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Genetic Manipulation of Proline Accumulation Influences the Concentrations of Other Amino Acids in Soybean Subjected to Simultaneous Drought and Heat Stress

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The effect of simultaneous drought and heat stress on free amino acid levels was compared in wild type and transgenic soybean (*Glycine max* (L.) Merr cv Ibis) plants transformed with the cDNA coding for the last enzyme of Pro biosynthesis, L-Δ^1-pyrroline-5-carboxylate reductase (EC 1.5.1.2), in sense and antisense directions. The most rapid increase in Pro content was found in the sense transformants that exhibited the least water loss, while the slowest elevation of Pro levels was detected in the antisense transformants that exhibited the greatest water loss during stress. Correspondingly, the level of the Pro precursors Glu and Arg was higher in sense transformants and lower in antisense ones compared to the wild type plants during the initial part of the stress. Interestingly, genetic manipulation of Pro levels also affected the stress-induced changes in the concentration of several other amino acids, which indicates the coordinated regulation of their metabolic pathways.

KEYWORDS: Drought; free amino acid; *Glycine max*; heat; proline; soybean.

INTRODUCTION

Pro plays an important protective role during drought stress since its accumulation, together with an increase in the concentration of other osmolites, leads to the decrease of osmotic potential (*1*). This process, known as osmotic adjustment, is one of the main mechanisms ensuring the adaptation of plants to limited water availability. It has been suggested that Pro also participates in the detoxification of reactive oxygen species (*2*, *3*). Furthermore, changes in Pro concentrations influence the solubility of various proteins because of its interaction with the hydrophobic residues of proteins (*4*).

Nayyar and Walia (*5*) demonstrated the adaptive role of Pro during drought stress. They observed a higher rate of Pro accumulation and utilization during water deficit in a drought-tolerant wheat genotype compared to a drought-sensitive one. In alfalfa a relationship between Pro levels and adaptability to drought has been observed (*6*). In addition, a greater ability to accumulate Pro correlated with decreased membrane injury in barley (*7*).

The drought-induced accumulation of Pro may be mainly the result of increased synthesis, since parallel to a 67-fold increase in Pro levels during drought in *Brassica napus*, a large elevation in the concentration of its precursor Glu (5.5-fold) and Arg (37-fold) was detected (*1*). A large increase in the Arg and Glu content was also observed in aspen during drought (*8*). Besides the increase in Pro synthesis during drought, reduced oxidation may also contribute to Pro accumulation during drought stress, since the expression of the gene coding for Pro oxidase, the enzyme involved in the oxidation of Pro to Glu, rapidly decreased in *Arabidopsis* during water deficit and this decline preceded the increase in Pro concentration (*9*). During recovery, the expression of this gene increased parallel to decreasing Pro levels. The active accumulation of Pro in response to stress conditions was also corroborated by Trotel-Aziz et al. (*10*), who observed both the stimulation of Pro synthesis via the enhancement of gene transcription and the activity of Δ^1-pyrroline-5-carboxylate synthase (EC 1.2.1.41) and the inhibition of Pro degradation via the inhibition of Pro dehydrogenase (ProDH, EC 1.5.99.8) in stressed canola plants. The role of ProDH in the control of Pro levels was also shown in *Arabidopsis* where the ProDH gene expression was down regulated by dehydration and was up regulated by rehydration after dehydration (*11*). The involvement of Pro in the response to water shortage was also demonstrated in transgenic tobacco overexpressing an enzyme of Pro biosynthesis, ornithine amino-Δ-aminotransferase (EC 2.6.1.13). These transgenic plants exhibited higher Pro levels and improved drought tolerance (*12*). The suppression of Pro synthesis in transgenic plants containing the gene coding for L-Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) in

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Table 1. Effect of Preliminary (PS) and Subsequent (After One Irrigation) Drought and Heat Stress on the Relative Water Content (RWC) of Soybean[a]

	RWC (%)					
		PS	stress			recovery
	start	10 d	4 d	7 d	10 d	10 d
wild type	95 ± 7 a	81 ± 6 b	83 ± 4 b	79 ± 5 b	64 ± 8 c	73 ± 8 bc
sense	93 ± 5 a	92 ± 4 a	89 ± 6 ab	81 ± 7 b	78 ± 9 b	88 ± 6 ab
antisense	97 ± 6 a	75 ± 7 bc	78 ± 11 bc	65 ± 6 c	58 ± 7 c	67 ± 5 c

[a] Values carrying different letters were significantly different at the $P < 5\%$ level.

the antisense direction resulted in increased sensitivity to water deficit (*13*, *14*).

