



Research article

Cloning and molecular characterization of a putative habanero pepper *SERK1* cDNA expressed during somatic and zygotic embryogenesisDoribet Jiménez-Guillen^a, Daniel Pérez-Pascual^a, Ramón Souza-Perera^b, José Juan Zúñiga Aguilar^{a,*}^a Instituto Tecnológico Superior de los Ríos, Km 3 carretera Balancán-Villahermosa, Balancán 86930, Tabasco, Mexico^b Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, Mérida 97200, Yucatán, Mexico

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ABSTRACT

Background: Plant gene homologs that control cell differentiation can be used as biotechnological tools to study the *in vitro* cell proliferation competence of tissue culture-recalcitrant species such as peppers. It has been demonstrated that *SERK1* homologs enhance embryogenic competence when overexpressed in transformed tissues; therefore, cloning of a pepper *SERK1* homolog was performed to further evaluate its biotechnological potential.

Results: A *Capsicum chinense* *SERK* full-length cDNA (*CchSERK1*) was cloned and characterized at the molecular level. Its deduced amino acid sequence exhibits high identity with sequences annotated as *SERK1* and predicted-*SERK2* homologs in the genomes of the *Capsicum annuum* CM-334 and Zunla-1 varieties, respectively, and with *SERK1* homologs from members of the Solanaceae family. Transcription of *CchSERK1* in plant tissues, measured by quantitative RT-PCR, was higher in stems, flowers, and roots but lower in leaves and floral primordia. During seed development, *CchSERK1* was transcribed in all zygotic stages, with higher expression at 14 days post anthesis. During somatic embryogenesis, *CchSERK1* was transcribed at all differentiation stages, with a high increment in the heart stage and lower levels at the torpedo/cotyledonal stages.

Conclusion: DNA sequence alignments and gene expression patterns suggest that *CchSERK1* is the *C. chinense* *SERK1* homolog. Significant levels of *CchSERK1* transcripts were found in tissues with cell differentiation activities such as vascular axes and during the development of zygotic and somatic embryos. These results suggest that *CchSERK1* might have regulatory functions in cell differentiation and could be used as a biotechnological tool to study the recalcitrance of peppers to proliferate *in vitro*.

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1. Introduction

According to the Product Complexity Index [1], peppers are among the world's most traded products mainly because of their high pungency levels, with habanero pepper (*Capsicum chinense* Jacq.) being one of the most pungent varieties [2]. Peppers belong to a genus in which all species are recalcitrant to *in vitro* proliferation through tissue culture techniques. Limitations in regenerating whole plants from a cell, tissue, and organ explants have delayed the biotechnological breeding of pepper species [3]. Most pepper regeneration and genetic transformation protocols are not

reproducible or cannot be applied to other species of the genus or are based in the *in vitro* regeneration of shoots [4,5]. On the other hand, enhancement of the regeneration competence in peppers through the embryogenic pathway has been achieved by the overexpression of master genes that regulate cell differentiation. Transient induction of the *Brassica napus* BABY BOOM gene in pepper transgenic plants produced a large number of somatic embryos that could be converted readily to seedlings [6]. In addition, the ectopic induction of an *Arabidopsis thaliana* gene encoding a Wuschel transcription factor in the transgenic hypocotyl explants of pepper promoted the development of embryo globular structures [7]. Other regulatory genes such as the *Somatic Embryogenesis Receptor-like Kinase 1* from *Coffea canephora* (*CcSERK1*) lead to an increase in the somatic embryogenesis competence when overexpressed ectopically and also induced the transcription of developmental regulatory genes [8].

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SERK1 homologs are broadly present in monocotyledonous and dicotyledonous plants as members of a multigene family (*SERK1*, *SERK2*, etc.) with different functions [9]; however, all the *SERK1* homologs have in common their accurate expression patterns in tissues with high cell differentiation activity. For example, *AtSERK1* is expressed during megasporogenesis and in all cells of the embryonic sac until the fertilization [10]. Further, during somatic embryogenesis, *SERK1* homologs analyzed are expressed in embryonic cells and embryo structures but not in non-embryonic tissues [10,11,12]. *SERK1* homologs are consistently expressed in somatic cells displaying embryogenic competence and later during early stages of zygotic and somatic embryogenesis [13]; these expression patterns have led to the proposal of *SERK1* as the best molecular marker of embryogenic competence [14].

