Chapter 4

Results
4. Results

The current thesis entails the study of SOS2 gene in two important crop plants i.e. *Brassica* and rice which may be playing a crucial role in response of plants towards salinity stress. **Part “A”** deals with isolation and functional characterization of *BjSOS2* gene isolated from salinity tolerant *Brassica* genotype-*B. juncea* var. CS52. Isolation and functional characterization of *OsSOS2* gene isolated from rice cultivar IR64 has been described in **part “B”**.

**PART “A”**

4.1 Natural variability of salinity tolerance among *Brassica* genotypes

Seeds of various *Brassica* species as shown in figure 5B were allowed to germinate and grow under normal conditions for 6 d, after which they were subjected to salinity stress (200mM NaCl). These seedlings were then used to score the natural variability in tolerance towards salinity stress. Within 24 h of stress treatment, seedlings of all cultivars started exhibiting the visual symptoms of stress-induced injury. Seedlings started losing 'greenness', which is a reflection of the adverse affects of stress on photosynthesis. However, the extent of loss in greenness was different in different cultivars. Seedlings of all the cultivars lost their turgidity within 48–72 h of salinity treatment except *Brassica nigra*, where turgidity was lost very early (within 2–3 h of stress). We used the plant's ability to maintain low Na\(^+\) inside the cells – as a basis for screening various *Brassica* cultivars for differential tolerance in our experiments. The analysis (Fig. 7A and 7B) indicated towards time-dependent differential Na\(^+\) accumulation amongst the various *Brassica* cultivars. For example, during the first 24 h, the three diploids accumulated relatively higher Na\(^+\) (2–4 mg/g fresh weight (FW)) than the amphidiploids (<2 mg/g FW) and wild germplasms (Fig. 7A). Barring one genotype (*Brassica carinata* var. HC210, which showed Na\(^+\)
accumulation comparable with diploids), this trend continued till 48 h of stress. However, after 72 h of stress, all the cultivars accumulated high Na⁺ (≥6 mg g⁻¹ FW), indicating that by this time, seedlings lose their ability to restrict the entry of Na⁺ ions and/or exclude them from the cytosol. Hence, they are facing ion toxicity in addition to osmotic stress. One of the key features of plant salt tolerance is their ability to maintain optimal K⁺/Na⁺ ratio in the cytosol. With this idea, when Na⁺ and K⁺ amounts were calculated, *Brassica juncea* var. CS52 exhibited the highest ratio of 4 (after 24 h of stress), while *Brassica carinata* (HC209 and 210) exhibited a ratio of 2–2.5 (Fig. 7B). The three diploids showed a sharp decline in this ratio (0.5), while the wild species (*Eruca sativa* and *Brassica tournefortii*) showed an intermediate response (a ratio of 1–1.5). During the extended period of stress (48 and 72 h), all the cultivars showed inability to maintain a ratio of ≥0.5. Taken together, this study clearly established the ability of amphidiploids to maintain higher K⁺/Na⁺ ratio than their counterpart diploids at least during the initial period (24 h) of salinity stress. The inability of the seedlings to maintain this favourable ratio indicated that seedlings at this early stage are very sensitive to salinity stress (200mM NaCl), though different *Brassica* genotypes showed different patterns for accumulation of specific ions. Other parameters such as shoot and root length, electrolyte leakage and endogenous proline were also studied.

4.2 Transcriptional profiling of BjSOS genes at seedling and reproductive stage in *Brassica* genotypes exhibiting contrasting salinity response

4.2.1 ‘Very early’ and ‘late responses’ in regulation of BjSOS genes in seedlings of contrasting *Brassica* genotypes
With the availability of contrasting salinity responsive *Brassica* genotypes, we were prompted to work out the fine regulation of expression of SOS genes. For this purpose, seedlings of contrasting genotypes (as revealed from above analysis) *B. juncea* var. CS52 (tolerant) and *B. nigra* (sensitive) were subjected to either very short durations (10, 20 or 30 min) or long durations (24, 48 or 72 h) of salinity stress to get an insight into regulation of BjSOS gene expression in very early (< 30 min) and late phase (< 72 h) of salinity stress.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 7:** Analysis of ion toxicity in seedlings of *Brassica* cultivars exposed to 200mM NaCl for up to 72 h. Endogenous Na⁺ content (mg g⁻¹ FW). (B) Ratio of K⁺/Na⁺. Data are means±SE. Each data set represents an average of minimum three separate experiments. Concentration of Na⁺ was found to be in the range of 0.5–1.0 mg g⁻¹ FW in all the cultivars before stress treatment.
This RNA gel-blot analysis (employing unfractionated seedlings) indicated clear differences in regulation of all BjSOS genes in the contrasting genotypes (Fig. 8).

Figure 8: Differential transcript accumulation for SOS genes during ‘very early’ and ‘late phase’ of salinity stress in seedlings (non-fractionated) of two contrasting Brassica genotypes (B. nigra and B. juncea var. CS52). Northern blots probed with A. BjSOS3 B. BjSOS2 and C. BjSOS1. D. Ethidium bromide (EtBr) stained RNA gel shown as the loading control. Same Northern blot was sequentially hybridized using BjSOS1, BjSOS2 and BjSOS3 cDNA as probe after deprobing the blot each time. Duration of 200 mM NaCl stress has been mentioned on top of each lane. Bar diagram for relative transcript abundance has been shown below the blots. Light shaded and dark filled bars represent relative transcript abundance for seedlings of B. nigra and B. juncea var. CS52 respectively.
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*BjSOS3* transcript was found to be induced within the very early phase in both genotypes; however, the tolerant genotype *B. juncea* exhibited higher transcripts than sensitive *B. nigra* under the conditions tested here (Fig. 8A). It is also interesting to note that during the late phase (especially at 48 and 72 h salinity stress); the *BjSOS3* transcripts were comparable in the two genotypes. No transcripts for *BjSOS2* could be detected in *B. nigra* under control conditions, however, the comparable sample from *B. juncea* showed a good transcript level, reconfirming the results presented in figure 8B. Though, *BjSOS2* was again found to be inducible by salinity stress in both the genotypes during the early as well as late phase; the tolerant genotype always exhibited higher transcripts than the sensitive (Fig. 8B). However, in this case, the transcript levels were seen to decline during 72 h of salinity stress in both the cultivars. The transcripts for *BjSOS1* could be detected in the two genotypes even without stress, with further upregulation under salinity stress (Fig. 8C). The kinetics of induction showed a great contrast as under the conditions tested here, the sensitive genotype *B. nigra* maintained a lower transcript level than the tolerant genotype *B. juncea*. However, this analysis does not cover the differences which may exist in the two cultivars because of post-transcriptional and/or post translational control operative in them. Nonetheless, the fine regulation of the various SOS genes in the two genotypes of *Brassica* presents before us an interesting gene regulatory model which warrants further detailed analysis.

4.2.2 Organ specific expression patterns of *BjSOS* genes in contrasting *Brassica* genotypes

*B. juncea* var. CS52 and *B. nigra* were grown to maturity employing standard agronomic practices. At the mature plant stage, tissue samples from various plant organs were collected and leaf discs were either incubated in nutrient solution only (control) or supplemented with NaCl (stress) before extracting total RNA for analysis. Northern blots prepared from these RNA samples were successively hybridized to various *BjSOS* gene probes. However, for comparison, blots corresponding to a given organ (for all the three *BjSOS* genes)
are placed below each other (Fig. 9; the organ position indicated w.r.t. to whole mature plant).

![Diagram of plant organs with Northern blots]

**Figure 9**: *BjSOS* transcripts accumulation is tissue specific in various vegetative and reproductive organs of field grown mature plants of salt sensitive *B. nigra* and salt tolerant *B. juncea* var. CS52. Analysis was performed on root, stem (upper, middle and lower), leaf (upper, middle and lower), flower and pods of the two genotype under control (C) and 200 mM NaCl, 5 h (S) conditions. Northern blots prepared from various samples were probed with *BjSOS1*, *BjSOS2* or *BjSOS3* as indicated towards the left side of the blots. Note the *BjSOS2* transcripts were only observed in root, flower and pod.
The phosphor-images of Northern blots were visually inspected for differences in the intensity of each BjSOS transcript within various tissues. Except BjSOS2, transcripts corresponding to members of SOS pathway (i.e. BjSOS1 and BjSOS3) could be detected in all the plant organs analysed in the two cultivars. Constitutive as well as salinity induced BjSOS3 transcripts could be detected in all organs for both the genotypes. However, as can be seen from figure 9, there are some organs which showed a contrasting pattern between the two genotypes. For example, roots of B. juncea showed relatively higher transcripts than roots of B. nigra under unstressed conditions which were found to be upregulated under salinity stress in the later genotype. As we move to other organs in the plants, a relative decrease (both constitutive and salinity induced) in the BjSOS3 transcript levels was noticed. In contrast, the reproductive parts of the plants (such as flower and pod) exhibited very high transcripts for BjSOS3 in the two genotypes. On an overall basis, BjSOS2 transcripts coding for S/T kinase were, very low in all the organs of mature Brassica plant analysed here. However, the reproductive organs (both flower and pod) and the selected vegetative organs (roots) showed relatively higher signal for BjSOS2 than other organs (after longer exposure of the blots). B. juncea var. CS52 showed relatively higher salinity induced transcripts for BjSOS2 in root and flower. In case of BjSOS1, the transcripts could be detected in almost all organs of both the cultivars being most abundant in roots of B. juncea. In case of upper stem samples, an additional cross reacting band was detected along with the main transcript. It was also observed that in certain organs, the transcript size differed slightly between the two genotypes (e.g. BjSOS3 in middle stem, upper stem and BjSOS1 in upper stem and upper leaf) which need to be looked into further.

