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Research Article

Characterization of the third SERK gene in pineapple (*Ananas comosus*) and analysis of its expression and autophosphorylation activity *in vitro*

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Abstract

Two somatic embryogenesis receptor-like kinase genes (identified as *AcSERK1* and *AcSERK2*) have previously been characterized from pineapple (*Ananas comosus*). In this work, we describe the characterization of a third gene (*AcSERK3*) in this family. *AcSERK3* had all the characteristic domains and shared extensive sequence homology with other plant *SERKs*. *AcSERK3* expression was studied by *in situ* hybridization and quantitative real-time PCR to analyze its function. Intense *in situ* hybridization signals were observed only in single competent cells and competent cell clusters; no hybridization signal was detected in the subsequent stages of somatic embryogenesis. *AcSERK3* was highly expressed in embryogenic callus compared to other organs, *e.g.*, 20-80 fold more than in anther but similar to that of non-embryogenic callus, which was 20-50 fold that of anther. *AcSERK3* expression in root was 80 fold higher than in anther and the highest amongst all organs tested. These results indicate that *AcSERK3* plays an important role in callus proliferation and root development. His-tagged AcSERK3 protein was successfully expressed and the luminescence of His6-AcSERK3 protein was only ~5% of that of inactivated AcSERK3 protein and reaction buffer without protein, and 11.3% of that of an extract of host *Escherichia coli* pET-30a. This finding confirmed that the AcSERK3 fusion protein had autophosphorylation activity.

Keywords: AcSERK3, autophosphorylation activity, expression analysis, prokaryotic expression, somatic embryogenesis.

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Introduction

Somatic embryogenesis (SE) is the process whereby somatic cells can develop into plants via characteristic morphological stages (Shah *et al.*, 2002) and is a notable example of plant totipotency. Somatic embryogenesis can be used for rapid propagation of valuable clones and also for gene transfer and regeneration of genetically modified plants (Schellenbaum *et al.*, 2008). The use of SE-based transformation systems may overcome the problem of chimera formation (Sanjeev *et al.*, 2008). Pineapple is a vegetative propagated crop that depends on suckers because it is self-sterile. Somatic embryogenesis is a highly efficient means of propagating pineapples and can be induced in this species by exposure to 2,4-dichlorophenoxyacetic acid (2,4-D), with the frequency of somatic embryo induction reaching 95%; the number of somatic embryos per callus (3x3x3 mm) after 40 days (d) of culture is ~48 (He *et al.*, 2007). The origin of SE in pineapple is unicellular (He *et al.*, 2010; Ma *et al.*, 2012a,b). Somatic embryogenesis thus provides a good way to genetically transform pineapple.

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The genetic and biochemical mechanisms leading to SE are not well understood (Nolan *et al.*, 2003; Pérez-Núnez *et al.*, 2009). The screening of differentially and specifically expressed genes in carrot embryogenic cell cultures resulted in the isolation of *DcSERK* (*Daucus carota* somatic embryogenesis receptor-like kinase). *DcSERK* was found to be a marker for single somatic cells capable of forming embryos and its expression continued up to the early globular stage but was absent in later stages of embryonic development (Schmidt *et al.*, 1997). A study in maize suggested that SERK protein may be involved in signal transduction and the induction of embryonic development since cells without *SERK* expression show no such response (Baudino *et al.*, 2001). *AtSERK1*, which is expressed during ovule development and early embryogenesis, is a component of the embryogenesis-signaling pathway (Hecht *et al.*, 2001) and a positive regulator that enhances embryogenesis after ectopic expression (Schellenbaum *et al.*, 2008). Studies of plant species such as *Arabidopsis*, maize and *Medicago truncatula* have suggested that some SERKs play pivotal roles in conferring embryonic potential to cells (Ito *et al.*, 2005). The absence of detectable *LsSERK* gene transcripts reduces the ability of plants to form somatic embryonic structures *in vitro*

(Santos *et al.*, 2009). *SERKs* are involved in the acquisition of embryogenic competence in plant cells (Schellenbaum *et al.*, 2008). The expression patterns of *ZmSERK1* (Baudino *et al.*, 2001) and *StSERK1* (Sharma *et al.*, 2008) suggested that *SERKs* may be involved in plant development, disease resistance or other procedures (Alam *et al.*, 2010; Huang *et al.*, 2010; Yang *et al.*, 2011). In many plants, SERKs form a gene family with several members (Baudino *et al.*, 2001; Hecht *et al.*, 2001; Hu *et al.*, 2005; Nolan *et al.*, 2003; Thomas *et al.*, 2004) that have variable roles in embryogenesis and other developmental processes (Sanjeev *et al.*, 2008; Song *et al.*, 2008; Alam *et al.*, 2010). For example, the five SERKs in *Arabidopsis* are involved in several independent pathways (Albrecht *et al.*, 2008), while the two *ZmSERKs* (Baudino *et al.*, 2001) and three *VvSERKs* (Schellenbaum *et al.*, 2008) have different expression patterns in maize (*Zea mays*) and grapevine (*Vitis vinifera*), respectively. We have previously shown that in pineapple (*Ananas comosus*) *AcSERK1* plays an important role in the induction and development of SE (Ma *et al.*, 2012b), and that *AcSERK2* is highly expressed only in embryogenic cells before the pro-embryonic stage (Ma *et al.*, 2012a). The characterization of other SERKs from pineapple and analysis of their expression and function in SE and other developmental processes should improve our understanding of the biological roles of the SERK gene family.

The perception and transduction of external stimuli frequently involves the interaction of membrane-associated receptor proteins with extracellular ligands and the subsequent transmission of information through kinase domains of the receptor that in turn leads to the phosphorylation of target proteins (Ullrich and Schlessinger, 1990; Posada *et al.*, 1993). Plant receptor-like kinases (RLKs) can autophosphorylate serine and/or threonine residues and play an important role in the perception and transmission of external signals (Shiu and Bleecker, 2001; Torii, 2004). The dephosphorylation of transmembrane receptor kinases catalyzed by phosphatases is an essential regulatory mechanism in receptor-mediated signaling (Shah *et al.*, 2002). Protein kinases play important roles in cellular signaling and metabolic regulation in plants (Shah *et al.*, 2001b). The autophosphorylation activities of five AtSERKs have been compared and their phosphorylation sites identified (Karlova *et al.*, 2009). Intramolecular autophosphorylation is required for AtSERK1 activation, although most of the receptors are in a non-phosphorylated state (Shah *et al.*, 2001b, 2002). *In vitro*, this phosphorylation can be achieved through the action of a minor population of catalytically active AtSERK1 molecules, while *in vivo* it can be obtained via ligand-induced conformational changes. The analysis of phosphorylation is thus an important tool for establishing the function of SERKs.

