Effect of Drought Stress at Supraoptimal Temperature on Polyamine Concentrations in Transgenic Soybean with Increased Proline Levels

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The effect of drought stress at supraoptimal temperature on free proline and polyamine levels was compared in wild type and transgenic soybean (*Glycine max* cv. Ibis) plants having increased proline levels. Since glutamate and arginine are precursors of both proline and polyamines, it was assumed that the genetic manipulation of proline levels would also affect the polyamine levels. The proline and spermine concentrations increased, while the putrescine concentration generally decreased or did not change after the treatments in both genotypes. Following drought higher proline and lower spermine levels were detected in the transgenic plants compared to the wild type ones, which could be explained by the increased use of their common precursors for proline biosynthesis in the transgenic plants.

Key words: Polyamine, Proline, Soybean

Introduction

Polyamines (PAs) are essential to all cells, since mutants lacking the ability to synthesize polyamines are unable to grow and develop normally (Galston and Kaur-Sawhney, 1990). As polycations they bind readily to cellular polyanions such as DNA, RNA, phospholipids and acidic protein residues, affecting their synthesis and activity. They are also involved in the reproductive activity (flower initiation, fruit growth) (Galston *et al.*, 1997) and stress responses of plants (Bouchereau *et al.*, 1999). Their accumulation during a mild stress period functions as a type of hardening and results in better survival in the case of subsequent stress. The ability of PAs to reduce stress-induced injuries can be explained by their participation in the removal of reactive oxygen species (Guerrier *et al.*, 2000) and their involvement in the maintenance of turgor (Islam *et al.*, 2003) and photosynthetic activity (Galston *et al.*, 1997). Polyamines can be synthesized in plants through both the ornithine decarboxylase and arginine (Arg) decarboxylase pathways, but the latter is much more important under stress situations (Tiburcio *et al.*, 1997; Cohen, 1998).

The involvement of PAs in the response to drought stress was reported in several publications (Erdei *et al.*, 1996; Zhang *et al.*, 1996; Rajasekaran and Blake, 1999; Guerrier *et al.*, 2000). Mannitol-induced osmotic stress increased the putrescine (Put), spermidine (Spd) and spermine (Spm) contents in wheat (Galiba *et al.*, 1993). In a time course experiment a great increase in their level was detected after one week of drought (Kubis and Krzywanski, 1989). Similarly, osmotic stress induced a greater increase in Put and Spd contents in the tolerant species *Lycopersicon pennellii* than in the sensitive *L. esculentum* (Santa-Cruz *et al.*, 1997). The withholding of water induced a greater increase in the Put synthesis (as shown by the greater activity of Arg decarboxylase and ornithine decarboxylase) in drought-tolerant sugarcane varieties than in sensitive ones (Zhang *et al.*, 1996). Further evidence for the adaptive role of

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Abbreviations: OPLC, overpressured layer chromatography; P5CR, L-Δ^1-pyrroline-5-carboxylate reductase; PA(s), polyamine(s); PC(A): principal component (analysis); PS, preliminary stress; Put, putrescine; RWC, relative water content; Spd, spermidine; Spm, spermine; Tym, tyramine.

polyamines during drought was obtained by comparing reed ecotypes, since the Arg decarboxylase activity and PA levels were higher in terrestrial reeds than in swamp reed ecotypes (Wang *et al.*, 1995). The transformation of tobacco with *S*-adenosylmethionine decarboxylase led to increased polyamine biosynthesis and improved drought tolerance (Waie and Rajam, 2003). Exogenous spermidine and spermine stimulated elongation growth and reduced membrane damage to jack pine seedlings under drought conditions (Rajasekaran and Blake, 1999).

