Coordinate induction of glutathione biosynthesis and glutathione-metabolizing enzymes is correlated with salt tolerance in tomato

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Abstract The acclimation of reduced glutathione (GSH) biosynthesis and GSH-utilizing enzymes to salt stress was studied in two tomato species that differ in stress tolerance. Salt increased GSH content and GSH:GSSG (oxidized glutathione) ratio in oxidative stress-tolerant *Lycopersicon pennellii* (Lpa) but not in *Lycopersicon esculentum* (Lem). These changes were associated with salt-induced upregulation of γ-glutamylcysteine synthetase protein, an effect which was prevented by pre-incubation with buthionine sulfoximine. Salt treatment induced glutathione peroxidase and glutathione-*S*-transferase but not glutathione reductase activities in Lpa. These results suggest a mechanism of coordinate upregulation of synthesis and metabolism of GSH in Lpa, that is absent from Lem.
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1. Introduction

Reduced glutathione (GSH), the most abundant low molecular weight thiol compound in plants, has unique structural properties and a broad redox potential. Its stability is derived from the γ-glutamyl linkage, whilst the strong nucleophilic nature of the central cysteine makes it a powerful cellular reductant. In this role, GSH protects against a range of peroxides, xenobiotics and heavy metals [1–3]. Glutathione peroxidases (GPXs) catalyze the GSH-dependent reduction of peroxides, a reaction that can also be catalyzed by glutathione-*S*-transferases (GSTs; [4,5]). GSH also participates in the ascorbate–glutathione cycle [6]. All the functions described above involve the oxidation of the thiol group, forming glutathione disulfide (GSSG). High cellular GSH:GSSG ratios, which are maintained by the action of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase (GR), make an important contribution to the redox state of the cell [7].

GSH is synthesized from its constituent amino acids in an adenosine triphosphate (ATP)-dependent, two-step reaction catalyzed by the enzymes γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase. These enzymes have been partially characterized and shown to exist in both chloroplastic and cytosolic compartments [2,8]. GSH biosynthesis has been extensively studied in transformed plants [9,10], which has demonstrated that GSH synthesis is controlled by three mechanisms: the amount of γ-ECS activity, substrate availability and, less importantly, feedback inhibition of γ-ECS by GSH. γ-ECS activity, in turn, is determined by transcriptional and translational controls and by the GSH:GSSG ratio [3,11].

Environmental factors alter both the size and the redox state of the glutathione pool. For example, salt stress [12–14], heavy metals [15], low temperature [16,17] and pathogens [18] all lead to GSH accumulation. Elevated GSH levels have been related to increased γ-ECS activity in plants subjected to a range of abiotic stresses [13,15,19]. Moreover, treatment with buthionine sulfoximine (BSO), an inhibitor of γ-ECS activity, leads to enhanced stress sensitivity [17]. One study has indicated that enhanced γ-ECS activity and GSH paradoxically led to increased stress sensitivity in transgenic plants [20], although this effect was subsequently attributed to changes in γ-EC, rather than GSH [21]. It is pertinent, therefore, to determine whether GSH accumulation and γ-ECS activity in particular are linked to salt stress responses in tomato, which have been related to changes in antioxidant status [22]. We have shown previously that two closely related tomato species that differ in their salt sensitivity are an excellent tool to explore the mechanisms of protection against salt-induced oxidative stress [14,22–24]. We therefore explored the role of GSH and associated enzymes in the acclimation of these species to salt. The results presented here show for the first time that γ-ECS protein is increased in the salt-tolerant species following exposure to salt. Confirmation that GSH is required for stress tolerance in both species is provided by experiments with BSO, a specific inhibitor of γ-ECS. Furthermore, these data show that there is considerable interspecific variation in the capacity to induce GSH synthesis in response to stress and that this might have an impact on the ability of different species to withstand salt.