As the transient overexpression of the *C. canephora SERK1* gene homolog during somatic embryogenesis promoted the differential expression of key cell differentiation regulatory genes, including *LECI1*, *BBM*, and *WUS* [8], it is possible that the *SERK1* product functions as a master coregulator of cell differentiation that could be used to study cell proliferation *in vitro*. Despite the large number of *SERK1* homologs isolated to date, no *SERK1* homologs from pepper have been cloned. Recently, the publication of the DNA genome sequences from three *Capsicum annuum* varieties has been reported online. Analyses of the Zunla-1 and CM-334 pepper varieties predicted the existence of *SERK1* homologs; however, only the predicted *SERK2* homolog in the Zunla-1 variety and the *SERK1* homolog in the CM-334 variety have been annotated, with no supporting experimental data. Because cloning of the *SERK1* homolog from peppers could be potentially exploited as a biotechnological tool to promote cell proliferation competence in these recalcitrant species, the objective of this work was to clone the *SERK1* full-length cDNA homolog from habanero pepper (*C. chinense* Jacq.) and to characterize it at the molecular level as a way to understand its possible role during the zygotic and somatic embryogenesis.

2. Materials and methods

2.1. Plant material and growth conditions

Habanero pepper plants of an orange Criollo variety were grown in pots under greenhouse conditions. Plant tissues (floral bud, flower, stem, root, and leaf) were collected at different stages of development, frozen immediately in liquid nitrogen, and stored at -80°C until needed for RNA extraction.

2.2. Habanero pepper somatic and zygotic embryogenesis

Direct somatic embryogenesis of habanero pepper was conducted with modifications to the method reported by Avilés-Viñas et al. [15]. In brief, hypocotyls were excised from 15-day-old *in vitro* germinated pepper seedlings (MS salts supplemented with $1.1\ \mu\text{M}$ GA3, 3% sucrose, 0.2% Gelrite, pH 5.8) and incubated in an embryogenesis induction medium (MS salts supplemented with $142.36\ \mu\text{M}$ cysteine-HCl, $554.93\ \mu\text{M}$ myo-inositol, $29.64\ \mu\text{M}$ thiamine, $4.50\ \mu\text{M}$ 2,4-dichlorophenoxyacetic acid, 3% sucrose, pH 5.8) at $25 \pm 2^{\circ}\text{C}$ under constant agitation (55 rpm) and light. Embryos at the globular, heart, torpedo, and hypocotyl developmental stages were collected within a period of 30–55 days after the initiation of culture.

To analyze *SERK1* expression during zygotic embryogenesis, tissues were collected at different developmental stages of the fruit [flower at anthesis; the whole fruit buds at 1 day after pollination (DAP); and whole seeds, placenta, and pericarp at 4, 10, 14, 20, 25, 30, 35, 40, and 45 days after anthesis], frozen immediately in liquid nitrogen, and stored at -80°C until they were processed for RNA extraction.

2.3. Cloning of the habanero pepper *SERK1* full-length cDNA

The *CchSERK1* full-length cDNA was cloned according to the protocol described by Pérez-Pascual et al. [8]. In brief, the SMARTer™ RACE cDNA protocol was performed using total RNA isolated from fully opened flowers, following the manufacturer's instructions (Clontech, California, USA). A 927 bp cDNA (GenBank accession CAP15761) previously cloned in our lab with degenerate primers (Table 1) was used as the RACE template. After *in silico* assembling and corroboration of the DNA sequence identities of the 5' and 3' clones in databases (<http://www.ncbi.nlm.nih.gov/BLAST/>), specific primers (Table 1), directed against the 5' and 3' ends, were designed to amplify the *CchSERK1* full-length cDNA by RT-PCR assays using Advantage 2 Polymerase Mix (Clontech, California, USA) and PCR conditions as follows: initial denaturation step at 95°C for 1 min; then 94°C for 30 s, 66°C for 30 s, and 72°C for 3 min, 30 cycles; and a final extension step at 72°C for 10 min. The identity of the cloned RT-PCR product was corroborated by DNA sequencing (Davis Sequencing, Piscataway, NJ) and sequence analysis using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.4. Phylogenetic analysis

The deduced amino acid sequence of *CchSERK1* was obtained using the ExPasy Translate Tool, an online service of the Swiss Institute of Bioinformatics (<http://expasy.org/tools/dna.html>). The sequence of the putative open reading frame was used to run a BLAST (Basic Local 7 Alignment Search Tool, NCBI) analysis to further corroborate the identity of the *CchSERK1* encoded protein, and then, its amino acid sequence was used to run a phylogenetic analysis with the most similar amino acid sequences reported, the five *SERK* Arabidopsis homologs, and the two *SERK* homologs annotated in the pepper genome sequences, using the 14 MEGA 7 program (<http://www.megasoftware.net>), the neighbor-joining (NJ) test, and the p-distance method 16 with 1000 bootstrap repeats.

