Characterization of *Salt Overly Sensitive 1* (*SOS1*) gene homoeologs in quinoa (*Chenopodium quinoa* Willd.)

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Abstract: Salt tolerance is an agronomically important trait that affects plant species around the globe. The *Salt Overly Sensitive 1* (*SOS1*) gene encodes a plasma membrane Na^+/H^+ antiporter that plays an important role in germination and growth of plants in saline environments. Quinoa (*Chenopodium quinoa* Willd.) is a halophytic, allotetraploid grain crop of the family Amaranthaceae with impressive nutritional content and an increasing worldwide market. Many quinoa varieties have considerable salt tolerance, and research suggests quinoa may utilize novel mechanisms to confer salt tolerance. Here we report the cloning and characterization of two homoeologous *SOS1* loci (*cqSOS1A* and *cqSOS1B*) from *C. quinoa*, including full-length cDNA sequences, genomic sequences, relative expression levels, fluorescent in situ hybridization (FISH) analysis, and a phylogenetic analysis of *SOS1* genes from 13 plant taxa. The *cqSOS1A* and *cqSOS1B* genes each span 23 exons spread over 3477 bp and 3486 bp of coding sequence, respectively. These sequences share a high level of similarity with *SOS1* homologs of other species and contain two conserved domains, a Nhap cation-antiporter domain and a cyclic-nucleotide binding domain. Genomic sequence analysis of two BAC clones (98 357 bp and 132 770 bp) containing the homoeologous *SOS1* genes suggests possible conservation of synteny across the *C. quinoa* sub-genomes. This report represents the first molecular characterization of salt-tolerance genes in a halophytic species in the Amaranthaceae as well as the first comparative analysis of coding and non-coding DNA sequences of the two homoeologous genomes of *C. quinoa*.

Key words: quinoa, *SOS1*, Na^+/H^+ antiporter, synteny.

Résumé : La tolérance à la salinité est un caractère agronomique important chez plusieurs espèces végétales partout sur le globe. Le gène *Salt Overly Sensitive 1* (*SOS1*) code pour un antiporteur Na^+/H^+ de la membrane plasmique qui joue un rôle important dans la germination et la croissance des plantes en conditions salines. Le quinoa (*Chenopodium quinoa* Willd.) est une culture halophyte allotétraploïde de la familles des amaranthacées qui affiche une valeur nutritionnelle impressionnante et dont le marché connaît un essor mondial. Plusieurs cultivars de quinoa présentent une tolérance considérable à la salinité et la recherche suggère que le quinoa utilise des mécanismes inédits pour obtenir une telle tolérance. Les auteurs rapportent ici le clonage et la caractérisation de deux locus *SOS1* homéologues (*cqSOS1A* et *cqSOS1B*) chez le *C. quinoa*. Cette caractérisation comprend les séquences complètes des ADNc, les séquences génomiques, les niveaux relatifs d'expression, l'hybridation in situ en fluorescence (FISH) ainsi qu'une analyse phylogénétique des gènes *SOS1* provenant de 13 espèces végétales. Les gènes *cqSOS1A* et *cqSOS1B* comptent tous deux 23 exons qui totalisent respectivement 3477 et 3486 pb de séquence codante. Ces séquences présentent une grande similarité avec les homologues *SOS1* identifiés chez d'autres espèces et contiennent deux domaines conservés, un domaine antiporteur de cations Nhap et un domaine de liaison à des nucléotides cycliques. L'analyse de la séquence génomique de deux clones BAC (de 98 357 pb et 132 770 pb) contenant des gènes *SOS1* homéologues suggère une possible conservation de la synténie au sein des sous-génomes du *C. quinoa*. Ce travail constitue la première caractérisation de gènes de tolérance à la salinité chez une espèce halophyte de la famille des amaranthacées ainsi que la première analyse comparée de séquences codantes et non-codantes au sein des génomes homéologues du *C. quinoa*.

Mots-clés : quinoa, *SOS1*, antiporteur Na^+/H^+, synténie.

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Introduction

The productivity of over one-third of all arable land worldwide is affected by soil salinity (Epstein and Bloom 2005). High Na^+ concentrations in soil or water lead to high cytosolic Na^+ concentration and subsequent plant death. Nearly all enzyme activity decreases dramatically at NaCl concentrations above 0.3 mol/L owing 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 (Hernández et al. 1995). When cytoplasmic Na^+ concentration increases, potassium (K^+) levels decrease, which in turn is directly correlated with lower growth rates (Ben-Hayyim et al. 1987; Katsuhara and Tazawa 1986). Plants employ 3 basic strategies to prevent and correct for high Na^+ concentrations, specifically (*i*) active Na^+ efflux, (*ii*) Na^+ compartmentalization in the vacuole, and (*iii*) Na^+ influx prevention (Niu et al. 1995). Sodium efflux and vacuolar sequestration mechanisms are coupled to pH gradients created at the plasma membrane or the tonoplast. Transporters on both membranes respond to salt stress by signaling increased transcription of transporter genes (Niu et al. 1993*a*, 1993*b*).

The ability to increase salt tolerance in crop plants is a major breeding objective in many areas where saline and sodic soils limit crop production and in areas that experience significant fluctuations in soil salinity, including 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). Genetic mapping experiments have identified many quantitative trait loci (QTL) involved in salt stress (Foolad 1999; Koyama et al. 2001; Quesada et al. 2002), 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. Several researchers have identified NaCl-sensitive mutants among *Arabidopsis thaliana* plants treated with ethylmethane sulfonate (Wu et al. 1996; Liu and Zhu 1997; Zhu et al. 1998). Genetic analysis of the salt-sensitive plants identified several alleles of the *Salt Overly Sensitive 1* (*AtSOS1*) gene (GenBank accession No. NM_126259) that were responsible for sensitivity of the mutant plants to high Na^+ environments. The *AtSOS1* gene contains 23 exons and encodes a plasma membrane protein of 1146 amino acids which acts as a Na^+/H^+ antiporter (Shi et al. 2000). *Arabidopsis* plants preferentially express *AtSOS1* in epidermal cells at the root tip and in parenchyma cells at the xylem–symplast boundary of roots, stems, and leaves. *AtSOS1* is expressed at low basal levels but is up-regulated in the presence of NaCl and has been shown to regulate other genes in response to salt stress (Gong et al. 2001). Homologs of *AtSOS1* have been identified for multiple plant species, including *Solanum lycopersicum* L. (GenBank acc. No. AJ717346), *Oryza sativa* L. (GenBank acc. No. AY785147), *Triticum aestivum* L. (GenBank acc. No. AY326952), *Cymodocea nodosa* Asch. (GenBank acc. No. AJ427294), and *Suaeda japonica* Makino (GenBank acc. No. AB198179).

