

RESEARCH ARTICLE

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# Different evolutionary histories of two cation/proton exchanger gene families in plants

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## Abstract

**Background:** Gene duplication events have been proposed to be involved in the adaptation of plants to stress conditions; precisely how is unclear. To address this question, we studied the evolution of two families of antiporters. Cation/proton exchangers are important for normal cell function and in plants, Na<sup>+</sup>,K<sup>+</sup>/H<sup>+</sup> antiporters have also been implicated in salt tolerance. Two well-known plant cation/proton antiporters are NHX1 and SOS1, which perform Na<sup>+</sup> and K<sup>+</sup> compartmentalization into the vacuole and Na<sup>+</sup> efflux from the cell, respectively. However, our knowledge about the evolution of *NHX* and *SOS1* stress responsive gene families is still limited.

**Results:** In this study we performed a comprehensive molecular evolutionary analysis of the *NHX* and *SOS1* families. Using available sequences from a total of 33 plant species, we estimated gene family phylogenies and gene duplication histories, as well as examined heterogeneous selection pressure on amino acid sites. Our results show that, while the *NHX* family expanded and specialized, the *SOS1* family remained a low copy gene family that appears to have undergone neofunctionalization during its evolutionary history. Additionally, we found that both families are under purifying selection although *SOS1* is less constrained.

**Conclusions:** We propose that the different evolution histories are related with the proteins' function and localization, and that the *NHX* and *SOS1* families are examples of two different evolutionary paths through which duplication events may result in adaptive evolution of stress tolerance.

## Background

It has been argued that gene duplications underlie mechanisms to achieve stress adaptation [1,2]. There is little evidence, however, to support this. Additionally, it is not known if there is one or multiple ways of achieving stress adaptation through gene duplication. To address this question, we focused our attention on salt tolerance, and the evolutionary histories of two gene families involved in this trait.

Salt tolerance is a complex trait that is thought to have originated multiple times in plants [3], and it is important to understand the nature and molecular evolution of salt tolerance mechanisms throughout the history of land plants. This will be key not only to our understanding of how plants adapt against the disruptive effects of high salt concentrations in the soil, but also to point out directions

for possible crop improvement. Indeed farmers are increasingly facing loss of crop production due to this abiotic stress, since currently > 20% of the world's arable land is affected by high salinity [4].

Cation/proton exchangers are essential to the normal function of the cell. Besides helping regulating internal pH, cell volume, and cytoplasmic ion homeostasis [5-7], these transporters have also been shown to be involved in vesicular trafficking and protein targeting [8,9]. In plants, Na<sup>+</sup>,K<sup>+</sup>/H<sup>+</sup> antiporters are also associated with salt tolerance [10,11], and a series of studies have targeted these cation exchangers to improve agronomically important crops [12,13].

All Na<sup>+</sup>,K<sup>+</sup>/H<sup>+</sup> exchangers belong to a superfamily of monovalent cation/proton antiporters (CPA) and are divided in two families, CPA1 and CPA2 [14]. The CPA1 family is composed of two main groups, one containing plasma membrane-bound proteins and the other intracellular proteins [14]. Within these two groups are well-characterized antiporters associated with salt tolerance

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in plants, the salt overly sensitive 1 (SOS1) and sodium hydrogen exchanger (NHX) proteins. SOS1 proteins are localized in the plasma membrane [15,16], and are responsible for Na<sup>+</sup> efflux from the cell. NHX proteins, on the other hand, are intracellular proteins that compartmentalize Na<sup>+</sup> and K<sup>+</sup> in the vacuole [17-19].

In *Arabidopsis thaliana*, the NHX protein family has six members that are classified into two classes [17]. The class I proteins, AtNHX1-4, are localized in the tonoplast [17] and have equal affinity for Na<sup>+</sup> and K<sup>+</sup> [7,20]. Class II proteins include AtNHX5 and 6, which are localized in the endosomal compartment of the Golgi and trans-Golgi network [18] and have a higher affinity for K<sup>+</sup> compared to Na<sup>+</sup> [6].

Previous reports have shown that the *Arabidopsis* NHX genes which encode these proteins have different expression patterns and responsiveness to abiotic stresses [21,22]. *AtNHX1* and 2 were shown to be expressed at high levels in all plant tissues, while *AtNHX3* and 4 were mainly expressed in root and flower tissues, respectively [21,22]. *AtNHX5* was also expressed in all tissues but at lower levels [21,22], while *AtNHX6* expression was detected only in shoots and roots by RT-PCR [22]. In addition, while *AtNHX1-3* were shown to be responsive to both salt stress and abscisic acid (ABA) [22,23], *AtNHX5* was only responsive to salt stress, suggesting that its response is ABA-independent [22]. Moreover, all NHX genes promoted the recovery of a salt sensitive yeast mutant [21,22] and numerous studies have shown that overexpression of *Arabidopsis* NHX1 and 5, or rice NHX1 (among others), resulted in increased salt tolerance in transgenic plants [10,24-27].

The other protein that characterizes this cation/proton exchanger family is SOS1, which belongs to the well-known salt tolerance Salt-Overly-Sensitive (SOS) pathway [28-30]. Salt stress elicits a cytosolic calcium signal, which functions as a major secondary-messenger signalling molecule. A myristoylated calcineurin B-like protein (SOS3) senses the salt-elicited calcium signal, and upon Ca<sup>2+</sup> binding SOS3 undergoes dimerization and enhances the serine/threonine protein kinase activity of SOS2. The SOS3/SOS2 complex is targeted to the plasma membrane and activates SOS1 [28-30].

SOS1 has been called NHX7 by several authors, and was thus thought to be part of the NHX gene family [12,16]. Previous studies, however, regarding the phylogeny of cation/hydrogen transporters [14,31] suggest that it is distinct from the other NHX proteins and should more appropriately retain its designation as SOS1. For this reason, we refer to the *Arabidopsis* proteins SOS1/NHX7 and NHX8 (highly similar to, but shorter than SOS1) as SOS1A and SOS1B, respectively. SOS1B, like SOS1A, is localized to the plasma membrane [15,16]. While SOS1A is a Na<sup>+</sup>/H<sup>+</sup> antiporter [32], however,

SOS1B seems to only transport Li<sup>+</sup> [16]. In addition, the tonoplast exchangers in *Arabidopsis* are also regulated by the SOS pathway, specifically by SOS2 [33], thus suggesting coordination between tonoplast and plasma membrane antiporters.

The importance of these two families of proteins in plant salt tolerance is well established. By promoting the efflux of Na<sup>+</sup>, SOS1A helps protect the cell from the deleterious effects of this ion. Additionally, SOS1A seems to have an important role in long-distance Na<sup>+</sup> transport, thus helping to regulate the Na<sup>+</sup>/K<sup>+</sup> ratio in roots and shoots [17]. On the other hand, NHX family members have been described as essential to Na<sup>+</sup> compartmentalization in the vacuole, protecting the cell from the deleterious effects of this ion and maintaining cytoplasmic ion homeostasis [10,34]. Recent studies, however, suggest instead that increased salt tolerance of NHX over-expressing plants result from an improved ability to retain K<sup>+</sup> after stress induction [35-37].

Despite the key roles these genes play in salt tolerance in plants, little is known about the evolutionary histories of both gene families. Understanding the evolution of Na<sup>+</sup>,K<sup>+</sup>/H<sup>+</sup> antiporters can help clarify the mechanisms leading to plant stress adaptation associated with gene duplication events [1,2,38]. In this study, we report a comprehensive molecular evolutionary analysis of both the NHX and SOS1 plant protein families. We reconstructed the phylogeny and the history of duplication events for each family, as well as determined the selection pressure on amino acid sites within these proteins. Our purpose here is not to identify novel NHX and SOS1 plant genes, or to do a phylogeny analysis of the entire monovalent CPA gene family, as in others [39]. Instead, we show that the NHX and SOS1 gene families have very different evolution trajectories and suggest that these divergent evolutionary histories are related to the evolution of their function and cellular localization. Finally, we suggest that the NHX and SOS1 families represent examples of two different paths in the molecular evolution of stress tolerance.