Although it has been observed in several plant species that osmotic stress-induced changes in total free amino acid content were mainly due to the increase in Pro content, the levels of other amino acids were also elevated as a result of water deficit (*1*, *15*, *16*). Apart from the considerable accumulation of Pro and its precursors in *B. napus*, drought also induced a more than 5-fold increase in Ile, Leu, and Asp levels, as well as an increase, albeit small, in the levels of all other amino acids (*1*). Water deficit resulted in the accumulation of Leu, Ile, Thr, Ala, and Val in bean (*17*). Contrary to the active Pro accumulation, the increase in Ala and Asp concentrations was not the result of the increased activity of the enzymes catalyzing their synthesis in *B. napus* (*1*) but appears to be due to decreased protein synthesis, as shown by the reduced incorporation of ^{35}-[S] methionine into proteins during water deficit (*1*). Similarly, the incorporation of radioactive Cys into proteins also decreased in the roots of drought-stressed wheat (*18*). It is thus possible that the drought-induced increase in the concentration of other amino acids is the result of a decrease in protein synthesis. The reduction in protein synthesis during osmotic stress was more pronounced in drought-sensitive wheat genotypes compared to the tolerant genotypes (*19*). These results could be explained by the higher exopeptidase activity in the sensitive genotypes.

Unlike the participation of Pro and other free amino acids in the response to drought stress, their role during high-temperature stress has not been studied so intensively. The protective role of Pro during heat stress was shown in cotton, since cultivars with a higher Pro content suffered less damage during heat stress (*20*). High-temperature treatment also resulted in Pro accumulation in barley (*21*). The involvement of free amino acids in the response to heat stress was shown in spring wheat, where a mutant having greater amino acid contents (especially Ser, Met, Ile, His, and Arg) was damaged to a lesser extent after heat stress treatment than the parent genotype (*22*).

The aim of the present research was to establish whether an increase or decrease in Pro synthesis in transgenic soybean plants transformed with the gene coding for P5CR, in the sense or antisense direction, affected the changes induced by drought and heat stress in the concentrations of other amino acids.

MATERIALS AND METHODS

Plant Material and Treatment. Studies were made on wild-type soybean [*Glycine max* (L.) Merr cv Ibis] and transgenic lines transformed with a construct containing a heat-shock-inducible promoter and the cDNA coding for P5CR in the sense (two lines) or antisense (one line) directions (*13*, *14*). Molecular analysis of the T3 transgenic plants confirmed the presence of three to five copies of the P5CR gene in the test plants and at least three integrations in the genome (*23*). Under control conditions the mRNA and protein levels were similar in the wild type and the transgenic plants. Following simultaneous heat and drought stress, the P5CR mRNA levels were 3−4-fold, and the protein levels 2−3-fold, higher in the sense transformants than in the wild type. In the antisense transformants the mRNA level decreased to 30−50% and the protein level decreased to 40−60% of the corresponding values in the wild type plants following stress treatments. The lines used in the present study were selected on the basis of their drought tolerance and Pro concentrations. The seeds were germinated between two layers of damp filter paper in the dark at 25 °C for 4 d. After germination, the seedlings were raised in pots containing a 2:1:1 mixture of garden soil, humus, and sand. The plants were grown in an autumn−winter type growth chamber (Conviron PGV-36, Controlled Env. Ltd., Winnipeg, Canada) at 25/15 °C day/night temperature for 6 weeks with 16 h of illumination at 400 μEinstein m^{-2} s^{-1}. The seedlings were subjected to a preliminary stress (PS) by withholding water for 10 d at 35/25 °C day/night temperature. Subsequently they were watered once and then further cultivated at 35/25 °C without irrigation for an additional 10 d. The drought stress was carried out at high growth temperature in order to switch on the heat-inducible promoter contained in the introduced gene construct. In a previous study a combination of drought and heat stress was efficient in activating this promoter and manipulating Pro levels (*13*), so a similar experimental system was used in the present study. The stress treatment was followed by a recovery period during which the plants were watered for 10 d at 25/15 °C day/night temperature. Samples were taken at the beginning of the experiment, after 10 d PS, after 4, 7, and 10 d stress, and after 10 d recovery. At least three plants were investigated from each line in each experiment, and the experiments were repeated three times. 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 of 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: $100(IW - DW)/(TW - DW)$.