We have previously shown that *AcSERK1* is effectively induced by 2,4-D during SE and can be used as a marker for embryogenically competent cells (Ma *et al.*, 2012b). *AcSERK2* expression was synergistically increased when 2,4-D was supplied in the culture medium but was not specifically associated with SE and may play a broader role in morphogenesis (Ma *et al.*, 2012a). In the present work, we analyzed the expression of *AcSERK3* based on *in situ* hybridization and quantitative real-time PCR (qRT-PCR). *AcSERK3* was expressed at high levels only in competent cells during SE and there was no apparent difference in the expression level between embryogenic and non-embryogenic callus. The highest expression was detected in roots. The His-tagged AcSERK3 fusion protein was expressed in *E. coli* and autophosphorylation was detected.

Material and Methods

Plant material

Calluses derived from the leaf-base of *Ananas comosus. cv.* Shenwan cultured in 2,4-D-free medium were randomly allocated to one of two groups. One group was transferred to 2,4-D-containing medium for SE induction while the other was maintained on 2,4-D-free medium for proliferation of non-embryogenic calluses. Batches of embryogenic calluses were periodically used to extract total RNA every 5 d after incubation on 2,4-D containing medium. Non-embryogenic control samples were obtained at 0, 15, 25, 35 and 45 days. Samples of organs (root, stem, leaf, calyx, bract, petal and anther) were also used to isolate RNA for qRT-PCR. The ovules and ovaries were periodically collected at relevant stages (a week before flower blooming, at flower blooming, a week after flower blooming, two weeks after flower blooming and four weeks after flower blooming) to study *AcSERK3* expression during the development of these organs. All of the tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Embryogenic calluses induced with 2,4-D were fixed in formalin acetic acid (FAA) for *in situ* hybridization.

RNA/DNA extraction and cDNA synthesis

RNA was extracted using TRIzol reagent (Takara) based on the manufacturers recommendations. Each RNA sample was digested with DNase (Takara) to remove any remaining DNA. The first strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen). Genomic DNA was extracted from calluses cultured *in vitro* by treating with cetyltrimethylammonium bromide (CTAB) as described by Murray and Thompson (1980) and treated with RNase A to eliminate RNA.

Isolation of *AcSERK3*

The primers used to isolate AcSERK3 are shown in Supplementary Table S1. The 5' and 3' end sequences of *AcSERK3* were amplified by rapid amplification of cDNA ends (RACE) using a3'-Full RACE Core Set v.2.0 kit and

5'-Full RACE Core Set v.2.0 kit (Takara), according to the manufacturer's instructions. Sequences were edited, aligned and analyzed using DNAMAN and CLUSTAL software tools. Specific primers designed based on the results of RACE were used to amplify the full-length cDNA sequence and genomic sequence.

Bioinformatics analysis

The isolated sequence was blasted against sequences deposited in GenBank sequences. The open reading frame (ORF) was predicted with the ORF Finder program. The protein parameters of the protein were computed by the Protparam tool of ExPASy. The signal peptide (SP) was predicted with the SignalP 3.0 Server tool and the transmembrane region (TM) was predicted by the TMpred tool. The structure and function of the protein were assessed using the ScanProsite software (Zdobnov and Apweiler, 2001). The SWISS-MODEL program was used to predict the tertiary structure (Arnold *et al.*, 2006) and phosphorylation sites were predicted by the KinasePhos tool. Multiple sequence alignment was done with DNAMAN software and phylogenetic trees were constructed using MEGA4 software.

Quantitative real-time PCR (qRT-PCR)

The relative expression of *AcSERK3* was assessed using a Thunderbird SYBR qPCR mix (Toyobo) according to the manufacturer's instructions. The assays were done using an iQ5 real-time PCR system (BioRad). qRT-PCR was done using gene-specific primers for *AcSERK3* (Table S1). A gene encoding pineapple β-actin was used as an internal (housekeeping gene) control. Triplicate quantitative PCR experiments were run for each sample and for each tissue and time point three biological replicates assayed. The results were analyzed using iQ5 system software. To prevent the amplification of any contaminating genomic DNA, RNA preparations were treated with DNaseI and the probe primers were designed over an exon/intron boundary in the cDNA sequence. The specificity of the amplifications was verified by electrophoresis and at the end of the PCR run by melting curve analysis.

RNA *in situ* hybridization and detection

RNA *in situ* hybridization was done using the procedures described in the Roche manual for *in situ* hybridization to chromosomes, cells and tissue sections. The samples were fixed, embedded, sectioned and hybridized as described elsewhere (Ma *et al.*, 2012a). The sense and antisense probes were transcribed with cDNA fragments cloned in the pSPT 19 vector (Roche) and were labeled with digoxigenin-UTP using an SP6 or T7 RNA polymerase *in vitro* transcription kit (Roche) according to the technical manual. Detection was done with anti-digoxigenin-AP and NBT/BCIP ready-to-use tablets (Roche) and the hybridization signal was observed by microscopy (Olympus).

Prokaryotic expression of *AcSERK3*

After digestion with two restriction enzymes (*Eco*RI and *Hind*III), full-length *AcSERK3* without the signal peptide was sub-cloned into the pET-30a vector. The recombinant plasmid (pET-*AcSERK3*) was then introduced into *E. coli* BL21 (DE) pLysS competent cells and sequenced from both sides. A single colony of *E. coli* strain BL21 (DE) harboring the pET-30a-*AcSERK3* plasmid was cultured at 37 °C overnight in Luria-Bertani liquid medium containing kanamycin (50 μg/mL). Subsequently, 1 μL of the cultured cells was transferred to 200 μL of fresh medium and shaken at 150 rpm for about 4 h until the optical density (OD_{600}) reached 0.5. The cultures were then induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and shaken for another 20 h at 18 °C. Bacteria were harvested by centrifugation (4000 rpm, 5 min), resuspended in 2 mL of splitting buffer and ultrasonicated at 4 °C. The ultrasonicated product was centrifuged (12,000 rpm, 10 min, 4 °C) and 50 μL of the precipitate and supernatant were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The protein bands were visualized after being stained with pre-cooled 0.1 M KCl solution. The target protein was obtained by His column chromatography. An empty pET-30a vector in *E. coli* BL21 (DE) was used as a control.

