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CELL BIOLOGY AND MORPHOGENESIS

Detection of a *SERK*-like gene in coconut and analysis of its expression during the formation of embryogenic callus and somatic embryos

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Abstract Somatic embryogenesis involves different molecular events including differential gene expression and various signal transduction pathways. One of the genes identified in early somatic embryogenesis is *SOMATIC EMBRYOGENESIS RECEPTOR*-like *KINASE* (*SERK*). *Cocos nucifera* (L.) is one of the most recalcitrant species for in vitro regeneration, achieved so far only through somatic embryogenesis, although just a few embryos could be obtained from a single explant. In order to increase efficiency of this process we need to understand it better. Therefore, the purpose of the present work was to determine if an ortholog of the *SERK* gene is present in the coconut genome, isolate it and analyze its expression during somatic embryogenesis. The results showed the occurrence of a *SERK* ortholog referred to as *CnSERK*. Predicted sequence analysis showed that *CnSERK* encodes a SERK protein with the domains reported in the SERK proteins in other species. These domains consist of a signal peptide, a leucine zipper domain, five LRR, the Serine-Proline-Proline domain, which is a distinctive domain of the SERK proteins, a single transmembrane domain, the kinase domain with 11 subdomains and the C terminal region. Analysis of its expression showed that it could be detected in embryogenic tissues before embryo development could be observed. In contrast it was not detected or at lower levels in non-embryogenic tissues, thus suggesting that *CnSERK* expression is associated with induction of somatic embryogenesis and that it could be a potential marker of cells competent to form somatic embryos in coconut tissues cultured in vitro.

Keywords *CnSERK* · Gene expression · Somatic embryogenesis · *Cocos nucifera* (L.)

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Introduction

Different genetic and physiological factors trigger somatic embryogenesis in vitro in several kinds of plant somatic cells. However, the molecular basis of genetic and biochemical mechanisms leading to somatic embryogenesis are not well understood. In the last 10 years, molecular and genetic studies focused in plant development have resulted in the identification of different genes involved in the regulation of this process.

Somatic embryogenesis involves different molecular events including differential gene expression and various signal transduction pathways for activating or repressing numerous gene sets (Chugh and Khurana 2002). Genes involved in somatic embryogenesis are stage specific and one of the genes identified in early somatic embryogenesis is *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*DcSERK*) that was originally isolated from embryogenic cells in suspension cultures of the dicot *Daucus carota* (Schmidt et al. 1997). It was found to be expressed in embryogenic but not in non-embryogenic cultures, in cells predicted to be embryogenic, in tissue explants induced by placing them under embryogenic culture conditions, and during somatic embryogenesis up the globular stage. During *D. carota* zygotic embryogenesis, *SERK* expression occurred up to the early globular stage, but no expression was

found in any other plant tissues, and cells transformed with a *SERK* promoter-luciferase reporter gene were able to form somatic embryos (Schmidt et al. 1997).

Similar findings have been obtained in other dicots. In Arabidopsis, the *AtSERK1* gene was expressed during the formation of embryogenic cells in culture, early embryogenesis, and in planta in developing ovules, specifically in all cells of the embryo sac up to fertilization, and in all cells after fertilization of the developing embryo until the heart stage (Hecht et al. 2001). Arabidopsis seedlings overexpressing *AtSERK1* exhibited a three- to fourfold increase in efficiency for initiation of somatic embryogenesis; therefore, an increase in the level of the *AtSERK1* conferred embryogenic competence in culture (Hecht et al. 2001).

The *SERK* gene has also been isolated in *Medicago truncatula* (Nolan et al. 2003), in which an association was found between somatic embryogenesis and a high expression of the *MtSERK1* gene. This expression was much higher when both the auxin NAA and the cytokinin BAP were in the medium than when the auxin was used alone. In *Helianthus* sp., a correlation was found between the competence of cells to form somatic embryos and an accumulation of *SERK* transcripts (Thomas et al. 2004). In *Theobroma cacao* (de Oliveira et al. 2005) it was found that *TcSERK* was highly expressed in the initial induced embryogenic calluses but also in mature somatic and zygotic embryos, suggesting a role of *TcSERK* in later stages of somatic embryogenesis.

Regarding monocot species, it has been reported that expression of genes of the *SERK* family in maize are associated with embryogenesis induction (Baudino et al. 2001). In *Dactylis glomerata SERK* was expressed in a subpopulation of cells competent to form somatic embryos during the induction of embryogenic cell formation. Expression of the *SERK* gene in *D. glomerata* was found during early embryo development up to the globular stage but also in later stages in meristematic zones such as the shoot apical meristem, scutellum, coleoptile and coleorhiza (Somleva et al. 2000). Recently, in *Oryza sativa* it was reported that a high expression level of *OsSERK1* was detected in callus tissue during somatic embryogenesis, indicating that the *SERK* gene plays a role in mediating somatic embryogenesis in this species as well (Hu et al. 2005).