Determination of Free Amino Acids and Total Soluble Protein Content. Soybean samples (300 mg) were extracted with 3 mL of 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 analyzer (ion-exchange chromatograph). The amino acids were detected with ninhydrin at 570 and 440 nm (for Pro). The amino acids Ser, Asn, and Gln could not be separated with the eluent system used. The amount of soluble proteins was determined as described earlier (*18*).

Statistical Analysis. Samples from three independent experiments were analyzed, and the data are presented as the mean. Standard deviation, principal component analysis (PCA), and analysis of variance comparison of treatment means (least significant difference, at the $P < 5\%$ level) were performed using STATISTICA 6.0 software for Windows.

RESULTS

Relative Water Content. The smallest decrease in RWC following the simultaneous drought and heat stress was observed in the sense transformants, while the greatest reduction in RWC occurred in the antisense transformants (**Table 1**). The RWC of the stressed wild type plants was intermediate. During the recovery phase, a slight increase in RWC was observed in all of the genotypes tested.

Total Free Amino Acid and Soluble Protein Accumulation. At the beginning of the experiment, the total free amino acid content of the antisense transformants (*A*) was the highest (10.24 mg/g FW), followed by those of the wild type plants (*W*, 7.36 mg/g FW) and the sense transformants (*S*, 5.62 mg/g FW) (data not shown). After the 10 d PS, a 3-fold increase was observed in total free amino acid content in the sense transformants, a small decrease (0.3-fold) in the antisense transformants, and no change in the wild-type soybean. The subsequent main stress (4 d, 7 d, 10 d) induced a greater increase in the total free amino amino acid content in the sense transformants (1.3-, 3.6-, 3.1-fold) compared to that observed in the two other genotypes (*W*: 0.5-, 1.3-, 2.6-fold; *A*: 0.2-, 1-, 2.6-fold). However, during recovery the concentrations of total free acids decreased to below the starting values (*W*: 0.6-fold, *S*: 0.5-fold, *A*: 0.6-fold).

Significant differences in the amount of total soluble protein (data not shown) were only observed between the three genotypes at the end of the stress treatment, when the level was 49, 71, and 56 μg/g FW in wild type plants, sense, and antisense transformants, respectively.

Changes in the Concentrations of Individual Amino Acids. As transgenic plants with manipulated Pro synthesis were used in the experiments, the changes in the concentrations of Pro and its precursor Glu and Arg (all three amino acids belong to the Glu family) were of special significance. The Pro and Glu contents increased and the concentration of Arg decreased as a result of simultaneous drought and heat stress (**Figure 1**). The most rapid drought-induced Pro accumulation was observed after the PS period in the sense transformants (**Figure 1a**) where the Pro concentration increased 124-fold, compared with 31-fold in the wild type and 22-fold in the antisense transformants. A significant increase in Pro content was observed following the 4-, 7-, and 10-d stress treatments in all three genotypes, the increase being 12-, 45-, and 110-fold in the wild type, 36-, 185-, and 178-fold in the sense transformants, and 8-, 59-, and 127-fold in the antisense transformants. The Pro concentrations declined to the starting levels during recovery. The relative Pro content, calculated as a percentage of the total free amino acid level, was very low before stress (1%) but increased to 40−60% during the subsequent stress (data not shown). The stress-induced increase in Glu was much smaller (maximum 7-fold) than that in Pro (**Figure 1b**). Similarly to Pro, the earliest increase in Glu concentration was observed in the sense transformants. Following the recovery period, the Glu levels decreased to that of the starting values. During PS, the Arg level decreased 13-fold in the wild type compared to that of the nonstressed plant, 2-fold in the sense transformants, and 14-fold in the antisense transformants (**Figure 1c**). The highest Arg content was detected during drought at high temperature in the sense transformants, except for the last sampling where the highest levels were observed in the antisense transformants. Glu can serve as precursor not only for Pro but also for GABA, the levels of which gradually decreased during water deprivation, except for a few deviating values, and increased during recovery (data not shown).

