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# SEQUENCE ANALYSIS OF A PARTIAL *HbSERK* GENE EXPRESSED DURING SOMATIC EMBRYO INITIATION IN *HEVEA BRASILIENSIS*

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Somatic Embryogenesis Receptor Kinase (*SERK*) genes are known to express during the transition of somatic cells to embryogenic cells, which have the capability to differentiate into somatic embryos at a later stage of development. In the past 20 years, *SERK* have drawn much research interest due to its crucial role in somatic embryogenesis. The aim of the current study was to isolate and characterize *HbSERK* gene through cDNA cloning and to perform comparative genomic analysis. The partial cDNA sequence of *HbSERK* was 408 bp in length and comparison with genomic sequence revealed the presence of two exons separated by one intron. *In silico* analysis of *HbSERK* predicted a protein of 136 amino acids that revealed high similarity (99%) to amino acid of *AtSERK1*. Multiple sequence alignment showed that *HbSERK* possessed partial sequences from the characteristic intracellular kinase domain containing the subdomains from V-X of the *SERK* family. Molecular phylogeny of *HbSERK* with *SERK* from other plant species showed that *HbSERK* was clustered most closely with *AtSERK1* and *MtSERK1*.

**Keywords:** *Hevea brasiliensis*, Intracellular kinase domain, *SERK* gene, Somatic embryogenesis

## INTRODUCTION

Somatic embryogenesis pathway is considered as the best choice for a wide range of *in vitro* approaches including mass propagation and genetic engineering in many plant species. In this pathway, a large number of genes specific to each stage of development have been found to express differentially. Under favourable *in vitro* conditions, the somatic callus acquires embryogenic competence and has been reported to proliferate as individual embryogenic cells (Dodeman *et al.*, 1997; Namasivayam, 2007; Gulzar *et al.*, 2020). *SERK* is not only an activator of the transitional state of plant somatic cells, but also a potential marker characterizing single embryogenic cells in suspension cultures of carrot (Schmidt *et al.*, 1997). The *SERK* gene belongs to a large plant receptor-like kinases (RLKs) family which consists of an extracellular domain having a minimum of five Leu-rich repeats (LRRs), a proline rich motif, a transmembrane domain and an intracellular kinase domain (Ma *et al.*, 2012). The role of *SERK* genes in mediating somatic embryo induction was demonstrated in carrot (*DcSERK*), for the first time (Schmidt *et al.*, 1997). Thereafter, a functional ortholog

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of *DcSERK* was isolated from embryogenic callus of *Arabidopsis thaliana* (*AtSERK1*) (Hecht *et al.*, 2001). Since then, many *SERK* homologs have been identified in both monocot and dicot species and their role in controlling somatic embryogenesis has been linked. A gymnosperm *SERK* was also characterized from *Araucaria angustifolia* showing significant similarity to angiosperm homologs and was found to be closely associated with somatic embryogenesis induction (Steiner *et al.*, 2012).

The rubber tree (*Hevea brasiliensis*) is one among the major commercial tree crops for natural rubber production. With the increase in global demand for natural rubber, development of improved clones with high latex yield and tolerance to environmental stresses has become the prime aim of breeding programmes. Greater efforts have been made to developing such clones. However, conventional crop improvement is limited mainly due to the highly heterozygous nature and long breeding cycle of the crop. Genetic improvement of the crop *via* transgenic technology could thus provide an alternate approach and somatic embryogenesis is the most suitable *in vitro* system for such genetic manipulation studies. Intensive research has been carried out for somatic embryogenesis for different clones in *H. brasiliensis* (Jayasree, 2017a). However, it was found that not all clones are amenable to somatic embryogenesis. Moreover, to date, only a few clones are amenable to genetic transformation. The effective utilization of somatic system in transgenic plant development needs better understanding of the molecular mechanism involved in this process. Several studies reported that *SERK* is a potential marker of embryogenic cells, an ideal target tissue for genetic transformation. A full length cDNA *SERK* gene has previously been reported from rubber (Wang *et al.*, 2011). Recently, a partial gene fragment of 612 bp was isolated from genomic DNA of *Hevea* and the role of *SERK* in *H. brasiliensis* somatic embryogenesis has been proved (Jayasree *et al.*, 2016; 2017b). In the current study, we have focused on the molecular characterization of *SERK* gene through cDNA cloning and *in silico* sequence analysis.

