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An Enhancer Mutant of *Arabidopsis salt overly sensitive 3* Mediates both Ion Homeostasis and the Oxidative Stress Response[▽]

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The myristoylated calcium sensor SOS3 and its interacting protein kinase, SOS2, play critical regulatory roles in salt tolerance. Mutations in either of these proteins render *Arabidopsis thaliana* plants hypersensitive to salt stress. We report here the isolation and characterization of a mutant called *enh1-1* that enhances the salt sensitivity of *sos3-1* and also causes increased salt sensitivity by itself. *ENH1* encodes a chloroplast-localized protein with a PDZ domain at the N-terminal region and a rubredoxin domain in the C-terminal part. Rubredoxins are known to be involved in the reduction of superoxide in some anaerobic bacteria. The *enh1-1* mutation causes enhanced accumulation of reactive oxygen species (ROS), particularly under salt stress. ROS also accumulate to higher levels in *sos2-1* but not in *sos3-1* mutants. The *enh1-1* mutation does not enhance *sos2-1* phenotypes. Also, *enh1-1* and *sos2-1* mutants, but not *sos3-1* mutants, show increased sensitivity to oxidative stress. These results indicate that ENH1 functions in the detoxification of reactive oxygen species resulting from salt stress by participating in a new salt tolerance pathway that may involve SOS2 but not SOS3.

High salinity is a major adverse environmental factor affecting plant growth and development, leading to large global yield losses in crops. In most saline soils, Na^+ is the major toxic ion. Excess salts are harmful to plants because of ion toxicity and osmotic stress (6). Salt stress causes disrupted ion homeostasis in plants, leading to excess accumulation of toxic Na^+ in the cytoplasm and a deficiency of essential ions, such as K^+ (22, 33).

It appears that several ion transporters act to facilitate Na^+ entry into and exit out of plant cells. Na^+ can also be compartmentalized in the vacuole through tonoplast-localized Na^+/H^+ antiporters (4). The uptake of Na^+ into plant cells appears to occur at least partly through the transporter HKT1 (30, 40, 51) and through nonselective cation channels (2). In *Arabidopsis thaliana*, Na^+ efflux is mediated by the plasma membrane Na^+/H^+ antiporter encoded by the *SOS1* gene (47, 48, 52, 53) aided by plasma membrane H^+-ATPases, which generate the proton gradient needed to drive Na^+ transport. SOS1 facilitates Na^+ efflux into the root medium, thereby delaying Na^+ accumulation in the cytoplasm to allow time for Na^+ sequestration in the vacuole. SOS1 appears also to control long-distance Na^+ transport between roots and leaves by mediating loading and unloading of Na^+ in the xylem and phloem. Overexpression of the *SOS1* gene appears to confer increased salt tolerance, at least in part, in transgenic *Arabidopsis* plants by these processes (54).

Activation of the Na^+/H^+ antiport activity of SOS1 by salt stress is controlled by the SOS3 and SOS2 proteins (47, 48). SOS3 is a myristoylated calcium-binding protein that is capable of sensing cytosolic calcium changes elicited by salt stress (24, 34). *SOS2* is a serine/threonine protein kinase with a unique regulatory domain at the carboxyl terminus and an amino-terminal catalytic domain (35). The carboxyl-terminal regulatory domain of SOS2 interacts with SOS3 through the FISL motif (19). In the presence of calcium, SOS3 activates the substrate phosphorylation activity of SOS2 (21). Recent data demonstrate that the SOS3-SOS2 protein kinase complex activates SOS1 via phosphorylation (47, 48).

Although failure to reestablish ion homeostasis is a major cause of salt-induced injury, the etiology of salt toxicity in plants is more complex (60) and includes oxidative damage caused by reactive oxygen species (ROS) elicited by high levels of salt. ROS such as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) are produced in aerobic cellular processes such as mitochondrial and chloroplast electron transport or oxidation of several metabolites, such as glycolate (photorespiration), xanthine, and glucose. Abiotic stresses, including salinity, significantly increase the rate of ROS production (8, 17, 23, 56).

To minimize the damaging effects of ROS, organisms have evolved detoxification mechanisms. Plants utilize nonenzymatic antioxidants, such as ascorbate, glutathione, flavonoids, alkaloids, and carotenoids, to scavenge ROS (3). Detoxifying enzymes employed in plants include superoxide dismutase, catalase, and enzymes of the ascorbate-glutathione cycle (3,

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12). The importance of oxidative protection in salt tolerance is indicated by the characterization of the *Arabidopsis pst1* (for *p*hotoautotrophic *s*alt *t*olerance *1*) mutant, which is more tolerant to both salt and direct oxidative stresses than the wild type. The *pst1* mutant also has significantly higher activities of superoxide dismutase and ascorbate peroxidase than wild-type *Arabidopsis* when treated with high levels of NaCl (57). Overexpression of genes involved in ROS scavenging, such as *NtGST*/*GPX* (*g*lutathione *S*-*t*ransferase [GST]/*g*lutathione *p*eroxidase) also improve salt and chilling stress tolerance by reducing ROS-mediated membrane damage (49, 50).

Emerging evidence indicates that ROS also can act as an important signal controlling salt adaptive responses mediated through mitogen-activated protein kinases (MAPKs). An *Arabidopsis* MAPK kinase kinase, ANP1, is also activated by H_2O_2. After activation by ROS, ANP1 initiates a phosphorylation cascade involving two MAPKs, AtMAP4 and AtMAP6 (14, 58). Transgenic tobacco plants expressing a constitutively active ANP1 ortholog, NPK1, show improved tolerance to multiple environmental stress conditions including salinity. An *Arabidopsis* nucleoside diphosphate (NDPK2) may also positively regulate H_2O_2-mediated MAPK signaling in plants (42). So far, the SOS pathway, which controls ion homeostasis, has not been linked to ROS detoxification or ROS-mediated signaling initiated by salt stress.

Here, we describe a second site mutation, *enh1-1* (*enh*ancer *1-1*), in the *sos3-1* mutant background of *Arabidopsis* ecotype Columbia. The *enh1-1* lesion causes salt sensitivity on its own and enhances the salt sensitivity of *sos3-1*. This indicates that ENH1 plays a role in salt tolerance in a pathway separate from SOS3. Further, genetic analysis revealed that the *enh1-1* mutation did not enhance the salt sensitivity of *sos2-1*, indicating that SOS2 and ENH1 may function in the same pathway. ROS hyperaccumulate in *enh1-1*, *sos3-1 enh1-1*, *sos2-1 enh1-1*, and *sos2-1* mutant plants but not in *sos3-1* plants. The *enh1* mutant thus reveals a new pathway involved in salt tolerance that appears to connect the ion homeostasis function of the SOS pathway via SOS2 with components of oxidative stress suppression signaling. The ENH1-mediated pathway may also be under the control of SOS1.

MATERIALS AND METHODS

Isolation of the *enh1-1* mutant. *Arabidopsis* (ecotype Col gl1) *salt overly sensitive 3* (*sos3-1*) (33) mutant plants were mutagenized with *Agrobacterium tumefaciens*-mediated transferred DNA (T-DNA) transformation (strain GV3101) with the activation tagging vector pSKI015 (61). Seeds from T_2 plants were screened for mutants that exhibited increased sensitivity to 75 mM NaCl. Four-day-old seedlings grown on cellophane membrane (Bio-Rad, Hercules, CA) placed over germination medium (1× Murashige and Skoog [MS] salts [43], 2% sucrose, 1.2% agar, pH 5.7) were transferred to MS medium supplemented with 75 mM NaCl. When appropriate, seedlings were transferred to soil and grown to maturity (61).

