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Characterization and expression analysis of *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) genes in sexual and apomictic *Paspalum notatum*

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Abstract The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) gene plays a fundamental role in somatic embryogenesis of angiosperms, and is associated with apomixis in *Poa pratensis*. The objective of this work was to isolate, characterize and analyze the expression patterns of *SERK* genes in apomictic and sexual genotypes of *Paspalum notatum*. A conserved 200-bp gene fragment was amplified from genomic DNA with heterologous primers, and used to initiate a chromosomal walking strategy for cloning the complete sequence. This procedure allowed the isolation of two members of the *P. notatum SERK* family; *PnSERK1*, which is similar to *PpSERK1*, and *PnSERK2*, which is similar to *ZmSERK2* and *AtSERK1*. Phylogenetic analyses indicated that *PnSERK1* and *PnSERK2* represent paralogous sequences. Southern-blot hybridization indicated the presence of at least three copies of *SERK* genes in the species. qRT-PCR analyses revealed that *PnSERK2* was expressed at significantly higher levels than *PnSERK1* in roots, leaves, reproductive tissues and embryogenic calli. Moreover, in situ hybridization experiments revealed that *PnSERK2* displayed a spatially and chronologically altered expression pattern in reproductive organs of the apomictic genotype with respect to the sexual one. *PnSERK2* is expressed in nucellar cells of the apomictic genotype at meiosis, but only in the megaspore mother cell in the sexual genotype. Therefore, apomixis onset in *P. notatum* seems to be correlated with the expression of *PnSERK2* in nucellar tissue.

Keywords *Paspalum notatum* · Apomixis · Apospory · *SERK* · qRT-PCR · In situ hybridization

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Introduction

Perception and transduction of external stimuli are essential for growth and development in multicellular organisms. Members of one of the most important groups of cell surface receptors, the receptor-like protein kinases (RLKs), display unique structural features that make them particularly suitable for cell-to-cell signaling. A typical RLK protein contains an extracellular receptor domain to perceive a specific signal, a single-pass transmembrane domain to anchor the protein within the membrane, and a cytoplasmic kinase domain to transduce the signal downstream via autophosphorylation, followed by further phosphorylation of specific substrates (Gou et al. 2010).

A small number of RLKs have been functionally characterized in plants and a few specific ligands have been identified. These RLKs play essential roles in plant growth, development, pathogen resistance and cell death (Gou et al. 2010). Plant RLKs were initially classified as serine/threonine protein kinases (Horn and Walker 1994), but more recent studies indicate that some RLKs may have serine/

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threonine and tyrosine dual kinase activities (Oh et al. 2009).

Based on their sequence and structural similarities *Arabidopsis thaliana* RLKs were classified into more than 10 subfamilies, among which the leucine-rich repeat RLKs (LRR-RLKs) comprise the largest subfamily, containing at least 223 members (Gou et al. 2010; Li 2010). About two-thirds of the genes belonging to this superfamily encode proteins with a typical N-terminal signal peptide and a hydrophobic transmembrane domain, which are consistent structural features of transmembrane RLKs. Despite the identification of a large number of LRR-RLKs in Arabidopsis, biological functions have only been defined for about 30 proteins (Gou et al. 2010), which play crucial roles in a variety of different physiological processes. For instance, ERECTA (ER) regulates organ shape and inflorescence architecture (Torii et al. 1996), CLAVATA1 (CLV1) determines the balance between undifferentiated and differentiated shoot and floral meristem cells (Clark et al. 1997), BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) are involved in brassinosteroid (BR) signaling (Li and Chory 1997; Li et al. 2002; Nam and Li 2002), HAESA controls floral organ abscission (Jinn et al. 2000), FLAGELLIN-SENSITIVE 2 (FLS2) contributes to plant defense/pathogen-recognition (Gómez-Gómez and Boller 2000), VASCULAR HIGHWAY 1 (VH1) influences leaf cell patterning (Clay and Nelson 2002), and EXCESS MICROSPOROCYTES 1 (EMS1), SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1) and SERK2 play important roles in microsporogenesis and male sterility (Zhao et al. 2002; Albrecht et al. 2005; Colcombet et al. 2005). Other LRR-RLKs of known function include RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1), which is involved in abscisic acid early signaling (Hong et al. 1997; Osakabe et al. 2005), TOAD2 and its redundant homologue RPK1, which are required for Arabidopsis embryonic pattern formation (Nodine et al. 2007), PXY, which is responsible for maintaining vascular tissue polarity (Fisher and Turner 2007), and GASSHO1 (GSO1)/GASSHO2 (GSO2), which are essential for normal development of the epidermal surface in Arabidopsis embryos (Tsuwamoto et al. 2008). Moreover, two LRR-RLKs, BIR1 and SOBIR1, were shown to regulate cell death and innate immunity in Arabidopsis (Gao et al. 2009).

The *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)* gene was first isolated from *Daucus carota* (carrot) as a marker gene to monitor the transition from somatic to embryogenic cells in carrot cell cultures (Schmidt et al. 1997). The main feature distinguishing SERK from other RLKs is the presence of a Pro-rich domain containing an SPP motif located between the LRR and the transmembrane domain. The presence of the SPP motif together with precisely five LRRs was used as a criterion to identify all members of the SERK family among numerous LRR-RLKs in the Arabidopsis genome (Hecht et al. 2001). Specifically, five homologues of DcSERK were identified in Arabidopsis, named SERK1 to SERK5 (Hecht et al. 2001). AtSERK1 is expressed in germline cells during megagametogenesis, as well as in pre-heart-stage developing embryos, and is thought to play an important role in embryogenesis (Hecht et al., 2001). AtSERK3 and AtSERK4 were also named BRASSINOSTEROID-INSENSITIVE 1 (BRI1) ASSOCIATED RECEPTOR KINASE1 (BAK1) and BAK1-like1 (BKK1), because of their functions in the BR signaling pathway (Li et al. 2002; Nam and Li 2002; He et al. 2007). In addition, the *SERK* gene family has at least three members each in maize (Baudino et al. 2001), wheat (Singla et al. 2008) and grapevine (Maillot et al. 2009), and eleven members in rice (Singla et al. 2009). Recently, a total of nine *SERK* or *SERK*-like genes have been identified in *Medicago truncatula* and potentially 17 in soybean (*Glycine max* (L.) Merr.). The presence of splice variants was suggested for one of these genes (Nolan et al. 2011). Interestingly members of the SERK family show both significantly overlapped and divergent functions (Albrecht et al. 2008). The identification of *SERK* genes with multiple roles has raised interesting questions about functional specificity and crosstalk among SERK-mediated signaling pathways (Li 2010).