4.2.3 Heatmap analysis of BjSOS genes in two contrasting Brassica genotypes

Based on the transcript abundance on Northern blots (Fig. 8 and 9), a heatmap was generated using the absolute value of expression using Mayday software (www. http://www.zbit.uni-tuebingen.de/pas/mayday/). For this purpose, both
control (un-induced) as well as salinity-induced transcript abundance of BjSOS genes in two contrasting, salt tolerant and sensitive, Brassica genotypes was scored and analysed (Fig. 10A and B).

Figure 10: The salinity tolerant genotype B. juncea var. CS52 show higher constitutive and salinity inducible transcript for various members at both seedling and reproductive stage. Heat map showing relative transcript abundance of BjSOS genes in two contrasting Brassica genotypes as revealed from Northern probing analysed using Mayday software. (A) In seedlings, BjSOS transcripts are more abundant in salt tolerant B. juncea var. CS52 even under control conditions and showed further induction in response to salinity. (B) In various vegetative and reproductive organs of mature plant, the level of BjSOS2 transcripts was comparatively lower than BjSOS1 and BjSOS3 in both the genotypes. BjSOS1 transcripts were relatively more abundant in roots of B. juncea var. CS52.

The left heatmap depicts the transcript abundance for BjSOS genes under very early (10, 20 or 30 min) or late phase (24, 48 or 72 h) of salinity stress. Salt tolerant, B. juncea var. CS52 showed constitutively higher BjSOS transcripts compared to salt sensitive B. nigra under control conditions and showed further induction in response to salinity (Fig. 10A). The heatmap on right indicates the transcript abundance for BjSOS genes in various organs of contrasting Brassica genotypes. The level of BjSOS2 transcripts was comparatively lower than
BjSOS1 and BjSOS3 in both the genotypes in all the plant organs analysed in the two cultivars (Fig. 10B). Another noticeable feature as revealed from this analysis is that leaf tissue has more BjSOS transcripts than stem tissues. Further, among the various leaf tissues analysed here, lower leaf has more levels for BjSOS1 possibly indicating the role of BjSOS1 in physiological partitioning. Root tissue of B. juncea var. CS52 exhibited higher BjSOS1, BjSOS2 and BjSOS3 transcript, again reflecting an ability towards efficient ion homeostasis in the tissues.

4.2.4 BjSOS2 expression analysis in seedlings of Brassica species under other abiotic stresses indicate it to be a multi-stress responsive gene

From the above analysis, BjSOS2 was found to be differentially regulated among the contrasting Brassica genotypes in response to salinity stress. To check if the BjSOS2 gene is one of the multi-stress responsive members of SOS family, its expression was analysed under various above stresses employing RNA gel blot analysis. BjSOS2 expression was analysed under different abiotic stresses, i.e. cold, heat, ABA and drought (Fig. 11). Total RNA was extracted from control and stress-treated shoot tissues. Seedlings kept in normal media were used as control. In the control, no transcript for BjSOS2 was detected in B. nigra but constitutive expression was observed in B. juncea var. CS52.

Effect of EGTA on the BjSOS2 expression at the seedling stage

It has been proved earlier that SOS pathway is regulated by secondary signaling molecule Ca^{2+}. Where salinity induced Ca^{2+} peaks are sensed by calcium sensing protein SOS3, which then activates the next molecule SOS2 of the cascade.
Calcium also appears to influence several other cellular mechanisms mediating salt tolerance in plant. We wanted to see the effect of Ca$^{2+}$ peaks on induction of BjSOS2 transcripts by using EGTA which is well known Ca$^{2+}$ chelator (Fig. 12).

Under control condition, BjSOS2 was found to be expressed constitutively which further gets enhanced in response to exogenous application of CaCl$_2$ as well as NaCl. It was observed that upon application of EGTA with NaCl, the expression of BjSOS2 was decreased. Similar kind of behavior was observed when the
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seedlings were exposed to heat or cold conditions. Abscisic acid (ABA) plays an important role in plant growth and development, as well as in the response of the plant to the environmental stresses. Similar kind of response was also observed when the seedlings were treated with EGTA and ABA. Thus, it can be safely concluded that exogenous application of EGTA is able to affect the stress induced accumulation of BjSOS2 transcript in seedling of B. juncea var. CS52. The results suggest that Ca\(^{2+}\) plays a major role in the signal transduction pathway leading to ion-homeostasis in plants.

4.3 Isolation and characterization of full-length BjSOS2 gene from Brassica juncea var. CS52

The results presented so far, were carried out employing partial cDNA clone (250bp) of BjSOS2. However, in order to characterize this gene in detail, we wanted to have full length cDNA clone of BjSOS2. Following the standard protocol, total RNA was extracted from the 6 d old seedlings (Fig.13A) treated with 200 mM NaCl for 24 h of B. juncea var. CS52. Using the cDNA synthesis kit (Fermentas, EU), first strand cDNA was synthesized following the standard protocol. The first strand cDNA obtained was later used as template for cloning of BjSOS2 gene from B. juncea var. CS52.

4.3.1 Cloning of full length BjSOS2 gene and its in-silico analysis

To isolate full length cDNA of BjSOS2 from Brassica juncea var. CS52, PCR amplification was done using BjSOSF2F and BjSOSF2R primers and cDNA synthesized from hydroponically grown 24 h salinity stressed 6d old seedlings of Brassica juncea var. CS52 (Fig.13). This 1.4 Kb amplicon was cloned into pGEMT vector and recombinant plasmids transformed into E. coli (DH5\(\alpha\)) (Fig.14A-C) and DNA sequencing was carried out at Macrogen Inc. (South Korea).
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Figure 13: Amplification of full length BjSOS2 employing gene specific primers using cDNA as template. (A) 6d old seedlings of Brassica juncea var. CS52. (B) Ethidium bromide stained gel showing amplification of full length BjSOS2 cDNA. Arrow indicates an amplicon of 1.4 Kb. Marker positions are shown on the left.

Figure 14: Cloning of full length BjSOS2 into pGEMT vector (A) Ligation of full length BjSOS2 cDNA into pGEMT-Easy cloning vector. (B) Recombinant plasmid after ligation. (C) Recombinant plasmids transformed into E. coli (DH5α) by freeze thaw method. (D) Ethidium bromide stained gel showing colony PCR for the confirmation of presence of 1.4 Kb BjSOS2 cDNA insert, M: Marker, Colonies: (1-2). Marker positions are shown on the left. Arrow indicates an amplicon of 1.4 Kb.

Agarose gel electrophoresis of amplified product showed a band of around 1.4kb after colony PCR (Fig. 14D). The full length cDNA (GenBank accession number
EF190471) of *BjSOS2* contains a 1.4 Kb open reading frame encoding 445 amino acids.

*In silico* analysis of deduced amino acid sequences corresponding to *BjSOS2* 1.4 Kb full length cDNA indicated that it possesses typical features such as kinase, FISL and regulatory domains. Multiple amino acid sequence alignment was performed using the ClustalW while phylogenetic and molecular evolutionary analysis was conducted using MEGA 4.

The tree was constructed using the neighbor-joining method. Overall identity of *BjSOS2* with *AtSOS2* is 88% at nucleotide level while it is 87% at amino acid level. Multiple alignment data revealed high conservation at N-terminal putative kinase catalytic and the C-terminal regulatory domains of SOS2 amongst other plant species (Fig. 15A). Phylogenetic unrooted tree analysis revealed *BjSOS2* of *Brassica* to be closely related to *AtSOS2* from *Arabidopsis* (Fig. 15B).

### 4.3.2 *BjSOS2* shows strong structural homology with *AtSOS2*

Since, *BjSOS2* was found to be close to *AtSOS2* in sequence, we wanted to compare their structure as well in order to comment on their functional conservation. For this purpose, the sequence of *BjSOS2* and *AtSOS2* were analysed using Visual Molecular Dynamics (VMD) software. Structure of *BjSOS2* showed the presence of kinase domain along with the FISL and PPI domain (Fig. 16A). The kinase domain in the SOS2 protein showed homology with protein kinase domain of yeast AMP-activated protein kinase Snf1 and Protein kinase domain of the Human AMPK protein.
Figure 15: Sequence analysis of BjSOS2 isolated from Brassica juncea var. CS52 (A) BjSOS2 ORF (1.4kb), encodes 445 amino acids. It shows all the conserved features of amino acid alignments for various SOS2 Ser/Thr protein kinase. A kinase domain (shown in red line), Regulatory domain (shown in black dotted line) and a FISL domain (shown in red rectangle). (B) Phylogenetic unrooted tree analysis revealed BjSOS2 of Brassica to be closely related to AtSOS2 from Arabidopsis.
Figure 16. Cartoon representation of the modeled BjSOS2 protein. (A) Showing the presence of Kinase, FISL and PPI domain. (B) Kinase domain of BjSOS2 (Blue colour), overlapped with the yeast Snf1 protein (Brown colour), with which the domain shows strong homology. Final figure was prepared using VMD (Visual Molecular Dynamics) software.