Ion content measurements. One-week-old seedlings ($n = 40$ to 60) grown on germination medium were transferred to a 250-ml flask containing 60 ml half-strength MS salts and 2% sucrose (pH 5.7). The flasks were shaken at 100 rpm under cool fluorescent light (16-h light, 8-h dark) at 22 ± 1°C. After 1 week, NaCl was added to give the desired final NaCl concentrations, and the seedlings were allowed to grow for an additional 2 days. The seedlings were then harvested, rinsed with an excess amount of deionized H_2O, dried in an oven at 65°C for 48 h, and weighed. Ground tissues were then extracted with 0.1 M nitric acid for 4 h and filtered through Whatman no. 1 filter paper. Na^+ or K^+ content was determined by use of an atomic absorption spectrometer (model SpectrAA-10; Varian, Springvale, Australia).

Cloning of the *ENH1* gene. Genomic DNA flanking the left border of the inserted T-DNA in *sos3-1 enh1-1* plants was isolated by thermal asymmetric interlaced PCR (TAIL-PCR) and subcloned into cloning vector pBluescript SK(+) as described elsewhere (61). The entire isolated fragment was sequenced. The primers used in the TAIL-PCR were as described previously (61). We also designed the following primer pair to examine whether the T-DNA insert is homozygous or heterozygous on chromosome 5 of plants that are homozygous for the *sos3-1 enh1-1* mutant: forward, 5′-ACTCACACATTTTCGTCAATTG G-3′; reverse, 5′-GAAGTAGTGTGATGTGTGCGTTAG-3′. We have used the primers enh1-2LP, 5′-TTTTTAACTCTTGCTGCTGCAC-3′, and enh1-2RP, 5′-TTTCCTTTGAATCCTGTGCAC-3′, to identify the mutation in the *enh1-2* (SALK_018190) allele.

RT-PCR and complementation of the *sos3-1 enh1-1* mutant. Total RNA was extracted from different wild-type (Col-0) plant tissues with the RNeasy plant mini kit (QIAGEN, Valencia, CA), and 3 μg of total RNA was used for the first-strand cDNA synthesis with the thermoscript reverse transcription-PCR (RT-PCR) system (Invitrogen, Carlsbad, CA). Gene-specific primers for *ENH1* were 5′-CAAAGCTTCTTCGTTCGATCACACTAG-3′ and 5′-GGTTTGCAA TTACAAAGCGGACTTG-3′. The RT-PCR product amplified from nontreated wild-type plant tissues was subcloned into the pGEM-T Easy vector (Promega, Madison, WI), resulting in the clone ENH1-4, and the identity of the insert was confirmed by sequencing.

The coding region of *ENH1* was amplified by PCR using ENH1-4 as a template with the primers 5′-AGCTGAGCTCATGGCAACGTCTGGTGCG-3′ and 5′-AGTCTCTAGATTATTGAAGACCATAGACAAGC-3′. The PCR fragment was cloned into the binary vector 99-1 (62) under the control of the CAM 35S promoter, and the identity of the cloned insert was confirmed by sequencing. The construct was introduced into *sos3-1 enh1-1* mutant plants through *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation.

***ENH1* promoter::*GUS* construct.** A genomic fragment promoter including 2,014 bp 5′ to the ATG start codon of the *ENH1* gene was amplified by PCR with the wild-type genomic DNA with the primer pair 5′-AGTCGGATCCGTCTCC AAAACCACACCATTGTC-3′ and 5′-AGTCCTGCAGCTTTCTTTCTAGTG TGATCGAACG-3′. The PCR fragment was cloned into the binary vector pCAMBIA1381Z between the BamHI and PstI sites, and the identity of the clone insert was confirmed by sequencing. The construct was introduced into *Arabidopsis* wild-type plants (ecotype Columbia) through an *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation. β-Glucuronidase (GUS) staining was performed on tissues or organs from transgenic plants in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Rose Scientific Ltd., Cincinnati, OH) in 0.1 M potassium phosphate buffer (pH 7.5) with 0.1% Triton X-100 (25). Chlorophyll was removed with 70% ethanol.

ENH1-GFP construct. The *ENH1*-coding region was amplified by PCR with the primers 5′-AGTCTCTAGAATGGCAACGTCTGGTGCG-3′ and 5′-AGT CGAATCCGTTGAAGACCATAGACAAGC-3′, with ENH1-4 used as a template. The PCR product was cloned into vector 326-SGFP between XbaI and BamHI sites, and the entire insert and the conjunction regions were sequenced. The resulting construct was then transformed into *Arabidopsis* protoplasts, and expression of the fusion constructs was monitored as described elsewhere (44).

GST-ENH1 recombinant protein construct. To produce *Escherichia coli*-expressed GST-ENH1 recombinant protein, the coding region of ENH1 was cloned in frame between the BamHI and XhoI sites of the expression vector pGEX-4T-1. The resulting construct was transformed into *E. coli* BL21(DE3) cells to obtain the GST-ENH1 fusion protein. Rubredoxin activity assays were performed as described previously (26).

ROS detection. One-week-old seedlings were used to determine the generation of ROS in the root as described elsewhere (55). For salt treatment, the seedlings were transferred to medium containing 100 mM NaCl and allowed to grow for 12 h. The untreated and treated seedlings were incubated with 50 μM 5- (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes) for 30 min. All fluorescence images were obtained with a Leica SP2 confocal microscope (Mannheim, Germany).

Quantitative measurement of H_2O_2 production was performed with 1-week-old seedlings with an Amplex red hydrogen/peroxidase assay kit (Molecular Probes) as described elsewhere (9). For salt treatment, the seedlings were transferred to medium containing 100 mM NaCl and allowed to grow for an additional 12 h.

The detection of superoxide was performed essentially as described previously (31) with modifications. One-week-old seedlings germinated on germination medium were transferred to medium containing 0 mM or 80 mM NaCl. These seedlings were allowed to grow for one additional day and vacuum infiltrated with 0.1 mg/ml nitroblue tetrazolium (Sigma) in 25 mM HEPES buffer (pH 7.6).