Chenopodium quinoa Willd. (quinoa) is a halophytic crop species in the family Amaranthaceae (formerly Chenopodiaceae). The Amaranthaceae has the highest proportion of halophytic genera (44%) among the angiosperms, constituting 321 species (Flowers et al. 1986). Quinoa is an ancient New World food staple commonly cultivated throughout the Andean region of South America. Quinoa seeds have a high protein content and a well-balanced essential amino acid profile, rivaling milk for nutritive value (Fairbanks et al. 1990; Brinegar et al. 1996; Prego et al. 1998). Botanically speaking, quinoa is an annual, self-fertile allotetraploid ($4x = 2n = 36$) exhibiting predominantly disomic morphological and molecular marker segregation (Gandarillas 1979). Quinoa thrives in sandy to loamy soils under a wide range of pH (4.8–8.5) and is tolerant of saline and sodic soils. Cultivated quinoa varieties can be separated into different ecotypes, including a highly salt-tolerant Salares ecotype that is traditionally grown around the margins of saline playas on the Altiplano (high plains) of Bolivia (Wilson 1988). Salares ecotypes are reported to have a germination rate of 75% at a salt concentration of 57 mS/cm, a significant feat considering sea water has a salt concentration of 40 mS/cm (Prado et al. 2000; Jacobsen et al. 2003). While little is known about the specific molecular mechanisms involved in quinoa's ability to thrive under high salt conditions, it has been suggested 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 (Wilson et al. 2002).

Here we describe the cloning and characterization of 2 homoeologous *SOS1* loci (*cqSOS1A* and *cqSOS1B*) from *C. quinoa*. In characterizing the *cqSOS1* loci, we (*i*) cloned, sequenced, and compared the cDNA and full-length genomic sequences of each *SOS1* homoeolog, (*ii*) predicted intron–exon boundaries via homoeolog-specific cDNA analysis and described the conserved domains of the SOS1 protein, (*iii*) analyzed the *SOS1* homoeolog expression via quantitative PCR analysis in root and leaf tissue, (*iv*) analyzed the phylogenetic relationship of *SOS1* genes from 13 plant taxa where the full *SOS1* gene sequence is available, and (*v*) aligned the overlapping homoeologous bacterial artificial chromosome sequences to characterize the genomic context of the homoeologous *SOS1* loci in quinoa.

Materials and methods

Plant materials and DNA extraction

Quinoa seed for the cultivars 'Real' and 'Ollague' (Salares ecotypes) was kindly provided by Angel Mujica (National University of the Altiplano, Puno, Peru). For DNA extraction, plants were grown at 25 °C with a 12 h photoperiod in Sunshine Mix # 2 (Sun Gro Horticulture, Bellevue, Washington) supplemented weekly with nitrogen fertilizer. All plants were grown in 6″ pots in greenhouses at Brigham Young University, Provo, Utah. 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).

For gene expression experiments, quinoa plants ('Ollague') were grown in a hydroponic growth chamber using a protocol

Table 1. Primers used to amplify *SOS1* genes in *Chenopodium quinoa*.

Primer name	Primer sequence (5′→3′)	Application
4F	ATGAATGATGGGACGGCDATTGTTGT	RT-PCR
5R	TCCAAACCATAGCCAAA	RT-PCR
48F	GATCCTTGGTCGAACCTTCA	Genomic amplification
422R	AGAACACCTTGTGCTATGACAG	Genomic amplification
SOS1AF2	TCACTTCAATTTCCATTTTCAGTGTCCTCA	Full-length cDNA amplification
SOS1BF2	CAATCTCCACTTCAATTTCCATTTCAACA	Full-length cDNA amplification
SOS1RU*	GGCAGACATTCGCCGGACTACAAAC	Full-length cDNA amplification
SOS1AF	CCTCATGATGCTTCCGACAA	Quantitative PCR
SOS1AR	CCGAGTCAAGTGCTTCATCA	Quantitative PCR
SOS1BF	ACCCTCATGATGCTTCTGATAC	Quantitative PCR
SOS1BR	TGCTTCATCAACTGATTGCAT	Quantitative PCR
GAPDHF	GGTTACAGTCATTCAGACACCATCA	Quantitative PCR
GAPDHR	AACAAAGGGAGCCAAGCAGTT	Quantitative PCR

*SOS1RU is a universal reverse primer used in combination with a locus-specific primer for the amplification of the full-length cDNA of *cqSOS1A* and *cqSOS1B* genes.

previously described by Camp et al. (1987). The growth chambers were maintained at 27 °C with a 13 h day length. Plants in the salt stress treatment group were supplemented with NaCl at 50 mmol/L increments on a daily basis (starting 12–14 days after germination) until the hydroponic solution reached a final concentration of 450 mmol/L NaCl. Leaf and root tissue was harvested 48–72 h after the final treatment concentration was reached. Plants in the non-stress treatment group were maintained at a final concentration of 50 mmol/L NaCl throughout the experimentation period.

