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Cloning and Characterization of the Salt Overly Sensitive 1 (SOS1) Gene in Chenopodium quinoa WILLD.

Taylor Brian Turner
Brigham Young University - Provo

CLONING AND CHARACTERIZATION OF THE *SALT OVERLY SENSITIVE 1* (*SOS1*) GENE IN *CHENOPODIUM QUINOA* WILLD.

by

Taylor B. Turner

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of a thesis submitted by

Taylor B. Turner

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

_____	_____
Date	P. Jeff Maughan, Chair
_____	_____
Date	Craig E. Coleman
_____	_____
Date	Brent L. Nielsen

BRIGHAM YOUNG UNIVERSITY

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_____ _____

Date P. Jeff Maughan
Chair, Graduate Committee

Accepted for the Department _____

Loreen A. Woolstenhulme
Graduate Coordinator

Accepted for the College _____

Rodney J. Brown
Dean, College of Life Sciences

ABSTRACT

CLONING AND CHARACTERIZATION OF THE *SALT OVERLY SENSITIVE 1* (*SOS1*) GENE IN *CHENOPODIUM QUINOA* WILLD.

Taylor B. Turner

Department of Plant and Wildlife Sciences

Master of Science

Salt tolerance is a commercially important trait that affects plant species around the globe. Cellular response to saline environments is a well studied but complex system that is far from being completely understood. The *SALT OVERLY SENSITIVE 1* (*SOS1)* gene is a critical component of salt tolerance in many species, encoding a plasma membrane Na^+/H^+ antiporter that plays an important role in germination and growth in saline environments. Here we report a preliminary investigation of salt tolerance in quinoa (*Chenopodium quinoa* Willd.). Quinoa is a halophytic grain crop of the *Chenopodiaceae* family with impressive nutritional content and an increasing world-wide market. Many quinoa varieties have impressive salt tolerance characteristics and research suggests quinoa may utilize novel mechanisms to confer salt tolerance. At this time there is no published data on the molecular characteristics of those mechanisms. We report the identification and sequencing of the *SOS1* gene in quinoa, including a full length cDNA

sequence of 3490 bp and a full length genomic clone of 21314 bp. Sequence analysis predicts the quinoa *SOS1* homolog spans 23 exons and is comprised of 3474 bp of coding sequence (excluding the stop codon). Introns comprise 17840 bp of the genomic clone and range in size from 77 to 2123 bp. The predicted protein contains 1158 amino acid residues and aligns closely with SOS1 homologs of other species. The quinoa SOS1 homolog contains two putative domains, a Nhap cation-antiporter domain and a cyclic-nucleotide binding domain. Sequence analyses of both cDNA fragments and intron fragments suggest that two *SOS1* loci are present in the quinoa genome that are likely orthologous loci derived from the ancestral diploid genomes of the modern allotetraploid quinoa genome. This report represents the first molecular characterization of a putative salt-tolerance gene in *C. quinoa*.

Key words: Chenopodium quinoa – salt tolerance – SOS1

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INTRODUCTION

The productivity of over one-third of all arable land world-wide is affected by soil salinity (Epstein, 1972). Sodium ions (Na^+) adversely affect plant growth when high concentrations are found in soil or water by leading to high cytosolic Na^+ concentration and subsequent plant death, likely due to a combination of adverse effects. Nearly all enzyme activity decreases dramatically above 0.3-0.5 M NaCl due to disruption of the electrostatic forces maintaining protein structure (Wyn Jones and Pollard 1983). NaCl stress also significantly damages photosynthetic mechanisms through a combination of superoxide-and H_2O_2-mediated oxidation (Hernandez et al. 1995). When cytoplasmic Na^+ concentration increases potassium (K^+) levels decrease—directly correlated with lower growth rates (Katsuhara et al. 1986; Ben-Hayyim et al. 1987). Plants employ three basic strategies to prevent and correct high Na^+ concentrations: 1) active Na^+ efflux, 2) Na^+ compartmentalization in the vacuole, and 3) Na^+ influx prevention (Niu et al. 1995). Sodium efflux and transport into the vacuole are coupled to pH gradients created at the plasma membrane and vacuolar membrane, respectively. Transporters on both membranes respond to salt stress with increased transcription of transport proteins, only plasma membrane transporters are activated when exposed to high salinity (Niu et al. 1993a, 1993b, 1995).

The ability to increase salt tolerance in crop plants is a major breeding objective in many world regions where saline and sodic soils preclude crop production and in other areas that experience significant fluctuations in soil salinity, such as estuaries and intertidal zones (de Leeuw et al. 1991). The determinants of salt tolerance include proteins that directly affect ion movement as well as those involved in maintaining

osmotic balance, gene regulation, and membrane integrity (Zhu 2002). Many of these processes, including the plants' coordinated ability to maintain osmotic balance and turgor pressure under abiotic stress, are not well understood. Genetic mapping experiments have identified many quantitative trait loci involved in salt stress (Quesada et al. 2002, Foolad et al 1999, Koyama et al. 2001), but the most active research targeting specific genes involved in salt tolerance has employed a forward genetics approach, including the analysis of induced mutant lines screened for their ability to grow on NaCl-infused media. Shi et al. (2000) used ethylmethane sulfonate (EMS)-treated *Arabidopsis thaliana* Heynh. plants to identify NaCl-sensitive mutants. Genetic analysis of the salt sensitive plants indicated that mutations in the *SALT OVERLY SENSITIVE 1* (*SOS1;* GenBank: NM_126259) gene rendered the *Arabidopsis* plants extremely sensitive to high Na^+ or low K^+ environments. Homozygous *sos1* plants failed to grow on 50 mM NaCl media whereas wild-type seedlings showed no growth inhibition. At 100 mM NaCl the germination rate of *sos1* mutants is reduced by nearly 65% relative to wild type (Wu et al. 1996). The *Arabidopsis SOS1* gene contains 23 exons and encodes a plasma membrane protein of 1146 amino acids that acts as Na^+/H^+ antiporter (Shi et al. 2000). *Arabidopsis* plants preferentially express *SOS1* in epidermal cells at the root tip, and parenchyma cells at the xylem/symplast boundary of roots, stems, and leaves. *SOS1* is expressed at low basal levels but is upregulated in the presence of NaCl and has been shown to regulate other genes in response to salt stress (Gong et al. 2001). *SOS1* homologs have been identified, sequenced and submitted to GenBank for multiple plant species in addition to *Arabidopsis*, including *Solanum lycopersicum* D. *(*GenBank: AJ717346*)*, *Oryza sativa* L. (GenBank: AY785147), *Triticum aestivum* L. (GenBank:

AY326952), *Cymodocea nodosa* A. (GenBank: AJ427294), and *Suaeda japonica* M. (GenBank: AB198179).

Here we report the cloning and characterization of an *SOS1* gene homolog from *Chenopodium quinoa* Willd. (commonly referred to as 'quinoa'), a halophytic species in the *Chenopodiaceae* family. The Chenopodiaceae family has the highest proportion of halophytic genera (44%) constituting 321 species (Flowers et al. 1986). Quinoa is an ancient New World food staple, commonly cultivated throughout Central and South America. The high protein content of quinoa seeds consists of a well-balanced essential amino acid profile, rivaling milk for nutritive value (Prego et al. 1998; Brinegar et al. 1996; Fairbanks et al. 1990). Botanically speaking, quinoa is an annual, self-fertile, hermaphroditic allotetraploid (4x=2n=36) predominantly exhibiting disomic morphological and molecular marker segregation (Gandarillas, 1979; Mason et al. 2005). Quinoa thrives from sandy to loamy soils under a wide range of pH (4.8-8.5) and is tolerant to saline and sodic soils. Cultivated quinoa varieties can be separated into different ecotypes, including Salares types that exhibit high salt tolerance when grown near salt beds on the Altiplano (high plains) of Bolivia (Wilson, 1988). While little is known about the specific mechanisms involved in quinoa's ability to thrive under salt stress conditions, several recent reports suggest that quinoa may deal with soil salinity using unique and as yet undescribed mechanisms involving salt ion accumulation in specialized tissues as well as the adjustment of leaf water potential. Salares varieties of quinoa are reported to germinate (75%) at a salt concentration of 57 mS/cm, a significant feat considering sea water has a salt concentration of 40 mS/cm (Christiansen et al. 1999; Jacobsen et al. 1999; Jacobsen et al. 2003). Wilson et al. (2002) states that quinoa

appears to regulate salt ions (Na^+ and K^+) differently than wheat. Here we report the cloning and characterization of the first specific gene (*SOS1*) known to be integral to salt tolerance from this halophytic species.

MATERIALS AND METHODS

Plant materials and nucleic acid extraction. Quinoa seed for the cultivar 'Real' (Salares ecotype) was kindly provided by Angel Mujica (National University of the Altiplano, Puno, Peru). For DNA extraction, plants were grown at 25^{o}C with a 12-hour photoperiod, in Sunshine Mix II^{TM} (Sun Grow, Inc., Bellevue, WA), supplemented weekly with nitrogen fertilizer. All plants were grown in 6" pots in greenhouses at Brigham Young University, Provo, Utah and DNA was extracted from leaf tissue from 30 day old plants according to procedures described by Sambrook et al. (1989) with modifications described by Todd and Vodkin (1996). The extracted DNA was quantified with a spectrophotometer and diluted to 30 ng μl^{-1} in TE (10mM Tris, 1 mM EDTA, pH 7.5). For RNA extraction, seedlings were grown for 7-10 days at 25°C with a 14-hr photoperiod on pre-wetted Whatman filter paper. The filter paper was kept damp throughout the growing period and a single application (~100mg) of powdered Hi-Yield Captan fungicide 50% WP was applied on the 3rd day. After 7-10 days the seedlings were transferred to Whatman filter paper soaked in 300mM NaCl for five hours prior to RNA extraction. Treated seedling roots were ground in liquid nitrogen and RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). Purified RNA was quantified with a NanoDrop spectrophotometer and stored at -80°C.