In the last few years, *SERK* expression has been associated with apomictic processes (Albertini et al. 2005; Dusi 2001). Apomixis includes a variable group of plant reproductive behaviors leading to the formation of progenies that are exact genetic replicas of the mother plant (Nogler 1984). Several apomixis mechanisms have been described (Koltunow 1993), however, all of them share common characteristics: neither a meiotic process nor fertilization of the egg cell are involved in progeny generation, so embryos invariably retain the maternal genotype. In particular, gametophytic apomixis is characterized by the formation of non-reduced embryo sacs (by avoiding meiosis), parthenogenetic development of the embryo, and endosperm formation that can be either autonomous or dependent on polar nuclei fertilization (pseudogamy) (Nogler 1984).

Since a key component of apomixis is the fertilization-independent formation of embryos, and the expression of *SERK* genes was reported to mark vegetative-to-embryogenic transitions (Schmidt et al. 1997), an examination of the activity of this gene family in apomictic species was of interest. Tucker et al. (2003) studied the expression of an *AtSERK1* homologue in sexual and aposporous *Hieracium* and reported the presence of *HpSERK1*-like mRNA transcripts from early ovule development until early seed development. However, similar levels of expression were

detected in sexual and apomictic plants. Moreover, transformation with a chimeric *AtSERK1*:*GUS* construct and in situ hybridization experiments using carrot *SERK1* as an antisense probe revealed the same spatial and temporal pattern of expression in both sexual and apomictic plants (Tucker et al. 2003). On the contrary, differential activity was observed between sexual and apomictic genotypes of *P. pratensis* (Albertini et al. 2005). Complete cDNA and genomic sequences for two *P. pratensis SERK* members (*PpSERK1* and *PpSERK2*) were identified (Albertini et al. 2005). The in situ expression pattern in ovules of the two members differed between apomictic and sexual genotypes at late premeiosis. In aposporous genotypes, expression was observed in isolated cells of the nucellus. In sexual genotypes, expression was observed only in the megaspore mother cell (MMC). Based on this evidence, the authors proposed that the expression pattern of *PpSERK* was compatible with its role in the specification of aposporous initials (Albertini et al. 2005).

Paspalum notatum Flügge (bahiagrass) is a rhizomatous species widely distributed from Central Eastern Mexico to Argentina and throughout the West Indies (Burton 1946). The species has an extremely versatile reproductive system with sexual self-fertile diploids and pseudogamous apomictic autopolyploids (Burton 1948; Forbes and Burton 1961). Tetraploid cytotypes (common bahiagrass) are important natural forage resources in tropical and subtropical areas of southern Brazil, Paraguay and northeastern Argentina; they are obligate or highly apomictic (Burton 1955; Ortiz et al. 1997). Apomixis in the species is characterized by aposporous development. In young ovules, the products of meiosis degenerate and embryo sacs develop from somatic cells of the nucellus. As a result, mature ovules bear one or usually several aposporous embryo sacs with cytologically unreduced nuclei. These sacs are characterized by a lack of antipodal cells and hence are easily distinguishable from meiotic sacs. In some ovules the meiotic sac remains together with aposporous sacs (Martínez et al. 2001).

The objective of this work was to isolate, characterize and analyze *SERK* expression patterns in aposporous and sexual genotypes of *P. notatum*. The analyses performed allowed identification of two different members of the *P. notatum SERK* family (*PnSERK1* and *PnSERK2*). *PnSERK1* invariably displayed a lower expression level with respect to *PnSERK2* in roots, leaves, reproductive tissues and embryogenic calli. *PnSERK2* showed a relatively low expression in roots, but a strong representation in leaves, reproductive tissues and embryogenic calli. Differential quantitative and spatial expression patterns were detected in immature ovules of sexual and apomictic genotypes. Moreover, a significantly higher expression level was detected in embryogenic calli from apomictic genotypes with respect to sexual ones. Our results suggest that *PnSERK2* differential expression could be associated with the occurrence of apospory in this species.

Materials and methods

Plant material

The following *P. notatum* genotypes were used: (a) Q4117, an obligate apomictic tetraploid plant (2n = 4x = 40) collected from southern Brazil (Ortiz et al. 1997); (b) Q4188, a fully sexual tetraploid genotype (2n = 4x = 40) of experimental origin (Quarin et al. 2003); and (c) JS-83 and JS-90, fully sexual tetraploid (2n = 4x = 40) hybrids and JS-40 a tetraploid apomictic hybrid, all derived from a cross between Q4188 (pistillate parent) × Q4117 (pollen donor) (Stein et al. 2004). Genotype Q4117 was used to construct six uncloned genomic DNA libraries and isolate *SERK* genomic sequences. Several clonal individuals originated by vegetative propagation from rhizomes of genotypes Q4117 and Q4188 were used in qRT-PCR and reproductive tissue in situ hybridization experiments. Mature seeds originating from self-pollination of Q4117 and from the cross between Q4188 × JS-83 were cultivated in vitro to obtain embryogenic calli (see below). A *P. notatum* reproductive calendar reported by Laspina et al. (2008) was used as a guide for selecting the appropriate developmental stage for both molecular and cytoembryological studies.

Calli induction

Mature seeds originating from self-pollination of Q4117 (apomictic) or the cross Q4188 × JS-83 (both sexual) were incubated at 28 °C for 48 h. They were then treated with concentrated H_2SO_4 for 30 min for dehusking, washed in distillated water and incubated at 40 °C for 24 h to break dormancy. Seeds were surface sterilized with commercial bleach containing 50 % v/v NaClO (35 g/L active chlorine) and 10 μL Tween 20 for 7 min, followed by washing with absolute ethanol for 7 min. Finally, they were washed three times with distilled sterile water and dried on sterile filter paper. Approximately 25–40 seeds were placed in Petri dishes containing MICS media (4.3 g/L Murashige and Skoog (1962) salts + B5 vitamins, 30 g/L sucrose, 5 μM BAP, 30 μM Dicamba) and cultured in the dark at 28 °C for approximately 10 days. Once compact, nodular, white-yellow, embryogenic calli were obtained, fractions of about 5 mm were transferred to the same media every 2 weeks.