The FISL/NAF motif plays role in binding to the SOS3 protein. Earlier reports showed that the PPI domains show similarity to the kinase associated domain-1 of the mouse map/microtubule affinity-regulating kinase 3 (MARK3) (Fig. 16B).

4.4 Construction of over-expression as well as RNAi cassettes for BjSOS2

Cloning of BjSOS2 into binary vector

For raising transgenic plants overexpressing BjSOS2, we cloned the BjSOS2 ORF into pCAMBIA1304 vector. BjSOS2 fragment was amplified using primers containing BjSOS2NcoIF and BjSOS2SpeIR primers and cloned into pCAMBIA1304 as Ncol and SpeI fragment. Vector and insert (1.4Kb BjSOS2) were digested with Ncol and SpeI and ligation was performed. The recombinant product was transformed to E. coli (DH5α). Colony PCR was done to confirm the presence of 1.4 Kb insert (Fig. 17). The resultant vector carrying BjSOS2 was
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named as pCAMBIA-BjSOS2. Restriction digestion was done using Ncol and SpeI which yielded a fall out of 1.4 Kb.

For raising transgenic plants underexpressing BjSOS2, a specific Kinase domain fragment (≈230 bp) of BjSOS2 was cloned into sense and anti-sense orientation into pFGC1008 vector under the control of 35S promoter. Using specific primer for 1st (Ascl and Swal) and 2nd (BamHI and SpeI) sites for vector pFGC1008, PCR amplification was carried out. PCR product obtained after amplification was digested with site-specific restriction enzymes and ligated.

Figure 17: Restriction analysis of pCAMBIA-BjSOS2 for confirmation of cloning. (A) Schematic representation of overexpressing BjSOS2 construct showing the cloning of BjSOS2 in pCAMBIA 1304 binary vector at multiple cloning sites. (B) Digested insert and vector were loaded on 0.8% agarose gel. (C) Recombinant plasmid pCAMBIA-BjSOS2 was confirmed by digested with Ncol and SpeI restricted endnuclease enzymes. Ud- Undigested plasmid, D- Digested plasmid, Arrow shows the 1.4 Kb product.

For first site ligation, (Ascl and Swal), digestion of both vector as well as insert was carried separately and ligated. The ligated product was then transformed into E. coli (DH5α). The colonies obtained were then confirmed through colony PCR. Colony PCR confirmed the presence of insert when amplified using vector
specific primer (ASF and ASR for first site), as expected, a PCR product of 476 bp was obtained when ligated and in absence of insert gave 226 bp products. This led to cloning of the partial SOS2 insert in the sense and antisense direction with a GUS intron region in between them which allowed the formation of hair pin loop required for siRNA production in plant system. For second site ligation, (BamHI and SpeI), digestion of both vector as well as insert was carried separately and ligated. The ligated product was transformed to E. coli (DH5α). The colonies obtained were then confirmed through colony PCR using vector specific primer (BSF and BSR) for amplification. As expected, a PCR product of 815 bp was obtained when ligated and in absence of insert gave 565 bp products (Fig. 18). Positive clone of Agrobacterium carrying the RNAi construct was also confirmed via colony PCR and sequencing. The resultant vector carrying BjSOS2 was as pFGC1008-RNAiBjSOS2 (Fig. 18).

These recombinant pCAMBIA-BjSOS2 overexpressing and pFGC1008-RNAiBjSOS2 constructs were further transformed into Agrobacterium strain GV3101 by freeze-thaw method and grown on YEB media containing antibiotic rifampicin (25μg/ml) and kanamycin (50 μg/ml) for pCAMBIA-BjSOS2, rifampicin (25μg/ml) and chlorempenicol (34 μg/ml) for pFGC1008-RNAiBjSOS2 construct. Agrobacterium culture was incubated at 28°C for 48 h. The Agrobacterium colony carrying pCAMBIA-BjSOS2 construct was further confirmed by colony PCR using vector specific forward and reverse primers (Fig. 19).

4.5 Raising of transgenic Brassica juncea var. CS52 plants with overexpressing (OXBjSOS2) or underexpression (RNAiBjSOS2)

For raising overexpressing plants, Agrobacterium containing pCAMBIA-BjSOS2 was used, while for raising underexpression (RNAi) plants, Agrobacterium strain GV3101 containing pFGC1008-RNAiBjSOS2 was used for transformation of Brassica juncea var. CS52 plant.
Figure 18: PCR confirmation of pFGC1008-RNAiBjSOS2 cassette using vector specific primer. M = marker; 1st lane = control of 1st site (pFGC1008 only); 2nd lane = 1st site ligation (ASF + ASR); 3rd lane = control of 2nd site (BSF + BSR); 4th lane = 2nd site ligation (ASF + BSR).

Figure 19: PCR to confirm the transformation of pCAMBIA-BjSOS2 into Agrobacterium strain GV3101. PCR was done using transformed colonies 1-8 using gene specific forward (BjSOS2F) and vector specific reverse (pCAMBIAR) primers. Lane 5, 7 and 8 shows the positive amplification of BjSOS2. Arrow shows the position of BjSOS2 amplified product.
The different stages of transformation and regeneration of transgenic plants for overexpressing and underexpression BjSOS2 gene are shown in figure 20. The transgenic plants were selected on MSB1N1 medium containing hygromycin (Fig. 20) and complete regenerated plants were transferred into pots for hardening (Fig. 21).

Figure 20: Steps involved in transformation and regeneration of Brassica juncea var. CS52 transformed with pCAMBIA-BjSOS2 and pPFGC1008RNAiBjSOS2 constructs which is present in Agrobacterium strain GV3101. (A) 6d old Brassica juncea var. CS52 seedlings geminated in MSO medium (B) Hypocotyls kept on selection hygromycin (25mg/l) containing medium (C) regenerating hypocotyls showing small plantlets (D-F) regenerated plant on rooting media (G) Transformed plants transferred to pots (H) Transformed plants transferred to green house.
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Figure 21: Putative transgenic plants of *Brassica juncea* var. CS52 (T₅) plants. Wild type, OXBjSOS2 (T₀) and RNAiSOS2 (T₀) transgenic. An account of difference in leaf morphology among WT (A), OXBjSOS2 (B) and RNAiBjSOS2 (C) plants of *Brassica juncea* var. CS52 is shown (inset).

4.5.1 Histochemical GUS analysis for confirmation of *Brassica juncea* var. CS52 transgenic plants

Putative transformed plants were screened for the expression of GUS gene present in the construct. GUS activity was noted predominantly in the leaves, stem, lateral roots and primary root tips (Fig. 22). Histochemical staining of different parts of overexpressing SOS2 (OXBjSOS2) revealed that, after incubation in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, GUS activity was detectable predominantly in both the primary and lateral roots (abundantly in the root tip and meristematic region). Significant levels of GUS activity were also detectable in the aerial parts. In leaves, GUS activity was detected in vascular tissues of the lamina and petiole (Fig. 22).
4.5.2 Molecular confirmation of transgenic overexpressing (OXBjSOS2) *Brassica juncea* var. CS52 plants

To confirm the incorporation of full BjSOS2 gene into genome of *Brassica juncea* var. CS52 overexpressing lines, tissue PCR was done using gene specific forward and vector specific reverse primers.

![Figure 22: Histochemical GUS analysis showing expression of BjSOS2. (A) GUS staining shows expression pattern of BjSOS2 in vivo in various tissues (Leaf, Stem, Root, Root tip) from the T<sub>0</sub> generation of OXBjSOS2 Brassica transgenic (B) GUS staining in OXBjSOS2 Brassica 6d old seedling (T<sub>1</sub>-generation).](image)

Genomic DNA from WT (non transgenic) *B. juncea* var. CS52 and putative OXBjSOS2 plants was analyzed for this purpose. The pCAMBIA-BjSOS2 plasmid was taken as positive control. A positive amplicon of 1.4 Kb (Fig. 23A) was obtained in overexpressing lines corresponding to positive control and the specificity of amplified band was further confirmed by Southern PCR hybridization using the radiolabeled DNA fragment amplified from pCAMBIA-BjSOS2 (using the same primer pairs) as probe. Southern PCR hybridization revealed the presence of a specific band in each plant (Fig. 23B).

4.5.3 Southern analysis of PCR positive BjSOS2 transgenic (OXBjSOS2) lines

To check the stable integration of the BjSOS2 transgene in *B. juncea* var. CS52, genomic Southern blot of PCR positive plants was done. Each sample of genomic DNA (10 µg) isolated from transgenic lines (L1, L2) and wild type (*B. juncea* var. CS52) was digested with HindIII and EcoRI. The digested DNA was
fractionated on 0.8% agarose gel (Fig. 23C) and transferred to the nitrocellulose membrane which was then probed with radiolabeled gfp probe. Only a single band was observed in transgenic lines indicating the presence of single copy of the T-DNA (Fig. 23D). However, no band was detected in wild type plants. All lines showed the band at different positions which suggests different position of integration of the transgene.

