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BRIEF COMMUNICATION

# Stress-induced changes in the free amino acid composition in transgenic soybean plants having increased proline content

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## Abstract

Following drought stress at supraoptimal temperature the increase in proline (Pro) content in transgenic (T) soybean [*Glycine max* (L.) Merr. cv. Ibis] plants overexpressing the gene coding for the last enzyme of Pro biosynthesis, L-$\Delta^1$-pyrroline-5-carboxylate reductase, was much greater than in wild type (W) plants (105-fold versus 19-fold after 7 d). Under control conditions arginine accounted for nearly 60 % of the total free amino acid content. After stress treatment the content of Pro was more than 50 % in both T and W genotypes, and at the end of recovery the γ-aminobutyrate content reached 27 and 53 % in the W and T plants, respectively. Without stress treatment there was only a 2-fold difference between T and W in the tyrosine content. However, during the stress period and the subsequent recovery a similar difference was found for many amino acids. The present results indicate that manipulating of the content of a single amino acid influences the whole free amino acid composition in soybean.

*Additional key words*: drought, *Glycine max*, heat.

Under drought and heat stress conditions, when the water availability is limited, the accumulation of low molecular mass compounds, such as free amino acids, ensures the osmotic adjustment of the plants (Good and Zaplachinski 1994, El Tayeb and Hassanein 2000, Trotel-Aziz *et al.* 2003). The contribution of proline (Pro) to improved drought tolerance was shown, *e.g.*, in barley, where greater ability to accumulate Pro correlated with decreased membrane injury (Bandurska 2000). In addition, a large increase in the Pro precursors, glutamate (Glu) and arginine (Arg), was observed in *Brassica napus* during drought (Good and Zaplachinski 1994). The suppression of Pro synthesis in transgenic plants containing the gene coding for L-$\Delta^1$-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) in the antisense direction resulted in increased sensitivity to water shortage (De Ronde *et al.* 2000). Apart from the considerable accumulation of Pro and its precursors in *B. napus*, drought also induced a more than 5-fold increase in isoleucine (Ile), leucine (Leu) and aspartate (Asp) contents, as well as an increase, albeit small, in the levels of all other amino acids (Good and Zaplachinski 1994). Water deficit resulted in the accumulation of Leu, Ile, threonine, alanine, and valine in bean (Raggi 1994). The involvement of free amino acids in the response to heat stress was shown in spring wheat, where a mutant having

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*Abbreviations*: Arg - arginine; Asn - asparagine; Asp - aspartate; DM - dry mass; Gaba - γ-aminobutyrate; Gln - glutamine; Glu - glutamate; His - histidine; Ile - isoleucine; Leu - leucine; Phe - phenylalanine; P5CR - L-$\Delta^1$-pyrroline-5-carboxylate reductase; Pro - proline; PS - preliminary stress, RWC - relative water content; Ser - serine; T - transgenic plants; Tyr - tyrosine; W - wild type.

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greater amino acid contents (especially serine, methionine, Ile, histidine and Arg) was damaged to a lesser extent after high temperature treatment than the parent genotype (Behl *et al.* 1991). The aim of the present research was to find out whether overexpression of the gene coding for P5CR in soybean affected the stress-induced changes in the amino acid composition.

Wild type soybean [*Glycine max* (L.) Merr. cv. Ibis] was compared with transgenic lines transformed with a construct containing a heat shock-inducible promoter and the cDNA coding for P5CR in the sense direction (De Ronde *et al.* 2000). 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 transgenic line used in the present study was selected on the basis of its drought tolerance and Pro concentration. After germination (25 °C for 4 d), the seedlings were raised in pots containing a 2:1:1 mixture of garden soil, humus and sand. The plants were grown for 6 weeks in a spring type growth chamber (*Conviron PGV-15, Controlled Env. Ltd.*, Winnipeg, Canada) at day/night temperature 25/15 °C, relative humidity 70/75 %, and 16-h photoperiod (irradiance of 400 μmol $m^{-2}$ $s^{-1}$). The seedlings were subjected to preliminary stress (PS) by withholding water for 10 d at 35/25 °C day/night temperature in order to switch on the heat-inducible promoter. Subsequently they were watered once and then further cultivated at 35/25 °C without irrigation for an additional 10 d. The stress treatment was followed by a recovery period during which the plants were watered for 10 d at 25/15 °C. Samples were taken at the beginning of the experiment, after 10-d preliminary stress, after 4-, 7- and 10-d stress, and after 10-d recovery. At least 3 wild type (W) or transgenic (T) plants were investigated in each experiment and the experiments were repeated 3 times.