Degenerate Primer Design. In order to identify conserved regions of the *SOS1* gene for PCR amplification, *SOS1* cDNA sequences from *Arabidopsis* (GenBank: NM_126259), *Solanum lycopersicum (*GenBank: *AJ717346)*, *Oryza sativa (*GenBank: *AY785147)*, and *Triticum aestivum (*GenBank: *AY326952)* were aligned using the multiple sequence alignment application, AlignX, of the computer program Vector NTI (Invitrogen, Carlsbad, CA). While no region showed complete homology across all species, 12 forward and 11 reverse degenerate primers, with significant sequence conservation, were identified and manufactured by Integrated DNA Technologies (Coralville, IA; Table 1).

Cloning and DNA Sequencing. A single primer pair (4F: 5'-ATG AAT GAT GGG ACG GCD ATT GTT GT and 5R: 5'-TCC AAA CCA TAG CCA AA) produced an amplicon near the predicted bp size. The resulting *SOS1* gene fragment (termed hereafter as 4F-5R) was cloned using the pGEM-T Easy Vector System II (Promega, Madison, WI, USA). Recombinant clones were identified by standard blue/white screening methods with IPTG and X-Gal. Plasmid DNA from a single recombinant clone was purified using a GenElute^{TM} plasmid miniprep Kit (Sigma, St. Louis, MO, USA) and was quantified using a spectrophotometer. Isolated plasmid DNA was sequenced bidirectionally using M13 (F/R) primers. DNA sequencing was performed at the Brigham Young University DNA Sequence Center (Provo, UT, USA) using standard ABI Prism Taq dye-terminator cycle-sequencing methodology. DNA sequence chromatograms were analyzed with the Contig Express program in the Vector NTI software suite (Invitrogen).

Rapid Amplification of cDNA Ends (RACE). The SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA) was used for RACE amplification reactions following the manufacturer's recommended protocols. Gene specific primers (5RP74, 5RP520, 3RP231, and 3RP498; Table 1) were designed from the 4F-5R internal fragment of the quinoa *SOS1* gene using the computer program Primer3 (Rozen and Skaletsky, 2000). RACE amplicons were purified with the Wizard SV system (Promega, Madison, WI) and cloned using the pGEM-T Easy Vector System II as described above. Multiple cloned RACE amplicons from both the 5' and 3' RACE reactions were sequenced using M13 forward and reverse primers. Primer walking strategies were employed to sequence complete 3' RACE amplicons. DNA sequence chromatograms were analyzed and assembled with the Contig Express program in the Vector NTI software suite (Invitrogen).

Bacterial Artificial Chromosome (BAC) Library Screening. A 9X BAC library (Stevens et al. 2006) consisting of 74,496 clones and developed from the quinoa ecotype "Real" was screened with the internal *SOS1* gene fragment (4F-5R) in order to identify BAC clones containing the genomic sequence of the *SOS1* gene homolog. The 4F-5R hybridization probe was labeled with α-dCTP^{32} using a Prime-a-Gene kit (Promega, Madison, WI) and membranes were hybridized using standard protocols as described by Sambrook et al. (1989). Clones with positive hybridization signals were picked from the local copy of the BAC library and grown in LB broth supplemented with 15ug/ml chloramphenicol. BAC plasmids were extracted using a NucleoBond BAC 100 kit (Macherey-Nagel, Easton, PA) and confirmed to contain the *SOS1* gene sequence via

PCR with the 4F and 5R primer combination. Genomic sequence of a single BAC clone (117:G8), confirmed to contain a quinoa *SOS1* homolog, was sent to Arizona Genomics Institute for 10X shotgun sequencing.

Computational Analysis. Sequence alignments were performed with ClustalW, using the default parameters (Chenna et al. 2003). The Spidey alignment program was used for identification of intron/exon boundaries using the default parameters for plant sequences (http://www.ncbi.nlm.nih.gov/spidey/). Assembly of contigs was performed with Contig Express from the Vector NTI software suite (Invitrogen) and hydrophobicity analysis and plots were generated by TMpred (Hofmann and Stoffel, 1993).

RESULTS

Using the 12 forward and 11 reverse consensus primers designed to amplify internal fragments of the quinoa *SOS1* gene we tested 76 unique primer combinations of which a single combination, termed 4F-5R, produced a single strong amplicon. The amplicon was 561 base-pairs (bp) and correlated closely with the predicted size of 559 bp, as determined by the multi-species alignment. BLASTN searches against the non-redundant (nr) GenBank database showed significant hits to 26 Na^+/H^+ antiporter sequences (E-value ≤2.0E-12), with the top two significant alignments being *SOS1* homologs from *S. japonica* (AB198179; E-value=0.0; 86% Max identity) and *Mesembryanthemum crystallinum* L. (EF207776; E-value=1e-173; 84% Max identity). BLASTX searches against the nr GenBank protein database identified 27 GenBank sequences with significant alignment to the translated 4F-5R sequence (E-value<1.0E-15). As with the

BLASTN results, the two most significant alignments with the BLASTX search were SOS1 homologs of *S.japonica* (BAE95196; E-value=1.0E-70; 86% identity) and *M. crystallinum* (ABN04858; E-value=8.0E-66; 82% identity). Twenty-one additional Na^+/H^+ transporter proteins also showed significant alignment (E-value<1.0E-15) to the translated 4F-5R fragment, including the *Arabidopsis thaliana* SOS1 protein (NP_178307; E-value=1.0E-55; 72% identity) identified by Shi et al. (2000). Based on these results we concluded that the 4F-5R fragment was likely a portion of a quinoa Na^+/H^+ antiporter homolog of the *SOS1* gene.

To clone the 5' and 3' portions of the quinoa *SOS1* cDNA homolog, gene specific primers (GSP) were designed from the 4F-5R cDNA fragment and used in a RACE reaction to amplify the 5' and 3' ends of the quinoa *SOS1* cDNA homolog (Table 1). 5' and 3' RACE reactions amplified two fragments of ~3000 bp and ~1200 bp fragments that captured the 5' and 3' ends of the cDNA, respectively, and overlapped with the 4F-5R internal SOS1 gene fragment. From the 5' RACE reaction, several clones were sequenced that when assembled formed two separate *SOS1*-like contigs, suggesting that the quinoa genome may encode two or more copies of the *SOS1* Na+/H+ antiporter gene. This was not surprising considering that quinoa is allotetraploid and therefore could encode two orthologs of the SOS1 gene, each originating from a separate ancestral diploid genome. Similarly, assembly of the DNA sequences from the 3' RACE reactions also suggested the presence of at least two separate SOS1 genes in quinoa. The two 5' RACE contigs show 95% identity, not including an 83 bp insertion in the 5' UTR of one contig. Fragment truncation in the 3' RACE sequences prevented a complete assembly or comparison, but an overlap of 451 bp with 98% identity was observed.

The presence of two distinct SOS1 genes in the quinoa genome was also supported by the analysis of genomic portions of the SOS1 gene. Primers 48F and 422R (Table 1) were designed from the 4F-5R fragment and were used in a PCR reaction to amplify a genomic portion of the SOS1 gene. The 48F and 422R primers are predicted to amplify a 375 bp fragment from the cDNA sequence, but when used with genomic DNA, two fragments were amplified that were significantly larger than expected based on the cDNA sequence. Both fragments were cloned and sequenced by primer-walking and two distinct contigs were assembled, including a 4309 bp and 4257 bp contig. Alignment of the genomic fragments and the 4F-5R cDNA fragment identified the presence of five introns which accounted for the significant increase in size between the cDNA and genomic sequences. The two largest introns (1290 and 1686 bp respectively) were later found to correspond to introns 7 and 9 of the complete genomic sequence (Figure 1). Significant nucleotide differences clearly distinguished the two genomic fragments, which showed only 85% identity and were differentiated by 10 gaps of ≥ 5 nucleotides, and confirmed the presence of two loci (tentatively termed Locus A and Locus B) as seen previously in the RACE fragments (Figure 2).

In order to obtain a complete genomic sequence of a quinoa *SOS1* homolog, a quinoa BAC library (Stevens et al. 2006) was probed with the 4F-5R fragment. Twenty putative hits were identified (Figure 3) and the strongest six BAC clones (117:G8, 120:I10, 146:N17, 150:P1, 152:L11, and 159:019) were isolated and were confirmed to contain the *SOS1* gene sequence via PCR analysis with the 4F and 5R primers. A single BAC clone (117:G8) was cultured, purified and shotgun sequenced at the Arizona Genomics Institute. Fragment assembly produced a single contig spanning 98357 bp

which has been submitted to GenBank (EU024570). Comparison of BAC sequence with 48F-422R genomic fragment sequences indicated the BAC sequence is homologous to the quinoa *SOS1* locus A (Figure 2).

Start and stop codons of the SOS1 gene were putatively identified based on BLASTX alignment of the BAC clone sequence with the *S.japonica SOS1* cDNA sequence (BAE95196). Primers LAstart and LAstop (Table 1) were designed from the BAC sequence to span the start and stop codons, respectively, and were used in a RT-PCR reaction to amplify a full length cDNA transcript of a single SOS1 homolog (presumably Locus A). PCR amplification with the LAstart and LAstop primers produced a strong amplification band of ~3500 bp. The amplification product was cloned and 33 independent clones were purified and sequenced. The consensus sequence produced a single contig of 3490-bp (Appendix I). Interestingly, several cDNA transcripts showed internal deletion events, including two transcripts with a 141 nt deletion at positions 1076 to 1216 of the consensus sequence and a single clone with a 60 nt deletion at positions 195 to 255 of the consensus cDNA sequence (Appendix I).

BLASTX searches of the non-redundant GenBank protein database using the consensus cDNA sequence showed significant hits to Na+/H+ antiporter sequences (E-value=0) for 14 different species, with the top two significant alignments being *SOS1* homologs from *S. japonica* (BAE95196; 82% identity) and *M. crystallinum* (ABN04858; 76% identity). Alignment of the consensus cDNA sequence and the 117:G8 BAC sequence using the mRNA-to-genomic DNA alignment program "Spidey" predicted the presence of 23 exons comprising 3474 bp of coding sequence (excluding the stop codon) and 17840 bp of intron sequence. Exon sizes ranged from 45 to 323 bp while intron sizes

ranged from 77 to 2123 bp. When translated, the coding sequence generated a protein of 1158 amino acids with a predicted molecular weight of 127.92 kd.