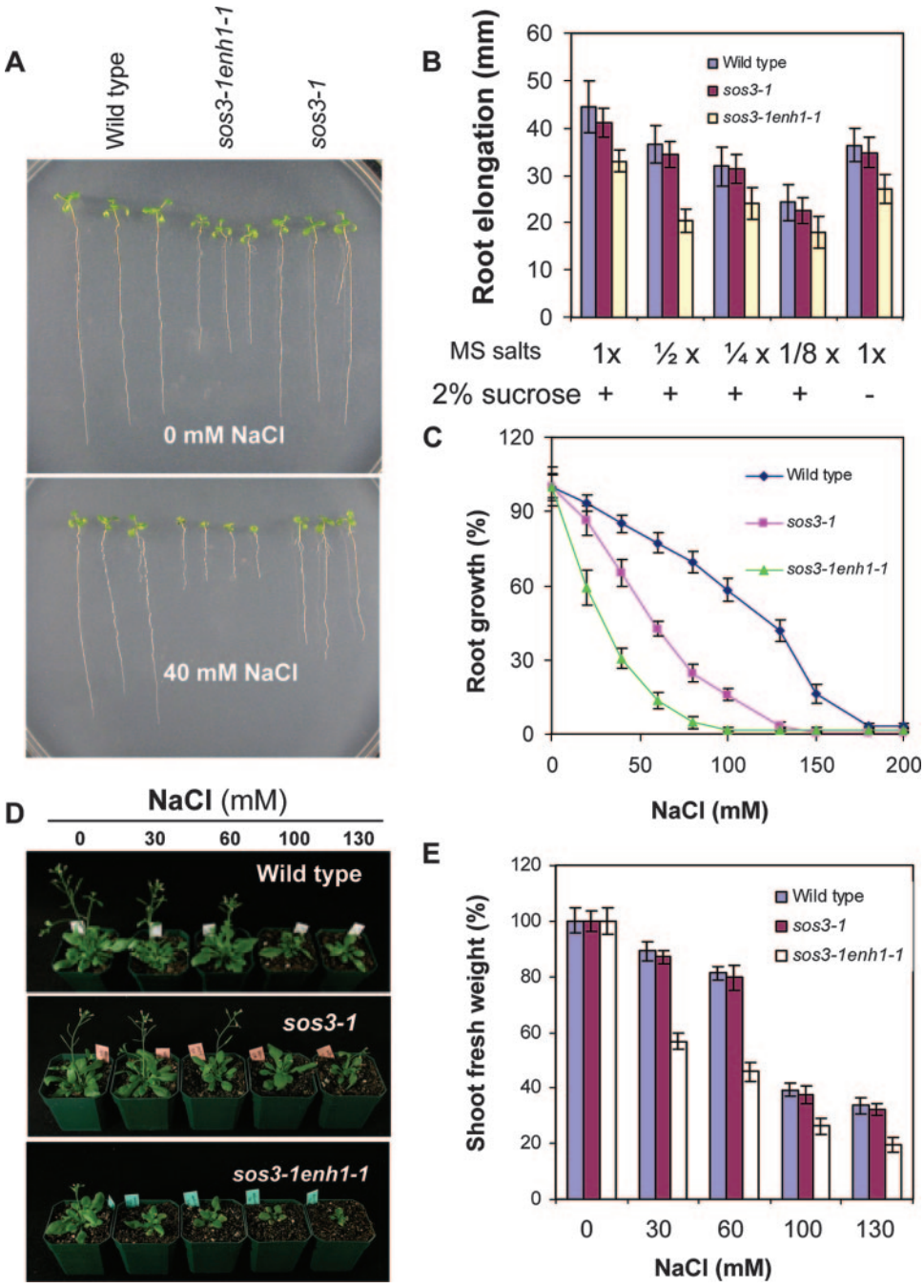

FIG. 1. The *enh1-1* mutation confers hypersensitivity of *sos3-1* to NaCl. (A) Root elongation of wild-type, *sos3-1*, and *sos3-1 enh1-1* plants with or without 40 mM NaCl. (B) Growth of plants shown in panel A in different strengths of MS medium or full-strength MS medium without sucrose. (C) Dose response of plants shown in panel A to NaCl. The root growth was presented as a proportion of growth compared to growth without NaCl (100%). (D) The *sos3-1 enh1-1* plants grown in soil are hypersensitive to salt treatment. Photographs were taken 18 days after the salt treatments. (E) Shoot fresh weight at the end of each treatment as shown in panel D. Shoot fresh weight data are shown as a percentage of shoot fresh weight in the absence of NaCl. Error bars in panels B, C, and E represent standard deviations ($n = 18$).

In a control treatment, 10 mM $MnCl_2$ and 10 units/ml superoxide dismutase were added to the buffer. Samples were incubated at room temperature in darkness for 2 h. Chlorophyll was removed with 70% ethanol.

Quantification of chlorophyll. Frozen seedlings were ground and extracted with 80% (vol/vol) acetone. Chlorophyll was quantified photometrically as described elsewhere (32).

Northern hybridization. Two-week-old seedlings grown on germination medium (1× MS salts, 2% sucrose, 0.6% agar, pH 5.7) were untreated or treated with NaCl (200 mM; 12 h). Total RNA was extracted from whole seedlings with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Total RNA was then separated on a formaldehyde-containing agarose (1.2% [wt/vol]) gel, transferred to a nylon transfer membrane (Schleicher & Schuell, Keene, NH) overnight and cross-linked. Blots were hybridized overnight (0.5 M phosphate buffer [pH 7.2], 7% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM EDTA, 2 mM bovine serum albumin) with ^{32}P-labeled probe at 60°C. Washes, 20 min each at 60°C, were performed first in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015

M sodium citrate), 0.1% SDS, then in 1× SSC, 0.1% SDS, and finally in 0.5× SSC, 0.1% SDS. The 26S rRNA gene was used as a loading control.

RESULTS

Identification of *enh1-1* mutant. T_2 seedlings from 65,000 individual T-DNA insertion lines in the *sos3-1* genetic background were screened for suppressors and enhancers of *sos3-1* hypersensitivity to NaCl. The *enh1-1* mutant was identified as conferring increased sensitivity of *sos3-1* to as little as 40 mM NaCl (Fig. 1A to C). The *sos3-1 enh1-1* double mutant grows slightly slower than *sos3-1* in medium without added NaCl (Fig. 1A). The severely sensitive phenotype of the *sos3-1 enh1-1* mutant to NaCl indicates that it even may be sensitive to salts or other aspects of the normal growth environment that affect ROS production. However, lowering the concentrations of MS salts or sucrose in the medium did not affect the growth of *sos3-1 enh1-1* relative to the other genotypes (Fig. 1B).

The *sos3-1 enh1-1* mutant plants were backcrossed with *sos3-1* plants. All resulting F_1 plants (21 of 21) exhibited a *sos3-1*-like phenotype in the presence of 60 mM NaCl. F_2 progeny from the self-crossed F_1 segregated 391:128 (*sos3-1*: *sos3-1 enh1-1*), which indicates that *enh1-1* is a monogenic recessive mutation in a nuclear gene.

The *sos3-1 enh1-1* mutant plants also displayed significantly increased sensitivity to NaCl treatment in soil compared to *sos3-1* (Fig. 1D). The double mutant gained much less fresh weight during 18 days of NaCl treatment compared to *sos3-1* plants (Fig. 1E). The salt-hypersensitive phenotype of *sos3-1* was not evident until more severe stress was applied (data not shown).

The *enh1-1* mutation enhances the K^+-deficient phenotype of the *sos3-1* mutant. Seedlings of the *sos3-1* mutant have impaired growth on medium containing low levels of K^+ (33) and require 10 mM K^+ for optimal root growth (Fig. 2A). Root elongation of *sos3-1 enh1-1* mutant seedlings was substantially less than that of *sos3-1* in medium with low K^+. The enhancement of the K^+ deficiency phenotype of *sos3-1* by the *enh1-1* mutation indicates that ENH1 is involved in controlling K^+ nutrition separately from SOS3 (Fig. 2A) and appears to function in a separate signal pathway that mediates stress tolerance.

External Ca^{2+} fails to improve the salt tolerance of the *sos3-1 enh1-1* mutant. High levels of external Ca^{2+} can ameliorate *sos3-1* sensitivity to NaCl stress, at least in part through improved K^+/Na^+ selectivity (33). Without added Ca^{2+}, the root growth of both *sos3-1* and *sos3-1 enh1-1* plants is arrested at 50 mM NaCl. Increasing the Ca^{2+} concentration to 0.5 mM or higher greatly improves the salt tolerance of *sos3-1* plants, but not the tolerance of *sos3-1 enh1-1* plants (Fig. 2B). These results further substantiate that ENH1 mediates salt tolerance by a new pathway, independent of SOS3.

