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Relationship between antioxidant defence systems and salt tolerance in *Solanum tuberosum*

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Abstract. A relationship between the antioxidant defence system and salt tolerance in two clones of potato (*Solanum tuberosum* L.) differing in salt sensitivity was studied. The antioxidant defence system of the sensitive clone responded differently to 100 and 150 mM NaCl. At 100 mM NaCl, growth, dehydroascorbate reductase and catalase activities remained unaltered, but chlorophyll and reduced glutathione content decreased (23% and 35%, respectively), while ascorbate content and superoxide dismutase activity were increased 34% and 63%, with respect to the control (0 mM NaCl). The superoxide dismutase increment was higher under 150 mM NaCl treatment, while a general decrease (except for dehydroascorbate reductase and catalase activities) in all the antioxidant parameters studied was observed in the sensitive clone. Reduced glutathione and ascorbate, the main antioxidant soluble defences, and all antioxidant enzymes (except catalase) were significantly elevated in the tolerant clone compared to the sensitive one when both were grown in the absence of NaCl. Under 100 and 150 mM NaCl treatments, no changes in the antioxidant stress parameters were detected in the tolerant clone. These results suggest a relationship between salt tolerance and the antioxidant defence system in the two clones.

Keywords: antioxidant defences, oxidative stress, salt stress, *Solanum tuberosum*.

Introduction

There is accumulating evidence that production of toxic oxygen species ($O_2^{\cdot-}$, H_2O_2, $HO^{\bullet}$) is a major damaging factor in plants exposed to different environmental stresses, including salinity (Monk and Davies 1989; Corpas *et al.* 1993; Hernández *et al.* 1993, 1995; Scandalios 1993; Fadzilla *et al.* 1997). A highly efficient antioxidant defence system is present in all plants, composed of non-enzymatic and enzymatic constituents, to control the potential reaction of activated oxygen, which could produce deleterious effects in the tissues. Ascorbate (ASC)* and reduced glutathione (GSH) are the main soluble antioxidants implicated in the adaptation of plants to environmental stresses (Wise and Naylor 1987; Alscher 1989; Smirnoff 1996). Hydrogen peroxide, the first stable product of oxygen reduction, is formed mainly by dismutation of the superoxide anion, either non-enzymatically or enzymatically by superoxide dismutase (SOD). In higher plants, H_2O_2 is scavenged by the ascorbate-glutathione pathway and/or by catalase and non-specific peroxidases (Asada 1994; Scandalios 1994).

In vitro selection of salt stress-resistant material has been used to investigate plant metabolism and the mechanisms of salt tolerance at the cellular level. Sodium chloride tolerance was accompanied by a significant increase in antioxidant activity in a cotton cell line grown under NaCl stress (Gosset *et al.* 1996). Ochatt *et al.* (1999) have recently reported the *in vitro* production of a stable salt-tolerant cell line of potato (*Solanum tuberosum* L. cv. Kennebec) by direct recurrent selection. The selected cell line was regenerated into whole plants and used for the present experiment. The aim of this study was to investigate whether a relationship exists between salt tolerance and the antioxidant defence system in the two *Solanum tuberosum* L. clones differing in their tolerance to sodium chloride.

Materials and methods

Chemicals

NADPH, GSH, GSSG, DTNB, GR, NBT, and 2-vinylpyridine were obtained from the Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade.

*Abbreviations used: APOX, ascorbate peroxidase (EC 1.11.1.11); ASC, ascorbate; CAT, catalase (EC 1.11.1.6); DHAR, dehydroascorbate reductase (EC 1.8.5.1); DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid (disodium salt); GR, glutathione reductase (EC 1.6.4.2); GSH, reduced glutathione; GSSG, oxidised glutathione; NBT, nitroblue tetrazolium; PVP, polyvinylpyrrolidone; SOD, superoxide dismutase (EC 1.15.1.1).

