water stress by maintaining the SML at 75, 50 and 25 % (represented as mild, moderate and severe stress). After 8 d leaf samples were collected from control and stressed plants and dry mass was measured. Chlorophyll content was estimated by measuring absorbance in 80 % cold acetone extracts at 645 and 663 nm (Arnon 1949). Extraction and estimation of free Pro was done according to Bates *et al.* (1973). Assay of P5C reductase activity was done according to Rena and Splittstoesser (1975). Frozen leaves were homogenized in 100 mM sodium phosphate buffer, pH 8.0 containing 0.1 mM cysteine and 0.1 mM EDTA. The homogenate was centrifuged at 18 000 *g* in a refrigerated high speed centrifuge (*Sigma*, Osterode, Germany) for 15 min and the supernatant collected. 0.1 cm^3 of this extract was added to the 1 cm^3 reaction mixture supplied with 128 μM NADH, 400 μM L-pyrroline-5-carboxylate (*Sigma*, St. Louis, USA) and 100 mM sodium phosphate buffer pH 7.4. This mixture was then incubated at 32 °C for 3 min. Reaction was started by the addition of L-pyrroline-5-carboxylate and the enzyme activity was followed for 3 min by measuring the variation in absorbance at 340 nm. The same system devoid of NADH and the enzyme extract was used as blank. P5C synthetase assay was determined by measuring the formation of γ-glutamyl hydroxamate followed by the addition of γ-glutamyl kinase (Hayzer and Leisinger 1980). Leaves were homogenized in 100 mM Tris HCl extraction buffer at pH 7.5 containing 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 4 mM dithiothreitol (DTT), 2 mM phenylmethane-sulphono fluoride (PMSF) and 2 % polyvinylpoly-pyrrolidone (PVPP). Extract was then centrifuged at 4 °C for 30 min at 10 000 *g* and 1 cm^3 of the supernatant was added to the reaction mixture provided with 50 mM Tris-HCl (pH 7.0), 50 mM L-glutamate, 20 mM $MgCl_2$, 100 mM hydroxylamine HCl and 10 mM ATP. The mixture was then incubated at 37 °C for 15 min and the reaction was stopped by adding 1 cm^3 of the stop buffer (150 μM $FeCl_3$ and 360 μM trichloroacetic acid in 2.5 M HCl). Precipitated proteins were removed by centrifu-gation and absorbance of the supernatant was read at 535 nm against the blank devoid of ATP and the enzyme extract. The amount of γ-glutamylhydroxamate complex produced was estimated by using molar coefficient of absorbance of 250 M^{-1} cm^{-1} reported for Fe^{3+}-hydroxamate complex. Enzyme activity unit represents the amount of enzyme required to produce 1 μM of γ-glutamylhydroxamate min^{-1}. Total protein content was determined according to Bradford (1976).

Total RNA was extracted according to the method described by Datta *et al.* (1989) and treated with DNase I (*Invitrogen*, Carlsbad CA, USA). First-strand cDNA was synthesized from 1 μg total RNA added to the reaction mixture supplied with 0.3 mM dNTP mix, 0.5 μg $Oligo(dT)_{12-18}$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 0.01 mM DTT, 40 U of RNase-OUT™ (*Promega*, Madison, USA), and 50 U of SuperScript™ II RT (*Thermo Scientific*, Epsom,UK). The reaction was carried out at 42 °C for 1 h, followed by incubation at 70 °C for 15 min. 1 U of RNase H (*Promega*) was finally added and incubated at 37 °C for 20 min to remove RNA templates from the cDNA:RNA hybrid molecules. Control reactions for each sample were performed simultaneously without reverse transcriptase. Based on the *Oryza sativa* sequence (GenBank accession No. BAA19916), specific primers (forward: 5'-TTC ATG GGA AAA AAT TGG T-3' and reverse: 5'-CCG CAA TAA GAT GCT TGT AC-3') were designed and used to amplify a fragment of *Ctp5cs* from safflower. 0.001 cm^3 of the RT reaction product was used as template in the 0.02 cm^3 reaction mixture. Amplification conditions for *Ctp5cs* was setup with initial 5 min denaturation at 94 °C followed by 30 cycles comprising of 94 °C 30 s, 61 °C 30 s, 72 °C 120 s and the whole reaction ended up with a final extension step at 72 °C for 7 min. Actin was the internal control used. PCR products were run on 1 % agarose gel and relative quantification was done based on the intensity of the bands using *Doc-It®LS* image analysis software. For Northern blotting, the RNA samples (20 - 25 μg) were allowed to run on 1.5 % agarose-formaldehyde gel and transferred to a nylon membrane (*Roche Diagnostics*, Indianapolis, USA) by capillary blotting. Hybridization probe was synthesized from our cDNA library clone containing the anther-specific pro-rich protein (GenBank accession no. EL611815) using random primer labeling kit (*Gibco-BRL*, Gaithersburg, MD, USA) and the hybridization was done at 65 °C overnight. The blot was washed twice with 2× saline sodium citrate buffer (SSC, pH 7.0) and 0.5 % sodium-dodecylsulphate (SDS) for 15 min at room temperature for 10 min followed by another two washes with 0.2× SSC and 0.1 % SDS for 15 min at 60 °C. Images captured on X-ray films were scanned using *Phospho-*

