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RAPID COMMUNICATION

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Salinity-induced glutathione synthesis in *Brassica napus*

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Abstract The role of S-assimilation and the biosynthesis of cysteine and glutathione were studied during the response to salt stress of wild-type and salt-tolerant transgenic *Brassica napus* L. (canola) plants overexpressing a vacuolar Na^+/H^+ antiporter. A 3-fold increase in cysteine and glutathione content was observed in wild-type plants exposed to salt stress, but not in the transgenic plants. The induction of cysteine and glutathione synthesis during salt stress in the wild-type plants suggests a possible protective mechanism against salt-induced oxidative damage. On the other hand, the salt-tolerant transgenic plants did not show significant changes in either cysteine or glutathione content, confirming the role of vacuolar Na^+ accumulation and ion homeostasis in salt tolerance.

Keywords Vacuolar Na^+/H^+ antiporter · *Brassica* (salt stress) · Cysteine · Glutathione · Salt stress · Salt tolerance

Abbreviations ATP-S: ATP sulfurylase · AOS: active oxygen species · γ-ECS: γ-glutamyl-cysteine synthetase · GR: glutathione reductase · GSH: glutathione · GSHS: glutathione synthetase · SAT: serine acetyl transferase · OAS-TL: *O*-acetylserine(thiol)lyase

Introduction

Glutathione (GSH), the tripeptide γ-glutamylcysteinylglycine, is the major source of non-protein thiols in most plant cells (Bergmann and Rennenberg 1993). GSH plays an important role in the response of plants to environmental stresses, including oxidative stress due to the generation of active oxygen species (AOS; Grant et al. 1996), xenobiotics (Marrs 1996), and some heavy metals (Grill et al. 1989). In addition, other physiological functions have been attributed to GSH in plants such as the regulation of inter-organ sulfur allocation (Lappartient and Touraine 1996) and gene expression (Baier and Dietz 1997). GSH may also be involved in the redox regulation of the cell cycle (Sánchez-Fernández et al. 1997). Biosynthesis of GSH in all organisms studied to date occurs in two steps: (i) the synthesis of γ-glutamylcysteine from L-glutamate, catalyzed by γ-glutamyl-cysteine synthetase (γ-ECS) (which is the rate-limiting step in GSH biosynthesis); (ii) the addition of glycine to the C-terminus of γ-glutamylcysteine to yield GSH, catalyzed by glutathione synthetase (GSHS; Hell and Bergmann 1990).

Elevated GSH levels, mediated by increased γ-ECS activities, have been measured in extracts of pea, tobacco, maize, and tomato subjected to different stresses (Rennenberg et al. 1982; Chen and Goldsbrough 1994; Kocsy et al. 1996). Recent studies support the notion that cysteine availability in plants also plays an important role in determining cellular GSH concentrations through the kinetic restriction of the reaction catalysed by γ-ECS (Noctor et al. 1997). Environmental conditions that stimulate synthesis of GSH will therefore place an increased demand upon S-assimilation into cysteine. Support for this argument comes from reports showing that the demand for increased GSH synthesis in plants treated with cadmium (Chen and Goldsbrough 1994) or herbicide safeners (Hell 1997) was mediated by the activation of γ-ECS activity and concomitant with substantial increases in the activities of key enzymes responsible for S-assimilation [ATP sulfurylase (ATP-S), serine acetyl transferase (SAT) and *O*-acetylserine (thiol)lyase (OAS-TL); Farago et al. 1994].

Increases in the GSH pool have been measured in response to chilling, heat shock, pathogen attack, AOS accumulation, air pollution and heavy metals (May et al. 1998; Noctor et al. 1998; Kocsy et al. 2001). Recently, Barroso et al. (1999) showed that the cytosolic isoform

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of OAS-TL, the enzyme that catalyses cysteine biosynthesis, is induced in leaves of *Arabidopsis thaliana* exposed to salt stress. In addition, the overexpression in yeast of the *Atcys-3A* gene, coding for an OAS-TL isoform, can support the growth of the yeast cells at high concentrations of NaCl (Romero et al. 2001). The authors suggested that the plant responded to the salt stress by inducing cysteine biosynthesis as a protection against high Na^+ concentrations, since the intracellular levels of cysteine and GSH increased up to 3-fold after salt treatment.

Here we show that S-assimilation and the synthesis of cysteine and GSH increased significantly when wild-type *Brassica napus* was exposed to salt stress. On the other hand, these changes were minimal in transgenic salt-tolerant *B. napus* plants, overexpressing a vacuolar Na^+/H^+ antiporter (Zhang et al. 2001). These results suggest that the processes leading to the biosynthesis of GSH are salt-stress-elicited and that the active accumulation of excess Na^+ in the vacuole of the transgenic plants minimizes the stress response.