2.5. Real-time quantitative analysis of gene expression

Total RNA was extracted from the corresponding tissue using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Synthesis of cDNA was performed with 300 ng of total RNA isolated from specific adult organs of the plant, whole seeds at different stages of development, or isolated embryos following the directions of the Maxima H Minus First Strand cDNA Synthesis Kit and dsDNase cat#K1681 (Thermo Fisher Scientific, California, USA). Analyses of relative gene expression were performed by quantitative real-time PCR using the Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific, California, USA) and the $2^{-\Delta\Delta\text{CT}}$ method [16] in a Rotor-

Table 1
Oligonucleotide primers employed in the RT-PCR experiments.

Amplification type	Primer name	DNA sequence (5' → 3')
Degenerate RT-PCR	SecoDir2 SecoRev4	GTCTTG CAGAGTTGGGATCCYAC CCRTTAGCCATGTADGG
3' RACE	CchSERK1-For.1 CchSERK1-Rev1	TTCACCGGTCCATCCGCACTTC TGGCGGTGGTGAACAAATGGAG
5' RACE	CchSERK1-For.2 CchSERK1-Rev.2	CAACCGTCTCAGGTGCTGTCCA TCCTGGGCAAGGACGTCAGTTACA
Full-length CchSERK1 cDNA synthesis	For1 Rev1	ACATGGGGACTTACTTTTTCTCT TTTTTTTCGGTTGTAATAATTTGCT
Real-Time RT-PCR	qChSERK1 For qChSERK1 Rev CchGAPDH For CchGAPDH Rev	CTCTCAGGTGCTGTCCAGA GTGGAGAGAGAATGGAGGA GGCCTGACGAAATCATTCAT TACCAACCCATGTGCTCTA

SERK1-specific signature Ser-Pro-Pro (SPP) motif located in the extracellular domain, the amino acid residues required to display a functional kinase activity in the intracellular domain, and the conserved threonine that defines the substrate specificity of most plant serine/threonine type RLKs [17,18] (Fig. 1). The encoded putative peptide has higher identity levels with the deduced amino acid sequences of the genomic annotated *CaSERK1* and *CaSERK2* homologs from pepper (*C. annuum* Zunla-1 and CM-334 varieties, respectively), and with the SERK1 and SERK2 protein homolog members of the Solanaceae family (Fig. 2).

3.2. Phylogenetic analysis

A dendrogram performed with *SERK1*, *SERK2*, and *SERK3* homologs from *C. annuum* and members of the Solanaceae family revealed four well-defined clades: the first clade groups *CchSERK1* with the *CaSERK1* and *CaSERK2* *C. annuum* homologs (from CM-334 and Zunla-1 varieties, respectively), the second clade includes exclusively *SERK1* homologs, the third clade includes *SERK2* homologs, and the fourth clade includes *SERK3* members exclusively (Fig. 2). The clade corresponding to *CchSERK1*, *CaSERK1*, and *CaSERK2* is much closer to the clade of *SERK1* homologs than to the *SERK2* homolog clade. The reported pepper genomes have not been annotated completely, but the available information suggests the existence of three distinct pepper homologs of the *SERK* gene family (<https://www.ncbi.nlm.nih.gov/genome/genomes/10896>). Interestingly, the phylogenetic analysis presented in the present work grouped the Zunla-1 annotated *SERK2* homolog with the *SERK1* homologs from *C. annuum* and *C. chinense* but not with the Solanaceae *SERK2* homologs. As the annotated Zunla-

1 *SERK2* homolog has no experimental evidence that supports its annotation, it is possible that it could be indeed the *SERK1* homolog. A multiple alignment of sequences and a phylogenetic comparison between *CchSERK1* and the five *SERK* members of *A. thaliana* demonstrated that *CchSERK1* is much closer to the *A. thaliana* *SERK1* and *SERK2* homologs (Fig. S1 and Fig. S2).