Degenerate primer design

To identify conserved regions of the *SOS1* gene for PCR amplification, *SOS1* cDNA sequences from *Arabidopsis* (GenBank acc. No. NM_126259), *Solanum lycopersicum* (GenBank acc. No. AJ717346), *Oryza sativa* (GenBank acc. No. AY785147), and *Triticum aestivum* (GenBank acc. No. AY326952) were aligned using the multiple sequence alignment application, AlignX, of the computer program Vector NTI (Invitrogen, Carlsbad, California). Based on sequence conservation across species, primer sequences were identified that were predicted to amplify an internal fragment of the *SOS1* gene (4F and 5R; Table 1). The amplified *SOS1* gene fragment was cloned using the pGEM-T Easy Vector System II (Promega, Madison, Wisconsin) and sequenced at the Brigham Young University DNA Sequence Center (Provo, Utah) using standard ABI PRISM *Taq* dye-terminator cycle-sequencing methodology. DNA sequence chromatograms were analyzed with the ContigExpress program in the Vector NTI software suite.

Bacterial artificial chromosome (BAC) library screening

A 9× BAC library (Stevens et al. 2006) consisting of 74 496 clones and developed from the quinoa ecotype 'Real' was screened with the 4F–5R *SOS1* gene fragment to identify BAC clones containing the genomic sequence of the *SOS1* gene. The *SOS1* gene hybridization probe was labeled with [α-^{32}P]dCTP using a Prime-a-Gene kit (Promega, Madison, Wisconsin), and membranes were hybridized using standard protocols as described by Sambrook et al. (1989). Clones with positive hybridization signals were picked from the BAC library and grown in LB broth supplemented with 15 μg/mL chloramphenicol. BAC plasmids were extracted using a NucleoBond BAC 100 kit (Macherey-Nagel, Easton, Pennsylvania) and confirmed via PCR to contain the *SOS1* gene sequence. Positive BAC clones (117:G8 and 150:P1) were 10× shotgun sequenced at the Arizona Genomics Institute (Tucson, Arizona). Sequences were base-called, cleaned of vector sequences, assembled, and visualized using cross_match (Ewing et al. 1998), Phrap (Green 2006), and consed (Gordon et al. 1998).

RNA extraction and quantitative PCR

Tissue samples (root and leaf) were ground to a fine powder in liquid nitrogen and total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, Valencia, California). DNA was removed using a TURBO DNase kit (Ambion, Austin, Texas). The RNA was quantified using a Quant-iT RiboGreen assay kit (Invitrogen, Carlsbad, California) and then run on an RNA Nano Chip (Agilent, Santa Clara, California) to verify RNA quality. Quantitative PCR primers were designed to specifically amplify each of the two *SOS1* homoeologs (Table 1). Gene expression was measured in Log_{10}(RQ) units using the *GAPDH* gene as an endogenous control and the non-stress treatment leaf sample as a baseline. Gene expression at the *SOS1* loci was quantified using a High Capacity RNA-to-cDNA Master Mix reagents kit (Applied Biosystems, Foster City, California) and a Fast SYBR Green Master Mix (Roche, Indianapolis, Indiana) on a 7300 Real-Time PCR System (Applied Biosystems) according to the manufacturer's recommendations. All RT-PCR reactions were done with 6 replications.

Computational analysis

Repetitive DNA elements in the BAC clone sequences were identified using RepeatMasker (Smit et al. 1996–2004) and a cross_match query to known elements in the *Arabidopsis* repeat database (RM-20080801). Putative gene sequences were identified using the masked BAC sequences and a BLASTx analysis using the RefSeq protein database (Pruitt et al. 2007) limited to Viridiplantae. VISTA plots were created using a SLAGAN global alignment to show

the relative nucleotide identity between the two homoeologous BAC clones (Frazer et al. 2004). The Spidey alignment program was used for identification of intron–exon boundaries of the *SOS1* loci (http://www.ncbi.nlm.nih.gov/spidey/). Sequences of *SOS1* genes across species were aligned using the Muscle multiple protein sequence alignment tool in Jalview (Clamp et al. 2004). Phylogenetic and molecular evolutionary analyses were conducted using MEGA v.4 (Tamura et al. 2007). Synonymous and non-synonymous distances were calculated using DNAsp v.4.50.3 (Rozas et al. 2003). Hydrophobicity analysis and plots were generated by TMpred (Hofmann and Stoffel 1993).

Fluorescent in situ hybridization

Somatic chromosome preparations and probe labeling using nick translation were done according to published protocols (Kato et al. 2004, 2006) with slight modifications. BAC clones 117:G8 and 150:P1 were nick translated using Alexa Fluor 488-5-dUTP (Invitrogen, cat. No. C11397) and Texas Red-5-dUTP (PerkinElmer, cat. No. NEL417), respectively. Root-tip meristems were prepared using 2 μmol/L 8-hydroxyquinoline instead of nitrous oxide. A cocktail containing 200 μg of each probe was applied to each slide for approximately 16 h at 55 °C and slides were washed at medium stringency (2× SSC, 55 °C, 20 min). Slides were examined on a Zeiss Axioplan 2 microscope and images were captured using Zeiss Image software with a Zeiss AxioCam HRc CCD camera. Images were processed using Adobe Photoshop CS2 using smart sharpen and moderate contrast enhancement.

Results and discussion

Cloning and sequencing of *cqSOS1A* and *cqSOS1B*

Degenerate primers were designed to amplify an internal fragment of the quinoa *SOS1* gene from reverse-transcribed cDNA. The RT-PCR primer pair, 4F and 5R, amplified a single strong amplicon of 561 bp. The multi-species alignment of the *SOS1* gene predicted that this primer pair would amplify a fragment of ~559 bp. Sequence analysis followed by BLASTn and BLASTx searches against the non-redundant GenBank database showed that the amplification product had significant sequence homology to several Na^+/H^+ antiporter-like sequences, with the top two BLASTn alignments being *SOS1* homologs from *Suaeda japonica* (AB198179; *E* value = 0.0; 86% Max identity) and *Mesembryanthemum crystallinum* L. (EF207776; *E* value = 1e–173; 84% Max identity). The high alignment score of a *C. quinoa SOS1* sequence fragment to the *SOS1* genes of *S. japonica* and *M. crystallinum* was not surprising, since all three species belong to the same order (Caryophyllales). Based on these results, we concluded that the 4F–5R fragment was likely a portion of a *SOS1* homolog in quinoa.

