Australian Journal of Crop Science AJCS
AJCS 6(4):632-640 (2012) ISSN:1835-2707

Differential response to elevated NaCl by antioxidant enzymes and gene transcripts in two contrasting lettuce genotypes

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Abstract

Enzyme activities and transcript profiles of major antioxidant enzymes in two contrasting cultivars of lettuce seedlings (*Lactuca sativa* L.) were profiled after a 15 day period growing in 100 mM and 200 mM NaCl. Total superoxide dismutase activity (SOD) was enhanced in the more saline-tolerant'Verte de Cobham' cultivar in response to NaCl, and this was accompanied by increased expression of *Fe-SOD* and *Mn-SOD* genes at the two salt concentrations. Transcripts for these two genes were limited in the more salt sensitive 'Romaine' cultivar, while *CuZn-SOD* transcription remained consistent across all salinity levels in both cultivars. Ascorbate peroxidase (APX) enzyme activity was more than 1000-fold lower than total SOD activity in both cultivars in roots and leaves, even though APX enzyme activity increased with salinity in 'Verte' leaves. Catalase (CAT) and gaiacol peroxidase (GPX) enzyme activity were ~100-fold lower than total SOD enzyme activity in both leaves and roots, but did not appear to be limiting in salt-sensitive 'Romaine' at the lower NaCl concentration. The pattern of root activity for these two latter enzymes differed between the two cultivars, and CAT transcripts disappeared in 'Romaine' roots at the higher salt concentration. Our findings suggest that lettuce could benefit from enhancement of antioxidant enzymes through molecular or traditional plant breeding, although clearly such an effort would be of greater benefit to 'Romaine'.

Keywords:, Catalase, CAT gene, *Lactuca sativa* L, peroxidase, superoxide dismutase, SOD isozymes, NaCl salinity
Abbreviations: APX: ascorbate peroxidase, CAT, catalase; DW, dry weight; DTT, 1,4-dithiothreitol; EDTA, ethylenediamine tetra acetate; FW, fresh weight; GPX, guaiacol peroxidase; PPHO, phospholipid hydroperoxide glutathione peroxidase; PVP, polyvinyl pyrrolidone; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; SOD, superoxide dismutase.

Introduction

Soil salinization is one of the most widely occurring environmental constraints that limit crop productivity (Hoshida et al. 2000). Under saline conditions, physiological, biochemical, and molecular processes are disrupted in sensitive plants (Hernandez et al. 2000; Nemoto and Sasakuma 2002; Zhu 2002; Mahmoudi et al. 2010), so that growth is reduced within hours of excess salt exposure, apparently because of the elevated osmolarity of the external solution (Munns 2002). Within the cell, excessively high Na^+ ion content is also responsible for deficiency of many nutrient ions (Silberbush and Ben-Asher 2001), and other environmental factors (such as drought) can exacerbate the severity of Na^+ toxicity. Deficiency in nutrient ions such as K^+ and Ca^{2+} can occur because elevated Na^+ accumulation and associated osmotic effects inhibit their uptake and root growth by interfering with transporters in root plasma membrane (Tester and Davenport 2003). Besides these well-known osmotic and ionic effects, salinity has been linked to induced oxidative responses in several plant species, including Arabidopsis (Katiyar-Agarwal et al., 2006) and lettuce (Mahmoudi et al., 2010). Reactive oxygen species (ROS) accumulate in different subcellular compartments, including chloroplasts, mitochondria, peroxisomes and apoplast, as a result of normal metabolic activities and can accumulate in response to a multitude of stress factors (Mittler, 2002). ROS are responsible for damage to membrane structure and other essential macro-molecules, such as photosynthetic pigments, proteins, nucleic acids and lipids (Halliwell and Gutteridge, 1989). Salt tolerant plants protect themselves against the impact of elevated salinity by Na^+ exclusion, Na^+ compartmentalization, and accumulation of a battery of antioxidant and osmoprotectant metabolites, such as carotenoids, phenolics, ascorbic acid, amino acids, glycine betaines, and sugars (Kim et al., 2007; Charkuzi et al., 2010; reviewed in Vijayan, 2009). They also rely on a cascade of antioxidant enzymes, including superoxide dismutases,

