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Genetic manipulation of proline levels affects antioxidants in soybean subjected to simultaneous drought and heat stresses

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It was assumed that the genetic manipulation of the proline (Pro) level would also affect the (homo)glutathione content as both compounds have a common precursor, glutamate. To test this hypothesis, the levels of Pro, reduced and oxidized (homo)glutathione [(h)GSH and (h)GSSG] and other antioxidants were compared under simultaneous drought and heat stress conditions and in a control treatment in a time course experiment on wild-type soybean (*Glycine max* cv. Ibis) and on transgenic plants containing the cDNA coding for L-Δ^1-pyrroline-5-carboxylate reductase (EC 1.5.1.2), the last enzyme involved in Pro synthesis, in the sense and antisense directions. At the end of the recovery period, the highest H_2O_2 and lipid hydroperoxide concentrations were observed in the antisense transformants, which exhibited the greatest injury, while the lowest H_2O_2 content was detected in the sense transformants, which exhibited the lowest injury percentage. During stress treatment, the highest Pro and ascorbate (AA) levels were detected in the sense transformants, while the highest GSH and hGSH contents, AA/dehydroascorbate (DHA) and (h)GSH/(h)GSSG ratios and ascorbate peroxidase (APX) activity were found in the antisense transformants. The greatest APX (EC 1.11.1.11) activity was observed in the first part of the stress treatment in the antisense transformants, and the greatest glutathione reductase (EC 1.6.4.2) activity was observed in the second part of the treatment in the same genotype. The present experiments indicate that the manipulation of Pro synthesis affects not only the (h)GSH concentrations, but also the levels of other antioxidants.

Introduction

When proline (Pro) accumulates during drought stress, it not only acts as an osmolyte, helping cells to maintain membrane integrity (Wyn Jones and Storeys 1978), but also affects the solubility of various proteins by interacting with their hydrophobic residues (Schobert and Tschesche 1978). The suggestion that it contributes to

Abbreviations – AA, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DW, dry weight; γEC, γ-glutamylcysteine; ESSE, γ-glutamylcystine; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; hGSH, reduced homoglutathione; hGSSG, oxidized homoglutathione; IW, initial weight; P5CR, L-Δ^1-pyrroline-5-carboxylate reductase; Pro, proline; RWC, relative water content; SOD, superoxide dismutase; TW, turgescent weight.

the detoxification of reactive oxygen species (Floyd and Nagy 1984) was confirmed recently, when an elevated Pro content was found to reduce free radical levels in response to osmotic stress in tobacco (Hong et al. 2000). In addition, when monitoring gene expression with a cDNA microarray during rehydration in *Arabidopsis*, Oono et al. (2003) observed that the promoter of several rehydration-inducible genes contained the ACTCAT sequence involved in Pro-inducible gene expression.

The drought-induced accumulation of Pro has been described in several plant species (Galiba et al. 1989, Irigoyen et al. 1992, Van Rensburg and Krüger 1994). Heat stress also results in increased Pro levels in barley and soybean (de Ronde et al. 2000, Georgieva et al. 2003). The adaptive role of Pro in the response to drought stress has been shown by Bandurska and Stroinski (2003), who found that water deficit resulted in Pro accumulation in a drought-tolerant wild accession of *Hordeum spontaneum*, but not in the sensitive *H. vulgare* cv. 'Maresi'. A higher Pro content was found in rice grown in a dry nursery compared with plants grown in a wet nursery (Zhao et al. 2001).

The role of Pro in the protection against drought and osmotic stress has also been shown by the genetic manipulation of its synthesis or degradation. The overexpression of the L-Δ^1-pyrroline-5-carboxylate synthetase (EC 1.2.1.41) gene, coding for the rate-limiting enzyme in the glutamate pathway of Pro synthesis, enhanced root biomass and flower development in tobacco under drought stress conditions (Kishor et al. 1995), and improved the tolerance to ionic osmotic stress in rice and wheat (Sawahel and Hassan 2002, Anoop and Gupta 2003). The transformation of tobacco with the gene coding for ornithine-Δ-aminotransferase (EC 2.6.1.13), involved in the ornithine pathway of Pro synthesis, also increased Pro levels and biomass production under osmotic stress (Roosens et al. 2002). The underexpression of L-Δ^1-pyrroline-5-carboxylate reductase (EC 1.5.1.2, P5CR), which controls the common step in the two pathways of Pro synthesis, decreased the Pro levels and survival of soybean (de Ronde et al. 2000). The inhibition of Pro degradation by transforming *Arabidopsis* with the gene coding for proline dehydrogenase (EC 1.5.99.8) in the antisense direction increased the Pro content and the tolerance to ionic osmotic stress, providing further evidence for the protective role of Pro (Nanjo et al. 1999).