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Plant culture and salt treatments

Solanum tuberosum L. cv. Kennebec plants regenerated from a salt-susceptible callus line (clone S) or from a line of the same genotype regenerated after recurrent selection for tolerance to 150 mM NaCl (clone 150) (Ochatt *et al.* 1999) were used.

For this experiment, the *in vitro* plants described above were grown by hydroponic culture for 14 d to acclimatise. They were then transferred to 800-mL plastic pots containing perlite, with 3 plants per pot and 20 replicates, and watered with Hoagland solution (Hoagland and Arnon 1953) for another 14 d. At this time, the plants were divided into three groups, each containing 10 replicate pots. Each group was watered with a half-strength Hoagland solution with 0, 100 or 150 mM NaCl added (saline treatments) for 14 d. The experiment was performed in a controlled temperature room (25/20ºC day/night) under a photosynthetic photon flux density of 150 µmol m^{-2} s^{-1}, with a photoperiod of 14 h and 50% relative humidity. The position of the pots was rotated at random every 4 d during the experiment to homogenise the environmental conditions. Plants were harvested and leaves were used for all biochemical determinations.

Chlorophyll determination

Chlorophyll was extracted by homogenising and boiling 1 g of fresh weight of leaves in 35 mL of 96% ethanol. After centrifugation for 10 min at 4000 *g*, the chlorophyll content was determined spectrophotometrically from the ethanolic supernatant at 654 nm, as described by Wintermans and De Mots (1965).

GSH assay

Non-protein thiols were extracted by homogenising 0.3 g of leaves in 3 mL of 0.1 N HCl (pH 2) and 1 g PVP (Schupp and Rennenberg 1988). After centrifugation at 10 000 *g* for 10 min at 4°C, the supernatant was used for analysis. Total glutathione (GSH plus GSSG) was determined in leaf homogenate by spectrophotometry at 412 nm, after precipitation with 0.1 N HCl, using yeast-GR, DTNB and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine, and GSH content was calculated from the difference between total glutathione and GSSG (Anderson 1985).

Ascorbate determination

Plant material (0.3 g of fresh weight of leaves) was homogenised in 3 mL of 2% (w/v) metaphosphoric acid and 1 mM EDTA. After centrifugation at 10 000 *g* for 15 min the ascorbate content was determined in the supernatant as described by Dalton *et al.* (1993).

Enzyme preparations and assays

Extracts for determination of CAT, SOD and APOX activities were prepared from 0.3 g of leaves homogenised under ice-cold conditions in 3 mL of extraction buffer containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP and 0.5% (v/v) Triton X–100 at 4°C. The homogenate was centrifuged at 10 000 *g* for 20 min and the supernatant fraction was used for the assays. CAT activity was determined in the homogenate by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k\,[CAT]$) of the decrease in H_2O_2 absorption was determined and the catalase content in pmol mg^{-1} protein was calculated by the equation $k = 4.7 \times 10^7$ M^{-1} s^{-1} (Chance *et al.* 1979). Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana *et al.* (1986). The reaction mixture consisted of 50–150 µL of enzyme extract and 3.5 mL $O_2^{\cdot-}$-generating solution containing 14.3 mM methionine, 82.5 µM NBT and 2.2 µM riboflavin. Extracts were brought to a final volume of 0.3 mL with 50 mM K-phosphate (pH 7.8) and 0.1 mM Na_2EDTA. Test tubes were shaken and placed 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was measured by reading absorbance at 560 nm. Blanks and controls were run in the same manner but without illumination and enzyme, respectively.