Cloning of a *PnSERK* conserved fragment using heterologous primers

DNA extraction was carried out using the CTAB method according to Martínez et al. (2003). Heterologous primers were designed based on consensus maize, rice, Arabidopsis and *P. pratensis SERK* sequences stored in the NCBI (http://www.ncbi.nlm.nih.gov/) database. Primers were designed from conserved regions of *PpSERK* exon 9 and had the following sequences: forward 5′-GCGGCTTTGGTGTTGTTTAT-3′ and reverse 5′-CCTTTCTTTCGAGGTCATGC-3′. These oligonucleotides were used to amplify specific sequences from genomic DNA of genotypes Q4117 and Q4188. Amplification reactions were carried out in a final volume of 25 µL containing 80 ng template DNA, 0.2 µM primers, GoTaq amplification buffer 1× (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 200 µM dNTPs and 1.5 U Taq polymerase (Promega). PCR conditions consisted of an initial denaturing step of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. Amplification products were electrophoresed in low-melting temperature agarose gels and stained with 10 mg/L ethidium bromide. Bands of interest were cut out with a scalpel, purified using phenol/chloroform and cloned into the pGEMT-easy vector (Promega). Clones were sequenced by Macrogen (Korea).

Extension of the original *PnSERK* fragment by chromosomal walking

The initial 200 bp *PnSERK* fragment was extended following the Genome Walker TM Universal Kit protocol (Clontech Laboratories, Inc. Mountain View, CA, USA). Briefly, six uncloned genomic libraries were generated by digesting Q4117 genomic DNA for 2–4 h at 37 °C with *Eco*RV, *Dra*I or *Pvu*II restriction enzymes. Digestion products were linked to the Genome Walker Adapters (see GenomeWalker™ Universal Kit User Manual) following the manufacturer's instructions. Ligation products were used as templates in two successive PCR amplification rounds. In the first reaction, the outer adapter primer AP1 (GenomeWalker™ Universal Kit) and an outer sequence-specific primer were used (Supplemental Material 1). PCR products then served as templates for a second PCR reaction using the nested adapter primer AP2 (GenomeWalker™ Universal Kit) in combination with sequence-specific primers (Supplemental Material 1). PCR products were electrophoresed on 2.0 % agarose gels. Discrete bands were isolated, cloned and sequenced as described previously. The VecScreen tool (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to identify and remove vector segments. The extended fragments were validated by searching the corresponding upper and lower primers used in the PCR amplifications, and aligning the overlapping segments with the original *PnSERK*OF sequence. Alignments were carried out with Sequencher 4.10.1 Demo Version (Gene Codes Corporation). Once a contig was identified, primers were designed at both edges to amplify the complete sequence from the *Paspalum* genome (Table 1). Amplified fragments were cloned and sequenced to confirm the assembly.

Table 1 Primer pairs used for amplifying *Pn*SERK1 and *Pn*SERK2 contigs from genomic DNA of *Paspalum notatum*

Primer name	Sequence
Pnserk1VF	ACTGACAAGGTGATATGTTTCAGTGAC
Pnserk1VR	AAGGTAGAGGAGGTAGGATGGGCAGTT
Pnserk2VF	CTCCTTTTCACTCTTCACCCCTATAA
Pnserk2VR	CTGAGGTTGTAGGTGGAGTCGACGAT

Southern hybridization analysis

To estimate the copy number of *PnSERK* sequences, 30 µg of genomic DNA each from genotypes Q4117, Q4188 and two F_1 hybrid progenies (one apomictic and one sexual) was digested with *Eco*RI, *Hae*III and *Pst*I, using 2.5 U of enzyme per µg of DNA, and incubated overnight at 37 °C. A 711-bp fragment of the conserved region of exon 9 was used as a probe. Labeling, hybridization and non-radioactive detection assays were performed as described by Martínez et al. (2003).

Sequence data analysis

All amplified fragments were queried against the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database using the BLAST 2.2.25 tool (Altschul et al. 1990, 1997) to identify similar sequences. BLASTn and BLASTx were used to compare nucleotides and translated sequences, respectively. Sequences showing the most significant nucleotide and amino acid sequence homology were used in multiple sequence alignments with ClustalW2 (Chenna et al. 2003). The matrix obtained from multiple alignments of plant SERK proteins was used to perform a phylogenetic analysis using PHYLIP version 3.68 (Felsenstein 2005). Ten thousand bootstrap replicates were performed to obtain an unrooted consensus tree. In silico mapping analyses were carried out using the BLASTn or tBLASTx tool at the Gramene (www.gramene.org) and Maizesequence (www.maizesequence.com) websites.

RNA isolation and cDNA synthesis

Young leaves and roots from Q4188 (sexual) and Q4117 (apomictic) genotypes were collected from tillers growing

Table 2 Primer pairs used for relative expression analyses of *PnSERK1* and *PnSERK2* genes in sexual and apomictic *Paspalum notatum*

Primer name	Sequence
Pnserk1F	GCGGCTTTGGTGTTGTTTAT
Pnserk1R	CCTTTCTTTCGAGGTCATGC
Pnserk2F	CTGGAGCTTATTACTGGACAGAGGGC
Pnserk2R	CTCCTACCCTCTTCTCCTTCAGCAGTC
TubF	GTGGAGTGGATCCCCAACAA
TubR	AAAGCCTTCCTCCTGAACATGG
Glu-6-PF	CATCAGAGATGAGAAAGTCAAGGTT
Glu-6-PR	AGCTTTAAGAATGAAAGGAACACCT

under greenhouse conditions. Flowers from both sexual and apomictic genotypes were classified into four different developmental stages: premeiosis, meiosis, postmeiosis and anthesis, based on pollen morphology as indicated by Laspina et al. (2008) and dissected under a stereo microscope. Embryogenic calli were collected after 15 days of culture. Samples were frozen in liquid nitrogen and stored at −80 °C. RNA was isolated from about 30 mg of fresh tissue using the SV Total RNA Isolation System (Promega) following the manufacturer's instructions. Total RNA concentration was determined spectrophotometrically at 260 nm and RNA quality was verified by agarose gel electrophoresis. Only total RNA without visible degradation of the 18S and 28S rRNA was used for subsequent experiments. cDNA samples were prepared from 1 μg of total RNA using the SuperScript™ II Reverse Transcriptase enzyme (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's protocols. The cDNA samples were stored at −80 °C until use.