Since quinoa is an allotetraploid ($2n = 4x = 36$; Maughan et al. 2006), we suspected that it harbored 2 homoeologous *SOS1* loci. To test this hypothesis, we attempted to amplify and sequence across introns to look for sequence divergence. Based on the sequence information from the 4F–5R *SOS1* fragment, 2 new primers were designed, 48F and 422R (Table 1), which were predicted to amplify a 375 bp cDNA fragment. When used to amplify genomic DNA, these pri-

Table 2. Comparison of two homoeologous *SOS1* loci in *C. quinoa*.

Locus	No. of exons	Coding length	Average exon length (range)	Average intron length (range)	Nucleotide identity	No. of variable nucleotide sites	No. of indel sites	Protein identity	Ks (no. of sites)	Ka (no. of sites)	Ka/Ks ratio
SOS1A	23	3477	152 (45–312)	812 (78–2124)	96.9%	100	1	96.5%	0.077 (62)	0.014 (38)	0.186
SOS1B	23	3486	152 (45–312)	842 (75–1998)							

Note: Coding, exon, and intron lengths are reported in base pairs.

mers amplified 2 distinct fragments that were both significantly larger than expected based on the cDNA sequence alone. Both fragments were cloned and sequenced and 2 distinct contigs of 4309 and 4257 bases were assembled. Alignment of the genomic fragments and the 4F–5R cDNA fragment identified the presence of 5 introns, which accounted for the significant increase in size between the cDNA and genomic sequences. Substantial nucleotide diversity (85% identity) within the predicted introns clearly distinguished the two genomic fragments and confirmed the presence of 2 *SOS1* loci (hereafter referred to as *cqSOS1A* and *cqSOS1B*).

To obtain the complete genomic sequences of the *cqSOS1A* and *cqSOS1B* genes, a 9× quinoa BAC library (Stevens et al. 2006) was probed with the 4F–5R *SOS1* cDNA fragment. Twenty positive BAC clones were identified and each was confirmed via PCR to contain the *SOS1* gene sequence, supporting the hypothesis that 2 unique *SOS1* gene sequences exist in the quinoa genome. Sequence analysis of the 48F–422R fragment was utilized to subdivide the positive BAC clones into *cqSOS1A* and *cqSOS1B* clones. BAC clones for *cqSOS1A* (clone ID 117:G8) and *cqSOS1B* (clone ID 150:P1) were cultured, purified, and 10× shotgun sequenced. Fragment assembly for each BAC clone produced a single contig. Clone 117:G8 (*cqSOS1A*) spanned 98 357 bp, while clone 150:P1 (*cqSOS1B*) spanned 132 770 bp. Full BAC sequences for the *cqSOS1A* and *cqSOS1B* clones have been submitted to GenBank (accession Nos. EU024570 and FJ755791, respectively).

Sequence comparisons between the putative *SOS1* homoeologs

Putative start and stop codons of the *SOS1* gene were identified based on a cDNA to genomic DNA alignment of the BAC clone sequences with the *S. japonica SOS1* cDNA sequence (BAE95196). Utilizing a unique 10 bp indel identified upstream of the start codon in the *SOS1B* homoeolog, primer pairs specific for each *cqSOS1* gene were designed (SOS1AF2–SOS1RU and SOS1BF2–SOS1RU; Table 1) and used to amplify the full-length cDNA transcript for each *SOS1* locus. Each primer pair produced a single amplicon that, when sequenced, spanned the start and stop codons of the *cqSOS1* loci. BLASTx searches of the non-redundant GenBank protein database using the full-length *cqSOS1* cDNA sequences identified significant sequence homology to the putative *SOS1* homologs of *S. japonica* (BAE95196; *E* value = 0.0) and *M. crystallinum* (ABN04858; *E* value = 0.0). Alignment of the cDNA sequences with the 117:G8 and 150:P1 BAC genomic sequences revealed the presence of 23 exons in both *cqSOS1* gene homologs. The *cqSOS1A* homolog consisted of 3 477 bp of coding sequence (including the stop codon) and 17 840 bp of intron sequence, while *cqSOS1B* consisted of 3 486 bp of coding sequence and 18 492 bp of intron sequence. Exon sizes in the *SOS1* homologs ranged from 45 to 312 bp, while intron sizes ranged more dramatically from 74 (*cqSOS1B* intron 21) to 2123 bp (*cqSOS1A* intron 1; Table 2). When translated, the *cqSOS1A* and *cqSOS1B* coding sequences generated proteins of 1158 and 1161 amino acids, respectively. The difference in size between the two *cqSOS1* protein sequences was due to a single in-frame 9 bp insertion/deletion that occurred in exon 15.

Sequence comparison between the two *SOS1* homoeologs indicated that *cqSOS1A* and *cqSOS1B* are 96.9% and 96.5% identical at the nucleotide and amino acid levels, respectively. A total of 62 synonymous (Ks: 0.077) and 38 nonsynonymous (Ka: 0.0145) substitutions were identified (Table 2). Using the Ks value and an averaged silent substitution rate of 6.1 substitutions per silent site per billion years in vascular plants (Lynch and Conery 2000), we cautiously estimate the speciation event between the two diploid progenitors of *C. quinoa* to have occurred approximately 6.31 mya. We note that this estimate is tentative, as it relies on a single gene pair and assumes an averaged silent substitution rate across vascular plants. Indeed, Zhang et al. (2002) showed that in *Arabidopsis*, synonymous substitution rates can vary by as much as 13.8-fold across duplicate gene pairs, although 90% differed by less than 2.6-fold. Ka and Ks values can also be used to detect adaptive changes at the molecular level (Miyata et al. 1980). Ka/Ks ratios > 1 are indicative of positive selection, while Ka/Ks ratios < 1 suggest that the sequences are under purifying selection. The Ka/Ks ratio identified here (0.186) suggests that the *cqSOS1* gene homologs are under purifying selection for retained function, suggesting that both homologs are actively expressed in the quinoa genome.