Figure 23: Screening of putative BjSOS2 overexpressing transgenic plants through tissue PCR and Southern analysis. (A) Ethidium bromide gel showing screening of putative transgenic Brassica plants. Positive transformants were selected using a combination of gene specific and vector specific primer for the presence of 1.4kb amplicon (marked by an arrow here). Genomic DNA from wild type plant was taken as negative control. (B) PCR southern showing specific hybridization of the amplicon with BjSOS2 probe as shown by an arrow. (C) Ethidium bromide stained gel showing completely digested DNA from wild type and transgenic lines. 10 μg genomic DNA from both wild type Brassica and transgenic Brassica lines (L1, L2) overexpressing BjSOS2 (OXBjSOS2) was digested with HindIII and EcoRI and transferred onto the nylon membrane. (D) Southern blot analysis of OXBjSOS2 transgenic plants of B. juncea var. CS52 to detect copy number of the transgenic BjSOS2. Radiolabeled full length gfp was used as probe.

4.6 Stress tolerance assay of transgenic B. juncea var. CS52 plants overexpressing (OXBjSOS2) and underexpressing (RNAiBjSOS2)
4.6.1 Enhanced tolerance of T₀ transgenic plants overexpressing BjSOS2 (OXBjSOS2) towards salinity stress

It was observed that the transgenic Brassica juncea var. CS52 plants OXBjSOS2 grew healthier as compared to RNAiSOS2 and WT plants even under control conditions (Fig. 24A).

Figure 24: Evaluation of performance of transgenic (T₀-generation) Brassica juncea var. CS52 overexpressing BjSOS2 (OXBjSOS2) and underexpression RNAiBjSOS2 under salinity stress and no stress (control) in comparison to wild type. (A) Wild type (Brassica juncea var. CS52), OXBjSOS2 and RNAiBjSOS2 plants showing normal growth under controlled conditions. (B) Leaf disc assay to compare stress tolerance in wild type (Brassica juncea var. CS52), transgenic plants (OXBjSOS2) and RNAiBjSOS2 either subjected to 200mM and 400mM salinity stress for duration of 5 days or no stress (control). (C) Histogram depicts the estimated chlorophyll values (in µg/gm fresh weight) from tissue obtained from leaf disc assay.
To investigate the effect of overexpressing of BjSOS2 gene in ameliorating the effects of salinity, leaf discs from T₀ transgenic lines and WT Brassica juncea var. CS52 plants were kept separately in Hoagland media supplemented 200mM and 400mM NaCl or Hoagland (as control) for 5 days. The BjSOS2 overexpressing lines (OXBjSOS2) were quite efficient in overcoming the deleterious effects of NaCl toxicity. The RNAiBjSOS2 transgenic lines showed extensive bleaching during salinity condition as compared to OXBjSOS2 transgenic lines and WT plants, thus reflecting the symptoms of injury during stress while the transgenic OXBjSOS2 lines were not affected under similar conditions (Fig. 24B). These results were confirmed biochemically by estimating the chlorophyll content in these leaf discs. The loss of chlorophyll content in the RNAiBjSOS2 and WT plants was observed to be more in comparison to OXBjSOS2 transformants under salinity condition (Fig. 24C). These observations clearly establish a positive relationship between the overexpressing of OXBjSOS2 and salinity tolerance.

4.7 Growth analysis of overexpressing transgenic plants (T₁-generation) of Brassica juncea var. CS52 (OXBjSOS2) under salinity stress

4.7.1 Transgenic lines of Brassica (T₁-generation) show tolerance towards salinity stress

We analysed the growth of the wild type (WT) and overexpressing (OXBjSOS2) transgenic plants (T₁) in Hoagland media. Consistent with the previous results, both WT and transgenic plants showed normal growth under control conditions. Five-days-old seedlings were transferred from normal Hoagland medium to media supplemented with 200mM NaCl. The root growth of OXBjSOS2 transgenic plants was slightly more as compared to WT during stress conditions (Fig. 25D). On an overall basis, the transgenic plants overexpressing BjSOS2 exhibited better growth under stress condition as compared to WT plants. WT seedlings showed drastic reduction in fresh weight as well as growth observed by shoot length and root length, whereas transgenic OXBjSOS2 seedlings showed
less reduction in fresh weight and shoot length under stress condition (Fig. 25B-D).

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![Image of seedling phenotypes and measurements](image)

**Figure 25:** Evaluation of performance of transgenic (T₁-generation) *Brassica juncea* var. CS52 overexpressing *BjSOS2* (OXBJsOS2) at seedling level. (A) Seedling phenotypes of wild-type (WT) plants and overexpressing OXBJsOS2 transgenic plants treated with 200 mM NaCl. These pictures were taken 2 days after the NaCl treatment. (B) Fresh weight of the seedlings under control and 200mM salt stress conditions. (C) Shoot length measurement of WT and transgenic plants. (D) Root length measurement for which five-day-old seedlings were transferred from normal Hoagland medium to media supplemented with 200mM NaCl and root elongation after 7 days was measured after 2 days of stress treatment. Error bars represent the standard deviation (n=10).
4.8 Confirmation of transgenic plants underexpressing *BjSOS2* (RNABjSOS2)

Putative transgenic plants underexpressing SOS2 (RNAiBjSOS2 plants) were further confirmed by tissue PCR using vector specific primers flanking the sense and antisense cloning sites (Fig. 26B). PCR Southern hybridization revealed the presence of a specific band in L-1 to L-5 RNAiBjSOS2 plants (Fig. 26C). These PCR positive plants were hardened and transferred to earthen pots. These plants were maintained in green house under optimal growth conditions for the purpose of seed collection.

![Diagram of transgenic plant confirmation]

**Figure 26:** Confirmation of Underexpressing *Brassica* juncea var. CS52 (RNAiSOS2) transgenic lines by tissue PCR and Southern PCR. (A) Schematic representation of pFGC1008-RNAiBjSOS2 cassette. (B) Tissue PCR was performed using vector specific forward and reverse primers with tissue of transgenic plants as template. M: DNA marker; L-1 to L-4: sense and antisense orientation of SOS2 fragment amplified in RNAiBjSOS2 transgenic *Brassica juncea* var. CS52, using ASF and ASR primers; BSF and BSR primers. (C) Southern hybridization of tissue PCR amplified products of (A) using labeled BjSOS2 full length gene as probe.
4.9 Transgenic *Brassica* plants (OXBjSOS2) performed better than WT or RNAiBjSOS2 lines

Transgenic *Brassica* (OXBjSOS2) plants were brought to maturity under standard agronomic conditions inside the glass house and analysed for various physiological parameters like the plant height, number of branches/plantlets, number of pods/branch and number of seeds/pods. Transgenic OXBjSOS2 plants performed better as compared to the RNAiBjSOS2 and WT (Fig. 27). This analysis indicated that these physiological parameters may contribute towards the increased yield of *Brassica* plants under control condition (Fig. 28).

![Image](image_url)

**Figure 27:** Performance of WT and (T₁) transgenic *Brassica juncea* var. CS52. The transgenic (OXBjSOS2) plants showed vigorous growth in contrast to RNAiBjSOS2 and WT plants under control conditions in green house. Various growth parameters (plant height, No. of branches, No. of pods and seeds per pods) were reported to be better in OXBjSOS2 plants as compared to wild type or RNAiBjSOS2 lines.

4.9.1 Transgenic lines of *Brassica juncea* var. CS52 (T₁-generation) showed tolerance towards salinity stress during seed germination
Under salinity stress, the germination and growth of WT *Brassica* seeds was reduced while the transgenic line (OXBjSOS2) showed better germination and growth. Salinity stress (150 mM NaCl) affected the germination and growth of WT plant severely while the performance of OXBjSOS2 line no. L-1 to L-4 was again much better (Fig. 29A).

![Graphs showing analysis of yield parameters in WT, OXBjSOS2 and RNAiBjSOS2 Brassica juncea var. CS52 transgenic plants (T1) under green house condition.](image)

**Figure 28:** Analysis of yield parameters in WT, OXBjSOS2 and RNAiBjSOS2 *Brassica juncea* var. CS52 transgenic plants (T1) under greenhouse condition.