Various antioxidants also have an important protective role, participating in the removal of the reactive oxygen species accumulated during drought and heat stress. Superoxide dismutase (SOD, EC 1.15.1.1) catalyses the detoxification of superoxide radicals. The H_2O_2 produced in this reaction is then degraded in the ascorbate–glutathione cycle, the components of which are glutathione disulphide (GSH, reduced glutathione), ascorbate (AA), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) (May et al. 1998, Noctor and Foyer 1998, Pastori and Foyer 2002). A drought-induced accumulation of H_2O_2 and lipid hydroperoxide has been shown in tobacco (Hideg et al. 2003), and a heat-induced increase in H_2O_2 content has been observed in mustard (Dat et al. 1998b). The role of AA and GSH in the case of water deficit has been demonstrated in resurrection plants, where they were oxidized during a dehydration experiment, but reduced during rehydration, indicating their effective participation in the AA–glutathione cycle (Kranner et al. 2002). The involvement of AA and GSH in the response to heat stress has been observed in wheat, where higher levels were recorded in stressed plants than in the control (Dash and Mohanty 2002). In mustard subjected to high-temperature stress, there was a transient decrease in the AA content and AA/dehydroascorbate (DHA) ratio (Dat et al. 1998a). The activity of SOD, APX and GR increased in tolerant wheat genotypes, but remained unchanged or decreased slightly in sensitive genotypes, during drought (Lascano et al. 2001). The activity of these three enzymes also increased after high-temperature stress in mulberry (Chaitanya et al. 2002). The higher GR activity in tolerant genotypes led to a high ratio of GSH to its oxidized form (GSSG) (Kocsy et al. 2001). GR is also able to reduce homoglutathione (hGSH), a homologue of glutathione, in Fabaceae (Klapheck 1988). In hGSH, glycine is replaced by a β-alanine. GSH and hGSH are also involved in the detoxification of lipid peroxides, a reaction catalysed by glutathione *S*-transferase (GST, EC 2.5.1.18) (Marrs 1996). The involvement of GST in the response to water deficit was shown by Bianchi et al. (2002), who found a drought-induced increase in the GST transcript level.

Stress-induced alterations in the Pro level may also influence the amount of GSH, as the two molecules have a common precursor, glutamate. Mannitol-induced osmotic stress increased the glutamate content in sensitive wheat genotypes, but decreased it in tolerant forms (Galiba et al. 1989). The reduction in glutamate content in the tolerant genotype may be the result of greater GSH and/or Pro synthesis. To test the possible interactions between Pro and antioxidant levels during osmotic stress, these parameters were measured in transgenic soybean transformed in the sense and antisense directions with the gene coding for P5CR, the last enzyme in Pro biosynthesis.