Hydrophobicity plot analysis of the consensus cDNA sequence predicted the presence of 11 transmembrane helices beginning at the N-terminus and continuing approximately until residue 500 (Figure 4). The remaining 658 residues (501 to C-terminus) are predicted to be an entirely hydrophilic region. The consensus cDNA sequence was compared to the NCBI Conserved Domain Database (v.2.11-17402 PSSMs) and two conserved regions were identified. Amino acids ~125 to 475 showed high similarity (E=5e-34) to Nhap, a Na^+/H^+ and K^+/H^+ antiporter domain (COG0025.2) and residues ~750-850 predicted (E=2e-9) a cyclic nucleotide-binding domain (pfam00027)

The complete BAC sequence was also screened for repeated elements with RepeatMasker v.3.1.8 against the *Arabidopsis* repeat database. The GC content was 36.15%. No transposons, SINEs, or LINEs were identified, however, several LTR elements were detected as were 26 simple sequence repeats (Table 2). BLASTX searches against the nr protein database (limited to *Viridiplantae* and masked for low complexity and repeat sequences*)* using the complete BAC clone sequence, identified two genes in addition to the SOS1 homolog. At position 608-1667 there was significant homology to a pre-mRNA splicing factor in *Oryza sativa* (Q6ZK48), and at position ~91000 to 94000 there was significant homology to a KUP-related K^+ transporter protein (Figure 5).

DISCUSSION

A full-length cDNA fragment of an SOS1 homolog was successfully cloned and sequenced from *C. quinoa*. The transcript contained a 3474 bp coding region encoding a 1158 amino acid protein with significant homology to SOS1 proteins from other plant species. Based on hydrophobicity plots, the predicted protein evidenced multiple N-terminal transmembrane helices and a long C-terminal hydrophilic region, consistent with the structure predicted for the *Arabidopsis* SOS1 (AtSOS1) protein. The predicted protein also contained two conserved domains common in SOS1, Nhap (a Na^+/H^+ and K^+/H^+ antiporter domain) and a putative cyclic nucleotide-binding domain in the C-terminal region. Work in *Arabidopsis* and other species has confirmed that the hydrophilic C-terminal region is essential for protein function, both *in vivo* and *in vitro* (Qui et al. 2002; Hamada et al. 2001). A SOS1 homolog (AY974336) that lacked 56 C-terminal residues was successfully used to complement salt-intolerant *E.coli* but showed reduced complementation as compared to the complete construct (Hamada et al. 2001). Recently the entire C-terminus of an SOS1 homolog (amino acids 440-1146) were shown to bind with RCD1, a regulator of oxidative-stress responses. In their study, Katiyar-Agarwal et al. (2006) showed RCD1 transportation from the nucleus to the cytosol during salt stress and RCD1 expression patterns similar to those of SOS1, indicating a high probability of RCD1-SOS1 interaction *in vivo*. Additionally, RCD-1 and SOS1 were found to regulate a similar set of genes, all involved in reactive oxygen species (ROS) detoxification. Salt stress has also been associated with ROS, particularly in superoxide- and H_2O_2-mediated damage of photosynthetic mechanisms (Hernandez et al. 1995).

It has also been hypothesized that the C-terminal region serves as a NaCl sensor (Zhu, 2002), a phenomenon seen in the yeast glucose transporters Snf3 and Rgt2; both serve as glucose transporters and gene regulators in glucose starvation (Ozcan et al. 1998). These data suggest SOS1, particularly the C-terminal region, may play a larger role in cellular salt-stress response. The possibility of a SOS-cNMP mediated response is supported by cyclic nucleotide involvement in Na^+ regulation as previously demonstrated in *Arabidopsis* (Maathuis and Sanders, 2001; Donaldson et al. 2004). cAMP or cGMP presence in the cytosol was shown to decrease the opening probability of voltage independent monovalent cation channels and cyclic nucleotide presence improved *Arabidopsis* salinity tolerance (Maathuis and Sanders, 2001). cGMP levels have also been shown to rapidly ($\leq$ 5 s) increase in response to salt stress (Donaldson et al. 2004). We hypothesize that the putative domain described here mediates a direct interaction between SOS1 and cyclic nucleotides as part of the cellular salt-stress response. SOS1 complementation tests performed in yeast and *E.coli* with the addition of cyclic nucleotides would provide a preliminary test of this hypothesis.

Several SOS1 cDNA transcripts cloned and sequenced in this study showed internal deletions that may have resulted during the RACE amplification or cloning procedure. One clone lacked the final 60 nucleotides of exon 1. This deletion represents 20 amino acids located within the predicted region of transmembrane helices, but upstream of the putative Nhap domain. Two transcripts also showed a 141 nt deletion that represents nucleotides 2-142 of exon 12 (total length 185 nt) and resulted in a 47 amino acid deletion from the putative Nhap domain. At least one other researcher has reported SOS1 transcript mutations, truncations, excisions and poor growth when cloning

SOS1 fragments from other species (Andres Belver, personal communication). Alternative splicing events are another possible explanation for these novel transcripts. Such a unique response to salt stress is an intriguing hypothesis and needs to be further investigated. We note that while partial exon-skipping is well characterized, it often involves mutation and activation of a cryptic splice site (McCullough et al. 1993). In the cDNA transcripts reported here the missing fragments do not appear to be flanked by cryptic splice sequences.

The presence of *SOS1* homologs in quinoa suggests that the SOS response to salt toxicity is conserved across many species. Whether or not the allelic variants of SOS1 identified in quinoa (which show only 70% identity to AtSOS1) or upstream promoter differences contribute to quinoa's extreme salt-tolerance still remains unanswered. The cloning and characterization of the genomic and cDNA sequence of the SOS1 gene homolog from quinoa, as reported here, provides the necessary framework to begin to answer these questions. Follow-up research, including the transgenic complementation of a mutant *sos1 Arabidopsis* line (CS3862) with combinations of promoter:SOS1 gene constructs (e.g., S35:cqSOS1; cqPromoter:AtSOS1; Atpromoter:cqSOS1) will undoubtedly be an important next step toward fully elucidating the SOS1 gene response in quinoa.

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FIGURES AND TABLES

Figure 1. Diagram representing the distribution of *SOS1* exons in 22kb of genomic quinoa DNA, as determined by cDNA alignment with Spidey. Grey arrows represent exons ≥150-bp in length. Black lines are exons ≤ 150-bp. The genomic region amplified by 4F-5R is boxed with a dotted line.

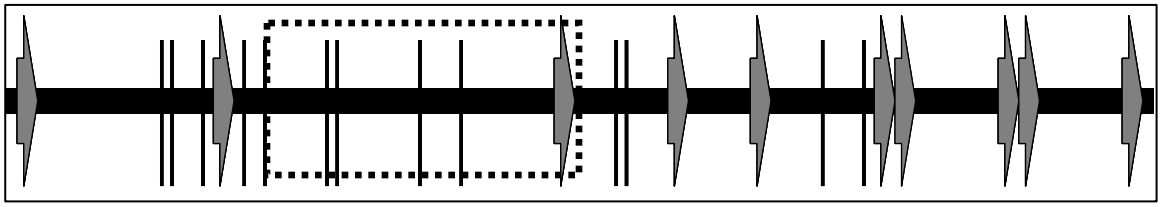
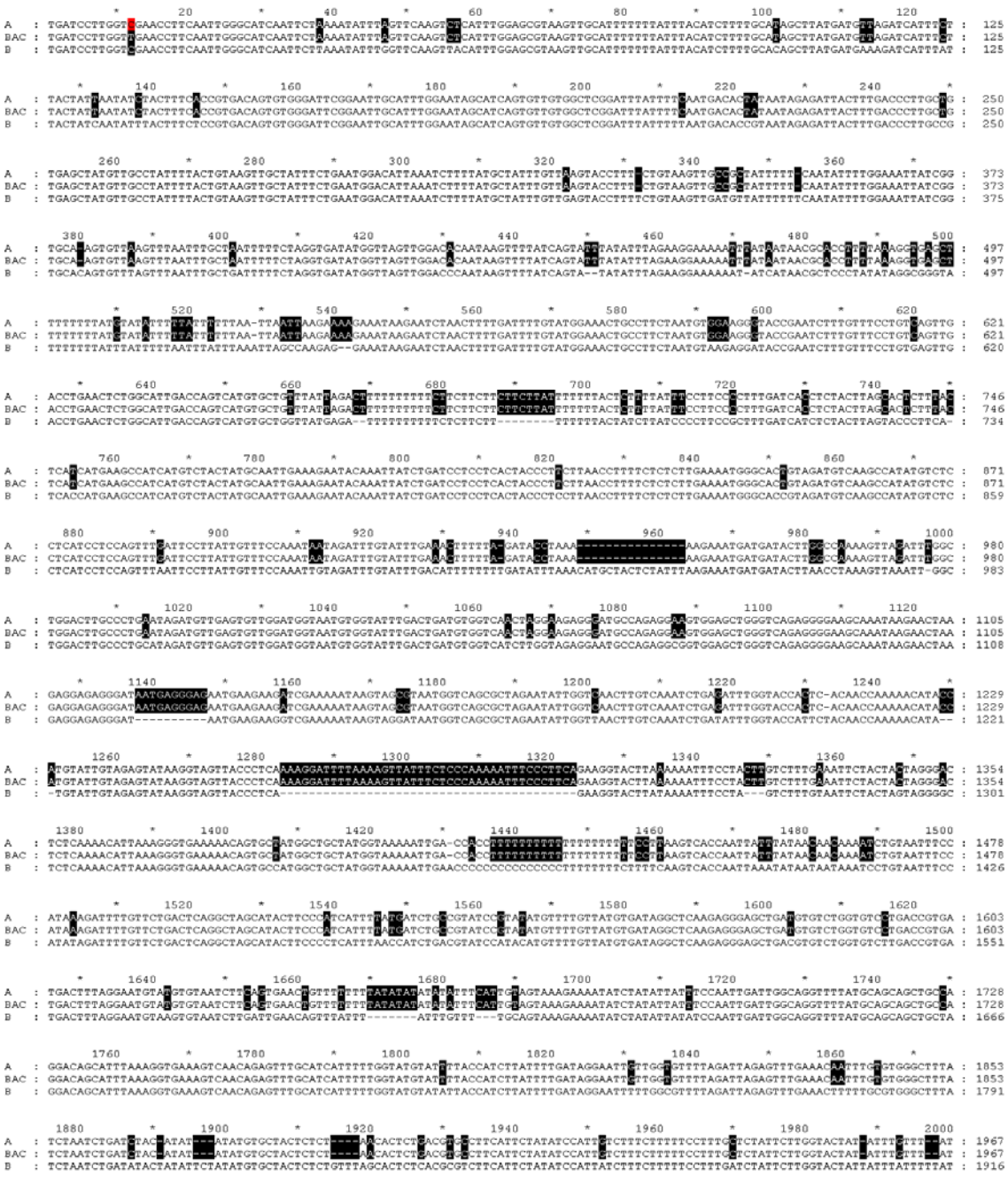


Figure 2. Alignment of the genomic sequences corresponding to the 4F-5R cDNA fragments. A and B represent the two genomic fragments sequenced at BYU. BAC is the 117:G8 BAC sequence. The BAC sequence has been trimmed to show only overlapping regions. Differences between A and the BAC are in red, between B and the BAC are in black.