The electrolyte leakage and endogenous Na\(^+\) and K\(^+\) levels also reflected the ability of OXBjSOS2 overexpressing lines to survive better under salinity stress conditions (Fig. 29B & C). A drastic decline in K\(^+\)/Na\(^+\) ratio was observed when WT and OXBjSOS2 seeds (T1) were subjected to 150 mM NaCl for 5 days (Fig. 29C). In contrast, the OXBjSOS2 transgenic seedlings had higher K\(^+\)/Na\(^+\) ratio and were able to maintain the ion homeostasis, also less electrolyte leakage as compared to WT seedlings under salinity stress condition.
4.10 Characterization of transgenic plants at molecular level with northern analysis for other genes involved in signal transduction

For this purpose, we planned to perform RNA gel blot analysis with OXBjSOS2 plants employing the probes from various genes known to play key role in stress perception and signal transduction. Figure 30 shows such analysis for various SOS pathway members and other important genes i.e. Glyoxalase I (BjG/yl) in stress response. As it can be seen clearly that the salinity induced expression of both SOS members and BjG/yl is more in OXBjSOS2 transgenic plants which are over expressing BjSOS2 as compared to WT plants. This observation indicates a

![Image of germination and physiological analysis]

Figure 29: Germination and physiological analysis of T1 seeds of Brassica juncea var. CS52 plants (OXBjSOS2) in comparison to WT Brassica juncea var. CS52 seeds under salinity (150 mM) stress. (A) Equal number of seeds of wild type and OXBjSOS2 lines (L-1 to L-4) was inoculated on Hoagland media supplemented with 150 mM NaCl. OXBjSOS2 Brassica seeds showed higher germination rate as well as better growth as assessed visually. (B) Electrolyte leakage was measured in salt stressed seedlings of both wild type and OXBjSOS2 lines. (C) K⁺/Na⁺ ratio was measured in salt stressed seedlings of both wild type and transgenic Brassica seedlings (OXBjSOS2).
Results

possible ability of these transgenic plants to regulate the expression of other salinity responsive genes. We are extending this work to other known genes also so that novel indications on how transgenic plants tolerate stress better than WT can be established.

![Northern blot analysis of the T1 transgenic seedlings and untransformed control plants of B. juncea var. CS52. RNA gel blot analysis indicating the transcript abundance for various SOS members and BjGlyl of Brassica juncea var. CS52. The analysis was carried out using RNA from WT and various BjSOS2 transgenic lines under control and stress conditions (200mM NaCl for 5h).]

4.11 Isolation and characterization of BjSOS2 promoter from Brassica juncea var. CS52

Isolation of BjSOS2 Promoter and Search for its Cis-Acting Elements

We isolated a 803 bp SOS2 promoter of BjSOS2 from B. juncea var. CS52. The isolated SOS2 promoter sequence was analysed for the presence of known consensus cis-acting elements by PLANTCARE software (Fig.31). There elements included ABRE (-793), TGA (-301) etc. To characterize promoter in greater details, series of 5’ deletions were made.
4.11.1 Generation of transgenic *Brassica juncea* var. CS52 plants expressing the GUS gene driven by full length or 5’ deleted BjSOS2 promoter

An 803 bp SOS2 promoter sequence was dissected from *Brassica juncea* var. CS52 by PCR-based Genome Walking protocol and sequenced which showed 94.8% homology with *AtSOS2*.

![Diagram of BjSOS2 promoter and deletions](image)

**Figure 31:** *In silico* analysis of *BjSOS2* 803 bp promoter from *Brassica juncea* var. CS52: Schematic diagram fused to GUS reporter gene showing putative cis acting elements in promoter of BjSOS2. Various 5’ deletions were made. The arrow indicates the transcriptional start site and the numbers indicate the length of each undeleted or deleted constructs from 5’ end of the promoter region.

The core promoter (803 bp) and also their corresponding deletion derivatives (D1 533 bp and D2 299 bp) were fused with beta-glucuronidase (GUS) reporter gene by cloning in pCAMBIA1391z promoter-less vector and transformed in *Brassica juncea* var. CS52 via *Agrobacterium tumifaciens* strain GV3101. To determine the function of SOS2 promoter, leaf tissues of T₀ and
seedlings of T1 transgenic lines of *B. juncea* var. CS52 were subjected to various treatments of abiotic stresses.

### 4.11.2 Activity of *BjSOS2* promoter in response to multiple abiotic stresses

To determine the function of promoter, independent transgenic lines consisting of core promoters and their derivatives were screened under treatment of different abiotic stresses. Leaf tissues of transgenic lines consisting of UD promoter exhibited activity 756.9 pmol 4-MU/min/mg protein under salt stress. Activity after ABA and drought treatment was 622.4 and 593.9 respectively. Temperature extremes (heat and cold) showed only 402.0 and 349.4 pmol 4-MU/min/mg protein activity. A sharp reduction in GUS expression quantified in leaf tissues of transgenic lines consisting D1 (661.4, 594.9, 563.6, 289.4 and 203.9) and D2 (358.6, 326.0, 282.8, 183.7 and 164.05) promoter regions under defined treatment of NaCl, ABA, drought, heat and cold. The fold reduction was evident in D2 (8.4, 7.7, 6.6, 4.3 and 3.8) as compared to UD (17.9, 14.7, 14.0, 9.5 and 8.2) after treatment with NaCl, ABA, drought, heat and cold respectively (Fig. 32B). Histochemical staining also mimic the same pattern of GUS expression in leaf tissues of transgenic lines (Fig. 32A). This concludes that the minimal promoter lies within SOS2-D1 sequence of *BjSOS2* promoter.

Stress treated seedling obtained from T1 seeds of above plants also showed several folds higher GUS activity as compared to the unstressed ones. GUS activity of core promoter (UD) in pmol 4-MU/min/mg protein was 690.0 with salt treatment, which was followed by ABA (605.7), drought (582.8), heat (386.3) and cold (356.9). GUS activity was found to considerably decrease in D1 (654.7, 587.5, 532.4, 280.5, 236.8) whereas D2 exhibited lowest induction in GUS activity after treating with NaCl, ABA, drought, heat and cold stresses respectively. T1 transgenic seedling showed 15.6 fold higher GUS activity for UD
after NaCl treatment compared to control (unstressed) which showed gradual reduction in D1 (14.8-fold) and in D2 (7.9-fold) respectively.

![Results](image)

Figure 32: GUS expression in leaf tissues of transgenic lines of *Brassica juncea* var. CS52 for SOS2 promoter (A) Independent transgenic lines containing UD-SOS2-GUS transgene and their deletion derivatives were analysed in T0 generation for GUS activity by histochemical staining (B) Fluorimetric mean of leaf tissues of 6-d old plant were used for GUS activity measurement after treatment with multiple abiotic stresses viz., NaCl (200 mM), ABA (100 µM), Cold (4°C), heat (40°C) and drought for 10h. The error bars indicate standard deviation from at least three independent experiments; the experiment was repeated three times.

The level of induction under ABA was 13.7, 13.3 and 6.1-fold in UD, D1 and D2 respectively, which was followed by drought (13.2, 12.1 and 6.3-fold) and heat (8.7, 6.3 and 3.4-fold). In contrast, slight induction (8.1, 5.3 and 2.7-fold) was observed after cold treatment (Fig. 33B). The GUS expression pattern for seedling observed after histochemical staining was more or less same. GUS expression was maximum observed in salt followed by drought and ABA condition. However, it reduced gradually from UD to D3. The control (unstressed) transgenic seedling exhibited either negligible or no expression (Fig. 33A).
Figure 33: GUS expression in transgenic seedlings of *Brassica juncea* var. CS52 for BjSOS2 promoter (A) Independent transgenic lines containing UD-SOS2-GUS transgene and their deletion derivatives were analysed in T1 generation for GUS activity by histochemical staining (B) Fluorimetric mean of 6-d old seedlings were used for GUS activity measurement after treatment with multiple abiotic stresses viz., NaCl (200 mM), ABA (100 μM), Cold (4°C), heat (40°C) and drought for 10h. The error bars indicate standard deviation from at least three independent experiments; the experiment was repeated three times.

The various abiotic stresses such as cold, heat, salt, drought and ABA, were able to induce strong activity in core promoter in these transgenic plants whereas its expression levels got reduced in SOS2-D1. Further in SOS2-D2, there is little/no visible activity of GUS indicating that all the necessary cis-elements are absent in this region. Thus SOS2-D1 seems to be a good minimal promoter in this case.
PART "B"

4.12 OsSOS2 transcripts are differentially regulated in contrasting cultivars of rice under salinity stress conditions

In order to study the constitutive (un-induced) and salinity induced transcripts abundance for OsSOS2 gene in salt tolerant Pokkali and salt sensitive IR64, RNA gel blot analysis was carried out. For this purpose total RNA was extracted from 4 d old seedlings treated with 200 mM NaCl for 10', 20' 30' (very early) and 24h, 48h and 72 h (late responses) of OsSOS2 transcript. This analysis showed that in O. sativa cv. Pokkali the OsSOS2 transcript intensity was high in late phase while it was low in O. sativa cv. IR64. We have extended this analysis to study the organ specific expression patterns of OsSOS2 genes in contrasting rice cultivars.

Figure 34: Differential transcript accumulation for OsSOS2 genes during ‘very early’ and ‘late phase’ of salinity stress in seedlings and tillering stages of two cultivars IR64 and Pokkali. RNA gel blots probed with OsSOS2. Ethidium bromide (EtBr) stained RNA gel shown as the loading control. Duration of 200mM NaCl stress has been mentioned on top of each lane. Heat map showing relative transcript abundance under control and salinity stress conditions of OsSOS2 genes in two contrasting rice cultivars as revealed from Northern probing analyzed using Mayday software.
OsSOS2 transcript was found to be induced within the very early phase (30 min.) in sensitive rice cultivar 'IR64'; however, the tolerant cultivar 'Pokkali' exhibited lower transcripts than IR64 under the conditions tested here (Fig. 34). It is also interesting to note that during the late phase (especially at 48 and 72 h salinity stress); the OsSOS2 transcripts were comparable in the two cultivars. Though, OsSOS2 was again found to be inducible by salinity stress in both the cultivars during the late phase, the tolerant cultivars always exhibited higher transcripts than the sensitive (Fig. 34). However, at tillering stage, the transcript levels were seen to decline during 24 h of salinity stress in sensitive IR64 cultivar but no significant changes in Pokkali was observed of SOS2.