Materials and methods

Plant material and treatment

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 (de Ronde et al. 2000, 2001a) were investigated. Molecular analysis of the T3 transgenic plants confirmed the presence of two to five copies of the *P5CR* gene in the test plants and at least three integrations in the genome (de Ronde et al. 2004). In the sense transformants, the P5CR mRNA levels were three- to four-fold and the protein levels were two- to three-fold higher than in the wild-type. In the antisense transformants, the mRNA level decreased to 30–50% and the protein level to 40–60% of the corresponding values in the wild-type plants. 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 paper in the dark at 25°C for 4 days. 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 a spring-type growth chamber (Conviron PGV-15, Controlled Env. Ltd, Winnipeg, Canada) at 25°C/15°C day/night temperature for 6 weeks with 16 h illumination at 400 μmol m^{-2} s^{-1} (metal halide lamps, Tungsram HgMIF 400W/DH, Budapest, Hungary). The seedlings were subjected to preliminary stress by withholding water for 10 days at 35°C/25°C day/night temperature. They were then watered once and further cultivated without irrigation at 35°C/25°C for an additional 10 days. The drought stress was carried out at high growth temperature in order to switch on the heat-inducible promoter of the introduced gene construct. The stress treatment was followed by a recovery period with watering at 25°C/15°C for 10 days. Samples were taken at the beginning of the experiment, after 10 days of preliminary stress, after 4, 7 and 10 days of stress and after 10 days of recovery. At least three plants were investigated from each line in each experiment and the experiments were repeated three times. The injury percentage and the relative water content (RWC) were also determined at the above sampling dates. The injury was scored on a 0–100% scale on the basis of the wilting and drying of the shoots (0%, no wilting; 25%, severely wilted plants and young leaves dried out; 50%, about half of the shoot dried out; 75%, only about one-quarter of the shoot turgescent; 100%, the whole shoot dried out). For the calculation of RWC, the weight of the leaf discs (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 then calculated using the formula: $100 \times (IW - DW)/(TW - DW)$.

Measurement of hydrogen peroxide and lipid hydroperoxide

Samples of 200 mg soybean leaves crushed in liquid nitrogen were homogenized in 1 ml of 10% H_3PO_4. The supernatant was used for the determination of H_2O_2 and lipid hydroperoxide by the methods of Wolff (1994). The reaction mixture for H_2O_2 analysis contained 100 μ*M* xylenol orange, 250 μ*M* ammonium ferrous sulphate, 100 m*M* sorbitol, 25 m*M* H_2SO_4 and 50 μl extract in a total volume of 1 ml. The following mixture was used for the measurement of lipid hydroperoxide concentration: 100 μ*M* xylenol orange, 250 μ*M* ammonium ferrous sulphate, 90% methanol (HPLC grade), 4 m*M* butylated hydroxytoluene, 25 m*M* H_2SO_4 and 50 μl extract in a total volume of 1 ml. For both compounds, calibration was performed using H_2O_2.

Determination of free proline content

Samples of 200 mg soybean leaves were crushed in liquid nitrogen before adding 4 ml of distilled water. After centrifugation, the free Pro content was determined from the supernatant following the procedure of Bates et al. (1973), as described by de Ronde et al. (2000).

Measurement of ascorbate and dehydroascorbate

Samples of 100 mg soybean leaves were ground with liquid nitrogen and homogenized with 1 ml of 5% trichloroacetic acid. The AA and DHA contents were measured spectrophotometrically from the supernatant by the method of Law et al. (1983). The calibration was performed using AA.

Detection of thiols

The plant material was ground with liquid nitrogen in a mortar, and 1 ml of 0.1 *M* HCl containing 1 m*M* Na_2EDTA was added to 100 mg of plant sample. For the determination of the total thiol content, the thiols present in the samples were coupled to monobromobimane after reduction with dithiothreitol (Kocsy et al. 2000). For the detection of oxidized thiols, the free thiols were blocked with *N*-ethylmaleimide, after which the excess *N*-ethylmaleimide was removed with toluol (Kranner and Grill 1996). The oxidized thiols

were reduced and coupled, like the total thiols, to monobromobimane. The derivatized samples were analysed as described by Schupp and Rennenberg (1988), modified by Rüegsegger and Brunold (1992), after separation by reverse-phase HPLC (Waters, Milford, MA) using a fluorescence detector (W474 scanning fluorescence detector, Waters). The qualitative and quantitative identification of the thiols and the recovery experiments were performed as described previously (Kocsy et al. 2000).