Hydrophobicity plot analysis (Hofmann and Stoffel 1993) predicted the presence of 11 strong transmembrane helices beginning with the N-terminus outside and continuing until residues 472 and 461 in *cqSOS1A* and *cqSOS1B*, respectively (Fig. S1).[2] The remaining amino acid residues (C-terminus) were predicted to comprise an entirely hydrophilic region. When the quinoa *SOS1* cDNA sequences were compared with the NCBI Conserved Domain Database (v.2.11 - 17402 PSSMs), 2 conserved regions were identified. Amino acids 122 to 476 showed high similarity (*E* = 5e–34) to Nhap, a Na^+/H^+ and K^+/H^+ antiporter domain (COG0025.2), while residues 775–860 predicted (*E* = 2e–9) a cyclic nucleotide-binding (cNMP) domain (pfam00027) (Fig. S1). Both the transmembrane helices and the hydrophilic tail were similarly identified in the *Arabidopsis* and *Cymodocea nodosa* SOS1 proteins (Shi et al. 2000), while the conserved domains are similar to those identified in *Thellungiella halophila* O.E. Schulz, *Arabidopsis*, and *M. crystallinum* (Oh et al. 2007). Work in *Arabidopsis* and other species has confirmed that the hydrophilic C-terminal region is essential for SOS1 function (Oh et al. 2007; Qiu et al. 2002). A SOS1 homolog (AY974336) from *Synechocystis* that lacked 56 C-terminal residues was successfully used to complement salt-intolerant *E. coli* but showed reduced complementation compared with the complete construct (Hamada et al. 2001). Indeed, the entire C-terminus of the *Arabidopsis*

[2] Supplementary data for this article are available on the journal Web site (http://genome.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3966. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

SOS1 protein (amino acids 440–1146) has been shown to bind with RCD1, a regulator of oxidative stress responses. RCD1 and SOS1 are known to regulate similar sets of genes 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 (Hamada et al. 2001). Zhu (2002) hypothesized that the C-terminal region serves as a NaCl sensor. The possibility of a SOS1-cNMP mediated response is supported by cyclic nucleotide involvement in Na^+ regulation as previously demonstrated in *Arabidopsis*. The presence of cAMP or cGMP in the cytosol was shown to improve *Arabidopsis* salinity tolerance via the regulation of voltage-independent monovalent cation channels (Zhu 2002). cGMP levels have also been shown to increase rapidly (≤5 s) in response to salt stress (Maathuis and Sanders 2001). The conservation of the cNMP domain in the *SOS1* gene across species suggests a direct interaction between SOS1 and cyclic nucleotides as part of the cellular salt-stress response.

Phylogenetic analysis

The translated cDNA sequences showed a high degree of sequence similarity to SOS1 Na^+/H^+ antiporters from several plant species (Fig. S1). A comparison of the SOS1 proteins from quinoa and 12 other species for which the complete coding sequence was available was performed to determine the phylogenetic relationship of the quinoa SOS1 proteins with homologous SOS1 proteins from other plant species (Fig. 1). The evolutionary history was inferred using the unweighted pair group method with arithmetic means. The phylogenetic tree was well resolved between closely related taxa, clearly placing the quinoa SOS1 sequences with those of other Caryophyllales (*M. crystallinum*, *S. japonica*, and *Limonium gmelinii* Kuntze) at bootstrap values of 100. Similar monophyletic groups were also identified at bootstrap values of 100 for the three Brassicales species (*Arabidopsis*, *T. halophila*, and *Brassica napus* L.) as well as the three Poaceae species (*Oryza sativa*, *Triticum aestivum*, and *Triticum turgidum* L.). *Cymodocea nodosa* (seagrass) is a monocotyledonous species of the class Liliopsida and, as expected, clustered closer to the Poaceae group than to any of the other groups. The SOS1 sequence from the bryophyte *Physcomitrella patens* (Hedw.) Bruch & Schimp. was placed into the most distal clade. The ability of the SOS1 sequence to resolve phylogenetic relationships between sequences from across angiosperm taxa is noteworthy. However, the lack of sufficient SOS1 sequence data to match the broad range of angiosperm species creates some challenges for assessing the SOS1 sequence as a phylogenetic tool. Without adequate sequence data, and with the added complication of the potential for paralogous duplications and multigene families, it is difficult to know whether homologous genes are being compared appropriately. However, it appears that there is sufficient phylogenetic signal, even from diverse species, to support future studies investigating the molecular evolution of *SOS1* genes in angiosperms.