## MATERIALS AND METHODS

### Establishment of embryogenic callus cultures

Embryogenic cell cultures from immature anther explants of *H. brasiliensis* clone RRII 105 were initiated as described earlier (Jayasree *et al.*, 1999). Immature anthers were sterilized, dissected out and inoculated on callus induction medium containing the auxin 2, 4-D (2 mg $L^{-1}$) and cytokinin kinetin (0.5 mg $L^{-1}$). Induced calli were subcultured on embryo induction medium and cultures were maintained in this medium for four months for embryogenic callus formation.

### Extraction of RNA and synthesis of cDNA

Total RNA was isolated from embryogenic callus (Venkatachalam *et al.*, 1999) and quantified using spectrophotometer. Integrity and quality of the total RNA was assessed by running an aliquot of the RNA sample in 1.0 per cent agarose gel stained with ethidium bromide. First strand cDNA was prepared using Improm-II™Reverse Transcription system (M/s. Promega, USA) with oligo-(dT) primer.

### Molecular cloning and characterization of *HbSERK*

PCR reactions were performed with primer combination, F (forward 5'-GCT TGT ATA TCC CTA TAT GGC-3'and R (reverse 5'- CGA GCA AGA TCA AAA GCC C-3') which were designed based on consensus

sequences reported in other plant species to amplify a fragment of 612 bp of *HbSERK* gene from genomic DNA (Jayasree *et al.*, 2016). PCR reactions consisted of an initial denaturation at 94°C for 3 min. followed by 32 cycles with denaturation for 1 min. at 94°C, annealing for 1 min. at 55°C, extension for 1 min. at 72°C and a final extension for 10 min. at 72°C (Perkin Elmer Thermal Cycler 480). Each reaction was repeated thrice. PCR amplicon was analyzed on 1.5 per cent agarose gel and viewed using a gel documentation system (Kodak EDAS 290). The amplified PCR fragment was purified and cloned into pGEM-T® Easy vector system (M/s. Promega, USA). After cloning, recombinant vector was transformed into *E. coli* competent cells. Positive clones were identified and plasmid DNA was isolated using Illustra Plasmid Prep Mini Spin Kit (M/s. GE Healthcare). Restriction digestion was performed with *Eco*RI restriction enzyme to confirm the presence of insert in recombinant plasmid. Amplified cDNA fragment was purified and subjected to DNA sequencing. Using the BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/), *HbSERK* nucleotide sequences were compared with sequences available in Genbank database. The retrieved sequences from BLAST analysis corresponding to domain structure and conserved motifs of known *SERK* genes were aligned using Clustal Omega multiple sequence alignment program to generate alignments between the sequences. According to the method of Saitou and Nei (1987), relatedness of *HbSERK* gene with other *SERK* gene sequences was compared using MEGA 4.0 software, an integrated tool for conducting sequence alignment to infer phylogenetic trees (Tamura *et al.*, 2011). The evolutionary distance was inferred from the branch length within similar units of tree drawn to scale. The Poisson correction method was used to