Real time PCR analysis

Real-time PCR reactions were prepared in a final volume of 15 μL, containing 200 nM of gene-specific primers (Table 1), 1× REALMIX qPCR (Biodynamics, Buenos Aires, Argentina) and 20 ng of cDNA. Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/input.htm) and synthesized by Operon Technologies, USA (www.operon.com/default.aspx) (Table 2). *β-TUBULIN* (*β-TUB*) and *GLUCOSE-6-PHOSPHATE DEHYDROGENASE* (*GPDH*) were used as reference genes, since they were reported to show equivalent expression patterns throughout the reproductive developmental stages in sexual and apomictic *P. notatum* (Ochogavía et al. 2011, Felitti et al. 2011). No-template controls were incorporated in all assays. At least three biological replicates were processed in triplicate (technical replicates). Amplifications were performed in a Rotor-Gene Q thermocycler (Qiagen), programmed as follows: 2 min at 94 °C, 40 cycles of 15 s at 95 °C, 30 s at 55 °C, 20 s at 72 °C. A melting curve (86 cycles of 10 s from 72 to 95 °C, the temperature was increased by 0.5 °C each cycle after cycle 2) was produced at the end of each cycling to control the specificity of amplification products. Amplification efficiency was controlled to be equivalent for targets and reference genes by constructing calibration curves with a dilution series for each gene and slope calculation (Pfaffl 2004). Relative expression of *PnSERK* genes in vegetative tissues and flowers at different reproductive developmental stages was estimated by the standard curve method for both sexual and apomictic genotypes (Pfaffl 2004). A calibration curve was constructed for each target gene (*PnSERK*1 and *PnSERK2*) by plotting Ct values versus log copy number (5, 50, 500, 5,000, 50,000, 5×10^6, 5×10^7 and 5×10^8 copies of cloned fragments). Six replicates were used to define each point of the curve. Calibration curves for the references genes were constructed by plotting Ct values versus log ng total RNA (1/2, 1/5, 1/10, 1/20 dilutions). The relative expression values of *PnSERK1* and *PnSERK2* in each genotype at each developmental stage were calculated by dividing the target gene results (copy number) by the corresponding reference gene results (ng of total RNA). Comparison of the relative expression values between genotypes Q4188 and Q4117 were performed by the Wilcoxon-rank test (Mann–Whitney U) using the statistical package Infostat (http://www.infostat.com.ar); p values lower than 0.05 were considered significant. Moreover, comparative C_t quantifications were calculated with the REST© software (Relative Expression Software Tool V 2.0.7 for Rotor Gene, Corbett Life Sciences) (Pfaffl et al. 2002) using the sexual genotype Q4188 as a calibrator. For analyzing the *PnSERK2* antisense strand expression, strand-specific qRT-PCR assays were performed. cDNA was prepared as described above from flowers of the genotypes JS-90 (sexual) and JS-40 (apomictic), but using the primer Pnserk2F which is complementary to the *PnSERK2* antisense strand (Table 2). In the same reaction, primer TubR (Table 2) complementary to the sense *β-TUBULIN* strand was added to use *β-TUBULIN* as reference gene.

In situ hybridization experiments

In situ hybridization experiments were performed to analyze the expression patterns of *PnSERK* genes in the ovaries of Q4117 (apomictic) and Q4188 (sexual) at different developmental stages. Hybridization was carried out following the protocol described by Laspina et al. (2008), with minor modifications. Briefly, inflorescences at premeiosis/meiosis (stages I/II) and anthesis (stage VII) were fixed in 4 % paraformaldehyde/0.25 % glutaraldehyde in 0.01 M phosphate buffer pH 7.2, dehydrated in an ethanol–xylol series and embedded in paraffin. Specimens were cut

into 7–10 μm thin sections and placed onto slides treated with 100 μg/ml poly-L-lysine. Paraffin was removed using an ethanol-xylol series. Plasmids containing the selected clones were linearized using the restriction enzymes *Nco*I or *Sal*I (Promega). Probes were labeled using the Roche Dig RNA Labeling kit, following the manufacturer's instructions. The probes were hydrolyzed to 150–200 bp fragments. Prehybridization was carried out in 0.05 M Tris–HCl pH 7.5 buffer containing 1 μg/mL proteinase K in a humid chamber at 37 °C for 10 min. Hybridization was carried out overnight in a humid chamber at 37 °C, in 10 mM Tris–HCl pH 7.5 buffer containing 300 mM NaCl, 50 % formamide (deionized), 1 mM EDTA pH 8, 1× Denhardt, 10 % dextransulphate, 600 ng/ml total RNA and 60 ng of the corresponding probe. Detection was performed following the Roche Dig Detection kit instructions, using anti-DIG AP and NBT/BCIP. Three independent experiments, involving at least 20 flowers each, were conducted per genotype and developmental stage.

