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Characterization of a Putative *Serk-Like* Ortholog in Embryogenic Cell Suspension Cultures of *Coffea arabica L.*

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Abstract The acquisition of embryogenic cell suspension cultures (ECS) has been the objective of studies on in vitro induction of somatic embryogenesis with biotechnological tools, due to the high efficiency of ECS as plant material for genetic transformation and large-scale production and cryopreservation of germplasm. The objective of this work was to identify and analyze one of the main gene families involved in somatic embryogenesis, somatic embryogenesis receptor-like kinase (SERK) in coffee (*Coffea arabica* L.). Coffee SERKs were identified by searching an EST (expression sequences tag) database generated by the Brazilian Coffee Genome Project starting from candidate sequences obtained from the NCBI database (National Center for Biotechnology Information) . In silico analysis and quantitative PCR results imply that the identified EST-contig C166 might directly be involved in somatic embryogenesis. The results suggest that C166 is the possible ortholog of *SERK* in *C. arabica* (*CaSERK*) and indicate that C166 might be a valuable bio-marker for ECS, and in that context can increase the methodological efficiency for ECS formation in *C. arabica*. Functional analysis of *CaSERK* with mutants of a more manageable species will lead to a better understanding of the molecular regulation as well as the specific functions of genes involved in somatic embryogenesis in coffee.

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Introduction

Somatic embryogenesis is defined as the formation of embryos from competent somatic cells. It is a natural asexual reproduction mechanism that occurs in some plant species which can be induced in vitro by tissue culturing. Experimental conditions to induce somatic embryogenesis are specific to the genotype, tissue, and developmental phase of the mother plant. Obtaining protocols for induction of somatic embryogenesis are therefore practically empirical (Namasivayam 2007).

Histological analyses might increase the methodological efficiency. Cells with embryogenic potential are characterized as being small, isodiametric, having a big nucleus, evident nucleoli, dense cytoplasm, and microvacuoles, and being rich in amiloplasts (Georget et al. 2000; Namasivayam 2007; Quiroz-Figueroa et al. 2006), thereby resembling meristematic cells (Guerra et al. 1999). In embryogenic cell aggregates, found in banana cell suspension cultures, the cells that form the embryo (embryonic) have few amyloplasts and they are surrounded by the embryogenic cells (Georget et al. 2000). Consequently, it makes an established source–sink relationship between cell aggregates. These histological observations were used to classify and evaluate the quality of embryogenic cell suspension cultures (ECS) in banana.

Although the histological analysis are of great methodological value to obtain ECS, it does not allow the detection of the molecular events that induce the morphological differentiation associated with embryogenic potential. Therefore, the detection of those events in the initial phases of the in vitro cultivation may be of fundamental importance to increase efficiency. Quantitative

expression analysis of marker genes during the embryogenic process could be used as a reliable and efficient approach to identify somatic embryogenic cells competent to produce high quality ECS. It is known that several genes are differentially expressed during the induction, development, and maturation phases of the zygotic and somatic embryo. Among all the genes involved in the acquisition of the embryogenic potential, the *SERK* genes (somatic embryogenesis receptor-like kinases) have been considered the most specific markers (Ma et al. 2012; Yang et al. 2011). The acquisition of the totipotent state coincides with the expression of *SERKs* (Ma et al. 2012; Hecht et al. 2001; Namasivayam 2007).

Plant transmembrane receptors are prime components of many signaling cascades and thus are involved in cell fate, growth, and differentiation. *SERKs* encode transmembrane proteins that contain a conserved intracellular kinase domain (Pkc-protein kinase) (Hanks et al. 1988) and an extracellular domain that contains leucine rich repeats (LRR), associated with protein–protein interactions (Diévart and Clark 2004; Kobe and Deisenhofer 1994). LRR domains are only found in plants and more than half of the receptor-like kinases (RLKs) found in *Arabidopsis* contain from 1 to 32 LRRs (Shiu and Bleecker 2001). The LRR domain characterizes the *SERK* proteins as plant-exclusive LRR-RLKs which are clearly different from other RLKs (Hecht et al. 2001; Schmidt et al. 1997). However, *SERK* proteins are distinguished from other LRR-RLKs, since they contain one more extracellular domain rich in proline (SPP) that might be associated with interactions with the cellulosic wall (Hecht et al. 2001).