Not only drought but also high temperature stress resulted in the accumulation of PAs, as described for beans (Kuznetsov and Shevyakova, 1997). Heat stress induced greater PA synthesis and accumulation in a tolerant rice genotype than in a sensitive one (Roy and Ghosh, 1996). In addition, the inhibition of chickpea seed germination at supraoptimal temperature was alleviated by exogenous Put (Gallardo *et al.*, 1996). The involvement of polyamines in heat stress response was also demonstrated in rice plants, in which high temperature treatment increased the cadaverine, Put and Spd contents (Shevyakova *et al.*, 2001).

Similarly to PAs, proline (Pro) has also an important role in reducing the damage caused by water deficit and high temperature (de Ronde *et al.*, 2004; Georgieva *et al.*, 2003). It also acts as an antioxidant (Hong *et al.*, 2000). Transgenic soybean plants containing the gene coding for the last enzyme of Pro biosynthesis, L-Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1. 5.1.2) in the sense direction, had higher Pro content and suffered less damage during simultaneous water deficit and heat stress than wild type plants (de Ronde *et al.*, 2004). Pro and PAs have two common precursors, arginine (Arg) and glutamate (Glu) (Cohen, 1998). It was therefore assumed that the manipulation of Pro concentration also resulted in changes in PA synthesis.

To test this hypothesis the Pro and PA levels were compared in wild type and transgenic soybean plants overexpressing the *P5CR* gene.

Materials and Methods

Plant material and treatment

Wild type *Glycine max* (L.) Merr. cv. Ibis and transgenic soybean plants transformed with a construct containing a heat shock-inducible promoter and the cDNA coding for P5CR in the sense direction were investigated (de Ronde *et al.*, 2004). Molecular analysis of the T3 transgenic plants confirmed the presence of 3 to 5 copies of the *P5CR* gene in the test plants and at least 3 integrations in the genome (de Ronde *et al.*, 2004). The P5CR mRNA levels were 3- to 4-fold, and the protein levels 2 to 3-fold higher in the transformants compared to the wild type plants. The two lines used in the present study were selected on the basis of their drought tolerance and Pro concentrations. Seeds were germinated between two layers of damp paper in the dark at 25 °C for 4 d. After germination the seedlings were raised in pots (one plant/500 mL pot) containing a 2:1:1 mixture of garden soil, humus and sand. The same amount of soil (500 g) was placed in each pot and the same amount of water (200 mL) was added at each irrigation. The plants were watered when the weight of pots was reduced by 200 g during cultivation and recovery compared to their starting weight. Their weight was adjusted to this value just at the start of the drought hardening and the subsequent stress. The plants were grown in a growth chamber (Conviron PGR-16, Controlled Env. Ltd., Winnipeg, Canada) at 25/15 °C day/night temperature for 6 weeks with 16 h illumination at 400 μmol m^{-2} s^{-1} (Tischner *et al.*, 1997). The seedlings were subjected to preliminary stress (PS) by withholding water for 10 d at 35/25 °C day/night temperature. Then they were watered once and were further cultivated at 35/25 °C without irrigation for an additional 10 d. The drought stress was carried out at higher growth temperature in order to switch on the heat-inducible promoter in the introduced gene construct. In a previous work a combination of drought and heat stress was efficient in activating this promoter and manipulating Pro levels (de Ronde *et al.*, 2004), so a similar experimental system was used in the present study. The stress treatment was followed by a recovery period with watering at 25/15 °C for 10 d. Samples (the youngest fully developed trifoliate leaves) were taken at the beginning of the experiment, after 10 d PS, after 4, 7 and 10 d stress, and after 10 d recovery. The injury percentage was also determined at these sampling points. The injury was scored on a 0 to 100% scale on the basis of the wilting and drying of the shoots (Kocsy *et al.*, 2005).

Determination of relative water content

For the calculation of relative water content (RWC) the weight of leaf disks (8 mm in diameter) was measured immediately after sampling (initial weight, IW), after 4 h immersion in deionized water (turgescent weight, TW) and after subsequent drying at 80 °C for 24 h (dry weight, DW). The RWC was calculated using the formula: $100 \cdot (IW - DW)/(TW - DW)$ (Kocsy *et al.,* 2005).