## Results and discussion

### Phylogeny of the NHX family exhibits multiple independent duplication events

We selected 121 genes in 33 taxa that appear, or are described, to belong to the NHX family. The distribution of NHX genes among the various species is shown in Table 1. Among these genes, 108 are found in 28 angiosperm species, 2 in 1 gymnosperm taxa, and 9 in 2 non-seed plant taxa. We also identified 1 sequence from the alga *C. reinhardtii*, and we used 1 sequence from the budding yeast *S. cerevisiae* as an outgroup. The NHX phylogeny agrees with the classification of these proteins in two distinct evolutionary groups. We obtained two main clades, with ~99% bootstrap support, which

**Table 1 Species and number of NHX and SOS1 sequences used in this study**

	Species	Number of putative NHX sequences	Number of putative SOS1 sequences
<b>Yeast</b>	<i>Sacharomyces cerevisiae</i>	1	-
<b>Alga</b>	<i>Chlamydomonas reinhardtii</i>	1	-
<b>Moss</b>	<i>Physcomitrella patens</i>	7	2
<b>Spikemoss</b>	<i>Selaginella moellendorffii</i>	2	2
<b>Gymnosperm</b>	<i>Picea sitchensis</i>	2	-
<b>Angiosperms</b>	<i>Brachypodium distachyon</i>	5	2
<b>Monocots</b>	<i>Oryza sativa</i>	5	1
	<i>Setaria italica</i>	5	1
	<i>Zea mays</i>	5	1
	<i>Sorghum bicolor</i>	4	1
<b>Angiosperms</b>	<i>Aquilegia coerulea</i>	3	2
<b>Dicots</b>	<i>Mesembryanthemum crystallinum</i>	3	1
	<i>Atriplex gmelinii</i>	1	-
	<i>Atriplex dimorphostegia</i>	1	-
	<i>Suaeda salsa</i>	1	-
	<i>Salicornia europaea</i>	1	-
	<i>Chenopodium glaucum</i>	1	-
	<i>Kalidium foliatum</i>	1	-
	<i>Mimulus guttatus</i>	6	1
	<i>Vitis vinifera</i>	5	1
	<i>Eucalyptus grandis</i>	4	2
	<i>Citrus clementina</i>	5	1
	<i>Citrus sinensis</i>	3	1
	<i>Carica papaya</i>	4	1
	<i>Thellungiella halophila</i>	1	2
	<i>Arabidopsis thaliana</i>	6	2
	<i>Prunus persica</i>	6	1
	<i>Cucumis sativus</i>	3	1
	<i>Glycine max</i>	8	1
	<i>Medicago truncatula</i>	3	-
	<i>Populus trichocarpa</i>	6	2
	<i>Ricinus communis</i>	5	1
	<i>Manihot esculenta</i>	7	2
<b>TOTAL</b>	<b>33</b>	<b>121</b>	<b>32</b>

showed evolutionary divergence of NHX protein groups according to their protein localization, as previously reported [22,40] (Figure 1 and Additional file 1: Figure S1 for more detail and unrooted tree). Proteins localized in the endosomal compartment of the Golgi and trans-Golgi

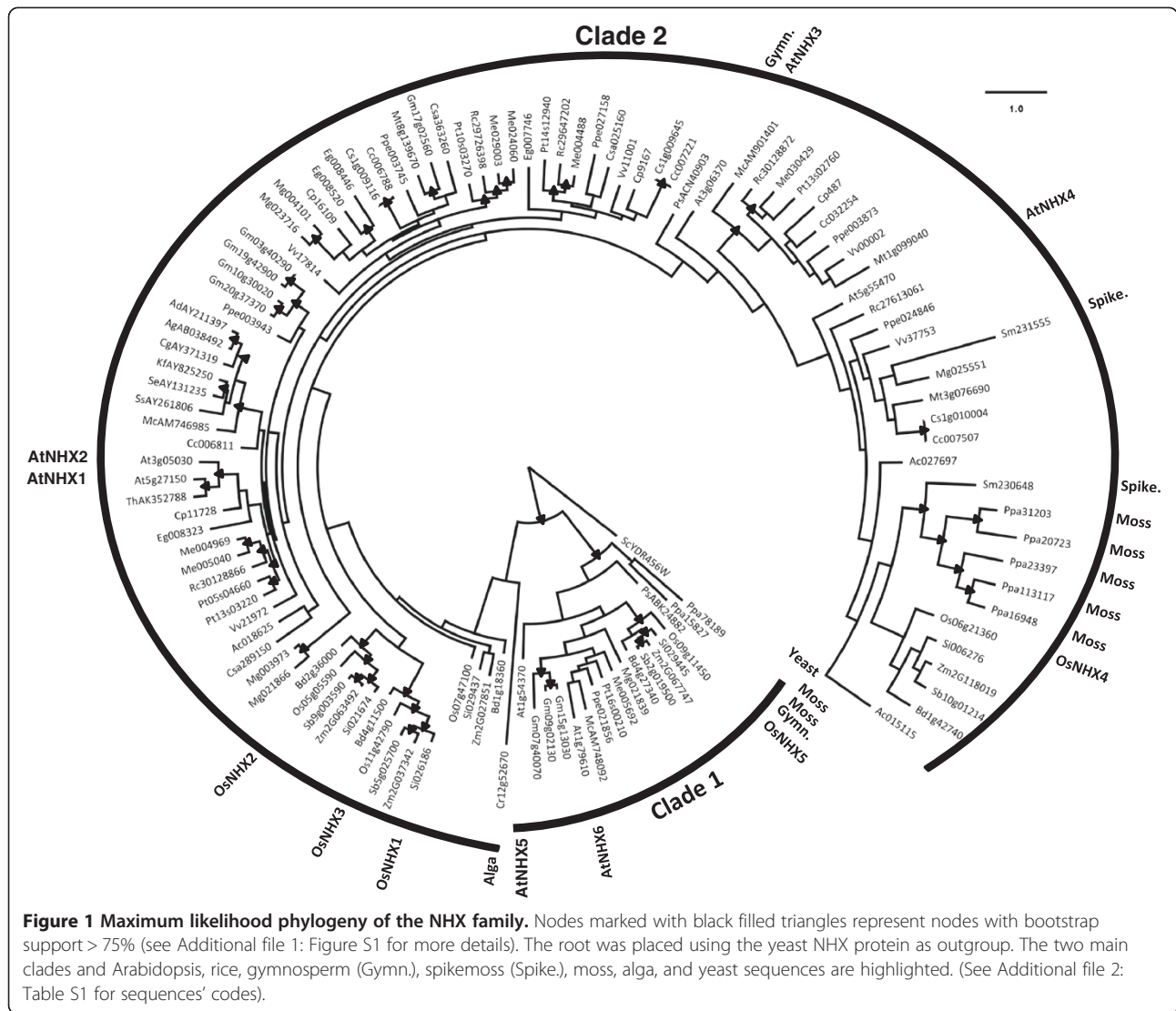
network grouped in clade 1 (closer to the yeast NHX1), while proteins localized in the tonoplast grouped in clade 2.

We observe that multiple independent duplication events have occurred throughout the evolutionary history of the NHX family. Based on the reconciled phylogeny (Figure 2), we estimate 27 independent gene duplication and 40 gene loss events during the diversification of this gene family. Gene loss events, however, are not displayed on the phylogeny since they might not represent true losses but may be due to partial gene inventories due to incomplete whole genome sequencing data (e.g., *Thellungiella halophila*) and to some genes still remaining to be identified. In this reconciled tree, all the branches with bootstrap support inferior to a set value (here 75%) are rearranged to achieve the most parsimonious duplication and loss history for the gene family. Nevertheless our results, together with the fact that non-plant species also have multiple NHX genes [9,39], suggest that multiple copies of NHX proteins were already present in a common ancestor of modern land plants (even though AtNHX1-4 and their orthologs are plant specific) [39].

Interestingly, orthologs of Arabidopsis NHX1 and 2 might be specific to (at least) seed plants. NHX1 and 2 proteins consistently grouped separately from proteins from basal plant lineages such as spikemoss (*Selaginella moellendorffii*), moss (*Physcomitrella patens*), and the green photosynthetic alga (*Chlamydomonas reinhardtii*) (Figure 1 and Additional file 1: Figure S1). Due to the limited number of gymnosperm sequences (only two NHX proteins from *Picea sitchensis*) we could not assess divergence from the more closely related species to the angiosperms. Sequences from more gymnosperm species are available as EST sequences in PlantGDB database, which coupled with greater availability of genomics resource data from other non-flowering plant taxa species, could potentially be used in the future to examine more closely the precise origins of specific gene family clades. However, our results suggest that NHX proteins grouping with AtNHX1 and 2 might be more recent and specific to seed plants. Nevertheless, bootstrap supports obtained for these branches were not very high (Figure 1 and Additional file 1: Figure S1) and more sequences will be necessary to be analyzed to confirm this.