Results

Cloning of partial *PnSERK* genomic sequences

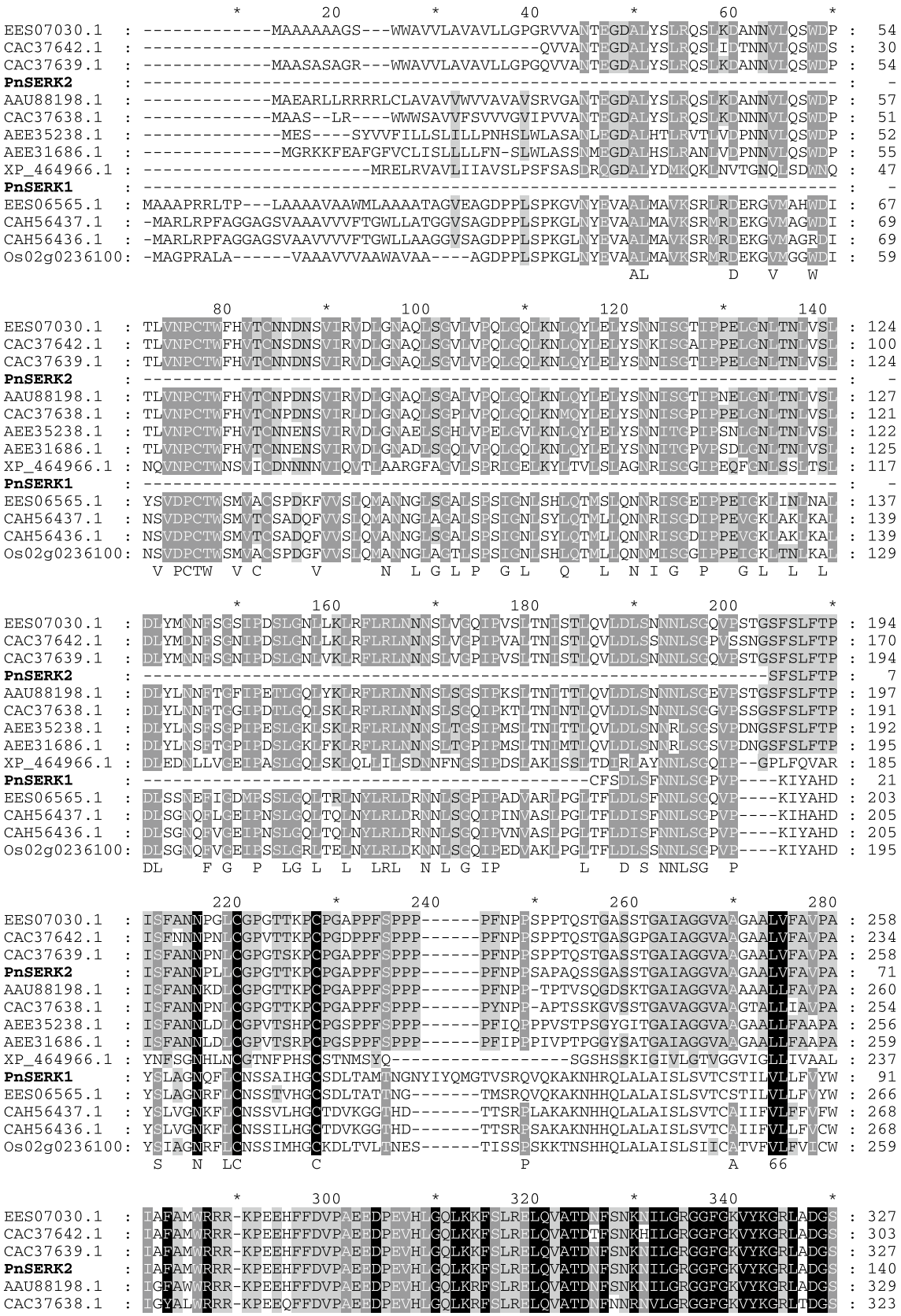
Thirteen known SERK amino acid sequences from *P. pratensis*, rice, Arabidopsis, and maize (AEE35238.1: AtSERK1; AEE31686.1: AtSERK2; AAB61708.1: DcSERK1; EES07030.1: SbSERK2; CAC37642.1: ZmSERK3; CAC37639.1: ZmSERK2; LOC_Os04g38480.1: OsBAK1; AAU88198.1: OsSERK1; XP_464966.1: OsSERK2; EES06565.1: SbSERK1; CAH56437.1: PpSERK1; CAH56436.1: PpSERK2; LOC_Os02g141201: OsBAK1) were aligned using ClustalW2. Initially, primers were designed at the most conserved *SERK* region corresponding to *P. pratensis* exon 9 (see section "Materials and methods"). The other exons were less conserved and did not align in all members of the family. Primers were used to amplify a 200 bp fragment from *P. notatum* genomic DNA, which was cloned and sequenced. The amplicons from both sexual and apomictic genotypes were identical. This small fragment had 93 % similarity to the *P. pratensis SERK2* gene AJ841697.1 (E-val: 4×10^{-70}), 92 % similarity to the *P. pratensis SERK1* gene AJ841698.1 (E-val: 7×10^{-67}) and 92 % similarity to the *Zea mays BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1* (LOC100285968) mRNA gb|EU974959.1| GENE ID: 100285968 LOC100285968 (E-val: 6×10^{-68}). It was named *PnSERK*OF after *Paspalum notatum SERK* Original Fragment.

From this known sequence, chromosomal walking was conducted towards both ends (5′ and 3′) to isolate the complete *SERK* sequences. Six different genomic libraries produced from apomictic genotype Q4117 were used in four rounds of nested amplification reactions to generate two different final contigs named *PnSERK1* and *PnSERK2*, of 1,760 and 2,050 nucleotides, respectively. The correct assembly of both contigs was validated by re-amplification of fragments from genomic DNA using specific primers designed at the edges of the sequences (Table 1), cloning the amplicons and re-sequencing. The sequences of the amplification products showed significant similarities in fragments delimited by the following nucleotide positions: *PnSERK1* 1,513–1,738 and *PnSERK2* 802–1,027 (74 % ID, E-val: 3×10^{-34}); and *PnSERK1* 953–1,269 and *PnSERK2* 1,195–1,516 (65 % ID, E-val: 2×10^{-18}), but were dissimilar in other regions. *PnSERK1* showed high similarity to the original 200 bp segment (*PnSERK*OF) between nucleotides 1,052–1,231 (98 % ID, E-val: 7×10^{-88}). However, *PnSERK2* showed a lower value, which needed a Blast2seq discontiguous megablast to reveal. Homology reached 76 % ID (E-val: 2×10^{-13}) in the following regions: *PnSERK2* 1,248–1,333 and *PnSERK*OF 23–108.

BLAST searches conducted at the NCBI webpage showed that *PnSERK1* (1,760 nucleotides) was highly similar to *P. pratensis SERK2* exons 6–10 (AJ841697.1, 82 % ID, E-val: 0.0), and was split up by the corresponding introns. No homology was detected within the introns. This sequence also aligned with *PpSERK1* (AJ841696.1, 81 % ID, E-val: 0.0), and the *Z. mays BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1* (LOC100285968) mRNA gb|EU974959.1| GENE ID: 100285968, 92 % ID; E-val: 3×10^{-134}). Our results indicated that *PnSERK1* originated from direct extension of the 200 bp original fragment *PnSERK*OF.