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2. Materials and methods

2.1. Plant species and growth conditions

Plants of the cultivated tomato *Lycopersicon esculentum* Mill. cultivar M82 (Lem) and its wild salt-tolerant relative *Lycopersicon pennellii* (Corr.) D'Arcy accession Atico (Lpa) were grown hydroponically as described previously [25]. Salt treatment started at the stage of four true leaves by successively increasing the NaCl concentration by 25 mM per day over a period of 4 days, to a final concentration of 100 mM. Samples were taken thereafter at the points indicated in the figures, with day 0 defined as the first day where the salt concentration was 100 mM.

2.2. BSO treatment

BSO (1 mM) was added to hydroponically grown plants at the stage of two true leaves. 5 days after addition of BSO, salt treatment was started by successively increasing NaCl concentration by 50 mM per day for 3 days to a final concentration of 150 mM. Day 0 was defined as the first day where the salt concentration was 150 mM. Leaves and roots were sampled on day 5.

2.3. Measurements of enzyme activities, glutathione and protein

Glutathione was assayed according to Griffith [26]. GR (EC 1.6.4.2) activity was determined according to Madamanchi and Alscher [27] and GST (EC 2.5.1.18) activity was assayed according to Drotar et al. [28], using 1-chloro-2,4-dinitrobenzene as the substrate. GPX (EC 1.11.1.9) activity was followed by the decrease in A_{340}, resulting from NADPH oxidation [28]. The reaction mixture contained: 50 mM potassium phosphate buffer, pH 7, 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM GSH, 0.09 mM H_2O_2, 0.1 mM NADPH, 2.5 units GR and 5–20 µl of enzyme extract. Similar results were obtained with tert-butyl hydroperoxide as substrate. Protein was assayed according to Bradford [29].

2.4. Antiserum preparation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

An 805-bp fragment was amplified from plasmid mGR/pBluescript SKII− (generously provided by Dr. F. Van Breusegem). The reaction contained: 30 ng plasmid, 2.5 pmol mGR forward primer (5′-GACG-GATCCATGTTTGCAACA-3′), 2.5 pmol universal T7 primer, 0.5 µl 10 mM deoxyribonucleoside triphosphate (dNTP) mix, $1 \times Pfu$ buffer and 0.5 U *Pfu* DNA polymerase (BRL) in a total volume of 20 µl. Polymerase chain reaction (PCR) was performed as follows: initial denature: 94°C, 1 min; 30 cycles of 94°C, 2 min; 50°C, 2 min; 72°C 2 min; final extension 72°C, 10 min. The PCR fragment was restricted with *Bam*HI/*Xho*I and ligated into *Bam*HI/*Sal*I-digested pMAL-c2 (New England Biolabs), to generate an in-frame fusion between maltose binding protein and the C-terminal 213 amino acids of maize GR (accession number: O64409). This construct was transformed into *Escherichia coli* strain XL1Blue (Stratagene), and maltose binding protein-GR fusion was purified according to the manufacturers' instructions, using amylose affinity chromatography. The fusion protein was dialyzed into phosphate buffered saline and concentrated to 2 mg ml⁻¹ prior to immunization. Anti-γ-ECS polyclonal antiserum was raised in rabbits immunized with a synthetic peptide (KNGLERRGYKEVGFLREV) based on the sequence of maize γ-ECS (accession number: Q8W4W3) and conjugated to keyhole limpet hemocyanin.

Crude extracts and chloroplasts were prepared from tissue that was harvested on day 14 of salt treatment as described in Mittova et al. [25]. Protein was separated by SDS–PAGE (12% acrylamide gel) and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). Membranes were blocked in Tris buffered saline (TBS) containing 5% (w/v) bovine serum albumin (BSA) for 2 h at room temperature and thereafter probed with polyclonal antibodies raised against maize GR or γ-ECS (1:5000 dilution) for 2 h. The membranes were washed five times in TBS, containing 0.05% (v/v) Tween 20 and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies. Following extensive washing as before, signals were visualized by enhanced chemiluminescence, according to the manufacturers' instructions (Amersham Pharmacia, Piscataway, NJ, USA). In preliminary experiments, the γ-ECS and GR antisera recognized single polypeptides of ca. 59 and 53 kDa, respectively in crude extracts from plants (data not shown).