The increased salt sensitivity of *sos3-1 enh1-1* involves imbalanced ion homeostasis. Without stress, both *sos3-1 enh1-1* and *sos3-1* plants accumulated slightly higher levels of Na^+ and K^+ compared to wild-type plants (Fig. 3A and B). When treated with 100 mM NaCl, *sos3-1 enh1-1* plants accumulated the highest amount of Na^+ but the lowest K^+ among the genotypes tested (Fig. 3A and B). Consequently, the ratio of K^+ to Na^+ in the *sos3-1 enh1-1* mutant plants is the lowest,

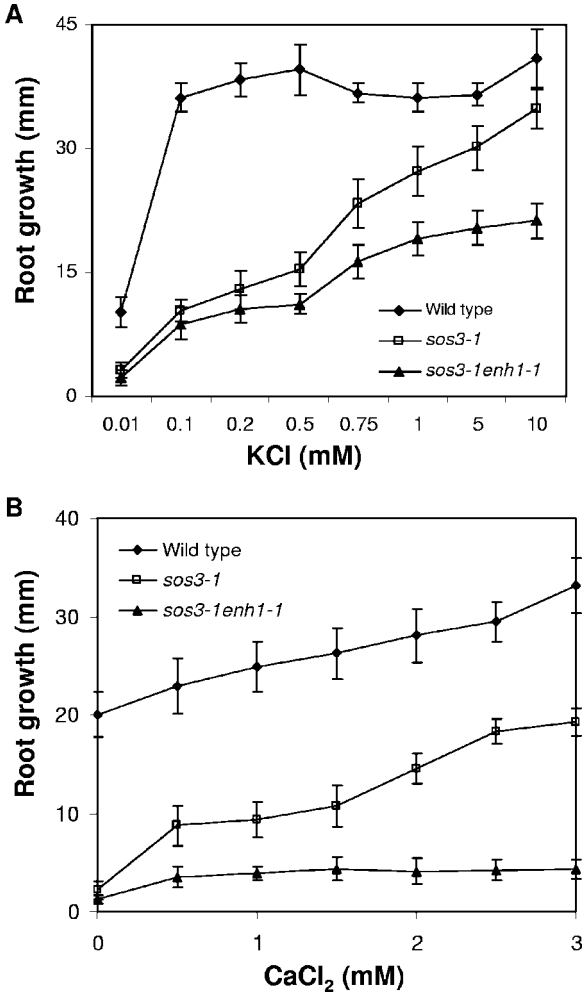

FIG. 2. Responses of *sos3-1 enh1-1* mutant plants to different levels of K^+ or Ca^{2+}. (A) Growth (over a period of 8 days) of *sos3-1 enh1-1* in medium supplemented with different levels of KCl. (B) Response (over a period of 8 days) of the *sos3-1 enh1-1* mutant to various levels of external Ca^{2+} in the presence of 50 mM NaCl. Error bars represent standard deviations ($n = 18$).

with or without NaCl stress (Fig. 3C and D). We isolated the *enh1-1* single mutant from the F_2 plants from a cross between the wild type and the *sos3-1 enh1-1* double mutant. The levels of Na^+ in *enh1* single mutants under NaCl stress were elevated compared to wild-type plants, whereas *enh1* mutants accumulated less K^+ than the wild type (Fig. 3E and F). Under normal conditions, the levels of K^+ and Na^+ accumulation as well as the ratios of K^+ to Na^+ in the *enh1* mutants are essentially the same as wild type. However, when treated with NaCl, the ratios of K^+ to Na^+ in the *enh1* mutants are significantly lower than wild type (Fig. 3H).

The *enh1-1* mutation does not enhance the salt sensitivity of *sos2-1* mutant plants. In the absence of NaCl, the growth of *sos2-1 enh1-1* is similar to that of *sos2-1* (data not shown). The growth response of *sos2-1 enh1-1* to NaCl is similar to that of *sos2-1* over various concentrations of NaCl (Fig. 4E). The inability of the *enh1-1* mutation to affect the salt sensitivity of

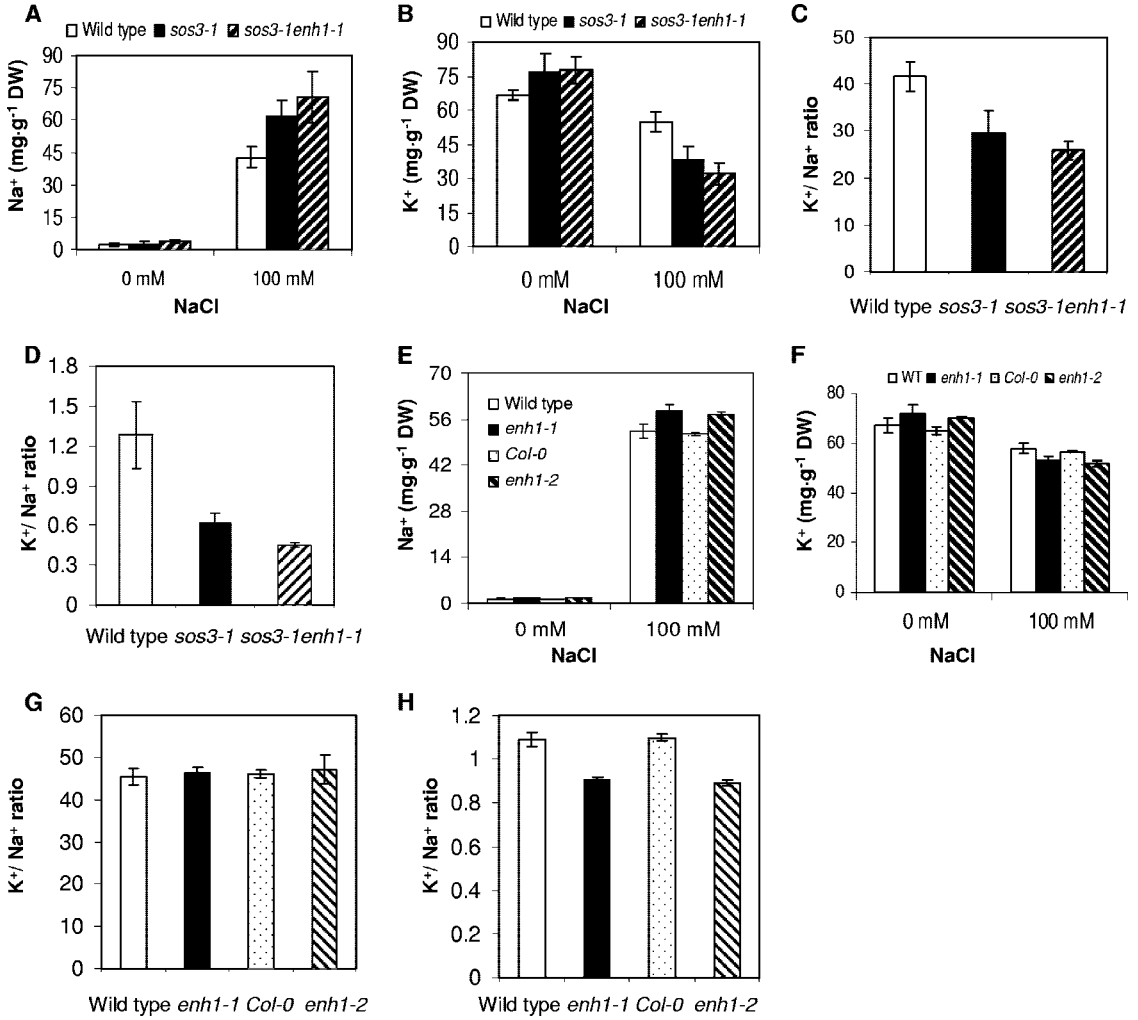

FIG. 3. Ion content in *sos3-1 enh1-1* mutant plants. (A) Na^+ content in *sos3-1 enh1-1* seedlings with or without NaCl in the medium. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test) under 100 mM NaCl. (B) K^+ content in *sos3-1 enh1-1* seedlings with or without NaCl in the medium. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test) under 100 mM NaCl. (C) Ratio of K^+ and Na^+ accumulation in the *sos3-1 enh1-1* seedlings without NaCl. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test). (D) Ratio of K^+ and Na^+ accumulation in *sos3-1 enh1-1* seedlings treated with NaCl. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test). (E) Na^+ content in *enh1* mutant seedlings with or without NaCl in the medium. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test) under 100 mM NaCl. (F) K^+ content in *enh1* mutant seedlings with or without NaCl in the medium. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test) under 100 mM NaCl. (G) Ratio of K^+ and Na^+ accumulation in *enh1* mutant seedlings without NaCl. The mean values between genotypes are not statistically significantly different ($P > 0.05$, paired *t* test). (H) Ratio of K^+ and Na^+ accumulation in *enh1* mutant seedlings treated with 100 mM NaCl. The mean values between genotypes are statistically significantly different ($P < 0.05$, paired *t* test). Error bars are standard deviations ($n = 7$). DW, dry weight.

sos2-1 plants indicates that SOS2 and ENH1 may function in a common pathway controlling salt tolerance.