LRR-RLKs function in several signal transduction mechanisms. In response to a signal, LRR-RLKs form homo- or heterodimers with other RLKs, causing the phosphorylation of the Pkc intracellular domain and triggering a signal transduction cascade (Becraft 1998, 2002; Hecht et al. 2001). Thus, LLR-RLKs probably are involved in several cellular processes, including embryogenesis. Cell–cell communication is important for embryo formation, and one of the communication mechanisms proposed is the signaling through LRR-RLKs (von Arnold et al. 2002). *SERK* function is associated with the perception of signaling molecules that activate the embryogenic process in somatic or zygotic cells (Ikeda et al. 2006; Schmidt et al. 1997). In fact, *SERK* genes are specifically expressed from the earliest stages of the somatic embryogenesis to the globular stage of the embryo (Schmidt et al. 1997). This expression pattern of SERKs is associated with the amount of exogenous auxin required for the undifferentiated cell multiplication to develop callus (Nolan et al. 2003; Sharma et al. 2008; Singla et al. 2008). This requirement may be connected with the thickening of the cell walls (Vasil 1988), which is a possible factor for the pre-embryo ectoderm formation. Pre-embryo ectoderm formation requires periclinal cellular divisions regulated by auxin, and this drives the tissue

organization of the embryo during development (von Arnold 2008).

SERK expression was identified for the first time in individual embryos forming somatic cells in carrot suspension cultures (Schmidt et al. 1997), and it includes the formation of pro-embryogenic masses to the globular stage of embryos in carrot (Schmidt et al. 1997), *Dactylis glomerata* (Somleva et al. 2000), *Arabidopsis thaliana* (Hecht et al. 2001), *Helianthus annuus* (Thomas et al. 2004), *Medicago truncatula* (Nolan et al. 2003), *Ocotea glomerata* (Santa-Catarina et al. 2004), *Citrus unshiu* (Shimada et al. 2005), *Theobroma cacao* (de Oliveira Santos et al. 2005), *Musa* spp. (Huang et al. 2010), *Ananas comosus* (Ma et al. 2012), and *Glycine max* (Yang et al. 2011) . In *Arabidopsis*, the overexpression of *AtSERK*1 caused an increase in the formation rate of somatic embryos (Hecht et al. 2001).

In the present work, putative *SERK* homologs were identified in silico and their expression was analyzed in materials histologically qualified as embryogenic callus (EC), non-embryogenic callus (NEC), and embryogenic cell suspension cultures (ECS) of *C. arabica* cv. Catiguá with the objective of evaluating the use of *SERK* expression as a putative molecular marker of the embryogenic potential of in vitro coffee cultivations during the development of ECS protocols for that species.

Materials and Methods

Finding Putative *SERK* Homologues in Coffee

Candidate *SERK* sequences were obtained from the EST (expressed sequence tag) bank generated by the Brazilian Coffee Genome Project (Vieira et al. 2006). Through the Gene Project interface of that project (http://www.lge.ibi.unicamp.br/cafe), it was possible to find reads and to form clusters from the consultation using the keywords "SERK" and "Somatic Embryogenesis Receptor-like Kinase" and BLAST (Basic Local Alignment Search) (Altschul et al. 1997) of eight nucleotide sequences (BLASTn) of *SERKs* and their corresponding amino acid sequences (tBLASTn) (accessions: AJ863559.1; AY570507.1; EF370120.1; AB188249.1; AF485384.1; AF485385.1; AF485386.1; EF623824.1) deposited in the database of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). From that search, 866 reads that showed significant homology (e-value $> 10^{-4}$) were selected. Those reads were grouped in clusters, forming 175 EST-contigs and 286 singlets. The amino acid sequences of those EST-contigs were deduced using the ExPASY interface (http://ca.expasy.org/tools/dna.html), and the integrity of the Pkc domain present in *SERKs* was verified using the NCBI Conserved Domain Search program. Sequences with the complete Pkc domain were

compared with other *SERK* sequences using the BLASTx algorithm. Of these, 18 EST-contigs that presented similarity were grouped in a dendrograma with *SERK* sequences and their in silico gene expression was analyzed in the libraries of the Brazilian Coffee Genome Project.

In Silico Analysis

Similarity Dendrogram

The sequences of 18 selected EST-contigs, similar to *SERKs* or unidentified genes with complete kinase domain, were aligned (ClustalW) (Thompson et al. 1994) and grouped (MEGA 4) (Tamura et al. 2007) using the *Neighbor-joining* comparison model (Saitou and Nei 1987) by the *p* distance and *pair-wise* suppression method. The validity of the dendrogram as to the distance of the clusters was given by probabilistic bootstrap testing (Sitnikova et al. 1995).

Electronic Northern

In silico gene expression analysis was performed as described by Silva (2013). The frequencies of the reads that form each expressed EST-contig in the libraries were normalized to correct for unequal library sizes. The normalization consisted of multiplying the frequency of each read by the ratio between the total number of reads of all the libraries and the total number of reads of the library in which the given read was expressed. With the normalization results, a matrix was processed using the Cluster and TreeView programs (Eisen et al. 1998), in which libraries and clusters of EST-contigs that were related were grouped by hierachial clustering. From the in silico results, three EST-contigs were selected for quantitative analyses of their expression in plant material.