Table 1. **Details of amino acid sequences included in phylogenetic analysis**

| Species | *SERK* proteins | Accession number |
|---|---|---|
| *Hevea brasiliensis* | *HbSERK* | AHY02134.1 |
| *Medicago truncatula* | *MtSERK1* | AAN64293.1 |
| *Medicago truncatula* | *MtSERK2* | ADO15291.1 |
| *Arabidopsis thaliana* | *AtSERK1* | NP_177328.1 |
| *Arabidopsis thaliana* | *AtSERK2* | NP_174683.1 |
| *Arabidopsis thaliana* | *AtSERK3* | AAK68074.1 |
| *Arabidopsis thaliana* | *AtSERK4* | NP_178999.2 |
| *Arabidopsis thaliana* | *AtSERK5* | NP_179000.3 |
| *Araucaria angustifolia* | *AaSERK1* | ACY91853.1 |
| *Pinus massoniana* | *PmSERK* | ACZ56417.1 |
| *Cocos nucifera* | *CnSERK* | AAV58833.2 |
| *Ananas comosus sus* | *AcSERK2* | AEC46976.1 |
| *Oryza sativa* | *OsB1SERK* | AAR26543.1 |
| *Zea mays* | *ZmSERK1* | NP_001105132.1 |
| *Zea mays* | *ZmSERK2* | NP_001105133.1 |
| *Saccharum hybrid* | *SoSERK1* | ACT22809.1 |

compute the evolutionary distance in which the units represented the number of amino acid substitution per site (Zuckerkandl and Pauling, 1965). The final data sheet had a total 136 positions after elimination of positions containing gaps and missing data. The analysis included 16 amino acid sequences (Table 1).

## RESULTS AND DISCUSSION

In the current study, during the culture of immature anther, explant swelling followed by callus induction was observed within 30-40 days. The induced callus then proliferated into profuse masses and numerous callus lines were produced. Upon further culturing in an embryo induction medium, pale yellow callus became brownish in colour and subsequently through frequent subculturing, embryogenic cells were produced. The calli were yellowish in colour and the texture was highly friable and mucilaginous in nature. Good quality RNA was extracted from these embryogenic calli and used for *HbSERK* gene isolation and cloning.

A cDNA fragment with 0.4 kb size was amplified by RT PCR (Fig. 1) using gene specific primers synthesized from the highly conserved regions of *SERK* gene sequences

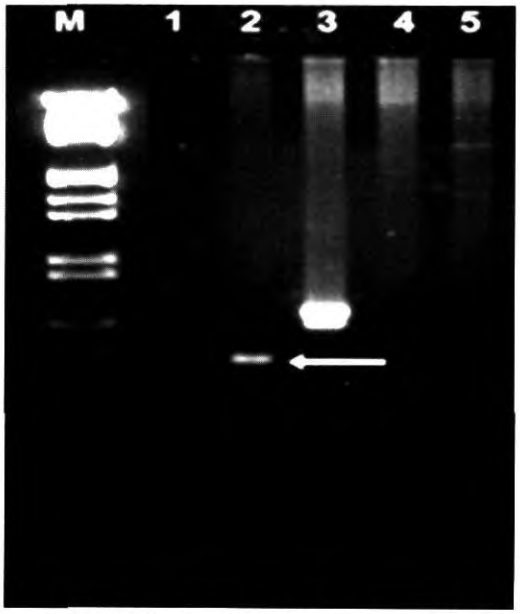


Fig. 1. Amplification of *SERK* gene from cDNA of embryogenic callus
Lanes M-Marker, 21kb (λ DNA *Eco RI/Hind III* double digest)
2- cDNA from embryogenic callus, 0.4kb
3- Genomic DNA
4- cDNA with Forward primer alone
5- cDNA with Reverse primer alone

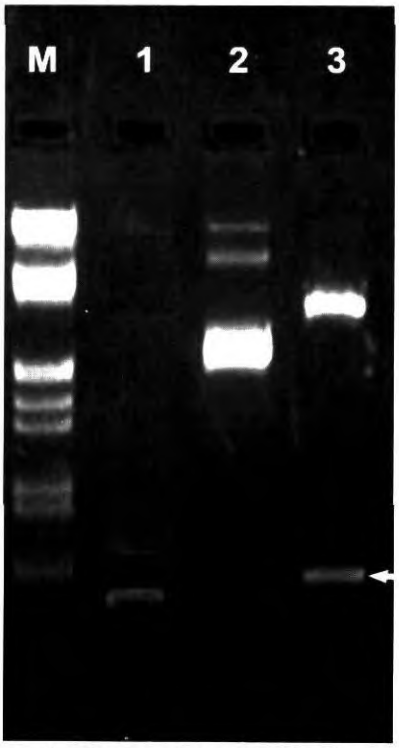


Fig. 2. Cloning of amplified *SERK*gene
Lanes M- Marker, 21kb (*Eco RI/ Hind III* double digest λ DNA marker)
1- Purified PCR product, 0.4kb
2- Plasmid with insert
3- Insert ( *SERK* gene released)