***ENH1* encodes a PDZ domain containing rubredoxin-like protein.** We isolated the genomic DNA fragment flanking the left border of a T-DNA insertion in *sos3-1 enh1-1* by TAIL-PCR. Sequence analysis indicated that the T-DNA was located 636 bp upstream of the start codon of an open reading frame for At5g17170/MKP11.2 (Fig. 5A). Constitutive expression of the *ENH1* coding sequence under control of the CaMV 35S promoter in *sos3-1 enh1-1* mutant plants reversed the effect of the *enh1-1* mutation (Fig. 4A and C).

The *ENH1* gene encodes a polypeptide containing two interesting domains, a putative PDZ domain in the N-terminal region that is potentially involved in protein-protein interactions and a domain at the C-terminal part resembling rubredoxin. ENH1 shows highest sequence similarity in the rubredoxin domain with rubredoxin proteins from *Pyrococcus furiosus* (26) and *Chlorobaculum tepidum* (15) (Fig. 5B). ENH1 also shares high sequence similarity in the PDZ domain with the human PDZ domain-containing guanine nucleotide exchange factor I (Fig. 5C).

Expression of *ENH1* and localization of ENH1-GFP. *ENH1* is constitutively expressed in wild-type plants, and its expression is abolished in *sos3-1 enh1-1*, *enh1-1*, and *enh1-2* mutant plants (Fig. 5E and G). The transcript of *ENH1* is more abundant in the shoot than root (Fig. 5F). Interestingly, we ob-

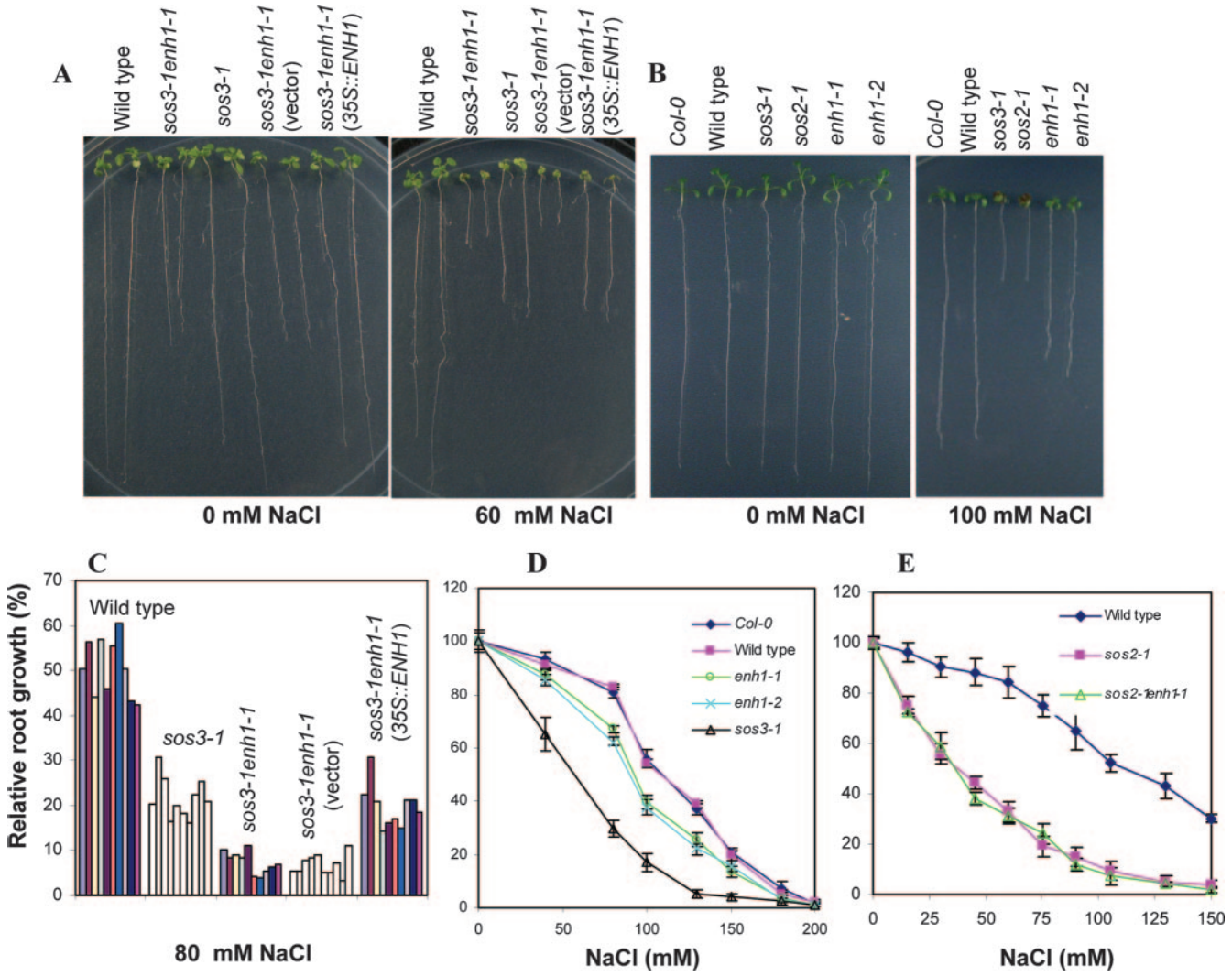

FIG. 4. Complementation of *sos3-1 enh1-1* mutant by constitutive expression of *ENH1* and *enh1* single mutants are more sensitive to NaCl than wild type. (A) Constitutive expression of *ENH1* complemented *sos3-1 enh1-1* mutant. (B) *enh1-1* and *enh1-2* plants are more sensitive to NaCl than wild type. (C) Quantification of root growth data of genotypes shown in (A). Each bar represents root growth of one individual line. (D) Growth of *Col-0*, wild type, *sos3-1*, *enh1-1* and *enh1-2* in media containing various levels of NaCl. (E) Response of *sos2-1 enh1-1* mutant plants to different concentrations of NaCl. Error bars are standard deviation ($n = 18$).

served that *ENH1* expression was also reduced in the *sos1-1* mutant under salt stress (Fig. 5G), indicating that SOS1 is a positive regulator of *ENH1* transcript accumulation. We also produced *Arabidopsis* transgenic plants expressing an *ENH1* promoter-*GUS* fusion gene. The expression of *ENH1*::*GUS* was observed in all tissues of seedlings, with weaker expression in the root tips (Fig. 5H). Strong GUS expression was also detected in the anther (Fig. 5H). The subcellular localization of ENH1 was determined by a transient expression assay of the ENH1-GFP fusion protein, which revealed that ENH1 accumulates in the chloroplast (Fig. 5D).