One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions (Giannopolitis and Ries 1977). Because APOX is labile in the absence of ascorbate, 5 mM ascorbate was included for the extraction of this enzyme. APOX activity was measured immediately in fresh extracts and was assayed as described by Nakano and Asada (1981) using 1 mL of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε: 2.8 mM^{-1} cm^{-1}). Extracts for determination of GR and DHAR activity were prepared from 0.3 g of leaves homogenised under ice-cold conditions in 3 mL of extraction buffer containing 50 mM Tris-HCl buffer (pH 7.6) and 1 mM EDTA. GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture contained leaf extract, 1 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 50 mM Tris-HCl buffer (pH 7.5) and 3 mM $MgCl_2$ (Shaedle and Bassham 1977). ß-Mercaptoethanol (2 mM) was included for the extraction of DHAR. DHAR activity was measured by formation of ascorbate at 265 nm (ε: 14 mM^{-1} cm^{-1}) in a reaction mixture containing 2.5 mM GSH, 0.1 mM EDTA, 0.2 mM dehydroascorbate and 50 mM potassium phosphate buffer (pH 7.0), as described by Nakano and Asada (1981).

Protein determination

Protein concentration was evaluated by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analysed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Plant growth and chlorophyll content

After 14 d of treatment, no significant differences were observed between clones S and 150 in fresh and dry weight when plants were grown in 0 and 100 mM NaCl. However, 150 mM NaCl produced a significant decrease in both fresh and dry weight in clone S, with a 30% net decrease in the FW/DW ratio, while clone 150 remained unaffected (Table 1).

When plants were cultivated at 0 mM NaCl, no significant differences were observed in the chlorophyll content of either clone. However, the chlorophyll content was significantly lower in clone S plants than in tolerant plants when both clones were grown at 100 and 150 mM NaCl. Chlorophyll content decreased 23% and 41%, respectively, in clone S under salt stress (Fig. 1).

Glutathione and ascorbate content

Reduced glutathione and ascorbate concentrations were measured in clones S and 150. Both antioxidant molecules were significantly elevated in clone 150 at 0 mM NaCl when compared with clone S. While GSH showed an 82% increment under basal culture conditions, ascorbate increased by about 36% compared to clone S in the absence of NaCl (Figs 2*A*, *B*). When plants were cultivated under

Table 1. Effect of salt treatments on fresh and dry weight of *Solanum tuberosum* leaves

Samples were taken at 14 d. Data are mean values of two independent experiments ± S.E. Each value represents 10 ± 2 replicates. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

Treatment		Fresh weight (g)	Dry weight (g)	FW/DW
0 mM NaCl	Clone S	1.5854 ± 0.0034[a]	0.1630 ± 0.0014[a]	9.7 ± 0.1[a]
	Clone 150	1.7322 ± 0.0045[b]	0.1732 ± 0.0015[b]	10.0 ± 0.1[b]
100 mM NaCl	Clone S	1.5783 ± 0.0035[a]	0.1643 ± 0.0014[a]	9.6 ± 0.1[a]
	Clone 150	1.7281 ± 0.0038[b]	0.1728 ± 0.0013[b]	10.0 ± 0.1[b]
150 mM NaCl	Clone S	0.9841 ± 0.0024[c]	0.1416 ± 0.0014[c]	6.9 ± 0.2[c]
	Clone 150	1.7249 ± 0.0040[b]	0.1715 ± 0.0015[b]	10.1 ± 0.2[b]