Determination of free polyamines

Soybean samples (300 mg) were extracted with 3 mL 7% $HClO_4$ for 1 h at room temperature using a shaking equipment (VEB MLW, Labortechnik, Ilmenau, Germany). Each sample was filtered through a 0.45 μm pore membrane filter (Sartorius, Göttingen, Germany).

The sample extracts (500 μL) were mixed with dansyl chloride (18.5 μmol mL^{-1} in acetone; 1000 μL) and saturated sodium carbonate (500 μL) was added. The mixture was incubated overnight in darkness at room temperature. Pro (868.5 μmol mL^{-1}; 200 μL) was added and the mixture was incubated for 30 min to remove excess dansyl chloride. The dansyl amines were extracted twice with toluene (500 μL).

The dansyl amines were analyzed chromatographically on 20 × 20 cm HPTLC (high performance thin layer chromatography) silica gel 60 F_{254} plates (Merck, Germany) using a Personal OPLC BS50 Chromatograph (OPLC-NIT Ltd., Budapest, Hungary). The parameters were as follows: mobile phase: eluent A (first step), *n*-hexane/*n*-butanol/triethylamine 90 : 10 : 8.1 (v/v), eluent B (second step), *n*-hexane/*n*-butanol 80 : 20 (v/v); development conditions: external pressure, 5.0 MPa; flow rate, 500 μL min^{-1}; rapid volume, 200 μL; eluent volume A, 11500 μL, eluent volume B, 800 μL; development time, 1576 s. Quantitative evaluation was accomplished at 313 nm using a CAMAG SC3 (CAMAG, Switzerland) densitometer (Kovács *et al.*, 1998).

All the chemicals used in this study were of analytical grade. The biogenic amine standards: putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Sigma (St. Louis, MO, USA). The standard solution was prepared in 0.1 M hydrochloric acid and dansylated in the same way as the samples.

Determination of amino acids

Selected amino acids (proline and its precursors arginine and glutamate) were determined from the same extract (7% $HClO_4$) as used for polyamine analysis on an amino acid analyser (Biotronik LC 3000, Germany) as described earlier (Galiba *et al.*, 1989).

Statistical analysis

Analyses were performed in triplicate, and the data are presented as mean ± SD. Statistical analysis, involving principal component analysis (PCA) and the comparison of treatment means at the 5% level, was performed using STATISTICA 6.0 software for Windows.

Results

Injury to the plants gradually increased during stress treatment and decreased slightly during recovery (Table I). During PS it was slightly lower in the transgenic plants, and this difference became significant during the second half of the subsequent stress and the recovery.

Table I. Effect of simultaneous drought and mild heat stress on injury percentage of wild type (W) and transformed (T) soybean plants. Values carrying different letters were significantly different at the $P < 5\%$ level.

	Injury percentage					
	Start	PS	Stress			Recovery
		10 d	4 d	7 d	10 d	10 d
W	6 ± 2^{a}	12 ± 3^{b}	17 ± 4^{bc}	27 ± 5^{d}	34 ± 5^{d}	32 ± 4^{d}
T	4 ± 1^{a}	8 ± 3^{ab}	11 ± 3^{b}	18 ± 4^{c}	25 ± 4^{cd}	21 ± 6^{cd}

The effect of drought stress on RWC was not as pronounced as on injury. Although RWC was slightly greater in the transgenic plants than in the wild type ones, a significantly higher RWC was only observed in transgenic soybean at the end of the recovery phase (Fig. 1).