***enh1* single mutations also confer reduced tolerance to salt.** The *enh1-1* single mutant seedlings are more sensitive to NaCl than the wild type (Fig. 4B and D). This result indicates that ENH1 is required for normal salt tolerance. A T-DNA insertion line (SALK_018190, referred to as *enh1-2* hereafter), which has a T-DNA insertion in the At5g17170 gene (Fig. 5A), is more sensitive to NaCl than its wild-type background (Col-0) (Fig. 4B and D). Consistent with their allelic nature, F_1 plants (18 of 18) from a cross between *enh1-1* and *enh1-2* showed a level of sensitivity to 100 mM NaCl that was similar to the parental plants. Both *enh1-1* and *enh1-2* seedlings grew nearly normally compared to their wild types in control medium without stress (Fig. 4B).

ENH1 and SOS2 are involved in the control of ROS detoxification. Rubredoxins are known to act as electron carriers for various enzymes. Reports have described the involvement of rubredoxins in oxidative stress protection in *Desulfovibrio gigas*, *Desulfovibrio vulgaris*, and *Treponema pallidum* (5, 11, 13). *ENH1* encodes a chloroplast protein with a putative rubredoxin domain that may also function as an electron carrier. We assayed ENH1 recombinant protein for possible rubredoxin activity and found that it does not appear to act as an electron donor or acceptor in a super-

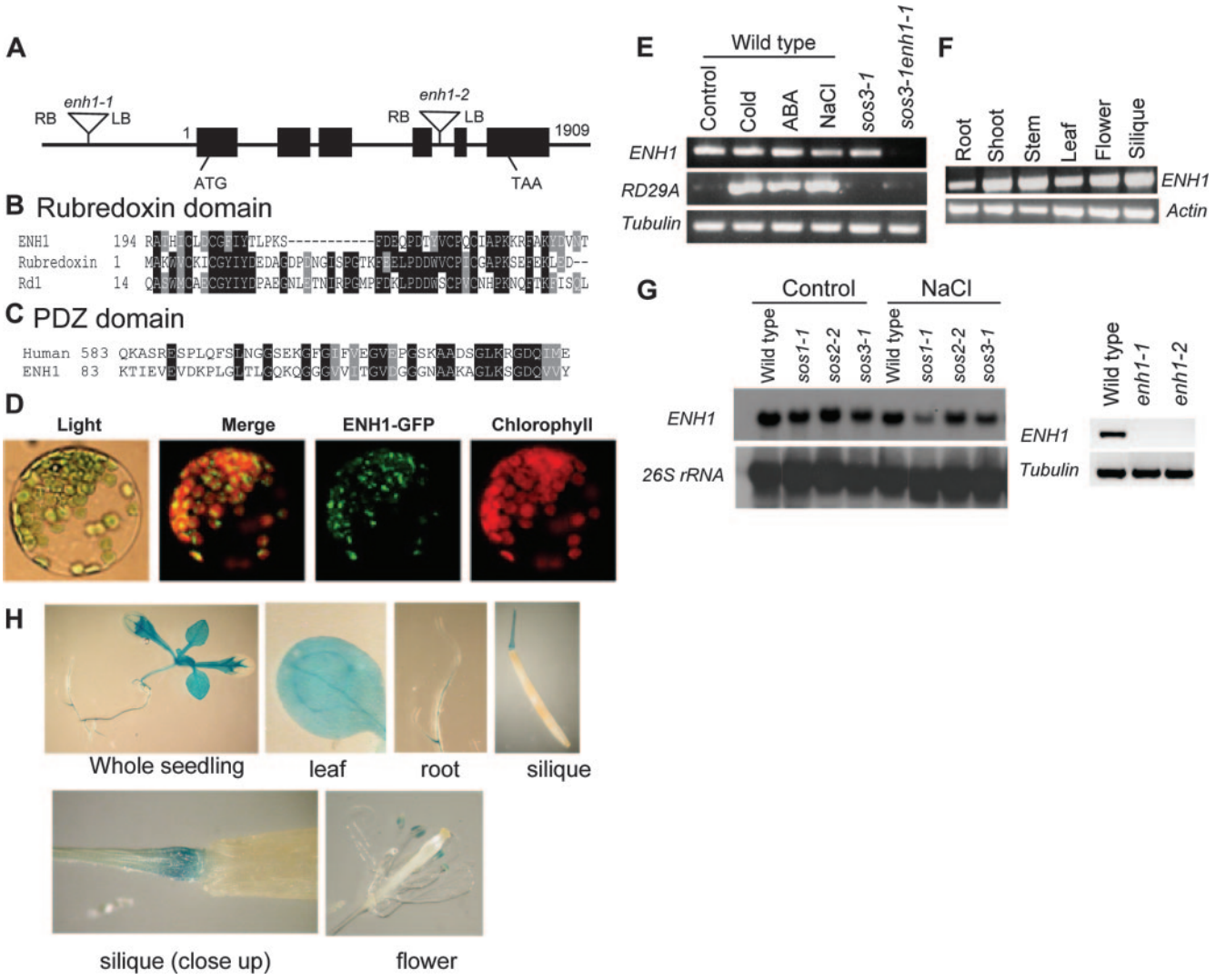

FIG. 5. *ENH1* encodes a PDZ and rubredoxin domain-containing protein. (A) Structure of the *ENH1* gene. Positions are relative to the translation start codon. Filled boxes represent the exons, and lines between filled boxes represent introns. (B) Alignment of ENH1 and other proteins in the rubredoxin domain. Proteins compared are ENH1 (accession number AAK59624) from *Arabidopsis thaliana*; rubredoxin (AAF03228), rubredoxin from *Pyrococcus furiosus*; and Rd1 (P58992), rubredoxin from *Chlorobaculum tepidum*. (C) Comparison of ENH1 with its homolog in the PDZ domain. Human (NP_057424), PDZ domain-containing guanine nucleotide exchange factor I human (*Homo sapiens*). (D) Subcellular localization of ENH1 protein. (E) The *ENH1* gene is constitutively expressed. Control, no treatment; cold, 0°C for 24 h; ABA, 100 μM for 3 h; NaCl, 300 mM for 5 h. (F) Transcript abundance of *ENH1* in different plant tissues. (G) *ENH1* expression in different genotypes by Northern analysis (left panel) and RT-PCR analysis (right panel). (H) *ENH1* promoter::*GUS* expression in transgenic *Arabidopsis* plants.

oxide reductase reaction system from *Pyrococcus furiosus* (26) (data not shown).

Nevertheless, an analysis of the ROS levels in *sos3-1 enh1-1* and *enh1-1* plants revealed that the mutants accumulate higher amounts of ROS as detected by CM-H_2DCFDA staining followed by confocal imaging (Fig. 6A). Stress treatment with 100 mM NaCl for 12 h stimulated the accumulation of ROS in wild type, *sos3-1*, *sos3-1 enh1-1*, and *enh1-1* plants. However, *sos3-1 enh1-1* and *enh1-1* plants accumulated ROS to much higher levels (Fig. 6A). Staining with nitroblue tetrazolium specifically detects superoxide radicals. As shown in Fig. 6B, under normal growth conditions, *sos3-1 enh1-1* and *enh1-1* plants have slightly more superoxide accumulation than *sos3-1* or wild-type plants. However, after treatment with 80 mM NaCl for 1 day, *sos3-1 enh1-1* and *enh1-1* plants accumulated much more superoxide than *sos3-1* plants or wild-type plants, indicating that hyperaccumulation of superoxide resulting from the *enh1-1* mutation is independent of *sos3-1* (Fig. 6B). Without stress, *enh1* plants displayed a low level of H_2O_2 similar to that in the wild type (Fig. 6C). When treated with 100 mM NaCl for 12 h, *sos3-1 enh1-1* and *enh1-1* plants all accumulated substantially higher levels of H_2O_2 than *sos3-1* or wild-type plants (Fig. 6C). Interestingly *sos2-1* mutant plants also accumulated more ROS than wild-type or *sos3-1* plants with or without NaCl stress (Fig. 6). However, the *enh1-1* mutation has no additive effect with the *sos2-1* mutation (Fig. 6), which indicates that genetically, ENH1 and SOS2 may function in a common pathway controlling ROS detoxification in response to NaCl stress.

