Distinct Roles for *SOS1* in the Convergent Evolution of Salt Tolerance in *Eutrema salsugineum* and *Schrenkiella parvula*

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Abstract

***Eutrema salsugineum* and *Schrenkiella parvula* are salt-tolerant relatives of the salt-sensitive species *Arabidopsis thaliana*. An important component of salt tolerance is the regulation of Na^+ ion homeostasis, which occurs in part through proteins encoded by the *Cation/Proton Antiporter-1* (*CPA1*) gene family. We used a combination of evolutionary and functional analyses to examine the role of *CPA1* genes in the salt tolerance of *E. salsugineum* and *Sc. parvula*, and found evidence that changes in *CPA1*-mediated Na^+ extrusion may contribute to the salt tolerance of both species. Specifically, we found that a member of the *CPA1* family, the Na^+/H^+ antiporter gene *Salt Overly Sensitive 1* (*SOS1*), evolved under positive selection in *E. salsugineum*. In the absence of activation by the SOS2 kinase/SOS3 calcium-binding protein complex, *SOS1* from *E. salsugineum* (*EsSOS1*) confers greater salt tolerance than *SOS1* from *Sc. parvula* (*SpSOS1*) and *Ar. thaliana* (*AtSOS1*) when expressed in a salt-sensitive strain of *Saccharomyces cerevisiae*. A single amino acid change in the putative autoinhibitory domain is required but not sufficient for the enhanced salt tolerance conferred by *EsSOS1*. When activated by SOS2 and SOS3, both EsSOS1 and SpSOS1 confer greater salt tolerance than AtSOS1. Enhanced *SOS1*-mediated Na^+ extrusion therefore appears to contribute to the salt tolerance of both *E. salsugineum* and *Sc. parvula*, although through apparently different mechanisms.**

Key words: **convergent evolution, *Eutrema salsugineum*, *CPA1*, salt tolerance, *Schrenkiella parvula*, *SOS1*.**

Article

Introduction

Arabidopsis thaliana has been a useful model for understanding mechanisms contributing to plant salt tolerance (Sanders 2000; Zhu 2000) despite its extreme sensitivity to low concentrations of salt (Orsini et al. 2010). In contrast, comparatively little is known about salt tolerance mechanisms in halophytes (salt-tolerant species) (Flowers and Colmer 2008), but mounting evidence indicates that the genes and pathways contributing to salt tolerance in glycophytic (salt-sensitive) species such as *Ar. thaliana* are present in halophytic species, and that differences in salt tolerance are at least in part attributable to modifications to these common genes and pathways (Amtmann 2009).

Understanding how halophytes evolved to tolerate saline conditions is an important first step in elucidating mechanisms that may be useful in crop species. *Eutrema salsugineum* (Pall.) Al-Shehbaz and Warwick (formerly *Thellungiella salsuginea* [Al-Shehbaz and Warwick 2005]) and *Schrenkiella parvula* (Schrenk) D. German and Al-Shehbaz (formerly *The. parvula* [German and Al-Shehbaz 2010]) are salt-tolerant relatives of *Ar. thaliana* (Orsini et al. 2010) and the agronomically important *Brassica* species, and as such have recently emerged as models for studying adaptation to salinity in plants (Bressan et al. 2001). In addition, sequenced genomes from both species now enable genome-wide studies to better understand how each species has evolved to tolerate salinity (Dassanayake et al. 2011; Wu et al. 2012; Yang et al. 2013).

The phylogenetic relationship between *E. salsugineum* and *Sc. parvula* has undergone recent revision (Koch and German 2013), altering our view of the evolution of salt tolerance in these species. *Thellungiella* housed both species (Al-Shehbaz and O'Kane 1995) before all members of the genus were transferred to *Eutrema* (Al-Shehbaz and Warwick 2005). This congeneric taxonomic designation implied a close evolutionary relationship between the species, raising the possibility of a single evolution of salt tolerance from a recent common ancestor. However, phylogenetic analyses with more dense species sampling (German et al. 2009) clearly placed *Sc. parvula* outside the genus *Eutrema*, and the species was subsequently transferred to the new genus *Schrenkiella* (German and Al-Shehbaz 2010). The phylogeny of German et al. (2009) did not include both *E. salsugineum* and *Sc. parvula*, leaving their true evolutionary relationship unclear. Recent phylogenetic analyses based on nuclear and chloroplastid markers aimed to resolve this discrepancy by including both species in a sample of over 100 Brassicaceae spanning all major lineages in the family. Results from this study indicate that *Sc. parvula* is more closely allied to the agronomically important *Brassica* species in what is designated as Lineage II, whereas *E. salsugineum* falls in a monophyletic *Eutrema* in expanded Lineage II (Yang et al. 2013). These analyses further indicate that the two species shared a common ancestor approximately 38.4 Ma. The majority of descendant species of this common ancestor are likely salt-

sensitive; therefore, the most parsimonious explanation is that *E. salsugineum* and *Sc. parvula* evolved salt tolerance independently.

A fundamental question in evolutionary biology is how species in diverse lineages can independently acquire the same trait (Wood et al. 2005; Gompel and Prud'homme 2009; Christin et al. 2010). For example, eyes (Fernald 2006; Kozmik et al. 2008; Yoshida and Ogura 2011), C_4 photosynthesis (Sage 2004; Christin et al. 2008, 2009), and secondary compound detoxification (Dobler et al. 2012) have evolved independently multiple times across several taxonomic levels. Many recent studies have begun to elucidate the molecular genetic mechanisms that underlie this convergent evolution of phenotypes. These studies have shown that convergent phenotypes can occur independently through changes at sites in nonhomologous loci (Wittkopp et al. 2003; Steiner et al. 2009), at different sites within homologous loci (Yokoyama and Radlwimmer 2001; Fernald 2006), or at the same sites within homologous loci (Christin et al. 2008; Li et al. 2010; Liu, Cotton, et al. 2010; Arnaud et al. 2011; Dobler et al. 2012). Given the likelihood that *E. salsugineum* and *Sc. parvula* evolved salt tolerance independently, we explored whether convergence plays a role in the evolution of salt tolerance in these species. More specifically, we examined genes involved in the important salt-tolerance mechanisms of Na^+ sequestration and Na^+ extrusion to test whether the same or similar changes have occurred in *E. salsugineum* and *Sc. parvula*.

The adverse effects of soil salinity on plant growth are predominantly caused by the accumulation of toxic sodium ions (Na^+) in the cytosol of plant cells. Sodium toxicity is primarily the result of inhibition of potassium (K^+)-based metabolism; tight regulation of the cytosolic K^+/Na^+ ratio is therefore critical for the plant's response to salinity stress (Niu et al. 1995; Serrano et al. 1999). Two of the primary mechanisms by which plant cells regulate the K^+/Na^+ ratio are sequestration of Na^+ in intracellular compartments such as the vacuole and extrusion of Na^+ from the cell (Munns and Tester 2008). Sodium sequestration is controlled in part through the action of proteins belonging to the *Na^+/H^+ Exchanger* (*NHX/NHE*) family. In *Ar. thaliana*, there are six *NHX/NHE* genes, designated *NHX1–NHX6*. Of these, NHX1–NHX4 are localized to the vacuolar membrane (Apse et al. 1999; Li et al. 2009; Liu, Tang, et al. 2010; Bassil, Tajima, et al. 2011) and NHX5 and NHX6 are localized to the Golgi and trans-Golgi network (Bassil, Ohto, et al. 2011). One of the most well-characterized *NHX/NHE* genes in *Ar. thaliana* is *NHX1*, with several studies showing NHX1 contributes to salt tolerance by transporting Na^+ and K^+ across the vacuolar membrane (Apse et al. 1999; Zhang and Blumwald 2001; Venema et al. 2002; Hernández et al. 2009; Leidi et al. 2010). Recent evidence suggests that *NHX1* may also be required for salt tolerance in *E. salsugineum* (Wu et al. 2009).

