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Isolation and Expression Analysis of a *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) Gene in *Curcuma alismatifolia* Gagnep.

Kannaporn Sucharitakul[1], Rungroj Rakmit[2], Yada Boonsorn[2], Oranuch Leelapon[2], Tharathorn Teerakathiti[2], Sumontip Bunnag[1] & Yindee Chanvivattana[2]

[1] Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

[2] National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand

Correspondence: Yindee Chanvivattana, National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Pathum Thani, 12120, Thailand. Tel: 66(0)-2564-6700. E-mail: yindee@biotec.or.th

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Abstract

Somatic embryogenesis provides a useful tool to facilitate efficient mass propagation in plants. The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) gene serves a fundamentally important role in somatic embryogenesis of many plant species. The isolation of a *SERK* gene homolog, namely *CaSERK*, from *Curcuma alismatifolia* Gagnep. cv. Blue Tung, was reported. Prediction of coding sequence showed that it encoded a protein of 628 amino acids showing high similarity to previously characterized SERK sequences and containing all the features shared by members of the SERK family, including five leucine-rich repeats and the distinctive proline-rich SPP domain. Investigation of *CaSERK* expression revealed that its transcripts were found throughout the whole somatic embryogenesis process with highest abundance in embryogenic callus. These results indicate that *CaSERK* might have somatic embryogenesis-associated functions in this economically important ornamental ginger. Detection of *CaSERK* transcript accumulation in flower and coma bract tissues is suggestive of its additional roles in other developmental signaling pathways.

Keywords: *Curcuma alismatifolia* Gagnep., *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), somatic embryogenesis, gene expression

1. Introduction

Members of the *Curcuma* genus in the monocotyledonous Zingiberaceae family have long been extensively cultivated for culinary and medicinal uses. Recently, however, *Curcuma* plants have been gaining attention for their ornamental values due to their conspicuous inflorescences. *Curcuma alismatifolia* Gagnep., known as Siam tulip, summer tulip or Patumma, is a native of Indochina with widespread occurrence throughout Thailand. It bears beautiful inflorescence containing large and colorful coma bracts, making it highly popular in the floriculture market. Due to its economic importance, *C. alismatifolia* has become a prime ornamental crop of Thailand and a breeding target for novel and improved characteristics (Prathepha, 2000).

C. alismatifolia is mainly vegetatively propagated using its underground rhizomes. However, the propagation rate is considerably slow, which makes the production difficult to keep up with increasing market demands. Moreover, rhizome production is hampered by serious damages caused by wilt disease (Elphinstone, 2005). In this regard, advances in plant biotechnology could provide alternative strategies for an efficient propagation of *C. alismatifolia*. In recent years, somatic embryogenesis has become a promising tool for mass propagation in a wide range of plant species. Establishment of *in vitro* propagation and regeneration via somatic embryogenesis also provides a platform for genetic engineering of desired traits. The widely recognized single-cell origin of the transformants generated by somatic embryogenesis route is considered advantageous over the traditional regeneration by organogenesis since chimerism is unlikely to occur (Ghosh et al., 2009). However, monocotyledonous plants are generally known to be recalcitrant towards somatic embryogenesis. In the genus *Curcuma*, there are very few reports to date on protocols for efficient and reliable regeneration system via somatic embryogenesis (Raju et al., 2013; He & Gang, 2014). Thorough understanding of the genetic mechanisms underlying various aspects of somatic embryogenesis could help overcome the barriers to the

process and thus lead to successful implementation of the technology in *in vitro* propagation of *Curcuma* species, including *C. alismatifolia*.

The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) genes have garnered a lot of research attention due to their taken out pivotal role during somatic embryogenesis in plants. They encode members of the leucine-rich repeat receptor-like kinases (LRR-RLKs), a sub-group of plant RLKs, which are known to be integral to the signal transduction machinery in diverse developmental processes (Cock et al., 2002). The involvement of SERK proteins in regulating somatic embryogenesis was first described for DcSERK in embryogenic cell suspension cultures of carrot more than 15 years ago (Schmidt et al., 1997). Since then *SERK* gene homologs have been identified and their role in mediating somatic embryogenesis demonstrated in several monocot and dicot species such as *Arabidopsis thaliana* (Hecht et al., 2001), *Medicago truncatula* (Nolan et al., 2003), *Triticum aestivum* (Singla et al., 2008), *Vitis vinifera* (Maillot et al., 2009), *Cocos nucifera* (Pérez-Núñez et al., 2009), *Ananas comosus* (Ma et al., 2012) and *Cyrtochilum loxense* (Cueva et al., 2012). Moreover, a gymnosperm *SERK* homolog has recently been discovered and its association with somatic embryogenesis was shown (Steiner et al., 2012). This finding and the high similarity found among the SERK members strongly suggest that the function of SERKs in embryogenic processes is likely conserved during the evolution of seed plants.