In plants, Asp is the precursor of four other amino acids via its conversion to aspartic β-semialdehyde (Lys) and homoserine (Met, Thr, Ile). Water deprivation induced a large increase in the Asp content of the sense transformants (**Figure 2a**). However, a sharp reduction in the level of this amino acid was observed in the antisense transformants during PS and the first part of the subsequent stress. In the wild type plants, only small changes in Asp content occurred, except after recovery. The Met content doubled during PS, but decreased to below its

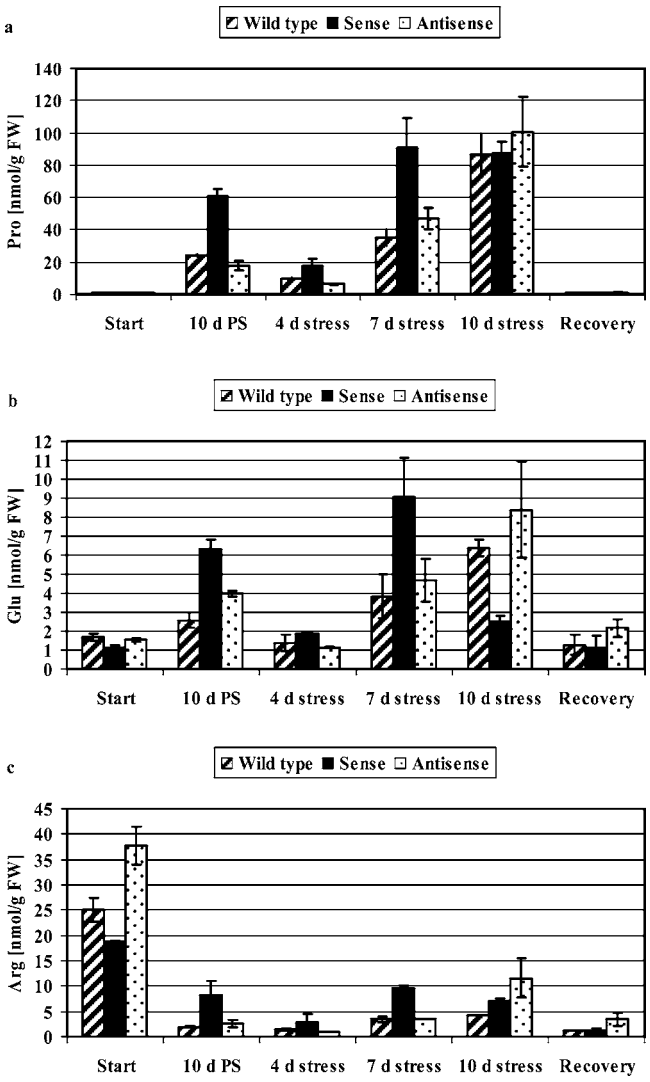

Figure 1. Effect of preliminary drought and heat stress (PS) and subsequent stress (after one irrigation) on Pro (**a**), Glu (**b**), and Arg (**c**) content in soybean. The bars represent standard deviation. Differences between any two values of Pro, Glu, and Arg content were significant at the $P < 0.05$ level if they exceeded 16.5 nmol/g FW, 1.69 nmol/g FW, and 6.08 nmol/g FW, respectively.

starting value during the subsequent stress in all the genotypes (**Figure 2b**). After PS, the Met levels were the highest in the sense transformants. The Thr content increased during both PS and subsequent stress in all the genotypes and declined to the original level following recovery (data not shown). A substantial increase in the Ile content was detected in all the genotypes after PS (*W*: 12-fold, *S*: 61-fold, *A*: 5-fold) and the subsequent stress (10 d *W*: 41-fold, *S*: 44-fold, *A*: 17-fold), and the Ile levels were 3-fold higher during PS in the sense transfomants compared to those of the other genotypes (**Figure 2c**). As observed for Thr and Ile, water deprivation resulted in greater Lys levels at the end of PS and stress compared to the starting values (data not shown).

With regard to the Ala family (Ala, Leu, Val), the content of Ala decreased during drought and heat stress in the wild type plants and showed only a slight change in the transformants (data not shown). However, during recovery Ala increased in the wild type plants and antisense transformants. After PS, the Val concentration increased 9-fold in the wild type, 29-fold in the sense, and 4-fold in the antisense transformants compared to the starting values, being 3.5-fold greater in the sense