On the other hand, *PnSERK2* (2,050 bp) displayed the highest homology to *Z. mays SERK2* exons 6–11 (GenBank: AJ400869.1; GENE ID: 542016 *SERK2*, 85 % ID, E-val: 0.0), split up by the corresponding introns. This sequence also showed homology to *ZmSERK1* (GENE ID: 542015 *SERK1*, 74 % ID, E-val: 0.0), but no significant similarity to the *P. pratensis SERK* genes was detected. These data indicated that *PnSERK*2 had been amplified from the extension of a fragment similar to the original fragment *PnSERK*OF, but must correspond to a related member of the *SERK* family. Based on these results, we concluded that we had cloned two gene fragments

Fig. 1 Alignment of PnSERK1 and PnSERK2 sequences to homologous SERK protein sequences from different species. EES07030.1: SbSERK2; CAC37642.1: ZmSERK3; CAC37639.1: ZmSERK2; AAU88198.1: OsSERK1; CAC37638.1: ZmSERK1; AAEE35238.1: AtSERK1; AEE31686.1: AtSERK2; XP_464966.1: OsSERK2; EES06565.1: SbSERK1; CAH56437.1: PpSERK1; CAH56436.1: PpSERK2; Os02g0236100: OsBAIK1 ▶


EES07030.1
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CAC37639.1
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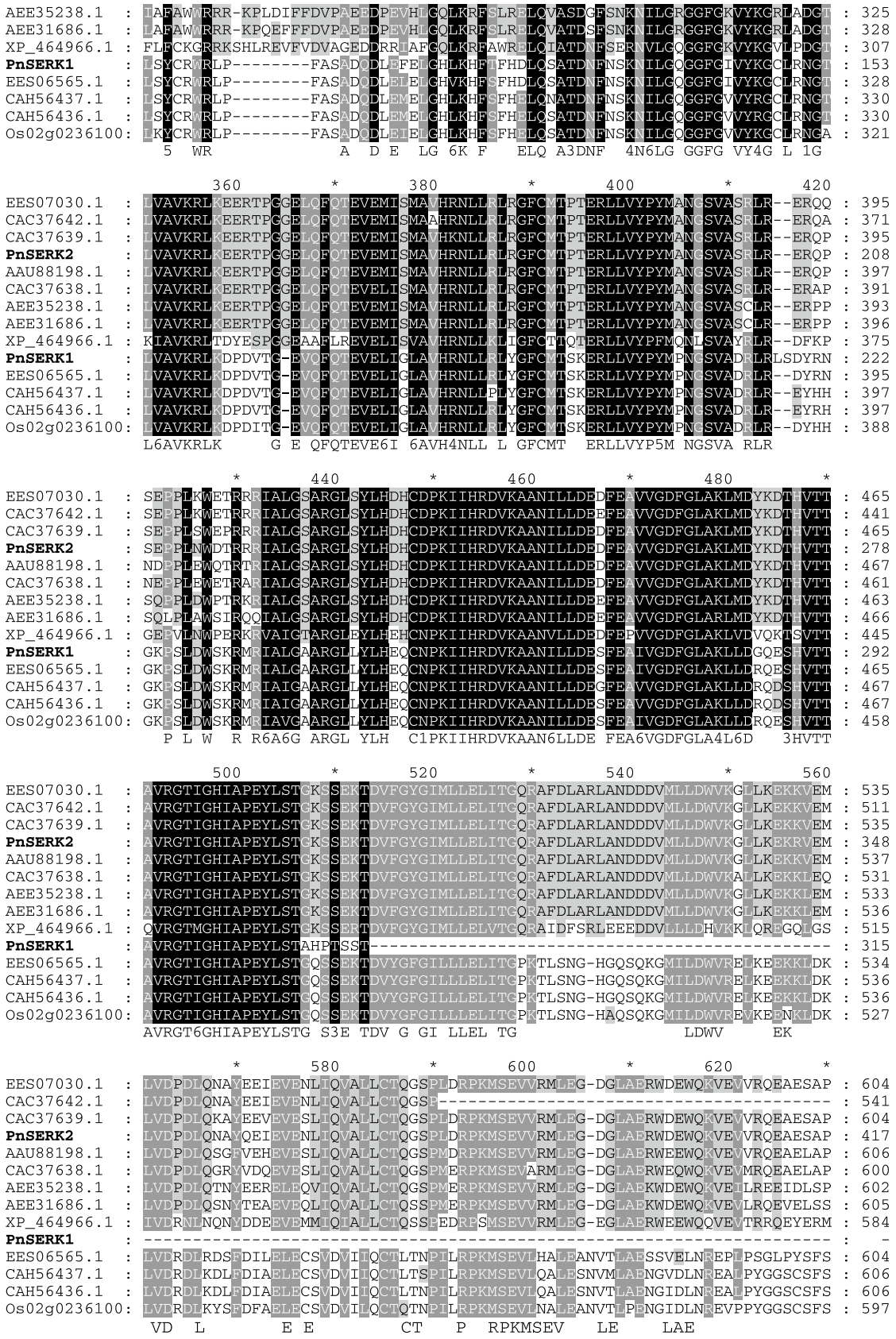

