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ARTICLE

The Impact of Genotype and Salinity on Physiological Function, Secondary Metabolite Accumulation, and Antioxidative Responses in Lettuce

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Salinity inhibits plant growth due to osmotic and ionic effects. However, little is known about the impact of genotype and salinity on biochemical and molecular processes in the leafy vegetable lettuce. We report here evaluations of two lettuce types, Verte (NaCl tolerant) and Romaine (NaCl sensitive), under iso-osmotic 100 mM NaCl and 77 mM Na_2SO_4 treatments. As compared to Romaine, NaCl-treated Verte displayed better growth, contained lower levels of inorganic cations in leaves, and possessed superior antioxidative capacity due to enhanced carotenoid and phenolics biosynthesis and more active antioxidative enzymes resulting in reduced membrane damage. Both genotypes had relatively similar growth patterns under Na_2SO_4 treatment, but Romaine showed enhanced root lignification, greater malondialdehyde formation, and suppressed Fe-superoxide dismutase expression in roots as compared with Verte.

KEYWORDS: Lettuce (*Lactuca sativa* L.); salinity; carotenoid; lignin; phenolics; antioxidative enzymes; malondialdehyde

INTRODUCTION

Salinity inhibits plant growth due to ionic and osmotic effects on metabolic processes and nutritional balance, leading to impaired physiological functions such as unfavorable water relations and disrupted photosynthetic machinery (*1*). At the subcellular level, salinity was reported to alter gene expression patterns in *Arabidopsis* (*2*) and protein distribution profiles in wheat cultivars (*3*). Although extensive research has been carried out under NaCl salinity (*4*), much less attention has been directed toward other types of salinity prevailing in certain geographical regions. In Western Canada, for instance, saline and saline−sodic soils mainly contain mixed salts of Na_2SO_4 and $MgSO_4$ (*5*). Gypsum treatment of oil sands tailings are highly saline (*6*). Declining growth in $NaHCO_3$-treated Butterhead lettuce (*Lactuca sativa* L.) has been attributed to HCO_3^- toxicity and high pH rather than osmotic effects (*7*).

The generation of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$), is enhanced in salinity-treated plants (*8*, *9*). If plants are poorly protected, these reactive molecules may damage macromolecules such as DNA, proteins, and membrane lipids and ultimately lead to cell death (*1*). The ROS scavenging ability relies on the primary antioxidant defense system, which is comprised of nonenzymatic and enzymatic components (*9*). The enzymatic component acts on the removal of ROS produced in chloroplasts by photosystem I. Nonenzymatic components of the antioxidant defense system include various secondary metabolites, such as hydrophilic phenolics and flavonols, lipophilic α-tocopherols and carotenoids, and water-soluble ascorbate and glutathione (*9*). Phenolics are known to accumulate in sugar cane under salinity stress (*10*). Phenolic reprofiling observed in brown-seeded *B. carinata* seedlings exposed to LiCl positively correlates with high levels of specific phenolics and greater salinity tolerance (*11*, *12*). Phenolic compounds are among a range of metabolites that accumulate in lettuce (*13*). For example, wild lettuce (*L. indica*) contains simple phenols, phenylpropanoids, and flavonoid derivatives, and their antioxidant properties have been demonstrated in animal cell models and in vitro DNA strand cleavage assays (*14*).

Hydroxycinnamic acid esters are involved in the formation of the lignin polymers and function in defense responses (*15*). Carotenoid pigments are more ubiquitous than phenolics in plants, fungi, and cyanobacteria (*16*). Carotenoids participate in dissipating excess energy in the form of heat and quench both triplet chlorophyll and singlet oxygen (*17*). Carotenoids contribute to the nutritive and visual properties of Romaine lettuce (*L. sativa* L.) growing in saline irrigation water (*18*). Carotenoid accumulation in plant tissues is controlled not only by the rate of de novo biosynthesis, that is, gene expression and enzyme activity, but also by other factors, including rate of degradation, translocation, sequestration, and the availability of cellular compartments for carotenoid storage (*16*).

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Although lettuce is harvested when fully developed, the early stages of development are also critical to long-term plant performance and vigor. This is the time when the plant establishes, and poor performance at this stage can impact later stages of agronomic and quality trait development. Evaluation of lettuce genotypes for salinity tolerance at the seedling stage is also important to farmers who start seedlings in peat pots in greenhouses prior to transplantation in the field (*19*, *20*). In addition, salt tolerance in seedlings has been reported to be an accurate indicator of tolerance at the mature plant stage (*21*, *22*).

The objective of the present study was to compare photochemical, secondary metabolite, and enzymatic antioxidative systems in seedlings of two lettuce varieties with differential responses to iso-osmotic NaCl and Na_2SO_4 salts. The two lettuce varieties employed in the study, Verte and Romaine, were selected as representatives of contrasting stress-responsive lettuce types out of a small-scale trial of four varieties belonging to Butterhead, Verte, and Romaine (also called Cos) types of lettuce evaluated for tolerance to increasing concentrations of NaCl.

MATERIALS AND METHODS

Growth Conditions and Salinity Treatments. Four lettuce varieties including Butterhead (*L. sativa* var. Augusta and var. Vista), Romaine (*L. sativa* var. longifolia), and Verte (*L. sativa* var. Verte de Cobham) were initially prescreened for differences in growth under 0−200 mM NaCl for 15 days. Verte (NaCl tolerant) and Romaine (NaCl sensitive) had the most contrasting differences and were selected for further analysis in this study. Seeds were surface sterilized in 70% ethanol for 5 min, rinsed five times with sterile water, germinated in Petri dishes at room temperature in the dark, and displayed equivalent (100%) rates of germination. Seedlings (7 days old) were moistened with Hoagland's nutrient solution during the first week of growth. Uniform seedlings (14 days old) (15 seedlings per genotype and salt treatment) subsequently were cultured in randomly arranged individual darkened (foil-covered) containers in Hoagland's control solution (*23*), diluted 8-fold, and maintained between pH 5.5 and pH 6.5 for each treatment by routine replacement of the hydroponic solution. Plants were maintained in a greenhouse with a 16 h photoperiod and a day/night cycle of 20/17 °C. Salinization treatment was initiated on seedlings (14 days) by incremental daily additions of 20 mM Na_2SO_4 and 25 mM NaCl until reaching the desired final concentrations of 100 mM NaCl and 77 mM Na_2SO_4 to avoid osmotic shock; then, plants were grown in the final salinity solutions for 12 days. Solutions were replaced every third day until harvest on day 26. Fresh weight (FW) and dry weight (DW) of roots and rosette leaves were separately recorded for six plants randomly selected from each genotype and salinity combination and used to calculate percent tissue water [(FW − DW)/FW] × 100. Tissues were also collected from six plants per genotype and salinity treatment and frozen at −80 °C for phytochemical and enzymatic analysis. To examine gene expression, seedlings (14 days) were treated with either control or saline solutions for 12 days, and then, transcript levels were measured, with the exception of polyphenol oxidase (PPO), which was measured over a shorter term (6, 12, and 24 h) after the initiation of salinization.

Chlorophyll Content and Net Photosynthetic Rate. The chlorophyll concentration was determined on 80% acetone (v/v) leaf extracts at 645 and 663 nm as described by Arnon (*24*). Net photosynthetic rates (CO_2 gas exchange) were measured with a portable LiCor 6200 photosynthesis system (LI-COR, Inc., Lincoln, NE) at the end of the salinity treatment on fully expanded healthy leaves randomly selected from five plants for each genotype and treatment combination (mean canopy photon flux density of 450 μmol m^{-2} s^{-1}, ranging from 350 to 550).