***cqSOS1* gene expression**

To examine the *cqSOS1A* and *cqSOS1B* gene expression profiles, quinoa plants ('Ollague') were grown hydroponically and subjected to NaCl stress. The cultivar 'Ollague' originates from the Salares (salt flats) of Bolivia and was previously reported to be salt tolerant (personal communication, Alejandro Bonifacio, La Paz, Bolivia). Plants in the salt stress treatment group were grown in a hydroponic solution containing 450 mmol/L NaCl, while the non-stressed plants were maintained at a concentration of 50 mmol/L NaCl throughout the experimentation period. Total RNA was extracted from leaf and root tissue and relative quantification of *SOS1* mRNA from the different treatments was done by quantitative PCR using the *GAPDH* gene as an endogenous control. Individual expression profiles for the *cqSOS1A* and *cqSOS1B* homoeologs were determined using primers specific to each locus. The specificity of each primer set was verified by sequence analysis of the quantitative-PCR products. Similar to results shown in *Arabidopsis*, relative to the leaf tissue, root tissue showed significantly more *SOS1* expression: nearly a 3- to 4-fold increase of both homoeologs regardless of the salt concentration. *SOS1* expression was significantly up-regulated by NaCl stress (450 mmol/L) in leaf tissue, but not in root tissue (Fig. 2). In fact, both *cqSOS1* homologs showed a slight but not significant decrease in expression in the root tissue under salt stress. This was unexpected, as *SOS1* was previously shown to be up-regulated by salt stress in *Arabidopsis* (Donaldson et al. 2004). Several differences between the two experiments may explain the apparent contradictions between our results and those reported by Shi et al. (2000). The two experiments utilized very different experimental species, one a known halophyte (quinoa) and the other a glycophytic model plant with limited salt tolerance (*Arabidopsis*). The experimental plants were stressed using considerably different methodologies. The *Arabidopsis* plants were salt "shocked" as 10-day-old seedlings by transferring them to Whatman filter paper soaked with 300 mmol/L NaCl. Seedlings were treated for 5 h, after which tissue was immediately taken for RNA extraction. In contrast, the quinoa plants were subjected to gradually increasing salt stress in a hydroponic solution that was supplemented with NaCl at 50 mmol/L increments on a daily basis until a final concentration of 450 mmol/L NaCl was reached. Two days after the hydroponic solution reached the final salt concentration, tissue samples were taken for RNA extraction. While both methods introduced salt stress, it is not unreasonable to suspect that the methods could produce different patterns of gene expression. What is curious, however, is that even under the non-stress treatment (50 mmol/L NaCl), quinoa root tissue exhibited a high level of expression of both *SOS1* homoeologs. Perhaps quinoa, being a native halophyte, has acquired constitutive expression of the *SOS1* gene homologs in root tissue but has retained inducible expression in leaf tissue upon exposure to excessive amounts of Na^+. Alternatively, the quinoa *SOS1* genes in root tissue may be hyper-inducible by even minimal salt concentration, as in our non-stress treatment. Follow-up experiments — including testing *SOS1* expression when no salt is present in the hydroponic solution, evaluating promoters of quinoa *SOS1* homologs via transgenic analysis, and analyzing *SOS1* expression in other plant tissue types — will be important next steps in elucidating the functional role of SOS1 in response to salt stress in quinoa.

Fig. 1. Evolutionary relationships of 14 SOS1 protein sequences inferred using the unweighted pair group method with arithmetic means. The bootstrap consensus tree was inferred from 1000 replicates. Bootstrap values are shown next to the branch points. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set.

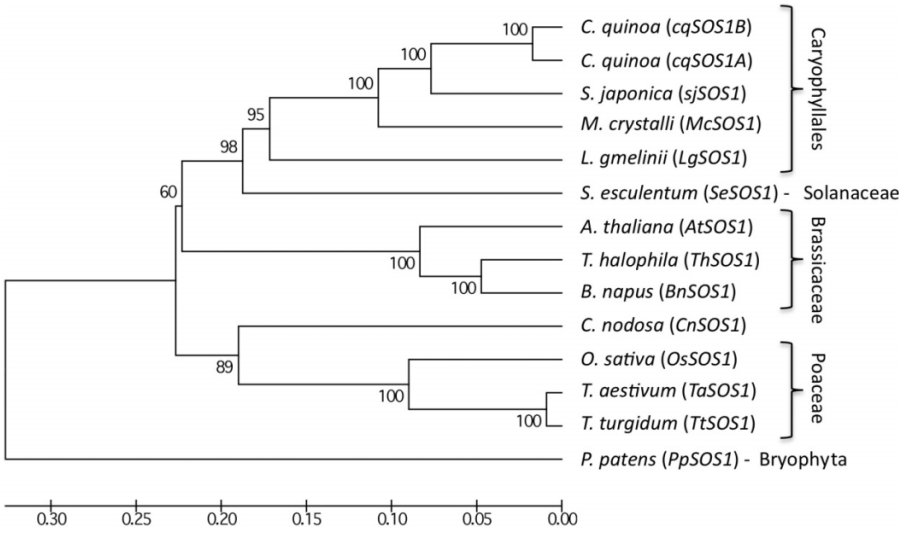
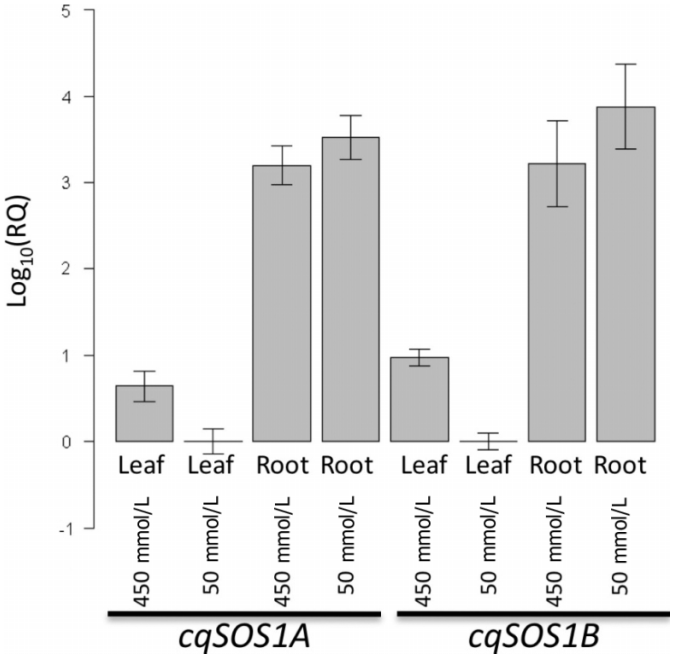


Fig. 2. *SOS1* expression in *C. quinoa* is up-regulated by NaCl stress (450 mmol/L) in leaf tissue, but not in root tissue. Gene expression is measured in Log_{10}(RQ) units using the *GAPDH* gene (not shown) as an endogenous control and the 50 mmol/L NaCl leaf samples as a baseline. Gene expression is shown at both homoeologous loci (*cqSOS1A* and *cqSOS1B*).