3. Results

We have shown previously that 14 days of salt treatment causes oxidative stress and increases lipid peroxidation in Lem and leads to salt-induced upregulation of antioxidative enzymes in Lpa [14,22–24,30]. The present experiments were designed to investigate the role of glutathione and glutathione-related enzymes in the acclimation process in both species. While there was little or no effect of salt treatment on GSH content in Lem leaves or roots (Fig. 1A), salt induced a decrease in the GSH:GSSG ratio in Lem roots (Fig. 1B). In contrast, salt treatment induced marked GSH accumulation in Lpa roots and leaves, together with an increase in the GSH:GSSG ratio in both organs of this species (Fig. 1). It is interesting to note the effect of these changes on the redox environment of the cell, as calculated by the method of Schafer and Buettner [7]. Control Lpa leaves were maintained at a lower redox potential than those of Lem (Table 1). However, acclimation to salt increased the redox potential of the Lpa leaves to a slightly higher value than that calculated for Lem, which was unchanged by salt. In both species, the redox po-
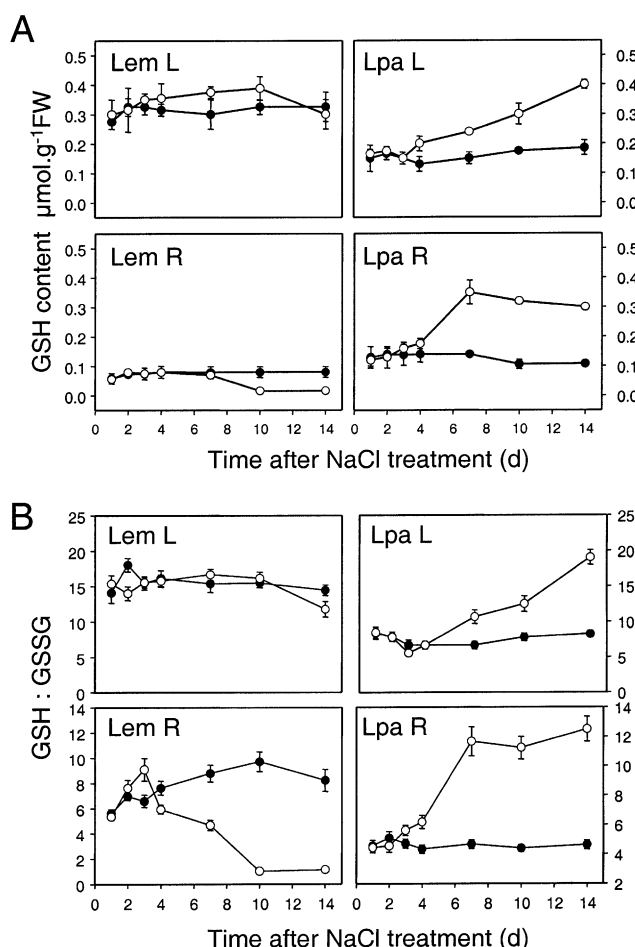

Fig. 1. Time course of glutathione accumulation in leaves (L) and roots (R) of the cultivated tomato (Lem) and the wild species (Lpa) under normal and salt stress growth conditions. A: GSH content. B: GSH:GSSG ratio. Closed symbols: control plants; open symbols: salt-stressed plants. Values represent the means of 18 measurements from three plants in each of two independent experiments ± S.D. Time represents days after the completion of salt stress (day 0).