Sodium extrusion from plant cells is mediated in part by members of the *Na^+/H^+ antiporter P/Salt Overly Sensitive 1* (*NhaP/SOS1*) family of Na^+/H^+ exchangers. In *Ar. thaliana*, there are two *NhaP/SOS1* genes: *SOS1* (also known as *NHX7*) and *SOS1B* (also known as *NHX8*), both of which encode proteins that localize to the plasma membrane (Shi et al. 2000; An et al. 2007). *SOS1* is the most studied *NhaP/SOS1* gene; in *Ar. thaliana*, it regulates both Na^+ extrusion from the cell as well as long-distance Na^+ transport (Shi et al. 2000, 2002, 2003). *SOS1* from *E. salsugineum* (*EsSOS1*) is required for salt tolerance (Oh et al. 2007, 2009). Sodium extrusion by SOS1 is activated by members of the SOS pathway, which include SOS3, a calcium-binding protein (Liu and Zhu 1998; Liu et al. 2000) that responds to Na^+-induced increases in cytoplasmic calcium and activates SOS2, a serine/threonine protein kinase (Liu et al. 2000). Activated SOS2 phosphorylates SOS1, which releases autoinhibition of SOS1, causing the electroneutral transport of one Na^+ ion out of the cell in exchange for moving one H^+ ion down its concentration gradient into the cell (Quintero et al. 2011). Much less is known about *SOS1B*, which in *Ar. thaliana* appears to regulate tolerance to lithium (Li^+) but not Na^+ (An et al. 2007).

Although *NHX/NHE* and *NhaP/SOS1* genes collectively make up the *Cation/Proton Antiporter-1* (*CPA1*) gene family in plants (Mäser et al. 2001), they represent distinct subfamilies whose most recent common ancestor appears to be prokaryotic (Brett et al. 2005; Pardo et al. 2006; Chanroj et al. 2012; Pires et al. 2013). Here, we combined evolutionary and functional analyses to test the hypothesis that changes in *NHX/NHE* and *NhaP/SOS1* genes contribute to the salt tolerance of *E. salsugineum* and *Sc. parvula*. Specifically, we investigated the relative importance of Na^+ sequestration and Na^+ extrusion by focusing on the roles of copy number variation (CNV) and positive selection in *NHX/NHE* and *NhaP/SOS1* genes. Changes in gene copy number arise frequently (Hastings et al. 2009) and are recognized as a major factor contributing to adaptation (Schrider and Hahn 2010; Kondrashov 2012). In addition, analyses of positive selection, as measured by the nonsynonymous-to-synonymous substitution rate ratio, are an effective method to identify the molecular targets of natural selection (Anisimova et al. 2001; Yang 2002). We complemented these analytical methods with functional validation by heterologous expression of genes in yeast, an approach that has been successfully used to identify and refine gene function (Quintero et al. 2002; Martínez-Atienza et al. 2007).

Results from the present study provided no evidence for altered or enhanced *NHX/NHE*-mediated Na^+ sequestration contributing to the salt tolerance of *E. salsugineum* or *Sc. parvula*. However, we found that both species appear to have converged on enhanced Na^+ extrusion via SOS1 as a means of increasing tolerance to salinity, although the underlying mechanism conferring tolerance is different between the two species. Specifically, we found that *SOS1* evolved under positive selection in *E. salsugineum*. When activated by the complex of the SOS2 kinase and SOS3 calcium-binding protein, *EsSOS1* and *SpSOS1* confer greater salt tolerance than *AtSOS1* when expressed in a salt-sensitive strain of

Saccharomyces cerevisiae, but only *EsSOS1* confers greater salt tolerance in the absence of activation.

Results

Identification of *NHX/NHE* and *NhaP/SOS1* Homologs in the Brassicaceae

As a first step in determining whether evolutionary changes in the *NHX/NHE* and *NhaP/SOS1* genes contributed to the convergent evolution of salt tolerance in *E. salsugineum* and *Sc. parvula*, we constructed a phylogenetic tree of all putative *NHX/NHE* and *NhaP/SOS1* sequences obtained from both species as well as from related Brassicaceae species. In all, *NHX/NHE* and *NhaP/SOS1* sequences were obtained from publicly available whole-genome sequence of 10 Brassicaceae species: *Aethionema arabicum*, *Ar. thaliana*, *Arabidopsis lyrata*, *Brassica rapa*, *Capsella rubella*, *E. salsugineum*, *Leavenworthia alabamica*, *Neslia paniculata*, *Sc. parvula*, and *Sisymbrium irio*. These species form a broad phylogenetic distribution within the Brassicaceae (Beilstein et al. 2006, 2010). *NHX/NHE* and *NhaP/SOS1* homologs were also identified in the sequenced genomes of the more distantly related non-Brassicaceae species *Carica papaya* and *Citrus sinensis*. For each species, sequences showing high similarity to the six *NHX/NHE* (*NHX1–NHX6*) and the two *NhaP/SOS1* (*SOS1* and *SOS1B*) genes annotated in *Ar. thaliana* were identified. To increase the statistical power of the tests of positive selection, *SOS1* sequences were also obtained from additional species representing the phylogenetic breadth of the Brassicaceae, including *Boechera stricta*, *Boechera laevigata*, *Cardamine hirsuta*, *Turritis glabra*, *Brassica napus*, *Hesperis matronalis*, *Thlaspi arvense*, and several species closely related to *E. salsugineum* in the tribe *Eutremeae*, including *Chalcanthus renifolius*, *Eutrema altaicum*, *Eutrema heterophyllum*, and *E. salsugineum* accessions Cracker Creek and Yukon. To reconstruct the evolutionary history of these genes, all nucleotide sequences were aligned, and a phylogeny was inferred using a maximum-likelihood approach (fig. 1). The resulting phylogeny included 10 major clades; seven of the clades had bootstrap support of 89% or greater, whereas the remaining three clades resolved in the tree had lower support (65–78%). Six of the clades contain previously annotated *NHXs* from *Ar. thaliana*, whereas two clades—herein designated *NHX9* and *NHX10*—are composed of *NHX* genes found only in *Cari. papaya* and *Ci. sinensis* (*NHX10*) or in *Cari. papaya*, *Ci. sinensis*, and *Ae. arabicum* (*NHX9*). The remaining two clades contain *NhaP/SOS1* genes homologous to the *SOS1* and *SOS1B* genes from *Ar. thaliana*. Two gene pairs, *NHX1–NHX2* and *SOS1–SOS1B*, appear to have arisen as a result of the α whole-genome duplication event (Bowers et al. 2003) that predated the radiation of the Brassicaceae (Beilstein et al. 2010) (fig. 1 and supplementary fig. S1, Supplementary Material online).

CNV and Positive Selection in *NHX/NHE* Genes

To determine whether *NHX/NHE*-mediated changes in Na^+ sequestration might contribute to the salt tolerance of *E. salsugineum* and *Sc. parvula*, we first analyzed CNV in *NHX1–NHX6*. Our results indicate that *NHX/NHE* genes are single copy in both *E. salsugineum* and *Sc. parvula* (fig. 2 and supplementary figs. S2–S7, Supplementary Material online). Among the sampled species, only *L. alabamica* and *Br. rapa*, which have undergone independent whole-genome triplication events (Haudry et al. 2013), contain more than one copy of any of the *NHX/NHE* genes. These results indicate that CNV correlates with whole-genome duplication events in glycophytic species but is not associated with salt tolerance.