Fig. 1 continued

```
                          640          *
EES07030.1   : LRN-DWIVDSTYNLRAVELSGPR : 626  SbSERK2
CAC37642.1   : ---------------------- :   -  ZmSERK3
CAC37639.1   : LRN-DWIVDSTYNLRAVELSGPR : 626  ZmSERK2
PnSERK2      : LRN-DWIVDSTYNL--------- : 430
AAU88198.1   : RHN-DWIVDSTYNLRAMELSGPR : 628  OsSERK1
CAC37638.1   : RHN-DWIVDSTYNLRAVELSGPR : 622  ZmSERK1
AEE35238.1   : NPNSDWILDSTYNLHAVELSGPR : 625  AtSERK1
AEE31686.1   : HPTSDWILDSTDNLHAMELSGPR : 628  AtSERK2
XP_464966.1  : QRRFDWGEDSVYNQEAIELSGGR : 607  OsSERK2
PnSERK1      : ---------------------- :   -
EES06565.1   : IRHEDPHDSSSFIIEPIELSGPR : 627  SbSERK1
CAH56437.1   : VRHEDPHDSSSFIIEPIELSGPR : 629  PpSERK1
CAH56436.1   : VRHEDPHDSSSFIIEPIELSGPR : 629  PpSERK2
Os02g0236100: VRHEDPHDSSSFIIEPIELSGPR : 620  OsBAIK1
                  D    S       ELSG R
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Fig. 1 continued

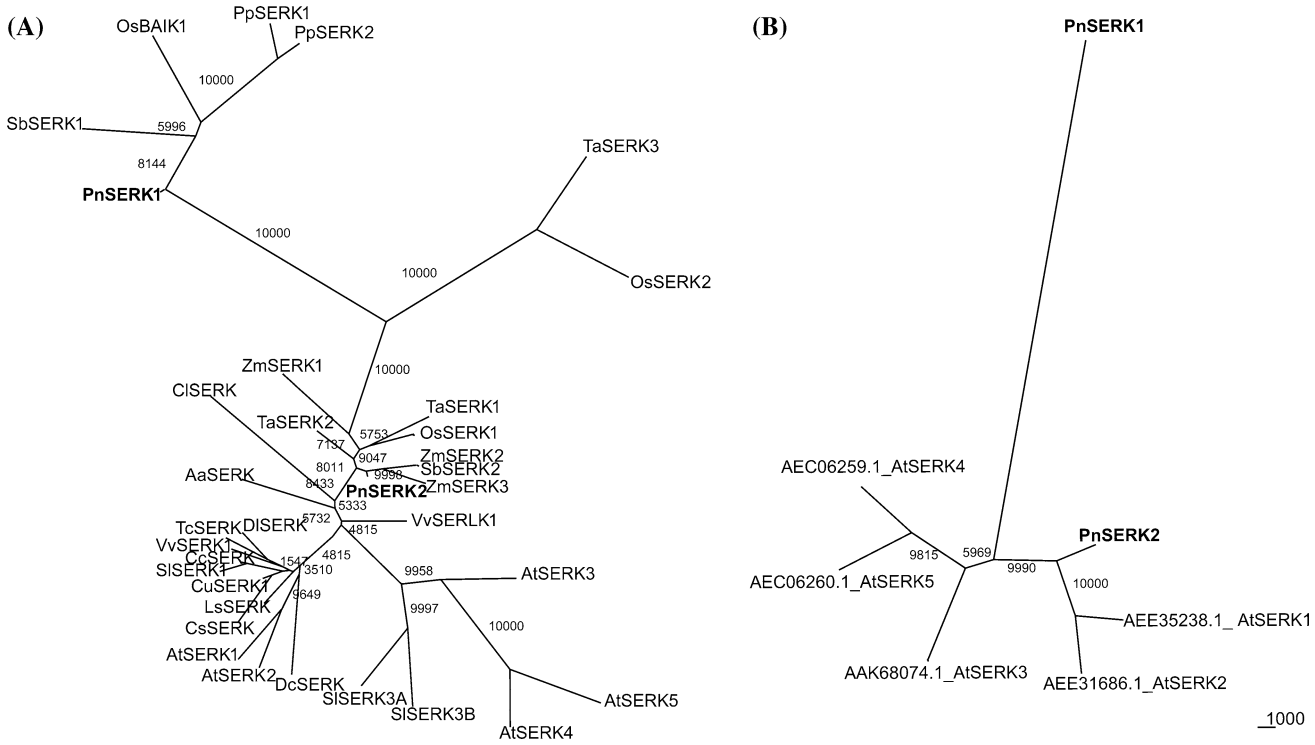


Fig. 2 Phylogenetic analysis of SERK sequences. **a** PnSERK1 and PnSERK2 cluster in different branches of the phylogenetic tree, which includes representatives of different plant species, indicating they represent two different paralogues. **b** PnSERK1 has no putative Arabidopsis orthologue, while PnSERK2 groups with AtSERK1 and AtSERK2

corresponding to different members of the *P. notatum SERK* family (GenBank numbers: *PnSERK1*, KC792581; *PnSERK2*, KC792582).

Phylogenetic studies

The translated amino acid sequences of *PnSERK1* and *PnSERK2* were compared to 12 SERK proteins to analyze the conservation levels of the different domains (Fig. 1). The consensus sequence consisted of 653 amino acids. Both PnSERKs represented partial fragments of the complete protein. The alignment indicated that the PnSERK1 sequence started at consensus sequence amino acid 187 and finished at consensus amino acid 514, while PnSERK2 sequence started at consensus sequence amino acid 204 and finished at consensus sequence amino acid 645. A low level

of conservation was detected in the region 1–44. In contrast, the region 44–300 displayed medium-level conservation and clearly defined two different groups of sequences. While PnSERK1 was included in a group together with SbSERK1, PpSERK1, PpSERK2 and OsBAIK1, PnSERK2 belonged to another group with SbSERK2, ZmSERK2, ZmSERK3, OsSERK1, ZmSERK1, AtSERK1, AtSERK2 and OsSERK2. The region 300–530 was highly conserved among all members (this was precisely the region encoded by *P. pratensis* exon 9 and corresponded to the kinase domain). The region 530–654 presented medium-level conservation, and the same two groups of genes already defined by region 44–300 were observed (Fig. 1).

A phylogenetic analysis was conducted by comparing the PnSERKs with 33 SERK protein sequences originating from different plant species (Fig. 2a). As expected, PnSERK1 clustered with PpSERK1, PpSERK2, OsBAIK 1.1 and SbSERK1. On the other hand, PnSERK2 clustered in a different branch with ZmSERK2, ZmSERK3, and SbSERK2. These results confirmed that PnSERK1 and PnSERK2 were encoded by different gene members.

In addition, to determine the putative Arabidopsis orthologues of *PnSERK1* and *PnSERK2*, we conducted a BLASTx analysis at TAIR (www.Arabidopsis.org). *PnSERK1* showed high similarity with LRR-receptor At5g45780 (E-val: 1×10^{-48}). It also showed homology to all Arabidopsis SERK sequences, but with higher E-values; it was most similar to AtSERK2 (E-val: 1×10^{-36}). *PnSERK2* showed the highest homology to AtSERK1 At1g71830.1 (E-val: 1×10^{-125}). A phylogenetic study conducted with the translated sequences and the program PHYLIP showed that PnSERK1 was not related to the Arabidopsis SERK proteins, but PnSERK2 was closely related to AtSERK1 and AtSERK2 (Fig. 2b).

Genomic hybridization analysis

A genomic hybridization analysis was performed to estimate the approximate copy number of the *SERK* family in *P. notatum* using a 711-bp fragment homologous to both *PnSERK* sequences as a probe. This fragment corresponded to a conserved segment of *PnSERK1*, flanked by positions 1,052–1,760. It matched *PnSERK2* at positions 802–1,027 and 1,195–1,333. The fragment showed 99 % ID (E-val: 0.0) and 74 % ID (E-val: 1×10^{-34}) to the *PnSERK1* and *PnSERK2* consensus sequences, respectively. A relatively low hybridization temperature (63 °C) was used to hybridize both gene members (*PnSERK1* and *PnSERK2*). The two parental genotypes Q4117 (apomictic) and Q4188 (sexual), as well as two F_1 