Table 1

Effect of salt stress on redox potentials of the GSH/GSSG redox pair in the cultivated tomato, Lem and its wild, salt-tolerant relative, Lpa

	Redox potential (mV)	
	Control	NaCl
Lem leaf	−200	−197
Lpa leaf	−186	−207
Lem root	−175	−129
Lpa root	−171	−198

Redox potentials were calculated from data obtained after 14 days exposure to salt, as described in [7].

tential was lower in roots than in leaves; whilst salt caused a marked decrease in the redox potential of Lem roots, there was a corresponding increase in that of Lpa roots (Table 1). These data show that the Lpa leaves and roots respond in a positive manner to salt stress, adjusting the cellular redox potential to higher values, while Lem leaves and roots appear to be unable to do so.

The induction of GSH in Lpa was accompanied by an increase in the amount of γ-ECS protein (Fig. 2A), but no effect of salt on γ-ECS protein was observed in Lem leaves or roots. The induction of γ-ECS protein was evident in chloroplasts isolated from the leaves of Lpa plants, whereas no increase was observed in Lem chloroplasts (Fig. 2B). No effect of salt on GR protein (Fig. 2C) or activity (Table 2) was observed. However, salt increased the activity of both GPX and GST in Lpa leaf and root extracts but not in Lem (Table 2).

To confirm the role of salt in the induction of γ-ECS protein accumulation in Lpa, and consequent increases in GSH, plants were subjected to BSO treatment, prior to exposure to salt stress. BSO is a specific inhibitor of γ-ECS, which has been widely used in animal studies and more recently in plants, as discussed in [32]. As in the experiment shown in Fig. 1A, salt induced GSH accumulation in Lpa but not Lem leaves or roots (Fig. 3A). In accordance with previous studies [31], BSO had a marked effect on tissue GSH content in both species (Fig. 3A). In the absence of salt, BSO had little or no effect on tissue levels of H_2O_2 and the lipid peroxidation product malondialdehyde (MDA) in both species. However, in the presence of BSO, salt caused a dramatic increase in leaf and root H_2O_2 in Lpa and a similarly large increase in MDA in its leaves but not roots.

4. Discussion

The amount of GSH in a given organ is the result of the combined action of biosynthesis, metabolism, transport and degradation [2,33]. In the present study, we have shown that at least two of these parameters – biosynthesis and metabo-
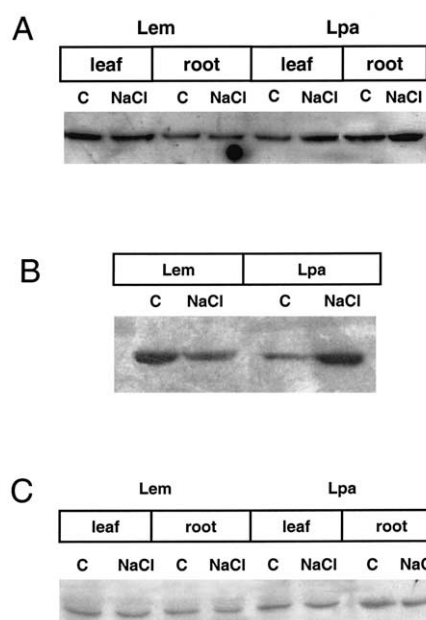

Fig. 2. Western blot analysis of γ-ECS and GR in tomato leaf and root extracts. Crude extracts and isolated chloroplasts were prepared from the cultivated tomato Lem and wild salt-tolerant species Lpa grown for 14 days in control (−) or salinized (+) media. Crude extracts (20 μg protein) were loaded onto 10% (w/v) denaturing acrylamide gels, transferred to PVDF membranes and probed with antibodies raised to either maize γ-ECS (A) or maize GR (C). B: Chloroplastic proteins (30 μg) were loaded onto 10% (w/v) denaturing acrylamide gels, transferred to PVDF membranes and probed with antibodies raised against maize γ-ECS.

lism – are differentially influenced by salinity in two tomato species which vary in their tolerance to salt-induced oxidative stress. Evidence that GSH biosynthesis is induced in the salt-tolerant Lpa but not in Lem is provided by the accumulation of γ-ECS protein in salt-treated leaves and roots of the former species, which is coincident with increased GSH content in these tissues. Conversely, in Lem, where no accumulation of γ-ECS was observed in salt-treated plants, GSH did not accumulate, and even decreased slightly in roots. Moreover, when GSH synthesis was inhibited by BSO pretreatment, the salt-induced accumulation of GSH in Lpa leaves and roots was prevented. Taken together, these results suggest that enhanced GSH synthesis and accumulation are important for the tolerance of salt-induced oxidation in Lpa. Furthermore, when these acclimatory processes are prevented, oxidative stress ensues in Lpa. It is interesting that this does not happen in Lem tissues treated with BSO.