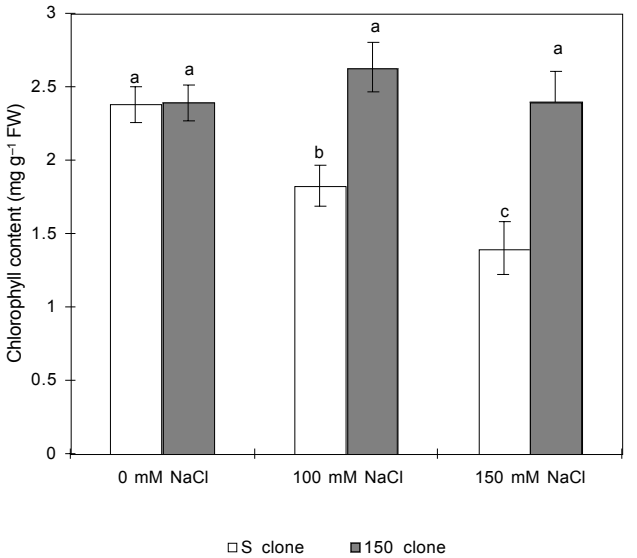

Fig. 1. Effect of salt treatments on chlorophyll content. Values are the mean of two different experiments with five replicated measurements, and bars indicate S.E. Different letters means significant differences ($P < 0.05$) according to Tukey's multiple range test.

100 mM NaCl, GSH decreased in clone S (35% with respect to the control) while ascorbate showed a 34% increase. However, under 150 mM NaCl, clone S showed a significant decrease in both soluble antioxidant compounds (57% for GSH and 29% for ASC) compared to the corresponding controls grown at 0 mM NaCl (Figs 2*A*, *B*). The tolerant clone did not show changes in GSH or ASC content when subjected to both salt concentrations, and the values observed were similar to those at 0 mM NaCl (Figs 2*A*, *B*).

Activated oxygen-scavenging enzymes

Antioxidant enzymes activities for clones S and 150 are shown in Table 2. Except for CAT, all enzymes activities increased significantly in clone 150 with respect to clone S under basal culture conditions (0 mM NaCl). The increments were 48% for SOD, 51% for APOX, 46% for GR and 27% for DHAR, and these values remained unchanged when the tolerant clone was subjected to salt stress. When plants were grown under 100 mM NaCl, clone S showed increased activities of SOD (63%), APOX (84%) and GR (61%), with respect to the control, reaching similar values to clone 150. DHAR and CAT activities remained unchanged in clone S compared to the control. Therefore, except for DHAR, no significant differences were detected between the clones at 100 mM NaCl (Table 2).

Nevertheless, when clone S was subjected to the 150 mM NaCl treatment, a significant decrease in APOX and GR activities was observed (33% and 37%, compared to the control, respectively), an impressive increment in SOD (100%) was found, and DHAR and CAT remained unaffected (Table 2).

Discussion

When growth was analysed, significant differences were observed between the two clones subjected to 150 mM NaCl, thus indicating that the higher NaCl concentration affected clone S and did not affect clone 150 (Table 1). This result was the first evidence of salt tolerance shown by clone 150. The increase in cellular Na^+ content has been reported by Pandey and Ganapathy (1984) in selected *Cicer arietinum* cells at high levels of salt and is often observed in other NaCl-tolerant cell lines and in some salt-resistant plants in comparison to sensitive ones (Piqueras 1992; Olmos *et al.* 1994).

The reduction in chlorophyll concentration is a general phenomenon in salt-sensitive plants growing under salt stress (Seeman and Critchley 1985). This took place in clone S at 100 and 150 mM NaCl, while clone 150 did not decrease its pigment content (Fig. 1). This could be further evidence of salt tolerance exhibited by clone 150.

An elevated constitutive glutathione content was observed in clone 150 at 0 mM NaCl, and its value was unaltered under 100 and 150 mM NaCl (Fig. 2*A*). This is in contrast to the results of Gosset *et al.* (1996), who reported that total glutathione was significantly lower in a NaCl-tolerant cell line grown at 150 mM NaCl than in the control cell line grown at 0 mM NaCl.