#### Purifying selection on NHX proteins

We examined the sequences of the plant NHX gene family to determine whether there was significant heterogeneity in selective pressure among amino acid sites. Selective pressure at the protein level can be measured as dN/dS, where dN and dS are the number of non or synonymous substitutions per non or synonymous site, respectively [41]. If non-synonymous mutations are favored by positive selection, non-synonymous mutations are fixed at a faster



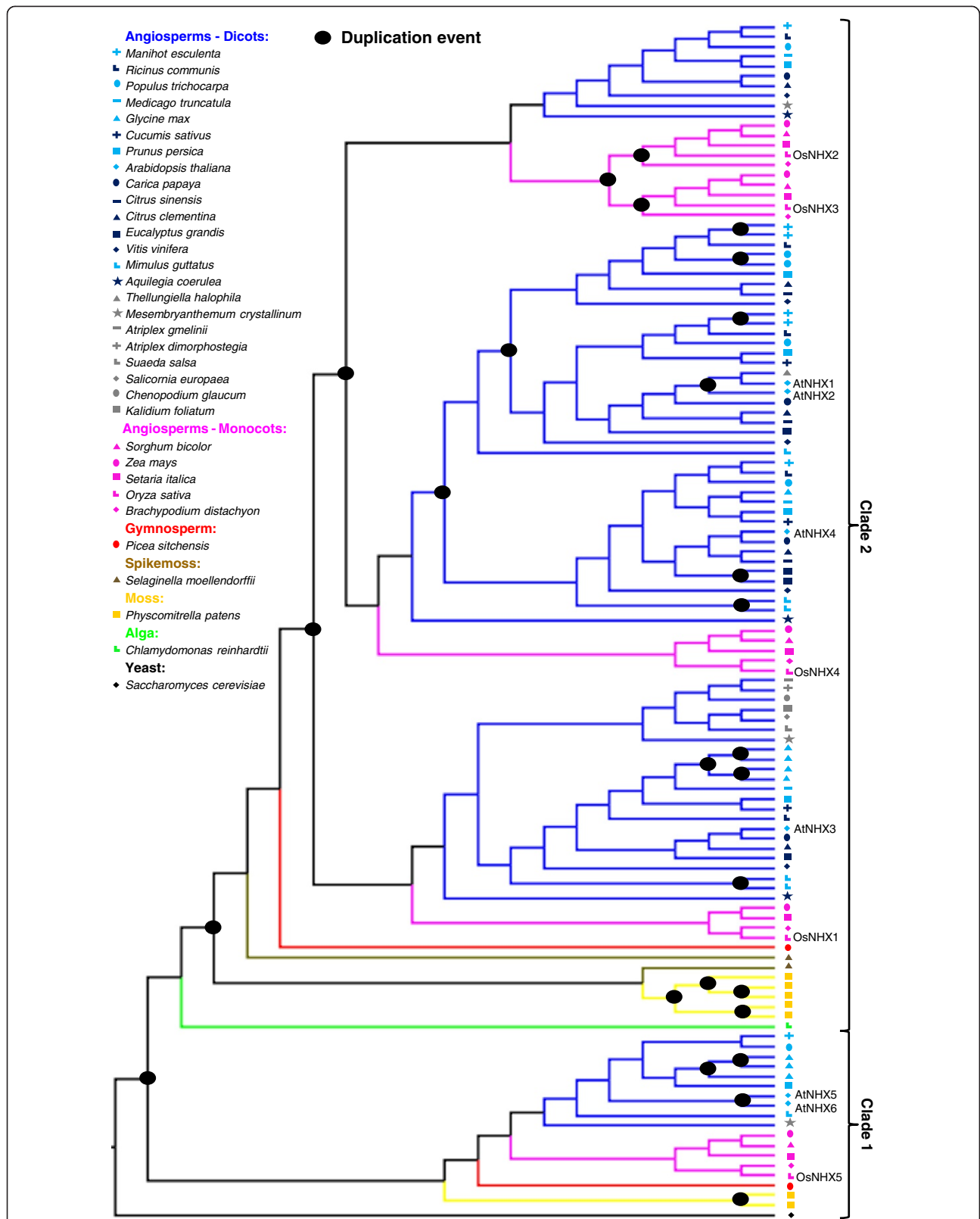
rate and  $dN/dS > 1$  [41]. In the case of purifying selection  $dN/dS < 1$ . However, if selection has no effect on fitness due to neutral evolution, both mutations are fixed at the same rate and  $dN/dS = 1$  [41].

For *NHX* coding region sequences from both clade 1 and 2, the average  $dN/dS$  was estimated at  $\sim 0.08$  and no sites were predicted to be under positive selection. Additionally, no difference in  $dN/dS$  between *NHX* genes was identified. We believe that these results might reflect the key function of *NHX* proteins, which besides salt tolerance [10,11] include normal cell functions in vesicular trafficking and protein targeting [8,9]. Additionally, *NHX* proteins are predicted to have  $\sim 12$  transmembrane domains [42] and are probably constrained at the structural level. Our results are consistent with the purifying selection expected for these proteins [43] and agree with results recently obtained by Hudson *et al.* [44].

### Phylogeny of the *SOS1* family exhibits few and recent duplication events

Unlike the *NHX* family, plant species appear to have lower numbers of *SOS1* genes in their genomes. We have selected 32 genes in 22 taxa that appear, or are described, to belong to the *SOS1* family. The distribution of *SOS1* genes among the various species is shown in Table 1. Among these genes, 22 are found in 16 angiosperm species, and 4 in 2 non-seed plant taxa. In this case we used 2 sequences from the moss *P. patens* as outgroup sequences.

We found that eight plant species, including *A. thaliana*, seem to have two *SOS1*-like genes (Table 2). At the time of submission of this manuscript, however, a new BLASTp search using Phytozome v8.0 (<http://www.phytozome.net/>, verified in June 2012) suggested that more species might possess more than one Putative *SOS1* protein. The



**Figure 2** Phylogeny of the NHX family that indicates multiple independent duplication events throughout its evolutionary history. The reconciled NHX tree obtained using NOTUNG v2.6, has a duplication/loss score of 60.5 and shows 27 independent gene duplication and 40 gene loss (not shown) events.

**Table 2 Plant species predicted to have two SOS1-like genes**

Species	Putative SOS1 – longer gene		Putative SOS1 – shorter gene		Δ Length (AA)
	Sequence code	Length (AA)	Sequence code	Length (AA)	
<i>Physcomitrella patens</i>	Pp1s12_8V6.1	1153	Pp1s15_101V6.1	1060	93
<i>Selaginella moellendorffii</i>	74518	1024	75049	940	84
<i>Brachypodium distachyon</i>	Bradi4g00290.1	1138	Bradi4g17380.1	719	419
<i>Aquilegia coerulea</i>	Aca_000299m	1150	Aca_002550m	588	562
<i>Eucalyptus grandis</i>	Egrandis_v1_0.001457m	1001	Egrandis_v1_0.008144m	547	454
<i>Arabidopsis thaliana</i>	At2g01980	1146	At1g14660	733	413
<i>Populus trichocarpa</i>	POPTR_0010s11130.1	1147	POPTR_0008s14030.1	1145	2
<i>Manihot esculenta</i>	cassava4.1_025141m	810	cassava4.1_002710m	703	107

Δ represents the difference in protein length.

existence of two *SOS1* genes has been described previously for *Arabidopsis thaliana* [16] and two other plant species not included in this study, namely Neptune grass (*Cymodocea nodosa*) [45] and quinoa (*Chenopodium quinoa*) [46]. Nevertheless, to confirm that these seven species have two *SOS1*-like proteins like in *Arabidopsis*, it is necessary to perform further functional studies.

As in *Arabidopsis*, the species with two *SOS1*-like proteins usually displayed differences in protein length (Table 2). However, two classes of length differences were identified - one in which both duplicates had similar lengths ( $\Delta < 110$  amino acid residues), and another in which the difference in duplicates length was substantially larger ( $\Delta > 400$  amino acid residues). Nevertheless, as in *Arabidopsis*, the differences in length were mainly due to shortening in the C-terminus instead of deletions within the protein primary sequence. This is important to notice, because it was shown by Quintero *et al.* [47] that it is within the C-terminus of *SOS1A* that lays the activation site and the auto-inhibitory domain of the protein. Additionally, *SOS1*-like proteins from *A. coerulea*, *E. grandis*, and *M. esculenta* have deletions in the N-terminus of the protein when compared to the *Arabidopsis SOS1* proteins. This suggests that *SOS1*-like proteins from these three species might have a smaller transmembrane region, but further biochemical analyses need to be performed to confirm this.