Identification of Common Grouping Motifs

The MEME program (Multiple Expectation Minimization for Motif Elicitation, http://meme.nbcr.net/meme/) version 4.9.0 (Bailey et al. 2009) was used to elucidate grouping motifs among *SERK* genes (Hecht et al. 2001; Ma et al. 2012; Schmidt et al. 1997) and the sequences of selected EST-contigs. Any number of replications, maximum number of motifs set to 7, and optimum width between 6 and 200 were used as parameters. Motif annotation regarding the functional domains present in *SERK* sequences were carried out using the NCBI Conserved Domain Search program. This program uses a superset of domains including NCBI-curated domains and data imported from Pfam, SMART, COG, PRK, and TIGRFAM.

Plant Material

Embryogenic callus (EC), non-embryogenic callus (NEC), and embryogenic cell suspension cultures (ECS) constituted the plant materials used for the isolation of RNA. These materials were obtained after 5 month of in vitro cultivation of foliar explants of *C. arabica* cv. Catiguá, according to the protocol established by Teixeira et al. (2004) and were characterized by histological analyses.

Histological Characterization

Samples of the plant materials were fixed with FAA_{50} (10 % formalin + 5 % acetic acid + 50 % ethanol, v/v) during 48 h at room temperature, dehydrated in ethanol series (60–100 %) and embedded in epoxy resin (Historesin®, Leica) according to manufacturer's protocol. Five-μm-thick sections were obtained in a manual rotary microtome (Reichert-Jung, 1130), stained with 0.05 % toluidine blue and observed under a light microscope (Zeiss, Axioscope).

Quantification of Gene Expression

The expression of the candidate genes was quantified by RT-qPCR with primers designed using the Primer Express 3.0 program (Applied Biosystems) starting from the sequences of the three in silico-selected EST-contigs and with *ACTIN* and *GAPDH* of coffee (Barsalobres-Cavallari et al. 2009) used as reference genes. For the assays, total EC, NEC, and ECS RNA were extracted in three biological repetitions using the NucleoSpin® Kit (Macherey-Nagel) according to manufacturer's protocol. The absence of DNA was verified with a PCR for the *ACTIN* control. The integrity of the extracted RNA and the elimination of DNA were appraised in 1.2 % agarose gel and the RNA samples were quantified by spectrophotometry (NanoDrop® 1000). The degree of sample purity was determined by the ratio between OD260/OD280 and OD260/OD230, considered the interval between 1.6 to 2.1 as ideal. The RNA isolations of the repetitions were grouped in an RNA pool at a final concentration of 100 ng/μL. Afterwards, cDNAs was synthesized in reverse transcription reactions using the High-Capacity® Kit (Applied Biosystems) according to the manufacturer's protocol. Those reactions were conducted with 10 μL of the kit reaction mix + 10 μL of the RNA pool (1 μg) for 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. With the obtained cDNAs, RT-qPCR reactions were performed (ABI PRISM 7500 Real-Time PCR software v.2.0.1; Applied Biosystems) using SYBR® Green, according to the manufacturer's protocol. The amplifications occurred in three replicas with approximately 10 ng of cDNA+10 ng of primer during 5 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The expression data, resultant from the amplifications,

were normalized [ΔCT=CT target gene) − CT(reference gene)] and relatively quantified (RQ=$2^{-\Delta\Delta CT}$) with NEC as reference sample. NEC samples [ΔΔ CT=ΔCT(sample) − ΔCT(reference sample)]. The efficiency of the reactions was verified by the equation $(1+E)=10^{(-1/slope)}$ (Ramakers et al. 2003). All the primers pairs had efficiency between 0.86 and 1.00.

## Results and Discussion

Histological Characterization of Embryogenic and Non-embryogenic Callus

Studies aiming to establish accurate protocols for somatic embryogenic callus acquisition have been conducted using different approaches. Cell differentiation is the major point that determines when plants enter an embryogenic stage. One amazing property of plants is their ability to regenerate very fast. Culturing the friable embryogenic *C. arabica* cv. Catiguá callus (Fig. 1a) produced fine granular cell suspension cultures (Fig. 1b). The yellowish color of the suspension culture resembled that of a typical embryogenic suspension culture in other coffee varieties (Teixeira et al. 2004). The suspension cultures were comprised of small, spherical embryogenic cells with dense cytoplasm and packed with starch grains (Fig. 1c). These characteristics have been widely used as the morphological markers to obtain somatic embryos in coffee (Ribas et al. 2011; Teixeira et al. 2004) and banana (Georget et al. 2000; Strosse et al. 2003). Non-embryogenic calli with a crystalline structure and large amount of water (Fig. 1a) were not suitable to obtain ECS. In histological analysis, calli constituted of this type of cells exhibit a large amount of vacuolated cells, giving rise to the hypothesis that they might