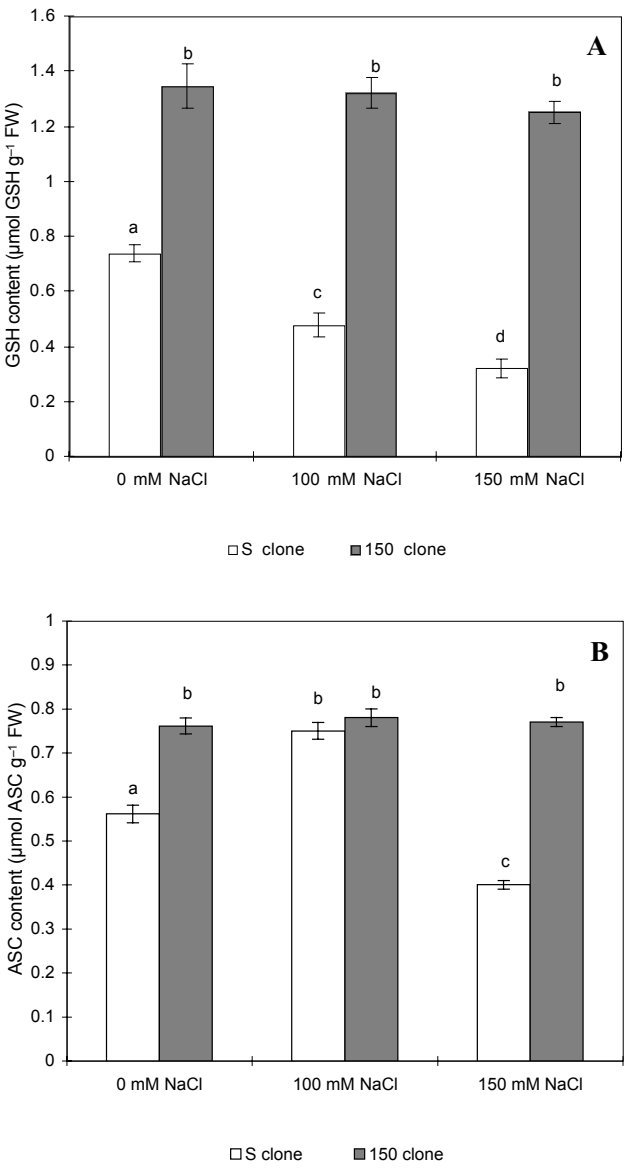

Fig. 2. Effect of salt treatments on GSH (*A*) and ascorbate (*B*) content. Values are the means of two different experiments with five replicated measurements, and bars indicate S.E. Different letters means significant differences ($P < 0.05$) according to Tukey's multiple range test.

All enzymatic activities measured at 0 mM NaCl were higher in clone 150 than in clone S (Table 2). This constitutive level of the antioxidant enzymatic system has been related to a higher resistance to oxidative stress (Wise and Naylor 1987; Monk and Davies 1989). The adaptive advantage of the tolerant clone could be its ability to respond rapidly to salt stress.

The elevated constitutive level of GSH, ascorbate and the antioxidant enzymes found in clone 150 might explain the tolerance to salt stress. This antioxidant defence system protects plant tissues against oxidative damage, the scavenging superoxide (SOD) anion and H_2O_2 (APOX), thus avoiding the formation of the most harmful radical species, $HO^{\bullet}$, and preventing tissue injury and chlorophyll breakdown (Table 1; Fig. 1).

Superoxide dismutase catalyses the conversion of the superoxide anion to H_2O_2. The higher basal SOD levels observed in clone 150 could be considered an advantage that allows the plant to resist the potential oxidative damage without the requirement to increase SOD activity like clone S. Considering the absolute enzymatic values at 100 mM NaCl, this result means that although clone 150 had a higher SOD basal level, both clones had similar dismutating capacity under moderate salt stress (Table 2).

The results observed with respect to the enzymes responsible for H_2O_2 detoxification (CAT and APOX), lead us to conclude that APOX was probably more important than CAT in this detoxification step in *Solanum tuberosum*. A parallel increment in SOD and the enzymes implicated in H_2O_2 detoxification is essential to avoid a metal-catalysed Haber–Weiss reaction. Both APOX and SOD activities increased in clone S under moderate salt stress (100 mM NaCl), while SOD increased only in clone S under higher salt treatment. However, similar absolute enzymatic activities were observed at 100 mM NaCl, indicating that both clones had similar H_2O_2-detoxifying capacities under this treatment (Table 2). These results are in agreement with those found by Gueta-Dahan *et al.* (1997) in citrus, and by Hernández *et al.* (1995) in chloroplasts of pea plants. In addition, increases in APOX activity were reported in salt-stressed radish plants (Lopez *et al.* 1996).