3.3. Gene expression of *CchSERK* in plant organs

The relative gene expression levels of *CchSERK1* were measured in different organs and tissues of the adult plant by quantitative RT-PCR. The results showed that *CchSERK1* was expressed at very low levels in leaves and floral buds and at higher levels in stems and placenta (Fig. 3). Interestingly, as compared to its expression levels in leaves, *CchSERK1* expression was almost 15-fold higher in open flowers and 130-fold higher in stems. These expression patterns of *CchSERK1* in whole plants are similar to those displayed by *SERK1* homologs in other plant species. For example, expression of *Citrus unshiu* *CitSERK1* was higher in leaf buds and in the vascular axes of seedling leaves but was lower in the leaves of adult plants [19]. Gene expression of *Ananas comosus* *AcSERK1* was observed in embryogenic tissues and in nonembryogenic calli, but in adult tissues, it was present in leaves and stems [20]. In *Cocos nucifera*, *CnSERK* transcripts were expressed at low levels in mature zygotic embryos, leaves, and roots and slightly higher in stems [12]. In *Medicago truncatula*, *in planta* studies revealed that expression of the *SERK1* homolog (*MtSERK1*) was associated with the primary meristems of the root and shoot and the newly formed meristems of the lateral roots and nodule; *MtSERK1* expression was also associated with the junction between one type of tissue or organ

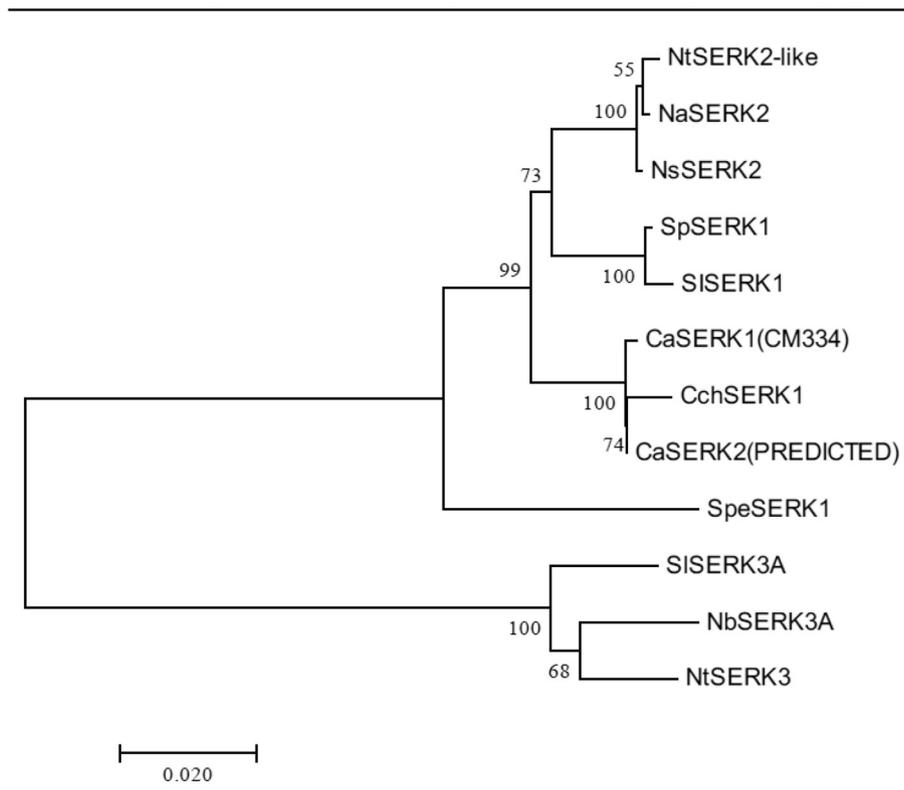


Fig. 2. Phylogenetic analysis of *CchSERK1*. A phylogenetic analysis was run with the deduced amino acid sequence of *CchSERK* and the amino acid sequences of the following *SERK* homologs delivered by a BLASTP analysis ran with the amino acid sequence of *CchSERK1* as probe: *Capsicum annuum* *SERK2* (GenBank accession number XP_016569384.1), *Capsicum annuum* *SERK1* (GenBank accession number PHT84123.1), *Nicotiana sylvestris* *SERK2* (GenBank accession number XP_009773846.1), *Nicotiana attenuata* *SERK2* (GenBank accession number XP_019237009.1), *Nicotiana tabacum* *SERK2*-like (GenBank accession number XP_016455663.1), *Solanum peruvianum* *SERK1* (GenBank accession number ABR18800.1), *Solanum pennellii* *SERK1* (GenBank accession number XP_015073305.1), *Solanum lycopersicum* *SERK1* (GenBank accession number NP_001233866.1). Three *SERK3* homologs from the Solanaceae family were included in the phylogenetic analysis, *Solanum lycopersicum* *SERK3A* (GenBank accession number NM_001247697.1), *Nicotiana benthamiana* *SERK3A* (GenBank accession number HQ332144.1), and *Nicotiana attenuata* *SERK3* (GenBank accession number XM_016586554). The analysis was performed as described in Materials and Methods.