Measurement of enzyme activities

The plant material was homogenized in 0.1 *M* Na–K-phosphate buffer, pH 7.5 (1 : 5, w/v), containing 0.2 m*M* diethylenetriaminepentaacetic acid and 4% (w/v) polyvinylpolypyrrolidone, in an ice-cooled glass homogenizer. After centrifugation, the supernatant was used for the measurement of soluble enzyme activities. SOD (EC 1.15.1.1) activity was determined according to the method of Elstner and Heuple (1976), based on the inhibition of nitrite formation from hydroxylammonium chloride by SOD. APX (EC 1.11.1.11) activity was measured by the method of Nakano and Asada (1987). The assay mixture contained 50 m*M* Na–K-phosphate (pH 7.5), 0.02 m*M* diethylenetriaminepentaacetic acid, 1 m*M* ascorbic acid, 0.25 m*M* H_2O_2 and 5 µl plant extract in a total volume of 500 µl. GR (EC 1.6.4.2) activity was measured according to Smith et al. (1988), and GST (EC 2.5.1.18) activity was detected by the method of Habig et al. (1974) as described previously (Kocsy et al. 1997). The activities are presented on a protein basis (measured according to Bradford 1976) and the measurements were performed using a Cary 100 UV–visible spectrophotometer (Varian, Middelburg, The Netherlands).

Statistics

The statistical analysis was performed using two-component (treatments, genotypes) analysis of variance (Microsoft Excel 2000). The significant differences were calculated using the *t*-test.

Results

Water deprivation resulted in increased injury (Fig. 1A) and decreased RWC (Fig. 1B) in all genotypes. Slightly less injury and increased water content was recorded after the recovery period. The injury percentage was significantly lower and the RWC was higher in the sense transformants than in the wild-type plants. The antisense transformants exhibited significantly greater injury and water loss compared with both the sense transformants and the wild-type plants.

During preliminary stress, the H_2O_2 content increased only in the antisense plants, but, during the first 4 days of the subsequent stress, a large increase was observed in all genotypes (Fig. 1C). Later, the H_2O_2 concentration decreased in the wild-type plants and the sense transformants, but, in the antisense plants, the H_2O_2 content increased after a transient decrease. At the end of the recovery period, the H_2O_2 concentration was 2- and 3.5-fold greater in the antisense transformants than in the wild-type plants and sense transformants, respectively. The changes in lipid peroxide concentrations were similar to those described for the H_2O_2 levels (Fig. 1D); the greatest differences

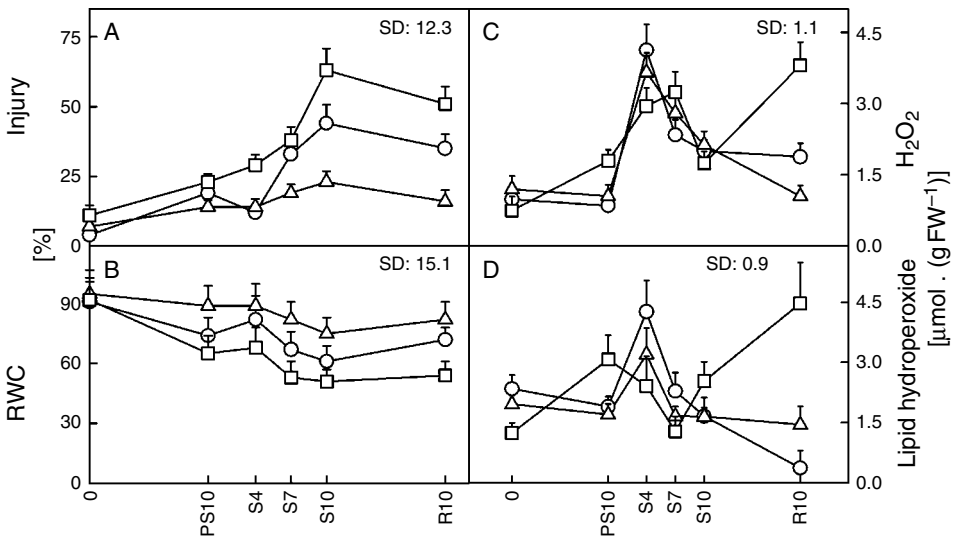

Fig. 1. Injury percentage (A) and relative water content (RWC; B) of shoots and H_2O_2 content (C) and lipid hydroperoxide content (D) in leaves of wild-type soybean plants (○) and sense (△) and antisense (□) transformants grown without watering for 10 days (preliminary stress, PS), rewatered once, further cultivated for an additional 10 days without watering (stress, S) and finally supplied with an optimal amount of water for 10 days (recovery, R). Mean values ± standard deviations of 12 measurements from three independent experiments are presented. SD, significant difference.

between the genotypes were observed at the end of the recovery period, when the concentration was 3.5- and 12.6-fold greater in the antisense transformants than in the sense transformants and the wild-type plants, respectively.