For the calculation of relative water content (RWC) the fresh mass of leaf disks (8 mm in diameter) was measured immediately after sampling (initial mass, IM), after 4 h immersion in deionised water (water-saturated mass, SM) and after subsequent drying at 80 °C for 24 h (dry mass, DM). The RWC was calculated using the formula: $RWC = (IM - DM)/(SM - DM) \times 100$.

For the determination of the free amino acid content the soybean leaves (300 mg) were extracted with 3 $cm^3$ 7 % $HClO_4$ for 1 h with gentle agitation at room temperature on a shaker (*VEB MLW*, *Labortechnik*, Ilnemann, Germany). Each sample was filtered through a 0.45 μm pore membrane filter (*Sartorius*, Göttingen, Germany). The analysis of the extracts was carried out on a *Biotronik LC 3000* (Frankfurt, Germany) amino acid analyser (ion-exchange chromatograph). The amino acids were detected with ninhydrin at 570 nm and 440 nm (for Pro). The amino acids serine (Ser), asparagine (Asn) and glutamine (Gln) could not be separated with the eluent system used and are indicated as $Ser^+$.

The stress treatment resulted in a much greater increase in the absolute Pro content in the T than in W plants: 90-fold versus 16-fold after PS and 105-fold

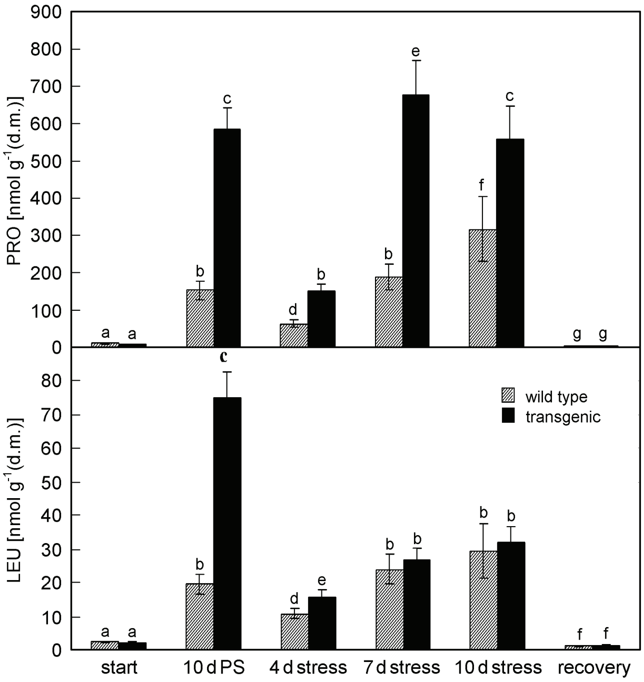

Fig. 1. Changes in Pro (*A*) and Leu (*B*) contents in soybean during preliminary drought stress (10 d PS) at high temperature (35/25 °C), the subsequent stress (4, 7, and 10 d after one irrigation), and after rehydration for 10 d. Values marked with different letters were significantly different at $P < 0.05$.

Table 1. Relative water content, RWC [%], and relative amino acid contents [% of total amino acid content] in soybean leaves under preliminary drought stress (PS) at high temperature (35/25 °C), subsequent stress (after one irrigation) for 4, 7 and 10 d, and after rehydration for 10 d. The selected amino acids exhibited the greatest changes in their relative amounts. The amino acids are listed in order of their relative contents in wild type plants before stress. W - wild type, T - transgenic, Gaba - γ-aminobutyrate, $Ser^+$ - Ser, Asn and Gln. Differences between any two values of RWC, Arg, $Ser^+$, Gaba, Asp, His, Glu, Phe, Pro, Tyr, Leu Val and Ile content were significant at the $P < 0.05$ level if they exceeded 10.21, 3.63, 3.73, 8.35, 2.90, 1.11, 2.54, 1.41, 10.93, 1.16, 0.85, 1.56 and 0.81 %, respectively.