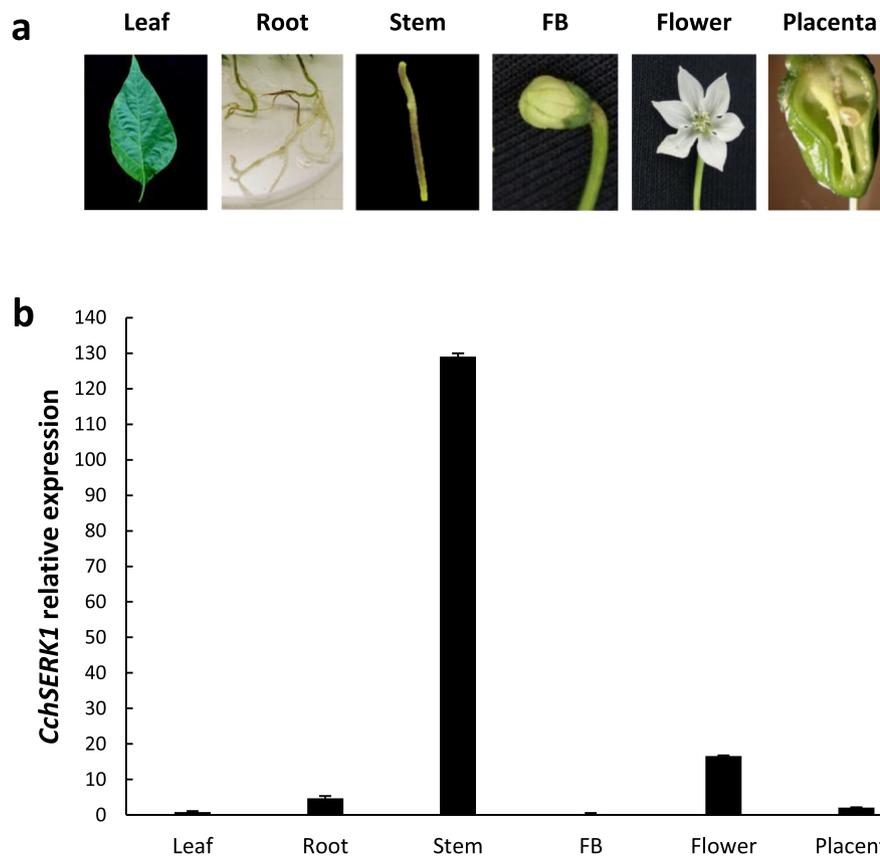


Fig. 3. *CchSERK1* transcription levels in adult organs of the plant. Transcript levels of *CchSERK1* were quantified by the $2^{-\Delta\Delta CT}$ qRT-PCR method (b) using specific oligonucleotide primers and total RNA extracted from leaves, roots, and stems of 2-week-old *in vitro* germinated seedlings, from 4-DAI floral buds, from open flowers of 3-month-old plants cultivated in the greenhouse, and from placenta extracted from 4-DAI fruits (a). The bars over the columns represent the mean value \pm SD of three independent experiments.

and another, and with the vascular tissue procambial cells. Based on these data, the authors concluded that gene expression of *MtSERK1* is associated with tissue developmental changes, possibly reflecting cellular reprogramming [21]. In other reports, gene expression of *AtSERK1* was found in the procambium of the vascular bundles in roots, hypocotyls, and inflorescence stems of *Arabidopsis* [10,22]. As procambium is regarded as a primary meristematic tissue, where procambium cells are differentiated as xylem and phloem cells [23], and *SERK1* expression becomes confined to the procambium, it is therefore proposed that *SERK1* expression marks the procambium vascular cell population to differentiate [22].

The similarity of the expression patterns developed by *CchSERK1* in the present work, with those reported for *SERK1* homologs in the literature suggests that the cloned *C. chinense SERK1* homolog could have similar functions in the regulation of cell differentiation in adult plants.