Free Pro accumulated in all genotypes during the withholding of water, and the levels decreased after recovery (Fig. 2A). Following preliminary stress and during the first half of the subsequent stress, the Pro concentration was much higher in the sense transformants and much lower in the antisense transformants than in the wild-type plants. This difference decreased during the second half of the drought stress and disappeared during recovery.

The AA content increased during preliminary stress in the wild-type plants and sense transformants, but decreased in the antisense transformants (Fig. 2B). During the initial part of the subsequent stress, the AA content increased in all genotypes, and then decreased. The highest level was observed in the sense transformants after 4 days of stress, and in the antisense transformants at the end of recovery. The AA/DHA ratio increased greatly during the first 4 days of stress treatment in the antisense transformants, but decreased in the other two genotypes (Fig. 2C). During the second part of the drought treatment, and during recovery, the highest AA/DHA ratio was detected in the antisense plants.

There was little or no change in the amount of reduced thiols during preliminary stress, but a large increase was observed during subsequent stress, except for cysteine, which reached a maximum after 4 days of drought stress and gradually decreased later on (Fig. 3A–D). In the recovery phase, the cysteine content decreased further and the concentrations of the other thiols also decreased. The amount of GSH was several orders of magnitude lower than that of homoglutathione (Fig. 3C, D). Although the cysteine level was higher in the sense transformants and lower in the antisense transformants compared with the wild-type plants at several sampling dates (Fig. 3A), the other three thiols showed the opposite pattern, resulting in higher concentrations in the antisense transformants and lower levels in the sense transformants (Fig. 3B–D).

The ratios of reduced to oxidized forms of the four thiols generally decreased during preliminary stress, except for cysteine in the sense transformants and γ-glutamylcysteine (γEC) in control plants (Fig. 3E–H). During subsequent stress, the ratio of cysteine to cystine first increased, then decreased (Fig. 3E). The γEC to glutamylcystine (ESSE) and GSH to GSSG ratios further decreased during the stress period, only increasing after 10 days and 7 days of drought, respectively (Fig. 3F, G).

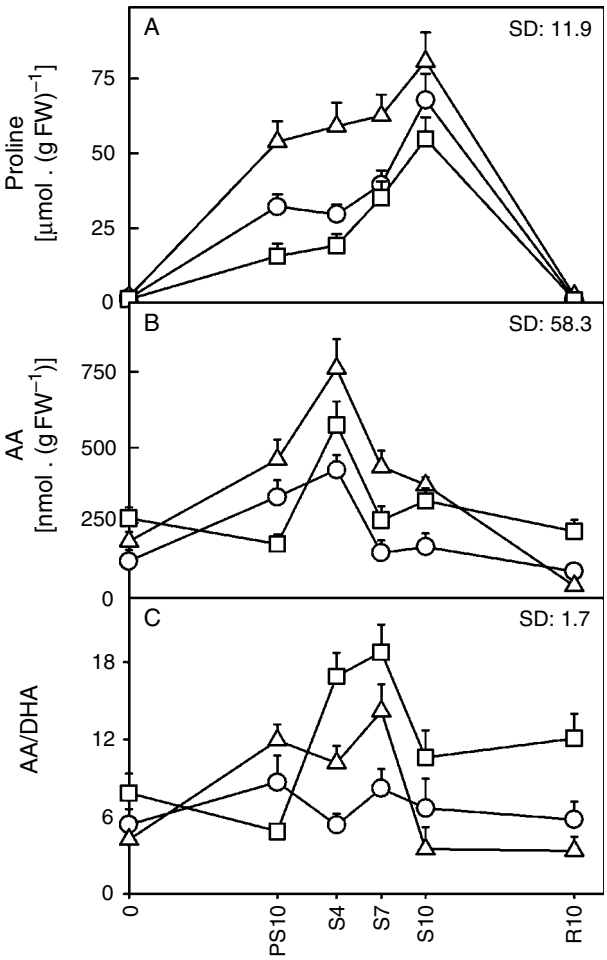

Fig. 2. Free proline content (A), ascorbate content (AA; B) and ratio of ascorbate to dehydroascorbate (AA/DHA; C) in leaves of wild-type soybean plants (○) and sense (△) and antisense (□) transformants grown without watering for 10 days (preliminary stress, PS), rewatered once, further cultivated for an additional 10 days without watering (stress, S) and finally supplied with an optimal amount of water for 10 days (recovery, R). Mean values ± standard deviations of nine measurements from three independent experiments are presented. SD, significant difference.