According to these data, in this work we considered to analyze *SERK* because its expression has been shown to be associated with individual embryogenic cells. Although the mechanism by which *SERK* promotes embryogenic competence is unknown, its expression appears to be strongly related to the process of cellular totipotency, which could be exploited in in vitro culture systems (Verdeil et al. 2007).

Cocos nucifera (L.), is one of the most recalcitrant species for in vitro regeneration, achieved so far only through somatic embryogenesis (Oropeza et al. 2005), although only a few embryos could be obtained from a single explant, plumule tissue being the most responsive. A recent protocol combining secondary somatic embryogenesis and embryogenic callus multiplication (Pérez-Núñez et al. 2006) allowed a significant increase of several thousand-fold in the efficiency of somatic embryo formation for this species. However, the number of embryos formed *per* explant remains low. In order to improve the efficiency of somatic embryo formation per explant, it is important to have a better understanding of this process in this species. No gene related to embryogenesis, has been reported so far in coconut. Therefore, it would be useful to learn if an ortholog of the *SERK* gene is present in the coconut genome, isolate it and analyze its expression during somatic embryogenesis and in somatic tissues, to determine if it can be used as a marker of somatic embryogenesis. These issues are addressed experimentally in this paper.

Materials and methods

Plant material

Embryogenic calluses used for nucleic acid isolation and expression analysis were obtained via secondary somatic embryogenesis as described by Pérez-Núñez et al. (2006). This process consisted of the isolation of somatic embryos formed on embryogenic callus. Isolated somatic embryos were used as explants and transferred to medium I for three months to form embryogenic callus, this medium consisted of Y3 salts (Eeuwens 1976) supplemented with 3 g l^{-1} Gelrite, 2.5 g l^{-1} activated charcoal and 600 µM 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic calluses obtained were transferred to medium II for 30 days to promote the formation of somatic embryos. This medium was the same as used to promote embryogenic callus growth but supplemented with 6 µM 2,4-D and 300 µM 6-benzyladenine (BA). Calluses were sampled at 15, 30, 45, 60, 75 and 90 days from medium I, and at 5, 10, 15, 20, 25 and 30 days in medium II. In order to verify the specificity of the expression of the *SERK* gene, tissues of leaves, roots, and young stems from in vitro plantlets at 6 months of culture as well as zygotic embryos, germinating somatic embryos and non-embryogenic callus were analyzed.

Histology

The histological procedures were carried out according to Buffard-Morel et al. (1992), with slight modifications. Tissue samples were fixed in 4% paraformaldehyde in

0.2 M phosphate buffer (pH 7.2) for 24 h under vacuum. Samples were dehydrated in a stepwise manner (1 h each step) using 30, 50, 70, 80, 90, 96 and 100% ethanol in water. This was followed by impregnation with JB-4 resin (Polyscience, USA). Three micrometer sections were prepared from the resin-impregnated tissues with a microtome (HM 325, MICROM), equipped with a steel knife blade. The sections were double stained with the Periodic Acid-Schiff reaction (PAS), combined with protein-specific naphthol blue-black. PAS stains starch reserves and cell walls in pink and naphthol blue black specifically stains soluble or reserve proteins dark blue (Fisher 1968).

Nucleic acid extraction

Total RNA was isolated from each sample with Trizol reagent (Invitrogen). Each RNA sample was analyzed by 1% agarose gel electrophoresis, and the quantity was determined by spectrophotometry (OD_{260}). All samples were treated with DNase I (Ambion) to avoid the presence of DNA. cDNA was synthesized using SuperScript II (Invitrogen) according to the directions of the manufacturer. DNA was extracted from tissues cultured in vitro using cetyl trimethylammonium bromide and treated with RNase A to eliminate the presence of RNA.

Isolation of *CnSERK* transcript

Degenerate primers based on conserved sequences were designed. Primer sequences were as follows: Forward 1: 5′-BCN ACT CTK GTT AAT CCA TG-3′; Forward 2: 5′-STT GAY CTT GGR AAT GCA M-3′; Reverse 1: 5′-YGC CAT RCT AAT CAK YTC RAC Y-3′ and Reverse 2: 5′-KGC YTC RAA STC YTC RTC C-3′. Combinations of these primers were used in the PCR amplification utilizing cDNA of embryogenic callus as template. Amplification program was: 94°C 3 min. 1 cycle; 94°C 1 min, 45°C 2 min, 72°C 3 min, 38 cycles; 72°C 15 min 1 cycle. PCR products were analyzed by 1% agarose gel electrophoresis and purified using Nucleo Spin Extract Kit (Macherey-Nagel) according to the directions of the manufacturer. Purified products were ligated in pGEM-T Easy vector (Promega) according to the directions of the manufacturer, cloned into *E. coli* and grown on LB medium using carbenicilline (50 mg ml^{-1}) as selective agent. White colonies were selected and grown in liquid LB medium for 24 h. Plasmids were isolated using the Qiagen Kit (Qiagen) and restricted with *Eco*RI enzyme.