Figure 35: OsSOS2 transcript accumulation is tissue specific in various vegetative and reproductive organs of field grown mature plants of salt sensitive IR64 and salt tolerant Pokkali cultivars. (A) Analysis was performed on stem (upper, middle and lower), leaf (upper, middle and lower), panicle of the two cultivars under control (C) and 200mM NaCl, 30min. and 24h (S) conditions. Northern blots prepared from various samples were probed with OsSOS2. (B) Heat map showing relative transcript abundance under control and salinity stress conditions of SOS2 genes in different tissue of two contrasting rice cultivars as revealed from Northern probing analyzed using Mayday software.
4.12.1 Regulation of OsSOS2 transcripts in mature field grown rice plants at various developmental stages

Plants of IR64 and Pokkali were grown to full maturity employing standard agronomic practices. At the mature plant stage, tissue samples from various plant organs were collected and leaf discs were either incubated in nutrient solution only (control) or supplemented with NaCl (stress) before extracting total RNA for analysis. OsSOS2 could be detected in all the plant organs analyzed in the two cultivars of rice. Constitutive as well as salinity induced OsSOS2 transcripts could be detected in all organs for both the cultivars. However, as can be seen from figure 35, there are some organs which showed a contrasting pattern between the two cultivars. For example, panicles of Pokkali showed relatively higher transcripts than panicles of IR64 under unstressed conditions. The reproductive organs (panicle) and the selected vegetative organs (leaf) showed relatively higher signal for OsSOS2 than other organs. Pokkali showed relatively higher salinity induced transcripts for OsSOS2 in all tissues as compared to IR64. In case of IR64, lower and middle stem samples showed relatively higher salinity induced transcripts for OsSOS2. But upper and middle leaf of IR64, in response to salinity stress showed down regulation of OsSOS2 transcripts.

4.12.2 OsSOS2 transcription is induced by different abiotic stresses

To study the induction of OsSOS2 gene under different abiotic stresses, RNA gel blot analysis was performed. For this purpose, total RNA was isolated from seedlings of O. sativa L cv IR64 and Pokkali grown on media containing NaCl (200 mM) for salinity stress, dehydration (dessication on tissue paper towel, cold (kept at 4°C) and ABA (100 μM) for 30 min, 2 h and 6 h. Seedlings kept in normal media were used as control. We found, transcripts of OsSOS2 to be inducible by salinity, drought, as well as in response to exogenous ABA application (Fig. 36).
However, no change in transcripts could be detected in response to low temperature in either cultivar. This accumulation of OsSOS2 transcripts was found to be differential in the two contrasting cultivars analyzed in the study. The tolerant cultivar Pokkali was found to have higher constitutive transcripts of this gene as compared to sensitive cultivar IR64.

![Northern blot showing expression of OsSOS2 transcripts under different stress conditions](image)

**Figure 36: Expression of OsSOS2 transcripts under different stress conditions in seedlings of contrasting rice cultivars.** Northern blot was performed using total RNA isolated from shoots of seedling subjected to salinity stress (200 mM NaCl); drought stress (kept on tissue paper); ABA (100 μM) and cold stress (4°C) for 30', 2 h and 6 h time period. C: control (untreated seedlings). OsActin gene was used as endogenous control.

### 4.12.3 Circadian control of SOS gene expression in two contrasting cultivars of rice

To further examine the pattern of the OsSOS2 mRNA accumulation over time, rice seedlings were grown under control conditions for 2 weeks and leaves were harvested at 3h intervals beginning at 8.30 am and lasting for 2.5 days. The results presented in figure 37 show that diurnal variation of the OsSOS2 transcript level occurred with minimal levels at day time and maximal levels in dark in both cultivars of rice. The periodic increase and decrease with a 24-h cycle suggests the involvement of a circadian rhythm. The calcium binding OsSOS3 transcripts also showed rhythmic variations but no rhythmic variation in OsSOS1 transcripts was reported in this analysis.
Figure 37: Circadian regulation of OsSOS genes expression. Leaves were harvested at the indicated times. Total RNA isolation and hybridization were performed by OsSOS3, OsSOS2 and OsSOS1. The hybridization signals were quantified and the relative mRNA levels were determined by the ratio of the OsSOS3, OsSOS2 and OsSOS1 as compared to OsActin. Ethidium bromide (EtBr) stained RNA gel shown as the loading control.

The hybridization signals were quantified by Fuji film software and the relative mRNA levels were determined by the ratio of the OsSOS3, OsSOS2 and OsSOS1 as compared to OsActin.

4.13 Isolation and characterization of OsSOS2 from Oryza sativa L. cv. ‘IR64’

Total RNA was extracted from the 6 d old seedlings of Oryza sativa L. ‘IR64’ (Fig. 38A) and treated with 200 mM NaCl for 24 h. Using the cDNA synthesis kit (Fermentas, EU), first strand cDNA was synthesized following the standard protocols and was later used as a template for cloning of OsSOS2 gene. Primer was designed by Primer-3-input software for isolating full length gene and then
PCR amplification was carried out using OsSOS2NcoIF and OsSOS2SpeIR primers. PCR amplification revealed an amplification product of around 1.4 kb from both root and shoot tissues (Fig. 38B).

Figure 38: Amplification of full length cDNA of OsSOS2. (A) 6 d old seedlings of rice cultivar ‘IR64’. (B) Ethidium bromide stained gel showing amplification of full length OsSOS2 gene through PCR using gene specific primers designed from both the terminals. An amplification product of 1.4 kb was obtained from root as well as shoot of rice seedlings. Marker positions are indicated on the left, M: marker.

Figure 39: Cloning of full length OsSOS2 into pGEMT vector (A) Ligation of full length OsSOS2 cDNA (1.4Kb) into pGEMT-Easy cloning vector. (B) Recombinant plasmid after ligation. (C) Recombinant plasmids transformed into E. coli (DH5α) by freeze thaw method. (D) Ethidium bromide stained gel showing colony PCR for the confirmation of presence of 1.4 Kb OsSOS2 cDNA insert, M: Marker, Colonies: (1-2). Marker positions are shown on the left. Arrow indicates an amplicon of 1.4 Kb.
Amplified product was eluted and cloned into pGEMT cloning vector. Recombinant plasmid was then transformed into E. coli (DH5α) by freeze and thaw method (Fig. 39C). DNA sequencing was carried out and obtained nucleotide sequence was used for BLAST search at GenBank. BLAST search exhibited significant homology with known OsSOS2 available in database. The OsSOS2 gene sequences have been submitted at NCBI under accession number DQ248963 (Fig. 40A).

Multiple amino acid sequences alignment was performed using the ClustalW. Multiple sequence alignments of these polypeptides indicated a high degree of colinearity among SOS2 in these amino acid sequences (Fig. 40B). In silico analysis of the deduced amino acid sequence corresponding to OsSOS2 showed that the isolated 1.4 Kb full length cDNA coded for a 50.65 KD OsSOS2 protein which was found to possess some typical features such as Kinase, FISL and regulatory domains (Fig. 41). The FISLN/AF motif plays an important role in binding of SOS2 to the SOS3 protein.

(A)  
5’ATGGAGGGGAGGGAAGAATGCAGGGACGGAGGAGTACTAGAGGGTTGGGAGGACCTAAGGGCAAGCCGCTGCAAGGCAGAAGCAGACCTTCAGTCCACACAGCAGATACATGGGAGGAGATATCAATAATGAAGATTGTAAGACACCCCAATATAGTTAGACTAAACGAGGTTCTAGCTGGAAGAGAAGCTTCGTGGAAAATGAAGCAAGGAAGTACTTCCAGCAGCTTATTGATGCCATCGATTATTGCCATAGCAAAGGAGTCTATCATAGAGATTTGAAGCCTGAAACCCTGCTTCTTGACTCGCGTGGAAAACTTGGGTTCTTGTAGTTACCTCTATGTTTTGAGTTTTGGTCATGTGGTGTTATTCTCTATGTTTTGATGGCTGGATACCTTCCTTTTGAGGAAGATGATCTTCCAACACTGTATGATAAGATTACTGCAGGTCAGTTTTCATGCCCTTATTGGTTCTCTCCAGGTGCTACCAGCTTTAACCAAGAATGACACTGATCAGACATAAGGGAAGACACATGGTTTAAGAAGATTATGAGCACATAAAGACGTGGAAGAATGAAAATGTTGATCTGGATGAGTTACAGGCTGTTTTTGACAATATTGAGGACAAGTACGTTTCTGAGCAAGTGACTCATAATGATGGTGAGCCCTCTTGATGAAATGCTTCGAGATGATTACATTATCTCAAGGTTTGGATCTTTTACGTTATTATTTGAGTTTGTCAAACGTCAAACTCGTTTTGTCTCAAGGAAACCAGCAAAGACTATAGTTACAGCTACAATTGAAGTTGTTGCCGAGACTATGGGTCTTAAGGTCCACCGTCCAGAATTACATGAGGTC

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Figure 40: In-silico analysis of OsSOS2 (A) Nucleotide sequences of OsSOS2 gene isolated from *oryza sativa* L. cv. 'IR64' and submitted at NCBI under Accession number DQ248963. Start and stop codons in the sequence are shown by green and red colour respectively. (B) Multiple alignment of OsSOS2 amino acids sequences of IR64 with SOS2 amino acids sequences of other plant species.