The ratio of reduced homoglutathione (hGSH) to its oxidized form (hGSSG) decreased throughout the experiment (Fig. 3H). The GSH/GSSG and hGSH/hGSSG ratios were higher in the antisense transformants and lower in the sense transformants compared with the wild-type plants in the second part of the treatment (Fig. 3G, H).

The activity of SOD, which was not significantly different in the three genotypes, first decreased, and then remained constant at the end of the stress treatment and during recovery (Fig. 4A). The activity of APX decreased greatly during the stress treatment in the sense

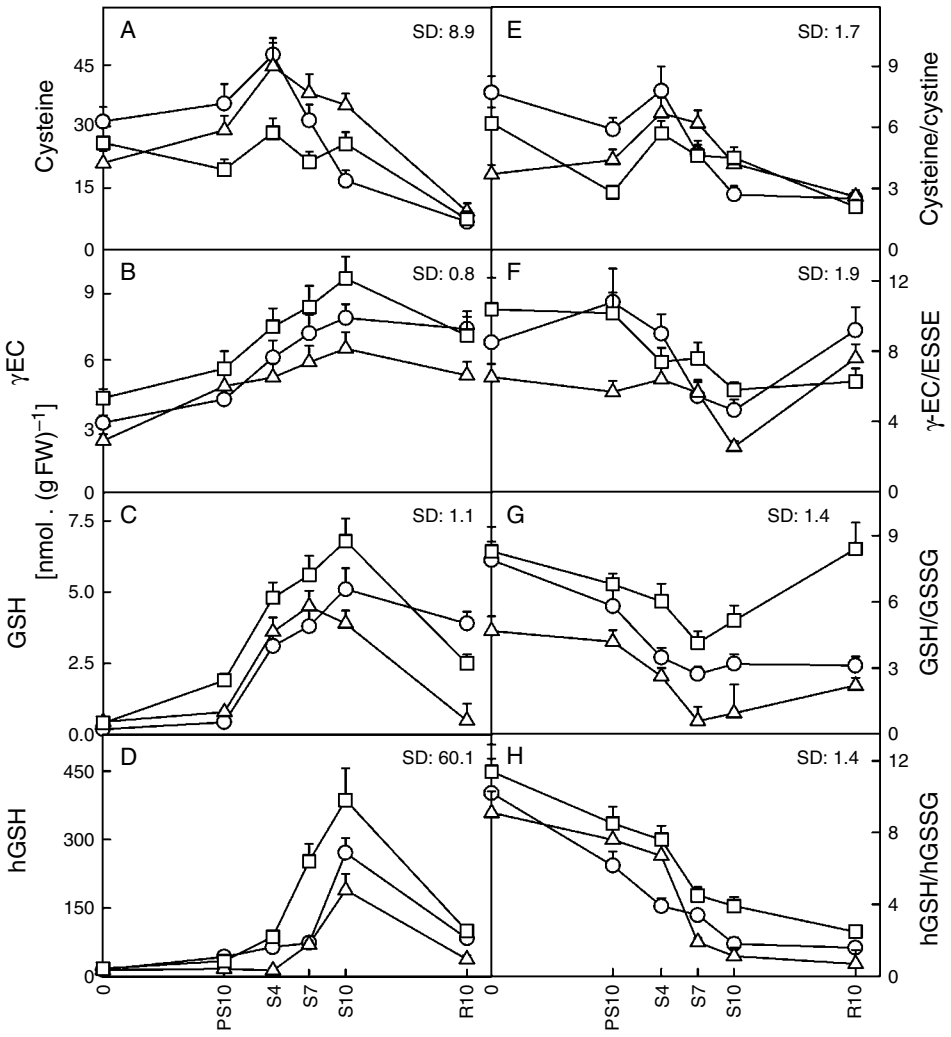

Fig. 3. Cysteine (A), γ-glutamylcysteine (γEC; B), reduced glutathione (GSH; C) and reduced homoglutathione (hGSH; D) contents and ratio of cysteine to cystine (E), γ-glutamylcysteine to γ-glutamylcystine (γEC/ESSE; F), reduced to oxidized glutathione (GSH/GSSG; G) and reduced to oxidized homoglutathione (hGSH/hGSSG; H) in leaves of wild-type soybean plants (○) and sense (△) and antisense (□) transformants grown without watering for 10 days (preliminary stress, PS), rewatered once, further cultivated for an additional 10 days without watering (stress, S) and finally supplied with an optimal amount of water for 10 days (recovery, R). Mean values ± standard deviations of nine measurements from three independent experiments are presented. SD, significant difference.