Leaf Ion Content. Major cations in dried leaf materials were extracted with 0.5% HNO_3 and were assayed by flame photometry as previously described (*25*).

Carotenoid Analysis. Frozen lettuce leaves (0.1 g) were ground in liquid nitrogen and extracted in 3 mL of hexane−acetone−ethanol (50:25:25, by volume), and the extract was mixed vigorously and centrifuged at 1800*g* for 10 min following a previously published method (*26*). The supernatant was transferred to a clean 16 mm × 125 mm Kimax glass centrifuge tube and dried. The dried residue was then resuspended in 5 mL of methanolic KOH (10% w/v in 80% MeOH) and incubated at room temperature for 30 min to saponify and partition the triacylglycerides into the aqueous phase. Phase separation was facilitated by mixing in 2 mL of H_2O and 3 mL of petroleum ether twice, followed each time by centrifugation. Combined ether extracts were dried at room temperature under a N_2 stream under dim light, resuspended in 200 μL of CH_3CN−$MeCl_2$−MeOH (50:40:10, by volume) containing 0.5% butylated hydroxytoluene (w/v), and filtered through a 0.2 μm nylon syringe filter. Carotenoids were analyzed in 20 μL cleared extracts using an Agilent high-performance liquid chromatography (HPLC) system equipped with a photodiode array (PDA) detection module (Waters, Mississauga, Ontario, Canada), a 5 μm YMC reverse-phase C_{30} column (4.6 μm × 250 mm; Waters) set at 35 °C, and a mobile phase of 1.2 mL/min starting at 95% A (MeOH) and 5% B (*tert*-methyl butyl ether), followed by a linear gradient to 35% A and 65% B over 25 min. Eluting peaks were identified and quantified by peak height at 450 nm, retention time, and absorption spectra as compared to those of authentic standards, which were of analytical grade and obtained from CaroteNature (Lupsingen, Switzerland).

Determination of Phenolics. Extraction and analysis of phenolics were carried out using a method adapted from Annalisa et al. (*27*). Frozen leaf tissue (0.5 g) was ground in liquid nitrogen and extracted four times each with 10 mL of 80% CH_3OH (v/v). The raw methanolic extract was evaporated to dryness under vacuum (Rotavapor) at room temperature and then dissolved in 1 mL of 100% CH_3OH (v/v). Phenolics in cleared extracts (200 μL) were analyzed using an Agilent HPLC system equipped with a PDA detection module (Waters), a 5 μm YMC reverse-phase C_{30} column (4.6 μm × 250 mm; Waters) set at 35 °C, and a mobile phase of 0.4 mL/min starting at 95% A (acetonitrile) and 0.3% B (formic acid), followed by a linear gradient to 35% A and 65% B over 60 min. Eluting peaks were identified by their retention times and UV absorption spectra as compared to authentic standards and quantified by integrating relative peak areas at 332 nm for phenolic acids (caffeic acid, chicoric acid, and chlorogenic acid) and 370 nm for flavonoids (quercetin 3-*O*-glucoside and quercetin 3-*O*-6- malonylglucoside).

Lignin Content. Lignin was extracted from frozen ground roots (0.1 g) and quantified using the thioglycolic acid method as described elsewhere (*28*).

Measurement of Malondialdehyde (MDA). MDA was determined for leaves and roots following a published procedure (*29*). Briefly, fresh tissue (0.2 g) was homogenized in 2 mL of a mixture containing 20% 2-thiobarbituric acid and 0.5% trichloroacetic acid. Extracts were incubated at 95 °C for 30 min, the reaction was stopped on ice and then centrifuged at 4000*g* for 30 min at 4 °C, and the absorbance of the cleared supernatant was measured at 532 and 600 nm. The MDA concentration (μmol g^{-1} FW) was calculated using a molar extinction coefficient at 532 nm (155 mM cm^{-1}). The absorption at 600 nm resulting from nonspecific obscurities was subtracted from the optimal absorption at 532 nm.

Antioxidant Enzyme Activity. Soluble proteins were extracted from frozen leaves (−80 °C) in an extraction buffer [50 mM phosphate buffer, pH 7.5, 100 mM ethylenediaminetetraacetic acid (EDTA), 5% PVP, 5% glycerol, and 1 mM dithiothreitol], and the protein concentration was determined from four independent leaf extracts using the Bradford dye-binding protocol (*30*). The catalase (CAT, EC 1.11.1.6) activity was measured in 25 mM phosphate buffer (pH 7.0) and 30 mM H_2O_2 following the method of Cakmak and Marschner (*31*), expressed relative to the initial rate of H_2O_2 decomposition for 1 min at 240 nm, and calculated using an extinction coefficient of 39.4 mM cm^{-1}. The guaiacol peroxidase (POD, EC 1.11.1.7) activity was determined as previously described (*32*) based on the formation of tetraguaiaco quinone over 2 min in a 1 mL reaction in 20 mM phosphate buffer (pH 7.0) and 30 mM H_2O_2 (optimal absorbance at 470 nm; extinction coefficient of 26.6 mM cm^{-1}), where one unit of peroxidase activity catalyzes the oxidation of 1 μmol of guaiacol. The total superoxide dismustase (SOD, EC 1.15.1.1) activity was measured at 560 nm as the enzymatic inhibition of the photochemical reduction of nitroblue tetrazolium (*33*) in a reaction mixture containing 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 2 μM riboflavin, 75 μM *p*-nitroblue tetrazolium, and 0.1 mM EDTA for 15 min at 70 μmol m^{-2} s^{-1} light produced by fluorescent lamps (15 W). Complete SOD reactions incubated in light without enzyme (extract) or in the dark with enzyme served as nonenzymatic and light-independent controls. One unit of SOD

Table 1. Growth, Water, and Chlorophyll Contents and Net Photosynthetic Rates in Verte and Romaine Lettuces Grown in 100 mM NaCl or 77 mM Na_2SO_4 Salts for 12 Days[a]

		physiological traits under salinity		
physiological parameter	genotype	no treatment	100 mM NaCl	77 mM Na_2SO_4
leaf FW (g) per plant (n = 6)	Verte	3.19 a ± 0.18	1.71 b ± 0.09	0.56 c ± 0.06
	Romaine	3.01 a ± 0.10	1.54 b ± 0.08	0.60 c ± 0.05
root FW (g) per plant (n = 6)	Verte	0.91 a ± 0.06	0.61 bc ± 0.07	0.12 d ± 0.01
	Romaine	0.78 ab ± 0.06	0.55 c ± 0.03	0.13 d ± 0.01
leaf DW (g) per plant (n = 6)	Verte	0.25 a ± 0.01	0.19 b ± 0.02	0.08 d ± 0.01
	Romaine	0.20 b ± 0.01	0.13 c ± 0.01	0.08 d ± 0.01
root DW (g) per plant (n = 6)	Verte	0.04 a ± 0.00	0.04 a ± 0.00	0.01 b ± 0.00
	Romaine	0.04 a ± 0.00	0.03 ab ± 0.00	0.01 b ± 0.00
leaf water content[b] (% per plant, n = 6)	Verte	91.96 b ± 0.51	88.96 c ± 0.25	85.00 e ± 0.96
	Romaine	93.40 a ± 0.20	91.78 b ± 0.43	86.17 d ± 0.41
root water content[b] (% per plant, n = 6)	Verte	94.89 a ± 0.37	92.71 c ± 0.22	91.44 d ± 0.24
	Romaine	95.16 a ± 0.10	94.73 b ± 0.47	91.09 d ± 0.14
chlorophyll content (mg g^{-1} FW leaf) (n = 4)	Verte	1.41 a ± 0.05	1.62 a ± 0.20	1.28 a ± 0.07
	Romaine	0.88 b ± 0.15	0.75 b ± 0.03	0.56 b ± 0.08
net photosynthesis (μmol CO_2 s^{-2} s^{-1}) (n = 5)	Verte	5.33 a ± 0.40	5.05 a ± 0.91	1.89 b ± 0.23
	Romaine	4.96 a ± 0.32	4.36 a ± 0.88	2.63 b ± 0.13

[a] Fourteen day old seedlings were exposed to diluted salt-free Hoagland's nutrient solution supplemented with either 0 mM salt, 100 mM NaCl, or 77 mM Na_2SO_4. Different letters represent significantly different means (±SEs) of indicated replicates across each physiological parameter using a Fisher's protected least square difference test, $p \leq 0.05$. [b] Leaf and root water contents were calculated as described in the Materials and Methods.

activity corresponded to the amount of enzyme that inhibited the reduction of nitroblue tetrazolium by 50%.