This study attempted to isolate a *C. alismatifolia SERK* sequence and analyze its predicted protein structure and expression patterns during *in vitro* somatic embryogenesis and in somatic tissues. The data obtained provides the first information on the molecular control of somatic embryogenesis in the genus *Curcuma*.

2. Materials and Methods

2.1 Plant Material & In vitro Cultures

Curcuma alismatifolia cv. Blue Tung plants grown in the greenhouse from commercially available rhizomes were used as material in this study. *In vitro* cultures were established using immature inflorescences as explants. The inflorescences were sterilized with 15% Clorox® and transferred onto MS medium (Murashige & Skoog, 1962) supplemented with 1 mg/L TDZ and 30 g/L sucrose for 3 months to generate *in vitro* plantlets. For shoot multiplication, plantlets were transferred and then subcultured onto MS medium supplemented with 1 mg/L kinetin and 30 g/L sucrose.

2.2 Establishment of Somatic Embryogenesis

For embryogenic callus induction, leaf sheath explants (2.0 cm long) from 3-month-old *in vitro* plantlets were placed on MS medium containing 4 mg/L picloram and 30 g/L sucrose for 1 month. Embryogenic calli were converted into plantlets by transferring to half strength MS medium supplemented with 0.2 mg/L NAA, 1 mg/L BA, 30 g/L sucrose, 300 mg/L casamino acids and 3 g/L Kelcogel (CP Kelgo, USA). Somatic embryo development was investigated. Samples from several stages of somatic embryogenesis were collected. For investigation of tissue-specific gene expression, samples were collected from leaves, flowers and coma bracts of blooming plants. All *C. alismatifolia* samples were immediately frozen in liquid nitrogen and stored at -80°C for later use in RNA isolation.

2.3 RNA Isolation and cDNA Synthesis

Total RNA was extracted from embryogenic callus, embryogenic tissues at different stages of somatic embryogenesis and various somatic tissues of *C. alismatifolia* cv. Blue Tung using PureLink® Plant RNA reagent (Ambion) following the instructions of the manufacturer. The RNA samples were treated with DNase I (Qiagen) to eliminate traces of genomic DNA. Spectrophotometric analysis was performed to determine the concentration of each RNA sample whose integrity was confirmed by 1.2% agarose gel electrophoresis. First-strand cDNA was prepared using SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies) with oligo $(dT)_{18}$ primer except for gene expression analysis, in which random pentadecamer primers (Bio Basic, Canada) were employed in the synthesis of cDNA.

2.4 Cloning of Full-Length cDNA of CaSERK

A pair of degenerate primers; SERK-F1 & SERK-R1 (listed in Table 1), were designed based on conserved domains found among plant *SERK* sequences and used to isolate a fragment of *CaSERK* in the PCR amplification with cDNA from globular embryos as template. The PCR conditions were as follows; 94 °C for 3 min, 50 cycles of amplification (94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s) and a final extension at 72 °C for 5 min. The 5' end and 3' end sequences were obtained using the GeneRacer™ kit (Invitrogen Life Technologies) following the instructions of the manufacturer. In 5'RACE, first round PCR amplification was carried out using a gene-specific primer BT-SERK-R1 and the nested PCR then followed by employing a second

gene-specific primer BT-SERK-R2. The thermal cycling conditions were 94 °C for 3 min, 35 cycles of amplification (94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s) and a final extension at 72 °C for 5 min. Another nested gene-specific primer pair; BT-SERK-F1 & BT-SERK-F2, were employed in 3'RACE with the amplification program; 94 °C for 3 min, 35 cycles of amplification (94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s) and a final extension at 72 °C for 5 min. PCR products of the expected size were cloned into pCR™4-TOPO® TA vector (Invitrogen Life Technologies) and subjected to DNA sequencing. All *CaSERK* sequences were analyzed and their identities determined by comparison with available sequences in GenBank databases using the BLAST program (http://www.ncbi.nlm.nih.gov). The sequence information obtained was used to design specific primers CaSERKfull-BT1-F1 & CaSERKfull-BT1-R1 to amplify the full-length *CaSERK* transcript.