transformants, whereas, in the other two genotypes, smaller changes were observed (Fig. 4B). The activity of GR and GST either decreased or remained constant throughout the experiment in all the genotypes, except for a transient increase in GR activity in the antisense transformants after 7 days of stress (Fig. 4C, D). Following 7 days of drought stress, the APX and GR activities were greater in the antisense transformants and lower in the sense transformants than in the wild-type plants.

Discussion

Simultaneous drought and heat stresses induced oxidative stress in soybean under controlled conditions, as shown by the accumulation of H_2O_2 and lipid hydroperoxide. The higher H_2O_2 content and lipid peroxidation coincided with greater injury and lower RWC at the end of recovery in the antisense transformants. These results are in good agreement with those obtained for wheat genotypes subjected to heat stress by late sowing in the field (Sairam et al. 2000).

The present results, similar to previous findings (de Ronde et al. 2000, 2001b), corroborated the role of Pro in the protection of plants against simultaneous drought and heat stress, as the sense transformants, which had higher Pro content, were less damaged, and the antisense transformants, with lower Pro levels, suffered greater injury than the wild-type plants. The overexpression of other enzymes involved in the synthesis of Pro (L-Δ^1-pyrroline-5-carboxylate synthetase or ornithine-Δ-aminotransferase) also corroborated the relationship between Pro levels and osmotolerance (Kishor et al. 1995, Roosens et al. 2002). In addition, the protective role of Pro during drought was demonstrated by the comparison of two mulberry genotypes with different