Semiquantitative Reverse Transcription−Polymerase Chain Reaction (RT-PCR) Analysis. The total RNA was isolated from frozen tissues (−80 °C) using a RNA extraction kit (Qiagen, Mississauga, Ontario, Canada), and contaminating DNA was removed by DNase I treatment. The total RNA was used in two-step, semiquantitative RT-PCR analysis of leaf genes encoding phytoene synthase (*PSY*), phytoene desaturase (*PDS*), and lycopene ε-cyclase (*ε-CYC*) and leaf and root genes encoding CAT (*CAT1*), manganese SOD (*MnSOD*), iron SOD (*FeSOD*), and copper/zinc superoxide dismutase (*Cu/ZnSOD*) on 26 day old seedlings 12 days after salinization. Root *PPO* expression was measured during short-term salinization of 14 seedlings over a 24 h period prior to the initiation of root browning. First strand cDNA was synthesized from oligo $(dT)_{12-18}$ primed total RNA (2 μg) in a 20 μL reaction mixture using the SuperScript RT-PCR kit (Invitrogen, Ontario, Canada) at 42 °C for 50 min, and the resultant cDNA was diluted 5-fold with water. RNA was pooled from 3 to 5 plants per biological replicate. At least three biological replicates (each from different plants), an internal RNA control (actin gene), and a mock reaction without reverse transcriptase were included in RT-PCR reactions.

Gene-specific primers spanning intron−exon junctions were designed for each gene wherever possible to distinguish between products amplified from cDNA and genomic DNA. The gene-specific primers were based on lettuce sequence data obtained from either Genbank or a lettuce EST database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=lettuce) and are as follows (5′ to 3′; F, forward primer; R, reverse primer): PSY-F, GAATGCTAGTAGGAAGCATAAGTG; PSY-R, GCCCATATAGCTTTTCGCC; PDS3-F, GAAGTTAGTCGGTGTTCC; PDS3-R, TTTAGTATAATCACCAGAT; ε-CYC-F, TCCAGATCTAATGGTGTTCATGGA; ε-CYC-R, CACAAT-GTTTCCCATGCTTGTT; CAT1-F, GAGGCCCAATCCTTCTTGAG; CAT1-R, GTGTTGACTCCTGAGCCTTCCA; MnSODII-F, ACACGAAGCACCATCAACTTAC; MnSODII-R, GAGGTAGTAGGCATGCTC; FeSOD-F, GAAGCACCACCAACTTATGTCG; FeSOD-R, CTTCCTCTGATGGACGTGG; Cu/ZnSOD-F, ATGGTGAAGGGAGTTGCAG, Cu/ZnSOD-R, GACAACTACAGCCCTTCCAA, PPO-F, TACGTGTCTCAACCGAGACTG; PPO-R, CCTGCGGTTACACGATTC; actin-F, TTTGCTGGGGATGATGCGCC; and actin-R, GTGGTACGACCACTGGCATA. One microliter of the diluted RT reaction mix (50 ng μL^{-1} cDNA) was used in gene-specific PCR reactions (25 μL) using an initial denaturation at 94 °C for 4 min, 25−35 cycles at 94 (30 s), 55 (30 s), and 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 that the reaction was not saturated (linear phase) and that differences in transcript abundance were readily discernible (30 cycle optimum for all genes except *PPO*; 35 cycle optimum for *PPO*).

Statistical Analysis. Data obtained from growth, physiological, and biochemical parameters were subjected to analysis of variance (ANOVA) using the Mixed Model procedures in a statistical analysis software (SAS9.1) package (SAS Institute, Cary, NC). Each treatment had 3−9 replicates as indicated. Means were compared with Fisher's protected LSD at $P \leq 0.05$ and expressed as means ± standard errors (SEs). Means denoted by different letters are significantly different from each other.

RESULTS

Impact of Genotype and Salinity on Physiological Responses. Growth and several whole-plant physiological parameters were measured for Verte and Romaine genotypes grown in the presence of iso-osmotic 100 mM NaCl and 77 mM Na_2SO_4 as a means to distinguish the ionic effects of these two treatments from their osmotic impacts. In the absence of salinity stress, Verte normally had 20% greater leaf biomass (DW), 1.6-fold higher leaf chlorophyll content, slightly less water content, but equivalent leaf photosynthetic rate and root biomass as compared with Romaine (**Table 1**). Leaf growth (DW) of both lettuce types was differentially inhibited under the two different salinity treatments, although chlorophyll and leaf color did not change for either lettuce type with either salinity type (**Table 1**). Verte leaf DW dropped 20% with NaCl and 65% with Na_2SO_4 treatments as compared with untreated plants, whereas Romaine leaf DW dropped 33% with NaCl and 60% with Na_2SO_4. The growth (DW) results suggested that Verte leaves were more tolerant to 100 mM NaCl than Romaine leaves, but both genotypes differed little in leaf tolerance to Na_2SO_4.

Root growth was equivalent to untreated plants for both lettuce types exposed to NaCl, and roots of both genotypes remained pale cream under this type of sodium salt. The root mass declined equally for both genotypes when exposed to Na_2SO_4, but prominent root browning was observed in Na_2S0_4-treated Romaine plants. In contrast, roots of Verte appeared similar in color to untreated and NaCl-treated roots (**Figure 1**).

Under control conditions, the leaf water content of Romaine lettuce was slightly higher than that of Verte, but the root water content was similar in both varieties. Leaves and roots of both genotypes were equally affected when exposed to Na_2SO_4 (**Table 1**).

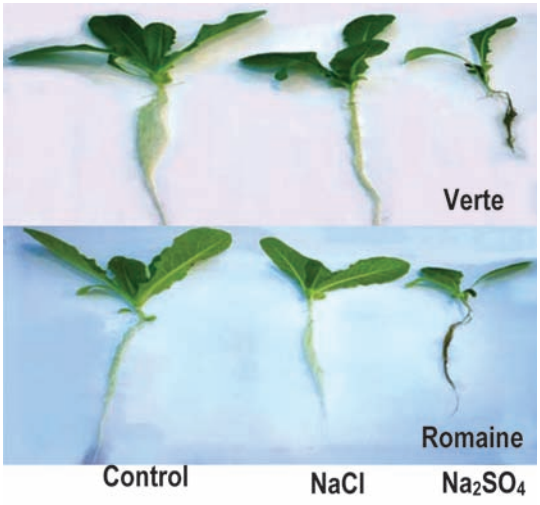

Figure 1. Phenotype of lettuce grown in 100 mM NaCl and 77 mM Na_2SO_4 salts for 12 days. Root browning occurred only in the presence of Na_2SO_4.