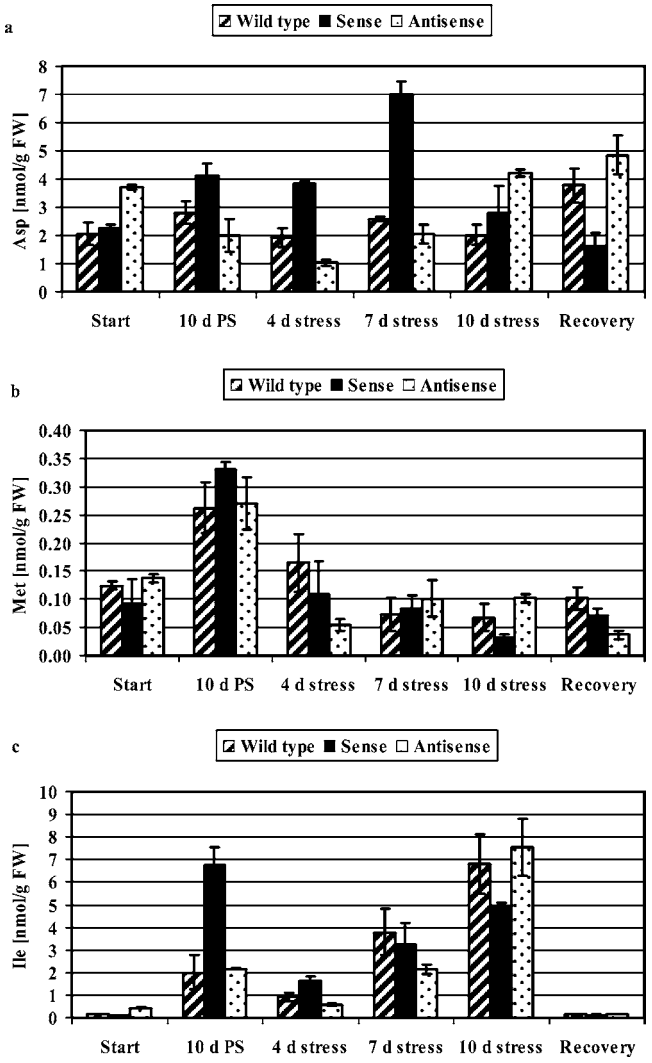

Figure 2. Effect of preliminary drought and heat stress (PS) and subsequent stress (after one irrigation) on Asp (**a**), Met (**b**), and Ile (**c**) content in soybean. The bars represent standard deviation. Differences between any two values of Asp, Met, and Ile content were significant at the $P < 0.05$ level if they exceeded 1.58 nmol/g FW, 0.11 nmol/g FW, and 2.44 nmol/g FW, respectively.

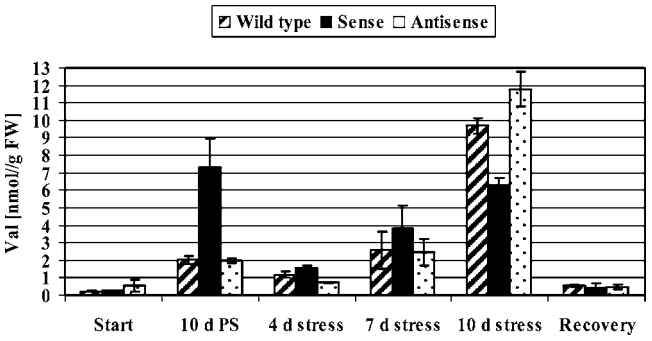

Figure 3. Effect of preliminary drought and heat stress (PS) and subsequent stress (after one irrigation) on Val content in soybean. The bars represent standard deviation. Differences between any two values of Val content were significant at the $P < 0.05$ level if they exceeded 2.73 nmol/g FW.

transformants than in the two other genotypes (**Figure 3**). Following a transient decrease as a result of rewatering at the end of PS, the Val content increased again during the subsequent stress (*W*: 41-fold, *S*: 25-fold, *A*: 22-fold increase after 10 d stress) and decreased to the initial value during recovery. A similar tendency was observed for Leu where there was a large increase after PS (*W*: 15-fold, *S*: 46-fold, *A*: 7-fold); then, after a transient decrease, its level increased again during the subsequent stress (*W*: 39-fold, *S*: 30-fold, *A*: 16-fold increase after 10 d stress), with levels decreasing to that of the initial values during recovery (data not shown).