3.4. Gene expression of *CchSERK1* during zygotic embryogenesis

Analysis of the *CchSERK1* gene expression during zygotic embryogenesis was performed in different tissues of the pepper fruit, as described in the **Materials and methods** section. When they are compared to the relative expression found in the open flowers, the expression patterns during the seed development showed a small reduction at the 10, 30, and 45-DAP stages and a small increment at the 14-DAP stage. The expression remained relatively constant in the other periods analyzed (Fig. 4). Gene expression analysis in nonembryogenic tissues during fruit development revealed that *CchSERK1* was expressed at early stages in the placenta and at low levels in the middle developmental stages in the pericarp (Fig. S3). The peaks of *CchSERK1* expression during fruit development coincide

with peaks at those stages when zygotic embryogenesis was triggered and when the globular to embryo transition occurred. These expression patterns are similar to those exhibited by other *SERK1* homologs during zygotic embryogenesis. In *Daucus carota*, *SERK1* transcripts were detected transiently in zygotic embryos up to the globular stage [14]. In flowers, these transcripts were expressed from 3-DAP, when fertilization occurs, to 20-DAP, which corresponds to the zygotic embryo early globular stage [24]. The authors did not find significant expression in leaves, stems, and roots [14]. In *Zea mays*, expression of *ZmSERK1* was detected in all cells of the embryo sac, except in the synergid, and it was found in zygotes by 36 h after pollination [25]. During the developmental stages of *Arabidopsis* zygotic embryogenesis, high levels of *AtSERK1* mRNA were found in closed floral buds and in pollinated flowers from stages 1 to 7. Quantitative analysis showed that *AtSERK1* transcripts in floral buds were 10-fold higher than those in leaf tissues [10]. The expression of *Medicago truncatula MtSERK1* showed different patterns compared with that in other species during zygotic embryogenesis because transcripts were detected in globular embryos but expression increased at the heart stage, to diminish later at torpedo and cotyledonal stages. In the late stages of embryo development, *MtSERK1* expression was relevant in cotyledons and radicle tips, especially in pro-vascular chain and epidermis. Expression was also detected in the cell layer that surrounds the embryo endosperm [21].

3.5. Gene expression of *CchSERK1* during somatic embryogenesis

Quantitative RT-PCR analysis of *CchSERK1* expression was also conducted during somatic embryogenesis. Results showed that the transcription levels of *CchSERK1* diminished from the globular to the cotyledonal stages but reached a remarkable fourfold peak of