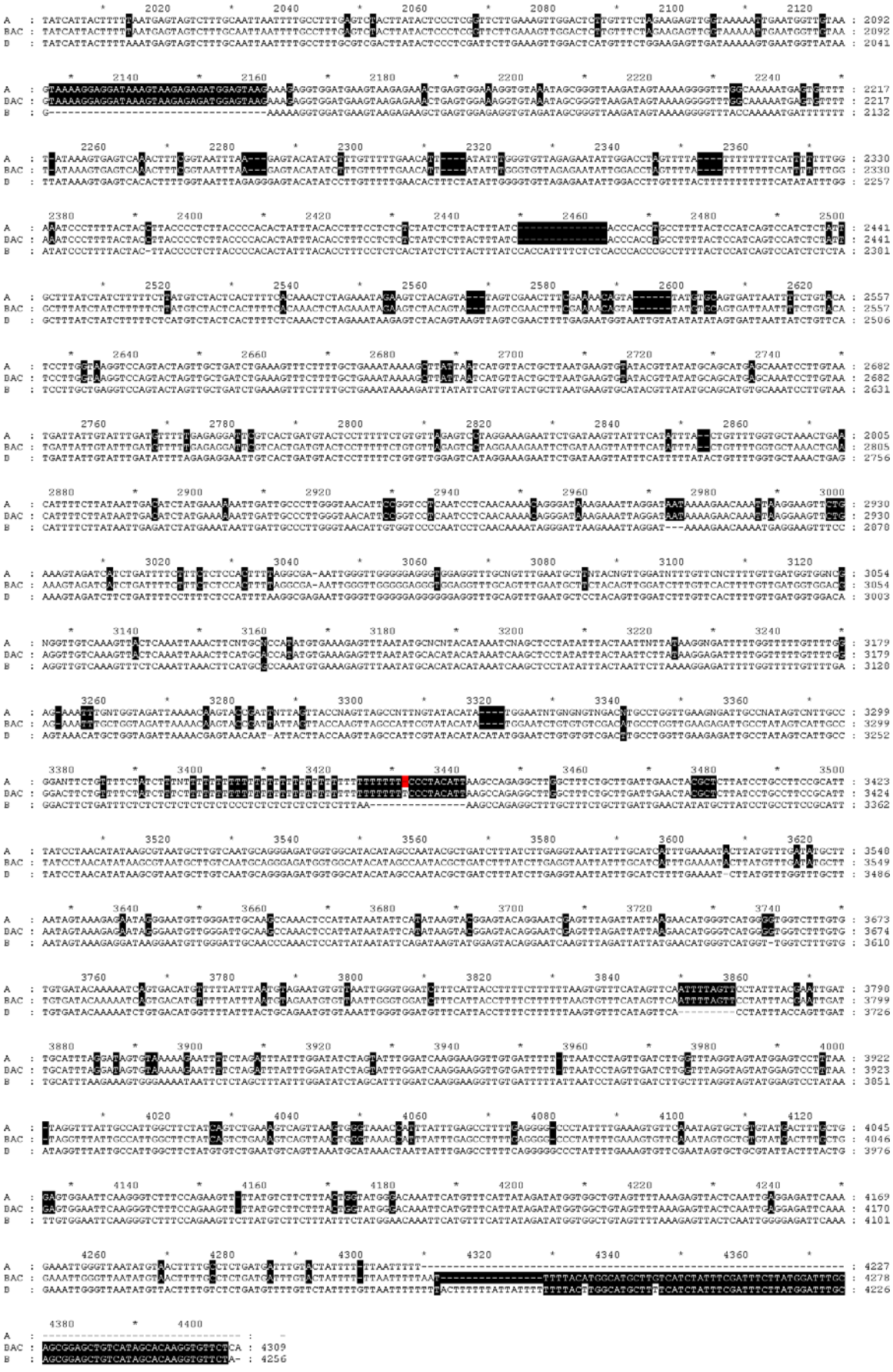


Figure 3. A single representative BAC hybridization blots, representing approximately 1/4 the entire library. Membranes are double stamped, placing each BAC clone on the filter twice. As such the presence of two hybridization events near each other represents true positives. See (http://www.genome.arizona.edu/information/protocols/addressnew.html) for complete address identification protocol.

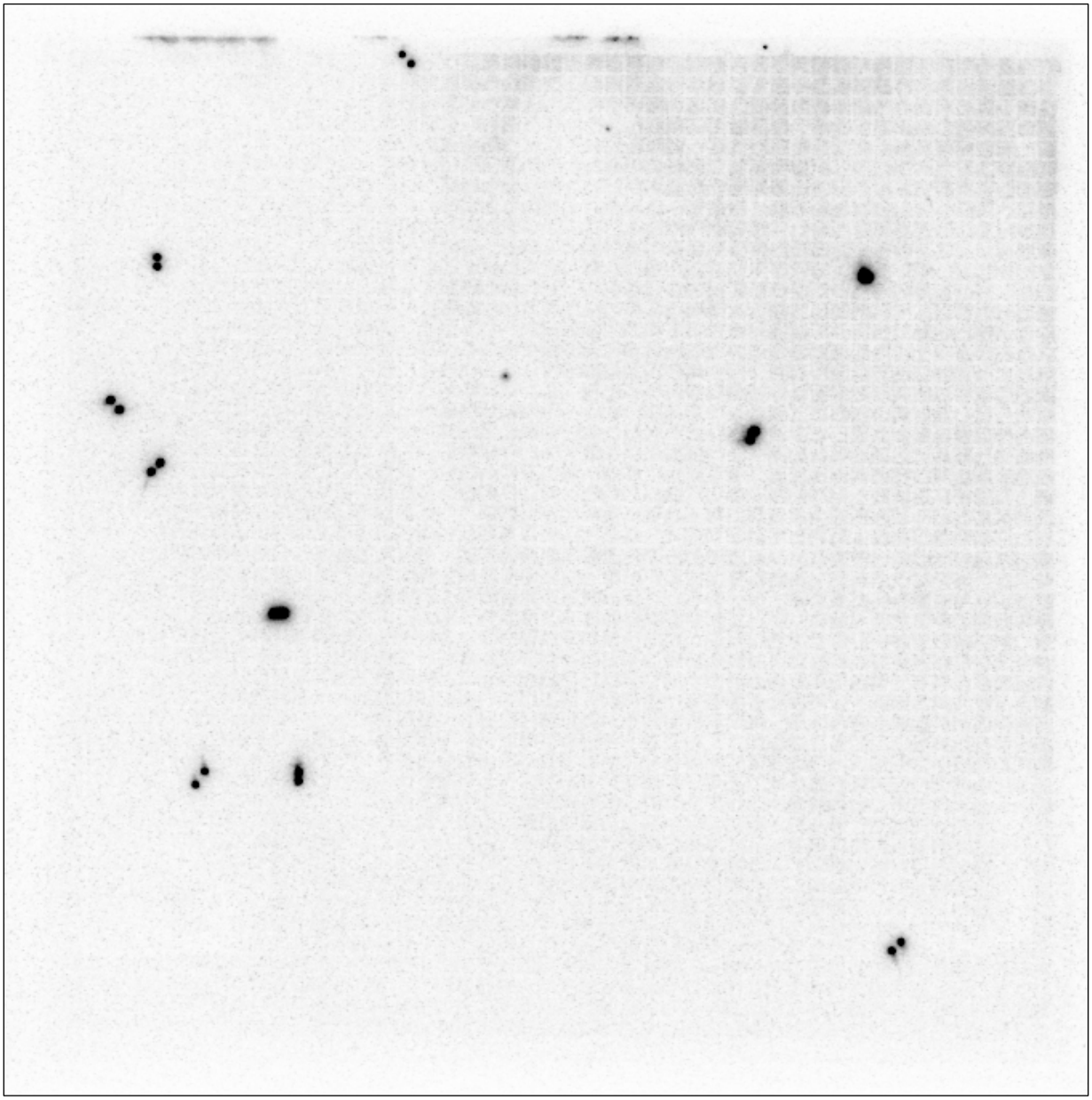


Figure 4. Hydrophobicity plot of the quinoa predicted protein indicating transmembrane regions and orientation. (http://www.ch.embnet.org/software/TMPRED_form.html)