Abiotic stresses have been shown previously to enhance GSH accumulation in leaves and roots of various species [15,17]. Salt stress induces increases in both GSH [13] and

Table 2

Effect of salt stress on activities of GSH-utilizing and regenerating enzymes in leaves and roots of the cultivated tomato, Lem and its wild, salt-tolerant relative, Lpa

	GR (μmol min^{-1} mg^{-1} protein)		GST (μmol min^{-1} mg^{-1} protein)		GPX (μmol min^{-1} mg^{-1} protein)	
	Control	NaCl	Control	NaCl	Control	NaCl
Lem leaf	0.059 ± 0.003	0.060 ± 0.007	0.041 ± 0.001	0.049 ± 0.012	0.065 ± 0.009	0.064 ± 0.002
Lpa leaf	0.057 ± 0.006	0.062 ± 0.006	0.066 ± 0.007	0.205 ± 0.008	0.070 ± 0.009	0.166 ± 0.012
Lem root	0.120 ± 0.015	0.117 ± 0.013	0.145 ± 0.015	0.145 ± 0.016	0.364 ± 0.054	0.240 ± 0.023
Lpa root	0.108 ± 0.006	0.091 ± 0.011	0.131 ± 0.010	0.280 ± 0.025	0.284 ± 0.022	0.575 ± 0.030

Measurements were taken 14 days after completion of salt treatment and represent the means ± S.D. of three independent experiments.

γ-ECS activity [34]. This agrees with other studies in the literature that link stress-induced increases in total GSH to enhanced γ-ECS activity, which is known to be a key factor controlling the amount of GSH in leaves [3,9]. The GSH biosynthetic pathway is located in both cytosol and chloroplast [2,8]. We show here that salt induces the chloroplastic isoform of γ-ECS in Lpa but not Lem leaves. However, this does not preclude a contribution from the cytosol.

γ-ECS activity has been shown to be regulated by transcriptional, translational and post-translational mechanisms [11]. The post-transcriptional control of γ-ECS protein abundance is determined largely by the redox state of the glutathione pool and/or the amount of H_2O_2, whereby low GSH:GSSG ratios and high H_2O_2 increase translation [35]. However, the observations that in Lpa the abundance of γ-ECS protein increased (Fig. 2A,B), despite increased GSH:GSSG ratios (Fig. 1B) and unchanged H_2O_2 (Fig. 3B) strongly indicate that salt-dependent upregulation of γ-ECS activity in this species occurs at the level of transcription. Moreover, the findings that the abundance of γ-ECS protein is not increased in Lem (Fig. 2A,B) in situations where GSH:GSSG ratios are low (Fig. 1B) and H_2O_2 is elevated (Fig. 3B) suggest that in this species, γ-ECS activity is not determined by post-transcriptional regulation. Although translational controls cannot be ruled out, a key hypothesis arising from the current study is that the salt-induced transcriptional regulation could differ in the two closely related tomato species but further studies are required to confirm this.