Fig. 1 Morphology of EC, NEC and ECS. [embryogenic material (*red arrows*); non-embryogenic material (*yellow arrows*)]. **a** EC and NEC after 5 months of cultivation; **b** granular cell suspensions; **c–f** histological sections of (**c**): detail of embryogenic cell in EC showing a prominent nucleus and dense cytoplasm; (**d**), detail of vacuolated cells NEC; heterogeneous ECS (**e**) and EC (**f**) showing embryogenic (densely stained) and non-embryogenic (vacuolated) cell clusters
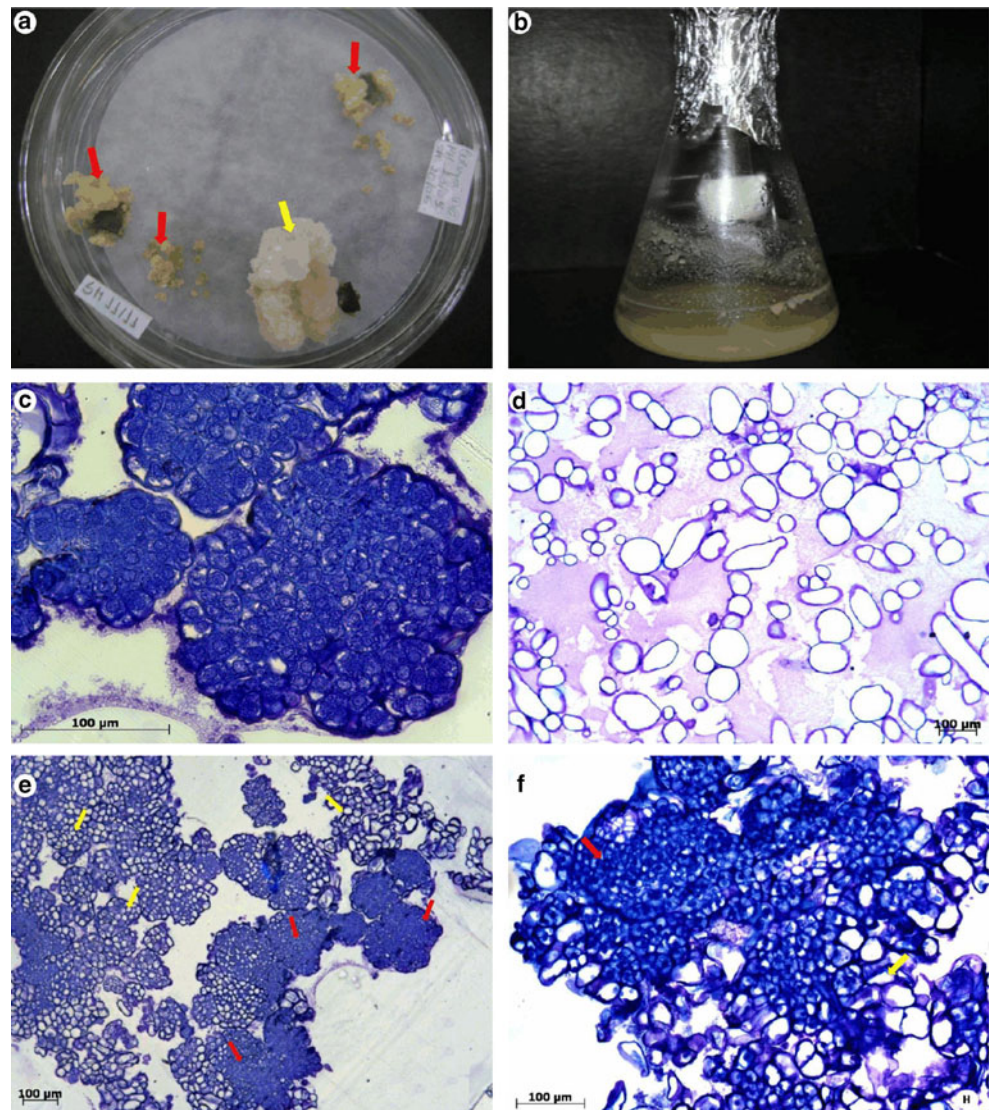
be undergoing a degeneration process (Fig. 1d), while yellowish cell aggregates show densely stained cells. These probably represent the proembryoid phase (Vries et al. 1988) that might be further converted into heart-shaped and cotyledonary embryos. These cells show a very large and intensely stained nucleolus (Fig. 1c) and have highly meristematic characters that are entirely similar to embryogenic cells which can be observed in the initial stages of zygotic embryogenesis (Bieysse et al. 1993). As a result of their rapid division, these cells establish a special area which evolves source–sink relationships that are associated with glycolytic enzymes and proteins involved in energy metabolism (Tan et al. 2013). This morphological structure is a subgroup of a few cells with few amyloplasts (embryonic) surrounded by a large amount of embryogenic cells, rich in amyloplasts (Georget et al. 2000; Recklinghausen et al. 2000). Histological analysis revealed that most active cells were observed in yellowish callus (Fig. 1a). Observation of primary embryogenic callus indicated a heterogeneous structure (Fig. 1e and f) with coexisting degenerating and active areas.
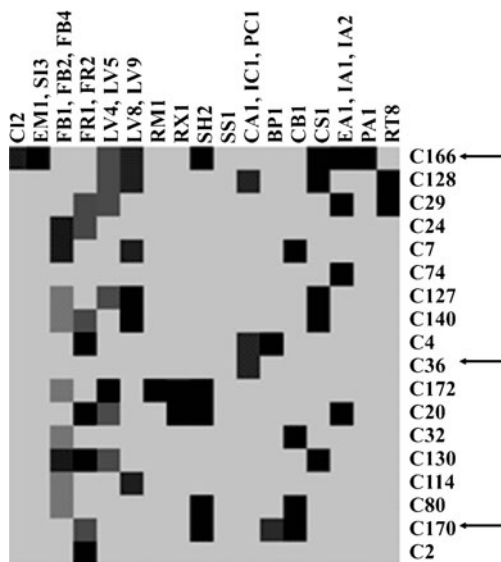