2.5 Protein Structure and Phylogenetic Analyses

The open reading frame (ORF) of *CaSERK* was determined using the "ORF Finder" program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The presence of a signal peptide and the transmembrane region were predicted using the "SignalP 3.0 Server" (http://www.cbs.dtu.dk/services/SignalP/) and "TMpred" (http://www.ch.embnet.org/software/TMPRED_form.html), respectively. Protein prediction was carried out using Scan Prosite tool (http://prosite.expasy.org/scanprosite/). Multiple sequence alignment of CaSERK with other plant SERK proteins was performed with ClustalW (http://www.genome.jp/tools/clustalw/). A phylogenetic tree was constructed with the MEGA4 software by the neighbor-joining (NJ) method with 1,000 bootstrap replicates.

2.6 CaSERK Expression Analysis

Semi-quantitative RT-PCR was performed for the profiling of *CaSERK* expression at different stages of somatic embryogenesis; namely embryogenic callus, globular embryos, club-shaped embryos, scutellum-shaped embryos and plantlets, as well as leaf, flower and coma bract tissues from intact plants. cDNA samples synthesized from total RNA were normalized against the housekeeping *C. alismatifolia 18S rRNA* gene (Accession No. KJ921785), then used as templates in amplification reactions using *CaSERK*-specific primers CaSERK1-F and CaSERK1-R. The PCR procedure was performed under the following non-saturating conditions; an initial denaturation step at 94 °C for 3 min, 29 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) and a final extension at 72 °C for 1 min. Each reaction was done in triplicate. The PCR amplicons were analyzed by running on a 1.4% agarose gel and visualized using a gel documentation system.

Table 1. Details of primers used in this study

Description	Primer sequence (5' - 3')
Degenerate primers	
SERK-F1	GDT TYC TSC GGC TYA ACA ACA AYA G
SERK-R1	TCH GCM AAR CCA TCW CCT TCA AGC ATT C
5'RACE primers	
BT-SERK-R1	CTT CAG CAG GTA CGT CAA AGA AAT G
BT-SERK-R2	GAA CAA ATG GAG GTG GCG GAG ATA AAG
3'RACE primers	
BT-SERK-F1	TGA TGA TGA TGT TAT GTT ACT TGA CTG G
BT-SERK-F2	GAG AAG AAG CTG GAA ATG TTG GTT GAT
Full-length cloning	
CaSERKfull-BT1-F1	GAA AAA TGA GCT CTG ATA AAA AAT GAA AGC
CaSERKfull-BT1-R1	ACA TGA ATC CAT TGC TCA TGG ACA AAT C
Gene expression analysis	
CaSERK1-F	CTC ATA CAA GTG GCT TTG CTA TGC
CaSERK1-R	AAC AAA GAT GAA AAT CAT GGA TCA CA
18S rRNA-F	GCG ACG CAT CAT TCA AAT TTC TG
18S rRNA-R	TGG ATG TGG TAG CCG TTT CTC A

3. Results

3.1 Somatic Embryogenesis and Regeneration

After one month of callus induction under dim light condition, rapidly proliferating embryogenic calli were derived from leaf sheath explants and numerous calli were produced (Figure 1a). Fresh embryogenic calli were then transferred to regeneration medium. In the first month, formation of globular embryos was initiated (Figure 1b). Later on, active globular embryos entered the transition phase and differentiated into club- and then scutellum-shaped embryos, which are characteristic of monocotyledonous embryos (Figures 1c-1d). Development of mature green somatic embryos then followed (Figure 1e). Subsequently, mature embryos were allowed to germinate into complete plantlets with shoot and root development (Figure 1f). The process of plantlet regeneration via somatic embryogenesis took approximately 6-7 months. Well-developed plantlets were subcultured in MS medium supplemented with kinetin for multiplication and maintenance of *in vitro* stock (Figure 1g). Samples were taken from embryogenic calli, somatic embryos at different stages and plantlets for *CaSERK* gene isolation and expression analysis purposes.