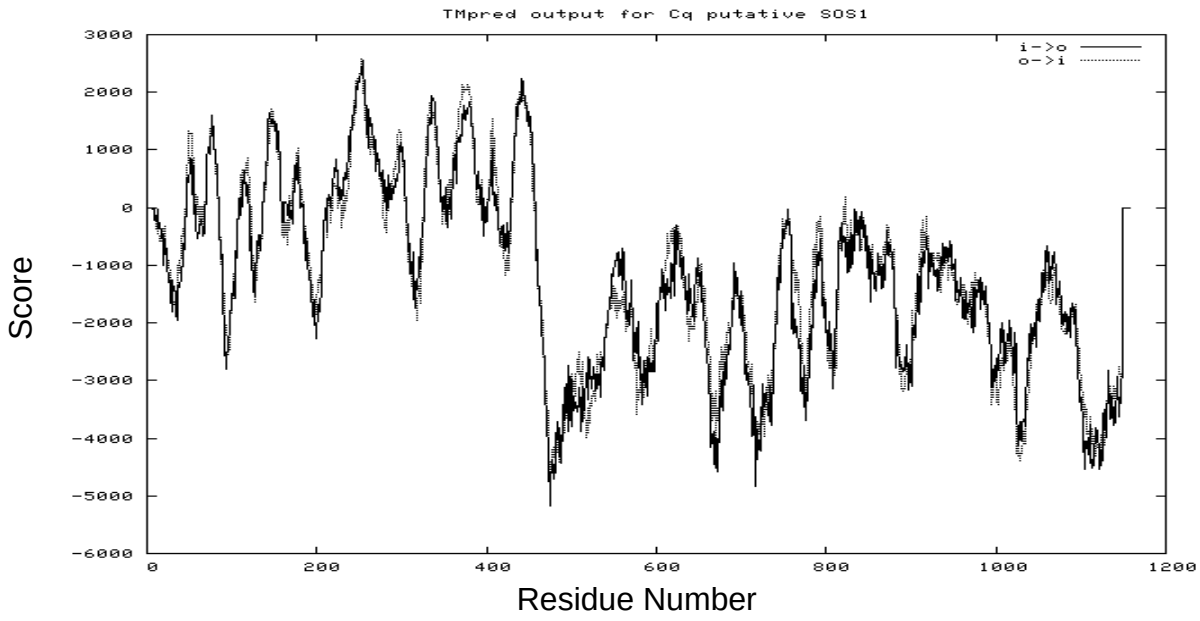


Figure 5. Summary of the significant Blastx alignments. The entire 117:G8 BAC was compared to the Viridiplantae (txid33090) database. RepeatMasker identified repetitive elements were masked during this alignment. Numbers indicate thousands of nucleotides from the left side of the BAC plasmid.

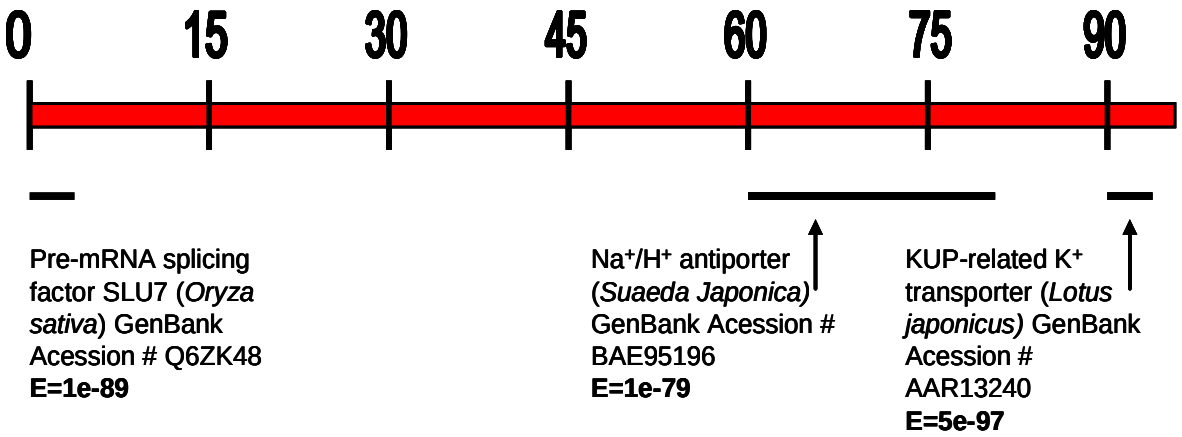


Table 1. Primers used in obtaining the *SOS1* cDNA sequence. All primers were ordered through IDT. Successful RT-PCR primers are in bold.

Name	Sequence	Application
5RP74	GCC CAA TTG AAG GTT CGA CCA AGG A	5' RACE Rxn
5RP520	CTC AAC AAC CAC TCC CCG AGC AAC	
3RP231	TGC TCA AGA GGG AGC TGA TGT GTC T	3' RACE Rxn
3RP498	TGC TCG GGG AGT GGT TGT TGG AGT T	
LAstart	AAG CAC CTA TGG CTG CAT ATC GAA	Full length cDNA amplification
LAstop	CAA AAG AGC TCT CAA GGT GCT TGG	
48F	GAT CCT TGG TCG AAC CTT CA	Genomic amplification
422R	AGA ACA CCT TGT GCT ATG ACA G	
1F	GTT TTT CTH CCB GCT CTT CTT TT	RT-PCR
2F	TTA CTT GCT GGV CCT GG	
3F	CTG GAA AAC ATC ATT GTT GCT TGG	
4F	**ATG AAT GAT GGG ACG GCD ATT GTT GT**	
5F	TCT GGT GTT TTG ACB GTC ATG AC	
6F	AAA GCT TGC ATC ACT TCT GGG AAA TGG TT	
7F	TTT GGC TAT GGT TTG GA	
8F	TGT TTG TTT TCT TCA CTG GTG G	
9F	GAT CTT GGA GAT GAT GAG GA	
10F	TAA ATG GTG TBC AAG C	
11F	CAT TTC CTC AGG TTC TCC G	
12F	CAT CTT CAT GAT GCT GTC CAG AC	
1R	CCA AGC AAC AAT GAT GTT TTC CAG	
2R	ACA ACA ATD GCC GTC CCA TCA TTC AT	
3R	GTC ATG ACB GTC AAA ACA CCA GA	
4R	AAC CAT TTC CCA GAA GTG ATG CAA GCT TT	
5R	**TCC AAA CCA TAG CCA AA**	
6R	CCA CCA GTG AAG AAA ACA AAC A	
7R	TCC TCA TCA TCT CCA AGA TC	
8R	GCT TGB ACA CCA TTT A	
9R	CGG AGA ACC TGA GGA AAT G	
10R	GTC TGG ACA GCA TCA TGA AGA TG	
11R	ACD AGT GCA CTT TCC TGC CA	

Table 2. Repeat elements identified by RepeatMasker (v.3.1.8) using 98357 nucleotides from quinoa BAC plasmid 117:G8. Sequence was screened against the Rep Database for *Arabidopsis*.

Class		Number	Length	% of Sequence
Retroelements		**7**	**3566**	**3.63**
LTR elements		7		
Ty1/Copia		2		
Gypsy/DIRS1		5		
Simple Sequence Repeats		**26**	**1789**	**1.82**
Mono-	$(T)_n$	7		
	$(A)_n$	4		
Di-	$(TA)_n$	2		
	$(TC)_n$	1		
Tri-	$(CAA)_n$	1		
	Other[a]	3		
Tetra-	$(TTTA)_n$	2		
	$(CCCA)_n$	1		
Penta-	$(TTGTG)_n$	2		
	$(CCGAA)_n$	2		
	$(CAGAT)_n$	1		

[a]Each other motif was found a single time

LITERATURE REVIEW OF THE SOS GENE FAMILY IN SALT TOLERANCE

INTRODUCTION

The productivity of over one-third of all arable land world-wide is affected by soil salinity (Epstein 1972). Sodium (Na^+) adversely affects plant growth when high concentrations are found in soil or water. Highly saline growing conditions lead to high cytosolic Na^+ concentration resulting in plant death, likely due to a combination of adverse effects. Nearly all enzyme activity decreases dramatically above 0.3-0.5 M NaCl due to disruption of the electrostatic forces maintaining protein structure (Wyn Jones and Pollard 1983). In addition Hernandez et al. (1995) found that NaCl stress significantly damaged photosynthetic mechanisms through a combination of superoxide-and H_2O_2-mediated oxidation. In the cytoplasm of salt treated *Nitellopsis obtusa* potassium (K^+) levels decrease with Na^+ concentration increase (Katsuhara et al. 1986); that decrease has been shown to directly correlate with lower growth rates (Ben-Hayyim et al. 1987). Salt stress has also been shown to alter phospholipid metabolism, inducing the expression of a phosphatidylinositol-specific phospholipase C (Hirayama et al. 1995) and increasing the synthesis of some phospholipids while increasing the turnover of others such as phosphatidylcholine (Pical et al. 1999). In 1995 Niu et al. determined that plants employ three basic strategies to prevent and correct high Na^+ concentrations: active Na^+ efflux, Na^+ compartmentalization in the vacuole, and Na^+ influx prevention. Sodium efflux and transport into the vacuole are coupled to pH gradients created at their respective membranes, and while both respond to salt stress with increased transcription of transport proteins, only plasma membrane transporters are activated when exposed to high salinity (Niu et al. 1995, 1993a, 1993b). However, regulation of plasma membrane transporters only occurs in plants un-adapted to saline conditions. Plants adapted to saline conditions

over hundreds of generations showed lower expression levels (Niu et al. 1993a, Perez-Prat et al. 1994). When plants are adapting to new salt conditions they increase expression levels of transporters and/or increase transporter activity, but once acceptable ion levels have been obtained under saline conditions they are maintained by passive mechanisms. These mechanisms likely include alterations in plasma membrane Na^+ permeability (Niu et al. 1995). Plants with little to no salt tolerance have missing, mutated, or insufficient mechanisms to counteract environmental salt concentration. Salt-sensitive mutants frequently lose all ability to grow in saline conditions, emphasizing the importance of salt tolerance pathways. The ability to increase salt tolerance in crop plants is desirable in many world regions where sodic soil precludes crop production and in other areas that experience significant fluctuations in soil salinity, such as estuaries and intertidal zones (de Leeuw et al. 1991).

Salt tolerance determinants include genes that directly affect ion movement as well as those involved in osmotic balance, gene regulation, and membrane integrity. Many of these processes, including the plants' coordinated ability to maintain osmotic balance and turgor pressure in a variety of environments, are not well understood. Identifying the individual genes and proteins of such processes reveals important patterns in biological ion transport and provides a basis of understanding to build upon. Understanding Na^+ transport also should shed light on the transport of other ions as they are likely to share similar cellular processing.

Plants with the ability to grow on saline soils are termed halophytes. Salt marsh grasses and other saline-environment plants fall into this category. While quantitative trait loci (QTL) have been successfully identified, the most active research done in salt

tolerance employs a forward genetics approach. Analysis of induced mutant lines of Arabidopsis screened for their ability to grow on NaCl-infused media has identified several genes related to salt-tolerance. This review briefly describes the QTL analysis on Arabidopsis salt-tolerance but focuses on the SOS family salt-tolerance genes, their respective functions, their interactions with each other, and their secondary roles.