Fig. 2 Electronic northern analysis representing expression levels of EST-contigs in the coffee libraries (the darker the *gray* tones, the higher the expression). *CL2* Hypocotyls treated with acilbenzolar-S-methyl; *EM1*, *SI3* germinating sSeeds (whole seeds and zygotic embryos); *FB1*, *FB2*, *FB4* floral buds in different development stages; *FR1*, *FR2* floral buds+fruitlets at the 1st stage+fruits at different stages; *LV4*, *LV5* young leaves of orthotropic branches; *LV8*, *LV9* mature leaves of plagiotropic branches; *RM1* mature leaves infected with rust and leaf miner; *RX1* branches infected with *Xylella* ssp.; *SH2* hydric stress in the field; *SS1*-well-watered field plants (pool of tissues); *CA1*, *IC1*, *PC1* non-embryogenic callus with and without 2,4-D; *BP1* cells in suspension treated with acilbenzolar-S-methyl; *CB1* cells in suspension treated with acilbenzolar-S-methyl and brassinosteroids; *CS1* cells in suspension treated with NaCl; *EA1*, *IA1*, *IA2* embryogenic calus; *PA1* primary embryogenic calli (*Coffea arabica* L.); *RT8* root and cells in suspension in the presence of aluminum. *Arrows* show the selected EST-contigs
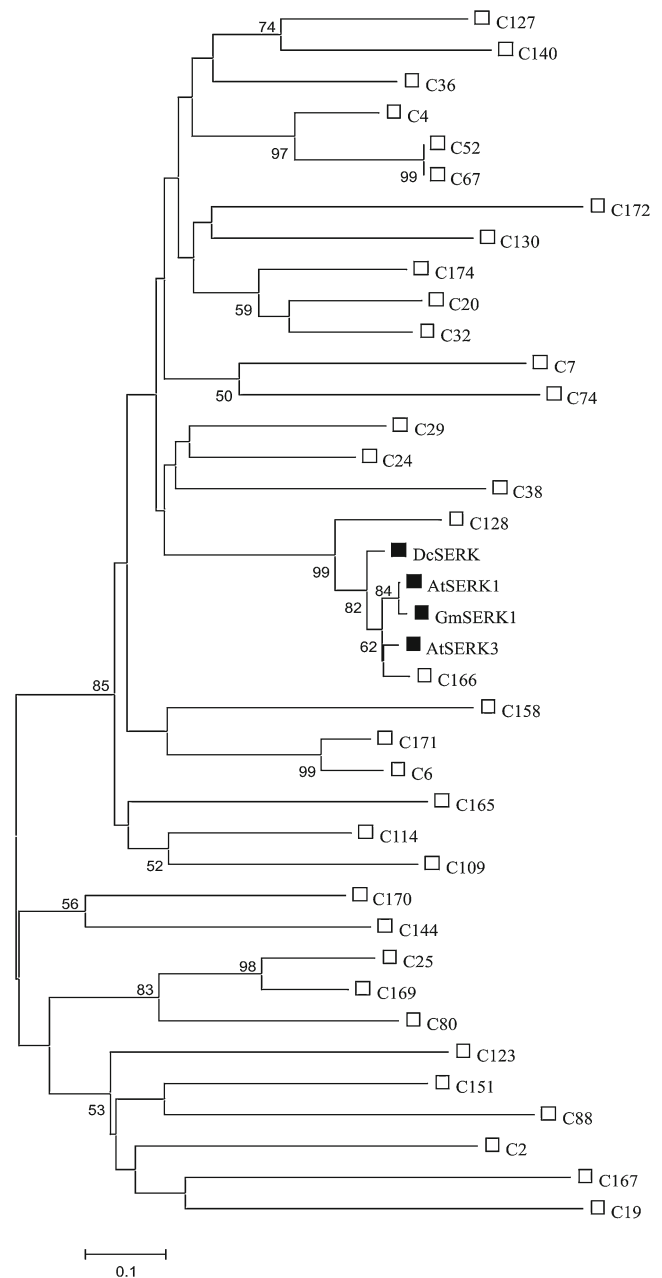