Western blot analysis of AcSERK3

Purified soluble recombinant proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Roche) using a Mini Trans-Blot apparatus (Bio-Rad) according to the manufacturer's instructions. The membrane was incubated in a blocking solution containing 5% skimmed milk overnight at 4 °C and then treated with anti-His polyclonal antibody (Amersham) at 37 °C for 2 h. After three washes in PBS, the membrane was incubated with a rabbit anti-goat IgG conjugated secondary antibody (Promega) at 37 °C for 1 h. After a further three washes in PBS, the membrane was treated with BeyoECL Plus (Beyotime) and the blots were exposed to X-ray film (Kodak).

Autophosphorylation assay

Purified fusion protein was coupled to γATP (50 μM, Promega) in a solid white 96-well plate and incubated for 60 min at room temperature in a volume of 50 μL of kinase reaction buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1 M $MgCl_2$ and 0.1 M DTT). After incubation with γATP, an equal volume of appropriate Kinase-Glo reagent (Promega) was added to each well, incubated at room temperature, and the luminescence then recorded using a 96-well plate multi-functional UV fluorescence microplate reader. The

luminescent signal correlated with the amount of ATP present and was inversely related to the amount of kinase activity. The prokaryotic expression and purification of the fusion protein was performed three times, and the autophosphorylation of each sample was assessed in triplicate.

Results

Cloning of *AcSERK3*

SERKs comprise a small gene family in many plants (Sharma *et al.*, 2008). The question arises as to whether there are other SERK genes in *A. comosus* that are closely related to *AtSERK1*. Based on the isolation of *AcSERK1* and *AcSERK2* (Ma *et al.*, 2012a,b) using the primers listed in Supplementary Table S1, the full-length DNA and cDNA sequences of *AcSERK3* were amplified. The full-length mRNA sequence of *AcSERK3* contained 2045 bp (GenBank accession number HM236377) and the full-length genomic DNA sequence contained 5299 bp (GenBank accession number JN672683).

Sequence analysis

The mRNA sequence of *AcSERK3* contained an 1890-bp open reading frame (ORF) that encoded 629 amino acids, with a calculated molecular mass of 69.6 kDa and a predicted pI of 5.55. The total number of negatively charged residues (Asp+Glu) was 71 and the total number of positively charged residues (Arg+Lys) was 60; the general formula was $C_{3092}H_{4889}N_{853}O_{921}S_{24}$. The extinction coefficient at 280 nm was 78,880 and the instability index (II) was 45.55, which classified the protein as unstable. The aliphatic index was 91.91 and the grand average of hydropathicity (GRAVY) was -0.220, indicating that AcSERK3 was a hydrophobin.

The *AcSERK3* mRNA sequence showed the highest identity with *AcSERK2* (*A. comosus*, HM236376, 82%), *CnSERK* (*Cocos nucifera*, AAV58833.2, 82%) and *AcSERK1* (*A. comosus*, HM236375, 79%) at the nucleotide level, and the predicted amino acid similarity of these sequences was 88% (Supplementary Figure S1). AcSERK3 aligned closely with some SERKs from other species and contained all the conserved domains present in these SERKs. A high similarity of AcSERK3 to other SERKs was observed in the leucine zipper region and kinase domains, while weak similarity was observed in the signal peptide and the serine-proline-proline (SPP) region. The SP sequence was detected using the SignalP3.0 program which predicted that the first 28 N-terminus amino acids of AcSERK3 (MAISTRQAVAPWFLWLLLLFNPVARVLR) formed a signal peptide, with a putative signal peptide cleavage site between residues 28 and 29 (Supplementary Figure S2A). The signal peptide of AcSERK3 has one amino acid less than that of AcSERK1 and five amino acids more than that of AcSERK2. This signal peptide was followed by a leucine-rich domain containing the ZIP motif. As in other SERKs, there were five leucine-rich repeat (LRR) consensus sequences with the typical 24 residues that are essential for correct localization of the AtSERK1 protein (Shah *et al.*, 2001a). The ZIP motif was followed by a proline-rich domain containing the SPP motif, an alanine-rich hydrophobic transmembrane domain (Supplementary Figure S2B), and a serine/threonine kinase domain comprising 11 sub-domains located at positions 305-592 (Supplementary Figure S2C). The 29 amino acid residue activation loop (A-loop) was also present in subdomains VII and VIII, which was defined as the active site of AtSERK1 (Shah *et al.*, 2001c). A protein kinase ATP-binding region was present at positions 311-333 and a serine/threonine protein kinase active site was present at positions 428-440 in subdomain VI with an active site at position 432, which indicated the function of AcSERK3 as a serine/threonine kinase (Singla *et al.*, 2008). The kinase region was followed by a leucine-rich C-terminal domain, as described by Schmidt *et al.* (1997) that was conserved among the three AcSERKs. The tertiary structure of AcSERK3 protein was predicted with SWISS-MODEL program (Supplementary Figure S2D). There were 13 serine phosphorylated sites, five threonine phosphorylated sits and four tyrosine phosphorylated sites in the AcSERK3 protein (Supplementary Figure S2E).

A comparative alignment of the predicted AcSERK3 coding regions with the corresponding genomic sequence revealed that AcSERK3 consisted of 11 exons and 10 introns (Supplementary Figure S3). This exon/intron structure was quite similar to that of *AcSERK1*, *AcSERK2* (Ma *et al.*, 2012a,b) and other *SERKs* (Sharma *et al.*, 2008; Stone *et al.*, 2001). The mRNA coding regions of *AcSERK1, 2, 3* were 1890, 1875 and 1890 bp long, respectively, while the corresponding full-length DNA sequences were 4845, 5707 and 5155 bp long, indicating that were important differences in the lengths of the intron sequences (Supplementary Figure S3). Alignment of the sequences of the corresponding introns indicated marked differences in their base pair composition (data not shown).