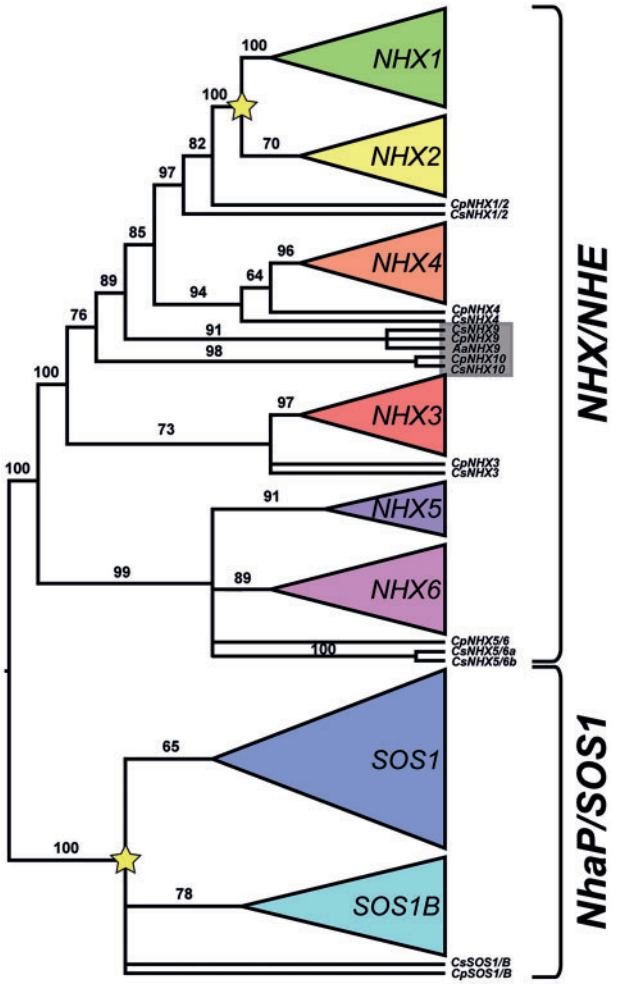


Fig. 1. Phylogenetic relationship among *NHX/NHE* and *NhaP/SOS1* orthologs in the Brassicaceae. Nucleotide sequences of genes from Brassicaceae species, *Carica papaya*, and *Citrus sinensis* were aligned by codons, and a phylogeny was inferred using maximum likelihood. Within each clade, branches for Brassicaceae species were collapsed, whereas the branches for *Cari. papaya* and *C. sinsensis* are indicated. *NHX9* and *NHX10* sequences are indicated with a gray box. Yellow stars indicate the At-α whole-genome duplication event. Values above branches represent the percent of 1,000 bootstrap replicates that support the topology. Branches with a bootstrap support value less than 60 have been collapsed. The tree was rooted on the branch containing *SOS1* and *SOS1B* and had a likelihood score of −73525.03.

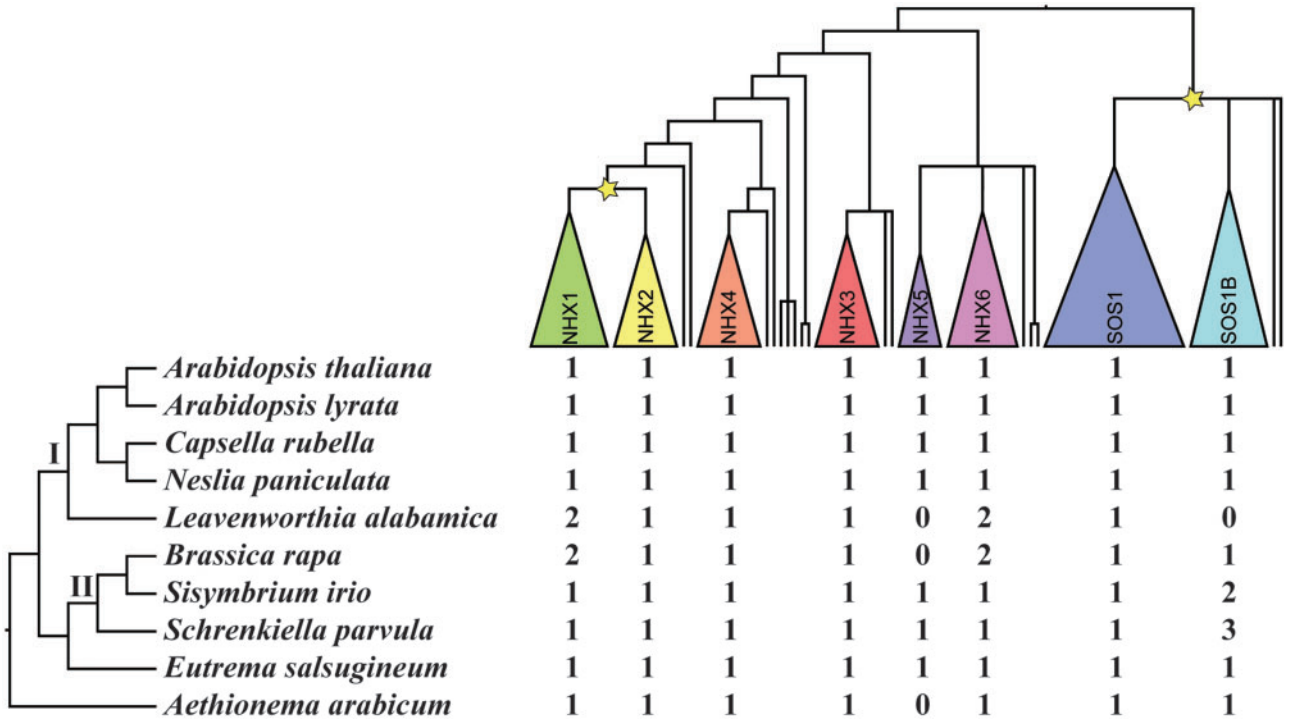


FIG. 2. *NHX/NHE* and *NhaP/SOS1* CNV in the Brassicaceae. The number of copies of each *NHX/NHE* and *NhaP/SOS1* gene for the indicated species is shown. The species are arranged in a tree that represents the accepted organismal phylogeny, with species from Lineage I (I) and Lineage II (II) indicated.

Table 1. Branch-Sites Tests for Positive Selection in Each of the *NHX1–NHX6* Lineages in *Eutrema salsugineum* and *Schrenkiella parvula*.

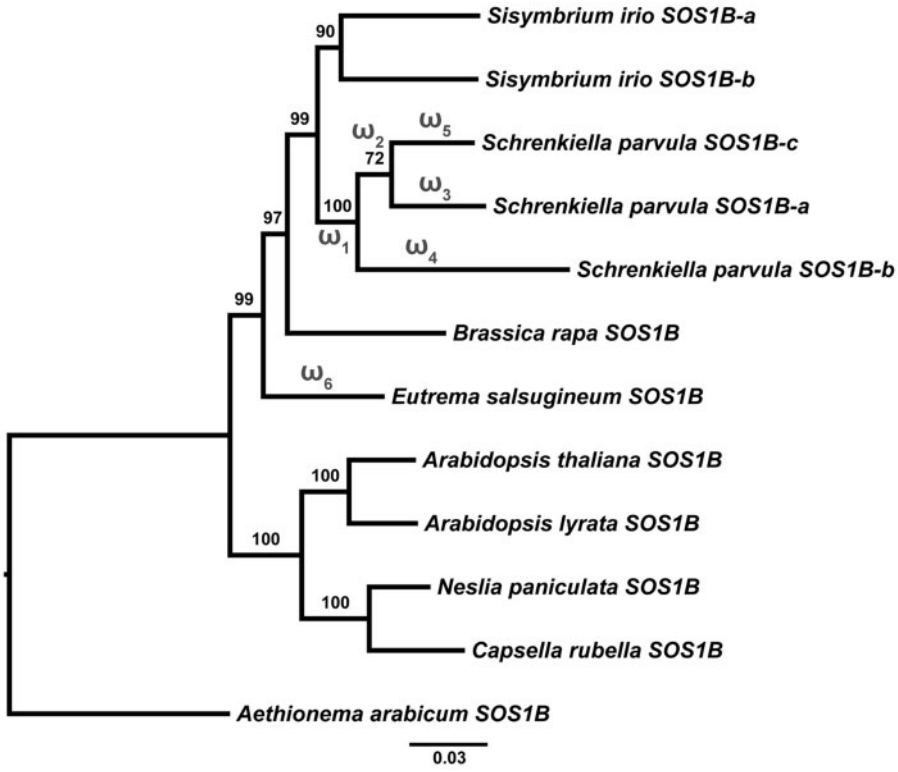
Species/Statistics	NHX1	NHX2	NHX3	NHX4	NHX5	NHX6
E. salsugineum						
Null ln L[a]	−6,013.22	−5,439.37	−5,573.84	−5,903.04	−3,407.84	−5,554.66
Alt ln L[b]	−6,013.22	−5,439.37	−5,573.84	−5,903.04	−3,407.84	−5,554.66
LRT[c]	0	0	0	0	0	0
P value[d]	1	1	1	1	1	1
Sc. parvula						
Null ln L[a]	−6,013.22	−5,439.91	−5,573.77	−5,900.04	−3,406.37	−5,553.50
Alt ln L[b]	−6,013.22	−5,439.91	−5,573.77	−5,900.02	−3,406.37	−5,553.50
LRT[c]	0	0	0	0.04	0	0
P value[d]	1	1	1	0.84	1	1

[a]Log-likelihood value of the null model.
[b]Log-likelihood value of the alternative model.
[c]Likelihood ratio test (2Δln L).
[d]P value based on 1 degree of freedom.