Figure 41: Secondary structure topology of OsSOS2 protein showing position of various structure motifs. OsSOS2 protein consist of two distinct structural domains namely kinase domain and NAF/FISL domain.
4.13.1 Cloning of OsSOS2 in plant transformation vector (Over-expression as well as RNAi cassettes for OsSOS2)

For rice transformation, the OsSOS2 cDNA containing a stop codon was cloned in plant transformation vector pCambia 1304 at Ncol and Spel sites, just upstream of mgfp gene (Fig. 42A). The recombinant vector pCambia1304OsSOS2 was then confirmed by PCR analysis. The recombinant pCambia1304OsSOS2 construct was further transformed into E. coli (DH5α).

![Restriction analysis of pCambia-SOS2 for confirmation of cloning.](image)

Figure. 42: Restriction analysis of pCambia-SOS2 for confirmation of cloning. (A) Schematic representation of overexpressing OsSOS2 construct showing the cloning of OsSOS2 in pCambia 1304 binary vector at multiple cloning sites. (B) Digested insert and vector were loaded on 0.8% agarose gel. (C) Recombinant plasmid pCambia-SOS2 was confirmed by digestion with Ncol and Spel restriction endnuclease enzymes. Arrow shows the 1.4 Kb product.
Transformed colony was confirmed by colony PCR and recombinant plasmid was checked by restriction digestion with Ncol and SpeI which yielded a fall out of 1.4Kb (Fig. 42C). For raising underexpression SOS2 plants, a specific Kinase domain fragment (∼230 bp) of OsSOS2 was cloned in sense and anti-sense orientation into pFGC1008 vector under the control of 35S promoter using specific primers for 1st (Ascl and Swal) and 2nd (BamHI and SpeI) sites (for vector pFGC1008). PCR product obtained after amplification was digested with site-specific restriction enzymes and ligated. The ligated product was then transformed into E. coli (DH5α). Colony PCR confirmed the presence of insert when amplified using vector specific primers (ASF and ASR for first site). As expected, a PCR product of 476 bp was obtained when ligated and in absence of insert gave 226 bp product. For second site ligation, (BamHI and SpeI) digestion of both vector as well as insert was carried individually and ligated. The ligated product was then transformed to E. coli (DH5α). The colonies obtained were confirmed through colony PCR using vector specific primer (BSF and BSR) for amplification. As expected, a PCR product of 815 bp was obtained when ligated and in absence of insert gave 565 bp product. Positive colony of Agrobacterium carrying the RNAi construct was also confirmed via colony PCR and sequencing. This resulted in cloning of the partial SOS2 insert in the sense and antisense direction with a GUS intron region in between, which allowed the formation of hair pin loop required for siRNA production in the plant system.

The recombinant pCAMBIA1304-OsSOS2 and pFGC1008-RNAiOsSOS2 plasmids were also transformed into Agrobacterium strain LBA4404 by freeze thaw method as described in materials and methods. Agrobacterium cells containing the recombinant plasmid construct were selected on YEM plate containing rifampicin (25 mg/l) and kanamycin (50 mg/l) or chloramphenicol (34 μg/ml) and incubated at 28°C for 48 h. These transformed colonies were further confirmed by colony PCR using vector specific forward and reverse primers (Fig. 43B).
Results

Figure 43: PCR analysis to confirm transformation of pCAMBIA-OsSOS2 into Agrobacterium strain LBA4404. (A) Schematic representation of overexpressing OsSOS2 construct. (B) PCR was done for transformed colonies using gene specific forward (OsSOS2F) and vector specific reverse (pCAMBIAR) primers. Agrobacterium colony shows the positive amplification of OsSOS2 (1.4 kb). Arrow shows the position of OsSOS2 amplified product.

4.14 Transformation of rice using Agrobacterium strain LBA4404

Agrobacterium cells containing recombinant pCAMBIA1304-OsSOS2 and pFGC1008-RNAiSOS2 plasmids were then used to transform rice plants cv. ‘IR64’. The transformed calli were selected on selection medium (MS medium containing 300 mg/l casein hydrolysate, 3.0 mg/l BAP, 1.5 mg/l NAA, 4g/l phytogel and 50 mg/l hygromycin). Figure 44A-L shows various stages of regeneration and development of transgenic plants.
Figure 44: Steps involved in transformation and regeneration of *Oryza sativa* L. cv ‘IR64’ with OsSOS2 constructs (pCAMBIA1304-OsSOS2 and pFGC1008-RNAiOsSOS2). (A) Seeds of *Oryza sativa* cv ‘IR64’ (B) Dehusked seeds of ‘IR64’. (C) Callus formation on callus induction media. (D) Subcultured rice calli kept on selection and regeneration media for 15-17 days after co-infection and co-cultivation of rice calli with *Agrobacterium* strain LBA4404 containing OsSOS2 construct. (E) Regenerated plantlets on fresh regeneration medium. (F) Regenerated plants on rooting medium. (K) Completely regenerated plants in culture tubes for hardening. (L) Regenerated plantlets transferred into earthen pot to complete life cycle in green house.

4.14.1 Screening of putative transgenic rice plants overexpressing OsSOS2

First round Screening of (T₀-generation) rice plants by PCR
To verify the incorporation of OsSOS2 gene into genome of ‘IR64’ overexpressing lines, tissue PCR was done (see materials and methods) using gene specific forward and vector specific reverse primers.
Results

Figure 45: Screening of putative OsSOS2 overexpressing transgenic plants through PCR and Southern PCR analysis. (A) Putative transgenic plants transferred to ½ Yoshida for hardening under control situation. (B) PCR southern showing specific hybridization of the amplicon with OsSOS2 probe as shown by an arrow (C) Ethidium bromide gel showing screening of putative transgenic rice plants (Oryza sativa L cv 'IR64') transformed with OsSOS2. Positive transformants were selected using a combination of gene specific and vector specific primer for the presence of 1.4kb amplicon (marked by an arrow here). Genomic DNA from wild type plant was taken as negative control.

Among a total of 30 lines screened, 13 lines were found to be PCR positive as they showed desired amplification from the genomic DNA of the putative transgenic plants (Fig. 45B). The specificity of amplified band was further confirmed by PCR. Southern hybridization using the DNA fragment amplified from pCAMBIA1304OsSOS2 with same primer pairs as probe. PCR Southern hybridization revealed the presence of a specific band in each plant (Fig. 45C).
Figure 46: Visual analysis of morphological features of rice transgenic plants (Overexpressing and Underexpression) lines in comparison to the wild type plants. (A) Overexpressing OsSOS2 and RNAiOsSOS2 rice transgenic lines shifted to the greenhouse environment along with the wild type plants (WT) for collecting T1-generation seeds. (B) Assessment of chlorophyll retention in OXOsSOS2, RNAiOsSOS2 rice transgenic plants in comparison with WT rice plants in response to salinity (200 mM) stress through leaf disc assay. (C) Chlorophyll estimation from wild type and transgenic rice plants subjected to no stress and salinity stress (200 mM) revealed better tolerance of transgenic plants in terms of chlorophyll retention.

The positive plants after hardening were transferred to earthen pots and maintained in greenhouse under optimal growth conditions (Fig. 46A). After maturity, plants were analysed for length of panicles, number of grains per panicle, number of filled grains per panicle and size of grain in transgenic
OXOsSOS2, RNAiOsSOS2 and WT plants (Fig. 47). Overexpressing OXOsSOS2 plants showed much better performance as compared to the wild type or underexpression RNAiOsSOS2 lines, thus indicating that OsSOS2 overexpressing may be contributing towards the increased yield of IR64.

Figure 47: Assessment of performance of overexpressing and underexpression OsSOS2 lines in comparison with WT Oryza sativa cv 'IR64' under control conditions. (A) Comparison of panicles length. (B) Filled grain per panicle. (C) Size of grains. (D) Percentage yield of transgenic (OXOsSOS2 and RNAiOsSOS2) plants vs WT under control condition.
4.14.2 Expression analysis of OXOoSOS2 transgenics through GUS assay

PCR confirmed transformed plants were also screened by the expression of GUS gene present in the construct. GUS fusion activity was noted predominantly in the anther, vascular bundle of leaves, vascular bundle of stem, vascular bundle of leaf sheath, aleuronic layer of rice grain, lateral roots and primary root tips. Histochemical staining of different parts of overexpressing SOS2 revealed that, after incubation in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, GUS activity was detectable predominantly in both the primary and lateral roots more widely in the root tip, in the meristematic region of the root tip (Fig. 48).