An unrooted phylogenetic tree was constructed using the deduced amino acid sequences of the three AcSERKs and 18 other SERKs (Supplementary Figure S4). In these SERKs, AtSERK1 and DcSERK were confirmed to be specifically expressed in embryogenic calluses and during the induction of embryogenesis. AcSERK1, AcSERK2, OsSERK, ZmSERK, CnSERK and MtSERK showed divergent expression patterns but were all regarded as markers of the embryonic competence of the cells. Four major groups or clusters were observed in the phylogenetic tree. Group 1 contained most of the dicots, Group 2 included monocots such as AcSERKs, ZmSERK and OsSERK, whereas tobacco NbSERKs belonged to Group 3. AtSERKs 3-5 were clustered in Group 4 while AtSERKs 1 and 2 belonged to Group I. AcSERK3 was closely associ-

ated with AcSERK2 and CnSERK (Pérez-Núnez *et al.*, 2009), while AcSERK1, which was closest to HvSERK and then to OsSERK1 and ZmSERK1, formed a separate cluster. All of these were grouped closer to AtSERKs 1 and 2 and DcSERK rather than to AtSERKs 3-5. AtSERK1 and DcSERK have been directly implicated in somatic embryogenesis and can be used as markers of competent cells (Schmidt *et al.*, 1997; Nolan *et al.*, 2003). AtSERKs 3-5 were distantly related to other SERKs because they showed very weak similarity in the SPP region, a unique feature of SERK proteins (Sharma *et al.*, 2008).

AcSERK3 expression during somatic embryogenesis

Our previous study confirmed that treatment with 2,4-D was important for the formation of embryogenic cells in activated pineapple calluses (He *et al.*, 2007). The presence of 2,4-D stimulates the formation of competent cells and a transition towards embryogenic cells. *In situ* hybridization was done with sections of 2,4-D-treated calluses to detect the expression pattern of *AcSERK3*. Intense expression signals were observed in a few of the small cytoplasm-rich cells (Figure 1A) and small clusters of these cytoplasm-rich cells (Figure 1B,C). The large vacuolated cells surrounding these small cytoplasm-rich cells were non-proliferating remnants of the callus. These *AcSERK3*-expressing cells were small, isodiametric, non-vacuolated, cytoplasm-rich and proliferating, indicating their high metabolic activity. We have confirmed that some of these cytoplasm-rich small cells are embryogenic cells and that they can form somatic embryos (He *et al.*, 2010). This finding indicated that these *AcSERK3*-expressing cells were competent cells that were involved in the formation of early stage pro-embryo through cell division (Figure 1D) in which the expression of *AcSERK3* apparently decreased. No hybridization signal was detected in the early stage of the globular embryo (Figure 1E). *AcSERK3* showed an essentially similar expression profile to that of *AcSERK2* during the early stage of pro-embryo formation (Ma *et al.*, 2012a), but different from that of *AcSERK1* which showed a high level of expression in the transition from single competent cells to the globular embryo (Ma *et al.*, 2012b). When the sense probe for hybridization was tested alone, no signal was detected in any region of the callus (Figure 1F).

To determine the abundance of *AcSERK3* during embryogenesis, qRT-PCR was done with RNA isolated from calluses cultured in the absence and presence of 2,4-D. The level of expression could be upregulated by short-term treatment (5 d) with 2,4-D and then maintained in a relatively low level. After culture with 2,4-D for 40 d, the expression of *AcSERK3* reached the highest level but then apparently decreased. In non-embryogenic calluses that were used as controls, the expression of *AcSERK3* gradually increased in the initial 25 d of culture and then decreased. Overall, except for the high level of expression on days 5 and 40 of the treatment with 2,4-D, there was no significant difference between the expression of *AcSERK3* in

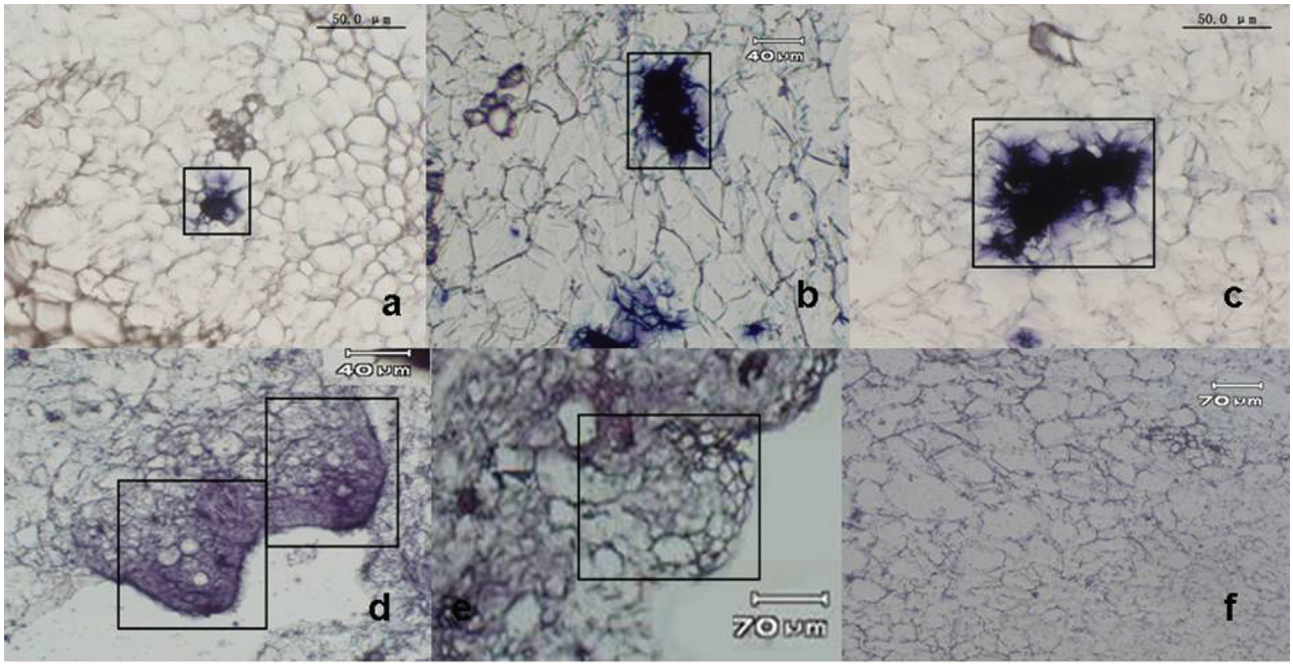

Figure 1 - *In situ* hybridization analysis of *AcSERK3* during embryogenesis in pineapple. (A) Single competent cell with intense hybridization signal; only a few of the small cytoplasm-rich cells showed *AcSERK3* expression. (B) Two competent cells with an intense hybridization signal that may have originated from one competent cell. (C) Cell clusters with an intense hybridization signal. (D) An early-stage pro-embryo with a very weak hybridization signal. (E) An early-stage globular embryo with no hybridization signal. (F) Negative control with no hybridization signal.

embryogenic and non-embryogenic calluses (Figure 2). The expression level in calluses was 5-10 times of that in other organs except root. This expression pattern indicated that *AcSERK3* may play a role in the proliferation of calluses beyond SE.