The treatment induced much more rapid Pro accumulation in the transformants than in the wild type plants (Table II). After PS the Pro content increased 31-fold in the wild type and 124-fold in transformed plants. During the subsequent stress the Pro content was higher in the transgenic plants than in the wild type ones. The Pro levels declined to the starting levels during recovery. The changes in the concentrations of Glu and Arg as precursors of Pro were of special interest. In general Glu content increased and the content of Arg decreased as a result of simultaneous drought and heat stress. The stress-induced increase in Glu was much smaller than that in Pro. Similarly to Pro, the in-

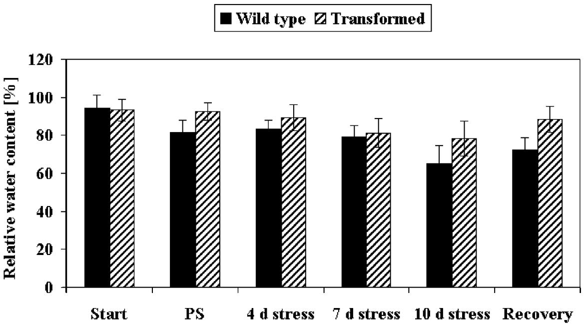

Fig. 1. Effect of simultaneous drought and mild heat stress on the relative water content (RWC) in wild type and transformed soybean plants. Bars represent standard deviation. Differences between any two values of RWC were significant at the $P < 0.05$ level if they exceeded 10.21%. PS, preliminary stress.

crease in Glu content during the subsequent stress was followed by a decrease in the recovery period. The decrease of Arg level was more pronounced in the wild type than in the transformed plant.

A nearly 3-fold increase in total PA level was observed at the end of PS in the wild type plants and after 7 d stress in the transformants (data not shown). At the other sampling points only slight changes were found in this parameter.

PS decreased the Put level, but during the first part of the subsequent stress the Put content increased again (Fig. 2A). This was followed by a decline after 7 d stress in wild type plants and after 10 d stress in transgenic ones. During recovery the Put content increased to the starting values.

The dominant polyamine was the triamine Spd, whose level exhibited a more than 50% increase

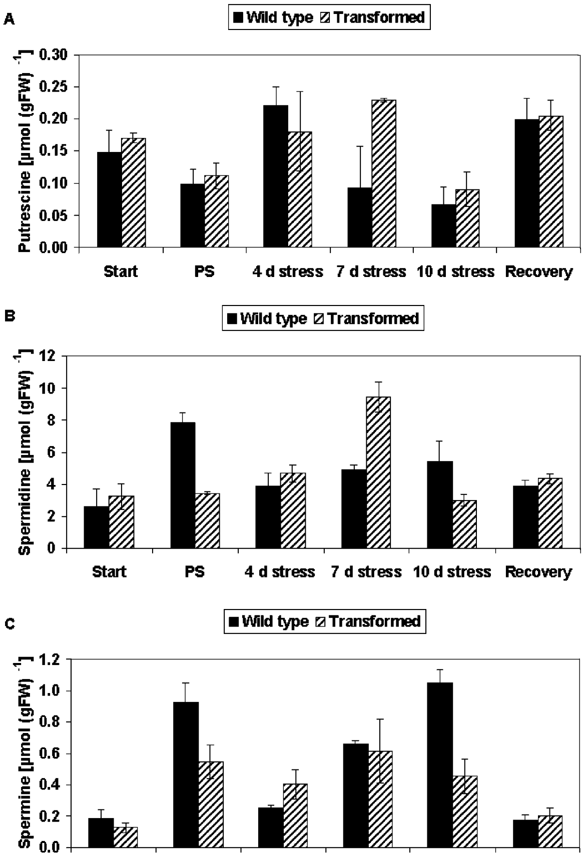

Fig. 2. Effect of simultaneous drought and mild heat stress on the putrescine (A), spermidine (B) and spermine (C) contents in wild type and transformed soybean plants. Differences between any two values of Put, Spd and Spm were significant at the $P < 0.05$ level if they exceeded 0.09, 2.02 and 0.27 μmol g^{-1}, respectively. PS, preliminary stress.

Table II. Effect of simultaneous drought and mild heat stress on the proline, glutamate and arginine contents in wild type (W) and transformed (T) soybean plants. Values carrying different letters were significantly different at the $P < 5\%$ level.