INITIAL GENETIC MAPPING OF SALT TOLERANCE IN ARABIDOPSIS AND OTHER SPECIES

Using germination percentages and vegetative growth rates, salt-tolerance in Arabidopsis accessions has been measured. Quesada et al. (2002) screened 200+ unique accessions and found that seeds germinated on 250mM NaCl media show a wide range of germination rates. Analysis of F2 germination percentages in segregating F2 populations in the most salt tolerant accessions indicates germination rate in saline conditions is polygenic. Germination rate and vegetative growth in salty conditions were found to be independently controlled (Quesada et al. 2002). Based on germination rates 6 QTL have been mapped in Arabidopsis to chromosomes 1, 2, 3, and 4. Vegetative growth analysis by Quesada et al. (2002) also yielded 5 QTL on chromosomes 1, 4, and 5. The highest LOD score of all 11 Arabidopsis QTL was 4.33, and the lowest 1.72. Only one QTL appears in both germination rate and vegetative growth analyses, indicating that the two salt-responsive systems function independently.

While this data was not used to identify the genes described below it did identify 11 genomic regions highly associated with various forms of salt tolerance. One region appears to correspond to SOS2, a major salt tolerance gene (see below), but the

remaining QTL still require fine mapping and gene identification.

Studies by Foolad et al. (1997) in Solanum esculentum (tomato) indicate similar polygenic control of germination during salt tolerance and a similar relationship between salt tolerance at germination and during vegetative growth. Two regions, on chromosomes 1 and 9, showed significant correlation to growth during both developmental stages, but overall the data indicates that the ability to germinate under salt stress is independent of the ability to continue vegetative growth under the same stress (Foolad 1999).

Salt tolerance traits have also been examined in *Oryza sativa* (rice). In 2001 Koyama et al. mapped regions that correlate significantly with sodium uptake, potassium uptake, and sodium:potassium selectivity. All three were shown to be independently controlled (Koyama et al. 2001).

GENE IDENTIFICATION

SOS1 Salt-intolerant Arabidopsis mutants can be identified by the root bending assay described by Howden and Collet (1992). (Figure 1) Wu et al. EMS-mutagenized M_2 seeds and identified 4 salt-tolerance mutants out of ~50,000 screened seedlings (1996). The four were crossed with one another and found to segregate in a pattern consistent with recessive single nuclear mutations and were found to be allelic to one another. This particular genotype was designated *SOS1*, for *salt-overly-sensitive 1*. However *sos1* mutant seeds were unable to germinate on salty media and were permanently damaged by salt treatment, being unable to germinate once transferred to standard media. Fully developed mutant plants' growth was also inhibited by salt treatment; these results

indicate a continued role of the *SOS1* genotype throughout development. The *SOS1* genotype was genetically mapped to chromosome 2, 29.5 ± 6.1 cM (Wu et al. 1996).

This region was fine mapped and candidate genes identified and sequenced by Shi et al. (2000). The *SOS1* locus was characterized by transgenic complementation assays in which the 35S::*SOS1* transcript completely rescued *sos1* mutant phenotypes. Sequence analysis predicted the candidate gene to be a membrane protein with 12 transmembrane regions and significant similarity to Na^+/H^+ antiporters of mouse and *Pseudomonas aeruginosa*. The *SOS1* gene contains 22 introns and 23 exons, and predicts a protein with 1,146 amino acid residues and a molecular mass of 127 kDa (Shi et al. 2000). The cellular location at the plasma membrane was confirmed by Shi et al. in 2002 with fluorescent fusion proteins. The same study indicates Arabidopsis plants preferentially express *SOS1* in epidermal cells at the root tip, and parenchyma cells at xylem/symplast boundary of roots, stems, and leaves (Shi et al. 2002b). Phylogenetic analysis using the full sequence of the SOS1 gene clusters the gene with four microbial Na^+/H^+ antiporters but not with plant or yeast tonoplast Na^+/H^+ antiporters. These results all suggest *SOS1* is a plasma membrane bound Na^+/H^+ antiporter essential in Na^+ efflux. Shi et al. (2000) also compared the sequences of all *SOS1* mutant alleles and identified several regions highly sensitive to mutations. Point mutations in transmembrane domains and the 5' cytosolic region significantly affect function of the entire protein, indicating both regions are essential for Na^+ transport. *SOS1* transcripts have weak basal expression but were found to be upregulated after salt treatment (Shi et al. 2000).

SOS1 has been identified and cloned in multiple plant species in addition to Arabidopsis, including in L*ycopersicon esculentum*, *Triticum aestivum*, and *Cymodocea*

nodosa (all unpublished data) *Oryza sativa* (Martinez-Atienza et al. 2007), and *Physcomitrella patens* (Benito and Rodriguez-Navarro, 2003).

SOS2 Zhu et al. screened plants from either ethyl methanesulfonate- or fast-neutron-mutagenized M_2 seeds with root-bending assays then crossed the mutants with *sos1* mutants and identified *SOS2* as a second salt tolerance locus. Backcrosses confirmed their *sos2* mutants are also allelic to each other, recessive, and single nuclear mutations. The *SOS2* locus was mapped to chromosome 5 of Arabidopsis. Mutant *sos2* lines were found to be hypersensitive to Na^+ and Li^+ but not to Cs^+ or mannitol (Zhu et al. 1998). These results indicate *SOS2* is not involved in general cation regulation but interacts with specific monovalent cations.

Liu et al. (2000) fine-mapped the *SOS2* locus on chromosome 5 and identified candidate genes based on predicted ORFs. Sequence comparison of various *sos2* mutants identified the *SOS2* coding sequence. The *SOS2* gene contains 13 exons and 12 introns and predicts a 446 amino acid-51 kDa protein (Liu et al. 2000). Database comparison predicted that an active kinase domain resides in the N-terminal region of the protein, indicating it's function as a kinase; but the C-terminal regulatory domain of *SOS2* shows low sequence homology with other kinases. Liu et al. (2000) found only minor homology with *Schizosaccharomyces pombe* (yCHK1) and human CHK1 (hCHK1) kinases and the C-terminal regulatory domain. However, several of the *sos2* mutant alleles contain single nucleotide polymorphisms (SNPs) in this regulatory domain, indicating the C-terminal domain does play a role in salt tolerance. In addition to its kinase activity, *SOS2* autophosphorylation is also required for salt tolerance. As with

SOS1, *SOS2* transcription is up-regulated by salt treatment. (Liu et al. 2000; Gong et al. 2002).

Gong et al. (2002) found that *SOS2*, like most kinases, requires a divalent cation to coordinate the phosphate groups of the nucleotide triphosphates (ATP or GTP) involved. *SOS2* shows a functional preference for Mn^{++} over Mg^{++} as the required divalent cation. The minimum Mn^{++} concentration required was 0.25mM; however, as intracellular Mn^{++} concentrations are in the micromolar range, this cation is unlikely to play a role in Arabidopsis cells *in vivo*. Gong et al. (2002) also found that the phosphate source for *SOS2* phosphorylation activity was limited to the nucleotide triphosphate ATP not GTP. The autophosphorylation activity of *SOS2* was shown to proceed by an intramolecular—not intermolecular—reaction, signifying that a single *SOS2* protein autophosphorylates itself, as opposed to *SOS2* proteins phosphorylating each other (Gong et al. 2002).

SOS3 Liu and Zhu (1997) identified the *SOS3* locus by root-bending assays on fast neutron-mutagenized M2 seedlings. Backcross analysis revealed the *sos3* mutation as nuclear, recessive, and unique from *SOS1*. Analysis of a segregating F2 population of 426 *sos3* mutants mapped the locus to chromosome 5. The *sos3* mutant phenotype is hypersensitive to Na^+ and Li^+ but not to general osmotic stress (created by KCl, CsCl, and mannitol). As with *SOS1* and *SOS2* the *sos3* mutants are also sensitive to low K^+ levels but to a lesser extent. To continue root growth in a bending assay *sos1* mutants require a minimum of 20mM K^+ media while *sos3* plants will grow normally on ~1mM K^+ media. However, the 1mM K^+ media requirement represents an order of magnitude

greater than the wild-type K^+ requirement. However, *sos3* K^+ sensitivity can be rescued by external treatment with Ca^{++}. This complementation is not exhibited in *sos1* mutants. Ca^{++} treatment also partially rescued the Na^+ sensitivity of *sos3* mutant plants, an observation not seen in *sos1* or *sos2* plants (Liu and Zhu, 1997).

Liu and Zhu (1998) determined the *SOS3* gene encodes a 222 amino acid residue protein encoded by a 8 exon—7 intron coding region. They confirmed the SOS3 locus through transgenic complementation tests and its predicted amino acid sequence was shown to be similar to other calcium-binding proteins, such as calmodulin and calcineurin B. The *sos3* mutant contains a deletion in one of the highly conserved Ca^{2+} binding regions, most likely disabling its binding function. The sequence similarities between calceneurin, neuronal calcium sensors and the SOS3 protein indicate SOS3 is likely a member of a Ca^{2+} signaling pathway in response to salt stress. This is consistent with the observed rise in cytosolic Ca^{2+} in response to salt stress (Lynch et al. 1989; Knight et al. 1997).

The SOS3 protein also requires myristoylation at the N-terminus in order to function (Ishitani et al. 2000). N-myristoylation is the covalent attachment of the lipid myristic acid to the N-terminal glycine by an amide bond. This form of covalent modification is common in signal transduction proteins, calcium binding proteins and many kinases (Johnson et al. 1994). When treated with myristoylation inhibitors wild-type plants assume *sos3* phenotypes (Ishitani et al. 2000).

SOS4 Shi et al. (2002a) identified a fourth salt-tolerance locus in Arabidopsis by screening ~60,000 seedlings from ethyl methanesulfonate— or fast neutron—

mutagenized M2 seeds. They also mapped this *SOS4* locus to chromosome 5. The *sos4* mutants also are nuclear, recessive mutations and have been confirmed non-allelic to *SOS1*, *SOS2*, and *SOS3* genes (Shi et al. 2002a). Mutant plants exhibit Na^+ and Li^+ hypersensitivity as with the other mutants, however, *sos4* mutations also cause K^+ hypersensitivity, a phenotype unique to these mutants. They do not show any change in Cs^+ or mannitol sensitivity, consistent with other SOS mutants (Shi et al. 2002a). These data indicate, as with the previously mentioned SOS genes, that salt-tolerance pathways are ion specific, not merely responding to general osmotic stress.