catalases and peroxidases. Superoxide dismutase (SOD; EC 1.15.1.1) catalyzes the conversion of superoxide anion (O_2^-) into H_2O_2 to ensure the removal of this type of ROS. Fe-SOD isozymes are localized in chloroplasts, Mn-SOD isozymes in mitochondria and peroxisomes, and Cu/Zn-SOD isozymes in chloroplasts, peroxisomes, the cytosol, and possibly the apoplast (extracellular space) (Alscher et al., 2002). These enzymes likely have evolved separately in eukaryotes (Smith and Doolittle 1992), and in some cases, are used to control oxidative processes like lignification (Ogawa et al., 1997). H_2O_2 is further converted to O_2 and H_2O by catalase (EC 1.11.1.6, CAT), which are present in the peroxisomes of nearly all aerobic cells, in glyoxysomes, and to a lesser extent in mitochondria (Longo et al., 1972; Shigeoka et al. 2002; del Rio et al., 2002), but are virtually absent in chloroplasts (Dionisio-Sese and Tobita 1998). In chloroplasts, H_2O_2 is degraded by ascorbate peroxidase (APX; EC 1.11.1.11) (Ashada, 1999), which is also present in peroxisomes and glyoxysomes (Nakano and Asada, 1987; Nito et al., 2001). Guaiacol peroxidase (EC 1.11.1.7, GPX) is also located in mitochondria and peroxisomes (Prasad et al., 1995; Mika and Lüthje, 2003) as part of a system that protects subcellular compartments and membranes from oxidative damage (Takahashi and Asada, 1983). Phospholipid hydroperoxide glutathione peroxidase (PPHO or PhGPx, EC 1.11.1.12) is a monomeric selenoenzyme localized in mitochondria and can directly reduce and remove phospholipid hydroperoxide from biological membranes (Chen et al. 2004; Eshdat et al. 1997; Yang et al., 2006). Lettuce seedlings tolerate very high concentrations of NaCl in irrigation water for short periods of time without developing detrimental symptoms (Kim et al. 2008). Our previous work has identified two lettuce varieties with contrasting physiological and biochemical responses to a 12 day period and a longer 15 day period of growth in 100 mM NaCl (Mahmoudi et al. 2010; 2011). In these studies, 'Verte de Cobham' is more tolerant than 'Romaine' to salinity if exposed at these two seedling growth stages However, the enzyme activity and transcriptional responses of antioxidant enzymes exposed to higher concentrations of NaCl are not established in lettuce at the later stage of seedling establishment. Here, we extend these earlier studies to report on the effect of sustained applications of moderate (100 mM) and high (200 mM) concentrations of NaCl over a 15 day period on the activities of four major antioxidant enzymes in the contrasting lettuce varieties, 'Verte de Cobham' and 'Romaine'. As well, we report on the transcript levels for five genes encoding antioxidant proteins under the same high salinity conditions. The study was undertaken to determine more clearly the basis for the differential tolerance to salinity displayed by the two contrasting lettuce varieties and to determine whether specific antioxidant-related genes should be targeted by plant breeders for selection or genetic manipulation to improve the ability of lettuce to establish and grow under saline irrigation.

Results

Effect of NaCl on activities of antioxidant enzymes

The activities of four key anti-oxidant enzymes that deconstruct O_2^- and H_2O_2, i.e. total SOD, CAT, APX, and GPX, were assessed in leaves and roots of 'Verte' and 'Romaine' lettuce grown in the presence and absence of NaCl (Fig. 1). Total leaf SOD activity was similar between the two cultivars in the absence of salt and significantly increased in both varieties with NaCl, but the profile differed with 'Verte'. SOD activity in 'Verte' peaked in leaves at two-fold higher activity than in 'Romaine' leaves when plants were grown in 100 mM NaCl (Fig. 1A). CAT leaf activity was at least 100-fold lower than total SOD leaf activity in both cultivars, and 'Romaine' had seven-fold higher leaf CAT activity (0.48 U mg^{-1} protein) compared to 'Verte' (0.07 U mg^{-1} protein) (Fig. 1B). In the presence of 100 mM NaCl, leaf CAT activity in 'Verte' rose to 0.5-fold of 'Romaine' and then peaked at the same level as "Verte' at 200 mM NaCl, while leaf CAT activity in both cultivars still remained 200-fold lower than total leaf SOD activity at this higher salt level. During both salt treatments, leaf CAT activity remained constant in 'Romaine' relative to the untreated control (Fig. 1B). APX activity in leaves of both cultivars was ~3000-fold lower than total SOD activity and 10-fold lower than CAT2 and GPX activities (Fig. 1C). APX activity in 'Verte' leaves rose in the presence of 200 mM NaCl to 23-fold higher than in untreated control plants, while 'Romaine' lagged behind (Fig. 1C). At 100 mM NaCl, APX activity was 8000-fold lower than total SOD in 'Verte' and ~1000-fold lower than 'Romaine' as a result of a higher relative increase in total SOD activity with salt treatment. In contrast, leaf GPX showed an opposite pattern compared with APX in that 'Romaine' enzyme levels rose at 200 mM NaCl, while 'Verte' lagged behind at this NaCl level (Fig. 1D). Lettuce roots are the first organs to experience salt stress in a plant; hence their protection is as critical as leaf protection. In roots, the only significant increase in total SOD activity was detected in 'Verte' at 200 mM NaCl (350.15 U mg^{-1} protein). This was a 1.8-fold increase compared to the non-saline control plants (Fig. 2A). NaCl treatment had no statistically significant impact on total root SOD activity in 'Romaine' due to high variability. Root CAT and root GPX activities in both cultivars were ~10/50-fold lower and root APX was 2000-fold lower than total root SOD activity. Between cultivars, root CAT activity was ~five-fold higher in 'Verte' than in 'Romaine' in the absence of added salt and rose to eight-fold higher in 'Verte' at 100 mM NaCl. 'Verte' CAT activity in roots rose even higher with 200 mM NaCl, at which point 'Romaine' CAT activity also increased to a small extent so that 'Verte' roots under the highest salt concentration had only four-fold higher CAT activity than 'Romaine' (Fig. 2B). The presence of salt did not seem to affect APX activity much in roots of either cultivar, although APX levels overall were 10-fold higher in roots than in leaves (Fig. 1C, 2C). Non-salinized 'Romaine' roots showed only 50% of the APX activity found in non-salinized 'Verte' roots, but salt treatment caused "Romaine' root APX activity to rise to the same level as in 'Verte' at both NaCl concentrations (Fig. 2C). A similar pattern between the two cultivars was observed for GPX root activity, except that overall activity level was much higher for GPX compared to APX.

Effect of NaCl on transcription of antioxidant-encoding enzymes using RT-PCR

Gene transcripts encoding most of the major antioxidant enzymes that eliminate O_2^- were determined relative to *ACTIN* using RT-PCR, specific primers, and cDNA from leaves and roots of both lettuce varieties grown for 15 days under the two salt conditions (Table 1). Lettuce *ACTIN* transcripts remained constant in both tissues in response to NaCl, hence was suitable as a gel-loading control for antioxidant-related transcript analysis (Fig. 3A,B). *Cu/Zn-SOD* transcripts were strongly represented in roots and leaves of both varieties, but expression was not significantly affected by salt treatment in either tissue (Fig. 3). In leaves, *Fe-SOD* transcripts were absent in 'Verte and strongly represented in 'Romaine' in the absence of NaCl (Fig. 3A). 'Verte' leaves strongly stimulated the expression of *Fe-SOD* at 100 mM and 200 mM, but leaf transcripts for this gene disappeared in 'Romaine' with increasing salinity. In contrast, *Fe-SOD*

Table 1. Primers used in RT-PCR of lettuce antioxidant-encoding genes.