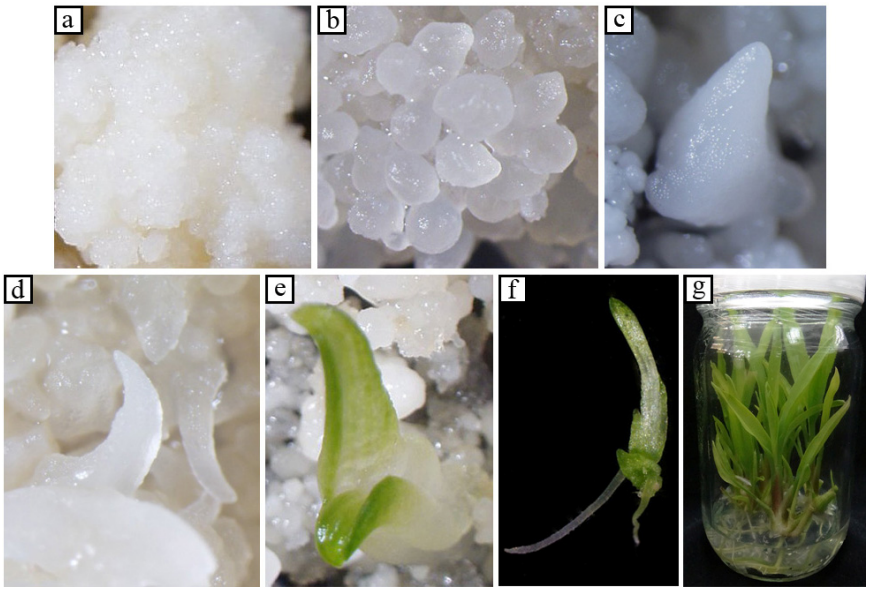

Figure 1. Somatic embryogenesis in *Curcuma alismatifolia*

(a) embryogenic callus formation; (b) cluster of globular embryos initiated after 1 month of culture on regeneration medium; (c) club-shaped embryo; (d) scutellum-shaped embryos; (e) mature green embryo; (f) plantlet developed via somatic embryogenesis; (g) proliferation of plants derived from somatic embryo.

3.2 Cloning of CaSERK

A 1,321 bp *SERK* fragment was amplified from *C. alismatifolia* with degenerate primers designed from *SERK* conserved regions. 5' RACE and 3' RACE reactions were carried out to complete the sequences at 5' and 3' ends, respectively. All fragments were assembled and used to design primers for full-length sequence verification. The corresponding full-length *SERK* cDNA was designated as *CaSERK* and its nucleotide sequence was submitted to GenBank under accession number KJ845677. The 2,521 bp of *CaSERK* sequence consisted of 305 bp of 5'UTR, 1,887 bp of ORF and 329 bp of 3'UTR. The ORF was found to encode a putative protein of 628 amino acids in length, with an estimated molecular weight of 69.54 kDa and a predicted pI of 5.48.

Comparison at the protein level revealed that CaSERK showed high identity to other characterized plant SERK sequences such as CnSERK (92%; Accession No. AAV58833.2), AcSERK2 (91%; AEC46976.1), OsSERK1 (91%; NP_001061108.1), HvSERK (88%; ABN05373.1), AtSERK1 (85%; NP_177328.1) and MtSERK1 (85%; AAN64293.1).

3.3 Analysis of CaSERK Sequence

Multiple sequence alignment analysis showed that CaSERK contained all the characteristic features of the SERK

family, including an extracellular domain containing an N-terminal signal peptide, a leucine zipper, five leucine-rich repeats (LRR), a proline-rich SPP region containing the SPP motif, a transmembrane (TM) domain, an intracellular kinase domain and a C-terminal domain (Figure 2); (Hecht et al., 2001). The SPP domain located next to the last LRR is a distinctive presence unique to SERK proteins. The schematic illustration depicting the organization of conserved structural domains in CaSERK is shown in Figure 3a.

Analysis of the signal peptide sequence at the amino terminal by SignalP indicated a possible cleavage site between positions 28 and 29 (Figure 3b). The presence of a putative TM spanning from amino acid residues 243 to 265 was revealed according to transmembrane topology prediction by TMpred (Figure 3c). In the kinase domain of CaSERK, a possible protein kinase ATP-binding region was found between amino acid residues 311 and 333. Additionally, a serine/threonine protein kinase active-site signature between amino acid residues 428 and 440 and an activation-loop (A-loop) region between amino acid residues 449 and 478 were also predicted. The 29 amino acid residues of the A-loop region of CaSERK were 100% identical to the AtSERK1 counterpart which was demonstrated to be the active site of AtSERK1 (Shah et al., 2001).