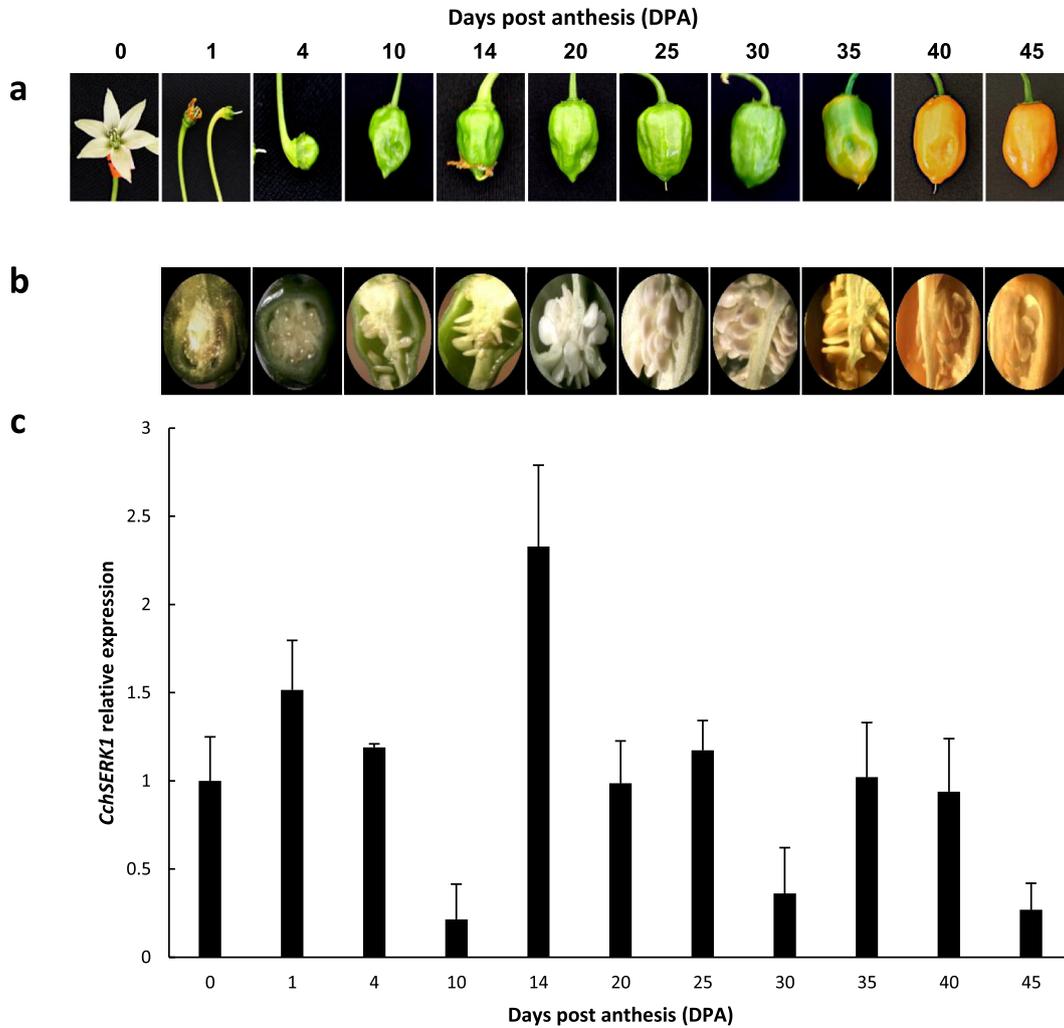


Fig. 4. *CchSERK1* transcription levels during different stages of seed development. Transcript levels of *CchSERK1* were quantified by the $2^{-\Delta\Delta CT}$ qRT-PCR method (c) using specific oligonucleotide primers and total RNA extracted from open flowers, the whole fruit body after 1 and 4-DAL, and from the whole seeds (b) isolated at different stages from 10 to 45 days of fruit development (a). The bars over the columns represent the mean value \pm SD of three independent experiments.

expression at the heart stage as compared to the expression levels found in the non-induced hypocotyl explants (Fig. 5). The expression pattern of *CchSERK1* during the development of somatic embryos was slightly different from those patterns exhibited by many other species, where higher expression was found at the globular stage. During the somatic embryogenesis processes occurring in *A. thaliana*, *D. carota*, and *M. truncatula*, the corresponding *SERK1* homologs showed consistent expression patterns, increasing at the globular stage and then diminishing at the heart and cotyledonal stages [10,14,21]. Nonetheless, gene expression patterns of *SERK1* homologs, which are distinct from this conserved behavior, are found in other species. In *Passiflora edulis*, the expression of *PeSERK*, as assessed by *in situ* hybridization, was predominantly high in the abaxial part of the cotyledonal explants from 10 to 30 days after embryogenic induction; interestingly, neither pro-embryogenic structures (20- to 22-day-old cultures) nor globular embryos (22- to 30-day-old cultures) showed *PeSERK* expression. These results suggested that in *P. edulis*, expression of *PeSERK1* is needed when embryogenic competence is acquired, but not later during embryo development [26], and also demonstrate that, as in *C. chinense*, *SERK1* homologs from different species may show different patterns of expression during somatic embryogenesis, essentially during the transition of the first stages.

While *SERK1* is probably the best molecular marker of embryogenesis, recent findings and reports in the scientific literature demonstrate that it

possesses wider biological functions. It is now accepted that the *SERK1* protein functions as a co-receptor that helps the main receptors to bind to their specific ligands and trigger the correspondent downstream signal transduction pathways [27]. Indeed, *SERK* receptors have been grouped as mode-I coreceptors, which can form complexes with different ligand-binding receptors, allowing *SERK1* receptors to participate in different biological processes [28]. Based in its ability to bind different receptors, it has been demonstrated that *SERK1* is also involved in the coregulation of different cell differentiation pathways, including the control of male sporogenesis [29], and the tapetum development and microspore maturation [30]. It has been demonstrated that *SERK1* complexes *in vivo* with the brassinosteroid receptor (*BRI1*), forming the true hetero-receptor binding site for the steroid. The heteromerization of *BRI1* with *SERK1* leads to the activation of a cytoplasmic signaling cascade, triggering plant growth and cell differentiation [31]. More recently, *in vivo* evidence demonstrated that *SERKs* (*SERK1*, *SERK2*, and *SERK3*) serve as co-receptors in *CLE41/TDIF-PXY* signaling to regulate plant vascular development. As *CLE* peptides have decisive roles in the regulation of proliferation and differentiation of plant stem cells, the authors deduced that *SERK1* acts as a coregulator of these cell functions [32].