The water content declined 7% in leaves and 4% in roots of both genotypes under the sulfate salt. With NaCl, the water content of leaves and roots dropped 1.5% less in Romaine than in Verte, although water loss due to NaCl in both tissues was below 3%.

Because salinity frequently disrupts photochemical capacity (net photosynthetic rate) (*7*, *25*, *34*), we compared leaf net CO_2 assimilation rates between Verte and Romaine in response to salinity treatments. The photochemical capacity was similar in both lettuce types with and without NaCl. The capacity was equally reduced for both lettuce types after Na_2SO_4 treatment.

Effect of Salinity on the Accumulation of Na^+, K^+, and Ca^{2+}. The concentration of major cations differed for leaves of Verte and Romaine in the absence of salt stress. (Because of limited tissue, roots were retained for other analyses and not analyzed for metal accumulation.) In the absence of salt, Verte leaves had a 2-fold higher capacity to accumulate Na^+ but a lower capacity to accumulate K^+ and Ca^{2+} (**Table 2**). Both lettuce types accumulated increased Na^+ at equivalent concentrations under NaCl treatment, but Verte leaves showed a stronger capacity than Romaine leaves to accumulate Na^+ under Na_2SO_4 treatment (**Table 2**). Reduced capacity to accumulate K^+ and Ca^{2+} occurred in leaves of both varieties under both salinity treatments but was more dramatic in Romaine (**Table 2**). Accumulation of K^+ under Na_2SO_4 was 2.5−3-fold lower and identical for leaves of both lettuce types and 2-fold lower with NaCl as compared to untreated tissue, although K^+ accumulation continued to be higher in Romaine than Verte under NaCl treatment. Accumulation of Ca^{2+} was 2-fold reduced in Verte for both salt types, but Ca^{2+} in Romaine was reduced 4-fold with NaCl and 7-fold with Na_2SO_4.

Effect of Salinity on the Accumulation of Carotenoid and Phenolic Antioxidants. The health and economic benefits of carotenoids and their potential for protecting plants against stress prompted us to evaluate ionic effects on carotenoid accumulation in Verte and Romaine under NaCl and Na_2SO_4 applications. Leaves of both varieties had similar total carotenoid levels in the absence of salt treatment, although the level of one major compound lutein was substantially higher in Verte than in Romaine. Under salinity, β-carotene (a vitamin A precursor), lutein, and total carotenoids were significantly elevated in Verte (**Table 3**). However, carotenoid levels (individual and total) did not significantly change in Romaine lettuce under either type of salt stress, except for a 3-fold increase in zeaxanthin under Na_2SO_4. This contrasted with a 0.3- and 0.8-fold increase in lutein and β-carotene in Romaine after 15 days of exposure to a low NaCl concentration (5 mM) (*18*) and demonstrates the marked influence of genotype, salinity type, and salinity concentration on carotenoid accumulation in lettuce.

Because soluble phenolics are also an important component of the antioxidant capacity of lettuce (*35*), we determined the inherent differences in phenolics between Verte and Romain. The total phenolics comprising phenolic acids and flavonoids were determined in leaves under different salinity conditions (**Table 4**). Both lettuce varieties contained mainly phenolic acids with only a minor fraction of flavonoids in the absence of salinity treatment (**Table 4**). While both classes of these compounds displayed differential changes in response to NaCl and Na_2SO_4 salinity, more profound increases in the minor class (flavonoids) were detected in Romaine (14.5- and 4.5-fold) as compared to Verte (4.2- and 2.4-fold) under NaCl and Na_2SO_4, respectively. In contrast, a 1.7-fold increase and a 0.8-fold decrease were found for the major class (phenolic acids) in Verte grown under the same two salinity conditions, respectively, while only a 1.2-fold increase and a 0.5-fold decrease were observed in Romaine, respectively. Both varieties were less capable of mustering a strong response from either compound class to Na_2SO_4 as compared with NaCl. Regardless of salinity type, Verte consistently maintained equivalent or significantly greater phenolic acids and overall total

Table 2. Leaf Cation Contents of Verte and Romaine Lettuces Grown in 100 mM NaCl or 77 mM Na_2SO_4 Salts for 12 Days[a]

		cation concentration (mmol g^{-1} DW)		
cation	genotype	no treatment	100 mM NaCl	77 mM Na_2SO_4
Na^+	Verte	0.08 a ± 0.01	1.61 b ± 0.04	2.80 c ± 0.08
	Romaine	0.04 a ± 0.01	1.69 b ± 0.03	1.62 b ± 0.10
K^+	Verte	1.39 b ± 0.11	0.60 d ± 0.02	0.53 d ± 0.04
	Romaine	1.75 a ± 0.04	0.91 c ± 0.07	0.57 d ± 0.04
Ca^{2+}	Verte	0.27 b ± 0.03	0.14 c ± 0.02	0.13 cd ± 0.02
	Romaine	0.5 a ± 0.03	0.12 cd ± 0.02	0.07 d ± 0.01
total ions ($Na^+/K^+/Ca^{2+}$)	Verte	1.75 a ± 0.11	2.34 b ± 0.04	3.47 d ± 0.13
	Romaine	2.29 b ± 0.05	2.72 c ± 0.08	2.26 b ± 0.12

[a] Fourteen day old seedlings were exposed to diluted salt-free Hoagland's nutrient solution supplemented with either 0 mM salt, 100 mM NaCl, or 77 mM Na_2SO_4. Different letters represent significantly different means (±SEs) of six plants across each cation parameter using a Fisher's protected least square difference test, $p \leq 0.05$.

Table 3. Contents of Major Carotenoid Fractions in Leaves of Verte and Romaine Lettuces Grown in 100 mM NaCl or 77 mM Na_2SO_4 Salts for 12 Days[a]

		carotenoid concentration (μg g^{-1} FW tissue)		
carotenoid species	genotype	no treatment	100 mM NaCl	77 mM Na_2SO_4
β-carotene	Verte	46.07 a ± 9.64	124.73 b ± 3.91	111.20 b ± 5.99
	Romaine	39.12 a ± 14.13	55.76 a ± 16.69	41.51 a ± 17.79
lutein	Verte	25.46 b ± 4.38	42.30 c ± 2.10	37.40 c ± 6.39
	Romaine	5.28 a ± 2.14	4.90 a ± 1.20	11.28 a ± 4.90
violaxanthin	Verte	9.60 a ± 2.27	11.27 a ± 1.01	12.18 a ± 2.87
	Romaine	15.89 a ± 5.99	14.90 a ± 2.11	22.99 a ± 8.54
zeaxanthin	Verte	0.73 ab ± 0.28	1.43 c ± 0.11	1.09 bc ± 0.25
	Romaine	0.19 a ± 0.19	0.25 a ± 0.12	0.55 ab ± 0.32
total carotenoids	Verte	89.26 a ± 11.37	180.09 b ± 6.00	162.04 b ± 8.90
	Romaine	60.54 a ± 22.49	75.82 a ± 18.16	76.52 a ± 31.22

[a] Fourteen day old seedlings were exposed to diluted salt-free Hoagland's nutrient solution supplemented with either 0 mM salt, 100 mM NaCl, or 77 mM Na_2SO_4. Different letters represent significantly different means (±SEs) of four plants across each carotenoid species using a Fisher's protected least square difference test, $p \leq 0.05$.