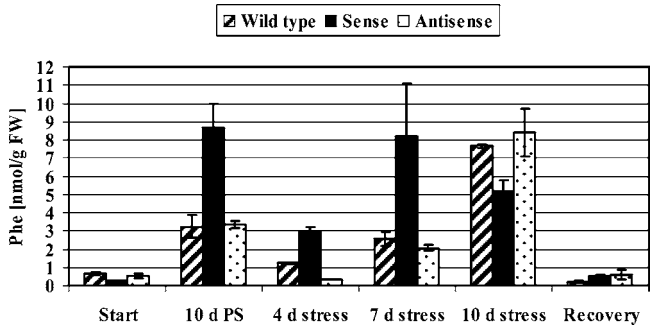

Figure 4. Effect of preliminary drought and heat stress (PS) and subsequent stress (after one irrigation) on Phe content in soybean. The bars represent standard deviation. Differences between any two values of Phe content were significant at the $P < 0.05$ level if they exceeded 1.75 nmol/g FW.

There was a large increase in the Phe content of the plants during the PS (*W*: 5-fold, *S*: 31-fold, *A*: 6-fold); then, following a transient reduction, the Phe levels increased again during the subsequent stress (*W*: 12-fold, *S*: 18-fold, *A*: 16-fold increase after 10 d stress) (**Figure 4**). After recovery, the Phe content decreased to the initial levels. The concentrations of the other aromatic amino acid, tyrosine, underwent similar changes during the experiment as those described for Phe (data not shown).

The Cys content gradually increased during water deprivation (*W*: 11-fold, *S*: 7-fold, *A*: 9-fold increase after 10 d stress) and declined to the starting level after recovery (data not shown). His content increased during water deprivation in all genotypes, especially during the main stress (*W*: 3-fold, *S*: 3-fold, *A*: 2-fold increase after 10 d stress) and declined to the starting value after the recovery period (data not shown).

DISCUSSION

Since Pro was the main component of the free amino acids in soybean during drought stress at high temperature, it is not surprising that a positive relationship was found between total free amino acid content and stress tolerance. Similarly, a positive correlation was observed between the drought-induced free amino acid accumulation and the drought tolerance of various soybean genotypes in field experiments (*24*).

In the present experimental system, using transgenic soybean, the protective role of Pro during drought stress, described earlier (*13*, *14*), was corroborated. The sense transformants, which demonstrated the earliest Pro accumulation, experienced the least water loss, while the antisense transformants, which possessed the slowest Pro accumulation, experienced the greatest water loss. The high Pro concentrations in the wild type plants and antisense transformants after the 10 d stress may be a result of its decreased degradation. Similarly to the great increase in Pro content (124-fold) in sense transformants in this study, a 100-fold increase in Pro level was observed in dehydrated tissues of *Arabidopsis* (*25*). The protective role of Pro against drought was also demonstrated using soybean cell suspension cultures, where Pro levels increased 40-fold in cells adapted to water stress, compared to only a 12-fold increase in Pro levels in nonadapted cells (*26*). There was also a positive relationship

between Pro accumulation and drought tolerance in wheat (*5*) and in alfalfa (*6*). Lazcano-Ferrat and Lovatt (*27*), however, reached the conclusion that Pro is only an indicator of plant water status, because they found an inverse relationship between Pro levels and drought tolerance in *Phaseolus* species. However, the drought treatments were performed on 5-day-old *Phaseolus* plants, and the reserves still present from the cotyledons might have influenced the Pro levels.

The genetic manipulation of Pro levels also affected the concentration of its precursors. In all genotypes tested the substantial decrease in the relative amounts of Arg, compared to the starting values, can be ascribed to its use in increased Pro synthesis during drought. The increased availability of Glu for Pro synthesis could be the result of its reduced use in GABA synthesis. The results of the present study appear to indicate that the differences observed in the drought tolerance of the genotypes at subraoptimal temperature could depend on the speed at which Pro synthesis is induced, which was rapid in the sense transformants. Unlike the situation in soybean in the present study, water deprivation at normal growth temperature resulted in an increased Arg concentration in wheat (*19*) and *B. napus* (*1*). As in soybean, water deficit also increased the Glu content in wheat (*19*) and bean (*17*).

Manipulation of the Pro levels affected not only the concentration of its precursors but also the concentrations of nearly all other amino acids. The levels of all members of the Asp family increased after water deficit in soybean, as observed in several other plant species (*1*, *17*, *19*, *25*). Interestingly, the stress treatments induced similar changes in the Asp and Glu concentrations.