Comparison of sequences

Sequence analysis was carried out at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) using the BLAST service. Sequence alignments were done using the ClustalW program (http://www.ebi.ac.uk/clustalw).

Rapid amplification of cDNA ends

5′ and 3′ ends of the *SERK* transcript were obtained from total RNA using the Gene Racer Kit (Invitrogen). The procedure consisted of dephosphorylation of RNA by Calf Intestinal Phosphatase (CIP) that acts only in non mRNA or truncated RNA but not in complete mRNA. Then, a step of elimination of the phosphate group by a pyrophosphatase enzyme at the 5′ end was necessary to add an RNA oligo. RNA oligo was ligated to the 5′ end by RNA ligase enzyme. cDNA was obtained using Super Script III and oligo dT's attached to a known sequence (provided in the kit). PCR reactions were performed with the kit Ampliqon III. To obtain the 5′ end 5′-Racer Primer and Reverse 2 *SERK* Specific Primer were used, and then a step of nested PCR was performed using 5′-Nested Racer Primer and Reverse 1 *SERK* Specific Primer. To obtain the 3′ end, the procedure was slightly different; first a PCR reaction using 3′-Racer Primer and random primers (Invitrogen) was done; finally, a nested PCR step was performed using 3′-Nested Racer Primers and random primers. PCR products were purified using Nucleo Spin Extract Kit (Macherey-Nagel), ligated to the TOPO vector (Invitrogen) and cloned in *E. coli* competent cells to sequence them.

Real time-PCR

Specific primers based on the sequence of *CnSERK* gene were designed. These primers were chosen to amplify a coding region of the cDNA of *CnSERK*. Primer sequences were: *CnSERK-Fw*: 5′-TAT CTG GTA CAT TGG TCC CTC-3′; *CnSERK-Rv*: 5′-CTC TTC AGC AGG CAC ATC-3′. These primers were used in the Real Time-PCR and in in situ PCR assays. The amplification parameters were as follows: 94°C 2 min 1 cycle; 94°C 50 s, 50°C 50 s, 72°C 1 min, 36 cycles; 72°C 10 min 1 cycle. Real Time-PCR reaction mix consisted of the reagents of Ampliqon III and SYBR GREEN I (Invitrogen). Expression of the 18S ribosomal subunit gene was used as internal control. Three independent assays were carried out. Real Time-PCR assays were developed in iCycler IQ real-time PCR detection system (BIORAD). Real time PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with appropriate validation experiments performed beforehand (Real-time PCR. Applications guide. BIORAD). Amplified cDNAs were cloned and sequenced to check the specificity of the amplification products.

In situ RT-PCR

Detection of specific gene transcripts of *CnSERK* in tissue sections was performed according with the methodology reported by Koltai and Bird (2000), with modifications. Briefly, callus samples at different stages of development were fixed in 4% paraformaldehyde in DEPC treated water for 24 h at 4°C, washed two times in 1× PBS for 5 min each, and kept in this buffer at 4°C until the processing. Samples were included in 5% agarose prepared with 1× PBS buffer and kept at 4°C for around 4 h. Sections of around 40–50 μm were obtained using a microtome (HM 325, MICROM). Three to four sections were placed in PCR tubes and treated with 100 μl of Proteinase K (1 μg ml^{-1}) and incubated at 37°C for 30 min. Subsequently, they were washed two times with PBS and incubated in 0.2% (w/v) glycine for 5 min. at room temperature. Sections were treated with DNase I (Ambion), incubating over night at 37°C, and washed three times with ultrapure water (Sigma). RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* (12574–026, Invitrogen) according to the directions of the manufacturer, adding 0.5 μl of Digoxigenin (Roche) in the reaction mix. After the RT-PCR reaction sections were washed two times in PBS and incubated with 1% (w/v) blocking reagent for 30 min, then sections were incubated with the antibody anti-Dig (Roche) coupled to alkaline phosphatase enzyme. Antibody was previously diluted 1:500 in a solution consisting of 0.1% (w/v) BSA and 0.3% (v/v) Triton X-100 for 1 h at room temperature. Sections were washed in the same solution without the antibody three times for 5 min. Then, sections were washed two times for 15 min each in a buffer consisting of 100 mM *Tris*-HCl, 100 mM NaCl and 50 mM MgCl pH 9.5. Sections were incubated in 100 μl of NBT-BCIP (Roche) for 10 min in the dark, washed in distilled water for 10 min and in ethanol for 10 min. Negative controls included elimination of the RT step, omission of primers or a preincubation with ribonuclease (RNAse A, Sigma R4642). Finally, sections were observed with a Nikon stereomicroscope.