AcSERK3 expression in various organs

Since the expression of *AcSERK3* in anther was the lowest amongst all of the organs screened, all other levels of expression were expressed as the fold change relative to that of anther. Root had the highest expression (80 fold that of anther), which suggested that *AcSERK3* played an important role in this organ. *AcSERK3* expression in leaf, calyx, bract and petal was 6-8 fold that of anther, with stem having a low expression similar to anther. *AcSERK3* expression during ovule development was stable and relatively high (~20 fold that of anther), perhaps because this protein has a role in the development of the zygotic embryo. The level of expression during ovary development was relatively low (~3 fold that of anther and similar to other organs, except root).

Prokaryotic expression, purification and western blot analysis of AcSERK3

The mRNA sequence of *AcSERK3* without the signal peptide was inserted into the pET-30a expression vector and transformed into *E. coli* BL21 (DE) Plys to express the N-terminally His6-tagged protein. SDS-PAGE of the fusion protein showed that AcSERK3 was expressed and migrated as a single band with the predicted molecular mass. The protein was detected in both supernatant and precipitate (Figure 3A). This result showed that the prokaryotically expressed ~68 kDa His6-AcSERK3 was a soluble protein. The soluble fraction of the fusion protein was purified by nickel-affinity column. The purified product was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The molecular mass of the purified protein

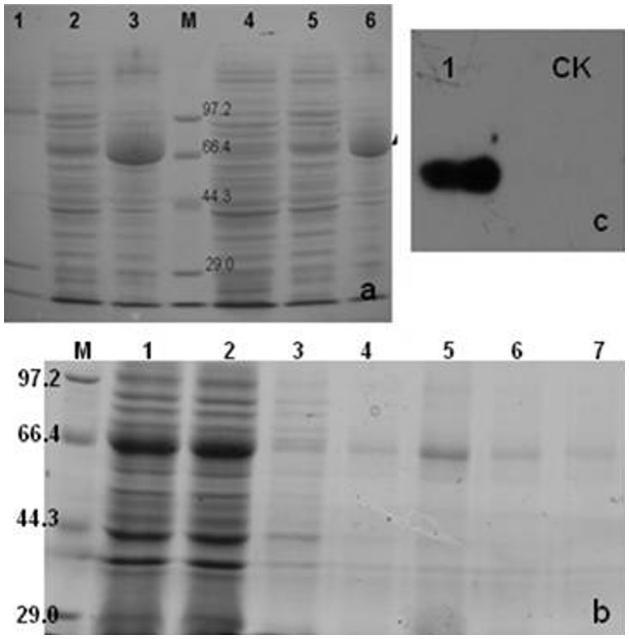

Figure 3 - Prokaryotic expression and western blot results of AcSERK3. (A) SDS-PAGE analysis of the expression product of pET-30a-AcSERK3 in *E. coli.* Lanes: M - low molecular mass marker, 1 - supernatant of cells carrying the pET30a vector, 2 - supernatant of non-induced cells carrying the pET30a-*AcSERK3* vector, 3 - supernatant of induced cells carrying the pET30a-*AcSERK3* vector, 4 - precipitate of induced cells carrying the pET30a vector, 5 - precipitate of non-induced cells carrying the pET30a-AcSERK3 vector, 6 - precipitate of induced cells carrying the pET30a-AcSERK3 vector. (B) Purification of expressed pET30a-*AcSERK3* protein. Lanes: M - low molecular mass marker, 1 - soluble fraction of induced cells containing pET30a-AcSERK3, 2 - pET30a-AcSERK3 flow-through, 3 - wash buffer, 4-7 - first, second, third and fourth washes with elution buffer. (C) Western blot of His6-AcSERK3 fusion protein. Lanes: *CK* - negative control (purified product of *E. coli* cells containing the pET30a vector), 1 - purified recombinant His6-AcSERK3.

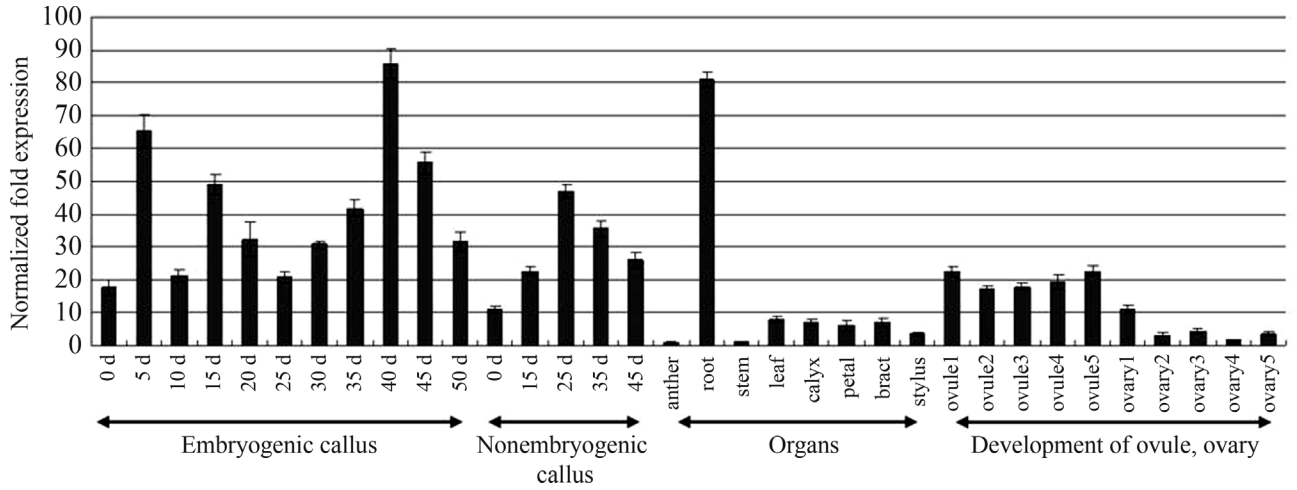

Figure 2 - *AcSERK3* expression assessed by qRT-PCR. The results are shown as the fold-change compared to the expression levels in anther and were normalized relative to the housekeeping gene AcACTIN in callus. *Embryogenic callus* - callus cultured in medium containing 2,4-D. *Non-embryogenic callus* - callus cultured in 2,4-D-free medium. For callus tissue, the time intervals are in days (d). The columns represent the mean ± SD of X determinations.