		Amino acid content [μmol/g FW]					
		Start	PS	Stress			Recovery
			10 d	4 d	7 d	10 d	10 d
Proline	W	0.79 ± 0.13^{a}	24.16 ± 0.87^{b}	9.24 ± 0.94^{c}	35.52 ± 5.15^{d}	86.54 ± 12.89^{e}	0.95 ± 0.07^{a}
	T	0.49 ± 0.04^{a}	60.64 ± 4.41^{f}	17.45 ± 4.67^{b}	90.78 ± 18.77^{e}	87.54 ± 7.17^{e}	0.55 ± 0.11^{a}
Glutamate	W	1.67 ± 0.19^{g}	2.58 ± 0.41^{g}	1.38 ± 0.41^{g}	3.83 ± 1.16^{h}	6.38 ± 0.45^{i}	1.26 ± 0.53^{g}
	T	1.14 ± 0.08^{g}	6.30 ± 0.49^{i}	1.91 ± 0.06^{g}	9.05 ± 2.09^{j}	2.49 ± 0.31^{g}	1.14 ± 0.61^{g}
Arginine	W	25.05 ± 2.43^{k}	1.93 ± 0.17^{m}	1.32 ± 0.23^{m}	3.45 ± 0.56^{po}	4.27 ± 0.03^{po}	1.23 ± 0.19^{m}
	T	18.80 ± 0.29^{l}	8.26 ± 2.79^{op}	2.87 ± 1.51^{m}	9.61 ± 0.46^{op}	7.08 ± 0.37^{op}	1.17 ± 0.58^{m}

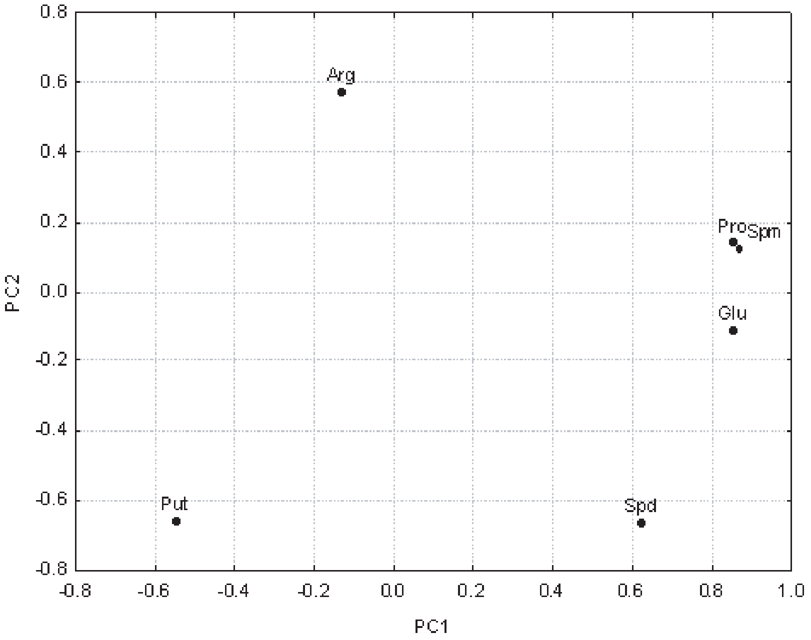

Fig. 3. Principal component analysis on the Arg, Glu, Pro, Put, Spd and Spm data. Unrotated principal component loadings, PC1 versus PC2.

after PS in the wild type plants and after 7 d stress in the transgenic plants (Fig. 2B). In the wild type plants the Spd content gradually decreased during the stress, and after 10 d its level was more than 50% lower compared to the transgenic plants.

The content of the tetramine Spm showed a great increase during the PS. Then after a transient decrease, the levels increased again, declining to the starting values during recovery (Fig. 2C). Spermine content increased 3-fold in both genotypes after PS. The Spm content was significantly greater after 10 d stress in the wild type and after 4 and 7 d stress in the transformants.