![Figure 48: Histochemical GUS analysis of OsSOS2 in vivo in transgenic rice plants. (A) GUS staining showed expression pattern of OsSOS2 in vivo in various tissues (a. Anther, b. vascular bundle of leaf, c. vascular bundle of stem, d. vascular bundle of leaf sheath, e. rice grain, f. Root tip, g. lateral root from the T0-generation of OXOoSOS2 Rice transgenic (B) 6-d old seedling (T1) of OxOSOS2.](image)

4.15 Transgenic lines of rice (T1) showed tolerance towards salinity stress during seed germination

Seeds of wild type, overexpressing OsSOS2 and underexpression RNAiOsSOS2 lines were kept in petridish containing cotton saturated with ½ Yoshida supplemented with 150 mM NaCl. We found that the germination rate of seeds on 150 mM NaCl was relatively more in case of overexpressing OsSOS2 lines (L-15 and L-19) and less in underexpression RNAiOsSOS2 lines (RNAi-4 and RNAi-7) as comparison to the wild type seeds (Fig. 49A). As we measured some other growth parameters like fresh weight, root and shoot
Results

Figure 49: Germination and growth assay of $T_1$ seeds of rice plants overexpressing OsSOS2 (OXOsSOS2) and underexpressing (RNAiOsSOS2) in comparison to wild type rice seeds under salinity (150 mM NaCl) stress. Equal numbers of OXOsSOS2, RNAiOsSOS2 and WT seeds were inoculated on % Yoshida media supplemented with 150 mM NaCl. (A) OXOsSOS2 rice plants showed higher germination and growth rate as compared to RNAiOsSOS2 and wild-type (WT). (B) 6 d old seedlings of rice WT, OXOsSOS2 and RNAiOsSOS2. (C) Fresh weight of the seedlings. (D) Root length measurement of WT and transgenic plants. (E) Shoot length measurement. Error bars represent the standard deviation (n=10).
length of germinated seedlings after 6 days of growth, we found that the performance was better in overexpressing lines, but less in underexpression lines as compared to the wild type under salinity stress clearly indicating a correlation between overexpressing of OsSOS2 and stress tolerance in rice seeds and seedlings (Fig. 49C-D).

4.16 Southern blot analysis of T-DNA copy number

To confirm the stable integration of the T-DNA in the rice genome; Southern blot analysis of T2 lines obtained from two PCR positive L-15 and L-19 plants (T1-generation) was done. The genomic DNA from each of the transgenic lines and WT rice plants was digested with BamHI and HindIII (Fig. 50C). The digested DNA was fractionated on 0.8% agarose gel and transferred to the nitrocellulose membrane and probed with radiolabeled gfp α-P32-radiolabeled probe (See materials and methods). Only a single band was observed in transgenic lines indicating the presence of single copy of the T-DNA integration (Fig. 50D). However, no band was detected in the wild type. All lines show the band at different positions suggesting different position of the integration of transgene.
Results

Figure 50: Screening of T₁-generation rice OsSOS2 overexpressing transgenic plants through PCR and copy number Southern analysis. (A-B) Ethidium bromide gel showing screening of T₂ lines obtained from two T₁ lines (L-15 and L-19) (Oryza sativa L.cv 'IR64') transformed with OsSOS2. Positive transformants were selected using a combination of gene specific and vector specific primers for the presence of 1.4kb amplicon (marked by an arrow here). Genomic DNA from wild type plant was taken as negative control. (C) Ethidium bromide stained gel showing completely digested DNA from wild type and transgenic lines.15 μg genomic DNA from both wild type rice and transgenic rice lines (L-15 and L-19) Overexpressing OsSOS2 (OXOsSOS22) was digested with BamHI and HindIII and transferred onto the nylon membrane. (D) Southern blot analysis of OXOsSOS2 transgenic plants of Oryza sativa L.cv 'IR64' to detect copy number of the transgenic OXOsSOS2. Radiolabeled full length gfp gene was used as probe.
4.17 Detection of siRNA in underexpression transgenic rice plants

It is anticipated that underexpression lines (RNAi) of OsSOS2 should generate detectable siRNA accumulated from the OsSOS2 transcripts. To identify siRNA in underexpression plant, total RNA was isolated from leaves and small RNA was precipitated using PEG/NaCl. Precipitated small RNA was resolved on 15% native PAGE and transmitted onto the nylon membrane. Hybridization was carried out using the OsSOS2 fragment used for making RNAi construct as probe. Appearance of a specific small size band in RNAi lines, which was absent in wild type and overexpressing lines (Fig. 51).

siRNA could be detected only in RNAi plants (shown by an arrow in Fig 51B), while full length transcript for OsSOS2 could be detected in overexpressing lines. These results clearly confirmed the underexpressing of OsSOS2 transcript due to production of siRNA specific to OsSOS2 transcript in OsRNAi transgenic rice lines.

![Figure 51: Detection of siRNA in RNAiOsSOS2 transgenic plants. (A) 50 μg of total RNA of WT, three OXSOS2 lines (L-4, L-15, L-19) and three RNAiOsSOS2 lines (R-4, R-7, R-9) was loaded into each well of denaturing PAGE. Hybridized with OsSOS2 probe.](image-url)
4.18 Stress tolerance test of overexpressing (OXOsSOS2) and underexpressing (RNAiOsSOS2) of rice plants at reproductive stage

All the transgenic plants overexpressing and underexpressing OsSOS2 matured normally under normal growth conditions. The seed setting of these transgenic plants was similar to that of the untransformed WT plants. The WT and transgenic rice plants were grown for 40d under control conditions followed by 1st salinization of 10 EC for 10d to the plants. The 2nd and 3rd salinization of 12 EC for 20-25d were allowed after short recovery for 5-7d (Fig. 52). It was observed that under stress conditions, numerous physiological and biochemical changes take place like low tillering, spikelet sterility, less florets per panicle, low grain weight. Some important symptoms were also observed like white leaf tip followed by tip burning, stunted plant growth, poor root growth etc. Ions and plant nutrients were examined after complete life cycle of rice plants under stress condition.

4.18.1 Leaf disc assay for chlorophyll retention of the T1-generation of transgenic rice plants

To study whether overexpressing of OsSOS2 in Oryza sativa cv 'IR64' can alter in their sensitivity towards salinity, leaf disc chlorophyll retention assay was performed in (T1) generation. We selected completely stretched vigorously growing leaves of WT rice plants and transgenic lines for leaf disc assay. Leaf
Figure 52: Stress tolerance test of transgenic plants during salinity stress at reproductive stage. Two independently confirmed transgenic T1 lines of OXOsSOS2, RNAiOsSOS2 plants and WT controls were grown in a greenhouse for 40 days and subjected to 1st salinization of 10 EC for 10d stress treatments, followed by 5d of recovery in the greenhouse. The 2nd and 3rd salinization of 12 EC for 20-25d was allowed after short recovery for 5-7d. Photographs were taken at the indicated time points. Note the typical yellowing of leaves in the RNAiOsSOS2 and WT plants while OXOsSOS2 transgenic plants continued green.

Discs of 1-1.5 cm were cut from each line and were kept in ½ Yoshida media supplemented with NaCl (200 mM) or half strength Yoshida media (control). Significant differences in the “greenness” of the leaf discs were observed among RNAiOsSOS2, WT and OXOsSOS2 transgenic plants treated NaCl within 72 h. OXOsSOS2 lines showed significant tolerance towards 200 mM NaCl (Fig. 53A). Chlorophyll estimation was performed for both wild type and transgenic rice plants under no stress and salinity stress 200 mM conditions. It was observed that OXOsSOS2 lines retained more chlorophyll then WT and RNAiOsSOS2 lines under control and salinity stress conditions (Fig. 53B).
Figure 53: Leaf disc assay for WT, OXOsSOS2 rice lines and RNAiOsSOS2 lines. 
(A) Leaf disc assay of SOS2 transgenic rice plants showing tolerance towards salinity stress. Leaf disc of WT (wild type 'IR64'), OXOsSOS2 (L-15 and L-19), RNAiOsSOS2 (L-4 and L-7), floated on ½ Yoshida (control) or ½ Yoshida containing 200 mM NaCl (salinity stress). Photograph was taken after 5d of stress treatment. (B) Chlorophyll estimation for SOS2 transgenic rice plants. Chlorophyll measurements were done after 5d of stress from WT (wild type IR64), RNAiOsSOS2 (L-4 and L-7), OXOsSOS2 (L-15 and L-19), under control (½ Yoshida) or salinity stress (½ Yoshida containing 200 mM NaCl).

4.18.2 Estimation of proline in T1-generation of OXOsSOS2 and RNAiOsSOS2 transgenic rice

To further confirm the accumulation of proline in transgenic plants under salinity conditions, proline estimation was done using ninhydrin method (Bates et al., 1970). We observed elevated amount of proline in shoots and roots of all three Overexpressing (OXOsSOS2) lines (L-8, L-15, L-19) as compared to the WT and Underexpression (RNAiOsSOS2) plants (Fig. 54).
Figure 54: Evaluation of proline in Transgenic Overexpressing (OXOsSOS2) and Underexpression (RNAiOsSOS2) as compared to the WT under salinity at reproductive stage. (A) 45 d old rice WT, Overexpressing and Underexpressing plants. (B) Endogenous proline accumulation in Root and Shoot of OXOsSOS2 transgenic lines (L-8, L-15, L-19), RNAiOsSOS2 lines (4, 6 and 7) as compared to the WT rice 'IR64'.