(~68 kDa) (Figure 3B) agreed well with the calculated molecular mass for AcSERK3. The identity of the His-tagged AcSERK3 protein was further confirmed by western blot analysis using anti-His antibody (Figure 3C).

Analysis of *AcSERK3* autophosphorylation

Protein phosphorylation is a critical part of signal transduction events that allow cells to respond to external stimuli. This is achieved by protein kinases that catalyze the transfer of the γ-phosphate from ATP to a target protein (Langer *et al.*, 2004). AtSERKs have been shown to have autophosphorylation activity and AtSERK1 was the most active kinase, with 24 autophosphorylated residues (Karlova *et al.*, 2009). An *in vitro* phosphorylation assay was used to assess autophosphorylation by incubating His6-AcSERK3 protein with γATP. The reaction products were then detected with a Kinase-Glo luminescent kinase assay kit (Promega). This method allows the measurement of kinase activity by quantifying the decrease in ATP levels at the end of the incubation. After incubation with purified His6-AcSERK3 protein, the luminescence decreased to 4.84% of that seen with inactivated AcSERK3 protein, 4.92% that of reaction buffer without protein, and 11.28% that of an extract of host *E. coli* cells containing empty pET-30a vector (Figure 4). The decrease in luminescence indicated that most of the γATP was consumed after incubation with AcSERK3 protein. This finding confirmed that AcSERK3 protein can catalyze the transfer of γ-phosphate from ATP to the phosphorylation sites of AcSERK3.

Discussion

AcSERK3 is a member of a small family of RLKs, all of which have a predicted signal peptide, five leucine-rich regions, a typical serine-proline rich juxtamembrane region, and a C-terminal kinase domain that is also conserved in all of the 11 subdomains, as described for serine/threonine protein kinases (Shah *et al.*, 2001a). The similarity of the deduced amino acid sequence of AcSERK3 with AtSERK1 (85%) suggested that the corresponding regions in AcSERK3 may play a similar role to AtSERK1. Alignment of the three AcSERKs showed that most of the divergent amino acids were located in the signal peptide, the SPP domain and the C-terminal region. The C-terminal regions showed less sequence conservation and is believed to generate docking sites for specific kinase substrates for autophosphorylation rather than a more general response to kinase activation (Pawson, 2004; Wang *et al.*, 2005). The difference in signal peptide of the three AcSERKs may affect the expression patterns and subcellular localization of AcSERKs. All three *AcSERKs* contained 11 exons and 10 introns; the highly conserved exons and exon/intron structure suggest that there is some functional significance for such organization in this gene family (Sharma *et al.*, 2008). However, the sequence length and base composition of the introns were quite different in the three *AcSERKs* and other *SERK*s (Stone *et al.*, 2001; Sharma *et al.*, 2008; Ma *et al.*, 2012a,b). Introns may play a role in mediating gene expression (Tosi, 1998; Gregoise and Romeo, 1999). Phylogenetic analysis showed that AcSERK3 grouped closely with AcSERK2 and, to a lesser extent, with CnSERK, whereas AcSERK1 was more closely associated with HvSERK, OsSERK1 and ZmSERK1 in a separate cluster. These relationships suggested that *AcSERK3* may be closer to *AcSERK2* in expression and function than to *AcSERK1*. The 22 phosphorylated sites predicted in AcSERK3 indicated that it was likely to have autophosphorylation activity.

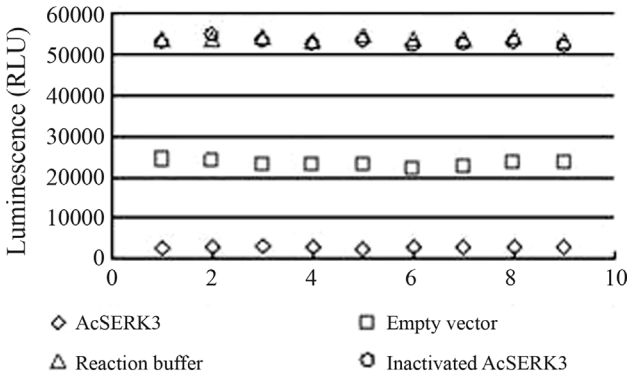

Figure 4 - Autophosphorylation of AcSERK3 fusion protein. The luminescence was inversely correlated with the amount of kinase activity. *AcSERK3* - purified His6-AcSERK3 fusion protein, *empty vector* - purified product of *E. coli* cells containing the pET-30a vector, *reaction buffer* - reaction buffer without protein, *inactivated AcSERK3* - AcSERK3 protein after boiling for 25 min. All phosphorylation assays involved incubation with 2.5 x 10^{-3} μmol γATP. RLU - relative luminescence units.

There are important gaps in our knowledge of the initial events involved in the transition of somatic cells to embryogenic cells. In our studies, we have focused our attention on a possible role of SERK in pineapple embryogenesis because *SERKs* seemed to play key roles in the initiation of embryogenesis. In previous work, we demonstrated that 2,4-D was effective in initiating SE in pineapple callus (He *et al.*, 2007) and therefore used calluses cultured in the presence of 2,4-D as a means of obtaining embryogenic calluses. We have also shown that some of the small cytoplasm-rich cells undergo asymmetrical division and subsequently progress to form the embryo (He *et al.*, 2010).

One of the goals of the present work was to determine the relationship between *AcSERK3* expression and the initiation of SE. The *in situ* hybridization results showed that *AcSERK3* was highly expressed in single competent cells and small competent cell clusters. These *AcSERK3*-expressing cells were small, isodiametric, non-vacuolated and rich in cytoplasm. Not all of these small cytoplasm-rich cells were competent cells and only a few of them expressed *AcSERK3*. The morphological characteristics of *AcSERK3*-expressing cells were similar to those of the cells observed to form embryos in calluses (He *et al.*, 2010). In a

manner similar to that observed here, *DcSERK* was also expressed in a few single cells and small clusters of 2-8 cells; the frequency of competent cells in enlarged cells of carrot was only 0.56% and *DcSERK* was expressed in single cells that were competent to regenerate through SE (Schmidt *et al.*, 1997).