Sequence alignment and characterization of the BAC clones containing *SOS1*

The complete BAC clone sequences were screened for repeated elements with RepeatMasker v.3.1.8 against the *Arabidopsis* repeat database. Both clones had equal GC content (36.2% GC). No transposons, SINEs, or LINEs were identified in either BAC clone; however, several LTR elements were detected, as were several simple sequence repeats (Table 3). The fraction (2.74% and 3.15%) of repeat elements identified by RepeatMasker in the BAC clones is low for a complex genome and suggests that *C. quinoa* may possess unique repeated sequences not detected by comparisons with the *Arabidopsis* repeat database. This conclusion is supported by FISH analysis using the BAC clones (Fig. 3). When the SOS1 BAC clones were individually used as FISH probes on quinoa chromosome spreads, not only were 2 distinct spots identifiable (Texas Red-labeled BAC 150:P1), but general background hybridization was also seen with both 150:P1 and 117:G8, suggesting the presence within the BAC clones of repeat elements even through few such elements were identified via our bioinformatic searches.

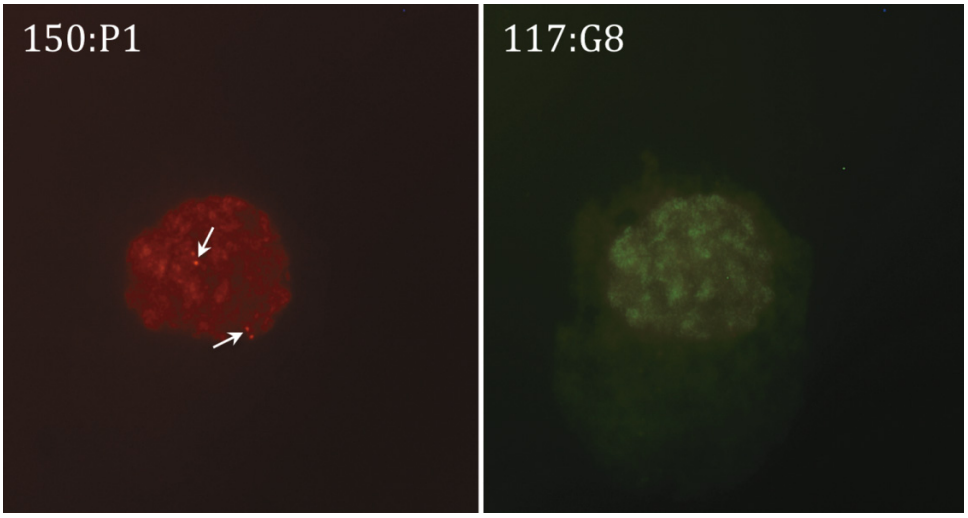
BLASTx searches against the RefSeq protein database (limited to Viridiplantae and masked for low-complexity and repeat sequences) identified 2 and 3 genes in the 117:G8 (*cqSOS1A*) and 150:P1 (*cqSOS1B*) BAC clones, respectively, in addition to their respective *SOS1* gene homologs. In BAC clone 117:G8, a protein belonging to the SLU7 zinc finger family (position 608–1667; Q6ZK48; 4e–90) and a K^+ transporter family protein (KUP; position 90774–95318; Q0J1D4; 8e–83) were identified. In BAC clone 150:P1, a K^+ transporter family protein (KUP; position 87280–100259; Q0J1D4; 1e–79), a FAR1 domain containing protein (position 77149–75035; Q0DYT9; 3e–121), and a HAT dimerization domain containing protein (position 124580–126898; e = 0) were identified. The identification of a second gene sequence (KUP) with shared homology between the BAC clones supports the conclusion that these two BAC clones are homoeologous and that *cqSOS1A* and *cqSOS1B* are homoeologs. Given the prospect that the BAC clones represented homoeologous DNA sequences, we used the global sequence alignment program VISTA (Shi et al. 2000) to examine the overall level of sequence conservation between the two BAC clones. Global pairwise alignment

Table 3. Summary of the plant repeat element content of the two homoeologous SOS1 BAC clones as determined using RepeatMasker (v.3.1.8) and the default repeat database for *Arabidopsis*.

	No. of elements[†]		Length (bp) and % of sequence		
Element*	117:G8	150:P1	117:G8	150:P1	Most common motif[‡]
Interspersed repeats					
Retroelements					
LTRs					
Ty1/Copia	2	3	332 (0.34%)	2991 (2.25%)	
Gypsy/DIRS1	6	2	2764 (2.81%)	648 (0.49%)	
SINEs	0	0			
LINEs	0	0			
DNA transposons	0	0			
Total	8	5	3096 (3.15%)	3639 (2.74%)	
Simple sequence repeats					
Mono-	11	16			$(T)_n$
Di-	3	3			$(TA)_n$
Tri-	4	3			—
Tetra-	3	6			$(TAAA)_n$
Other	4	8			—
Total	25	36	1432 (1.46%)	1876 (1.41%)	

*SINEs, short interspersed nuclear elements; LINEs, long interspersed nuclear elements; LTRs, long terminal repeats.
[†]Most repeats fragmented by insertions or deletions have been counted as one element.
[‡]—, no common motif.

Fig. 3. FISH on a somatic interphase cell of quinoa (*C. quinoa* 'KU-2') using nick-translated BAC clones 150:P1 (left, Texas red, red) and 117:G8 (right, Alexa Fluor 488, yellow). Areas of discrete hybridization of 150:P1 are indicated with arrows. Both probes hybridized to sequences dispersed throughout the quinoa genome.