The *SOS1* phylogeny (Figure 3 and Additional file 1: Figure S2) exhibits three distinct clades: (1) one that contains the moss and spikemoss proteins (~94% bootstrap support), (2) another that has monocot proteins (~91% bootstrap support), and (3) finally, one clade with dicots *SOS1* sequences. The *SOS1* protein phylogeny is almost identical to the accepted species phylogeny of the study species, although there appears to be some long-branch attraction that alters the position within the dicot clade of one *Eucalyptus grandis* and one *A. thaliana* protein.

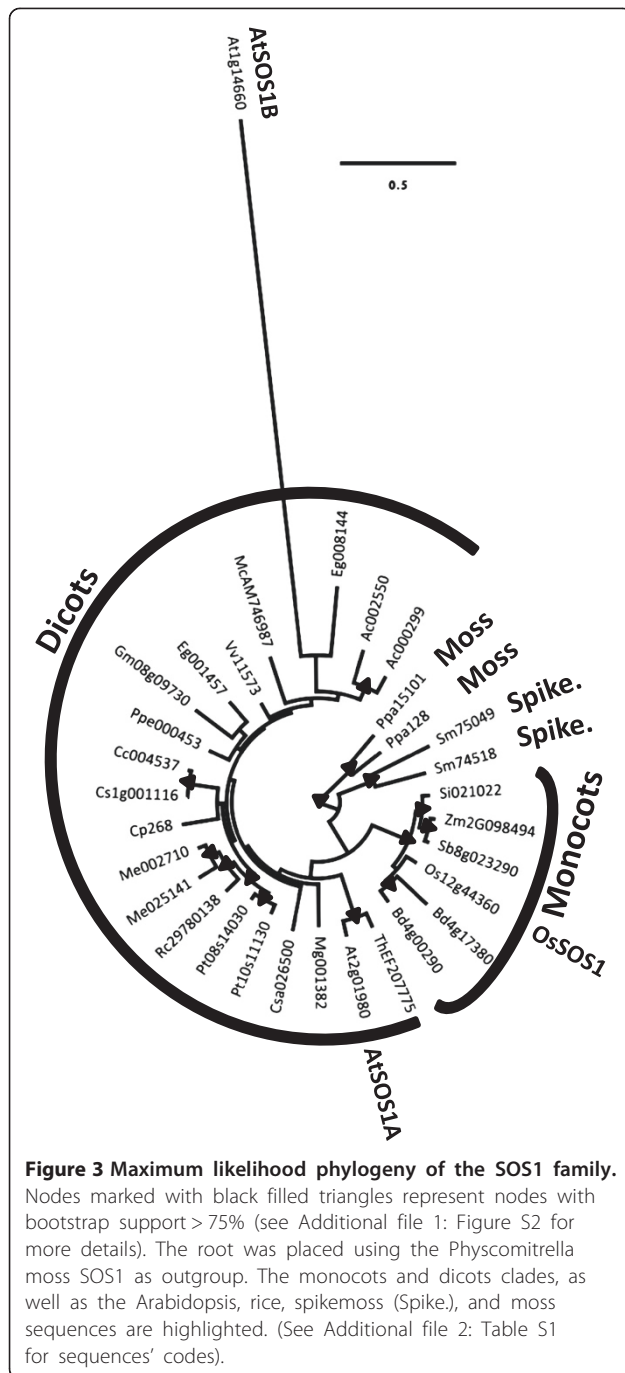
Unlike the *NHX* family, the *SOS1* family has undergone fewer gene duplication events. Reconciliation of the *SOS1*

tree with the species tree (Figure 3 and Additional file 1: Figure S3) estimates that 8 independent gene duplication events and one loss have occurred within the land plants (Figure 4). Once again, gene losses may simply represent unidentified genes; in fact Oh *et al.* [2] recently reported that *Thellungiella parvula* has three copies of the *A. thaliana SOS1B* and that these result from recent tandem duplications.

Interestingly, gene duplication events in the *SOS1* family appear toward the terminal nodes of the reconciled tree, indicating that these events were recent in evolutionary time. The analyses of some species that have a second copy of *SOS1* further support this observation. For example, *Populus trichocarpa* is thought to have undergone a recent whole genome duplication event [48] and monocots such as *Brachypodium distachyon* are thought to be more prone to tandem duplications which suggests that any gene duplication in one monocot species is probably recent and not shared with the common ancestor of this group [49].

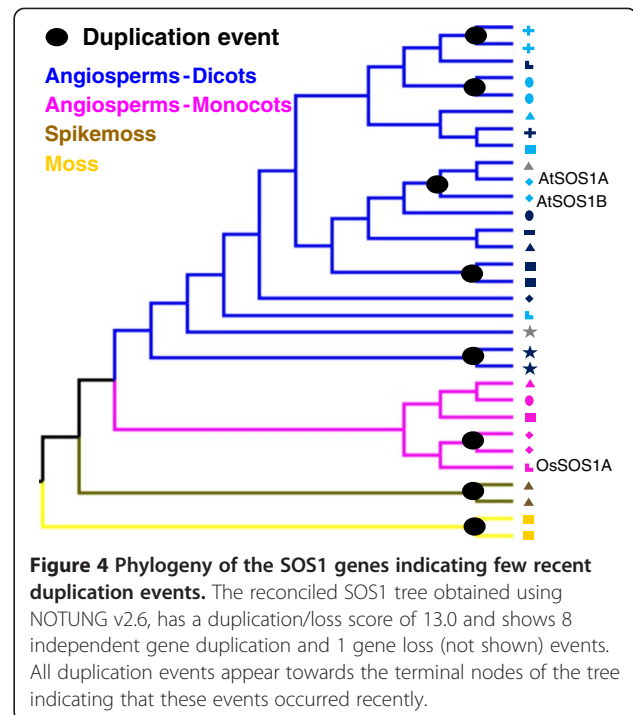
#### Purifying selection on *SOS1* proteins with few amino acids under positive selection

Like the *NHX* proteins, the *SOS1* proteins are generally subject to purifying selection, but our analysis indicates that some amino acid sites appear to be under positive selection. Among the land plants the *SOS1* proteins had an average dN/dS of ~0.16. Using alignments that largely represented orthologous sequences, the glutamine at position 958 (Q958) in *A. thaliana* is evolving neutrally (dN/dS ~ 1.00) and two amino acid residues are under positive selection (dN/dS ~ 1.23) - a valine at position 366 (V366) and a serine at position 738 (S738) (Figure 5). Other residues (L843, C902, and P915 in *A. thaliana*) were identified in our analysis as strong candidate residues to be under neutral or positive selection, but these were not as well supported as the other three sites. Nevertheless, these other residues might be indeed under neutral



or positive selection since the tests designed to detect positive selection are very stringent.

In general, structurally constrained sites should be under stronger purifying selection, while unstructured sites have higher levels of amino acid replacements [43]. According to different prediction tools available at ExPASy (<http://expasy.org/tools/>), however, the positively selected residue V366 is in a predicted transmembrane helix. Amino acid site Q958 is predicted to be in a transition