Materials and methods

Plant material

Transgenic *Brassica napus* L. cv. Westar was obtained as described before (Zhang et al. 2001). For the experiments described here, T3 seeds obtained from three lines of transgenic *Brassica* plants overexpressing AtNHX1, the *Arabidopsis thaliana* (L.) Heynh. vacuolar Na^+/H^+ antiporter, were pooled and used as described below. These lines displayed high transgene expression, high leaf sodium content (up to 6% of the leaf dry weight) and were able to grow, flower and set seed when irrigated with high NaCl concentrations (Zhang et al. 2001). Wild-type and transgenic seeds overexpressing the vacuolar Na^+/H^+ antiporter were germinated in 250-ml pots containing pro-mix BX peat moss, perlite, and vermiculite medium (Premier Brands, New Rochelle, N.Y., USA) and grown in the greenhouse. Four weeks after germination the plants were watered with solutions containing no NaCl (control), and 75 mM and 150 mM NaCl for 15 days. After this, the mature leaves with a length larger than 10 cm were collected. The experimental design was a randomized complete block with three treatments, arranged in individual pots with six plants per treatment, and three replications. All the plants received a nutrient solution of: 4 mM NH_4NO_3, 2 mM Ca_2NO_3, 2 mM KH_2PO_4, 4 mM K_2SO_4, 1.5 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$ and 5 μM H_3BO_3. The nutrient solution (pH 5.5–6.0) was renewed every 3 days. Day temperature was maintained at 28 ± 20 °C and night temperature was 20 ± 20 °C. Relative humidity was maintained at $50 \pm 10\%$. Plants were grown under a 14-h/10-h light/dark photoperiod. Supplemental lighting consisted of high-pressure sodium lamps and resulted in a total flux (sunlight and supplemental light) of ≈1,200 μmol photons m^{-2} s^{-1}.

Plant analysis

ATP-S activity was measured from leaf homogenates as described previously (Lappartient and Touraine 1996) using the molybdate-dependent formation of pyrophosphate (Osslund et al. 1982). SAT and OAS-TL activities were measured from leaf extracts as described by Nakamura et al. (1987). γ-ECS and GSHS activities were assayed as described previously (Rüegsegger and Brunold 1992; Cobbett et al. 1998). The activity of glutathione reductase (GR) was determined by monitoring the GSH-dependent oxidation of NADPH at 340 nm in a reaction mixture containing 950 μl of 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and 3 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.5) and 50 μl leaf extracts. The protein content of the extracts was measured according to Bradford (1976) with BSA as the standard. For determination of cysteine, leaf tissue was extracted in a mixture of 80 mM sulfosalicylic acid, 1 mM EDTA and 0.15% sodium ascorbate (w/v) as described by Maas et al. (1987). The determination of cysteine was based on the reactivity of its sulfhydryl group with methylglyoxal as described by De Kok et al. (1988). Total GSH (measured as total thiol) was determined according to Brehe and Burch (1976) as modified by Anderson et al. (1992). For determination of total GSH, leaf tissue was extracted in 6% *m*-phosphoric acid (pH 2.8) containing 1 mM EDTA. Total GSH was measured in a reaction mixture consisting of 400 μl reagent A [110 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 15 mM EDTA, 0.3 mM 5,5-dithiobis (2-nitrobenzoic acid)], 320 μl reagent B [1 mM EDTA, 50 mM imidazole, and an equivalent of 1.5 units GR activity (baker's yeast, Type III; Sigma)], and 400 μl of a 1:50 dilution of the leaf extract in 5% Na_2HPO_4 (pH 7.5) prepared immediately prior to starting the assay. The reaction was initiated by the addition of 80 μl of NADPH and monitored by measuring absorbance changes at 412 nm.

Statistical analysis

Standard analysis of variance techniques were used to assess the significance of treatment means.

Results and discussion

Assimilation and incorporation of S into organic compounds require the reduction of SO_4^{2-}, which in turn binds ATP, forming adenosine-5′-phosphosulfate (APS). This reaction, catalyzed by ATP-S (Leustek et al. 1994), is the rate-limiting step enabling and initiating sulfur metabolism. Overexpression of ATP-S led to an 8-fold increase in the enzyme activity in Indian mustard (*Brassica juncacea*) and to increases in GSH levels (Pilon-Smits et al. 1999). Moreover, ATP-S activation is correlated with an increase in SO_4^{2-} absorption, and a similar regulation for both SO_4^{2-} uptake and ATP-S activity has been proposed (Lappartient and Touraine 1996). Although the expression and activity of ATP-S has been studied under conditions of S-starvation (Takahashi et al. 1997) and upon exposure of plants to heavy metals, such as cadmium (Heiss et al. 1999), its role during salt stress has not been studied. In our experiments, the exposure of *Brassica* plants to 150 mM NaCl resulted in increased ATP-S activity in both wild-type and transgenic plants (Fig. 1a), with a 280% and 55% activity increase in wild-type and transgenic plants, respectively.