of other reported species. Following cloning, plasmid isolation was done and double digestion released the insert fragment of *SERK* gene (Fig. 2). Sequencing of the partial cDNA clone of *HbSERK* revealed presence of 408 bp region corresponding to the ORF region of *SERK* gene. Sequence of the isolated cDNA was compared with genomic sequence (Jayasree *et al.*, 2016) which showed the presence of two exons separated by one 204 bp large intron at position of 51 -nucleotide (Fig. 3). The *in silico* analysis of *HbSERK* gene predicted a protein of 136 amino acids (Fig. 4). Sequence comparison revealed similarity with *SERK* genes of earlier reported species such as *Arabidopsis thaliana, Medicago truncatula, Cocos nucifera* and *Araucaria angustofolia*. Amongst these comparisons, nucleotide sequence of *HbSERK* was 78 per cent identical to that of *AtSERK1* (Fig. 5) and at amino acid level *HbSERK* showed 99 per cent homology to *AtSERK1* (NP_177328.1). This homology between *HbSERK* and *AtSERK1* at amino acid level indicated that these two genes could be orthologs. BLASTn analysis of the *HbSERK* sequence showed 100 per cent identity to earlier reported *HbSERK* at the amino acid level (Wang *et al.*, 2011).

Multiple sequence alignment of *HbSERK* protein displayed the presence of partial sequences from intracellular protein kinase. This region containing V-X subdomains characteristic of the catalytic core of Ser/Thr protein kinases, had a high level of protein domain homology (Fig. 6a-c) with other *SERK* sequences. Figure 6a compares deduced amino acid sequences of *HbSERK* with amino acids of *AtSERK*1 between 374 to 509. In the kinase domain of *HbSERK*, the A-loop (activation loop) with 29 amino acid residue was located in VII and VIII subdomains, which is considered as the active site of *AtSERK*1 (Shah *et al.*, 2001a;

GCTTGTATATCCCTATATGGCAAATGGAAGTGTTGCATCATGTTTAAGAG↓AGCGCCCGCCATCTCAACCTCCTC
TTGATTGGCCAACACGGAAGCGAATTGCACTGGGATCTGCTAGGGGTCTATCTTATTTGCATGATCATTGTGA
CCCAAAGATTATTCATCGTGATGTTAAAGCTGCAAATATTTTATTGGATGAGGATTTTGAGGCTGTTGTTGGG
GATTTTGGGTTGGCTAAGTTGATGGACTACAAGGATACTCATGTCACTACTGCCGTCCGTGGTACAATAGGGC
ATATAGCTCCAGAGTACCTCTCTACTGGAAAATCATCGGAAAAAACTGATGTTTTTGGGTATGGGATCATGCTT
CTGGAGCTAATTACTGGACAGAGGGCTTTTGATCTTGCTCG

Fig. 3. Partial cDNA sequence of *HbSERK*, 408 bp (→ indicates position of intron)