GR and DHAR participate together with ascorbate peroxidase in the ascorbate-glutathione cycle. Both catalyse the reactions that maintain the large pool of glutathione and ascorbate. These are essential for the appropriate functioning of the antioxidant system in chloroplasts. GR and DHAR were constitutively higher in clone 150 in the absence of the stress factor, but only GR increased in clone S as a result of 100 mM salt treatment, in contrast to results seen in *Citrus sinensis* by Gueta-Dahan *et al.* (1997). The increased antioxidant enzyme activities observed in clone S under lower salt concentration correlated with the absence in growth alterations. However, chlorophyll and GSH levels were found to decrease. Despite this, these increments may be attributed to an early response to the osmotic stress generated (Munns 1993). Lower concentrations of sodium chloride induce antioxidant-soluble and enzymatic (ASC, SOD, APOX and GR) responses in clone S leaves, and this induction was correlated with the lack of salt damage in this clone.

When both clones were subjected to higher salt concentration, their behaviour was completely different. While all the antioxidant parameters studied were not affected in clone 150, clone S showed symptoms of salt stress injury. In this way, growth, chlorophyll content, GSH and ASC levels, and SOD, APOX and GR activities were reduced. DHAR and

Table 2. Effect of salt treatment on antioxidant enzyme activities

Enzymatic activities were assayed as described in 'Materials and methods'. Data are mean values of two independent experiments ± S.E. Each value represents five replicates. Different letters within lines indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. SOD, APOX, GR and DHAR were measured as U mg^{-1} protein; CAT was determined as pmol mg^{-1} protein. One unit of APOX forms 1 μmol of ascorbate oxidized min^{-1} under the assay conditions. One unit of GR oxidizes 1 μmol of NADPH min^{-1} under the assay conditions. One unit of DHAR forms 1 μmol of ascorbate min^{-1} under the assay conditions

	0 mM NaCl		100 mM NaCl		150 mM NaCl	
Enzyme	Clone S	Clone 150	Clone S	Clone 150	Clone S	Clone 150
SOD	2.22 ± 0.21^a	3.29 ± 0.22^b	3.61 ± 0.31^b	3.18 ± 0.25^b	4.51 ± 0.31^c	3.48 ± 0.25^b
APOX	0.43 ± 0.03^a	0.65 ± 0.05^b	0.79 ± 0.05^b	0.78 ± 0.08^b	0.29 ± 0.05^c	0.76 ± 0.08^b
GR	0.041 ± 0.003^a	0.060 ± 0.005^b	0.066 ± 0.005^b	0.071 ± 0.005^b	0.026 ± 0.005^c	0.070 ± 0.005^b
DHAR	1.38 ± 0.09^a	1.75 ± 0.11^b	1.40 ± 0.09^a	1.71 ± 0.15^b	1.40 ± 0.09^a	1.70 ± 0.15^b
CAT	0.36 ± 0.03^a	0.37 ± 0.02^a	0.44 ± 0.03^a	0.40 ± 0.03^a	0.44 ± 0.03^a	0.38 ± 0.03^a

CAT activities remained unaltered, and SOD was significantly increased over control values. However, this increment was not enough to prevent the deleterious effects of 150 mM NaCl on clone S.

These results clearly demonstrate the relationship between salt tolerance and the antioxidant defence system in *Solanum tuberosum* clones.

Our study indicates that acquisition of salt tolerance may be a consequence of improving resistance to oxidative stress, via increased soluble and enzymatic antioxidant systems.

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