Table 4. Contents of Major Phenolic Fractions in Leaves of Verte and Romaine Lettuces Grown in 100 mM NaCl or 77 mM Na_2SO_4 Salts for 12 Days[a]

		phenolic concentration (mg g^{-1} FW tissue)		
phenolic fraction	genotype	no treatment	100 mM NaCl	77 mM Na_2SO_4
phenolic acids	Verte	0.55 b ± 0.06	0.79 c ± 0.02	0.42 b ± 0.04
	Romaine	0.70 c ± 0.09	0.37 b ± 0.11	0.15 a ± 0.00
flavonoids	Verte	0.06 a ± 0.01	0.25 c ± 0.01	0.16 b ± 0.01
	Romaine	0.04 a ± 0.00	0.58 d ± 0.05	0.19 bc ± 0.01
total phenolics	Verte	0.61 b ± 0.07	1.04 c ± 0.01	0.58 b ± 0.05
	Romaine	0.74 b ± 0.09	0.95 c ± 0.09	0.35 a ± 0.01

[a] Fourteen day old seedlings were exposed to diluted salt-free Hoagland's nutrient solution supplemented with either 0 mM salt, 100 mM NaCl, or 77 mM Na_2SO_4. Different letters represent significantly different means (±SEs) of three plants across each phenolic species using a Fisher's protected least square difference test, $p \leq 0.05$.

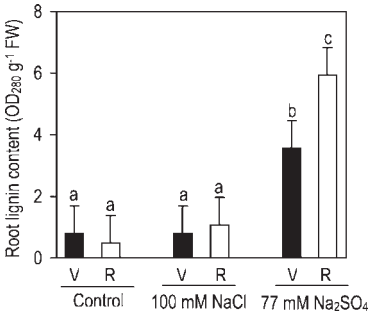

Figure 2. Root lignin content of Verte (V, filled bar) and Romaine (R, empty bar) lettuces grown under 100 mM NaCl and 77 mM Na_2SO_4 salts for 12 days. Error bars represent SEs of the mean of three independent lignin assays.

phenolics as compared to Romaine, suggestive of a considerably greater antioxidative potential in Verte.

Effect of Salinity on Root Lignification. The root lignin content was examined to determine the potential involvement in salinity adaptation for the two lettuce types (**Figure 2**). The relative root lignin content was increased 5.9-fold in Romaine under Na_2SO_4 treatment but only 3.6-fold in Verte with this type of salt. No measurable changes in lignin content were observed for either lettuce type in NaCl-treated roots (**Figure 2**), suggesting a Na_2SO_4-specific lignin induction in lettuce.

Effect of Salinity on Membrane Integrity in Verte and Romaine. Membranes are the primary sites of salinity injury, since excessive ROS can react with and damage membrane unsaturated fatty acids (UFAs). Membrane damage was detected by measuring the peroxidation product of UFAs, MDA, which is a representative biomarker for this type of damage (*36*). Both Verte and Romaine had greater MDA levels in leaves and roots after Na_2SO_4 treatment than after NaCl treatment (**Figure 3**). Root MDA levels and root lignin were strongly correlated (correlation coefficient of 0.86), especially with the more toxic Na_2SO_4 compound. MDA levels were much higher in leaves than in roots irrespective of the genotype, and the response in leaves of both genotypes was similar regardless of the salt type. However, Verte accumulated less MDA in roots as compared to Romaine. These results suggest stronger membrane sensitivity to salinity-based oxidative stress in leaves as compared with roots. They also suggest that Verte may have a more robust protection system than Romaine.

Impact of Genotype and Salinity on Activities of Antioxidative Enzymes. Because leaf membranes were more sensitive than roots

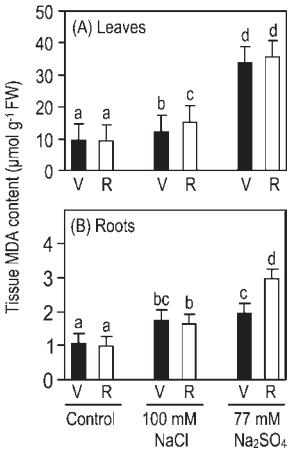

Figure 3. Tissue MDA contents of Verte and Romaine lettuces grown under 100 mM NaCl and 77 mM Na_2SO_4 salts for 12 days. (**A**) Leaves and (**B**) roots. Verte (V, filled bar) and Romaine (R, empty bar). Error bars represent SEs of the mean of nine independent MDA assays.

Table 5. Leaf Antioxidative Enzyme Activities of Verte and Romaine Lettuces Grown in 100 mM NaCl or 77 mM Na_2SO_4 Salts for 12 Days[a]

		enzyme specific activity (activity units mg^{-1} protein)		
enzyme activity	genotype	control	100 mM NaCl	77 mM Na_2SO_4
CAT (n = 4)	Verte	1.15 abc ± 0.12	2.81 d ± 0.28	1.44 bc ± 0.17
	Romaine	0.88 a ± 0.11	1.56 c ± 0.19	0.96 ab ± 0.09
POD (n = 4)	Verte	0.70 a ± 0.10	0.80 a ± 0.08	0.38 b ± 0.10
	Romaine	0.96 a ± 0.09	0.71 a ± 0.14	0.65 ab ± 0.09
SOD (n = 3)	Verte	65.87 a ± 2.17	85.38 b ± 7.57	176.65 d ± 7.72
	Romaine	90.28 b ± 5.52	85.33 b ± 5.17	126.12 c ± 6.40

[a] Fourteen day old seedlings were exposed to diluted salt-free Hoagland's nutrient solution supplemented with either 0 mM salt, 100 mM NaCl, or 77 mM Na_2SO_4. Different letters represent significantly different means (±SEs) of indicated plants across each enzyme activity using a Fisher's protected least square difference test, $p \leq 0.05$.

to salinity (i.e., leaves accumulated higher amounts of MDA), activities of three key leaf antioxidant enzymes (CAT, POD, and SOD) were assessed under salinity and no salt conditions. Without salt, both lettuce types showed similarly low CAT and POD activities. In contrast, the SOD activity was >65-fold higher in Verte leaves and >90-fold higher in Romaine leaves than the other two enzymes (**Table 5**). Higher activities for two out of the three antioxidant enzymes (CAT and SOD) were detected in both NaCl-treated and Na_2SO_4-treated Verte leaves, whereas Romaine leaves only exhibited increased SOD activity with Na_2SO_4. Although the POD activity was low and its activity did not change in leaves of either lettuce variety under NaCl treatment, Romaine leaves maintained 2-fold higher leaf POD activity than Verte under Na_2SO_4 (**Table 5**).