Results

In vitro culture

Callus used in these studies was obtained via secondary somatic embryogenesis and multiplication of embryogenic calluses. Isolated embryogenic structures were used as initial explants. At 15 days in medium I, the formation of initial callus was observed, which showed the presence of meristematic cells with densely stained cytoplasm in peripheral tissues (Fig. 1a). At 30 days calluses started to form translucent structures (Fig. 1b), characterized by the presence of densely stained small meristematic cells located in their periphery (Fig. 1c). After 60 days globular and then elongated structures formed on the surface of the translucent structures (Fig. 1d) with the presence of meristematic cells in the periphery of the callus (data not shown). These structures were completely formed at 90 days of culture (Fig. 1e) showing meristematic centers formed from meristematic cells, which were located along the periphery of the embryogenic structures below a layer of unstained cells forming the protoderm (pd) (Fig. 1f). Meristematic nodules eventually developed into somatic embryos and because of this they are called embryogenic structures; calluses reaching this stage of development are referred as embryogenic.

Embryogenic calluses were transferred to medium II where they started to form proembryos around 15 days of culture (Fig. 1g). Proembryos were formed from embryogenic structures on the periphery of the calluses. Globular somatic embryos were visible after 20 days of culture (Fig. 1h) and calluses bearing germinating somatic embryos were observed after 45 days of culture (Fig. 1i). Calluses representing each stage of development both in media I and II were used for the RNA extraction and isolation of the *CnSERK* transcript and finally for the expression analysis.

Cloning and sequence analysis of *CnSERK* gene

A partial clone of a *SERK* gene ortholog was obtained by PCR using degenerate primers designed on highly conserved sequences of the *SERK* cDNA sequences of Arabidopsis (*AtSERK1, AtSERK2* and *AtSERK3*), maize (*ZmSERK1*, *ZmSERK2* and *ZmSERK3*) and *Helianthus* sp. (*SERK1, SERK2, SERK3* and *SERK4*) (GenBank accession numbers: NM_105841, AF384969, AF384970, AJ277702, AJ277703, AJ400870, AF485384, AF485385, AF485386 and AF485387, respectively). The partial *CnSERK* sequence obtained was 999 bp in length. Reactions of 5′-RACE and 3′-RACE were carried out to generate *CnSERK* mRNA sequence that was 2,240 nt in length; the predicted amino acid sequence is 629 amino acids (GenBank accession number AY791293). Amino acid alignment showed that *CnSERK* aligns with *SERK* genes from other species (Fig. 2), and at the aminoacid level *CnSERK* showed 88% similarity with *AtSERK1* and *AtSERK2* (CAB42254 and AAK68073, respectively), 89% similarity with *DcSERK*, *ZmSERK1* and *ZmSERK2* (AAB61708, CAC37638 and CAC37639, respectively) and the highest similarity was 93% with *OsSERK1* and *BISERK1* (AY652735 and AY463361 respectively).