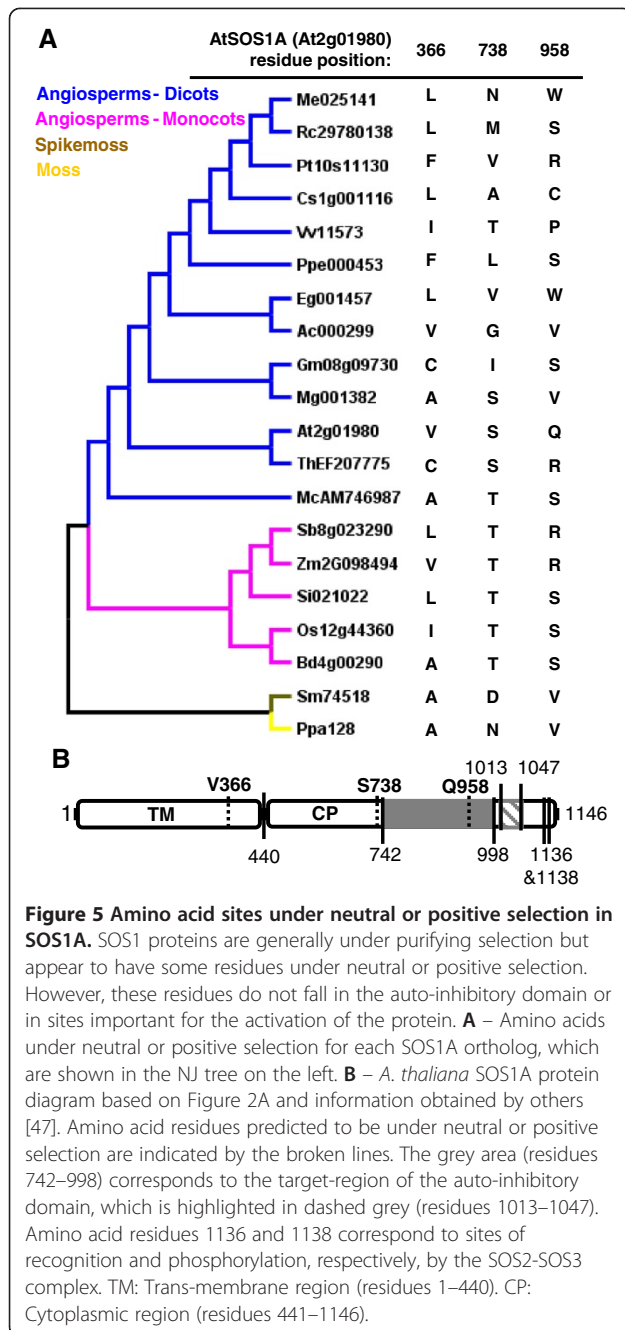


zone between coiled and beta-sheet regions, and residue S738 is predicted to be in a random coil.

Two of these residues, V366 and S738, are in the cytoplasmic domain of AtSOS1A (Figure 5) but do not fall in the auto-inhibitory domain (amino acids 1013–1047) or near sites known to be relevant for protein activation by phosphorylation (S1138 and S1136) [47]. Additionally, mutations of these three residues under neutral or positive selection are not predicted to result in changes that affect secondary or tertiary structure, or create new sites for putative posttranslational modifications (data not shown). Nevertheless, residue S738 is in close proximity to residues S742 and V743, which were shown to affect the activity of the *A. thaliana* protein when mutated [47]. Like S742 and V743, S738 is not well conserved between species. Mutations within the transmembrane pore were also shown to affect AtSOS1A activity [47]. Thus, it may be that the roles of residues S738 and V366 in the activity of the SOS1A protein are worth study.

#### Relaxation of selection on SOS1 gene duplicates

The SOS1 family apparently exhibits a relaxation of purifying selective pressure upon duplication, as previously reported in other gene families [50] and, specifically for ion transporters [44]. This relaxation of selection, however, seems to be present in only one duplicated gene copy, and only in species that have two SOS1-like proteins differing in length. Although the free-ratio model [51] is not generally considered to be a good method to predict dN/dS because it has too many free parameters, the comparison



between values of dN/dS is suggestive (Additional file 1: Figure S4). Species which have two Putative *SOS1*-like proteins similar in length ( $\Delta < 110$  amino acid residues) showed a difference in dN/dS values less than 0.1, while species that retain two putative *SOS1*-like proteins with very different length ( $\Delta > 400$  amino acid residues) displayed a difference in dN/dS values always greater than 0.2. Furthermore, in the cases where the two putative *SOS1*-like proteins substantially differed in length, the shortest appeared to be evolving faster than the longer protein from the same species (Additional file 1: Figure S4).

We also confirmed that these results were not affected by a saturation of nucleotide substitutions on the shorter sequences (Additional file 1: Figure S5), except possibly for *AtSOS1B*.

More data is needed to verify if this trend is statistically significant. Nevertheless, we can speculate that the possible relation between the differences in protein length and dN/dS might be due to the relative recent occurrence of the duplications in *SOS1*, which could indicate that not enough time has elapsed for differentiation between duplicated genes to have occurred (e.g., woody plants, such as *Populus*, have a slower evolution rate than *Arabidopsis*) [48]. Another hypothesis to explain the different dN/dS values observed between *SOS1* proteins that differ in length is that the duplication of some *SOS1* genes did not result in functionally equivalent gene copies, and hence functional divergence between copies is immediately observed.

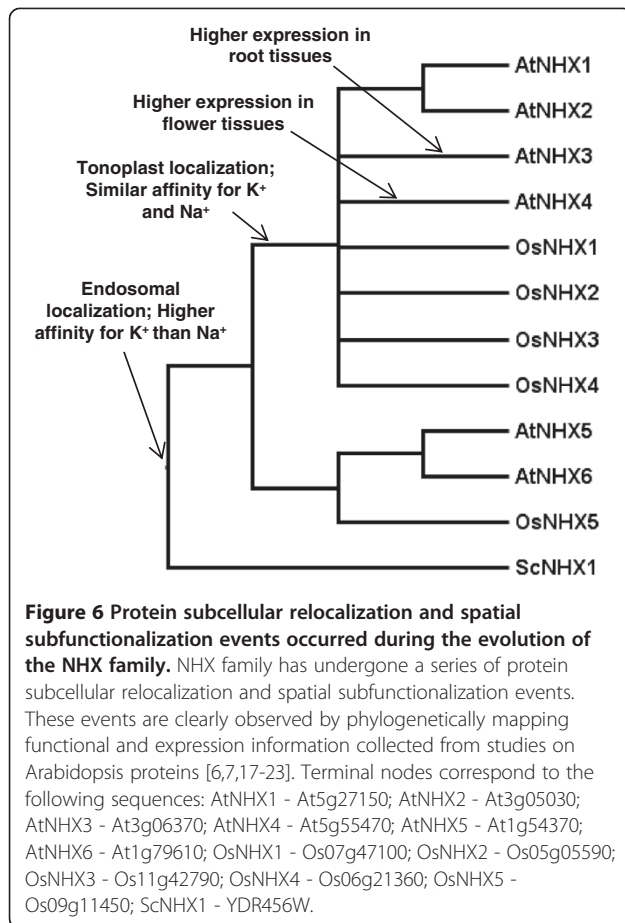
## Conclusions

### Contrasting evolutionary histories between the *NHX* and *SOS1* gene families

*NHX* and *SOS1* plant gene families exhibit markedly different evolutionary histories - while the *NHX* family expanded and developed functionally specialized members throughout the history of the land plants (Figures 1 and 2), the *SOS1* family remained a low copy gene family (Figures 3 and 4). It is clear that after gene duplication events, the *NHX* family members have undergone a series of protein subcellular relocalization and spatial subfunctionalization events. This can be observed by phylogenetically mapping functional and expression information collected from studies on *Arabidopsis* proteins (Figure 6) [6,7,17-23]. Rice (*Oryza sativa*) proteins also exhibit functional differentiation, although they are not always functionally equivalent to their *Arabidopsis* homologues. For example, both *AtNHX5* and *OsNHX5*, which are both part of clade 1 (Figure 1), have higher affinity to  $K^+$  compared to  $Na^+$  [6,40], although *AtNHX5* appears to be unresponsive to ABA [22] while *OsNHX5* responds to this hormone [40].

In contrast, the *SOS1* genes are found only as single- or low-copy genes in most plant species, suggesting either low duplication rates or a higher rate of gene deletion after gene duplication events for these loci. Although it is unclear why *SOS1* is constrained to be a single- or low-copy gene family, the few cases where a second *SOS1*-like gene has remained in the genome, it appears to have undergone neofunctionalization. For example, *Arabidopsis SOS1B* is only able to transport  $Li^+$  [16], and the *C. nodosa SOS1B* is described as having a different function from *CnSOS1A* [45]. Moreover, we found a difference in dN/dS between proteins that are diverging in protein length, thus supporting the observed neofunctionalization or possible loss of function of a second copy of *SOS1*. This difference





in dN/dS seems to imply that one of the *SOS1* gene copies suffers, more frequently, a non-synonymous mutation resulting in probable loss of function, and more rarely in neofunctionalization.