We show that salt also affects glutathione-utilizing protec-

tive processes catalyzed by GPXs and GSTs in Lpa but not in Lem. Both GPXs and certain GST isoforms are capable of scavenging lipid hydroperoxides [1,36,37]. Accordingly, Roxas et al. [38,39] have shown that overexpression of a GST with GPX activity in transgenic plants afforded protection against stress-induced oxidative damage. Although GSTs and GPXs are encoded by multigene families in plants, effects of salt on the total activities measured in this study indicate a general upregulation of glutathione-based defense metabolism in the salt-tolerant species. These results indicate that salt tolerance is linked to the ability to upregulate enzymes of GSH synthesis and utilization and that this is absent from the salt-sensitive species. A comparison of the total amounts of GSH and the GSH:GSSG ratios in the tissues of both species under salinity indicates that the dramatic decrease in the GSH:GSSG ratio in salt-stressed Lem roots is due to other factors such as NADPH deficits, rather than limitations on GR activity. Our results indicate that GR does not limit the regeneration of GSH from GSSG under salt stress, since neither the activity nor protein level of this enzyme was enhanced by salt.

In conclusion, our data demonstrate a salt-responsive, coordinated regulation of γ-ECS, GSTs and GPXs but not GR in Lpa and its absence from Lem. While further investigation is needed to determine the nature of this regulation, our present results suggest the hypothesis that members of the first three classes of genes could belong to a salt-responsive transcriptional network that is triggered in Lpa but not in Lem. We suggest, therefore, that Lem and Lpa employ markedly
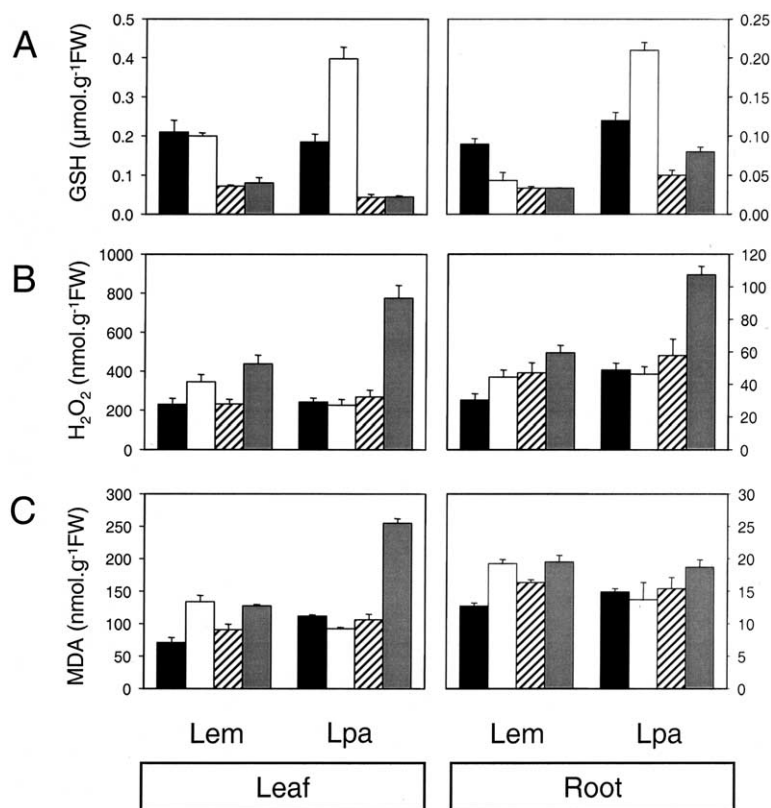

Fig. 3. The effect of BSO on GSH, MDA and H_2O_2 contents in leaves (L) and roots (R) of the cultivated tomato (Lem) and the wild species (Lpa) in the absence and presence of salt. Black bars: control; open bars: 150 mM NaCl; hatched bars: 1 mM BSO; gray bars: 1 mM BSO, 150 mM NaCl. Plants were pretreated with 1 mM BSO from the two leafs stage. Salt treatment was as described in Section 2, with samples harvested after 5 days of treatment. Note the different scales on the *y* axes. Values represent the means of three independent experiments ± S.D.

different mechanisms to combat the potentially harmful oxidative effects of high salt. We have demonstrated clearly that GSH is of intrinsic importance to prevention of salt-induced oxidative stress in Lpa, a mechanism that may also be employed by other salt-tolerant species.

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