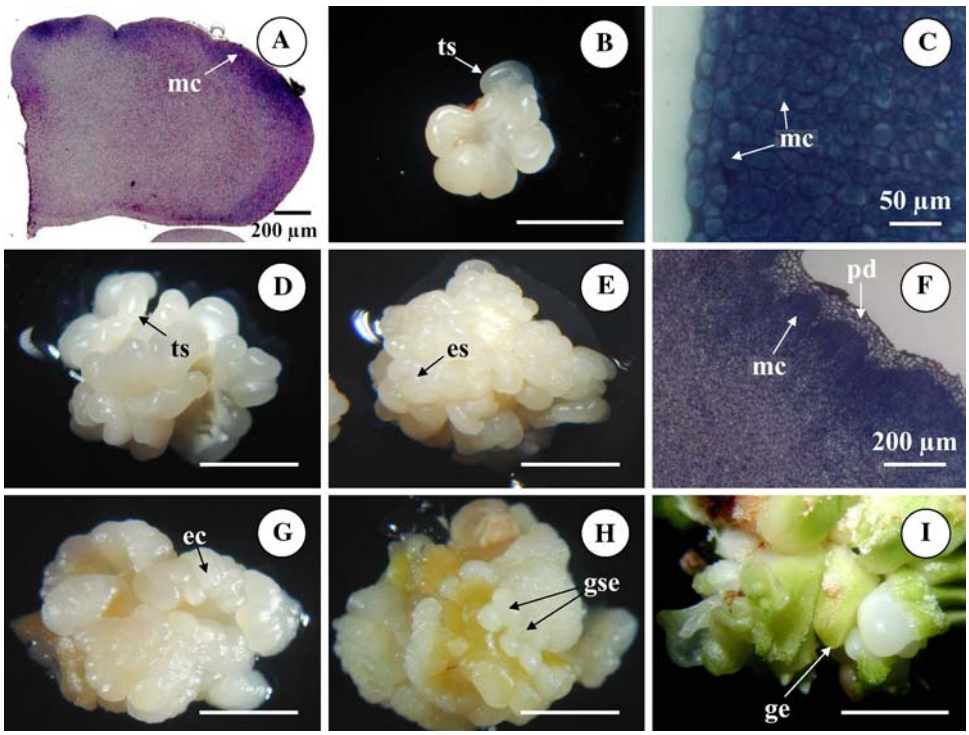


Fig. 1 In vitro development of coconut embryogenic calluses. Histological section of the initial explant at 15 days of culture in medium I showing meristematic cells (**a**). Callus formed from the initial explant at 30 days of culture in medium I showing the formation of translucent structures (**b**). Histological section of the translucent structures showing densely stained small meristematic cells in the periphery of the calluses (**c**). Calluses at 60 days of culture in medium I showing developed translucent structures (**d**). Calluses at 90 days of culture showing the presence of embryogenic structures (**e**). Histological section of callus in **e** showing meristematic cells at the periphery of the callus, under the protoderm (**f**). Embryogenic calluses at 15 days of culture in medium II (**g**). Embryogenic calluses at 25 days of culture showing the formation of globular somatic embryos from the embryogenic structures (**h**). Germinating somatic embryos after 45 days of culture in medium II (**i**). *mc* Meristematic cells, *ts* translucent structures, *es* embryogenic structures, *pd* protoderm, *ec* embryogenic callus, *gse* globular somatic embryos, *ge* germinating somatic embryos. *Bar* (**a**, **f**) 200 µm, (**c**) 50 µm, (**b, d, e, g, h, i**) 1 mm

Analysis of the putative SERK protein of *Cocos nucifera* showed that it contains the domains found in other SERK proteins such as the five Leucine Rich Repeats (LRR) domains (Hecht et al. 2001), the Pro-rich domain containing the Ser-Pro-Pro (SPP) motif, a feature that distinguishes the SERK protein from other LRR-RLK proteins (Hecht et al. 2001), the transmembrane domain and the 11 sub-domains of the kinase domain (Fig. 3).

Phylogenetic analysis

Phylogenetic analysis was done using the Phylip Program. Results showed that the *CnSERK* gene was grouped with the other monocot species and more closely with *OsSERK1* and *BISERK1* (Fig. 4).

Expression analysis of the *CnSERK* gene

Specific primers from the *CnSERK* sequence were designed. These primers were used in the expression analysis of *CnSERK* during one cycle of secondary somatic embryogenesis as well as in different somatic tissues of young plants.

Results of Real Time-PCR analysis showed three peaks of expression during somatic embryogenesis, the first one occurred at 30 days of culture in medium I (callus induction medium). The second peak was observed at 90 days of culture in the same medium and conditions. Finally, a third peak occurred 15 days after embryogenic calluses were transferred to medium II (somatic embryo induction medium).

CnSERK expression in somatic embryos during the germination stage (about 50 days in medium II) was drastically decreased. In non-embryogenic calluses expression of the *CnSERK* gene occurred at similar levels observed in somatic tissues such as leaves and roots. When the expression of *CnSERK* was analyzed in mature zygotic embryos a very low expression was found. The expression of *CnSERK* in different somatic tissues such as leaves, roots and stems was also low, although in the stem tissues the expression was slightly higher (Fig. 5).