Primer Name	Accession #	Source	Primer Sequence (5' to 3')	References and Use
Fe-SOD-F	AJ370450	*L. sativa* db[1]	GAAGCACCACCAACTTATGTCG	Ruiz-Lozano et al., 2001
Fe-SOD-R	AJ370450	*L. sativa* db[1]	CTTCCTCTGATGGACGTGG	Ruiz-Lozano et al., 2001
Mn-SODII-F	AJ310448 (designed for 1 isoform)	*L. sativa* db[1]	ACACGAAGCACCATCAACTTAC	Ruiz-Lozano et al., 2001
Mn-SODII-R	AJ310448 (designed for1 isoform)	*L. sativa* db[1]	GAGGTAGTAGGCATGCTC	Ruiz-Lozano et al., 2001
Cu/Zn-SOD-F	CY984840	*L. sativa* db[1]	ATGGTGAAGGGAGTTGCAG	Richards et al., 1998
Cu/Zn-SOD-R	CY984840	*L. sativa* db[1]	GACAACTACAGCCCTTCCAA	Richards et al., 1998
CAT1-F	DW136607	*L. sativa* db[1]	GAGGCCCAATCCTTCTTGAG	Yu et al., 2007
CAT1-F	DW136607	*L. sativa* db[1]	GTGTTGACTCCTGAGCCTTCCA	Yu et al., 2007
PPHO-F	TC9259[2]	Lettuce citation	GCCCCTAAAACCCCTCCTCT	Klerks et al., 2007
PPHO-R	TC9259[2]	Lettuce citation	AACCCCTCCTTCTAGCGATTCA	Klerks et al., 2007
ACTIN-F	NA	Lettuce citation	TTTGCTGGGGATGATGCGCC	Keita et al., 2007
ACTIN-R	NA	Lettuce citation	GTGGTACGACCACTGGCATA	Keita et al., 2007

[1]Lettuce db, database of lettuce sequences at (*http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=lettuce*)
[2]PPHO amplicon using primers based on TC9259 was further verified as a PPHO transcript by its homology to PPHO entry DY980238 in the database after TC9259 was withdrawn. NA, not applicable.

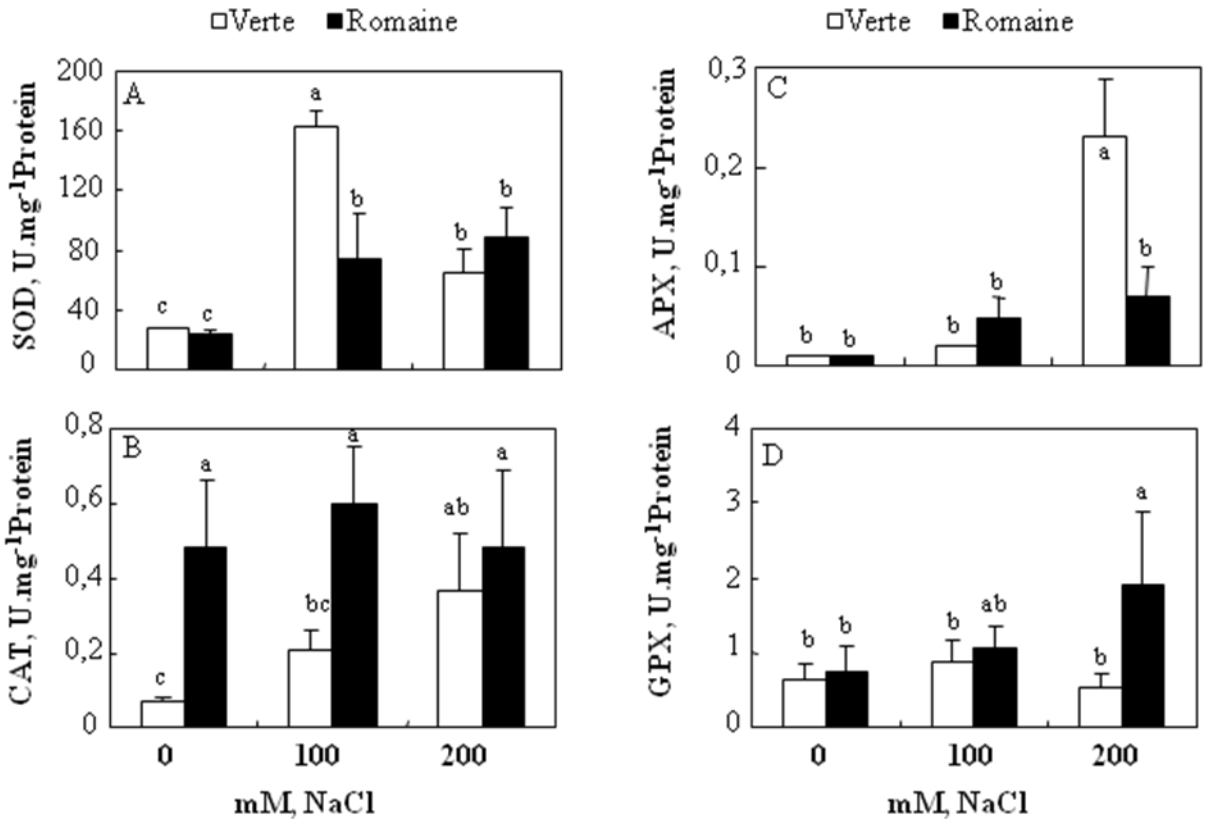

Fig 1. Leaf anti-oxidative enzyme activity (U mg^{-1} protein) of 'Verte' and 'Romaine' seedlings (14 d) grown in Hoagland nutrient solution supplemented with 0 (control), 100 or 200 mM NaCl for 15 d. Mean of replicates (± standard error) followed by different letters are significantly different at $p \leq 0.05$ level as determined by analysis of variance (ANOVA) and Fisher's LSD test, where n=3 for SOD and n= 4 for CAT, APX and GPX.