3.4 Phylogenetic Analysis

The evolutionary relationships between CaSERK and other SERKs were inferred by a phylogenetic tree (Figure 4) constructed based on the alignment of full-length amino acid sequences. The tree represented two subfamilies; plant SERK1/2 and SERK3/4/5. The SERK1/2 clade was further divided into 3 sub-clades; monocotyledonous plant SERK, dicotyledonous plant SERK and gymnosperm SERK. CaSERK was, expectedly, placed inside the monocot sub-clade with closest alliance to CnSERK from *C. nucifera*.

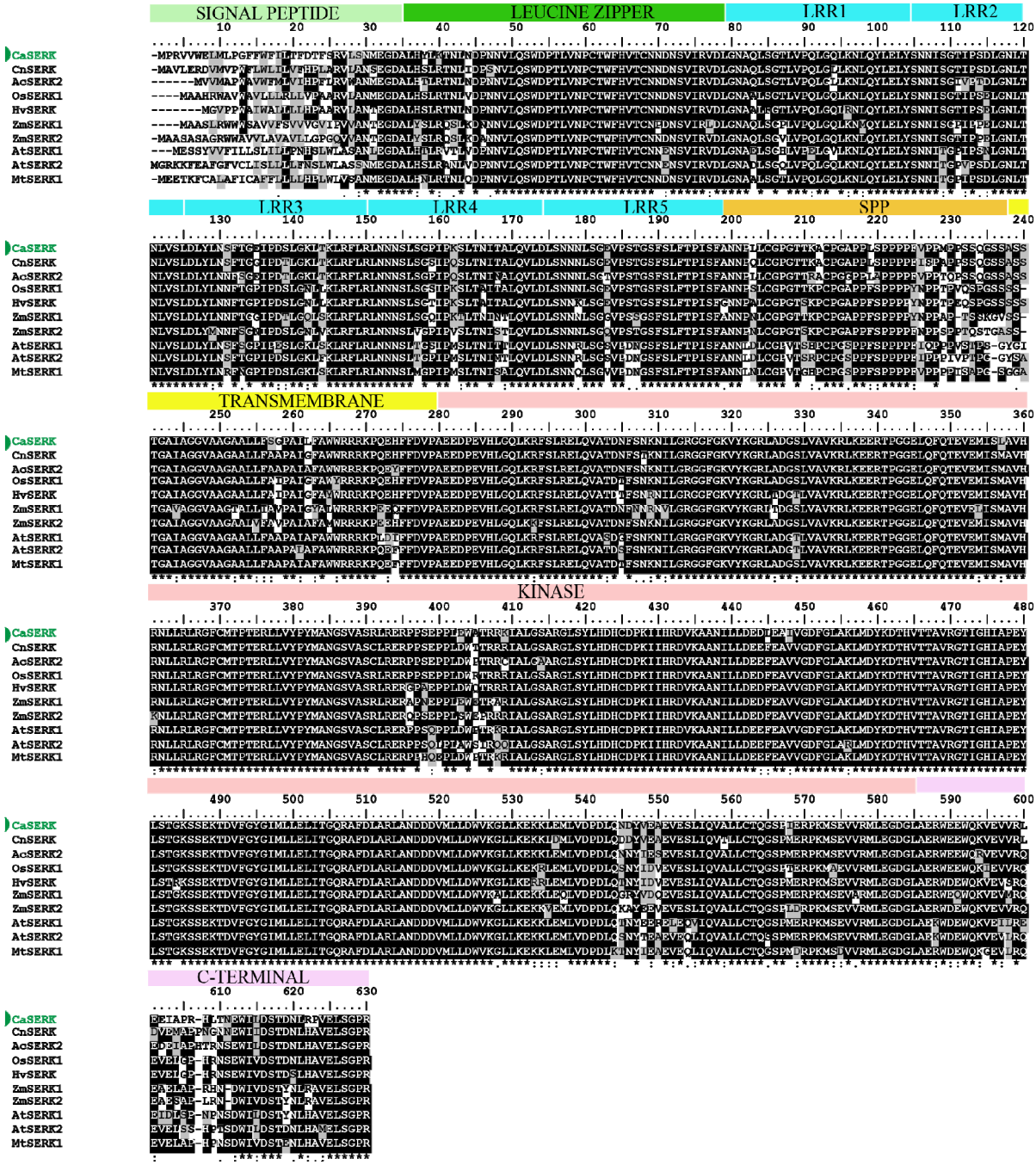

Figure 2. Sequence alignment of predicted amino acid sequences of CaSERK with other plant SERK family protein kinases