In our work, we do not detect a relaxation of selective constraint after duplication in the *NHX* gene family, in agreement with a previous study [44]. Furthermore, the difference in average dN/dS between *NHX* (~0.08) and *SOS1* proteins (~0.16) supports previous results [50,52] indicating that in eukaryotes, old duplicated genes evolve slower than singletons, despite an initial relaxation of constraint right after duplication. Our hypothesis is that while *NHX* proteins can be duplicated and subfunctionalized, they must retain their basic function and thus are under stronger purifying selection. In contrast, *SOS1* proteins cannot be as easily subfunctionalized and any duplicate copy seems to be preferentially lost and thus exhibiting more relaxed selective pressures.

Additionally, it may be that the evolutionary constraint on *SOS1* proteins arises from fewer possibilities for subfunctionalization, in contrast to cytoplasmic membrane proteins such as the *NHX*s. In a simplistic view, this might occur because intracellular membrane proteins can

have multiple locations within a cell without detriment to the function it performs, while a plasma membrane protein that is not localized to the plasma membrane would be unable to perform its desired function. Moreover, after subcellular relocalization within the cell, it may be easier to vary transporter affinity towards different ions, and intracellular membrane proteins can then be further subfunctionalized according to their specific location within the cell. This is observed for the *NHX* family in which AtNHX1-4 have equal affinities towards Na<sup>+</sup> and K<sup>+</sup>, while AtNHX5 and 6 have a higher affinity towards K<sup>+</sup> (Figure 6).

#### Different means of achieving stress adaptation through *NHX* and *SOS1* duplications

*NHX* and *SOS1* plant protein families have two contrasting evolutionary histories that seem to be related to their protein function and location within the cell. It is especially interesting to assess how the evolution of these two gene families, while different, has resulted in plant adaptation to stress conditions. Our hypothesis is that the first *NHX* protein was localized in the endosomal compartment of the Golgi and trans-Golgi membranes, corroborating a previous suggestion by Chanroj et al. [39]. The *NHX* duplications, however, allowed the appearance of other *NHX* proteins that were targeted to the tonoplast. Consequently, an organism with an improved ability to tolerate salt stress arose, since it was able to better retain K<sup>+</sup> after stress induction [36,37].

On the other hand, the duplication followed by neofunctionalization of *SOS1* has generated an increased capacity to tolerate soils with high Li<sup>+</sup> content in the case of Arabidopsis and related species. This is especially relevant for the Arabidopsis wild relative, *Thellungiella* spp., that lives in habitats with naturally high Li<sup>+</sup> concentrations [2]; indeed, *Thellungiella parvula* appears to have three copies of *SOS1B*, probably due to environmental adaptation [2]. Altogether, our results represent two examples of different molecular evolutionary trajectories in land plant genomes that result in organismal stress adaptation through gene duplication.

#### Methods

##### *NHX* and *SOS1* protein sequences

We used rice protein sequences OsNHX1 (Os07g47100) and OsSOS1A (Os12g44360), in Node Consensus BLASTp of Phytozome v7.0 [53] to obtain the homologous sequences from several plant species (from mosses to eudicots). Sequences belonging to families with an e-value lower than 10<sup>-100</sup> were used in the analysis. Independent BLASTp searches in Phytozome v7.0 and in NCBI (<http://www.ncbi.nlm.nih.gov/>) allowed retrieval of homologous sequences of OsNHX1 from the algae *Chlamydomonas reinhardtii* and the gymnosperm *Picea sitchensis*. In these particular

cases, sequences with an e-value lower than  $10^{-10}$  were used. Additionally, we also retrieved from NCBI sequences identified as being NHX- or SOS1-like from the yeast *Saccharomyces cerevisiae* (ScNHX1 – YDR456W) and from salt tolerant plant species (in grey background in Additional file 2: Table S1).

In order to improve the quality of the alignments obtained, sequences shorter than 500 or longer than 940 or 1200 amino acids from the NHX and SOS1 families, respectively, were removed from the study (38 out of 158 NHX like sequences, and 6 out of 38 SOS1 like sequences, were excluded). This exclusion was performed because the alignments these sequences produced had a large number of discontinuous aligned stretches and we had low confidence on the homology assignments from these alignments. The identifiers of all the sequences used, and their respective database origin, are listed in the Additional file 2: Table S1.

### Phylogenetic analysis

The phylogeny of NHX proteins was obtained from 121 sequences in 33 different species (Table 1), with the NHX1 protein from the yeast *Saccharomyces cerevisiae* as the outgroup sequence. The SOS1 phylogeny was estimated from 32 sequences in 23 plant species (Table 1), with the sequences from the moss *P. patens* used as outgroups. These outgroups were chosen because the *S. cerevisiae* sequence (NHA1) most similar to the plant SOS1 genes proved difficult to align with the rest of the plant genes.

Alignments of protein sequences were obtained using MUSCLE [54] and the alignments were refined using Gblocks (default settings) [55,56] in order to minimize the number of positions with missing information (gaps). For each cleaned or non-cleaned alignment the best-fit amino acid substitution model was selected using ProtTest from MEGA5 [57]. Since the Jones-Taylor-Thornton model [58] together with a discrete approximation of the gamma distribution (JTT +  $\Gamma$ ) was always within the top five best-fit models, we selected this model to obtain the phylogenies using the neighbor-joining (NJ) method [59]. We ran the NJ analysis with 1000 bootstrap replicates in MEGA5. Majority-rule consensus trees of 100 bootstrap replicates were also obtained from maximum likelihood (ML) phylogenies obtained using the JTT model plus a gamma rate distribution approximated using 5 categories. For this we used the SEQBOOT, PROML and CONSENSE programs from PHYLIP 3.69 (<http://evolution.genetics.washington.edu/phylip.html>). Branches with a good support (>75% bootstrap) were generally the same in the consensus trees obtained using either NJ (data not shown) or ML, although generally ML resulted in higher bootstrap support values.

### Estimation of duplication history

Using the information on species relationships available at the Angiosperm Phylogeny Site ([www.mobot.org/mobot/research/apweb/](http://www.mobot.org/mobot/research/apweb/)) we constructed a cladogram representing the species tree (Additional file 1: Figure S3). Estimation of the gene duplication and loss history of both protein families was performed, using NOTUNG v2.6 [60,61], through reconciliation of the species tree with the NHX and SOS1 ML gene trees (Figures 1 and 2 and Additional file 1: Figure S1 and S2). In order to obtain the most parsimonious estimation of duplication and loss events, the reconciliation of the species and gene trees was followed by rooting, and rearranging branches with less than 75% bootstrap support.

### Determination of selective pressure on amino acid sites

NHX protein sequences were separated into two groups according to the Arabidopsis proteins with which they grouped in the estimated phylogeny. Sequences grouping with Arabidopsis NHX1-4 formed group I, and sequences grouping with Arabidopsis NHX5 and 6 formed group II. Each group was used to obtain a new alignment and a new NJ tree as previously explained. Additionally, for SOS1 sequences three other alignments were obtained in which only orthologs were included or sequences deleted at the N- or C-terminus were excluded. All these alignments were cleaned and analyzed in order to maximize the alignment positions tested. Afterwards, in order to obtain codon-cleaned alignments, we mapped the coding sequences (CDS) of *NHX* and *SOS1* genes to protein alignments.

The codon-cleaned alignments and the NJ phylogenetic trees (both rooted and unrooted), were further used in Codeml from the PAML package [62] to determine heterogeneous selective pressure on amino acid sites. Codon substitution models [41] M0, M1a, M2a, M3, M7, and M8 were applied to the alignments. Each model builds on the preceding one by adding new dN/dS classes. M0 assumes that all sites in an alignment are under purifying selection ( $dN/dS < 1$ ), M1a allows for some sites to be under neutral selection ( $dN/dS = 1$ ), and M2a allows for some sites to be under positive selection ( $dN/dS > 1$ ). M3, M7, and M8 have increased classes of sites that are allowed to be under different selection pressures, being M8 the most complex model with 13 classes of sites. All models were tested at least twice to check for convergence problems and all alignments analyzed had an average synonymous substitution (dS) rate less than 14, which is near the lower limit of the dS range estimated to be disruptive for this type of analyses [63,64]. Afterwards, Likelihood Ratio Tests (LRT) [65] and Akaike Information Criteria (AIC) [66] methods were used to determine the model that best described the data. Neutral or positive selected sites were accepted when model M8 was the

best-fit model to the alignment and when sites came up both in model M2 and M8, with a probability  $\geq 95\%$  in model M8.