To understand whether changes in *NHX/NHE*-coding sequence might be correlated with salt tolerance in *E. salsugineum* and *Sc. parvula*, we performed tests of positive selection. Specifically, we tested the likelihood of different models of evolution using phylogenies (supplementary figs. S2–S7, Supplementary Material online) of each of the six *NHX/NHE* genes. For each *NHX/NHE* gene, we designated the branch leading to either *E. salsugineum* or *Sc. parvula* as the foreground branch in the branch-sites test of PAML. We found that under all tested scenarios, models incorporating a site-class where $\omega > 1$ (positive selection) did not produce statistically significant improvements in likelihood scores over models in which this site class was not present (table 1). Therefore, salt tolerance does not correlate with an elevated ratio of nonsynonymous-to-synonymous substitution rates in *NHX/NHE* genes for these species.

SOS1B Has Been Triplicated and Evolved under Positive Selection in *Sc. parvula*

We next looked for CNV in *NhaP/SOS1* genes, and found that while *E. salsugineum* and *Sc. parvula* each contain only one copy of *SOS1*, *Sc. parvula* contains three tandemly triplicated copies of *SOS1B* (figs. 2–4). However, *Sc. parvula* is not the



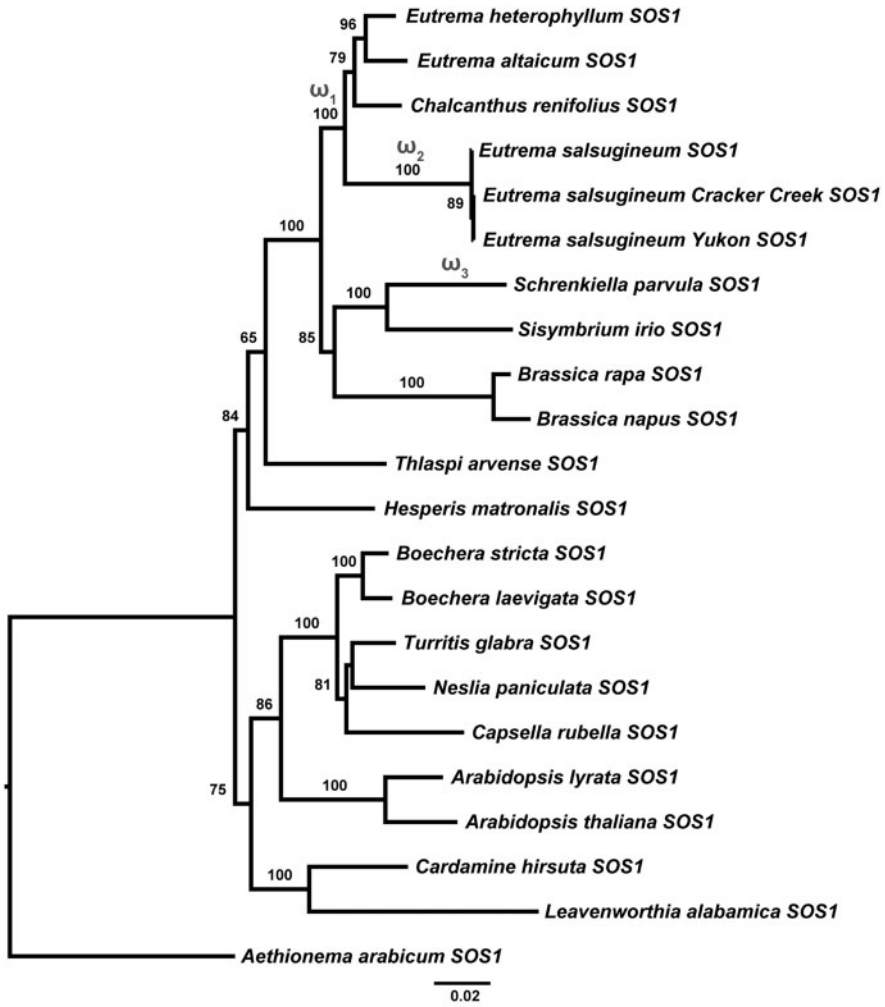
	ω_1:Sp-abc	ω_2:Sp-ac	ω_3:Sp-a	ω_4:Sp-b	ω_5:Sp-c	ω_6:Es
null lnL	-11426.41	-11424.78	-11424.12	-11423.96	-11426.41	-11426.28
alt lnL	-11426.41	-11403.13	-11426.03	-11419.88	-11426.41	-11426.28
LRT	0	43.30	0.18	8.16	0	0
p-value	1	<<0.01	0.67	<0.01	1	1

Fig. 3. Phylogenetic relationship among and tests of selection in *SOS1B* orthologs in the Brassicaceae. Nucleotide sequences were aligned by codons, and a phylogeny was inferred using maximum likelihood. The tree is rooted on *Aethionema arabicum*. Values above branches represent the percent of 1,000 bootstrap replicates that support the topology. Scale bar indicates the number of substitutions per site. The branches tested in branch-sites tests of selection for *EsSOS1B* and the *SpSOS1B* genes are indicated. The inset table shows the results of the branch-sites tests, with a *P* value based on 1 degree of freedom. ln *L*, log-likelihood values; alt, alternative model; LRT, likelihood ratio test ($2\Delta \ln L$).

only species in which *SOS1B* shows evidence of gene copy expansion; *Si. irio* also contains two copies of *SOS1B* that appear to have arisen through an independent duplication event (figs. 2 and 3). We performed branch-sites tests of positive selection to determine whether neofunctionalization in one or more copies of *Sc. parvula SOS1B* (*SpSOS1B*) might explain the retention of paralogs. Although we found no evidence of positive selection on the branch leading to all three *SpSOS1B* genes (fig. 3 and table 2), we identified positive selection on the single branch leading to *SpSOS1B-b*. Similarly, a statistically significant signature of positive selection was inferred along the branch uniting *SpSOS1B-a* and *SpSOS1B-c*. In contrast, neither branch leading to each of these genes showed evidence of positive selection. We found no evidence of positive selection along the branch leading to *E. salsugineum SOS1B*. These results indicate that evolution in *SpSOS1B* is characterized by a history of duplication followed by positive selection.

SOS1 Evolved under Positive Selection in *E. salsugineum*

SOS1 is a single-copy gene in *E. salsugineum*, *Sc. parvula*, and *Ar. thaliana*. Previous results indicate that *EsSOS1* confers greater salt tolerance in yeast than *SOS1* from *Ar. thaliana* (*AtSOS1*) (Oh et al. 2009). We looked for evidence of positive selection in *EsSOS1* and *SOS1* from *Sc. parvula* (*SpSOS1*) to determine whether changes at the protein level might underlie the enhanced salt tolerance conferred by *EsSOS1*, and to determine whether *SpSOS1* might also confer greater salt tolerance. Using branch-sites tests, we found statistically significant evidence of positive selection on the branch leading to *EsSOS1* but not on the branch leading to *SpSOS1* (fig. 4). This selection occurs specifically on the branch leading to *E. salsugineum* and does not occur in the deeper branch leading to all *Eutrema* species. This suggests that the salt tolerance of *E. salsugineum* may at least in part be derived from changes in cellular extrusion of Na^+ mediated by *SOS1*.



	ω_1:Eutrema	ω_2:Es	ω_3:Sp
null lnL	-18046.76	-18046.69	-18045.09
alt lnL	-18046.76	-18041.32	-18045.09
LRT	0	10.74	0
p-value	1	<<0.01	1

Fig. 4. Phylogenetic relationship among and tests of selection in *SOS1* orthologs in the Brassicaceae. Nucleotide sequences were aligned by codons, and a phylogeny was inferred using maximum likelihood. The tree is rooted on *Aethionema arabicum*. Values above branches represent the percent of 1,000 bootstrap replicates that support the topology. Scale bar indicates the number of substitutions per site. The branches tested in branch-sites tests of selection for the *Eutrema* clade, *EsSOS1*, and *SpSOS1* are indicated. The inset table shows the results of the branch-sites tests, with a *P* value based on 1 degree of freedom. ln *L*, log-likelihood values; alt, alternative model; LRT, likelihood ratio test (2Δln *L*).

Table 2. Amino Acid Sites Showing a Signature of Positive Selection in the *SpSOS1B-ac* Branch or in the *SpSOS1B-b* Branch.