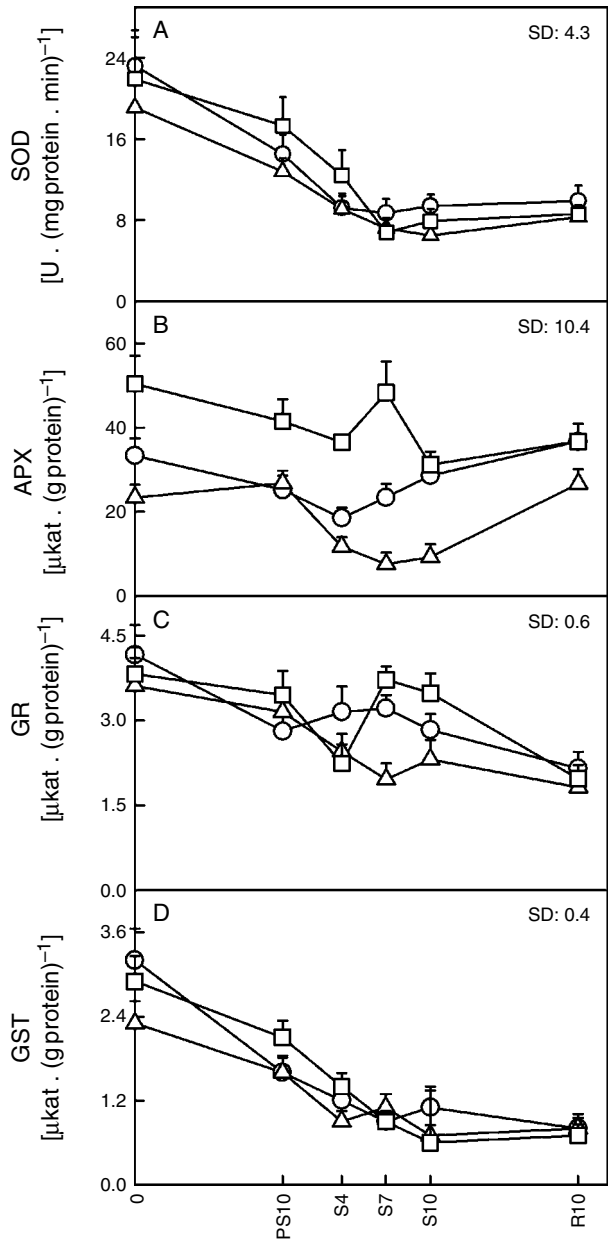

Fig. 4. Activity of superoxide dismutase (SOD; A), ascorbate peroxidase (APX; B), glutathione reductase (GR; C) and glutathione *S*-transferase (GST; D) in leaves of wild-type soybean plants (○) and sense (△) and antisense (□) transformants grown without watering for 10 days (preliminary stress, PS), rewatered once, further cultivated for an additional 10 days without watering (stress, S) and finally supplied with an optimal amount of water for 10 days (recovery, R). Mean values ± standard deviations of nine measurements from three independent experiments are presented. SD, significant difference.

drought tolerance, where a higher P5CR activity resulted in greater Pro content in the tolerant genotype (Ramanjulu and Sudhakar 2000). The importance of Pro in the heat stress response has been demonstrated in cotton, where cultivars with higher Pro content suffered less damage during heat treatment (Ashraf et al. 1994).

The lower Pro content in the antisense transformants compared with the wild-type plants was accompanied by higher γEC, GSH and hGSH levels following drought stress. However, these thiols showed lower concentrations in the sense transformants than in the wild-type plants. This can be explained by the increased demand for glutamate for Pro synthesis in the sense transformant, and the lower utilization of glutamate for Pro synthesis in the antisense transformant. The availability of glutamate may, in turn, influence the rate of GSH and hGSH synthesis, as was shown in the case of the other GSH precursor, cysteine, in maize (Kocsy et al. 1996). A higher cysteine content coincided with increased GSH content in chilling-tolerant maize genotypes compared with the sensitive genotype. In contrast with the γEC, GSH and hGSH contents, cysteine levels were lower in the antisense and higher in the sense transformants than in the wild-type plants. The low concentration of GSH and hGSH in the sense transformants may induce a greater rate of cysteine synthesis, as Hartmann et al. (2004) have demonstrated that changes in GSH content have an opposite effect on cysteine synthesis. Despite the higher cysteine content, the limited availability of glutamate as the result of increased Pro synthesis may reduce the rate of GSH synthesis.

The AA content increased in soybean in the present experiments, as previously observed in heat-stressed wheat (Dash and Mohanty 2002). Although the AA content reached the highest level in the sense transformants during the stress treatment, the AA/DHA ratio increased to a higher level in the antisense transformants than in the other two genotypes, which could be explained by the greater APX activity. Similar to the AA/DHA ratio, higher GSH/GSSG and hGSH/hGSSG ratios were detected in the antisense transformants, which could be the result of differences in GR activity. According to Schwanz and Polle (2001), if the increased Pro levels are ignored, the lower GSH/GSSG and hGSH/hGSSG ratios in the sense transformants should have resulted in decreased drought tolerance, as they found lower GR and APX activity in drought-avoiding pine than in drought-tolerant pedunculate oak. In addition, osmotic stress induced an increase in APX and GR activity in tolerant wheat varieties, but not in sensitive ones (Lascano et al. 2001). However, in the present experimental system, it seems likely that the higher Pro and AA contents in the sense transformant were sufficient for the effective reduction of the drought-induced accumulation of reactive oxygen species.

In summary, the genetic manipulation of the Pro level affected the stress-induced changes in the GSH and

hGSH contents, probably because of their common precursor, glutamate. The better stress tolerance of the sense transformants, observed despite the lower (h)GSH content and GR and APX activity, could be the result of both a higher Pro content and greater AA concentration during stress treatment.

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