The stress-induced increases in the Ile, Val, and Leu concentrations may be due to an increase in carbohydrate metabolism, as observed in osmotically stressed wheat and soybean (*28*, *23*), since the metabolic pathways of these amino acids are linked to the pentose-phosphate pathway via pyruvate. As in soybean, the concentration of Ala decreased, while the concentrations of Val and Leu increased during drought stress in bean (*17*).

The changes in Phe levels were similar to those described for Ile, Val, and Leu, which is not surprising, since the synthesis of Phe is also linked to the pentose-phosphate pathway (through phospho-enolpyruvate). As in soybean, drought stress also increased the amount of aromatic amino acids in *B. napus* (*1*).

The drought-induced changes in Cys content could be the result of the intensive use of Cys for glutathione synthesis, as described for wheat subjected to osmotic stress (*18*). Considering that the other sulfur-containing amino acid, Met, plays a central role in the biochemical and regulatory processes of the cell and the Met concentrations are tightly regulated, the 2-fold increase in the Met amount may have a great effect on stress-induced changes in the metabolism.

As observed for soybean, drought induced the accumulation of Ser and Gly in poplar (*29*) and *B. napus* (*1*). The increase detected in His level in soybean after simultaneous drought and heat stress corroborates the data obtained in cotton, where higher His contents were observed following water deficit (*30*).

The observed changes in the concentrations of several amino acids demonstrate that the manipulation of the level of a single amino acid (in this case Pro) affects stress-induced changes in the concentrations of other amino acids. The results of principal component analysis (PCA) on the absolute amino acid concentrations are shown in **Figure 5**. Various clusters can be observed in the figure. The first principal component (PC), which explains 51.3% of the total variance, negatively correlated with Thr, Glu, Pro, Val, Leu, Ile, Phe, His, and total amino acids. This cluster is formed by amino acids which behaved similarly to Pro during stress. The similarity in the stress-induced changes in the concentrations of these amino acids was also confirmed by the high correlation coefficient (greater than 0.85%) between the concentrations of Pro and Thr, Glu, Val, Leu, Ile, Phe, and His (data not shown). The second PC (16.4% of the total variance) correlates positively with the other amino acids. The regulation of the content of an individual amino acid could be a very complex process, since, besides their involvement in protein synthesis, they also participate in the synthesis of many other cellular compounds.

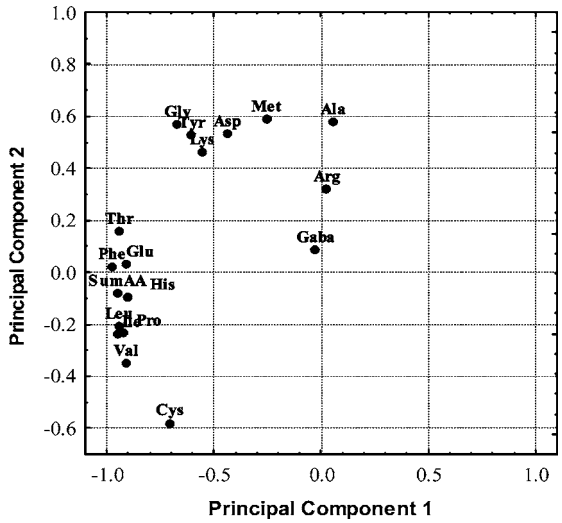

Figure 5. Principal component analysis of the free amino acid concentrations based on data for individual amino acids determined at all sampling dates. Gaba is γ-amino butyrate; SumAA is the total amino acid concentration.

In conclusion, the genetic manipulation of Pro levels induced various changes, not only in the concentrations of Pro and its precursors in wild type and transgenic plants, but also in the levels of other amino acids not directly involved in Pro metabolism. The differences in the stress-induced concentration changes within amino acid families indicate that each amino acid has its own system of checks and balances operating. In addition, the present results show that after chemical or genetic manipulation of the level of a certain compound the possible changes in the concentrations of other compounds important for food quality should be checked before using the products obtained from the transgenic organisms in the food chemistry.

ABBREVIATIONS USED

A, antisense transformant; ANOVA, analysis of variance; DW, dry weight; FW, fresh weight; GABA, γ-amino butyrate; IW, initial weight; P5CR, L-Δ^1-pyrroline-5-carboxylate reductase; PC(A), principal component (analysis); ProDH, proline dehydrogenase; PS, preliminary stress; RWC, relative water content; S, sense transformant; SumAA, total amino acid concentration; TW, turgescent weight; W, wild type plant.

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