Fig. 3 Similarity dendrogram between amino acid candidate sequences for *SERKs*. (□) EST-contigs; (■) *SERK* sequences (Hecht et al. 2001; Schmidt et al. 1997; Ma et al. 2012); the phylogenetic tree was constructed by the neighbor-joining method and evaluated by 1,000 bootstrap analysis; bootstrap values less than 50 % were omitted

In Silico Analysis and Gene Expression of the Putative *SERK* Homolog

The *SERK* genes belong to a multigenic family that encodes plant-specific membrane receptor-like kinases. A total of 175 EST-contigs with significant similarity to *SERKs* (e -value > 10^{-4}) were obtained from the EST database of the Brazilian Coffee Genome Project. We selected 18 EST-contigs that showed the complete Pkc domain and/or functional
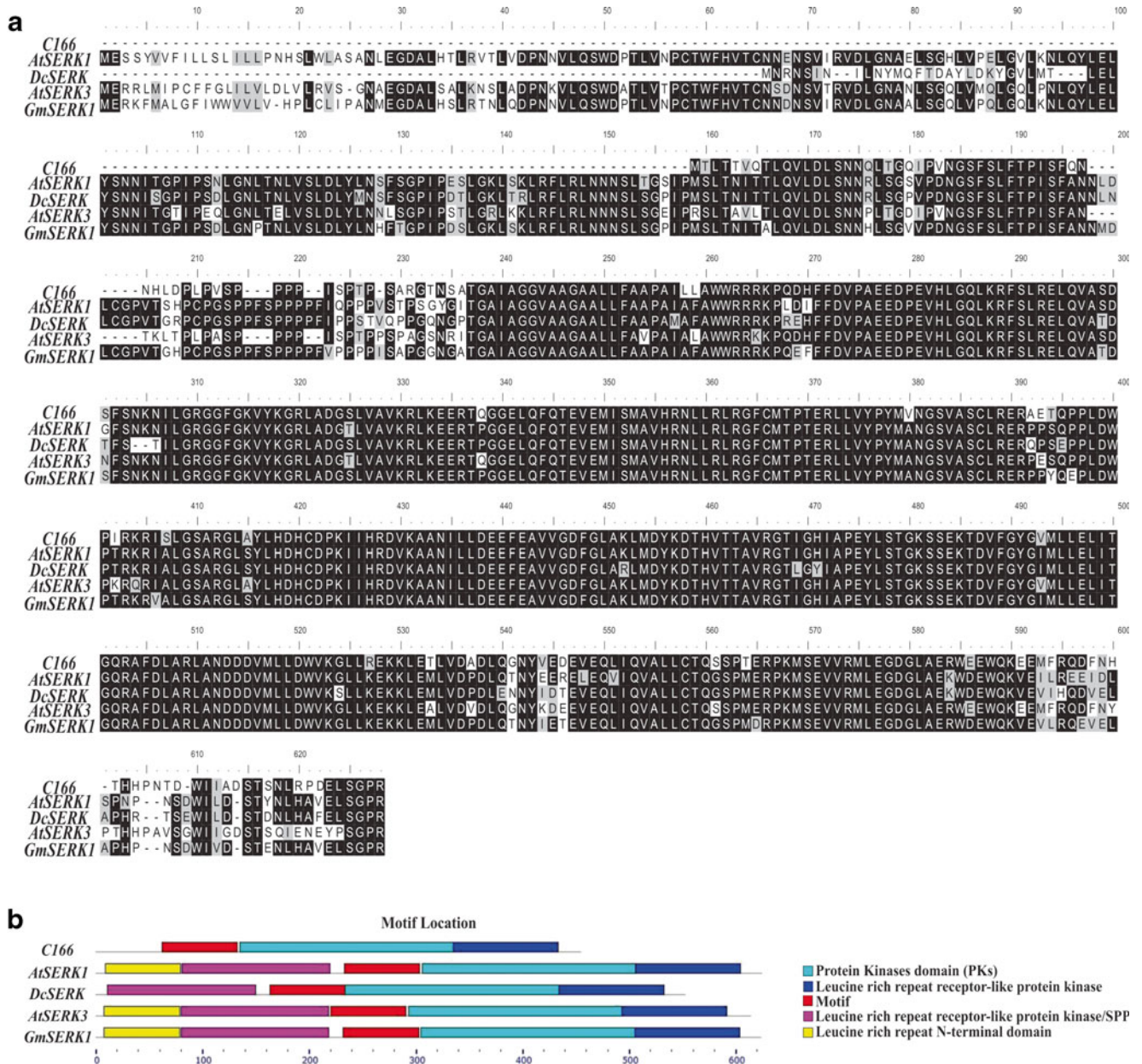
Fig. 4 **a** Multiple alignment of C166 amino acid deduced sequences with homologs *AtSERK* 1, *DcSERK*, *AtSERK3*, and *GmSERK1* showing a high homology of the *SERK* protein. The residues marked in *black* indicate 100 % similarity. **b** Putative domains of the C166 protein compared to other SERK proteins. The different domains are indicated by different *colors*