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        10         20         30         40         50
LVYPYMANGS VASCLRERPP SQPPLDWPTR KRIALGSARG LSYLHDHCDP
        60         70         80         90        100
KIIHRDVKAA NILLDEDFEA VVGDFGLAKL MDYKDTHVTT AVRGTIGHIA
       110        120        130
PEYLSTGKSS EKTDVFGYGI MLLELITGQR AFDLAR
```

Fig. 4. Amino acid sequence of partial *HbSERK* gene

2001b). This exhibited 100 per cent identity to *AtSERK1* sequence at positions 447-475 (Fig. 6b). As in *AtSERK1*, in *HbSERK* also, the activation loop in subdomain VII started with amino acid-Aspartic acid (D), and ended with amino acid- glutamic acid (E) of the subdomain VIII. Similarly, in comparison with *AtSERK1*, A-loop of *HbSERK* also contained 4 threonine residues, Thr-459, Thr-462, Thr-463 and Thr-468 and the tyrosine, Try-456. This A-loop portion of the kinase domains of *SERK* gene played critical roles in both autophosphorylation and phosphorylation of artificial substrates (Shah

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AtSERK1     -CTTGTGTATCCTTACATGGCCAATGGAAGTGTTGCTTCGTGTCTCAGAGAGAGGCCACC 59
HbSERK      GCTTGTATATCCCTATATGGCAAATGGAAGTGTTGCATCATGTTTAAGAGGTAAGCCGCC 60
             ***** ***** ** ***** ************** ** *** * ****  * *** **

AtSERK1     GTCACAACCTCCGCTTGATTGGCCAACGCGGAAGAGAATCGCGCTAGGCTCAGCTCGAGG 119
HbSERK      ATCTCAACCTCCTCTTGATTGGCCAACACGGAAGCGAATTGCACTGGGATCTGCTAGGGG 120
             ** ******** ************** ****** **** ** ** ** ** *** * **

AtSERK1     TTTGTCTTACCTACATGATCACTGCGATCCGAAGATCATTCACCGTGACGTAAAAGCAGC 179
HbSERK      TCTATCTTATTTGCATGATCATTGTGACCCAAAGATTATTCATCGTGATGTTAAAGCTGC 180
             * * *****  * ******** ** ** ** ***** ***** ***** ** ***** **

AtSERK1     AAACATCCTCTTAGACGAAGAATTCGAAGCGGTTGTTGGAGATTTCGGGTTGGCAAAGCT 239
HbSERK      AAATATTTTATTGGATGAGGATTTTGAGGCTGTTGTTGGGGATTTTGGGTTGGCTAAGTT 240
             *** **  * ** ** ** ** ** ** ** ** ******** ***** ******** *** *

AtSERK1     AATGGACTATAAAGACACTCACGTGACAACAGCAGTCCGTGGCACCATCGGTCACATCGC 299
HbSERK      GATGGACTACAAGGATACTCATGTCACTACTGCCGTCCGTGGTACAATAGGGCATATAGC 300
             ******* ** ** ***** ** ** ** ** ******** ** ** ** ** ** **

AtSERK1     TCCAGAATATCTCTCAACCGGAAAATCTTCAGAGAAAACCGACGTTTTCGGATACGGAAT 359