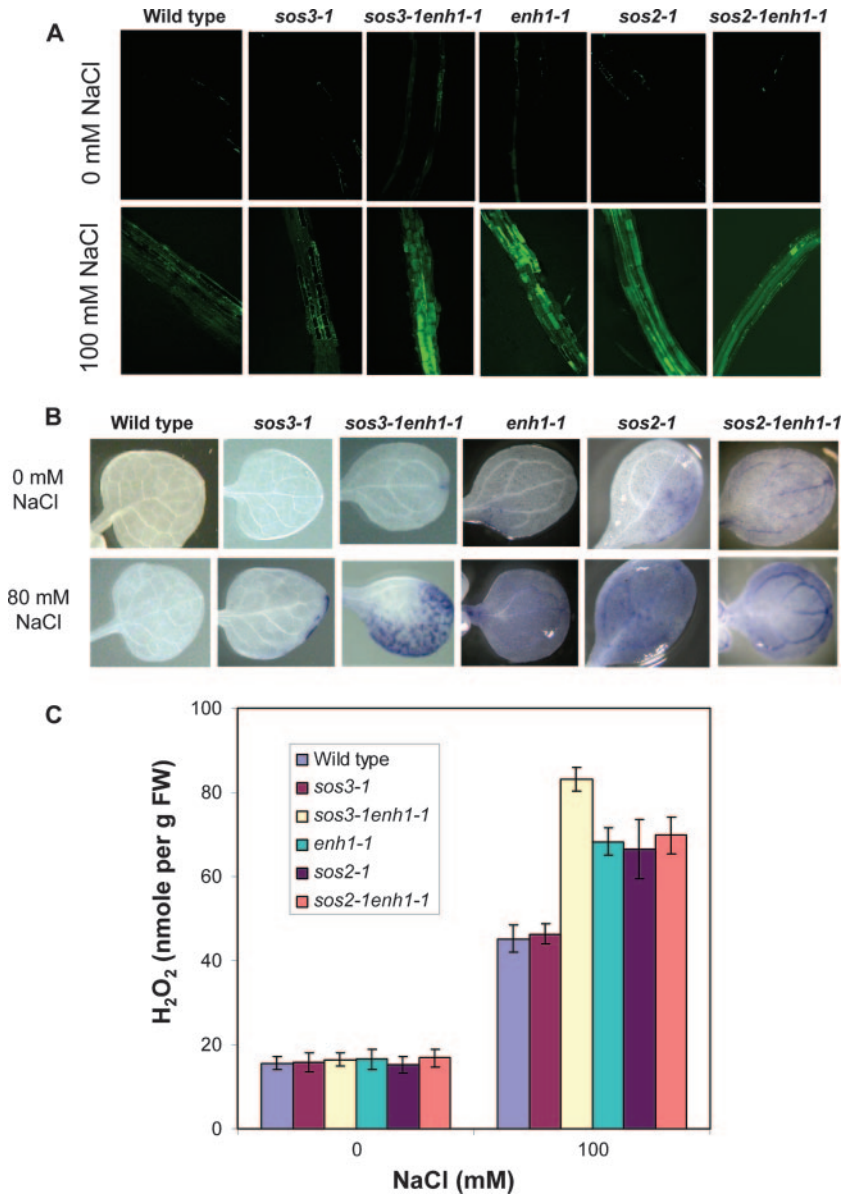

FIG. 6. Detection of ROS. (A) ROS levels in roots in different mutants after CM-H_2DCFDA staining. (B) Nitroblue tetrazolium staining for superoxide in unstressed or salt-treated (80 mM NaCl) seedlings. (C) H_2O_2 production under salt treatment. Error bars represent standard deviations ($n = 6$). FW, fresh weight.

***SOS2* and *ENH1* mediate tolerance to general oxidative stress.** We examined the tolerance of *enh1* mutants to oxidative stress caused by conditions other than NaCl stress. Wild-type, Col-0, and *sos3-1* plants can tolerate 0.5 and 1.0 μM methyl viologen treatment as indicated by their abilities to maintain chlorophyll in leaves (Fig. 7A and B). The *enh1-1* mutation enhances the sensitivity of *sos3-1* to both 0.5 and 1.0 μM methyl viologen (Fig. 7A and B). The *enh1-1*, *enh1-2*, and *sos2-1* mutant plants all showed a hypersensitive response to these stress conditions (Fig. 7A and B). In addition, the germination and postgermination growth and development of *sos3-1 enh1-1* and *enh1-1* mutant plants are more inhibited by H_2O_2 compared to *sos3-1* or wild-type control plants (Fig. 7C and D).

DISCUSSION

Several observations here indicate that ENH1 participates in a new pathway involved in salt tolerance that is at least partially separate from the SOS pathway. ENH1-mediated salt tolerance appears to involve maintaining Na^+ and K^+ ion homeostasis, since the *sos3-1 enh1-1* mutant has a decreased ability to control ion homeostasis (Fig. 3). The *enh1-1* mutation does not enhance the salt sensitivity of *sos2-1* mutant plants (Fig. 4E). This result, together with the evidence for ROS accumulation and oxidative stress sensitivity phenotypes of *sos2-1* plants (Fig. 6 and 7), suggests that the ENH1 pathway is connected with SOS2 and that ENH1 may be controlled directly or indirectly by SOS2.