expression in 'Verte' roots was strong without salt and was reduced to equivalent low, detectable levels by 100 mM and 200 mM NaCl treatment (Fig. 3B). Expression of this gene in 'Romaine' roots without salt was detectable ,but weak, then transcripts accumulated strongly at 100 mM NaCl and then disappeared completely at the highest salt level. Both cultivars showed substantial leaf *Mn-SOD* transcript levels in the presence and absence of salt, but transcripts increased somewhat in Verte at 100 mM NaCl. In contrast, NaCl treatment had no impact on root *Mn-SOD* expression in 'Verte' and strongly repressed *Mn-SOD* transcripts in 'Romaine' roots. Transcripts coding for hydrogen peroxide-eliminating enzymes were also evaluated in the two lettuce cultivars. Unfortunately, transcription profiles for *APX* and *GPX* could not be developed due to the lack of specific lettuce sequences at the time of this investigation and difficulties developing primers from Arabidopsis. However, *CAT1* primers were easily developed and used to test the ability of each cultivar to maintain or induce transcripts for H_2O_2-catalyzing activity. Leaf *CAT1* expression in both cultivars remained constant in the presence and absence NaCl, but activity was always slightly higher in 'Romaine' leaves compared with 'Verte' leaves under these conditions (Fig. 3A). A similar pattern for CAT1 transcription occurred for roots of both cultivars under non-saline and 100 mM NaCl conditions. However at 200 mM NaCl, *CAT1* transcripts were strongly repressed (disappeared completely) in 'Romaine' roots while remaining stable and high in 'Verte' roots. Since primers could not be developed to test transcription of lettuce APX and GPX peroxidase genes, we turned our attention to a peroxidase gene which appears critical for membrane integrity and for which lettuce sequence data was available. Transcript amplicons were developed using primers to a lettuce *PPHO*/*PHGPX (GPX4)* gene accession TC9259 (encoding phospholipid hydroperoxide glutathione peroxidase) from Klerks et al. (2007). These amplicons were re-confirmed as PPHO by their 100% homology over 242 nt to accession DY980238 (nt 584-825) in the lettuce database (Table 1). [Subsequent to RT-PCR data acquisition, the sequence for TC9259 was removed from the lettuce public EST database.] PPHO transcripts were barely detectable in 'Verte' leaves and only slightly more detectable in 'Romaine' leaves in the absence of salt. Application of salt enhanced transcription for leaf PPHO to much higher levels in 'Verte' at 100 mM NaCl compared with 'Romaine', but levels were equivalent for both cultivars at the higher salt concentration (Fig. 3A). Under non-saline conditions, root *PPHO* transcripts were much more abundant in both cultivars than in leaves, but on a cultivar basis were relatively more abundant in 'Romaine' roots than in 'Verte' roots (Fig. 3B). Curiously, root *PPHO* transcripts in 'Verte' were up-regulated by 100 mM NaCl to the level in 'Romaine' roots, while PPHO transcripts declined slightly with the higher salt concentration in roots of both cultivars.

Accumulation of Na^+ ions in leaves of 'Verte' and 'Romaine'

Levels of Na^+ ion were determined earlier for a slightly larger sampling of 'Verte' and 'Romaine' seedling leaves and roots (Mahmoudi et al., 2011) grown also for 15 days under 100 mM and 200 mM NaCl. These data are presented in Table 2 (with permission) to compare with samples tested above for antioxidant enzyme activity (Figs 1, 2). In this previous study, leaf Na^+ levels rose equivalently in both 'Verte' and 'Romaine' leaves to ~40-fold higher at 100 mM and ~100-fold higher at 200 mM NaCl, respectively (Table 2). In contrast, the pattern of root Na^+ ion accumulation differed between the two cultivars. Na^+ accumulated two-fold higher in 'Verte' roots at 100 mM NaCl than in 'Romaine' roots and then levelled off in both cultivars at a higher level after growth in 200 mM NaCl. These leaf Na^+ accumulation patterns correlated with leaf APX activity for both cultivars and for SOD and GPX leaf activity (but not for CAT) in 'Romaine'. Leaf Na^+ accumulation correlated with leaf CAT activity in 'Verte' but not with total SOD or GPX activity. In roots, Na^+ accumulation patterns did not correlate well with antioxidant enzyme activity patterns as a function of NaCl treatment except for CAT.