| Parameters | Plants | Control | PS | Stress | | | Recovery |
|---|---|---|---|---|---|---|---|
| | | | 10 d | 4 d | 7 d | 10 d | 10 d |
| RWC | W | 94.28 ± 6.73 | 81.48 ± 6.37 | 83.51 ± 4.21 | 79.18 ± 6.23 | 64.81 ± 9.72 | 72.45 ± 6.48 |
| | T | 93.32 ± 5.67 | 92.57 ± 4.75 | 89.13 ± 6.87 | 81.25 ± 7.42 | 78.47 ± 9.18 | 88.34 ± 6.87 |
| Arginine | W | 59.25 ± 5.76 | 4.35 ± 0.38 | 5.72 ± 1.02 | 6.31 ± 1.03 | 3.81 ± 0.03 | 7.92 ± 0.12 |
| | T | 58.22 ± 0.18 | 8.16 ± 2.76 | 6.87 ± 3.60 | 8.16 ± 0.39 | 7.15 ± 0.37 | 7.25 ± 3.60 |
| $Ser^+$ | W | 12.35 ± 2.24 | 6.55 ± 1.33 | 8.32 ± 1.93 | 5.44 ± 1.52 | 1.37 ± 0.16 | 12.31 ± 1.41 |
| | T | 10.99 ± 0.86 | 4.45 ± 0.78 | 18.02 ± 3.51 | 4.49 ± 0.24 | 2.34 ± 0.68 | 5.90 ± 0.30 |
| Gaba | W | 7.46 ± 1.04 | 9.80 ± 1.43 | 15.21 ± 0.76 | 3.14 ± 1.00 | 3.00 ± 0.07 | 27.41 ± 6.12 |
| | T | 8.75 ± 1.25 | 4.11 ± 0.29 | 6.82 ± 2.00 | 4.21 ± 0.52 | 2.40 ± 0.73 | 53.04 ± 13.23 |
| Aspartate | W | 3.73 ± 0.73 | 4.83 ± 0.70 | 6.37 ± 1.06 | 3.62 ± 0.10 | 1.38 ± 0.24 | 18.51 ± 3.03 |
| | T | 5.37 ± 0.25 | 3.10 ± 0.34 | 7.00 ± 0.16 | 4.53 ± 0.32 | 2.15 ± 0.72 | 7.71 ± 2.16 |
| Histidine | W | 3.41 ± 0.02 | 2.59 ± 0.30 | 4.58 ± 0.30 | 5.73 ± 0.43 | 3.77 ± 0.26 | 4.24 ± 0.89 |
| | T | 4.03 ± 0.21 | 3.22 ± 0.51 | 6.17 ± 1.22 | 3.74 ± 0.38 | 4.15 ± 0.28 | 2.08 ± 0.09 |
| Glutamate | W | 3.33 ± 0.38 | 4.91 ± 0.78 | 5.06 ± 1.52 | 5.91 ± 1.79 | 4.81 ± 0.34 | 6.83 ± 2.86 |
| | T | 2.98 ± 0.21 | 5.26 ± 0.41 | 3.85 ± 0.05 | 6.49 ± 1.50 | 2.12 ± 0.27 | 5.97 ± 3.21 |
| Phenylalanine | W | 1.42 ± 0.14 | 6.92 ± 1.30 | 5.03 ± 0.09 | 4.47 ± 0.68 | 6.48 ± 0.10 | 1.12 ± 0.28 |
| | T | 0.82 ± 0.01 | 8.12 ± 1.23 | 6.72 ± 0.59 | 6.62 ± 2.31 | 4.96 ± 0.58 | 3.25 ± 0.26 |
| Proline | W | 1.23 ± 0.21 | 36.00 ± 1.80 | 26.52 ± 2.68 | 42.95 ± 6.22 | 51.09 ± 7.61 | 4.04 ± 0.30 |
| | T | 1.01 ± 0.08 | 39.60 ± 2.88 | 27.60 ± 7.39 | 51.96 ± 10.54 | 58.37 ± 4.78 | 2.27 ± 0.47 |
| Tyrosine | W | 0.97 ± 0.72 | 3.64 ± 0.11 | 1.70 ± 0.12 | 1.04 ± 0.11 | 0.48 ± 0.16 | 0.59 ± 0.06 |
| | T | 0.48 ± 0.11 | 2.69 ± 0.35 | 2.08 ± 1.28 | 0.82 ± 0.10 | 0.94 ± 0.43 | 0.83 ± 0.10 |
| Leucine | W | 0.37 ± 0.03 | 5.25 ± 1.55 | 5.15 ± 0.85 | 6.24 ± 1.44 | 5.41 ± 1.09 | 1.22 ± 0.07 |
| | T | 0.40 ± 0.03 | 5.81 ± 0.66 | 3.30 ± 0.23 | 2.29 ± 0.54 | 3.79 ± 0.04 | 0.80 ± 0.14 |
| Valine | W | 0.37 ± 0.01 | 3.06 ± 0.41 | 3.48 ± 0.44 | 3.20 ± 1.30 | 5.82 ± 0.25 | 2.37 ± 0.29 |
| | T | 0.53 ± 0.04 | 4.87 ± 1.07 | 2.56 ± 0.22 | 2.19 ± 0.73 | 4.28 ± 0.26 | 1.62 ± 1.12 |
| Isoleucine | W | 0.29 ± 0.02 | 3.40 ± 1.28 | 3.02 ± 0.64 | 5.21 ± 1.42 | 4.57 ± 0.88 | 0.73 ± 0.15 |
| | T | 0.26 ± 0.03 | 5.03 ± 0.60 | 2.95 ± 0.31 | 2.07 ± 0.61 | 3.71 ± 0.16 | 0.46 ± 0.21 |