There is not much difference in the expression profiles of *AcSERKs* in single competent cells. The expression of *AcSERK3* decreased in the early stage of pro-embryos, when only a very weak hybridization signal was detected. This expression pattern was similar to that of *AcSERK2*, whereas *AcSERK1* was expressed at a high level from the competent single cell stage to the globular embryo stage (Ma *et al.*, 2012b). This expression pattern agreed with the phylogenetic analysis. There was an increase in *AcSERK3* expression after 5 d of induction with 2,4-D, as shown by *in situ* hybridization in competent cells. The results presented here, together with cytological observations (He *et al.*, 2010), demonstrate that *AcSERK3* may play a role in the transition of somatic to competent cells. This expression profile was similar to that of *AcSERK2*, but the fold increase in *AcSERK3* was only half that of *AcSERK2* (Ma *et al.*, 2012a).

Studies with *Arabidopsis* suggest that members of the SERK family have partially redundant functions, can act as co-receptors with different main receptors, and individual members can act in different signaling pathways (Karlova *et al.*, 2009). In contrast to *AcSERK1* and *AcSERK2*, the expression of which was very low in non-embryogenic callus, *AcSERK3* showed relatively high expression in this tissue. *ZmSERK* from maize (Baudino *et al.*, 2001) and *OsSERK* from rice (Ito *et al.*, 2005) were also expressed in non-embryogenic and embryogenic cells. This expression pattern suggested that *AcSERK3* may play a role in the proliferation and differentiation of somatic cells in the callus. Studies of *SERK* in other species have also suggested that their function is not limited to embryogenesis (Baudino *et al.*, 2001; Nolan *et al.*, 2003; Colcombet *et al.*, 2005; Ito *et al.*, 2005; Singla *et al.*, 2008). As with *Arabidopsis* SERKs that can act in different signaling pathways (Karlova *et al.*, 2009), the three *AcSERKs* may play different roles in SE and plant development.

SERK is a member of the diverse family of serine/threonine receptor kinases in plants. Expression of plant RLKs in *E. coli* cells yields proteins that are suitable for biochemical studies (Shah *et al.*, 2001b). As shown for the SERK family, the kinase domains of various plant receptor kinases are highly conserved, although their phosphorylation properties vary considerably (Karlova *et al.*, 2009). The molecular mass of prokaryotically expressed His6-AcSERK3 protein agreed well with the calculated molecular mass of AcSERK3 and the identity of the fusion protein was confirmed by western blot analysis using anti-His antibody. AcSERK3 was predicted to have 22 phosphorylation sites. In agreement with this, the autophosphorylation assay showed that purified His6-AcSERK3 was able to catalyze the transfer of γ-phosphate from ATP to the phosphorylation sites of AcSERK3, indicating that this protein was indeed a protein kinase. The autophosphorylation activity of AtSERK1 has been demonstrated and the catalytic site and phosphorylated sites have been identified (Shah *et al.*, 2001b; Karlova *et al.*, 2009).

In conclusion, the results of this study show that the *AcSERK3* sequence amplified from pineapple could be used to generate a His-tagged AcSERK3 fusion protein in *E. coli* cells and that the prokaryotically expressed protein had autophosphorylation activity. Further elucidation of the biochemical properties of AcSERK3 and identification of the role of this protein in intracellular signaling could provide valuable insights into the process of plant embryogenesis.

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References

Alam MM, Sharmin S, Nabi Z, Mondal SI, Islam MS, Nayeem SB, Shoyaib M and Khan H (2010) A putatuve leucine-rich repeat receptor-like kinase of jute involved in stress response. Plant Mol Biol Rep 28:394-402.

Albrecht C, Russinova E, Kemmerling B, Kwaaitaal M, de Vries SC (2008) Arabidopsis somatic embryogenesis receptor kinase proteins serve brassinosteroid-dependent and-independent signaling pathways. Plant Physiol 148:611-619.

Arnold K, Bordoli L, Kopp J and Schwede T (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 22:195-201.

Baudino S, Hansen S, Brettschneider R, Hecht Valérie FG, Dresselhaus T, Lorz H, Dumas C and Rogowsky PM (2001) Molecular characterisation of two novel maize LRR receptor-like kinases, which belong to the SERK gene family. Planta 213:1-10.

Colcombet J, Boisson-Dernier A, Ros-Palau R, Vera CE and Schroeder JI (2005) *Arabidopsis* somatic embryogenesis receptor kinases 1 and 2 are essential for tapetum development and microspore maturation. Plant Cell 17:3350-3361.

Gregoise JM and Romeo pH (1999) T-cell expression of the human *GATA-3* gene is regulated by a non-lineage-specific silencer. J Biol Chem 274:6567-6578.

He YH, Fang SQ, Ma J, Hu ZY, Lu M and Peng B (2010) Histocytology observation on the somatic embryogenesis in *Ananas comosus* callus. Acta Hort Sin 37:689-696 (in Chinese).

He YH, Luo J, Wu HT, Wang RX and Gao AP (2007) Somatic embryogenesis from leaf base callus of *Ananas comosus*. J Fruit Sci 24:59-63 (in Chinese).

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt DL, Boutilier K, Grossniklaus U and de Vries SC (2001) The *Arabidopsis* somatic embryogenesis receptor kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127:803-816.

Hu H, Xiong L, Ynag Y (2005) Rice SERK1 gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. Planta 222:107-117.

Huang X, Lu XY, Zhao JT, Chen JK, Dai XM, Xiao W, Chen YP, Chen YF and Huang XL (2010) *MaSERK1* gene expression associated with somatic embryogenic competence and disease resistance response in banana (*Musa* spp.). Plant Mol Biol Rep 28:309-316.

Ito Y, Takaya K and Kurata N (2005) Expression of SERK family receptor-like protein kinase genes in rice. Biochim Biophys Acta 1730:253-258.

Karlova R, Boeren S, van Dongen W, Kwaaitaal M, Aker J, Vervoort J and de Vries S (2009) Identification of *in vitro* phosphorylation sites in the *Arabidopsis thaliana* somatic embryogenesis receptor-like kinases. Proteomics 9:368-379.