Varied Gene Expression between Verte and Romaine under Salinity Treatments. To aid in the interpretation of leaf carotenoid and antioxidation enzyme activity changes, root lignification responses, and MDA changes, we analyzed the expression of several genes involved in molecular adaptations to salinity using semiquantitative RT-PCR (**Figure 4**). The selection of genes encoding *PSY*, *PDS3*, and *ε-CYC* and testing leaf rather than both tissues was based on the central roles of these genes in carotenoid biosynthesis. Without salt treatment, *ε-CYC* was the most strongly expressed of these three genes, and *PSY* and *PDS3* were quite weakly expressed in Verte. In contrast, the most strongly expressed gene without salt treatment in Romaine was *PDS3*. For the NaCl-tolerant Verte, none of these three leaf genes

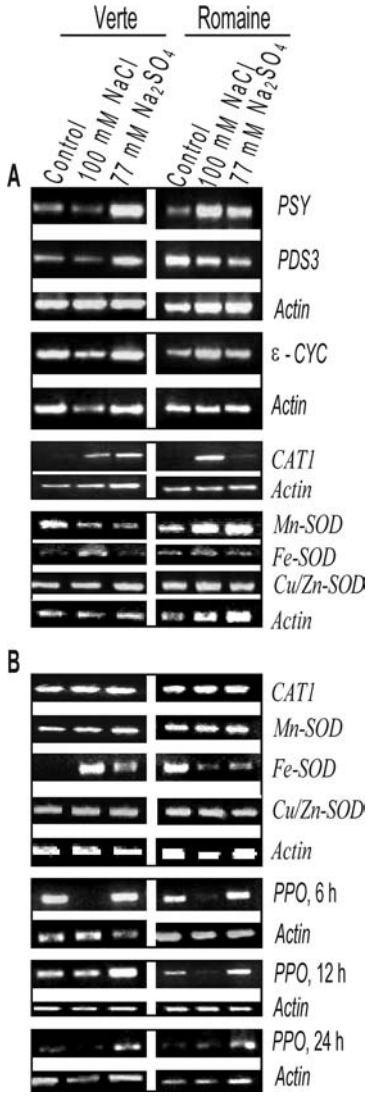

Figure 4. Representative semiquantitative RT-PCR analysis of carotenoid and antioxidative gene expression in Verte and Romaine lettuces growing in NaCl or Na_2SO_4. (**A**) Leaf expression after 12 days of salinity treatment. (**B**) Root expression after salinity treatment for short (24 h) or long (12 days) periods. *CAT1*, *MnSOD*, *FeSOD*, and *Cu/ZnSOD* for 12 days. *PPO* expression following salinity treatment for 6, 12, and 24 h. The lettuce *ACTIN* gene (cDNA loading control) is directly below each set of corresponding antioxidant or carotenoid gene assays. Genes other than *PPO* were amplified using 30 cycles; *PPO* was amplified using 35 cycles. Data are representative of RT-PCR reactions from at least three independent RNA extractions.

changed in expression with NaCl treatment, even though a 2-fold increase in Verte leaf carotenoids (β-carotene and lutein) occurred under NaCl. In contrast, *PSY* and *ε-CYC* were strongly stimulated in the NaCl-sensitive Romaine leaves under NaCl treatment, while *PDS3* was reduced in expression, even though leaf carotenoids did not change in Romaine. The *ε-CYC* transcripts in Verte leaves did not change, but *PSY* and *PDS3* were strongly stimulated in Verte in the presence of Na_2SO_4 (**Figure 4**). This was consistent with the 2-fold overall increase in carotenoids with Na_2SO_4 (**Table 3**). *PSY* transcripts were modestly stimulated, and *PDS3* transcripts were modestly reduced in Na_2SO_4-treated Romaine plants, and the sum total of these changes was consistent with the lack of significant change in carotenoid content in Romaine.

Expression of genes encoding well-characterized antioxidative enzymes (*37*) was also examined for leaves and roots, since MDA levels rose in these tissues. In leaves, differences between the two lettuce types were not obvious without salt treatment. Leaf *CAT1* transcripts were induced modestly in both genotypes with NaCl, but leaf *CAT1* transcript accumulation only occurred in the tolerant variety Verte and was absent in the susceptible variety Romaine under Na_2SO_4 treatment. These data correlated with the increase in leaf CAT enzyme activity in both varieties under NaCl treatment and with the trend toward leaf CAT activity increase in Verte under Na_2SO_4. They suggest that the Verte leaf peroxisomes (the usual site of CAT activity) and the Verte transcription response to salt stress may be more effective than equivalent systems in Romaine.

Because the SOD enzyme activity was 30% increased in Verte under NaCl treatment and 300% or 50% increased in Verte and Romaine, respectively, under Na_2SO_4 treatment and SOD was the strongest activity of the three antioxidative enzymes that we measured, transcript patterns of specific isozymes of SOD were also compared between the two genotypes. The strong leaf *Cu/ZnSOD* transcript abundance normally present in leaves of these two genotypes did not change in either genotype after either salt treatment (**Figure 4A**), suggesting that *Cu/Zn*SOD activity, which is found in many plant compartments (cytosol, chloroplasts, peroxisomes, and apoplast), may not contribute to the change in total SOD activity observed after saline exposure. *FeSOD*, a chloroplast SOD, showed no increase in leaf transcripts in Romaine under either salt treatment and none in Na_2SO_4-treated Verte leaves, but *FeSOD* transcripts were abundant in Verte leaves under NaCl treatment. *MnSOD*, a mitochondrial and peroxisome SOD, showed reduced transcript abundance in Verte and increased abundance in Romaine under both salt treatments. These *FeSOD* and *MnSOD* transcript expression patterns are inconsistent with the increased SOD enzyme activity in Verte as compared with Romaine.

Because distinct differences were observed for SOD isozyme transcripts in leaves under saline treatment (**Figure 4A**) and MDA was shown to increase in roots during exposure to salt (**Figure 3B**), antioxidant transcript abundance was further measured in the roots of contrasting lettuce types. In roots, both *CAT1* and *MnSOD* transcripts were moderately abundant in both lettuce types before salt treatment (**Figure 4B**). The signature genotype pattern for roots was the lack of *FeSOD* transcripts in Verte roots and the strong accumulation in Romaine roots in the absence of salt. No substantial differences were detected for *CAT1* or *MnSOD* expression in roots of either genotype following NaCl or Na_2SO_4 treatment. In contrast, *FeSOD* was strongly enhanced in Verte roots but repressed in Romaine by both salinity treatments (**Figure 4B**). Our results indicate that NaCl and Na_2SO_4 differentially affect antioxidative gene expression within these two genotypes and between their leaves and roots.

More prominent root browning was observed in Romaine lettuce plants treated for 12 days with Na_2SO_4 (**Figure 1**). Hence, we examined the root transcript profile of one *PPO* isoform in 14 day old seedlings exposed to short-term (24 h) salinity treatment before browning became apparent (**Figure 4B**). *PPO* transcripts were abundant in both Verte and Romaine roots in the absence of salinity. *PPO* transcripts declined over the following 24 h test period at a faster rate in Romaine roots than in Verte roots. Even though lignin and MDA were higher in Romaine than in Verte roots under the long-term salt treatment (12 days), both lettuce types had a similar profile of *PPO* transcripts over a short-term (24 h) of Na_2SO_4 treatment as compared with untreated seedlings. However, NaCl treatment showed a completely different profile between lettuce types. *PPO* was repressed at all three assessment times in Romaine roots during NaCl treatment, while *PPO* transcripts in Verte roots initially were

down-regulated by NaCl at 6 h, then showed a strong transient increase by 12 h, and subsequently disappeared by 24 h.

DISCUSSION

Despite the economic and dietary importance of lettuce, relatively little research has been undertaken to investigate the impact of different salinity types and genotypes on secondary metabolite accumulation, cellular antioxidative responses, and gene expression. Previous reports in the literature showed varied growth responses for two Butterhead lettuce varieties grown under moderate levels of Na_2SO_4 (0−60 mM) and low levels of $NaHCO_3$ (0−7.5 mM) (*7*). A recent study demonstrated that Romaine lettuce maintained moderate growth after 2 days of exposure to high NaCl concentrations (up to 1 M) but was stunted after 15 days of treatment with 200 mM NaCl (*18*). These reports were consistent with a report that several spruce ecotypes exhibited mixed physiological responses to 25 mM NaCl and 25 mM Na_2SO_4 (*34*) and suggested that genetic background and duration under salinity would influence sensitivity or tolerance to different salts.