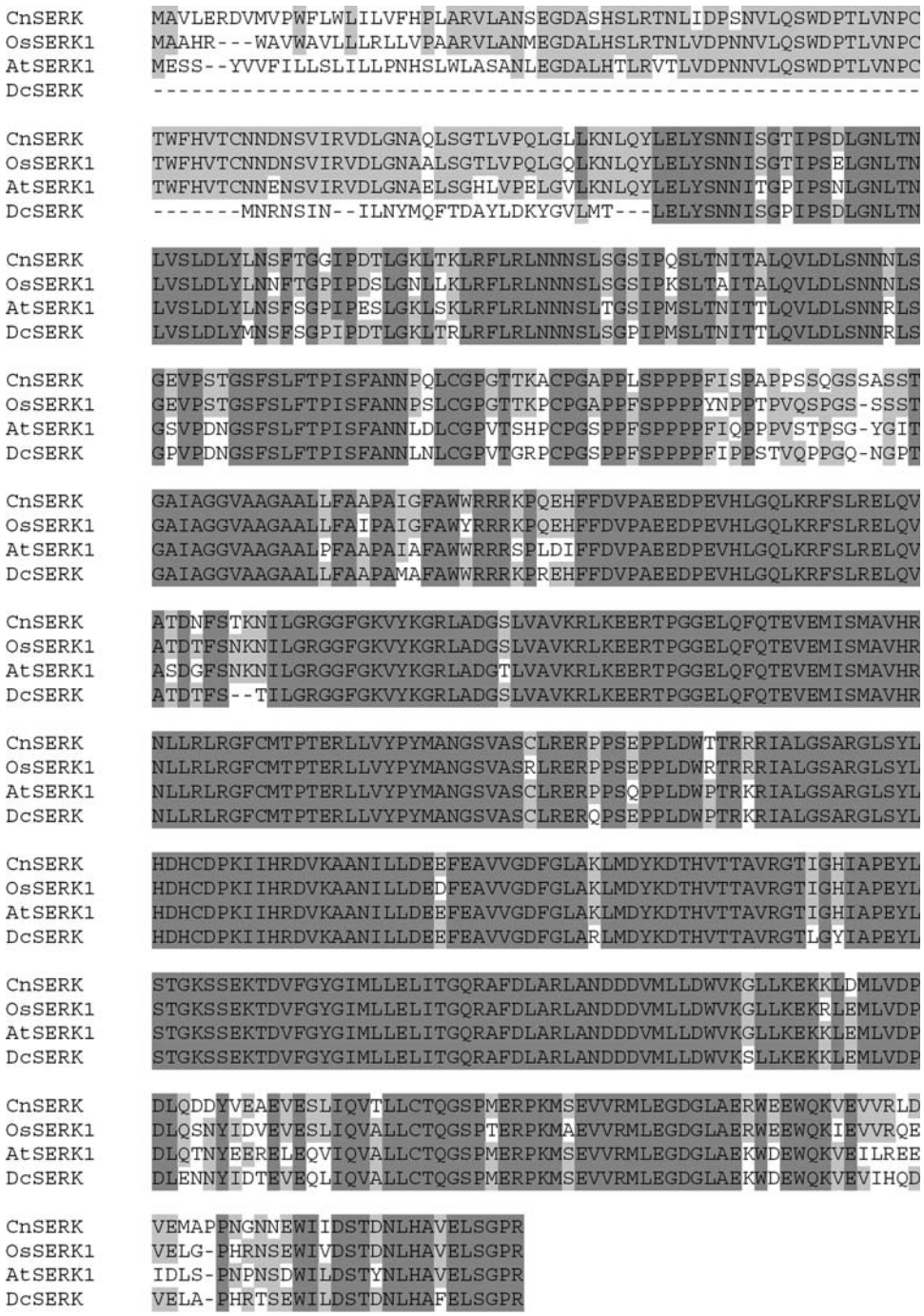


Fig. 2 Alignment of the deduced amino acid sequences of CnSERK with OsSERK1 from rice, AtSERK1 from Arabidopsis and DcSERK from carrot

Results from in situ RT-PCR showed that spatial expression of *CnSERK* occurred basically in regions in the embryogenic structures of the calluses that contained meristematic centers (Fig. 6a), which consist of small cells in active division located under the protoderm (Fig. 6b). When primers were omitted from the reaction no signal was detected in any region of the calluses (Fig. 6C). Similar results were obtained with the elimination of the RT step and a preincubation with a ribonuclease (data not shown).

Discussion

The complete sequence of the transcript of a *SERK* like gene was obtained from coconut in vitro cultures during the present work. The predicted sequence analysis showed that *CnSERK* encodes a SERK protein with the domains reported in the SERK proteins in other species, these domains consist of a signal peptide, a leucine zipper domain, five LRR, the SPP domain, which is a distinctive domain of the SERK proteins that distinguishes SERK

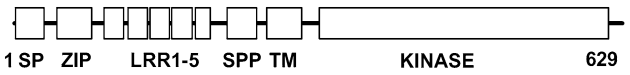


Fig. 3 Graphical illustration of the putative SERK protein in coconut. *SP* Signal peptide, *ZI*: Leucine zipper, *LRR* leucine rich repeat, *SPP* proline-rich region, *TM* transmembrane region, *Kinase* kinase domain

proteins from other proteins belonging to the LRR-RLK family, a single transmembrane domain, the kinase domain with 11 subdomains and the C terminal region.