Discussion

Plants adjust to environmental stresses, such as salinity, through activating and regulating their defence mechanisms (Stevens et al. 2006). In a previous study, we reported that 'Verte' seedlings were more tolerant to moderate NaCl levels (100 mM) compared to the more sensitive 'Romaine' cultivar over a 12 day stress period based on tissue water content, net photosynthesis, better growth, lower leaf Na^+ content, higher total leaf carotenoids, higher total flavonoids and phenolic compounds, and elevated activities of three SOD isozymes, CAT1 and polyphenol oxidase (POD) (Mahmoudi et al. 2010). Evaluation of transcript levels for antioxidant genes after this 12 day period of moderate NaCl stress showed that transcript levels for three carotenoid biosynthetic genes, three SOD isozymes, and CAT1 were enhanced in shoots and roots of 'Verte' compared with 'Romaine' (Mahmoudi et al., 2011). Some differences existed with this shorter (12 day) stress period compared to the longer (15 day) period of 100 mM NaCl stress we applied in our current study. Leaf MnSOD was only induced by 100 mM NaCl after the longer stress period (in our current report). CAT transcript levels were weak and FeSOD was strongly induced at 12 days (Mahmoudi et al., 2011) and then declined by 15 days (in our current study); otherwise transcript patterns for SOD isozymes and CAT were similar between the shorter treatments (Mahmoudi et al., 2011) and longer stress treatments (this report). In addition, we established that 'Verte' seedlings grown in 100 mM and 200 mM NaCl over the longer 15 day period accumulated lower H_2O_2 levels, lower MDA, higher melanoidin, and lower electrolyte levels (indicating less lipid peroxidation) than 'Romaine' (Mahmoudi et al., 2011). 'Verte' leaf growth, photosynthetic rate, electrolyte leakage, and stomatal conductance were less compromised and this cultivar accumulated higher levels of proline, higher total (and major) carotenoids, and higher chlorogenic acid levels (Mahmoudi et al., 2011; Mahmoudi, unpublished).

Antioxidant enzymes: new breeding targets for lettuce

In our current study, we document that total SOD enzyme activity is elevated in 'Verte' leaves at moderate NaCl levels and then declines to the level of the more salt-sensitive 'Romaine' at the higher NaCl concentration, whereas total root SOD (which would be devoid of chloroplast-specific FeSOD) was raised in 'Verte' at the higher NaCl level. APX, the main chloroplastic peroxidase activity, was far stronger at the highest NaCl concentration in 'Verte' leaves compared with 'Romaine' leaves, but was still 1000-fold lower overall compared with leaf SOD activity. Leaves contain chloroplasts and the increase in leaf APX activity suggests that the ascorbate-dependent chloroplast system of H_2O_2 removal functions better in 'Verte' than in 'Romaine' and is contributing to the greater tolerance one observes in 'Verte' under the stronger, more prolonged NaCl treatments. At the same time, such a low H_2O_2-

Table 2. Effect of NaCl salinity on **Na^+** content of Verte and Romaine seedlings grown in the presence of 0, 100 and 200 mM NaCl for 15 d.

Organ	Cultivars	Na^+ concentration (mmol g^{-1} DW)		
		0 mM NaCl	100 mM NaCl	200 mM NaCl
Leaf Na^+	Verte	0.07 c ± 0.02	1.73 b ± 0.26	4.41 a ± 0.47
$LSD_{0.05}$= 0.67	Romaine	0.04 c ± 0.01	1.71 b ± 0.27	4.26 a ± 0.25
Root Na^+	Verte	0.14 d ± 0.01	2.27 b ± 0.28	3.75 a ± 0.06
$LSD_{0.05}$= 0.44	Romaine	0.20 d ± 0.01	1.20 c ± 0.11	3.11 a ± 0.24

Means (± SE, n=6) followed by different letters are significantly different ($P \leq 0.05$) as determined by analysis of variance (ANOVA). Data is reproduced with kind permission from Springer Science + Business Media: (Table 2 in Mahmoudi H, Kaddour R, Huang J, Nasri N, Olfa B, M'Rah S, Hannoufa A, Lachaaˆl M, Ouerghi Z (2011) Varied tolerance to NaCl salinity is related to biochemical changes in two contrasting lettuce genotypes. Acta Physiologica Plantarum. Vol. 33 pg. 1618**).**