The biosynthesis of cysteine constitutes the final step of the sulfur-reduction pathway in plants (Leustek and Saito 1999). Consequently, the formation of cysteine is the crucial step for assimilation of reduced sulfur into organic compounds such as GSH (Noctor et al. 1998). During cysteine biosynthesis, an acetyl-group from acetyl-CoA is transferred to L-serine, creating the highly

reactive compound *O*-acetyl-L-serine. This reaction is catalysed by SAT. The subsequent synthesis of cysteine in plants in accomplished by the sulfihydrilation of *O*-acetyl-L-serine in the presence of sulfide. This reaction is catalysed by OAS-TL. Studies have shown that SAT activity is the limiting step in the regulation of cysteine synthesis (Blasczcyk et al. 1999; Höfgen et al. 2001). Nevertheless, the few studies that characterized cysteine synthesis with salt stress have centered on the analysis of OAS-TL activity. Our results are in agreement with those reported by Barroso et al. (1999) and Romero et al. (2001) since the increase in NaCl in the growth medium led to an increase in SAT and OAS-TL activities both in wild-type and transgenic canola plants (Fig. 1b, c). A marginal increase in SAT and OAS-TL (10% and 20%, respectively) was seen in the transgenic plants growing in the presence of 150 mM NaCl. On the other hand, wild-type plants grown in the presence of 150 mM NaCl displayed a marked increase in SAT and OAS-TL activities (236 and 181%, respectively). Notably, the transgenic plants displayed SAT and OAS-TL basal activities (i.e. in the absence of NaCl) that were 2-fold

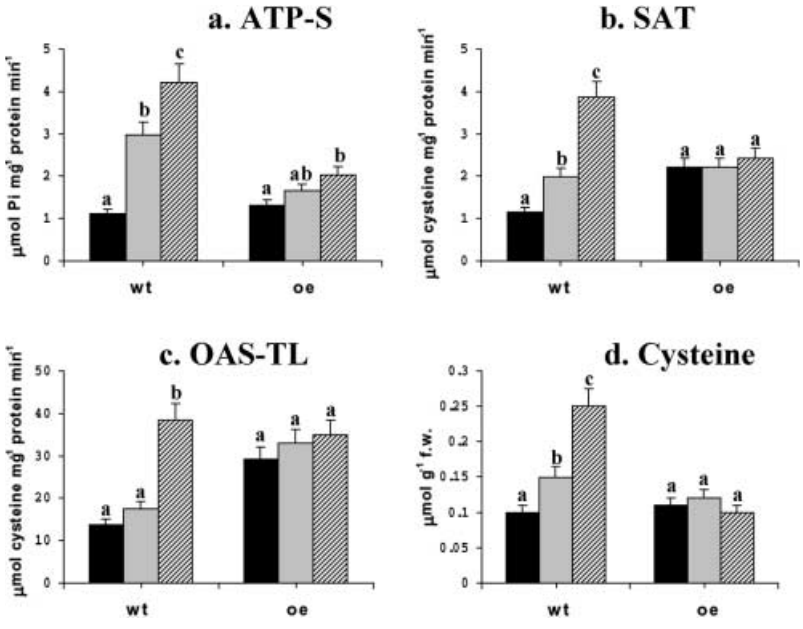

Fig. 1 Assimilation of S and biosynthesis of cysteine in wild-type (*wt*) and transgenic (*oe*) canola (*Brassica napus*) plants exposed to 0 mM NaCl (*black bars*), 75 mM NaCl (*grey bars*) or 150 mM NaCl (*hatched bars*). Values are the mean ± SE ($n = 6$). Based on *t*-test analysis, values for bars headed by different letters differ significantly ($P < 0.05$)