SpSOS1B-ac		*SpSOS1B-b*	
Site in *SpSOS1B-a*	BEB	Site in *SpSOS1B-b*	BEB
142	0.565	340	0.909
144	0.990	622	0.544
145	0.893	737	0.849
146	0.600	738	0.835
147	0.836	739	0.507
148	0.978		
688	0.646		
734	0.546		

NOTE.—BEB, Bayes emphirical Bayes.

The branch-sites test of PAML employs a Bayes empirical Bayes (BEB) approach to determine sites that are likely to have experienced positive selection. In total, there are 29 sites in which the amino acid encoded by *EsSOS1* is unique; 9 of these occur at sites in which the amino acid is conserved in all other species, whereas 20 occur at variable sites (table 3). Two of the nine unique sites occurring at conserved positions showed a weak signature of positive selection by BEB (table 4 and supplementary fig. S8, Supplementary Material online); these were the only two sites identified in the BEB analysis. In comparison, 36 total sites in *SpSOS1* encode unique amino acids; 12 of these occur at positions in which the amino acid is conserved in all other species, whereas 24 occur in positions where several different amino acids are encoded by the other

Table 3. The Number and Type of Conserved and Variable Sites in the *SOS1* Amino Acid Alignment.

Category	Number	Positions
Conserved[a]	684	
Variable	487	
Unique in *Esa*, conserved in all other taxa	9	
Unique in *Esa*, variable in all other taxa	20	
Unique in *Spa*, conserved in all other taxa	12	
Unique in *Spa*, variable in all other taxa	24	
Shared, unique in both *Esa* and *Spa*, conserved in all other taxa	0	
Shared, unique in both *Esa* and *Spa*, variable in all other taxa	2	1061, 1073 (*Esa*) 1063, 1075 (*Spa*)
Different, unique in both *Esa* and *Spa*, conserved in all other taxa	2	953, 997 (*Esa*) 955, 999 (*Spa*)
Total	1,171	

NOTE.—*Esa*, *Eutrema salsugineum*; *Spa*, *Schrenkiella parvula*.

[a]Includes any site represented by only one amino acid, even if some taxa include gaps.

Table 4. Amino Acid Sites Showing a Signature of Positive Selection in *EsSOS1*, and the Corresponding Orthologous Sites in *AtSOS1*.

Site	aa	Site	aa	BEB
EsSOS1		*AtSOS1*		
944	L	948	M	0.522
1044	E	1046	V	0.745

NOTE.—aa, amino acid; BEB, Bayes emphirical Bayes.

species (table 3). The inability to detect positive selection in *SpSOS1* is therefore not likely due to a lack of unique changes in *SpSOS1*.

EsSOS1 Confers Greater Salt Tolerance than *SpSOS1* in Yeast in the Absence but not in the Presence of *SOS2* and *SOS3*

Evidence of positive selection in *EsSOS1* suggests that its function might be enhanced in this salt-tolerant species. To determine whether *EsSOS1* is able to confer greater salt tolerance than *SOS1* from other species—even from other salt-tolerant species such as *Sc. parvula*—we compared the ability of *EsSOS1*, *SpSOS1*, and *AtSOS1* to confer salt tolerance to a salt-sensitive mutant strain of the budding yeast *Sa. cerevisiae*. The *Sa. cerevisiae* strain AXT3K (*Δena1::HIS3::ena4*, *Δnha1::LEU2*, *Δnhx1::KanMX4*) (Quintero et al. 2002) lacks the P-type ATPases ENA1–ENA4, the $Na^+(K^+)/H^+$ antiporter NHA1, and the vacuolar Na^+/H^+ antiporter 1, and is consequently extremely sensitive to Na^+ ion stress. AXT3K has been used as a system for characterizing the ability of different Na^+ transporters to restore yeast growth on media containing Na^+ (Quintero et al. 2002; Martínez-Atienza et al. 2007; Quan et al. 2007; Oh et al. 2009). To compare the salt tolerance conferred by *EsSOS1*, *SpSOS1*, and *AtSOS1*, cDNA sequences from all three genes were expressed in AXT3K. In plants, the SOS1 antiporter exhibits the highest Na^+ transport capability when activated by SOS2 and SOS3 (Qiu et al. 2002), so *AtSOS2* and *AtSOS3* cDNA sequences were also transformed into yeast for coexpression with the *SOS1* genes. In the absence of NaCl, there was no difference in growth among yeast coexpressing *AtSOS2* and *AtSOS3* with *EsSOS1*, *SpSOS1*, or *AtSOS1* (fig. 5*A*). When grown in the presence of 125 or 200 mM NaCl, *EsSOS1* conferred greater salt tolerance than *AtSOS1*. However, *EsSOS1* did not confer substantially greater salt tolerance than *SpSOS1*. To verify that this was not an artifact of altered activation of EsSOS1 and SpSOS1 by SOS2 and SOS3 from a different species (*Ar. thaliana*), each *SOS1* gene was also coexpressed with its native *SOS2* and *SOS3* genes. When activated by their own *SOS2* and *SOS3* genes, *EsSOS1* and *SpSOS1* conferred similar levels of salt tolerance (both still greater than the salt tolerance conferred by *AtSOS1*) (fig. 5*A*).

EsSOS1 has also been shown to confer greater salt tolerance in yeast than *AtSOS1* in the absence of activation by *SOS2* and *SOS3* (Oh et al. 2009). We therefore tested whether *EsSOS1* confers greater salt tolerance than *SpSOS1* in the absence of activation by *SOS2* and *SOS3*. In the presence of 25 mM NaCl, *EsSOS1* conferred greater salt tolerance than both *AtSOS1* and *SpSOS1* (fig. 5*B*). Together, these results indicate the important yet distinct roles of *EsSOS1* and *SpSOS1* in two aspects of Na^+ extrusion: both *EsSOS1* and *SpSOS1* contribute to enhanced Na^+ extrusion mediated by activated SOS1, whereas *EsSOS1* additionally contributes to enhanced Na^+ extrusion in the absence of activation by SOS2 and SOS3.

One Site under Selection in *EsSOS1* Is Required but not Sufficient for Enhanced Salt Tolerance Conferred by *EsSOS1* in the Absence of Activation by *SOS2* and *SOS3*

We used site-directed mutagenesis to determine whether the sites under selection in *EsSOS1* (table 4) contribute to the enhanced salt tolerance conferred by unactivated EsSOS1 in yeast. Specifically, the leucine (L) at position 944 and the glutamic acid (E) at position 1044 were mutated to encode for the methionine (M) and valine (V), respectively, that are found in all other species in our data set (L944M/E1044V). We also made corresponding changes to the orthologous sites in

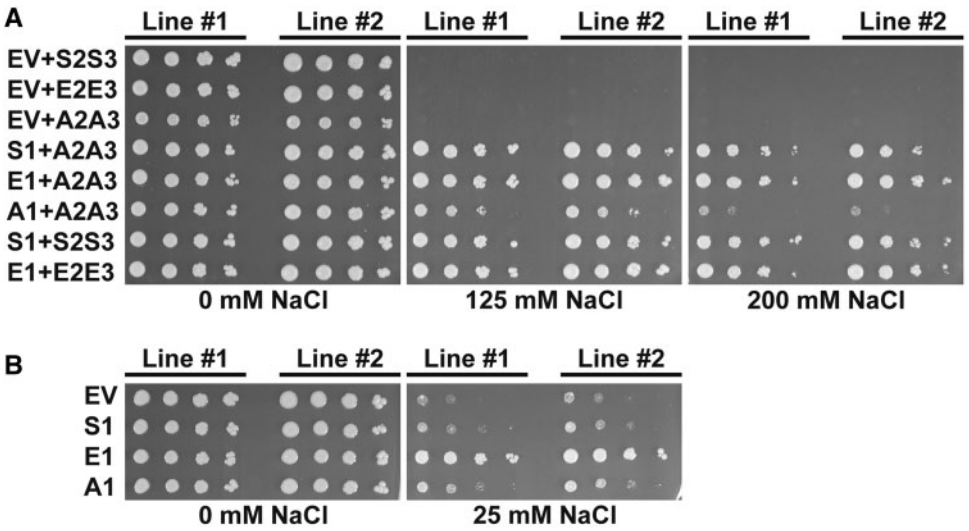


Fig. 5. Functional comparisons of *AtSOS1*, *EsSOS1*, and *SpSOS1* in salt-sensitive yeast. The salt-sensitive *Saccharomyces cereviseae* strain AXT3K was transformed with empty vector (EV) or vector containing *SpSOS1* (S1), *EsSOS1* (E1), or *AtSOS1* (A1). For each construct, two lines were plated in serial dilutions on control media (0 mM NaCl) or media containing the indicated concentrations of NaCl for 4 days. (*A*) Complementation of AXT3K with *SpSOS1*, *EsSOS1*, or *AtSOS1* coexpressed with *AtSOS2* (A2) and *AtSOS3* (A3), *SpSOS2* (S2) and *SpSOS3* (S3), or *EsSOS2* (E2) and *EsSOS3* (E3). (*B*) Complementation with *SpSOS1*, *EsSOS1*, or *AtSOS1* alone.