versus 19-fold after 7 d stress (Fig. 1*A*). During stress the Pro content was at least 2-fold higher in T plants compared to W plants except for the last sampling (10-d stress). Similarly to previous findings (De Ronde *et al.* 2004), transgenic soybean plants with higher Pro content were less damaged by drought at supraoptimal temperature as indicated by their higher RWC (Table 1*A*). This is in correspondence with a positive relationship between Pro accumulation and drought tolerance found in wheat and *Stylosanthes* (Nayyar and Walia 2003, Chandra *et al.* 2004). The absolute amounts of several other amino acids – Glu, Ile, Val, Phe – exhibited similar stress-induced changes to those as described for Pro (data not shown). As in the case of Pro, there was a great difference (nearly 3-fold) in the Leu concentrations between T and W at the end of PS, but their contents were only slightly different or not at all during the subsequent stress (Fig. 1*B*). The present observations are corroborated with the results of Raggi *et al.* (1994), who also observed an increase in the concentration of Ile in water-stressed bean.

The relative contents of individual free amino acids were similar in the T and W under control conditions; a two-fold difference between them was only observed in the case of Tyr, the content being higher in W (Table 1*B*). Before treatment Arg accounted for nearly 60 % of the free amino acids. Following PS the percentage of Gaba, Glu, Leu and Ile was two-fold greater in W, while that of Ser+ was two-fold greater in T. During stress the relative Pro content exhibited a great increase, and reaching 51 % in W and 58 % in T. The relative amount of Arg, on the other hand, exhibited a great decrease. Both amino acids are members of the Glu family. Interestingly, only slight changes were observed in the proportion of the main Pro precursor, Glu, which may indicate that Pro is synthesized mainly using Arg as a precursor under stress conditions. In addition, under stress only the Arg pathway is used for polyamine synthesis, which could be also a reason for the great decrease of relative Arg content (Galiba *et al*. 1993). Unlike the situation in soybean, water deprivation resulted in an increased Arg concentration in wheat (Galiba *et al.* 1989) and *B. napus* (Good and Zaplachinski 1994).

After 10 d recovery the relative Gaba and Phe contents were two-fold higher in the T than in W, while the opposite relationship was found in the case of the Ser+, Asp and His, which were higher in W than in T. Gaba became the most abundant amino acid, reaching a value of 27 % in W and 53 % in T plants.

According to principle component analysis on the relative amounts of all amino acids, the greatest difference between the two genotypes in the amino acid composition was observed at the end of PS and after 7 d stress (data not shown), indicating that after a longer stress period even wild type plants were able to adjust their amino acid metabolism to the unfavourable conditions, but less efficiently than the transgenic ones.

It can be concluded that the manipulation of a single amino acid affected not only this amino acid and those directly related to its metabolism, but also the amino acid composition in general, demonstrating the complex regulation of amino acid levels.

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