Langer T, Vogther M, Elshorst B, Betz M, Schieborr U, Saxena K and Schwalbe H (2004) NMR backbone assignment of a protein kinase catalytic domain by a combination of several approaches: Application to the catalytic subunit of cAMP-dependent protein kinase. Chembiochem 5:1508-1516.

Ma J, He YH, Hu ZY, Xu WT, Xia JX, Guo CH, Lin SQ, Cao L, Chen CJ, *et al.* (2012a) Characterization and expression analysis of AcSERK2, a somatic embryogenesis and stress resistance related gene in pineapple. Gene 500:115-123.

Ma J, He YH, Wu CH, Liu HP, Hu ZY and Shun GM (2012b) Cloning and molecular characterization of a SERK gene transcriptionally induced during somatic embryogenesis in *Ananas comosus*. cv. Shenwan. Plant Mol Biol Rep 30:195-203.

Nolan KE, Irwanto RR and Rose RJ (2003) Auxin up-regulates *MtSERK1* expression in both *Medicago truncatula* root-forming and embryogenic cultures. Plant Physiol 133:218-230.

Pawson T (2004) Specificity in signal transduction: From phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell 116:191-203.

Pérez-Núnez MT, Souza R, Sáenz L, Chan JL, Zúniga-Aguilar JJ and Oropeza C (2009) Detection of a *SERK*-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos. Plant Cell Rep 28:11-19.

Posada J, Yew N, Ahn NG, Vande Woude GF and Cooper JA (1993) Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase. In Vitro Mol Cell Biol 13:2546-2553.

Sanjeev KS, Steve M, Ingo H and Glenn JB (2008) Cloning and molecular characterisation of a potato SERK gene transcriptionally induced during initiation of somatic embryogenesis. Planta 228:319-330.

Santos MO, Romano E, Vieira LS, Baldoni AB and Aragão FJL (2009) Suppression of *SERK* gene expression affects fungus tolerance and somatic embryogenesis in transgenic lettuce. Plant Biol 11:83-89.

Schellenbaum P, Jacques A, Mailot P, Bertsch C, Mazet F, Farine S and Walter B (2008) Characterization of VvSERK1, VvSERK2, VvSERK3 and VvL1L genes and their expression during somatic embryogenesis of grapevine (*Vitis vinifera* L.). Plant Cell Rep 27:1799-1809.

Schmidt ED, Guzzo F, Toonen MA and de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124:2049-2062.

Shah K, Gadella Jr TWJ, van Erp H, Hecht V and de Vries SC (2001a) Subcellular localization and oligomerization of the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 protein. J Mol Biol 309:641-655.

Shah K, Vervoort J and de Vries SC (2001b) Role of threonines in *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 activation loop in phosphorylation. J Biol Chem 276:41263-41269.

Shah K, Schmidt ED, Vlak JM and de Vries SC (2001c) Expression of the *Daucus carota* somatic embryogenesis receptor kinase (DcSERK) protein in insect cells. Biochimie 83:415-421.

Shah K, Russinova E, Gadela Jr TWJ, Willemse J and de Vries SC (2002) The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1. Gene Dev 16:1707-1720.

Sharma SK, Millam S, Hein I and Bryan GJ (2008) Cloning and molecular characterization of a potato SERK gene transcriptionally induced during initiation of somatic embryogenesis. Planta 228:319-330.

Shiu SH and Bleecker AB (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA 98:10763-10768.

Singla B, Khurana JP and Khurana P (2008) Characterization of three somatic embryogenesis genes from wheat, *Triticum aestivum*. Plant Cell Rep 27:833-843.

Song DH, Li GJ, Song FM and Zheng Z (2008) Molecular characterization and expression analysis of *OsBISERK1*, a gene encoding a leucine-rich repeat receptor-like kinase, during disease resistance responses in rice. Mol Biol Rep 35:275-283.

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB and Harada JJ (2001) *LEAFYCOTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc Natl Acad Sci USA 98:11806-11811.

Thomas C, Meyer D, Himber C and Steinmetz A (2004) Spatial expression of a sunflower SERK gene during induction of somatic embryogenesis and shoot organogenesis. Plant Physiol Bioch 42:35-42.

Torii KU (2004) Leucine-rich repeat receptor kinase in plants: Structure, function, and signal transduction pathways. Int Rev Cytol 234:1-46.

Tosi M (1998) Molecular genetics of C1 inhibitor. Immunobiology 199:358-365.

Ullrich A and Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:103-212.

Wang X, Goshe MB, Soderblom EJ, Phinney BS, Kuchar JA, Li J, Asami T, Yoshida S, Huber SC and Clouse SD (2005) Identification and functional analysis of in vivo phosphorylation

sites of the *Arabidopsis* brassinosteroid-insensitive 1 receptor kinase. Plant Cell 17:1685-1703.
Yang C, Zhao TJ, Yu DY and Gai JY (2011) Isolation and functional characterization of a SERK gene from soybean (*Glycine max* (L.) Merr.). Plant Mol Biol Rep 29:334-344.

Internet Resources

ORF Finder program, http://www.ncbi.nlm.nih.gov/gorf/gorf.html (accessed June 20, 2013).
Protparam program for computation of protein parameters, http://web.expasy.org/protparam/ (accessed June 20, 2013).
SignalP 3.0 Server tool for signal peptide prediction, http://www.cbs.dtu.dk/servicesSignalP/ (accessed June 20, 2013).
TMpred software for transmembrane region prediction, http://www.ch.embnet.org/software/TMPRED_form.html (accessed June 20, 2013).
Zdobnov and Apweiler (2001) ScanProsite program for assessment of protein structure and function, http://us.expasy.org/prosite/ (accessed June 20, 2013).
Arnold *et al.* (2006) SWISS-MODEL program for prediction of tertiary structure, http://swissmodel.expasy.org/ (accessed June 20, 2013).
KinasePhos program for prediction of phosphorylation sites, http://kinasephos.mbc.nctu.edu.tw/ (accessed June 20, 2013).

Supplementary Material

The following online material is available for this article:
Table S1 - Details of the primers used in this study.
Figure S1 - Multiple sequence alignment of SERK family protein kinases.
Figure S2 - Sequence analysis of AcSERK3.
Figure S3 - Exon/intron structure of *AcSERK3*.
Figure S4 - Phylogenetic tree of SERK family proteins.
This material is available as part of the online article from http://www.scielo.br/gmb.

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