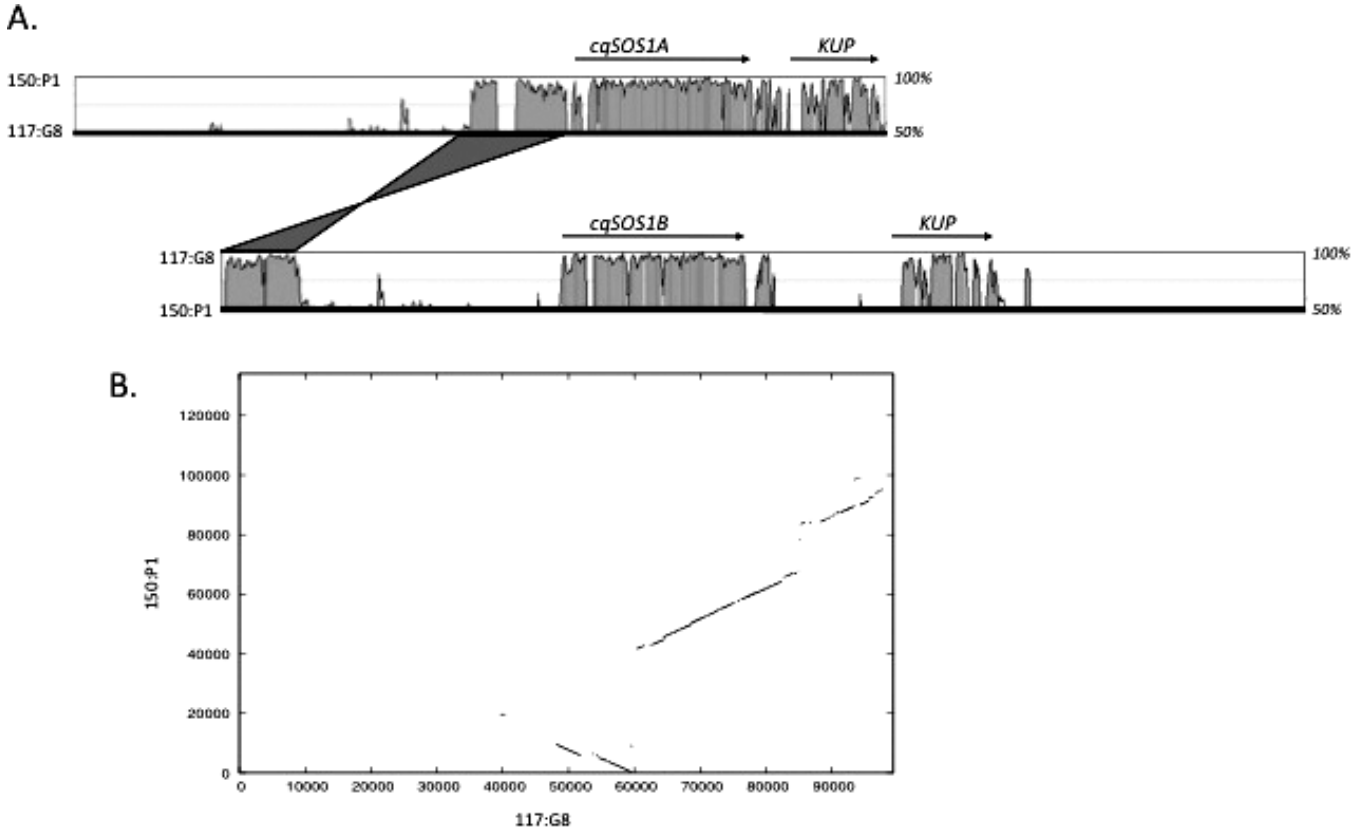


identified 7 conserved regions spanning 38 405 bp with an average nucleotide identity of 82.5%. Inspection of the VISTA global pairwise alignment plot (Fig. 4) suggests the potential for the conservation of microsynteny across the *C. quinoa* sub-genomes, but this is tempered by several dramatic changes evident between the clone sequences. In clone 150:P1, the distance between *SOS1* and *KUP* is nearly 25 kb, whereas in clone 117:G8 this region has decreased to less than 10 kb. Moreover, the *FAR1* gene, present in clone 150:P1, is absent in clone 117:G8. Notable also is the apparent inversion that is seen immediately upstream of the *SOS1* gene homologs in both the VISTA alignment plot and the dot plot (Fig. 4).

Conclusions

Full-length cDNA and genomic *SOS1* homologs (*cqSOS1A* and *cqSOS1B*) were successfully cloned and sequenced from *C. quinoa*. Gross sequence analyses of the two *SOS1*-containing BAC clones identified a second gene sequence (*KUP*) conserved upstream from the *SOS1* homologs in an overlapping region of both BAC clones, supporting the conclusion that these *SOS1* loci are homoeologous. Quantitative PCR analysis confirmed the expression of each *cqSOS1* homoeolog in quinoa and suggests that the SOS response to salt stress is a conserved cellular response across many plant species. Whether quinoa's ability to tolerate ex-

Fig. 4. Pairwise alignment of homoeologous BAC clones 117:G8 and 150:P1 as shown via VISTA plots alignments (panel A) using the SLAGAN algorithm that detects rearrangements, and dot plots (panel B). The calculation window was set to 100 bp with a consensus identity of 70%. The positions of *SOS1* and *KUP* (K^+ transporter family protein) are shown with arrows.



treme saline conditions is a unique property of its *SOS1* allelic variants or the result of differential control of its *SOS1* genes via promoter differences remains unclear. The cloning and characterization of the genomic and cDNA sequences of the *SOS1* gene homoeologs from *C. quinoa*, as reported here, provides the necessary framework to begin to answer these questions. Follow-up experiments, including the transgenic complementation of a mutant *sos1 Arabidopsis* line (ABRC: CS3862) with combinations of quinoa *SOS1* gene constructs (e.g., S35:*cqSOS1*; *cq*Promoter:*AtSOS1*; Atpromoter:*cqSOS1*), will undoubtedly be important steps toward fully elucidating *SOS1* gene function in quinoa.

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