The above reports provide strong evidence that *SERK1* homologs participate as coregulator of the cell differentiation processes of plants, including embryogenesis. Thus, it is possible that the gene expression patterns of the habanero pepper *SERK1* homolog observed in this work,

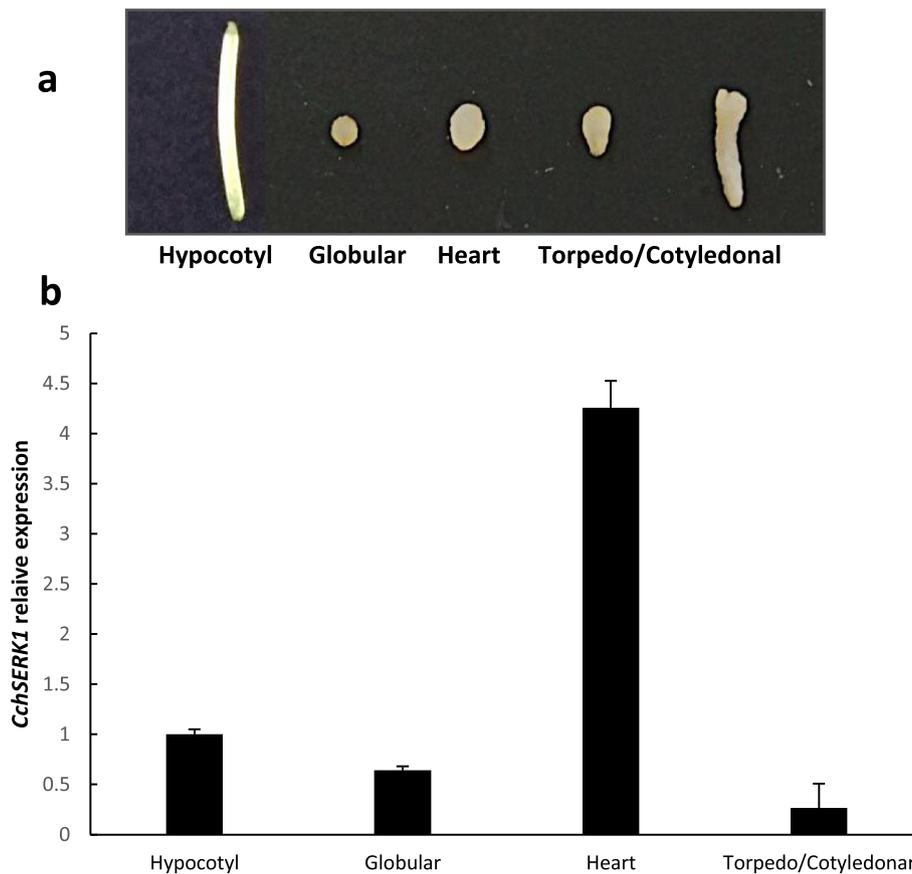


Fig. 5. *CchSERK1* transcription levels during somatic embryogenesis. Transcript levels of *CchSERK1* were quantified by the $2^{-\Delta\Delta CT}$ qRT-PCR method (b) using specific oligonucleotide primers and total RNA extracted from non-induced hypocotyl explants and globular, heart, and torpedo/cotyledonal whole embryo structures (a). The bars over the columns represent the mean value \pm SD of three independent experiments.

both in whole plants and during somatic and zygotic embryogenesis, could be related to its function as a general coregulator of cell differentiation, which is in turn needed to regulate the production of differentiated cells in roots, stem, leaves, and flowers and to trigger the somatic-embryogenic transition and the differentiation of tissues during the transition between every embryogenesis stage.

4. Conclusion

The alignment of the *CchSERK1* cDNA sequence with the DNA sequences of different *SERK1* homologs and the similarity in their gene expression patterns constitute indirect evidence that supports the identity of *CchSERK1* as the *C. chinense* *SERK1* homolog. The expression patterns in tissues with a high differentiation activity suggest that *CchSERK1* has functions in the co-regulation of both zygotic and somatic embryogenesis, as well as in the production of vascular cells. As the deduced amino acid sequence of the encoded protein presents all the protein residues and domains conforming a *SERK1* functional receptor-like kinase enzyme, the cloned cDNA constitutes a powerful tool that can be used to ectopically manipulate the expression of the *CchSERK1* gene; this could be useful to overcome the absence of *SERK1* mutants in peppers and can be used to analyze the molecular regulation of somatic embryogenesis and the recalcitrance of the genus *Capsicum* to proliferate *in vitro* by tissue culture techniques.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary material

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