The high sequence similarity between CnSERK and the SERK proteins of other species such as AtSERK1 (Hecht et al. 2001), ZmSERK1 (Baudino et al. 2001), and OsSERK1 (Hu et al. 2005) suggest that CnSERK1 could play a role in somatic embryogenesis as has been proposed for the orthologs in these species and specifically for AtSERK1. The highest sequence similarity was found with the monocot species.

We analyzed the expression of the *CnSERK* gene in different stages during the process of somatic embryogenesis in coconut tissues cultured in vitro. The results obtained showed that *CnSERK* gene is expressed during this process with three peaks of expression at different times. The first peak of expression occurred at 30 days of culture in medium I, this result coincides with the appearance of the first characteristic morphological features of calluses with embryogenic potential, such as the formation of translucent structures. Also, Islas-Flores et al. (2000) reported the

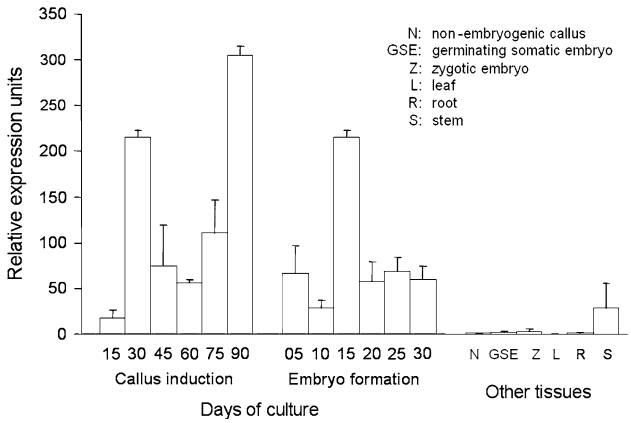
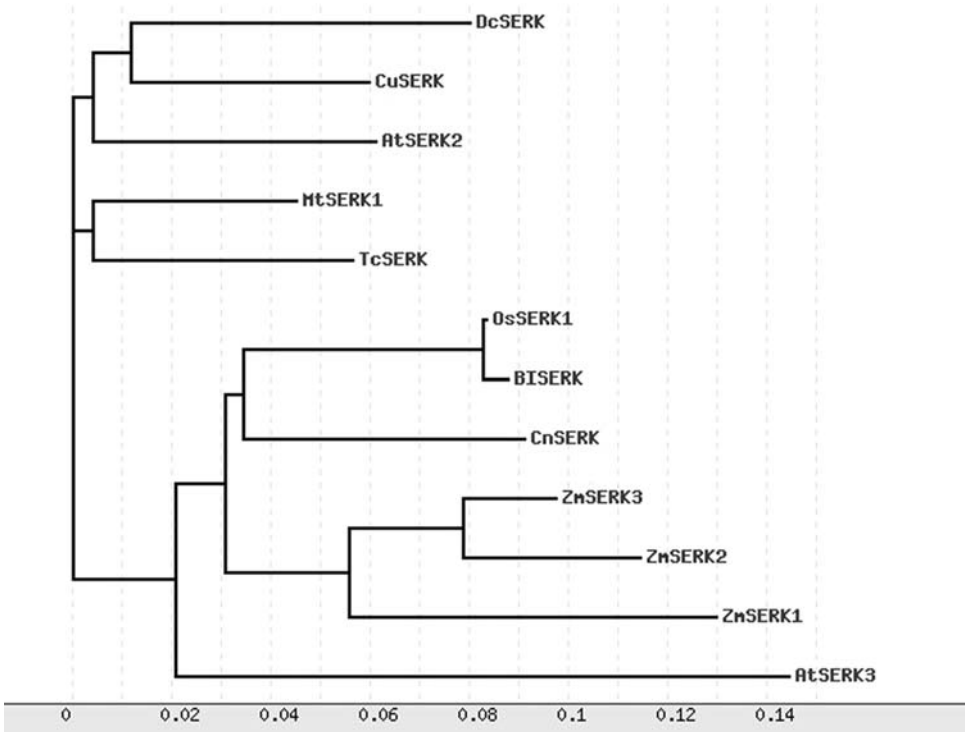


Fig. 5 Real time-PCR of *CnSERK* gene expression in coconut tissue cultures and in somatic tissues

presence of tyrosine phosphorylated proteins and tyrosine kinase activity in coconut plumules cultured in vitro. The enzyme activity was highest in callus formed from the plumule at 30 days, whereas at later stages of development the activity decreased to values close to 0. This peak of tyrosine kinase activity coincides with the peak of expression of the *CnSERK* gene observed at 30 days of culture in the present study. The stage of development at 30 days was the same in both studies since they used the same culture system. Furthermore, it has been reported that SERK protein phosphorylates serine, threonine and tyrosine residues (Shah et al. 2001), so it could be interesting to analyze if the