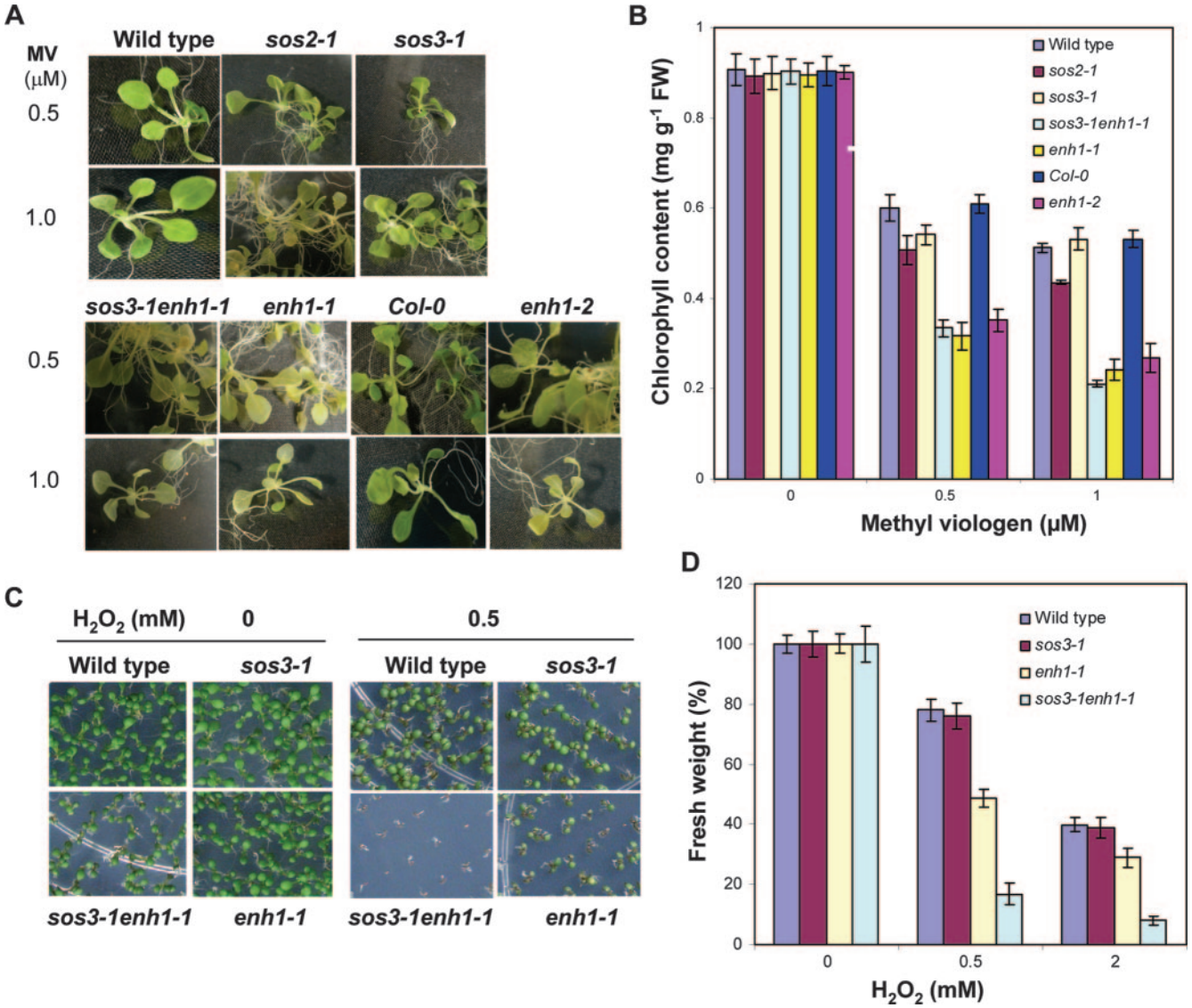

FIG. 7. *enh1* mutations cause hypersensitivity to oxidative stress. (A)Three-week-old seedlings grown in germination medium were treated with 0.5 or 1.0 μM methyl viologen under light for 4 days. Damaged seedlings exhibited a loss of chlorophyll content. (B) Quantification of chlorophyll levels of each genotype shown in (A). Error bars represent standard errors ($n = 6$). FW, fresh weight. (C) Tolerance of *enh1* mutant plants to H_2O_2. Seeds were directly sown in media containing 0.5 mM H_2O_2. (D) Fresh weight of 30 seedlings per genotype shown in (C). Error bars represent standard error ($n = 6$).

Although the salt hypersensitivity of *sos3-1* can be ameliorated by an external calcium supply (33), the rescuing effect of external calcium could not be observed with the *sos3-1 enh1-1* (Fig. 2B) mutant or *sos2* mutants. Therefore, both SOS2 and ENH1 are required for the pathway mediating the amelioration effect of external calcium. Yeast two-hybrid interaction studies have identified several SOS3-like proteins that interact with SOS2 (20). It is possible that the ENH1 pathway is important for the rescuing effect of external calcium, and it involves not only ENH1 but also SOS2 and another member of the family of SOS3-like calcium-binding proteins that may interact and activate SOS2.

The *ENH1* gene encodes a chloroplast-localized rubredoxin-like protein that shows greatest sequence similarity to rubredoxin proteins from *Pyrococcus furiosus* and *Nostoc* sp. strain PCC 7120 (Fig. 5). Rubredoxins are known as nonheme iron proteins in which a single Fe atom is covalently attached by four S atoms from cysteine residues (10). The suggested biochemical role for rubredoxin proteins is to act as a mediator of electron transfer for various enzymes (41). The rubredoxin from *Pseudomonas oleovorans* has been reported to be involved in the electron transfer pathway for the ω-hydroxylation of alkanes and fatty acids in the presence of NADH and O_2 (46), whereas the rubredoxin from *Desulfovibrio gigas* functions as an electron donor to a favohemoprotein, rubredoxin-oxygen oxidoreductase (11).

Recently, an alternative pathway for superoxide reduction has been proposed for anaerobic microorganisms, whereby the reduction of superoxide to H_2O_2 is carried out by the catalytic action of a mononuclear iron-containing enzyme, superoxide

reductase (SOR) (5, 13, 26, 27, 36–38). H_2O_2, the only product of the SOR reaction, is then likely reduced to H_2O by peroxidases or other enzymes (1, 26). This role is in sharp contrast to the role of superoxide dismutase from aerobic organisms, which converts superoxide to H_2O_2 and O_2. Rubredoxins from various anaerobes, including *Pyrococcus furiosus* and *Desulfovibrio vulgaris*, appear to function as an electron donor to SOR to avoid the production of O_2 in superoxide detoxification (7, 13, 26, 38). Rubredoxin is then reduced by the enzyme NADPH: rubredoxin oxidoreductase, with NADPH as the electron source (18, 39).

No rubredoxin activity could be detected using recombinant ENH1 protein in vitro with the SOR reaction system (26) from *Pyrococcus furiosus* (data not shown). However, we observed that the *enh1* mutant plants accumulated much higher levels of ROS than their wild-type parental plants (Fig. 6). This finding indicates that ENH1 somehow plays an important role in ROS detoxification that is most likely related to chloroplast function where ENH1 is localized.

From our results, it is clear that the ion homeostasis pathway is connected to a pathway that controls salt stress-elicited ROS detoxification. This connection involves not only ENH1 and SOS2 but also SOS1, because SOS1 appears to control *ENH1* expression under salt stress (Fig. 5G). Besides being an ion transporter regulated by SOS2, some evidence suggests that SOS1 may also have a regulatory role. SOS1 interacts with RCD1, a regulator of oxidative stress (28), and *sos1* mutant plants are also more sensitive to H_2O_2. How SOS1 regulates *ENH1* expression under salt stress is not known at the present time, although this regulation may involve RCD1.

The ENH1 pathway may also have a role in ion homeostasis regulation, since the *enh1* mutation enhances the ion homeostasis defect of *sos3-1* and blocks the rescuing effect of external calcium (Fig. 2 and 3). How ENH1 and salt stress-induced ROS regulate ion transport is unclear. However, the function of ROS in signal transduction has been extensively studied (3), and ROS signaling targets several mediators of ion transport (16, 29, 45, 55). The activity of ROS to control K^+ channels could explain both the hypersensitivity of *enh1* mutants to K^+ deficiency, and possibly even to excess Na^+, by interfering with K^+/Na^+ transport selectivity. The role of ROS in ion transport control of stomatal behavior has been particularly examined in detail also, and the function of stomata in *enh1* mutants needs to be examined more closely. In addition, ROS have been shown to affect cytoplasmic pH (59), which is crucial to the cell response to excessive Na^+. Entry of Na^+ increases cytoplasmic pH, which must be readjusted. This crucial adjustment in response to excess Na^+ may be substantially affected by excess ROS production. Although ENH1 may affect ROS and subsequently Na^+ and K^+ responses in one or more of these general manners, it may also involve a more specific function through RCD1, since RCD1 interacts with SOS1. Regardless of the exact mechanisms, the discovery of the ENH1 ROS detoxification pathway and of the cross talk between the two salt tolerance pathways opens up new approaches of research that are important for a full understanding of salt tolerance in plants. The connection between SOS1 and both ENH1 and RCD1 suggests that SOS1 may have multiple roles in stress adaptation besides its role in ion transport. The apparent multiple roles of SOS1 and SOS2 in both ion homeostasis and ROS regulation emphasize the central role of the SOS pathway in salt stress tolerance.

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