Accession numbers of SERK sequences used in the alignment are as follows: CnSERK (*Cocos nucifera*), AAV58833.2; AcSERK2 (*Ananas comosus*), AEC46976.1; OsSERK1 (*Oryza sativa*), NP_001061108.1; HvSERK (*Hordeum vulgare*), ABN05373.1; ZmSERK1 (*Zea mays*), NP_001105132.1; ZmSERK2, NP_001105133.1; MtSERK1 (*Medicago truncatula*), AAN64293.1; AtSERK1 (*Arabidopsis thaliana*), NP_177328.1; AtSERK2, NP_174683.1.

3.5 Expression Study

Expression profile of *CaSERK* was studied using semi-quantitative RT-PCR. Transcript levels were determined during somatic embryogenesis and also in leaf, flower and coma bract tissues (Figure 5). The results showed that *CaSERK* was expressed in all stages of somatic embryogenesis examined, although at varying levels. Highest

expression was detected in embryogenic callus. Subsequently, the expression decreased at early embryo-transition phase in globular and club-shaped embryos. Further down-regulation was observed during later stages of somatic embryogenesis in scutellum-shaped embryos and plantlets. Analysis of *CaSERK* transcript accumulation in somatic organs revealed that no signals were found in leaves while a low level of expression was detected in flowers. Expression level in coma bracts was, however, remarkably high and comparable to those detected in early stages of embryogenesis.

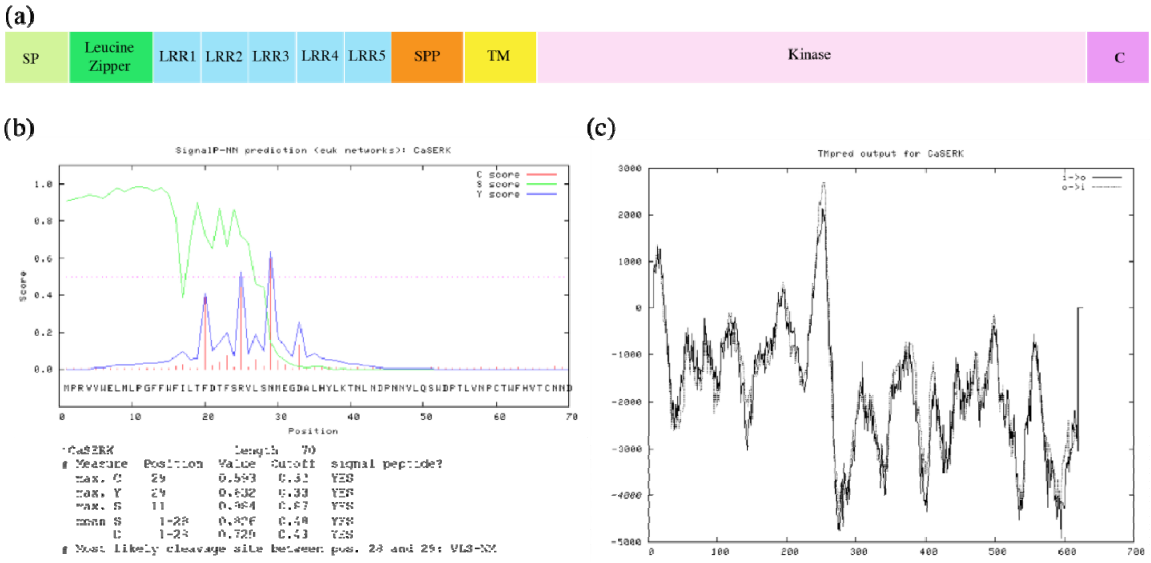

Figure 3. CaSERK sequence analysis

(a) A schematic illustration showing the organization of conserved domains in CaSERK. *SP* (N-terminal signal peptide), *LRR* (leucine-rich repeat), *SPP* (proline-rich SPP region containing the SPP-motif), *TM* (Transmembrane), *kinase* (kinase domains), *C* (C-terminal domain); (b) Signal peptide sequence prediction by SignalP; (c) Transmembrane region prediction by TMpred.