Mechanisms of response to sodium ion stress were rarely investigated systematically or in sufficient detail in these studies; hence, these mechanisms have remained unclear. For example, longer term exposure (15 days) to low levels of NaCl (5 mM) weakly stimulated the levels of carotenoids in Romaine, but higher NaCl concentrations did not (*18*). Moreover, responses other than carotenoid accumulation, such as the antioxidation enzyme system, sodium exclusion, and increases in the K^+/Na^+ and Ca^{2+}/Na^+ ratios (*1*, *4*, *7*, *25*), were overlooked in these studies.

Our study focused on elucidating physiological and biochemical functions that contribute to the contrasting responses of two lettuce types from widely different germplasm sources, Verte (tolerant to NaCl) and Romaine (sensitive to NaCl), to two different sodic salts, NaCl and Na_2SO_4. These include detailing differences in physiological parameters (growth, water and ion contents, and photochemical capacity), membrane oxidative damage, leaf carotenoids, phenolics, root lignin contents, and antioxidative enzyme responses (CAT, POD, and SOD activities) and gene expression for several of these parameters. Mechanistically, Verte was more tolerant to NaCl based on greater biomass (DW) accumulation during salt stress due to its more effective protection systems (carotenoids, polyhenols, and antioxidation enzymes). In contrast, Romaine appeared to be more adaptable to Na_2SO_4 salt as manifested by relatively better root growth, ion accumulation, and root lignification. Both germplasm types responded to different salinity treatments by differentially accumulating Na^+ ions rather than excluding them, but overall, Verte was more tolerant to salt-induced oxidative stress and could accumulate a higher level of Na^+ ions than Romaine by virtue of its greater leaf carotenoid content and a more robust response from its antioxidation enzyme system. These types of protection in Verte resulted in less root membrane disruption as manifested by the lower content of MDA as compared to Romaine roots. These variations highlight the divergent functional specializations during evolutionary adaptation of these two lettuce types.

The enhanced salt tolerance of Verte relative to Romaine is partly reflected in the differential accumulation of carotenoid and phenolic antioxidants. Verte accumulates higher levels of total carotenoids and most individual carotenoids (except for violaxanthin). It must be noted, however, that changes in carotenoid contents of the two lettuce types grown under salinity treatments did not correlate with the expression profiles of *PSY*, *PDS*, and *ε-CYC* genes that encode key enzymes in the pathway. The discrepancy between carotenoid accumulation (**Table 3**) and transcript abundance of these genes (**Figure 4**) may be explained by the complex nature of the regulation of carotenoid accumulation in plant tissues (*16*). On the other hand, the differential accumulation of phenolic fractions under different salt treatments in the two lettuce varieties suggests that these varieties may employ phenolic acids and flavonoids differentially to adapt to contrasting salinities, a novel mechanism not yet well understood.

Our focus on the analysis of carotenoid and antioxidant accumulation in the leaves is due to their pertinence to the health of the photosystem and, consequently, to seedling vigor. However, other attributes were determined in the roots, including browning, MDA level, lignin content, and antioxidative gene expression, which collectively contribute to integral salinity tolerance in the root (*1*). Root growth of both genotypes appeared to be equally slow in Na_2SO_4, but Romaine roots exhibited less damage by this type of salinity than Verte roots. Interestingly, Romaine roots produced more MDA and showed evidence of prominent browning under Na_2SO_4. In iceberg lettuce, root browning was found to be caused by the oxidation of *O*-quinones produced by PPOs released from plastids in salinity-damaged plants (*38*). Suppressed *FeSOD* expression may contribute to greater ROS and MDA accumulation in Romaine roots, and the result may be enhanced browning and lignification in Romaine. Similar to wound-induced leaf browning, which is coordinately catalyzed by phenylalanine ammonia lyase, various PPO isoenzymes, and POD (*38*), root browning may serve as a defense mechanism in sealing off the outer layer cells to prevent from further damage to the inner cells. It is conceivable that the greater increase in root lignin content of Romaine relative to Verte in response to Na_2SO_4 treatment (**Figure 2**) may contribute, in concert with root browning and other factors, to the enhanced tolerance of Romaine to this salt.

MDA content is nearly 10-fold lower in roots than in leaves (**Figure 3**), suggesting a tissue-dependent difference in the membrane lipid peroxidation process as a result of salinity-induced membrane damage. This could be related to the constant photochemical activity resulting in high ROS production in the leaves and/or more effective ROS scavenging machinery in the roots. In NaCl-exposed lettuce roots colonized by arbuscular mycorrhizal fungi, less perturbation in expression of dehydration-regulated genes and metabolite accumulation was documented (*39*). These aspects may also be involved in root adaptation to NaCl and Na_2SO_4, but they were not the focus of this study. However, we noticed that the abundance of *FeSOD* transcript increased under NaCl but decreased under Na_2SO_4 salinity in Romaine roots, while both salts enhanced *FeSOD* transcription in Verte roots (**Figure 4**). This suggests that the SO_4^- anion may be inhibitory to some root proteins regulating the transcription of the *FeSOD*. Overall, the choice of two iso-osmotic salts in this study allowed the differentiation of ionic from the osmotic effects on lignin (**Figure 2**), root MDA (**Figure 3B**), expression of leaf *CAT1* in Romaine and leaf PSY in Vert (**Figure 4A**), as well as root $FeSO_4$ in Verte and root PPO in both varieties (**Figure 4B**).

In conclusion, our study showed that moderate salinity could cause altered leaf carotenoid, lignin, phenolic, and flavonoid levels without noticeable changes in the green leaf color and morphology. Findings from this study further demonstrate that Verte lettuce seedlings are more protected (high carotenoids and antioxidants) from the oxidative stress associated with exposure to NaCl as compared with Romaine. On the other hand, Romaine is very tolerant to heat stress (http://en.wikipedia.org/wiki/Lettuce). As lettuce is amenable to classical and molecular genetic manipulations (*40*, *41*), it would be advantageous to

transfer the nutritive and hardy traits between diverse *Lactuca* lettuce types (Crisphead, Butterhead, Looseleaf, Redhead, Chinese lettuce, and Summer Crisp) to further improve the leaf quality and agronomic performance through traditional breeding or genetic engineering. Our findings along with previous reports (*18*) demonstrate the feasibility of expanding the production of Verte in saline soils or other marginal lands where nonhardy lettuce types are poorly adapted.