HbSERK      TCCAGAGTACCTCTCTACTGGAAAATCATCGGAAAAAACTGATGTTTTTGGGTATGGGAT 360
             ****** ** ***** ** ******** ** ** ***** ** ***** ** ** ** **

AtSERK1     CATGCTTCTAGAACTAATCACAGGACAAAGAGCTTTCGATCTCGCTCGG 408
HbSERK      CATGCTTCTGGAGCTAATTACTGGACAGAGGGCTTTTGATCTTGCTCG- 408
             ********* ** ***** ** ***** ** ***** ***** *****
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Fig. 5. Clustal W alignment of *HbSERK* with *AtSERK1* (nucleotide level)

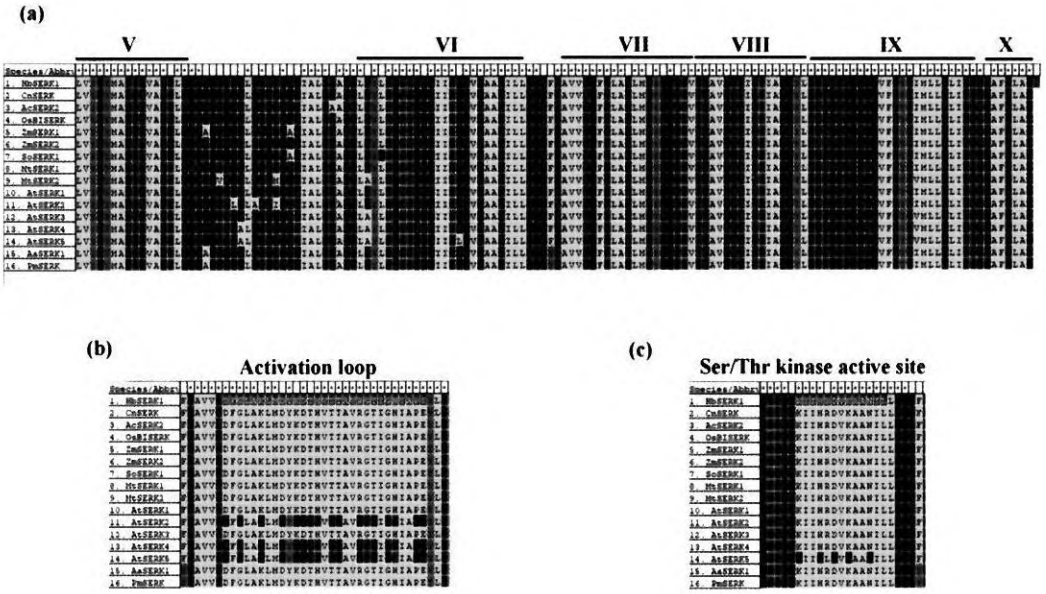


Fig. 6a-c. Multiple sequence alignment of partial amino acid sequence of *HbSERK* with other reported *SERK* sequences. (a) Kinase domain with subdomains V – X ; (b) activation-loop at amino acid positions 447-475 of *ATSERK1* ; (c) Ser/Thr kinase active–site at amino acid positions 424-436 of *ATSERK1*

*et al.*, 2001a). Additionally, *HbSERK* also contained another important site, the active site signature of Ser/Thr protein kinase, in subdomain VI similar to *AtSERK*1 at positions 424-436 of *AtSERK*1 with 100 per cent homology, which is indication of its function as a serine/threonine kinase (Fig. 6c). As in all plant *SERK* families, the kinase domains of *SERK* proteins are highly conserved in *HbSERK* protein indicating that *HbSERK* is an ortholog of *SERK* and could exhibit a positive relationship with somatic embryogenesis. These results had corroborated our previous findings that *HbSERK* is closely associated not only with the transition of somatic cells but also with the induction of somatic embryogenesis in *H. brasiliensis* (Jayasree *et al.*, 2016; 2017b).

The unrooted phylogenetic tree constructed with partial amino acid sequences from other reported *SERK* genes revealed the relationship between other *SERK* proteins and the analysis clearly indicated that *HbSERK* was tightly clustered with *AtSERK1* and *MtSERK1* (Fig. 7). An optimal phylogenetic tree with a sum branch length of 0.359314282 is depicted here and the percentage value given next to the branches represents the cluster of associated taxa in the bootstrap test (Felsenstein, 1985) conducted with 100 replicates.

## CONCLUSION

A partial *HbSERK* gene of 408 bp length was amplified from embryogenic callus and corresponded to a protein of 136 amino acids. Comparison with its genomic DNA