The *SOS4* gene encodes a 13-exon transcript that exhibits alternative splicing at the first intron, predicting two unique proteins that differ by 34 amino acids. Both predicted proteins have been modeled as cytoplasmic pyridoxal (PL) kinases with significant similarity to PL kinases in humans, yeast, and bacteria. PL kinases are involved in the production of PL-5-phosphate, and important enzyme cofactor and ligand for certain ion transporters (Shi and Zhu 2002a). *SOS4* transcripts introduced into *E. coli* complement mutants defective for a PL kinase. PL kinase in *E. coli* functions in the biosynthesis pathway of pyridoxal-5-phosphate (PLP), which is well documented as a coenzyme in animal cells. PLP function in plant cells has not been identified, nor has any connection with ion transporter activity. Shi et al. (2002a) created a *SOS4* promoter—β-glucuronidase fusion construct and demonstrated *SOS4* is expressed ubiquitously; however, expression levels do vary between tissues. As with all SOS family genes, expression of *SOS4* is mediated by salt stress (Shi et al. 2002a).

INTERACTION BETWEEN GENES AND PROTEINS

The three primary genes characterized in Arabidopsis salt-tolerance pathways are *SOS1*, *SOS2*, and *SOS3*. *SOS4* is clearly involved in salt tolerance, but has not been identified as part of the *SOS1*, *2*, and *3* pathway. The double mutant experiments described below have not been carried out with the *SOS4* locus. Each of the first three genes has been mapped and cloned, confirming they are non allelic. In addition, interactions between the three genes have been examined. Double mutants of *sos1* and *sos2* were created and did not exhibit a unique phenotype from the *sos1* single mutant, indicating they function in the same pathway (Zhu et al. 1998). Analysis of the three gene products also precludes the possibility of each having a similar function in different pathways as duplicate gene products. *SOS1* codes a transmembrane Na^+/H^+ antiporter, *SOS2* a protein kinase, and *SOS3* a calcium sensor (see above).

SOS2—SOS3 Data from Halfter et al. (2000) has elucidated the functional relationship between SOS2 and SOS3. Double mutants of *sos2* and *sos3* exhibit a *sos2* phenotype, and SOS2 and SOS3 physically interact in yeast two-hybrid system as well as *in vitro* (Halfter et al. 2000). Halfter et al. found this interaction takes place specifically at the C-terminal regulatory domain and does not involve the N-terminus. They report SOS2 is activated by SOS3 through this physical interaction and the activation is dependent on the presence of Ca^{2+} (Halfter et al. 2000). However, Guo et al. (2001) reported interaction between the N-terminal kinase domain and the C-terminal regulatory domain within SOS2. They found by removing the regulatory domain of SOS2, including the SOS3 binding motif, resulted in constitutively activated SOS2 protein kinase. A similar result

was produced by changing Thr^{168} to Asp in the activation loop of the SOS2 kinase domain. A superactive SOS2 kinase was produced when the two modifications were combined, and all three of the activated SOS2 proteins were no longer dependent on SOS3 binding for activation (Guo et al. 2001).

Recently Guo et al. (2004) found that an autoinhibitory FISL motif found in the C-terminal regulatory domain of SOS2 (Guo et al. 2001) plays a particular role in salt tolerance. SOS2 mutants activated by Thr^{168}-to-Asp mutations partially rescued salt hypersensitivity in *sos2* and *sos3* mutant plants, but SOS2 proteins lacking only the FISL domain rescued only the *sos2* mutation (Guo et al. 2004). SOS2 proteins lacking the entire C-terminus regulatory domain failed to rescue either mutant (Guo et al. 2004).

SOS1—SOS2 AND SOS3 To analyze the relationship between *SOS1*, and *SOS2* and *SOS3* Qui et al. (2002) extracted highly purified plasma membrane vesicles from Arabidopsis and examined them for Na^+/H^+ transport. *sos1* mutant plants show decreased Na^+/H^+ transport activity that cannot be rescued by constitutively activated SOS2. However, in wild-type plants the activated SOS2 protein does significantly increase Na^+/H^+ transport up to 2-fold. Activated SOS2 did increase Na^+/H^+ transport in *sos2* and *sos3* mutants (Qui et al. 2002). The activated SOS2 protein used does not require SOS3 or Ca^{2+} in order to function (Guo et al. 2001). These results identify SOS1 as a plasma membrane antiporter regulated by SOS2 and SOS3 (see Figure 2). The ability of SOS2 to increase Na^+/H^+ transport in *sos3* mutants indicates it is regulated by SOS3. The current model proposed by Qui et al. places SOS1 at the plasma membrane as a phosphorylation target for SOS2. SOS2 activity is mediated by SOS3 and Ca^{2+} binding

(Qui et al. 2002). The patterns described in this model are consistent with Na^+/H^+ transport regulation in animals (Fliegel et al. 1992, Takahashi et al. 1999).

SOS GENES AS INDIRECT SALT TOLERANCE MEDIATORS

Gong et al. (2001) identified 84 salt-regulated genes by repeated rounds of differential subtraction screening, followed by sequencing and northern blotting. Expression of those 84 salt-regulated genes was analyzed to investigate the possible role of SOS genes as transcriptional regulators. Six of the 84 showed differential regulation between wild-type and *sos3* mutant plants; (AD06C08/unknown, AD05E05/vegetative storage protein 2 [VSP2], AD05B11/S-adenosyl-l-Met:salicylic acid carboxyl methyltransferase [SAMT], AD03D05/cold regulated 6.6/inducible2 [COR6.6/KIN2], salt tolerance zinc finger [STZ], and (AD05C10/ circadian rhythm-RNA binding1 [CCR1]). Two of these were down-regulated (VSP2 and AD06C08/unknown) and the remaining 4 were up-regulated in the *sos3* mutants (compared to wild type). All except VSP2 show identical expression patterns between *sos1, sos2*, and *sos3* mutants. VSP2 shows similar expression in wild-type and *sos1*, indicating SOS1 is not involved in its regulation. These results suggest the model shown in Figure 2, as reported by Gong et al. (2001). In brief salt stress upregulates expression of *SOS1*, *SOS2* and *SOS3*, which function together in increasing transcription of the five genes listed. VSP2, the sixth gene, is solely regulated by *SOS3* and *SOS2* however it can be affected by jasmonic acid (JA) (Gong et al. 2001). In wild type and *sos3* plants Methyl jasmonate increases VSP2 transcript levels; however a connection between JA and salt-stress has not been discovered. These data clearly demonstrate the SOS family of proteins functions to directly and indirectly

mediate salt-tolerance. Their role as both positive and negative transcriptional regulators is also shown.

SOS MEDIATION OF VACUOLAR Na^+ TRANSPORT

AtNHX1 is a vacuolar Na^+/H^+ transporter in Arabidopsis that shows significant coordination with the SOS family (Shi and Zhu, 2002b; Shi et al. 2002b). Studies by Qui et al. (2004) showed that in homozygous *sos1* mutant plants AtNHX1 transport activity increased by 50% relative to the wild-type but decreased 60% in *sos2* plants. The *sos3* plants show no significant change in AtNHX1 activity. Activated SOS2 restored AtNHX1 transport to wild-type levels in *sos2* plants, but had no effect in wild-type, *sos1*, or *sos3* plants. *In vitro* phosphorylation assays were performed to analyze the mechanism of this effect, but no *SOS2*-mediated AtNHX1 phosphorylation was observed (Qui et al. 2004).. However active *SOS2* is required to restore AtNHX1 activity in the mutant, as inactive SOS2 has no effect. However, the results may only reflect the inability to create adequate conditions to sustain *in vitro* phosphorylation of AtNHX1 by SOS2, implying that activated SOS2 may in fact be phosphorylating AtNHX1 directly. The alternative hypothesis predicts that one or more intermediates exist in the phosphorylation pathway between SOS2 and AtNHX1 (Qui et al. 2004).

SALT TOLERANCE IN *Chenopodium quinoa*

C. quinoa has a natural salt tolerance, having adapted to growth on the high plains of the Andean region, which experiences drought, frequent frosts, and high soil salinity (Coles et al. 2005). Quinoa germination rates under salt stress have been quantified by Prado et

al. (2000). In the presence of 400 mM NaCl quinoa seeds showed 14% germination rate, compared to 87% in the control (Prado et al. 2000). However, in similar studies of arabidopsis germination rates, 26 of 102 Arabidopsis accessions show comparable decreases at only 250mM NaCl (Quesada et al. 2002). Quinoa has been observed to germinate up to 75% at a salt concentration of 57 mS/cm (Christiansen et al. 1999; Jacobsen et al. 1999), a significant feat considering sea water has a salt concentration of 40 mS/cm (Jacobsen et al. 2003). These data suggest an above-average salt tolerance in quinoa, compared to the available data on Arabidopsis. However, as of yet no research has been reported on the quinoa SOS gene family.

CONCLUSIONS

Salt tolerance is an economically important trait for crop species. In Arabidopsis it has been shown to be under polygenic control. The SOS genes already identified in Arabidopsis have provided a strong starting point from which a more complete understanding can be obtained in other plant species. In addition many of the components of salt-mediated cellular pathways remain undiscovered. We know that in Arabidopsis two responses to salt stress, germination and vegetative growth, are independently controlled. Additional investigation of the SOS pathway in multiple plant species across the plant kingdom represents an intriguing opportunity to better understand salt stress response and ion transport. Successful identification of the SOS genes in other species would allow genetic and functional comparisons across species, which could potentially elucidate different roles of the SOS pathway in different plants' salt-tolerance mechanisms.