### Comparison of non-synonymous/synonymous rate ratio between clades

Branch-site model [67] from Codeml (PAML) was used to compare the non-synonymous/synonymous substitution rate ratio (dN/dS) between clades or sequences. Bonferroni correction [68] was used to examine significance under these multiple tests. For the SOS1 alignment, the free-ratio model [51] was also used to estimate an independent dN/dS per branch. In both cases, models were tested at least twice to check for convergence problems. Convergence might be an issue, especially for parameter rich models, when there is lack of information in the data, normally resulting from highly similar or divergent sequences (see PAML manual).

### Additional files

**Additional file 1: Figures S1-S5.** Figures depicting NHX and SOS1 phylogenies; the species tree used for reconciliation of gene trees; SOS1 phylogenetic tree where branch lengths represent non-synonymous/synonymous rate ratios (dN/dS); and SOS1 phylogenetic tree where branch lengths represent the rate of synonymous substitutions.

**Additional file 2: Table S1.** List of all sequences used in this study with reference to the database from which they were retrieved. Known salt tolerant plant species are highlighted in grey background. A simplified version (without special characters and limited to 10 characters) of the original sequences' identifiers was used in this study, since the majority of phylogenetic software have limitations for sequence names.

### Abbreviations

NHX: Sodium hydrogen exchanger; SOS: Salt overly sensitive; dN: Number of non-synonymous substitutions per non-synonymous site; dS: Number of synonymous substitutions per synonymous site; dN/dS: Non-synonymous/synonymous substitution rate ratio.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

ISP participated in the design of the study, carried out the computational analysis, and drafted the manuscript. MMP helped carrying out the computational analysis. IAA performed the analysis of the *Arabidopsis thaliana* SOS1A secondary and tertiary structure, looking specifically at the sites predicted to be under neutral or positive selection in the SOS1 proteins. SN, MMO, and MDP conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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### References

1. Dassanayake M, Oh D-h, Hong H, Bohnert HJ, Cheeseman JM: **Transcription strength and halophytic lifestyle.** *Trends Plant Sci* 2011, **16**(1):1–3.
2. Oh D-H, Dassanayake M, Bohnert HJ, Cheeseman JM: **Life at the extreme: lessons from the genome.** *Genome Biol* 2012, **13**:241.
3. Flowers TJ, Galal HK, Bromham L: **Evolution of halophytes: multiple origins of salt tolerance in land plants.** *Funct Plant Biol* 2010, **37**:604–612.
4. Zhu J-K: **Plant salt tolerance.** *Trends Plant Sci* 2001, **6**(2):66–71.
5. Apse MP, Blumwald E: **Na<sup>+</sup> transport in plants.** *FEBS Lett* 2007, **581**(12):2247–2254.
6. Venema K, Belver A, Marín-Manzano MC, Rodríguez-Rosales MP, Donaire JP: **A Novel Intracellular K<sup>+</sup>/H<sup>+</sup> Antiporter Related to Na<sup>+</sup>/H<sup>+</sup> Antiporters Is Important for K<sup>+</sup> Ion Homeostasis in Plants.** *J Biol Chem* 2003, **278**(25):22453–22459.
7. Apse MP, Sottosanto JB, Blumwald E: **Vacuolar cation/H<sup>+</sup> exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1, the Arabidopsis vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter.** *Plant J* 2003, **36**:229–239.
8. Bowers K, Levi BP, Patel FI, Stevens TH: **The Sodium/Proton Exchanger Nhx1p Is Required for Endosomal Protein Trafficking in the Yeast *Saccharomyces cerevisiae*.** *Mol Biol Cell* 2000, **11**:4277–4294.
9. Brett CL, Tukaye DN, Mukherjee S, Rao R: **The yeast endosomal Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> exchanger Nhx1 regulates cellular pH to control vesicle trafficking.** *Mol Biol Cell* 2005, **16**(3):1396–1405.
10. Apse MP: **Salt Tolerance Conferred by Overexpression of a Vacuolar Na<sup>+</sup>/H<sup>+</sup> Antiport in Arabidopsis.** *Science* 1999, **285**(5431):1256–1258.
11. Hernandez A, Jiang X, Cubero B, Nieto PM, Bressan RA, Hasegawa PM, Pardo JM: **Mutants of the Arabidopsis thaliana cation/H<sup>+</sup> antiporter AtNHX1 conferring increased salt tolerance in yeast: the endosome/prevacuolar compartment is a target for salt toxicity.** *J Biol Chem* 2009, **284**(21):14276–14285.
12. Li T, Zhang Y, Liu H, Wu Y, Li W, Zhang H: **Stable expression of Arabidopsis vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene AtNHX1, and salt tolerance in transgenic soybean for over six generations.** *Chin Sci Bull* 2010, **55**(12):1127–1134.
13. Cao D, Hou W, Liu W, Yao W, Wu C, Liu X, Han T: **Overexpression of TaNHX2 enhances salt tolerance of 'composite' and whole transgenic soybean plants.** *Plant Cell Tissue Organ Cult* 2011, **107**(3):541–552.
14. Brett CL, Donowitz M, Rao R: **Evolutionary origins of eukaryotic sodium/proton exchangers.** *Am J Physiol Cell Physiol* 2005, **288**:C223–C239.
15. Shi H, Ishitani M, Kim C, Zhu JK: **The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na<sup>+</sup>/H<sup>+</sup> antiporter.** *PNAS* 2000, **97**(12):6896–6901.
16. An R, Chen QJ, Chai MF, Lu PL, Su Z, Qin ZX, Chen J, Wang XC: **AtNHX8, a member of the monovalent cation: proton antiporter-1 family in Arabidopsis thaliana, encodes a putative Li/H antiporter.** *Plant J* 2007, **49**(4):718–728.
17. Pardo JM, Cubero B, Leidi EO, Quintero FJ: **Alkali cation exchangers: roles in cellular homeostasis and stress tolerance.** *J Exp Bot* 2006, **57**(5):1181–1199.
18. Bassil E, Ohto MA, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E: **The Arabidopsis intracellular Na<sup>+</sup>/H<sup>+</sup> antiporters NHX5 and NHX6 are endosome associated and necessary for plant growth and development.** *Plant Cell* 2011, **23**(1):224–239.
19. Bassil E, Tajima H, Liang Y-C, Ohto M-a, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E: **The Arabidopsis Na<sup>+</sup>/H<sup>+</sup> Antiporters NHX1 and NHX2 Control Vacuolar pH and K<sup>+</sup> Homeostasis to Regulate Growth, Flower Development, and Reproduction.** *Plant Cell* 2011, **23**(9):3482–3497.
20. Venema K, Quintero FJ, Pardo JM, Donaire JP: **The arabidopsis Na<sup>+</sup>/H<sup>+</sup> exchanger AtNHX1 catalyzes low affinity Na<sup>+</sup> and K<sup>+</sup> transport in reconstituted liposomes.** *J Biol Chem* 2002, **277**(4):2413–2418.
21. Aharon GS, Apse MP, Duan S, Hua X, Blumwald E: **Characterization of a family of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters in Arabidopsis thaliana.** *Plant Soil* 2003, **00**:245–256.

22. Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM: **Differential expression and function of *Arabidopsis thaliana*  $\text{Na}^+/\text{H}^+$  antiporters in the salt stress response.** *Plant J* 2002, **30**(5):529–539.
23. Li HT, Liu H, Gao XS, Zhang H: **Knock-out of *Arabidopsis* *AtNHX4* gene enhances tolerance to salt stress.** *Biochem Biophys Res Commun* 2009, **382**(3):637–641.
24. Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, Hirochika H, Tanaka Y: **Function, Intracellular Localization and the Importance in Salt Tolerance of a Vacuolar  $\text{Na}^+/\text{H}^+$  Antiporter from Rice.** *Plant Cell Physiol* 2004, **45**(2):146–159.
25. Fukuda A, Nakamura A, Tanaka Y: **Molecular cloning and expression of the  $\text{Na}^+/\text{H}^+$  exchanger gene in *Oryza sativa*.** *Biochimica et Biophysica Acta - Gene Structure and Expression* 1999, **1446**(1–2):149–155.
26. Li M, Lin X, Li H, Pan X, Wu G: **Overexpression of *AtNHX5* improves tolerance to both salt and water stress in rice (*Oryza sativa* L.).** *Plant Cell Tissue Organ Cult* 2011, **107**(2):283–293.
27. Xue ZY, Zhi DY, Xue GP, Zhang H, Zhao YX, Xia GM: **Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L.) expressing a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf  $\text{Na}^+$ .** *Plant Sci* 2004, **167**(4):849–859.
28. Mahajan S, Pandey GK, Tuteja N: **Calcium- and salt-stress signaling in plants: shedding light on SOS pathway.** *Arch Biochem Biophys* 2008, **471**(2):146–158.
29. Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK: **Regulation of *SOS1*, a plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger in *Arabidopsis thaliana*, by *SOS2* and *SOS3*.** *PNAS* 2002, **99**(12):8436–8441.
30. Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM: **Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for  $\text{Na}^+$  homeostasis.** *PNAS* 2002, **99**(13):9061–9066.
31. Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D, et al: **Phylogenetic relationships within cation transporter families of *Arabidopsis*.** *Plant Physiol* 2001, **126**:1646–1667.
32. Guo KM, Babourina O, Rengel Z:  **$\text{Na}^+(\text{H}^+)$  antiporter activity of the *SOS1* gene: lifetime imaging analysis and electrophysiological studies on *Arabidopsis* seedlings.** *Physiol Plant* 2009, **137**(2):155–165.
33. Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK: **Regulation of vacuolar  $\text{Na}^+/\text{H}^+$  exchange in *Arabidopsis thaliana* by the salt-overly-sensitive (*SOS*) pathway.** *J Biol Chem* 2004, **279**(1):207–215.
34. Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR: **The *Arabidopsis thaliana* proton transporters, *AtNHX1* and *Avp1*, can function in cation detoxification in yeast.** *Proc Natl Acad Sci USA* 1999, **96**(4):1480–1485.
35. Leidi EO, Barragan V, Rubio L, El-Hamdaoui A, Ruiz MT, Cubero B, Fernandez JA, Bressan RA, Hasegawa PM, Quintero FJ, et al: **The *AtNHX1* exchanger mediates potassium compartmentation in vacuoles of transgenic tomato.** *Plant J* 2010, **61**(3):495–506.
36. Barragan V, Leidi EO, Andres Z, Rubio L, De Luca A, Fernandez JA, Cubero B, Pardo JM: **Ion exchangers *NHX1* and *NHX2* mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis*.** *Plant Cell* 2012, **24**(3):1127–1142.
37. Jiang X, Leidi EO, Pardo JM: **How do vacuolar *NHX* exchangers function in plant salt tolerance?** *Plant Signal Behav* 2010, **5**(7):792–795.
38. Hastings PJ, Lupski JR, Rosenberg SM, Ira G: **Mechanisms of change in gene copy number.** *Nat Rev Genet* 2009, **10**(8):551–564.
39. Chanroj S, Wang G, Venema K, Zhang MW, Delwiche CF, Sze H: **Conserved and diversified gene families of monovalent cation/ $\text{H}^+$  antiporters from algae to flowering plants.** *Frontiers in Plant Science* 2012, **3**:1–18.
40. Fukuda A, Nakamura A, Hara N, Toki S, Tanaka Y: **Molecular and functional analyses of rice *NHX*-type  $\text{Na}^+/\text{H}^+$  antiporter genes.** *Planta* 2011, **233**(1):175–188.
41. Yang Z, Nielsen R, Goldman N, Pedersen AMK: **Codon-substitution models for heterogeneous selection pressure at amino acid sites.** *Genetics* 2000, **155**:431–449.
42. Sato Y, Sakaguchi M: **Topogenic properties of transmembrane segments of *Arabidopsis thaliana* *NHX1* reveal a common topology model of the  $\text{Na}^+/\text{H}^+$  exchanger family.** *J Biochem (Tokyo)* 2005, **138**(4):425–431.
43. Brown CJ, Takayama S, Campen AM, Vise P, Marshall TW, Oldfield CJ, Williams CJ, Dunker AK: **Evolutionary rate heterogeneity in proteins with long disordered regions.** *J Mol Evol* 2002, **55**:104–110.
44. Hudson CM, Puckett EE, Bekaert M, Pires JC, Conant GC: **Selection for higher gene copy number after different types of plant gene duplications.** *Genome Biol Evol* 2011, **3**:1369–1380.
45. Garcíadeblas B, Haro R, Benito B: **Cloning of two *SOS1* transporters from the seagrass *Cymodocea nodosa*. *SOS1* transporters from *Cymodocea* and *Arabidopsis* mediate potassium uptake in bacteria.** *Plant Mol Biol* 2007, **63**(4):479–490.
46. Maughan PJ, Turner TB, Coleman CE, Elzinga DB, Jellen EN, Morales JA, Udall JA, Fairbanks DJ, Bonifacio A: **Characterization of *Salt Overly Sensitive 1* (*SOS1*) gene homoeologs in quinoa (*Chenopodium quinoa* Willd.).** *Genome* 2009, **52**(7):647–657.
47. Quintero FJ, Martínez-Atienza J, Villalta I, Jiang X, Kim WY, Ali Z, Fujii H, Mendoza I, Yun DJ, Zhu JK, et al: **Activation of the plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter *Salt-Overly-Sensitive 1* (*SOS1*) by phosphorylation of an auto-inhibitory C-terminal domain.** *PNAS* 2011, **108**(6):2611–2616.
48. Smith SA, Donoghue MJ: **Rates of molecular evolution are linked to life history in flowering plants.** *Science* 2008, **322**(5898):86–89.
49. Duarte JM, Wall PK, Edger PP, Landherr LL, Ma H, Pires JC, Leebens-Mack J, dePamphilis CW: **Identification of shared single copy nuclear genes in *Arabidopsis*, *Populus*, *Vitis* and *Oryza* and their phylogenetic utility across various taxonomic levels.** *BMC Evol Biol* 2010, **10**:61.
50. Jordan IK, Wolf YI, Koonin EV: **Duplicated genes evolve slower than singletons despite the initial rate increase.** *BMC Evol Biol* 2004, **4**:22.
51. Geng S, Zhao Y, Tang L, Zhang R, Sun M, Guo H, Kong X, Li A, Mao L: **Molecular evolution of two duplicated CDPK genes *CPK7* and *CPK12* in grass species: a case study in wheat (*Triticum aestivum* L.).** *Gene* 2011, **475**(2):94–103.
52. Davis JC, Petrov DA: **Preferential duplication of conserved proteins in eukaryotic genomes.** *PLoS Biol* 2004, **2**(3):0318–0326.
53. Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, et al: **Phytozome: a comparative platform for green plant genomics.** *Nucleic Acids Res* 2012, **40**(Database issue):D1178–1186.
54. Edgar RC: **MUSCLE: multiple sequence alignment with high accuracy and high throughput.** *Nucleic Acids Res* 2004, **32**(5):1792–1797.
55. Castresana J: **Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis.** *Mol Biol Evol* 2000, **17**:540–552.
56. Talavera G, Castresana J: **Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments.** *Syst Biol* 2007, **56**(4):564–577.
57. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: **MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.** *Mol Biol Evol* 2011, **28**(10):2731–2739.
58. Jones DT, Taylor WR, Thornton JM: **The rapid generation of mutation data matrices from protein sequences.** *CABIOS* 1992, **8**(3):275–282.
59. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees.** *Mol Biol Evol* 1987, **4**(4):406–425.
60. Durand D, Halldórsson BV, Vernot B: **A hybrid micro-macroevolutionary approach to gene tree reconstruction.** *J Comput Biol* 2006, **13**(2):320–335.
61. Vernot B, Stolzer M, Goldman A, Durand D: **Reconciliation with non-binary species trees.** *J Comput Biol* 2008, **15**(8):981–1006.
62. Yang Z: **PAML: a program package for phylogenetic analysis by maximum likelihood.** *CABIOS* 1997, **13**(5):555–556.
63. Yang Z: **On the best evolutionary rate for phylogenetic analysis.** *Syst Biol* 1998, **47**(1):125–133.
64. Anisimova M, Bielawski JP, Yang Z: **Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution.** *Mol Biol Evol* 2001, **18**(8):1585–1592.
65. Yang Z: **Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution.** *Mol Biol Evol* 1998, **15**(5):568–573.
66. Le SQ, Gascuel O: **An improved general amino acid replacement matrix.** *Mol Biol Evol* 2008, **25**(7):1307–1320.
67. Zhang J, Nielsen R, Yang Z: **Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level.** *Mol Biol Evol* 2005, **22**:2472–2479.
68. Anisimova M, Yang Z: **Multiple hypothesis testing to detect lineages under positive selection that affects only a few sites.** *Mol Biol Evol* 2007, **24**(5):1219–1228.

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