Imager (*Fujix BAS 1500*, *Fuji*, Tokyo, Japan).

Significant growth inhibition and reduction in chlorophyll content were observed in plants under water stress in plants of both cultivars (Table 1) and more pronounced decrease was observed in cv. Nira compared to cv. A1. Chlorophyll content estimation is one of the indices for estimating resistance to dehydration (Khidse *et al.* 1982). There are reports of reduction in the total chlorophyll content of leaves in water stressed plants (Ramanjulu and Sudhakar 2000, Reddy *et al.* 2008). On the other hand, increase in chlorophyll content under osmotic stress has also been reported (Wang *et al.* 2000, Silva-Ortega *et al.* 2008). Compatible solutes like Pro play an important role in preventing thylakoid membrane damage during dehydration (Schwab and Heber 1984) and since Pro is also having the function of a protein stabilizer, it can protect the enzymes involved in the chlorophyll biosynthesis during stress conditions.

Table 1. Effect of different soil moisture (SML 100 - 25 %) on leaf dry mass [g(d.m.) plant^{-1}], chlorophyll and proline contents [mg g^{-1}(d.m.)] and activities of P5C reductase and P5C synthetase [U mg^{-1}(protein) min^{-1}] on two safflower cultivars. Means from 5 experiments ± SE. The mean values in a row followed by a different letter for each plant species are significantly different ($P \leq 0.05$) according to Duncan's multiple range (DMR) test.

Cultivars	A-1				Nira			
Parameters/SML	100 %	75 %	50 %	25 %	100 %	75 %	50 %	25 %
Leaf dry mass	0.25±0.01	0.22±0.02	0.16±0.01	0.13±0.02	0.25±0.01	0.21±0.01	0.15±0.02	0.11±0.02
Chlorophyll content	3.34±0.14	3.12±0.14	2.52±0.12	2.13±0.14	3.24±0.12	2.93±0.16	2.31±0.14	1.85±0.14
Proline content	0.41±0.06	0.62±0.03	0.97±0.08	1.39±0.10	0.36±0.06	0.53±0.04	0.79±0.08	1.02±0.07
P5C reductase	11.03±2.41	19.81±2.63	28.60±3.21	42.80±3.23	10.56±2.21	18.90±2.43	25.50±3.24	33.90±3.43
P5C synthetase	6.21±0.63	8.66±0.81	8.83±0.51	8.11±0.63	5.90±0.81	8.10±0.65	8.40±0.59	7.90±0.65

Free Pro content in the leaves of both the cultivars was significantly higher in stressed plants than in the control plants under all stress levels (Table 1) and cv. A1 showed the higher Pro content than cv. Nira. This increase in Pro content is proved to be essential for stress tolerance because of its active role in osmotic adjustment, protection of the enzyme structure, stabilization of membranes and defense against hydroxyl radicals (Silva-Ortega *et al.* 2008, Reddy *et al.* 2008). Gene transfer experiments also suggest the possibility that Pro biosynthesis can enhance osmotic tolerance (Kishor *et al.* 1995, Yamada *et al.* 2005).

P5C reductase is the key enzyme in Pro biosynthesis and activity of P5C reductase was higher at 50 % SML than in control plants and increased further at 25 % SML (Table 1). A relatively higher activity of P5C reductase was observed in the leaves of cv. A1 than cv. Nira presenting evidence for higher rate of synthesis thereby leading to the increased accumulation of Pro in cv. A1. Similar increase in P5C reductase in relation to the Pro accumulation has been reported in *Brasssica juncea* (Madan *et al.* 1995), mulberry (Ramanjulu and Sudhakar 2000, 2001, Kumar *et al.* 2003) and foxtail millet (Veeranagamallaiah *et al.* 2007).