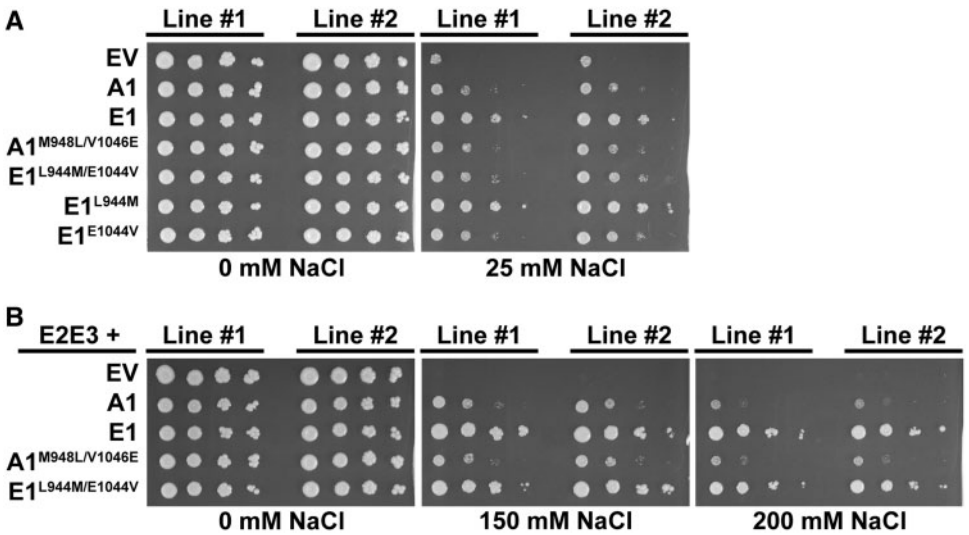


Fig. 6. Mutagenesis of sites under selection in *EsSOS1* and expression in yeast. *AtSOS1* (A1) was mutagenized at positions 948 and 1046 ($A1^{M948L/V1046E}$) to encode the amino acids from the orthologous 944 and 1044 sites in *EsSOS1* (E1); likewise, *EsSOS1* was mutagenized at sites 944 and 1044 ($E1^{L944M/E1044V}$) to encode the amino acids from sites 948 and 1046 in *AtSOS1*. (*A*) Wild-type *SOS1*, mutagenized *SOS1*, or *EsSOS1* mutagenized individually at sites 944 ($E1^{L944M}$) or 1044 ($E1^{E1044V}$) was expressed in the salt-sensitive *Saccharomyces cereviseae* strain AXT3K. (*B*) Wild-type or the mutagenized *SOS1* genes were coexpressed with *EsSOS2* and *EsSOS3* in AXT3K. For (*A*) and (*B*), two lines per construct were plated in serial dilutions on control media (0 mM NaCl) or media containing the indicated concentrations of NaCl for 4 days. EV, empty vector.

AtSOS1 (positions 948 and 1046) to encode for the *EsSOS1* amino acids (M948L/V1046E). If these sites contribute to the enhanced growth conferred by *EsSOS1* in the absence of *SOS2* and *SOS3*, then the mutated $EsSOS1^{L944M/E1044V}$ gene should confer less salt tolerance than *EsSOS1*, whereas the mutated $AtSOS1^{M948L/V1046E}$ should confer greater salt tolerance than *AtSOS1*. We expressed *AtSOS1*, *EsSOS1*, $AtSOS1^{M948L/V1046E}$, and $EsSOS1^{L944M/E1044V}$ in yeast in the absence of *SOS2* and *SOS3*. In the presence of 25 mM NaCl, there was no difference in growth between yeast expressing *AtSOS1* and yeast expressing $AtSOS1^{M948L/V1046E}$; however, we found that yeast expressing $EsSOS1^{L944M/E1044V}$ grew less than yeast expressing wild-type *EsSOS1* and similarly to wild-type *AtSOS1* (fig. 6*A*). To determine whether changes at each site individually are sufficient to alter growth, we mutated sites 944 and 1044 individually. When these mutated $EsSOS1^{L944M}$ and $EsSOS1^{E1044V}$ genes were expressed in yeast, the former grew similarly to yeast expressing wild-type *EsSOS1*, whereas

yeast expressing the latter grew less, again similarly to *AtSOS1*. These results indicate that the amino acid change at position 1044 in *EsSOS1* is required but not sufficient for the enhanced growth conferred by unactivated EsSOS1 in yeast.

EsSOS1 also confers enhanced salt tolerance in the presence of activation by SOS2 and SOS3. To determine whether the sites showing a signature of positive selection in *E. salsugineum* (table 2) underlie this trait, we coexpressed the wild-type and mutated *SOS1* sequences with *EsSOS2* and *EsSOS3* in yeast and found that there was no difference in growth in the presence of NaCl between yeast expressing *EsSOS1* and yeast expressing $EsSOS1^{L944M/E1044V}$ or between yeast expressing *AtSOS1* and yeast expressing $AtSOS1^{M948L/V1046E}$ (fig. 6*B*). These results suggest that these two sites alone do not underlie the enhanced growth conferred by activated EsSOS1 in yeast. *SpSOS1* also confers enhanced salt tolerance in the presence of activation by SOS2 and SOS3. It is possible that the sites underlying this trait are shared between *EsSOS1* and *SpSOS1* but not among the other species in our data set. By analyzing the *SOS1* multiple sequence alignment, we identified four sites in which *EsSOS1* and *SpSOS1* encode for either the same unique amino acid or two different yet unique amino acids relative to all other *SOS1* sequences (table 3). These four sites are candidates for future functional studies to determine whether they affect salt tolerance.

Discussion

We present evolutionary and functional evidence that *E. salsugineum* and *Sc. parvula*, two Brassicaceae species that diverged approximately 38 Ma (Yang et al. 2013), likely converged on salt tolerance in part through enhancement of Na^+ extrusion mediated by *NhaP/SOS1* genes. We showed that both *EsSOS1* and *SpSOS1* are able to confer enhanced salt tolerance when coexpressed with *SOS2* and *SOS3* (fig. 5*A*). This is an example of convergent evolution in which changes in the phenotype (enhanced Na^+ extrusion mediated by activated SOS1) converged on the same orthologous gene (*SOS1*) in two species. We also showed that *EsSOS1* confers greater salt tolerance than *SpSOS1* in the absence of activation by SOS2 and SOS3 (fig. 5*B*). This is an example of evolution in which the phenotype (enhanced Na^+ extrusion mediated by unactivated SOS1) is unique to one species (*E. salsugineum*) and involved changes to a gene (*EsSOS1*) without corresponding changes to a homologous locus in a second species. One of the sites under selection in *EsSOS1* (site 1044) is required but not sufficient for the enhanced growth conferred by unactivated EsSOS1 (fig. 6*A*). Second, we found that *SOS1B* has been triplicated in *Sc. parvula* (fig. 3). The retention of the triplicated *SpSOS1B* copies may be due to altered function consistent with a signature of positive selection (fig. 3). Third, in contrast to the results of our analysis of *NhaP/SOS1* genes, we found no variation in copy number and no correlation between positive selection in *NHX/NHE* genes and salt tolerance in either *E. salsugineum* or *Sc. parvula* (fig. 2 and table 1).