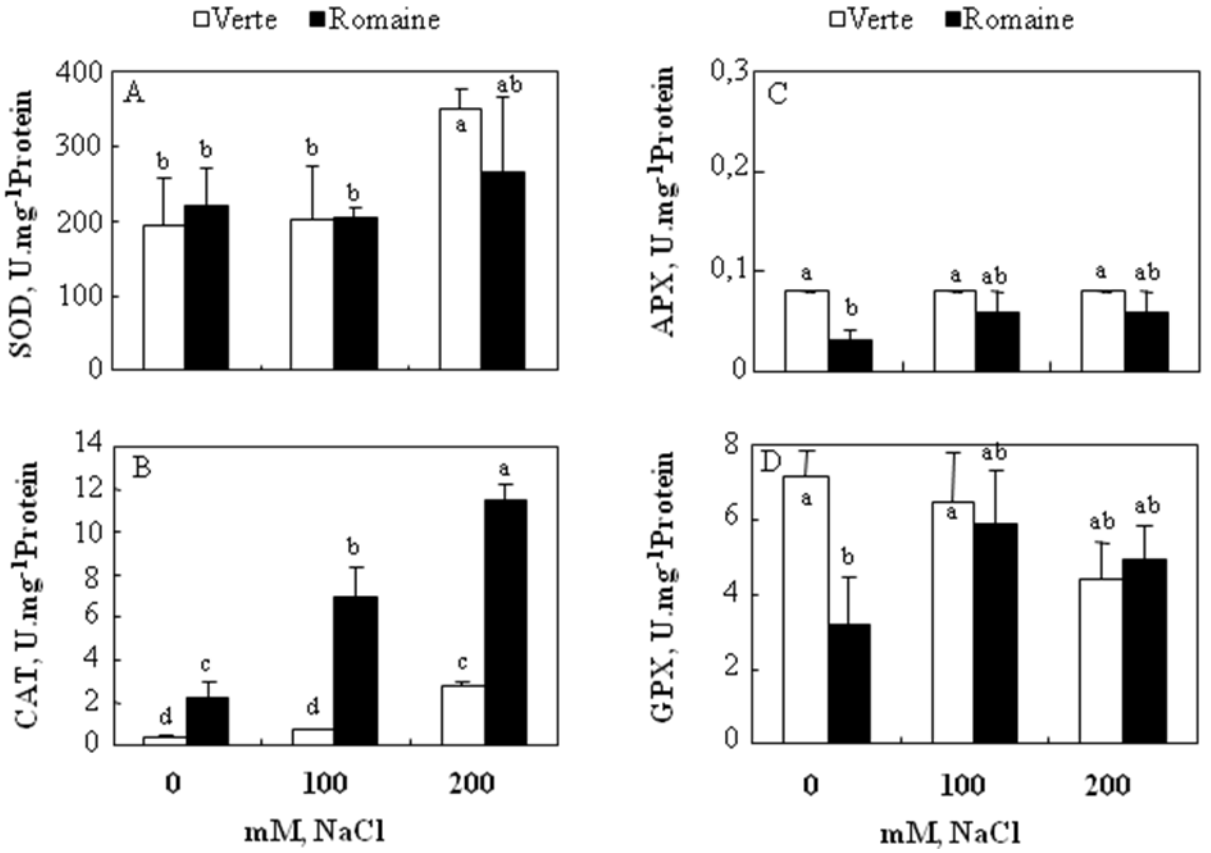

Fig 2. Root anti-oxidative enzyme activity (U mg^{-1} protein) of 'Verte' and 'Romaine' seedlings (14 d) grown in Hoagland nutrient solution supplemented with 0 (control), 100 or 200 mM NaCl for 15 d. Means of replicates (± standard error) followed by different letters are significantly different at $p \leq 0.05$ level using analysis of variance (ANOVA) and a Fishers LSD test, where n=3 for SOD and n= 4 for CAT, APX and GPX.

scavenging APX activity level in chloroplasts coupled with a high SOD activity at 100 mM NaCl, together with a decreased leaf SOD activity in 'Verte' at the highest level of NaCl and the similarity between the cultivars for root SOD activity, suggests two things: 1st that SOD is regulated differently between leaves and roots, and 2nd that one or both of the SOD isozymes in 'Verte' chloroplasts may be more affected negatively than in 'Romaine' by excess H_2O_2 presumably available as a result of higher SOD and lower APX activity levels. Both CuZnSOD and FeSOD proteins are known to be susceptible to damage by reactive oxygen (hydroxyl radical and H_2O_2) even while they actively degrade superoxide anions (Zhong and Li, 1996; Casano et al., 1997; dos Santos et al., 2000), while MnSOD proteins are not susceptible in this way. Although transcript levels for CuZnSOD remained high and were not affected by increased salinity in either lettuce cultivar in our study, the rising transcript levels for the FeSOD gene in 'Verte' should provide "backup" support for any H_2O_2-related damage to its cognate chloroplast enzyme activity levels as salinity increases. This transcription support does not appear to hold for 'Romaine' leaves in that FeSOD transcripts strongly declined in this cultivar as salinity increased. APX enzyme activity also remained weak in 'Romaine' as salinity increased. Hence, enhancing APX activity for both cultivars and for FeSOD in the case of Romaine are logical traditional or molecular breeding goals for improvement of lettuce, particularly to

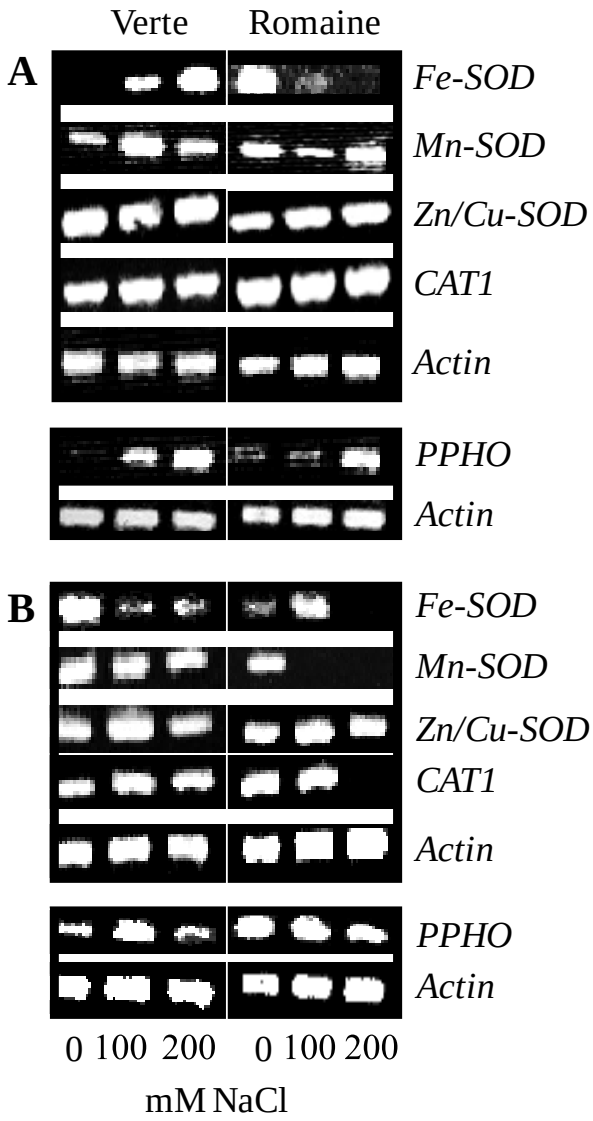

Fig 3. Differential transcript levels of five genes coding for antioxidant proteins in 'Verte' and 'Romaine' grown lettuce under medium (100 mM) and high (200 mM) NaCl concentrations for 15 d. Representative semi-quantitative RT-PCR reactions are shown for Fe-, Mn-, and Zn/Cu-superoxide (SOD) isozymes, catalase (CAT1), and phospholipid hydroperoxide glutathione peroxidase (PPHO) in response to increasing salinity levels in the two lettuce cultivars. (A) Gene expression in leaves. (B) Gene expression in roots. The lettuce *ACTIN* control reaction below each set of corresponding antioxidant gene reactions was conducted on equivalent cDNA batches to verify equivalent loading of reaction volumes on the gel. Antioxidant genes were amplified for 30 cycles. Data are representative of at least three independent RNA extractions.