However, the activity of P5C synthetase was only slightly higher in stressed plants than in control plants and remained similar at all stress levels (Table 1). In parallel to P5C synthetase, we studied γ-glutamyl kinase and found only a negligible change under reduced soil moisture. Also in aquatic macrophyte *Hydrilla verticellata* (Rout and Shaw 1998), wheat (Wang *et al.* 2007) and *Opuntia* (Silva-Ortega *et al.* 2008) the P5C synthetase activity was not significantly affected under stress. However, there are reports of P5C synthetase activity increase in response to salinity (Delauney and Verma 1993, Zhang *et al.* 1995).

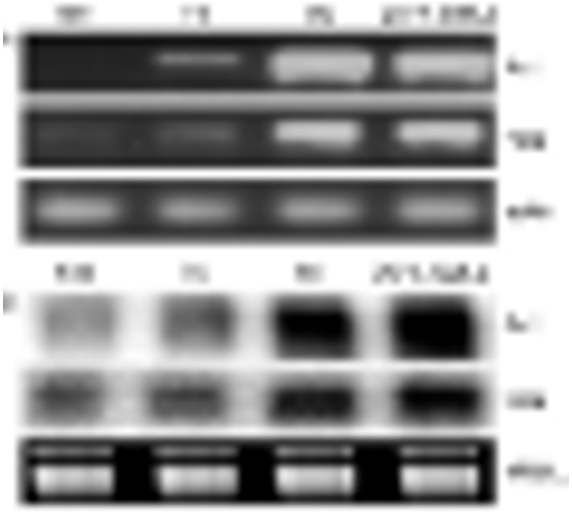

Fig. 1 Changes in gene expression in relation to drought stress. *A* - *Ctp5cs* band at the different levels of stress. *B* - Northern blot probed with anther specific Pro-rich protein.

Our experiments in safflower suggested a rate-limiting role for P5C reductase in Pro synthesis from glutamate under water stress conditions. Generally P5C synthetase gene is strongly induced by drought and salt stresses. RT-PCR results show that the *Ctp5cs* expression was significantly upregulated in both cultivars at 50 and 25 % SML. Among the two cultivars, the highest transcript level was observed in the drought-tolerant A1 cultivar (Fig. 1*A*). In *Arabidopsis* and rice, *p5cs* was over-expressed under high salinity and dehydration and a

simultaneous accumulation of Pro was also observed (Yoshiba *et al.* 1995, Hein *et al.* 2003). In *Arabidopsis* there are two P5CS isoforms playing specific roles in the control of Pro biosynthesis (Fabro *et al.* 2004, Szekely *et al.* 2008) and in few other cases, P5CS is reported to be encoded by two genes (Fujita *et al.* 1998, Ginzberg *et al.* 1998). Among the two closely related *p5cs* genes recently identified in *Arabidopsis thaliana, p5cs1* is the major one functioning under stress (Fabro *et al.* 2004, Szekely *et al.* 2008) and its expression is usually controlled by different signaling pathways (Szekely *et al.* 2008). P5CS activity is controlled by feedback inhibition and transcriptional regulation (Yoshiba *et al.* 1995, Silva-Ortega 2008).

Northern blot analysis show differential expression of anther specific Pro-rich protein in the two safflower cultivars and the expression levels were significantly increased with the increase in stress severity (Fig. 1*B*). The observed increase in transcript level was even up to 5.5 fold in drought-tolerant cv. A1 than the drought-sensitive cv. Nira (results not shown).

In the case of safflower, increased mRNA levels observed in the drought resistant cv. A1 were directly proportional to the Pro content whereas the drought sensitive cv. Nira did not show that much increase in the mRNA as well as the Pro contents. From the gene expression profiles of the Pro biosynthesis pathway, it can be concluded that the stress tolerance mechanism in safflower is controlled by Pro. During development, the levels of free Pro vary among plant organs independent of the amino acid pool size. Highest Pro contents are found in flowers, especially in pollen grains, seeds and the lowest in roots. Pro content is also dependent on age of the plant, leaf age, position or part of the leaf (Chiang and Dandekar 1995, Verbruggen and Hermans 2008).

In conclusion, Pro accumulation mostly due to increased activity of P5CS can be considered as an index for water stress tolerance in safflower. Transcriptional level regulation is found to be the major regulatory mode. The difference in stress tolerance observed among the cultivars A1 and Nira may be due to the variability in expression pattern of the genes associated to Pro biosynthesis. Further characterization of *Ctp5cs* by gene transfer experiments can conclude the mode of Pro mediated stress tolerance in safflower.

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