These results have important implications for understanding the role of *CPA1* genes in general—and *SOS1* in particular—in plant salt tolerance, and establish several hypotheses that can now be functionally tested. First, the observation that *EsSOS1* confers greater salt tolerance than either *AtSOS1* or *SpSOS1* when expressed in yeast in the absence of *SOS2* and *SOS3*, together with the finding that the amino acid change at site 1044 is required but not sufficient for this phenotype, raises the possibility that *EsSOS1* confers greater salt tolerance due to differences in protein activation. Based on orthology to *Ar. thaliana*, site 1044 resides in the autoinhibitory domain that regulates the activation of SOS1 by SOS2 and SOS3 (Quintero et al. 2011). In *Arabidopsis*, the autoinhibitory domain binds to the activation domain in the absence of salt stress, limiting Na^+ transport through the transmembrane domains. In the presence of NaCl, the SOS2/SOS3 complex phosphorylates a conserved serine residue in the autoinhibitory domain of SOS1, which releases autoinhibition and allows for increased Na^+ transport. Similar evidence for autoinhibition from the C-terminus has been found in SOS1 from wheat (Feki et al. 2011). Because site 1044 contributes to enhanced salt tolerance in the absence of activation by SOS2 and SOS3, it may alter the interaction between the activation and autoinhibitory domains, resulting in relaxed autoinhibition and an increased ability of unactivated EsSOS1 to transport Na^+. This hypothesis is consistent with Oh et al. (2009), which found no difference in the Na^+/H^+ exchange rate between yeast plasma membrane vesicles expressing *AtSOS1* or *EsSOS1*.

The present study also has important implications for understanding the function of *SOS1B*. Following the duplication event that gave rise to *SOS1* and *SOS1B* in the Brassicaceae, the *SOS1B* lineage evolved under positive selection whereas the *SOS1* lineage did not (supplementary fig. S1 and tables S1 and S2, Supplementary Material online). Although very little is known about the function of *SOS1B*, this result is consistent with a model of neofunctionalization (Innan and Kondrashov 2010). We found that *SOS1B* from *Sc. parvula* has been triplicated and the resulting copies evolved under positive selection. In *Ar. thaliana*, *SOS1B* appears to primarily transport Li^+ (An et al. 2007), although it is unknown whether the SpSOS1B proteins also transport Li^+. Whether *SOS1B* copy expansion confers increased Li^+ tolerance in *Sc. parvula* is not known; however, the current distribution of *Sc. parvula* is in a region containing soils reportedly high in Li^+ (Hamzaoğlu and Aksoy 2009; Oh et al. 2012).

Finally, the lack of CNV and positive selection in *NHX/NHE* genes in either *E. salsugineum* or *Sc. parvula* raises the possibility that Na^+ sequestration mediated by these genes does not play as important a role in salt tolerance in these species as Na^+ extrusion mediated by *NhaP/SOS1* genes. However, because the ability to detect positive selection is dependent on taxon sampling, it is possible that the inclusion of additional taxa would enable detection of positive selection in these genes. Moreover, we cannot rule out that adaptive mechanisms that are undetectable by analyses of CNV and positive selection—such as changes in gene regulation—may still play a role in the evolution of salt tolerance via *NHX/NHE* genes in *E. salsugineum* and *Sc. parvula*. Although the contribution of *NHX/NHE* genes to salt tolerance in these species is unclear, our results suggest that future functional studies

should be focused on characterizing differences in regulation rather than differences in function.

The results presented here suggest that alterations to *SOS1*-mediated Na^+ extrusion may contribute to the ability of *E. salsugineum* and *Sc. parvula* to grow in the presence of salt, although through apparently different mechanisms. Future studies focusing on in planta validation of these findings will contribute to our understanding of mechanisms that can be altered in crop species to enhance salt tolerance. The importance of research to increase the salt tolerance of crop species has risen dramatically over the last several decades. Soil salinity is an important agricultural problem that is estimated to affect more than 800 million hectares of land worldwide, including 45 million hectares of the 230 million hectares of irrigated farmland (Munns and Tester 2008).

Materials and Methods

Species Sampling

Sequences for all *NHX*/*NHE* and *NhaP*/*SOS1* genes were identified from the complete genome sequences of *Ae. arabicum*, *Ar. lyrata*, *Ar. thaliana*, *Br. rapa*, *Cap. rubella*, *Cari. papaya*, *Ci. sinensis*, *E. salsugineum*, *L. alabamica*, *N. paniculata*, *Sc. parvula*, and *Si. irio*. For *Ar. lyrata* (Hu et al. 2011), *Br. rapa* (Wang et al. 2011), *Cari. papaya* (Ming et al. 2008), *Ci. sinensis* (Xu et al. 2013), and *E. salsugineum* (Yang et al. 2013), sequences were retrieved from the Phytozome database (www.phytozome.net, last accessed May 8, 2014). For *Ar. thaliana* (Arabidopsis Genome Initiative 2000) and *Sc. parvula* (Dassanayake et al. 2011), sequences were obtained from the arabidopsis.org and thellungiella.org websites, respectively. Sequences from the recently published genomes (Haudry et al. 2013; Slotte et al. 2013) of *Ae. arabicum*, *Cap. rubella*, *L. alabamica*, and *Si. irio* were obtained from GenBank. Sequences from the draft genome assembly of *N. paniculata* (Slotte et al. 2013) were kindly provided by Dr Stephen Wright. For all species, *NHX*/*NHE* and *NhaP*/*SOS1* genes were identified through reciprocal BLAST searches with *Ar. thaliana* using a significance threshold *E* value of 10^{-20}, and homology was ultimately assigned by aligning the sequences and inferring their phylogenetic relationship, as described below.

Additional *SOS1* sequences were obtained for *Br. napus* and *Bo. stricta* from GenBank, and for *Bo. laevigata*, *Card. hirsuta*, *Ch. renifolius*, *E. altaicum*, *E. heterophyllum*, *H. matronalis*, *Thl. arvense*, and *Tu. glabra* from in-house sequencing. *SOS1* was also sequenced in-house from *E. salsugineum* accessions Cracker Creek and Yukon (seed kindly provided by Dr Thomas Mitchell-Olds). Information about all sequences used in this study, including accession numbers, is available in supplementary table S3, Supplementary Material online.

DNA Extraction, Amplification, and Sequencing

DNA was isolated as previously described (Beilstein et al. 2008). All in-house sequencing was performed by first amplifying *SOS1* in the species described above using degenerate primers designed from the *Ar. thaliana*, *Br. napus*, and *E. salsugineum SOS1* cDNA sequences using the default parameters of Primaclade (Gadberry et al. 2005). Primer sequences are listed in supplementary table S4, Supplementary Material online. Two overlapping fragments were amplified from genomic DNA for each *SOS1* gene. Amplification was performed using a step-down protocol modified from Beilstein et al. (2008), as follows: 1) 94 °C for 5 min, 2) 94 °C for 1 min, 3) 63 °C for 2 min, 4) 72 °C for 3.5 min, 5) go to Step 2 two more times, 6) 94 °C for 1 min, 7) 60 °C for 2 min, 8) 72 °C for 3.5 min, 9) go to Step 6 two more times, 10) 94 °C for 1 min, 11) 57 °C for 2 min, 12) 72 °C for 3.5 min, 13) go to Step 10 two more times, 14) 94 °C for 1 min, 15) 54 °C for 2 min, 16) 72 °C for 3.5 min, 17) go to Step 14 two more times, 18) 94 °C for 1 min, 19) 51 °C for 2 min, 20) 72 °C for 3.5 min, 21) go to Step 18 two more times, 22) 94 °C for 1 min, 23) 48 °C for 1 min, 24) 72 °C for 3.5 min, 25) go to Step 22 twenty-four more times, 26) 72 °C for 20 min, and 27) 4 °C hold. The purified products from at least two independent polymerase chain reaction (PCR) reactions were either directly sequenced or sequenced after being cloned into the TOPO-XL plasmid (Life Technologies, Grand Island, NY, USA). Sequences were obtained from only one strand.