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LITERATURE CITED

(1) Greenway, H.; Munns, R. Mechanism of salt tolerance in nonhalophytes. *Annu. Rev. Plant Physiol.* **1980**, *31*, 149–190.
(2) Zhu, J. K. Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.* **2000**, *124*, 941–948.
(3) Ouerghi, Z.; Rémy, R.; Ouelhazi, L.; Ayadi, A.; Brulfert, J. Two-dimensional electrophoresis of soluble leaf proteins, isolated from two wheat species (*Triticum durum* and *Triticum aestivum*) differing in sensitivity towards NaCl. *Electrophoresis* **2000**, *21*, 2487–2491.
(4) Gratton, S. R.; Grieve, C. M. Salinity-mineral nutrient relations in horticultural crops. *Sci. Hortic.* **1999**, *78*, 127–157.
(5) Redmann, R. E.; Fedec, P. Mineral ion composition of halophytes and associated soils in western Canada. *Commun. Soil Sci. Plant Anal.* **1987**, *18*, 559–579.
(6) Renault, S. C.; Lait, J.; Zwiazek, J.; Mackinnon, M. Effect of high salinity tailings waters produced from gypsum treatment of oil sands tailings on plants of the boreal forest. *Environ. Pollut.* **1998**, *102*, 177–184.
(7) Bie, Z.; Ito, T.; Shinohara, Y. Effects of sodium sulphate and sodium bicarbonate on the growth, gas exchange and mineral composition of lettuce. *Sci. Hortic.* **2004**, *99*, 215–224.
(8) Bowler, C.; Van Montagu, M.; Inze, D. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 83–116.
(9) Asada, K. The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 601–639.
(10) Wahid, A.; Ghazanfar, A. Possible involvement of some secondary metabolites in salt tolerance of sugarcane. *J. Plant Physiol.* **2006**, *163*, 723–730.
(11) Li, X.; Gao, P.; Gjetvaj, B.; Westcott, N.; Gruber, M. Y. Analysis of the metabolome and transcriptome of *Brassica carinata* seedlings after lithium chloride exposure. *Plant Sci.* **2009**, *177*, 68–80.
(12) Marles, M. A.; Gruber, M. Y.; Scoles, G. J.; Muir, A. D. Pigmentation in the developing seed coat and seedling leaves of *Brassica carinata* is controlled at the dihydroflavonol reductase locus. *Phytochemistry* **2003**, *62*, 663–672.
(13) Sobolev, A. P.; Brosio, E.; Gianferri, R.; Segre, A. L. Metabolic profile of lettuce leaves by high-field NMR spectra. *Magn. Reson. Chem.* **2005**, *43*, 625–638.
(14) Wang, S. Y.; Chang, H. N.; Lin, K. T.; Lo, C. P.; Yang, N. S.; Shyur, L. F. Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica*. *J. Agric. Food Chem.* **2003**, *51*, 1506–1512.
(15) Dixon, R. A.; Lamb, C. J.; Masoud, S.; Sewalt, V. J.; Paiva, N. L. Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defence responses—A review. *Gene* **1996**, *179*, 61–71.
(16) Cunningham, F. X., Jr.; Gantt, E. Genes and enzymes of carotenoids biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 557–583.
(17) Zhou, Y. H.; Zhang, Y. Y.; Zhao, X.; Yu, H. J.; Shi, K.; Yu, J. Q. Impact of light variation on development of photoprotection, antioxidants, and nutritional value in *Lactuca sativa* L. *J. Agric. Food Chem.* **2009**, *57*, 5494–5500.
(18) Kim, H. J.; Fonseca, J. M.; Choi, J. H.; Kubota, C.; Kwon, D. Y. Salt in irrigation water affects the nutritional and visual properties of Romaine lettuce (*Lactuca sativa* L.). *J. Agric. Food Chem.* **2008**, *56*, 3772–3776.
(19) Martorell, M.; Soliva, M.; Cañameras, N.; Martínez, F. X.; dos Reis, M. Lettuce seedling growth on substrate mixes using peat, cork, forest litter and sand. *Acta Hortic.* (*ISHS*) **1993**, *342*, 167–174.
(20) Maltais, A.; Gosselin, A.; Tremblay, N.; Van Winden, D. Effects of temperature and fertigation on lettuce seedling production using peat blocks. *Acta Hortic.* (*ISHS*) **2008**, *782*, 367–374.
(21) Al-Khatib, M.; McNeilly, T.; Collins, J. C. The potential of selection and breeding for improved salt tolerance in Lucerne (*Medicago sativa* L.). *Euphytica* **1993**, *65*, 43–51.
(22) Veatch, M. E.; Smith, S. E.; Vandemark, G. Shoot biomass production among accessions of *Medicago truncatula* exposed to NaCl. *Crop Sci.* **2004**, *44*, 1008–1013.
(23) Hoagland, D. R.; Arnon, D. I. The water-culture method for growing plants without soil. *Calif. Agric. Exp. Station Circ.* (*Berkley*) **1950**, *347*, 1–32.
(24) Arnon, D. I. Copper enzymes in chloroplasts phenol oxidase in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1–15.
(25) M'rah, S.; Ouerghi, Z.; Berthomieu, C.; Havaux, M.; Jungas, C.; Hajji, M.; Grignon, C.; Lachaâl, M. Effects of NaCl on the growth, ion accumulation and photosynthetic parameters of *Thellungiella halophila*. *J. Plant Physiol.* **2006**, *163*, 1022–1031.
(26) Marx, M.; Schieber, A.; Carle, R. Quantitative determination of carotene stereoisomers in carrot juices and vitamin supplemented (ATBC) drinks. *Food Chem.* **2000**, *70*, 403–408.
(27) Annalisa, R.; Patrizia, P.; Carlotta, G.; Graziano, S.; Antonio, C.; Daniela, H. Polyphenols in greenhouse and open-air-grown lettuce. *Food Chem.* **2002**, *79*, 337–342.
(28) Huang, J.; Bhinu, V. S.; Li, X.; Dallal Bashi, Z.; Zhou, R.; Hannoufa, A. Pleiotropic changes in Arabidopsis *f5h* and *sct* mutants revealed by large-scale gene expression and metabolite analysis. *Planta* **2009**, *230*, 1057–1069.
(29) Heath, R. L.; Packer, L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198.
(30) Bradford, M. M. A rapid and sensitive method for quantitation of microgram of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(31) Cakmak, I.; Marschner, H. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiol.* **1992**, *98*, 1222–1227.
(32) Srinivas, N. D.; Rshami, K. R.; Raghavarao, K. S. M. S. Extraction and purification of a plant peroxidase by aqueous two-phase extraction coupled with gel filtration. *Process Biochem.* **1999**, *35*, 43–48.
(33) Beauchamp, C.; Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **1971**, *44*, 276–287.
(34) Nguyen, H.; Calvo Polanco, M.; Zwiazek, J. J. Gas exchange and growth responses of ectomycorrhizal *Picea mariana*, *Picea glauca*, and *Pinus banksiana* seedlings to NaCl and Na_2SO_4. *Plant Biol.* (*Stuttgart, Ger.*) **2006**, *8*, 646–652.
(35) Nicolle, C.; Carnat, A.; Fraisse, D.; Lamaison, J.-L.; Rock, E.; Michel, H.; Amouroux, P.; Remesy, C. Characterisation and variation of antioxidant micronutrients in lettuce (*Lactuca sativa* folium). *J. Sci. Food Agric.* **2004**, *84*, 2061–2069.
(36) Karabal, E.; Yücel, M.; Öktem, H. A. Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. *Plant Sci.* **2003**, *164*, 925–933.
(37) Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **2002**, *7*, 405–410.
(38) Ke, D.; Saltveit, M. E. Plant hormone interaction and phenolic metabolism in the regulation of Russet Spotting in iceberg lettuce. *Plant Physiol.* **1988**, *88*, 1136–1140.
(39) Jahromi, F.; Aroca, R.; Porcel, R.; Ruiz-Lozano, J. M. Influence of salinity on the in vitro development of *Glomus intraradices* and on the

in vivo physiological and molecular responses of mycorrhizal lettuce plants. *Microb. Ecol.* **2008**, *55*, 45–53.
(40) Grube, R. C.; Wintermantel, W. M.; Hand, P.; Aburomia, R.; Pink, D. A.; Ryder, E. J. Genetic analysis and mapping of resistance to lettuce dieback: a soilborne disease caused by tombusviruses. *Theor. Appl. Genet.* **2005**, *110*, 259–268.
(41) Mazier, M.; Botton, E.; Flamain, F.; Bouchet, J. P.; Courtial, B.; Chupeau, M. C.; Chupeau, Y.; Maisonneuve, B.; Lucas, H. Successful gene tagging in lettuce using the Tnt1 retrotransposon from tobacco. *Plant Physiol.* **2007**, *144*, 18–31.

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