Regarding the possible relationship between the amounts of Pro, PAs and their precursors, Glu and Arg, principal component analysis (PCA) showed that two principal components explained 70% of the total variance in the data (Fig. 3). The data demonstrated a significant negative correlation between the content of Put and Spm and between those of Put and Spd, which could be the result of the conversion of Put into the other two PAs. On the other hand, the points representing changes in the Pro and Spm contents were very close to the Glu values in this figure, indicating that the greater Pro or Spm synthesis induced a corresponding increase in the level of the precursor Glu.

Discussion

The transformation did not affect the growth of the plants under control conditions since there was no significant difference in the injury and RWC of the plants. The PS treatment efficiently prevented major water loss during the subsequent stress, as shown by RWC data and previously reported in wheat (Sgherri *et al.*, 2000). This in turn ensured the appropriate functioning of protective metabolic pathways, as demonstrated by the increased Pro and polyamine synthesis. The efficiency of the protection against drought-induced damage is corroborated by the fact that even in the case of wild type plants the injury percentage did not exceed 32%.

Regarding the protective role of Pro against drought and heat stress, the present findings corroborate previous results where greater Pro accumulation was observed during drought in a tolerant wheat genotype compared to a sensitive one (Nayyar and Walia, 2003), and heat stress resulted in smaller injuries in cotton genotypes with a higher Pro content (Ashraf *et al.*, 1994).

The involvement of PAs in the response to drought and heat stress was also confirmed in soybean, since increased Spm content was detected in stressed plants and the level of the other PAs was also higher compared to their starting values at certain sampling points. Similarly to the present increase of the Put content in transformed soybean after 4 d simultaneous heat and drought stress, higher Put content was also detected in drought-stressed poplar (Guerrier *et al.*, 2000) and heat-stressed rice (Roy and Ghosh, 1996). As described in the present experimental system, drought also increased the Spm and Spd contents in rape (Aziz and Larher, 1995), while heat induced their accumulation in rice (Roy and Ghosh, 1996). While in soybean a parallel increase was found in the Pro and Spm levels, osmotic stress resulted in a simultaneous increase in the Pro and Put contents in rape leaf discs (Aziz and Larher, 1995) and in barley leaves (Turner and Stewart, 1988).

The manipulation of Pro synthesis affected not only Pro, but also the PA levels, as found by comparing wild type soybean plants with transgenic lines overexpressing the gene coding for P5CR. Because of the much greater increase in Pro in the transformants (124-fold) than in the wild type (31-fold) during PS, the initial pool of the common precursors (Arg, Glu) was probably unable to ensure the parallel induction of PA accumulation, as shown by the decreased Spd and Put levels in the transgenic plants. In wild type plants, on the other hand, a parallel increase in the Pro and Spd levels during PS was possible due to their smaller Pro accumulation. The simultaneously high Pro and Spm levels after 10 d stress in the wild type plants might have been due to increased protein degradation (Simon-Sarkadi, unpublished results), which resulted in greater injury during recovery. This assumption is corroborated by the results of Galiba *et al.* (1989), who found greater exopeptidase activity in a drought-sensitive wheat variety compared to a tolerant one after treatment with 13% mannitol. Similarly to the present findings, an interaction between Pro and PA synthesis was also observed by Larher *et al.* (2003).

The much greater increase in Pro levels during PS in transformed plants could be more important in the reduction of stress-induced damage than the large initial increase in PA levels observed in the wild type plants, since the transgenic plants recovered better after the treatments. However, the increased PA levels in transgenic plants in certain stress periods may also make an important contribution to their greater ability to reduce stress-induced damage. Although the increase in Put content induced by salicylic acid was not accompanied by improved drought tolerance in maize (Németh *et al.*, 2002).

Taken together, the fact that the genetic manipulation of the Pro concentration also affected PA levels may be due to their common precursors. The present results indicate that the time course of changes in Pro and PA concentrations may be more important for better stress response than their level at the end of a long period of treatment.

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