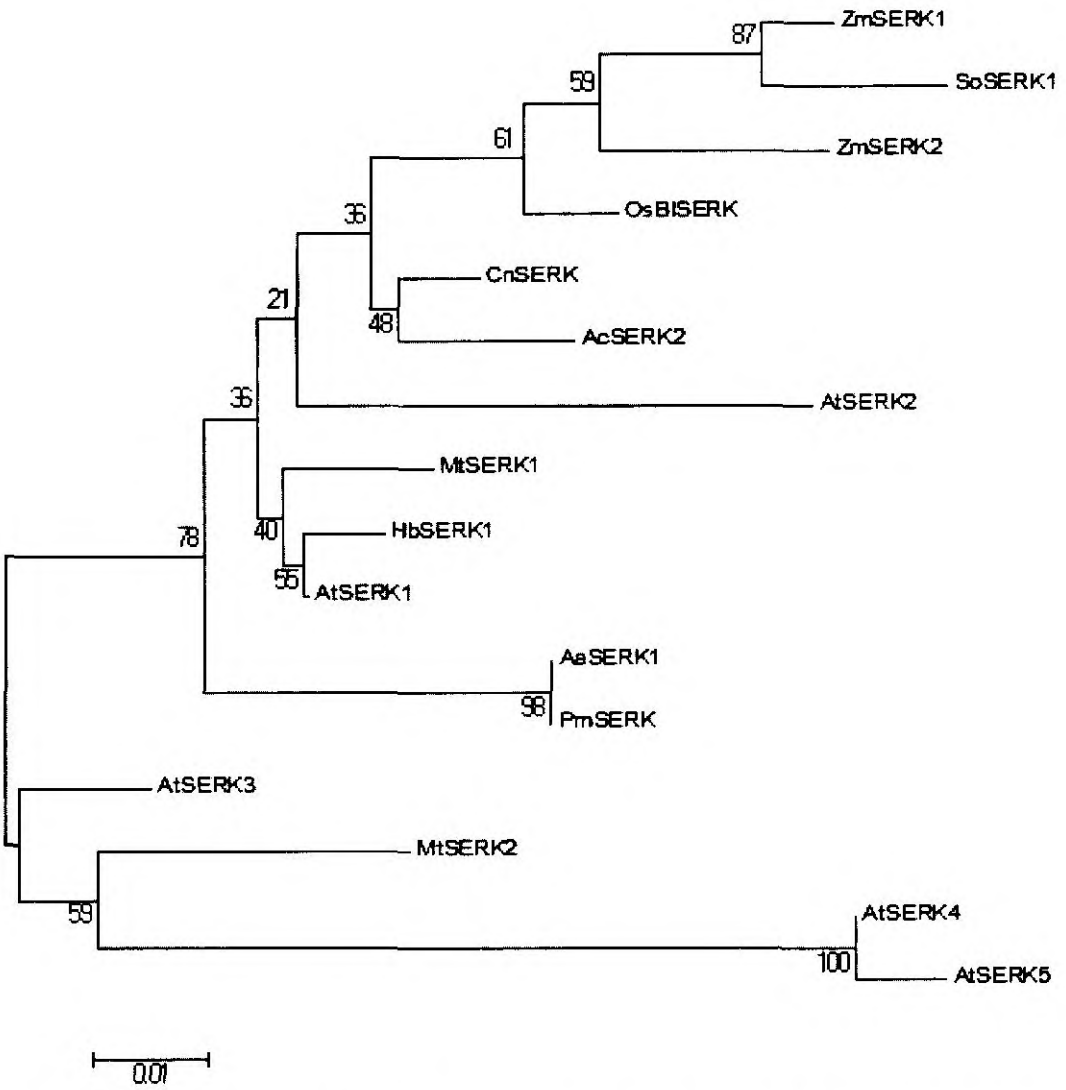


Fig. 7. Phylogenetic tree constructed based on the alignment of partial amino acid sequence of *HbSERK* with the other reported *SERK* sequences

sequence showed two exons and an intronic region of 204 bp. Clustal W alignment of the isolated cDNA sequence with *AtSERK1* revealed 78 per cent similarity at nucleotide level and 99 per cent at the amino acid level. *HbSERK* protein displayed presence of intracellular protein kinase domain. Phylogenetic relationships of *HbSERK* protein with other *SERK* proteins revealed high sequence identity and maximum

similarity with *AtSERK1* and *MtSERK1*. This indicates a strong evidence that the partial *HbSERK* gene encodes a *SERK* protein with characteristic intracellular kinase domain of *SERK* gene of other reported plant species. This could play a central role in somatic embryogenesis, which would help to establish a highly effective transformation system in *H. brasiliensis*.

## REFERENCES


Dodeman, V.L., Ducreux, G. and Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany*, **48**(313): 1493-1509.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**(4): 783-791.

Gulzar, B., Mujib, A., Malik, M.Q., Sayeed, R., Mamgain, J. and Ejaz, B. (2020). Genes, proteins and other networks regulating somatic embryogenesis in plants. *Journal of Genetic Engineering and Biotechnology*, **18**: 31.

Hecht, V., Vielle-Calzada, J.P., Hartog, M.V., Schmidt, E.D., Boutilier, K., Grossniklaus, U. and de Vries, S.C. (2001). The *Arabidopsis* somatic embryogenesis receptor kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology*, **127**: 803-816.

Jayasree, P.K., Asokan, M.P., Sobha, S., Sankariammal, L., Rekha, K., Kala, R.G., Jayashree, R. and Thulaseedharan, A. (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell. Arg.). *Current Science*, **76**(9): 1242- 1245.

Jayasree, P.K., Jose, S.M., Supriya, R. and Mathew, S.A. (2016). Molecular characterization and expression analysis of a somatic embryogenesis receptor kinase (*SERK*) gene from *Hevea brasiliensis*. *Rubber Science*, **29**(2): 159-167.

Jayasree, P.K.(2017a). Rubber (Regeneration Systems) In: *Biotechnology of Plantation Crops* (Eds. P. Chowdappa, Anitha Karun, M.K. Rajesh and S.V. Ramesh). Daya Publishing House, Astral International Pvt. Ltd, New Delhi, pp. 3-29.