Multiple Sequence Alignments and Phylogenetic Inference

Individual multiple sequence alignments of Brassicaceae sequences were made for *NHX1*, *NHX2*, *NHX3*, *NHX4*, *NHX5*, *NHX6*, *SOS1*, and *SOS1B*. In addition, *SOS1* and *SOS1B* Brassicaceae sequences were combined and aligned together with the *Cari. papaya* and *Ci. sinensis* homologs. All of these alignments were initially performed using ClustalX 2.1 (Larkin et al. 2007) and were then manually edited in Mesquite 2.75 (Maddison and Maddison 2011) based on the intron/exon boundaries of *AtSOS1*. Phylogenies based on the coding regions (exons) of these multiple sequence alignments were inferred with RAxML 7.2.6 (Stamatakis 2006) using maximum likelihood and assuming a general time reversible + gamma (GTR + Γ) model of nucleotide evolution. Support for nodes in the tree was assessed by inferring phylogeny in 1,000 bootstrap replicates. Additionally, the nucleotide alignments of individual genes were translated to amino acid sequences, and all combined amino acid sequences from the sampled Brassicaceae species as well as from *Cari. papaya* and *Ci. sinensis* were aligned using MAFFT 7 (Katoh and Standley 2013) and manually edited in Mesquite. This amino acid alignment was then used to guide the alignment of the corresponding nucleotide sequences. A phylogeny was inferred from nucleotide sequences of all combined genes using RAxML with the GTR + Γ model of nucleotide evolution. All multiple sequence alignments and trees are available on the TreeBase database (http://purl.org/phylo/treebase/phylows/study/TB2:S14872, last accessed May 8, 2014).

Analyses of Positive Selection

Analysis of the ratio (ω) of nonsynonymous substitutions per nonsynonymous site (d*N*) to synonymous substitutions per synonymous site (d*S*) is a common method to identify the type of selective pressure acting on protein-coding genes

(Kimura 1980; Li et al. 1985; Nei and Kumar 2000), with $\omega > 1$ indicative of positive selection, $\omega = 1$ indicative of neutral evolution, and $\omega < 1$ indicative of purifying selection. We performed branch-site tests of positive selection (Zhang et al. 2005) using maximum-likelihood models (Yang and Bielawski 2000; Yang 2002) implemented in the codeml program of PAML 4 (Yang 2007). In this test, the alternative model (Model A) allows for four classes of sites in both the lineage being tested for positive selection (the foreground branch) and the remaining lineages (the background branch). Site classes 0 and 1 allow for codons with $0 < \omega_0 < 1$ and $\omega_1 = 1$, respectively, in both the foreground and background branches. The two remaining site classes, designated 2a and 2b, allow for codons with $0 < \omega_0 < 1$ and $\omega_1 = 1$, respectively, in the background branch while both 2a and 2b allow for $\omega_2 > 1$ in the foreground branch. The null model for this test is Model A with $\omega_2 = 1$ fixed. For the individual phylogenies of each of the eight *NHX*/*NHE* and *NhaP*/*SOS1* genes (lacking *Cari. papaya* and *Ci. sinensis* sequences), the branch sites test of PAML 4 was used to test for positive selection in either *E. salsugineum* or *Sc. parvula* by designating branches leading to each species as the foreground branch. The likelihood of these alternative models was compared with that of null models by performing a likelihood ratio test (LRT; defined as twice the log likelihood difference between the null and alternative models) and calculating corresponding *P* values for each LRT. Sites showing a signature of positive selection were identified by calculating the posterior probability that a site belongs to a class with $\omega > 1$ using a BEB approach (Yang et al. 2005) in PAML 4.

Tests of selection were also performed with the duplicated gene pair *SOS1*–*SOS1B* using the combined phylogeny of *SOS1* and *SOS1B* (including *Cari. papaya* and *Ci. sinensis*). The branch test of selection (Yang 1998; Yang and Nielsen 1998) was implemented in PAML 4 using the codeml program to test different models of evolution following the duplication event. The likelihood of models was compared using the LRT and by calculating corresponding *P* values. Because the branch test supported a model in which *SOS1* and *SOS1B* have evolved differently post-duplication, additional tests of positive selection were performed using the branch-site test in PAML 4, and putative sites under positive selection were identified using a BEB approach, as described above.

Yeast Salt Screens

To compare the salt tolerance conferred by *AtSOS1*, *EsSOS1*, and *SpSOS1*, RNA was isolated from 2-week-old seedlings of *Ar. thaliana*, *E. salsugineum*, and *Sc. parvula* (seeds kindly provided by Dr Dong-Ha Oh), respectively, using the Qiagen RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). RNA samples were treated with TURBO DNase (Ambion, Austin, TX, USA) and purified using the Qiagen RNeasy MinElute Cleanup Kit. cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies, Grand Island, NY, USA), and RNA was removed using RNaseH (Life Technologies).

Full-length coding sequences of *AtSOS1*, *EsSOS1*, and *SpSOS1* were amplified from cDNA prepared from *Ar. thaliana*, *E. salsugineum*, and *Sc. parvula*, respectively. Purified *AtSOS1*, *EsSOS1*, and *SpSOS1* PCR products were cloned into *Xba*I–*Xma*I, *Xba*I–*Kpn*I, and *Xba*I–*Eco*RI sites, respectively, of pYPGE15 (Brunelli and Pall 1993) (kindly provided by Dr Alonso Rodriguez-Navarro), under control of the yeast *PGK1* promoter. The pFL32T plasmid containing full-length *AtSOS2* and *AtSOS3* cDNA sequences (Quintero et al. 2002) was kindly provided by Dr Javier Quintero. This plasmid was modified to create a plasmid containing *EsSOS2* and *EsSOS3* (pFLE3E2T) or *SpSOS2* and *SpSOS3* (pFLS3S2T). To create pFLE3E2T, full-length cDNA coding sequences of *EsSOS2* and *EsSOS3* were amplified with flanking *Eco*RI–*Eco*RI and *Xho*I–*Not* sites, respectively, and were subsequently cloned into pGEM-T Easy (Promega, Madison, WI, USA). *EsSOS3* was then subcloned into *Xho*I–*Not*I sites of pDR195 (Rentsch et al. 1995) (kindly provided by Dr Alonso Rodriguez-Navarro). A *Not*I–*Age*I fragment from the resulting plasmid was subsequently used to replace the *AtSOS3*-containing *Not*I–*Age*I fragment from pFL32T to create pFLE32T. Finally, *Eco*RI was used to release *AtSOS2* from pFLE32T and replace it with the *EsSOS2*-containing *Eco*RI fragment from pGEM-T Easy to create pFLE3E2T. An identical approach was used to create pFLS3S2T using *SpSOS2* and *SpSOS3* amplified from *Sc. parvula* cDNA. All PCR amplifications were performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA) and primers listed in supplementary table S4, Supplementary Material online. The sequences of all cloned genes were verified by sequencing to ensure that all sequences were correct.

Plasmids containing the cloned *SOS* genes were transformed into AXT3K cells. For experiments involving only *SOS1*, the pYPGE15 plasmids containing *AtSOS1*, *EsSOS1*, or *SpSOS1* were transformed separately into AXT3K, and transformed cells were selected on media lacking uracil. For experiments involving coexpression of *SOS1* with *SOS2* and *SOS3*, AXT3K containing one of the pYPGE15 plasmids was transformed separately with pFL32T, pFLE3E2T, or pFLS3S2T, and transformed cells were selected on media lacking both uracil and tryptophan. Salt-stress assays were carried out in the alkali cation-free medium AP (Rodríguez-Navarro and Ramos 1984) containing 1 mM KCl and the designated concentrations of NaCl. Two independent lines of yeast containing each construct were spotted in serial dilutions of 10^4, 10^3, 10^2, and 10^1 cells on each plate and cultured at 30 °C for 4 days.

Site-Directed Mutagenesis

The nucleotides encoding for amino acids 948 and 1046 in *AtSOS1* and 944 and 1044 in *EsSOS1* were mutated using the GeneArt Site-Directed Mutagenesis System (Life Technologies, Grand Island, NY, USA), following the manufacturers guidelines. Specifically, the ATG codon at positions 2842–2844 (amino acid 948) in the *AtSOS1* cDNA sequence cloned into pYPGE15 (see above) was changed to CTG; the GTT codon at positions 3136–3138 (amino acid 1046) in

AtSOS1 was changed to GAA. In the *EsSOS1* cDNA sequence cloned into pYPGE15, the CTG and GAA codons at positions 2830–2832 (amino acid 944) and 3130–3132 (amino acid 1044), respectively, were changed to ATG and GTT, respectively. The mutagenized sequences were verified by sequencing. Primers used for mutagenesis are listed in supplementary table S4, Supplementary Material online.

Supplementary Material

Supplementary tables S1–S4 and figures S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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