annotation similar to the *SERK* family. To elucidate the in silico expression of these contigs in the different coffee libraries (Vieira et al. 2006), electronic northern analysis was performed (Fig. 2). EST-contigs that showed expression in both embryogenic materials (EC and ECS) and in non-embryogenic calli (NEC) were discarded for the subsequent in vitro analysis. Based on in silico gene expression analysis (electronic northern), three EST-contigs were selected for further in vitro expression analysis (C36, C166, and C170) based on the fact that (1) C36 only showed expression in the libraries of non-embryogenic calli treated and non-treated

with 2,4-D (CA1, IC1, PC1; Fig. 2) and therefore might be useful as a negative control for embryogenic calli; (2) C166 showed higher expression in the five libraries with embryogenic material [cells in suspension treated with NaCl (CS1), primary embryogenic calli (PA1), and embryogenic calli (EA1, IA1, IA2; Fig. 2); and (3) C170 was only expressed in the EST libraries of embryogenic cell suspension cultures treated with acibenzolar-S-methyl and brassinosteroids (BP1 and CB1; Fig. 2), which might indicate a role in ECS rather than in the embryogenic callus. The EST-contig 166 showed similarity (Fig. 3) and function annotation related to *SERK*
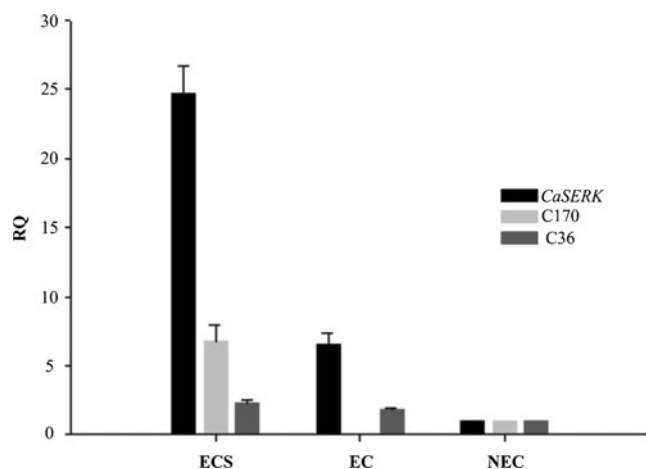
Fig. 5 Profile of relative quantitative expression (*RQ*), obtained by qRT-PCR, from EST-contigs (*C36*, *CaSERK* (*C166*) and *C170*) identified in coffee libraries. The columns represent the gene expression in different embryogenic materials (*ECS* embryogenic cell suspensions culture; *EC* embryogenic calli; *NEC* non-embryogenic calli) of *C. arabica* cv. Catiguá. Expression values = average of three technical replicates; reference genes are *ACTIN* and *GAPDH*; reference sample = NEC

genes. Although C166 probably does not present a complete SERK sequence (Fig. 4a), its identity as SERK is confirmed by the fact that the predicted protein includes main domains observed in SERK proteins of other species (Fig. 4b) (Schmidt et al. 1997; Hecht et al. 2001; Ma et al. 2012). These domains consist of the protein kinase domain, which is responsible for the phosphorylation of the Pkc intracellular domain (2002; Hecht et al. 2001) and leucine-rich repeated domains (LRR) which in general are involved in protein–protein interactions (Kobe and Deisenhofer 1994). Consequently, C166 is termed *CaSERK* as being a SERK-family gene in coffee.