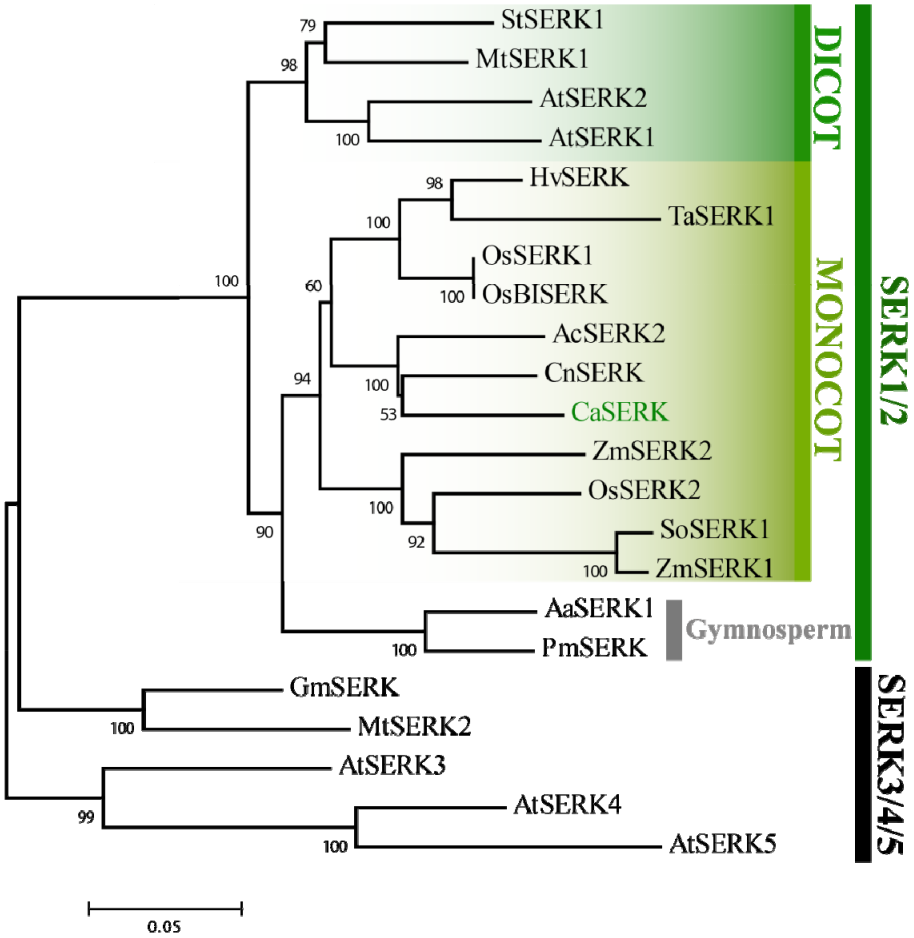

Figure 4. Phylogenetic tree constructed based on the alignment of full-length amino acid sequences indicating the evolutionary relationships among CaSERK, CnSERK (*Cocos nucifera*), AAV58833.2; AcSERK2 (*Ananas comosus*), AEC46976.1; OsSERK1 (*Oryza sativa*), NP_001061108.1; OsSERK2, NP_001052975.1; OsBISERK, AAR26543.1; HvSERK (*Hordeum vulgare*), ABN05373.1; TaSERK1 (*Triticum aestivum*), ACD49737.1; ZmSERK1 (*Zea mays*), NP_001105132.1; ZmSERK2, NP_001105133.1; SoSERK1 (*Saccharum* hybrid), ACT22809.1; MtSERK1 (*Medicago truncatula*), AAN64293.1; MtSERK2, ADO15291.1; StSERK1 (*Solanum tuberosum*), ABO14173.1; GmSERK (*Glycine max*), ACM89473.1; AtSERK1 (*Arabidopsis thaliana*), NP_177328.1; AtSERK2, NP_174683.1; AtSERK3, AAK68074.1; AtSERK4, NP_178999.2; AtSERK5, NP_179000.3; AaSERK1 (*Araucaria angustifolia*), ACY91853.1; PmSERK (*Pinus massoniana*), ACZ56417.1. Bootstrap percentages from 1,000 replicates are shown at each node