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FIGURES

Figure 1 Root bending assay mutant identification. Seedlings are transferred to a salty medium and positioned vertically, oriented opposite of their previous orientation. Plants uninhibited by high salt concentrations continue directional root growth as determined by gravitropism, and the new root begins to curve downward. This method allows for easy mutant identification due to the obvious phenotypic difference. Taken from (Wu S et al. **SOS1, a Genetic Locus Essential for Salt Tolerance and Potassium Acquisition**. *The Plant Cell* 8:617-627 1996)

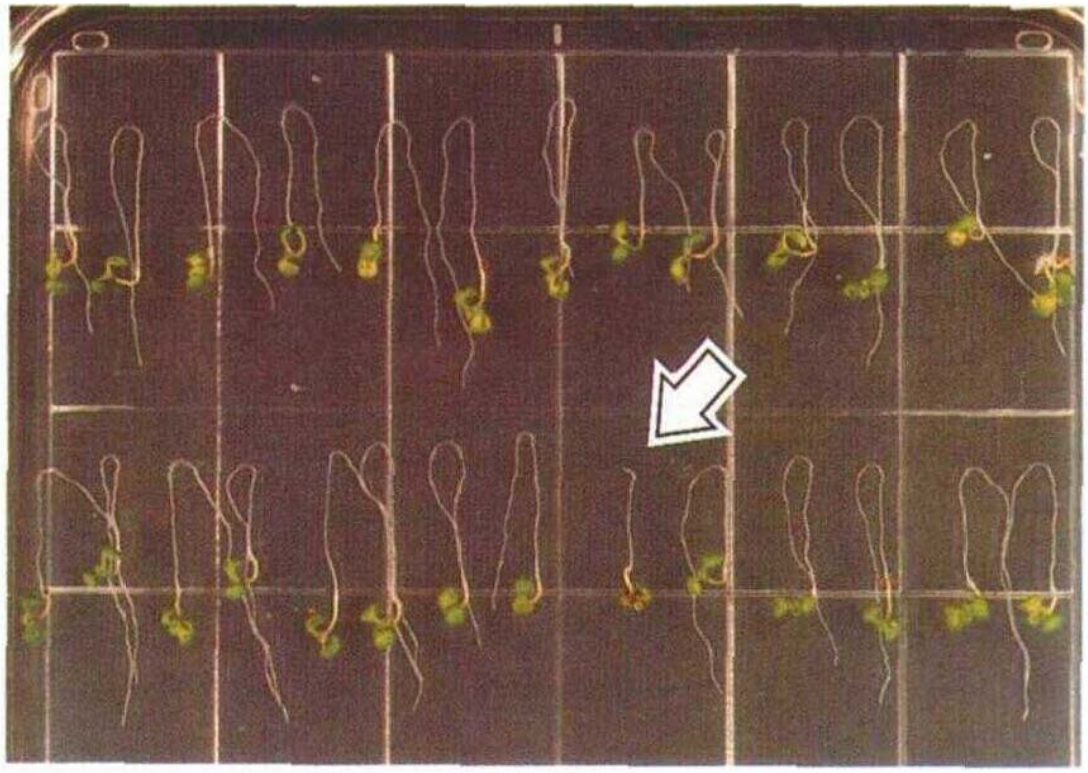
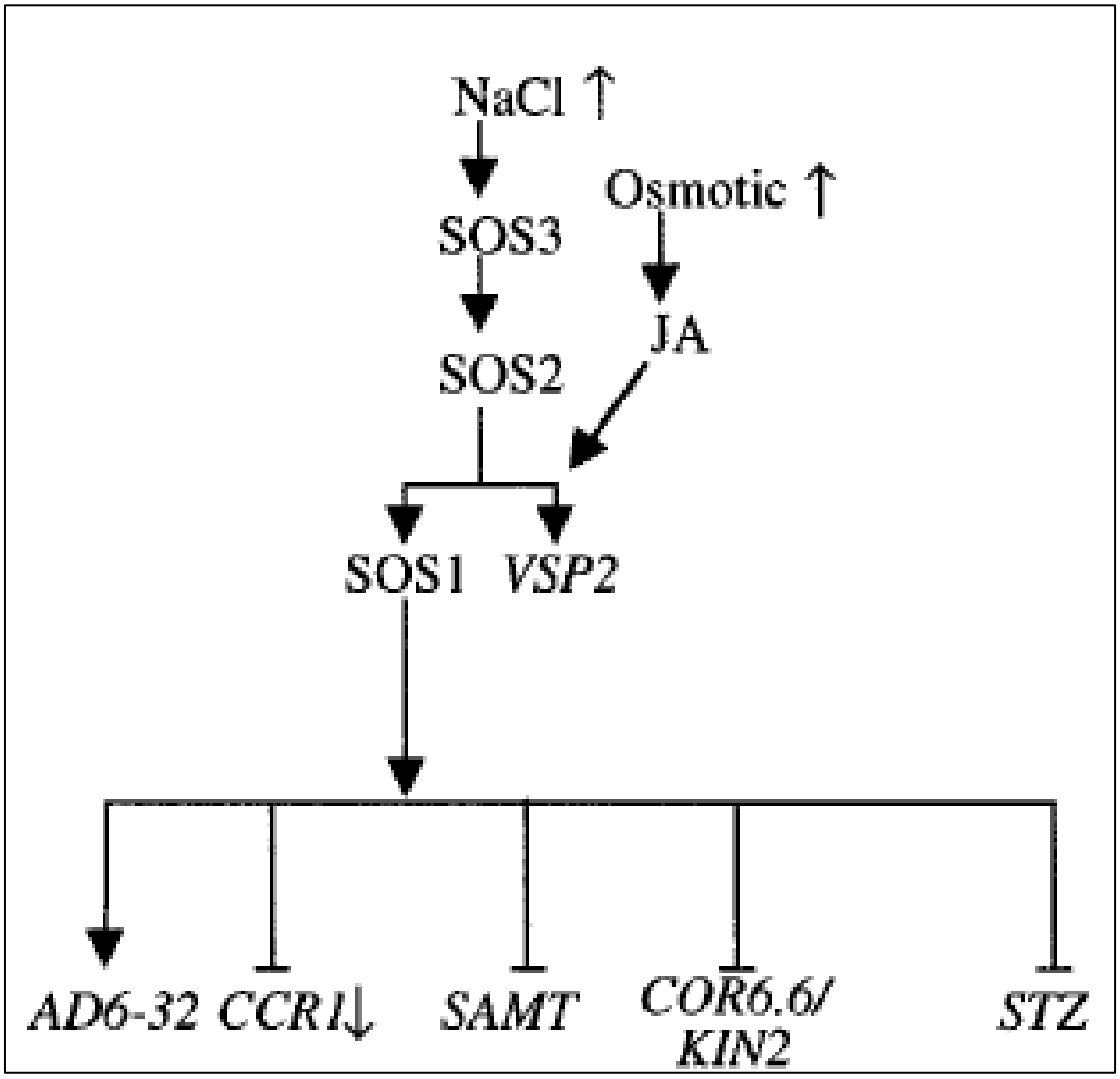


Figure 2. Proposed model of regulatory activity of SOS family genes. Positive (↓) or negative (⊥) regulation by the SOS genes is indicated in the figure. Taken from (Gong D, et al **Genes That Are Uniquely Stress Regulated in Salt Overly Sensitive (sos) Mutants** *Plant Physiol* **126**: 363-375 2001



Appendix

The complete quinoa SOS1 cDNA sequence. Start and stop codons are enlarged and in bold. The 4F-5R fragment originally identified is highlighted. Underlined-bold sections represent the two deletions seen in full-length clone alignment.

CT**ATG**GCTGCATATCGAATTGCGGTTCCGTTTCCGCTAATGTTAGCGGAAGTCGGAGCCGAAACT
ACCGCCCCAATGAATTCTACTATTTCTGCTTCTGCTATGGCGGAAGAATCCGAGTCTAATCCAACTG
ACGCCGTTATCTTTTTCGGCGTCAGTTTAATTCCAGGGATTGCTTGTCGTCACTTTCTTCGAG**GTACT**
CGCGTTCCTTATACTGTCGCTTTGCTCATCATTGGTATTGGTCTTGGTTCTTTAGAATATGGTA
CAAAGCATGGTTTGGGAAGGTTTGGGGATGGTATCCGCATTTGGGAAAATATTGATCCAGAGCTCC
TTTTGGCTGTATTTCTCCCTGCCCTTCTGTTTGAAAGTTCCTTTTCAATGGAAATTCACCAAATAAAG
AGATGTGCAGCTCAAATGATTCTGCTTGCTGGACCAGGTGTTCTGATATCAACTTTTTGTCTTGGAG
CAGCACTAAAGCTTTCATTTCCTTATGACTGGAGCTGGAAGACATCATTGTTGCTCGGTGGACTTCT
CAGTGCAACTGATCCTGTAGCTGTTGTAGCTCTCTTGAAAGAACTTGGGGCAAGCAAAAAATTGAG
TACAATAATTGAGGGGGAATCCTTGATGAATGATGGGACTGCGATTGTGGTTTATCAACTGTTCTTA
AAAATGATCCTTGGTCGAACCTTCAATTGGGCATCAATTCTAAAATATTTAGTTCAAGTCTCATTTG
GAGCTGTGGGATTCGGAATTGCATTTGGAATAGCATCAGTGTTGTGGCTCGGATTTATTTTCAATGA
CACTATAATAGAGATTACTTTGACCCTTGCTGTGAGCTATGTTGCCTATTTTACTGCTCAAGAGGGA
GCTGATGTGTCTGGTGTCCTGACCGTGATGACTTTAGGAATGTTTTATGCAGCAGCTGCCAGGACAG
CATTTAAAGGTGAAAGTCAACAGAGTTTGCATCATTTTTGGGAGATGGTGGCATACATAGCCAATA
CGCTGATCTTTATCTTGAGCGGAGCTGTCATAGCACAAGGTGTTCTCAGCAGTGATAACATTTTTGA
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GAGTTGGCCTGAGAATCAATACAAGTCATATCAGCATCTACCAGACGGTCAAGAAATAGATGATAG
TCAAAACTTGTCAGCCAAAGCTATGCGGTTGAGCATCTATGGCAGCACGGCAAAAGATGTCCCTGT
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CCTATCGGCCAGAAACAACTTCTTCTCACTTCAGTAAAATCAGAAGGATCTAATACTGTTAGGAAG
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GCAGTAGATGATTCAAGCTCTGAATCTGGTGGTGAAGATGAAGTTTTTGTGAGAATCGATTCGCCA
AGCAAGCTCTCTTTCCGCCAAGCACCT**TGA**GAGCTCTTTTG