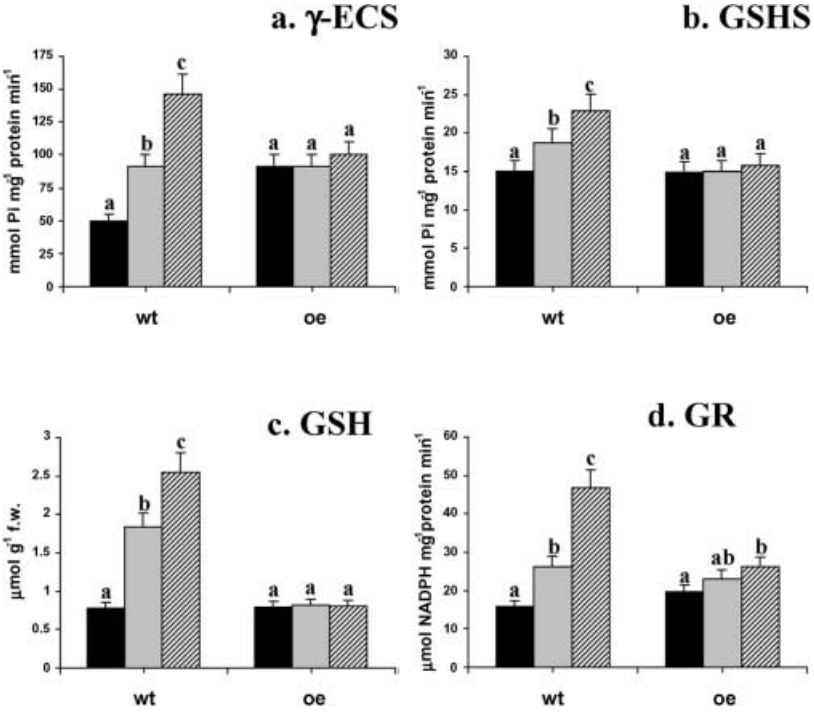

Fig. 2 Biosynthesis of GSH and glutathione reductase activity in wild-type (*wt*) and transgenic (*oe*) canola plants exposed to 0 mM NaCl (*black bars*), 75 mM NaCl (*grey bars*) or 150 mM NaCl (*hatched bars*). Values are the mean ± SE ($n = 6$). Based on *t*-test analysis, values for bars headed by different letters differ significantly ($P < 0.05$)

higher than those from wild-type plants. Whether these changes were due to a modification of the enzymes in the transgenic plants remains to be elucidated. Nevertheless, the salt-stress-induced differences in ATP-S, SAT and OAS-TL activities between wild-type and transgenic plants correlated with the leaf cysteine concentrations (Fig. 1d). While no differences in cysteine content were seen in the transgenic plants, a 150% increase in cysteine content was observed in wild-type plants.

Taking into consideration the results described above, we can conclude that salt stress induced a sulfur demand for cysteine synthesis. Since cysteine is required for GSH synthesis, the activation of GSH synthesis in the wild-type plants would be expected. Salt stress induced a 3-fold increase in γ-ECS (Fig. 2a), and only a marginal increase in GSHS activity (Fig. 2b), supporting the notion that γ-ECS activity is the critical step in GSH synthesis in plants. Transgenic plants exposed to 150 mM NaCl displayed a minimal increase in γ-ECS while GSHS activity was not affected (Fig. 2a, b). Similar to what was observed with SAT and OAS-TL (Fig. 1b, c), GSHS and γ-ECS basal activities (i.e. for plants grown in the absence of NaCl) were higher in the transgenic plants, and whether this increase was due to modification in the enzymes remains to be elucidated. Nonetheless, the salt-stress-induced increase in γ-ECS activity correlated with a 230% increase in leaf GSH content of wild-type plants, while the GSH content of the transgenic plants remained unchanged during salt stress (Fig. 2c).

Plants under salt stress displayed an increase in the generation of H_2O_2 and other AOS (Gueta-Dahan et al. 1997; Roxas et al. 2000). The major substrate for the reductive detoxification of H_2O_2 is ascorbate, which must be continuously regenerated from its oxidized form. A major function of GSH in protection against oxidative stress is the reduction of ascorbate via the ascorbate-glutathione cycle, where GSH acts as a recycled intermediate in the reduction of H_2O_2 (Foyer and Halliwell 1976; Noctor et al. 1998). Efficient recycling of GSH is ensured by GR activity (Noctor et al. 1998). Several groups have shown that GR activity increases during oxidative stress (Pastori and Trippi 1992; Xiang and Oliver 1998). Our results suggest that this is also the case in *Brassica* wild-type plants during salt stress, since GR activity increased by 2-fold in the presence of 150 mM NaCl (Fig. 2d). On the other hand, transgenic plants displayed a relatively minor increase in GR activity (Fig. 2d).

In conclusion, our results showed that in wild-type *Brassica* plants, salt stress induced an increase in the assimilation of S and the biosynthesis of cysteine, and GSH aimed to mitigate the salt-induced oxidative stress. The small changes seen in the salt-tolerant transgenic *Brassica* plants overexpressing the vacuolar Na^+/H^+ antiporter (Zhang et al. 2001) suggest that the accumulation of excess Na^+ in the vacuoles greatly diminished the salt-induced oxidative stress, highlighting the important role of Na^+ homeostasis during salt stress.

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