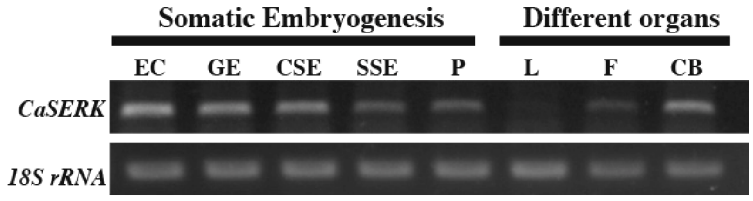

Figure 5. RT-PCR analysis of *CaSERK* expression at different stages of somatic embryogenesis and in different plant organs. *C. alismatifolia 18S rRNA* gene was used as an internal control. *EC* (embryogenic callus), *GE* (globular embryos), *CSE* (club-shaped embryos), *SSE* (scutellum-shaped embryos), *P* (plantlets), *L* (leaves), *F* (flowers), *CB* (coma bracts)

4. Discussion

In recent years, achieving high-frequency somatic embryogenesis and regeneration has become a research focus

in many crop species as it could facilitate their rapid mass propagation, trait modification by genetic transformation as well as applications in long-term germplasm conservation. Substantial efforts have been made to uncover the regulation of somatic embryogenesis at the molecular level to gain a better view of this key process, and thus generate the possibility for the establishment of efficient regeneration system via somatic embryogenesis in the target plants.

In the present study, a full-length transcript of a *SERK* gene, designated as *CaSERK*, was isolated from well-established *in vitro* embryogenic cultures of *Curcuma alismatifolia*, a commercially valuable ornamental ginger. Analysis of the predicted amino acid sequence showed that the encoded protein is a SERK member belonging to the LRR-RLK superfamily (Walker, 1994). All the domains characteristic of the SERK proteins reported in other plant species are present in CaSERK. These include the hallmark proline-rich SPP motif which has been proposed to act as a hinge to give flexibility to the extracellular portion of the SERK protein and is not found in other plant LRR-RLKs (Hecht et al., 2001). High sequence identity to other SERKs further confirmed CaSERK identity. The deduced amino acid sequence of CaSERK shared highest similarity to SERKs in other monocots. Close relationship to other SERKs was also suggested by the placement of CaSERK in the SERK1/2 clade of the SERK phylogenetic tree. High degree of structural conservation and extensive sequence similarity suggested that CaSERK is a SERK ortholog and could share similar functions in somatic embryogenesis.

In several species, *SERK* expression has been linked to acquisition of embryogenic competence (Hecht et al., 2001; Thomas et al., 2004; Pérez-Núñez et al., 2009; Cueva et al., 2012; Talapatra et al., 2014). Monitoring of expression during somatic embryogenesis in *in vitro* cultures revealed that *CaSERK* was most strongly expressed in embryogenic callus. Lower levels of transcripts were detected in globular and club-shaped embryos. Expression continued in scutellum-shaped embryos and plantlets but at further reduced levels. Up-regulation of *CaSERK* in the early stages suggested that it is involved in somatic embryogenesis initiation in *Curcuma alismatifolia*. However, the continuous expression of *CaSERK* in the subsequent embryo-transition phase and up to the plantlet stage was indicative of additional roles during development and regeneration of embryos. Transcript accumulation detected in flowers and particularly coma bracts also suggested a wider role of *CaSERK* in development. Studies of *SERK* in rice (Ito et al., 2005), maize (Baudino et al., 2001), *Citrus unshiu* (Shimada et al., 2005) and rose (Zakizadeh et al., 2010) have also reported a broad profile of *SERK* expression in various somatic tissues which suggested that *SERK* functions are not restricted to somatic embryogenesis. In fact, *SERK* genes have been implicated in a diverse array of processes such as defense responses (Hu et al., 2005; Song et al., 2008; Santos et al., 2009) and apomixis (Albertini et al., 2005; Podio et al., 2014) in addition to their notable role in somatic embryogenesis.

The identification of *CaSERK* is, to our knowledge, the first report of a *SERK* sequence in the Zingiberaceae plant family, which consists of more than 1,200 species. The expression patterns of *CaSERK* strongly suggest a crucial role in somatic embryogenesis. Detailed understanding of *CaSERK* function could offer new avenues for large-scale propagation, introduction of novel traits by gene transfer and also long-term germplasm conservation and maintenance in *Curcuma alismatifolia* and other members of the ginger family.

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