Jayasree, P.K., Saseendran, S. and Mathew, S.A. (2017b). Embryogenic competence and *HbSERK* gene expression during somatic embryogenesis. *Rubber Science*, **30**(1): 76-81.

Ma, J., He, Y., Wu, C., Liu, H., Hu, Z. and Sun. G. (2012). Cloning and molecular characterization of a *SERK* gene transcriptionally induced during somatic embryogenesis in *Ananas comosus* cv. Shenwan. *Plant Molecular Biology Reporter*, **30**: 195-203.

Namasivayam, P. (2007). Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell Tissue and Organ Culture*, **90**(1): 1-8.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*, **4**: 406-425.

Schmidt, E.D.L., Guzzo, F., Toonen, M.A.J. and deVries, S.C. (1997). A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development*, **124**(10): 2049-2062.

Shah, K., Gadella, T.W.J., Van Erp, H., Hecht, V. and de Vries, S.C. (2001a). Subcellular localization and oligomerization of the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 protein. *Journal of Molecular Biology*, **309**(3): 641-655.

Shah, K., Vrevoort, J. and de Vries, S.C. (2001b). Role of threonines in the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 activation loop in phosphorylation. *Journal of Biological Chemistry*, **276**(44): 41263-41269.

Steiner, N., Santa- Catarina, C., Guerra, M.P., Cutri L., Dornelas, M.C. and Floh, E.I.S. (2012). A gymnosperm homolog of somatic embryogenesis receptor-like kinase1 (*SERK1*) is expressed during somatic embryogenesis. *Plant Cell Tissue and Organ Culture*, **109**(1): 41-50.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution*, **28**(10): 2731-2739.

Venkatachalam, P., Thanseem, I. and Thulaseedharan, A. (1999). A rapid and efficient method for isolation of RNA from bark tissues of *Hevea brasiliensis*. *Current Science*, **77**(5): 635-637.

Wang, Y., Chen, J., Li, H., Guo, D. and Peng, S. (2011). Cloning and expression of *HbSERK*1 from *Hevea brasiliensis*. *Chinese Journal of Tropical Science*.

Zuckerkandl, E. and Pauling, L. (1965). Evolutionary divergence and convergence in proteins. In: *Evolving Genes and Proteins*. (Eds. V. Bryson and H.J. Vogel). Academic Press, New York, pp. 97-166.