protect chloroplasts from the effects of salinity. To some extent, though, compensatory mechanisms apparently can occur with FeSOD at the post-translational level according to a study in alfalfa leaves (Rubio et al., 2001). The value of such a strategy has been shown for tobacco, alfalfa, and cotton. Over-production of Arabidopsis Fe-SOD in chloroplasts increased tolerance to oxidative stress in transgenic tobacco (Van Camp et al. 1996) and improved winter survival in alfalfa (McKersie et al. 2000). Cotton leaves with chloroplast over-production of APX show lower cellular H_2O_2 and less photo-inhibition when subjected to low temperature stress (Komyeyev et al., 2003). Root APX enzyme activity remained very low in both lettuce cultivars as a function of increased salinity. This data incdicates that the APX enzyme, which is present in peroxisomes and glyoxysomes, may be less important at removing H_2O_2 in root than GPX and CAT. Still, enhancement of APX has been shown to be effective at combating oxidative stress in non-chloroplast compartments. For example, ROS accumulation was enhanced in transgenic tobacco cells when cytosolic APX activity was suppressed (Ishikawa et al. 2005). CAT leaf and root transcript levels appear substantial for both cultivars at modest NaCl levels, but disappear in 'Romaine' roots at the higher salt concentration. Hence, improving CAT levels in 'Romaine' may prove beneficial to 'Romaine'. Some post-transcriptional regulatory differences must exist between the two cultivars for CAT1 because of the high uniform levels of CAT activity in 'Romaine' leaves and the induction in 'Verte' leaves as salinity increases. Increasing stable expression of MnSOD, which predominates in the mitochondria and peroxisomes, also could serve as a suitable target since the leaf transcript levels for this enzyme decline in 'Verte' at the higher salt levels and the gene appears to be under some type of regulatory repression in 'Romaine' with exposure to salt. Up-regulated Fe-, Mn- and Cu/Zn-SOD activities by NaCl were documented in a halophyte plant (Wang et al. 2004). PPHO (or PhGPx) can directly reduce and remove phospholipid hydroperoxides from biological membranes in mitochondria (Chen et al. 2004; Eshdat et al. 1997; Yang et al., 2006). PPHO transcripts increased in lettuce leaves as NaCl increased, but 'Romaine' appeared less efficient at PPHO leaf induction than 'Verte' and roots were less effective at inducing PPHO transcripts than leaves. These results point to the possibility that PPHO is a part of the stronger leaf defence system present in 'Verte' seedlings compared with 'Romaine' seedlings. Proline, but not sugars, also play a strong role in leaf tolerance to salinity in lettuce and have been identified as a breeding target during the screening of 16 cultivars (Bartha et al., 2010; Bartha, 2012). Further proof of the role of PPHO in lettuce salinity tolerance will come with the analysis of its enzyme activity, which has been demonstrated in plants by Wang et al. (2007). With the advent of Next Generation Sequencing (Metzker, 2010), a more complete evaluation of transcript profiles as a function of salinity and their co-expression with other genes should enable lettuce breeders to determine a full set of antioxidant enzyme breeding targets.

Variability and selection of antioxidant enzyme targets

Some of our lettuce antioxidant enzyme activities showed higher than usual standard error bars, eg. CAT1 in 'Romaine' leaves, GPX in 'Romaine' leaves at 200 mM NaCl, GPX in roots of both cultivars, total SOD in 'Romaine' leaves and roots at 100 mM NaCl, and APX in 'Verte' at 200 mM NaCl. This variability appeared more prevalent in 'Romaine' than in 'Verte', but did not prevent us from distinguishing differences between the two cultivars for these antioxidant enzyme assays. The variability could point to the need for more robust sampling due, potentially to a negative impact of salt on these enzymes. Stability and conformation should be tested with recombinant enzymes under high salt conditions using X-ray crystallography. Most important, the variability highlights an opportunity for plant breeders to select lettuce plants with enhanced antioxidant enzyme capacity directly from 'Verte' and 'Romaine'. Even though lettuce is a self-pollinating species and can easily be developed into homozygous pure breeding lines, some North American lettuce cultivars also appear to have residual

variability, such that new varieties have been selected directly from a single cultivar without crossing (Mikel, 2007). Screening large numbers of lettuce accessions in germplasm collections around the world, such as

the US National Plant Germplasm System (*http://www.ars-grin.gov/npgs/searchgrin.html*) and Plant Gene Resources Canada (*http://pgrc3.agr.gc.ca/acc/search-recherche_e.html*), would also contribute additional genetic resources to lettuce improvement.

Conclusion

In conclusion, our data indicates that salt-induced oxidative damage occurs in *Lactuca sativa* L. despite concomitant increases in the activities and transcription of antioxidant enzymes. Enhanced total SOD in 'Verte' in response to NaCl was accompanied by increased expression of *Fe-SOD* and *Mn-SOD* genes, transcription and enzyme activities were limited in the more salt sensitive 'Romaine' cultivar. APX activity was 1000-fold lower than SOD in both cultivars even though it increased in 'Verte' as a function of higher salt concentration, CAT appears less limiting in either cultivar at moderate salt concentrations, but is limited at higher concentrations. Our findings clearly suggest that lettuce could benefit from enhancement of these specific enzymes through plant selection or genetic engineering (particularly to protect chloroplasts), although this type of effort would be of greater benefit to 'Romaine'.