Fig. 4 Phylogenetic relationship of CnSERK and representative plant SERKs



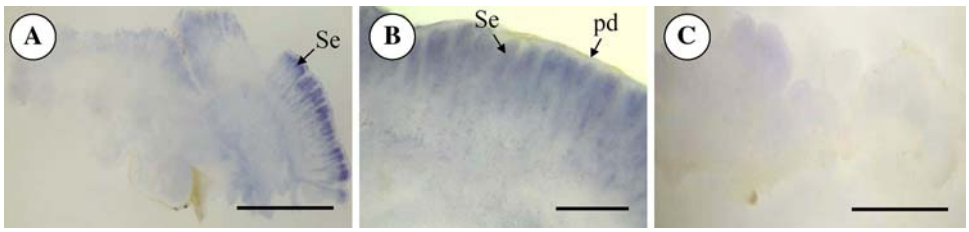


Fig. 6 In situ RT-PCR of the *CnSERK* transcript in embryogenic callus of coconut. Section of an embryogenic callus showing *CnSERK* expression in meristematic centers (**a**). Amplified section of a callus showing *CnSERK* expression in meristematic centers under the protoderm (**b**). Negative control of a callus section (**c**) similar to (**a**). *Se* *CnSERK* expression, *pd* protoderm. *Bar*: (**a, c**) 500 μm, (**b**) 200 μm

tyrosine kinase activity observed by Islas-Flores et al. (2000) could correspond to the SERK protein.

Furthermore, it has been reported that between 20 and 30 days a higher accumulation of 2,4-D occurs in the coconut tissues cultured in vitro (Sáenz et al. 2005). These results support the idea that accumulation of 2,4-D is sensed by cells and could trigger a signaling pathway that induces the expression of genes involved in somatic embryogenesis, including genes that encode for protein kinases such as *SERK*.

The second peak of *SERK* expression was observed at 90 days of culture in medium I. At this time, embryogenic calluses are fully developed, showing meristematic nodules in the periphery of the embryogenic structures, below a layer of unstained cells forming the protoderm, and they are ready for the formation of embryos.

Fifteen days after embryogenic calluses were transferred to medium II, a third peak of *CnSERK* expression occurred, when pro-embryos are forming, and a few days before globular somatic embryos are visible, which occurs between 25 and 30 days of culture in medium II. Therefore these results agree with previous observations in other species, reporting that the expression of the *SERK* gene occurs in the early stages of somatic embryogenesis and can be detected until somatic embryos reach the globular stage (Schmidt et al. 1997; Hecht et al. 2001).

In situ expression analysis showed the presence of the transcripts of *CnSERK* in the periphery of the calluses, in the regions known as meristematic centers that are located under the protoderm. Cells expressing the SERK gene were small, non-vacuolated and with a densely stained cytoplasm indicating a high metabolic activity (Pérez-Núñez et al. 2006; Sáenz et al. 2005). It is interesting to note that considering the quantity of cells expressing the *CnSERK* gene in coconut during in situ expression analysis, the number of somatic embryos is very small (no more than 10 per callus). The same occurs in *D. glomerata* (Somleva et al. 2000) and the authors suggested that this could be related to an arrest at late stages of somatic embryogenesis.

In several monocot and dicot plant species the *SERK* gene has been used as a marker of somatic embryogenesis, and at the tissue level, as a marker of cells competent to form somatic embryos (Schmidt et al. 1997; Somleva et al. 2000; Hecht et al. 2001). Recent data suggest that in Arabidopsis *SERK1* is expressed in the procambium and in the Transit Amplifying (TA) cells, and apparently *SERK1* functions in maintaining the pluripotent fate of these cells. In the presence of 2,4-D this state could be changed to a totipotent state, in which cells have the ability to proliferate and/or regenerate an embryo. This suggests that *SERK1* expression marks and possibly maintains a pluripotent cell population residing in plant vascular tissues (Kwaaitaal and de Vries 2007; Verdeil et al. 2007).

The results obtained in the expression analysis of *CnSERK* gene in somatic tissues, as well as in non-embryogenic calluses, showed that in all cases *CnSERK* expression occurred at lower levels than those observed in embryogenic tissues, thus suggesting that *CnSERK* is associated with somatic embryogenesis induction in coconut. Also, the results of the in situ expression analysis showed that *CnSERK* expression occurred in meristematic centers, where the embryogenic structures are formed, and in turn these structures eventually formed the somatic embryos, thus suggesting that *CnSERK* expression could be used as a marker of cell competence to form somatic embryos in coconut tissues cultured in vitro.

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