Based on the in silico expression analysis, primers were designed for the sequences of the three selected EST-contigs to evaluate their expression in ECS, EC, and NEC of *C. arabica* cv. Catiguá. The data show (1) high expression of *CaSERK* in EC and especially in ECS; (2) no expression of C170 in EC and relative little expression in ECS; and (3) practically basal expression of C36 in all materials. *CaSERK* shows significant expression in ECS and EC of *C. arabica* cv. Catiguá (Fig. 5). Although *SERK*2 homologs show constitutive expression in rice (Ito et al. 2005), maize (Baudino et al. 2001), and *Vitis vinifera* (Schellenbaum et al. 2008), the higher expression of *CaSERK* in ECS (Fig. 5) implies a differential role of this gene in coffee. Furthermore, *CaSERK* shows homology with *AtSERK*1, *AtSERK*3, *GmSERK1*, and *DcSERK*, which are also expressed in embryogenic materials (Hecht et al. 2001; Schmidt et al. 1997; Ma et al. 2012). Vv*SERK*1 and Vv*SERK*3 in *Vitis vinifera* were also expressed in embryogenic materials and also show homology with *AtSERK* genes (Schellenbaum et al. 2008).

Therefore, considering (1) that C166 might be a true ortholog of *SERKs* due to the prediction of the *SERK* domains

(Hanks et al. 1988; Ma et al. 2012) and its high homology with *AtSERK*1, *AtSERK*3, *GmSERK1*, and *DcSERK* (Figs. 3, 4a), and (2) its in silico expression in embryogenic material (Fig. 2) and in vitro in ECS and EC samples (Fig. 5), it is suggested that *CaSERK* is an important molecular marker candidate for the embryogenic competence of coffee ECS.

Structural analysis of the predicted sequence determined that *CaSERK* encodes for a protein of 455 amino acids that clusters with the *SERK* genes (Fig. 3). EST-contigs 36 and 170 also contain the kinase domain. However, they do not group together with C166 and the other *SERK* genes (Fig. 3). C170 only shows expression in the library of cells in suspension treated with acibenzolar-S-methyl and brassinosteroids (Fig. 2). The in vitro expression of C170 was 3.6-fold lower than *CaSERK* in ECS and it was not expressed in EC (Fig. 5). Regarding the heterogeneity of the plant material (Fig. 1e, f), it was expected that C170 would also be expressed in EC. The fact the we do not observe this is in agreement with the in silico expression, where C170 was only expressed in cell suspension cultures treated with acibenzolar-S-methyl and brassinosteroids (Fig. 2). Taken together, the expression of C170 in silico and in vitro implies that C170 might be associated with the signaling mechanism via brassinosteroids that control the innate immunity of genotypes resistant to pathogens (Karlova et al. 2006, 2009). Brassinosteriod insensitive (BRI1)-associated kinase1 (BAK1), which is a member of the SERK family, also known as *AtSERK3*, is the coreceptor of the brassinolide (BR)-perceiving receptor BRI1, a function that is BR-dependent and partially redundant with *AtSERK1* (Albrecht et al. 2008; Heese et al. 2007). In *Musa* spp, *MaSERK1* is associated with the embryogenic competence of ECS, as well as with resistance against *Fusarium* (Huang et al. 2010). In this study, a cultivar known to be resistant to *Fusarium* expressed high levels of *MaSERK1* while a susceptible cultivar showed no expression. As C170 does not belong to the *SERK* family, it represents a kinase-like protein that could be involved as a cofactor in the probable signaling function of *CaSERK* in homology with the interaction between *AtSERK*1 and *AtSERK3*, since both were expressed in ECS (Fig. 5).

It was expected that C36 would be significantly expressed in NEC since it was restricted to libraries of non-embryogenic calli in the in silico expression analysis (CA1, IC1, PC1; Fig. 2), and it was chosen as a negative control for the embryogenic process. However, the practically basal and constant expression in the ECS and EC heterogeneous material indicate that the expression of C36 might be constitutive (Fig. 5). The most plausible explanation for the detected lowest level of C36 expression in NEC (Fig. 5) is that a large part of the NEC cells in this work were apparently found in an advanced stage of degradation, given the high vacuolation degree and cellular destructuring (Fig. 1d).

The results obtained with EST-contigs 36 and 170 were not conclusive regarding their biological function, nor the proposed use. However, their expression responses might be different in less heterogeneous plant material and/or viable non-embryogenic calli. On the other hand, when obtaining ECS for biotechnological purposes, the plant materials under in vitro development are always heterogeneous, and under these conditions expression of *CaSERK* in *C. arabica* is probably a good evaluation parameter for the embryogenic potential of coffee materials under in vitro development.

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