Materials and methods

Plant material and growing conditions

Seeds of two contrasting lettuce (*Lactuca sativa* L.) cultivars, 'Verte' de Cobham' (more seedling tolerance to NaCl) and 'Romaine' (less seedling tolerance to NaCl) were obtained from the Ministry of Agriculture of Tunisia (Mahmoudi et al. 2010; Mahmoudi et al. 2011). Seeds were germinated in petri plates and irrigated with distilled water for seven days in the dark, then transferred to an 8-fold diluted aerated Hoagland nutrient solution (Hoagland and Arnon, 1950) for one week in a greenhouse with a 16 h d^{-1} photoperiod and a 20/17^{o}C day/night temperature regime. At 14 d, acclimated seedlings (one uniform plant in one pot per rep), were transferred to diluted Hoagland nutrient solution supplemented with increasing NaCl concentrations (0, 100, and 200 mM) and grown for an additional 15 days under the same greenhouse conditions. Fresh weight was determined at harvest and tissues were submersed in liquid N_2 and then stored at -80°C and sampled once per replicate (number of replicates are indicated in the statistical analysis sub-section).

Antioxidant enzyme assays

Soluble proteins were extracted at 4°C by homogenizing independent replicate batches of flash frozen tissue (0.5 g) in 50 mM potassium phosphate buffer containing 100 mM EDTA, 5% PVP, 5% glycerol and 1 mM DTT, pH 7, then centrifuging at 15,000 *g* for 15 min, and the supernatant collected. Protein concentration to calculate enzyme specific activity was determined using a dye-binding protocol (Bradford 1976). Catalase (CAT, EC 1.11.1.6) activity was determined as the disappearance of H_2O_2 at 240 nm (25°C) for 1 min according to the method of Cakmak and Marschner (1992). APX activity (1 unit) was determined as the decrease min^{-1} in ascorbic acid at 290 nm (E = 2.8 $mM^{-1}cm^{-1}$) in a 1 cm^3 reaction mixture according to Cakmak and Marschner (1992). Guaiacol peroxidase (GPX, EC 1.11.1.7) activity (1 unit) was assayed as the increase in absorbance at 470 nm due to the oxidation of 1 µmole guaiacol to tetraguaiacol as described by Srinivas et al. (1999). Total SOD activity (1 unit) was measured as the 50% decrease in optical density of nitro-blue tetrazolium dye at 560 nm for 15min at 25°C (Sairam and Srivastava 2002) using several volumes (µL) of enzyme extract. Illumination with 70 µmol m^{-2} s^{-1} fluorescent light was used to initiate the SOD reaction, and identical reaction were kept in the dark to serve as controls.

Semi-quantitative RT-PCR of antioxidant gene transcripts

Total RNA was isolated from root and shoot tissues (0.1 g per sample) using a RNA extraction kit according to the manufacturer's instructions (Qiagen, Mississauga, Ontario, Canada). Semi-quantitative real-time RT–PCR was performed on using gene specific primers (Table 1) designed from sequences retrieved from citations or the lettuce EST database (*http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=lettuce*) (Ruiz-Lozano et al., 2001). Primers were synthesized through a contract with Invitrogen (Burlington, ON, Canada). First strand cDNA synthesis was performed in a 20 µl reaction containing 5 µg total RNA using the SuperScript RT-PCR kit (Invitrogen, Ontario, Canada). PCR reactions (25 µL) were initiated by adding 1 µL of the diluted reverse transcription reaction mix (50 ng μL^{-1} cDNA) using an initial denaturation at 94°C for 4 min, 25-35 cycles at 94°C (30 s), 55 °C (30 s), 72°C and a final extension at 72°C for 10 min. RT-PCR reactions were optimized for individual genes by amplifying at 25, 30, and 35 cycles to ensure the reaction was not saturated (in linear phase), such that differences in transcript abundance were readily discernible (30 cycles were optimum for all genes). Non-template control assays and gDNA template reactions were included to ensure that only cDNA rather than contaminating gDNA was amplified. Amplified products were verified by DNA sequencing, BLAST analysis to the non-redundant lettuce database, and confirmation of sequence identity to the accessions detailed in Table 1. At time of publishing, the PPHO accession TC9259 used to develop PPHO primers was no longer available for comparison in the lettuce EST db (accessions for entries prior to TC15939 had been removed). Hence, the lettuce PPHO amplicon was further verified as a PPHO transcript by its homology to another PPHO entry DY980238 in the database.

Measurement of Na^+ accumulation

Na^+ was extracted from oven-dried roots and leaves with 0.5% HNO_3 and levels were assayed using flame photometry as described in Mahmoudi et al. (2010).

Statistical analysis

Each treatment pot (one plant cultivar and one unique salinity level) was replicated four times for enzyme assays and gene expression assays and randomly arranged within a block. Pots were also moved into different positions each day during the experimental period. All four replicates were

sampled once per CAT, APX and GPX enzyme assay and RNA extraction, while only three replicates were sampled for SOD activity. Na^+ accumulation assays were conducted on six replicates in a randomized design. Results were expressed as the means of replicates (± standard error) across each parameter. Analysis of variance (ANOVA) was independently applied to the collected data using SAS ver.9.2 (SAS Institute, Cary, NC, USA). Significant differences of the means were declared at $P \leq 0.05$ using a Fishers' protected least significant difference test.

Acknowledgements

This work was supported through a Tunisian-Canadian Cooperation Initiative. Ministry of Agriculture of Tunisia provided the lettuce seeds. We thank Dr. Yong-Bi Fu (AAFC, Saskatoon Research Centre) for assisting with SAS9.2 program.

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