progenies (one sexual and one apomictic) were analyzed, after digesting genomic DNA with three restriction enzymes (*Pst*I, *Eco*RI and *Hae*III). Neither *Pst*I nor *Eco*RI digested the 711-bp sequence internally. However, there was one internal *Hae*III restriction site. Hybridization signals showed a total of 2–3 bands in the samples digested with the enzymes that had no internal restriction sites (Fig. 3). The hybridization pattern in DNA samples digested with *Hae*III displayed two fragments of low molecular weight, indicating that all copies presented an internal restriction site at approximately the same location (Fig. 3). Based on the hybridization results, we concluded that at least three copies corresponding to different alleles/gene members were present in the *P. notatum* genome.

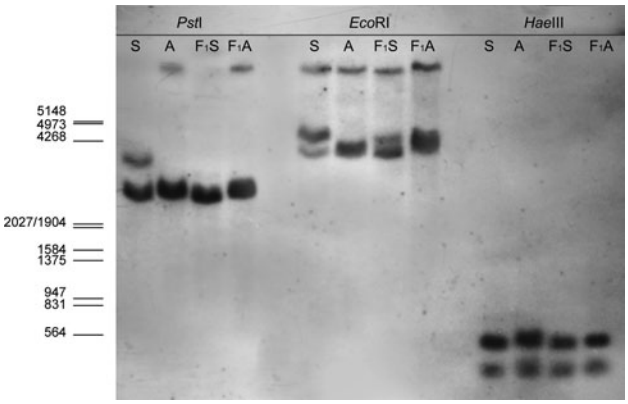


Fig. 3 Genomic hybridization analysis. Genomic DNA from plants Q4188 (S), Q4117 (A), a sexual F_1 hybrid (F_1S) and an apomictic F_1 hybrid (F_1A) was digested with enzymes *Pst*I, *Eco*RI and *Hae*III. Hybridization with a conserved *SERK* segment revealed 2–3 bands, indicating the presence of at least three copies of the *SERK* gene in the *P. notatum* genome. The migration of the molecular markers (as calculated from the gel image before capillary transfer to the nylon membrane) is indicated on the left

In silico mapping analysis

In silico mapping analysis onto the rice genome revealed that one putative *PnSERK1* rice orthologue sequence mapped to chromosome 2 at position 7,711,946–7,713,149 (E-val: 2.2×10^{-167}). *PnSERK2* matched two putative rice orthologues, one located on chromosome 4 at position 22,682,669–22,684,503 (E-val: 3.1×10^{-213}) and the other located on chromosome 8 at position 4,347,085–4,348,253 (E-val: 8.9×10^{-71}). A similar analysis with the maize genome revealed two putative *PnSERK1* maize orthologues, which mapped to maize chromosomes 5 and 6, at positions 146,839,257–146,840,472 (E-val: 0.0) and 102,396,444–102,397,239 (E-val: 1.4×10^{-257}), respectively. Likewise, *PnSERK2* has two putative orthologues in maize, located in chromosomes 4 and 5, at positions 124,636,274–124,638,004 (E-val: 0.0) and 176,215,947–176,217,905 (E-val: 0.0), respectively.

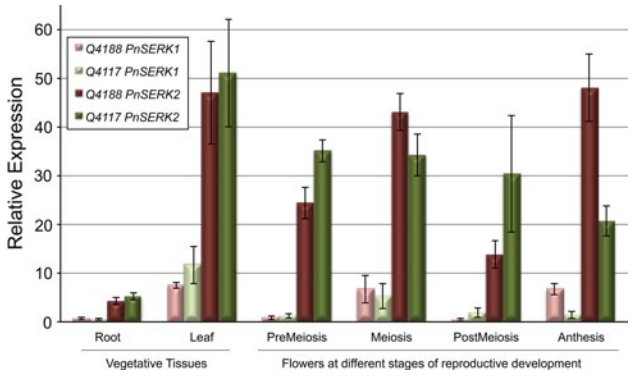


Fig. 4 Relative expression of *PnSERK1* and *PnSERK2* transcripts in roots, leaves and inflorescences (at different developmental stages) of genotypes Q4188 (sexual) and Q4117 (apomictic). Amplification efficiencies were equivalent for primers amplifying both genes. *PnSERK1* showed lower expression compared to *PnSERK2* in both sexual and apomictic plants. The chronological expression patterns in the apomictic and the sexual genotype were contrasting for both paralogues

Quantitative expression analysis in vegetative tissues, reproductive tissues and embryogenic calli

The expression of *PnSERK1* and *PnSERK2* was evaluated in roots, leaves and flowers of sexual (Q4188) and apomictic (Q4117) plants by quantitative real time PCR. Flowers were collected at four reproductive development stages: premeiosis, meiosis, postmeiosis and anthesis. Analyses were carried out with specific primers for *PnSERK1* and *PnSERK2*, allowing independent amplification of each gene (Table 2). Comparison of the expression levels of both reference genes (*β-TUB* and *GPDH*) showed no significant differences between sexual and apomictic plants neither in vegetative tissues nor in flower at any of the four reproductive development stages evaluated ($p > 0.05$). Relative expression analysis showed that *PnSERK2* was expressed 6–15 times higher than *PnSERK1*, depending on the tissue and/or the developmental stage considered, in both Q4117 and Q4188 genotypes (Fig. 4). In roots, *PnSERK2* showed a higher expression level in the apomictic genotype ($p < 0.006$), but its general expression was lower in comparison with the other tissue types analyzed. In leaves, *PnSERK2* showed similar expression levels in the apomictic and sexual genotypes ($p > 0.300$). In flowers, both genes (*PnSERK1* and *PnSERK2*) showed activity increases at meiosis and anthesis in the sexual genotype (Q4188) (Fig. 4). However, different levels of expression were observed in the apomictic genotype (Q4117); while *PnSERK1* showed a peak of activity only at meiosis, *PnSERK2* showed similar activity levels at premeiosis, meiosis and postmeiosis, but a decreased level at anthesis (Fig. 4). Comparison of expression levels between

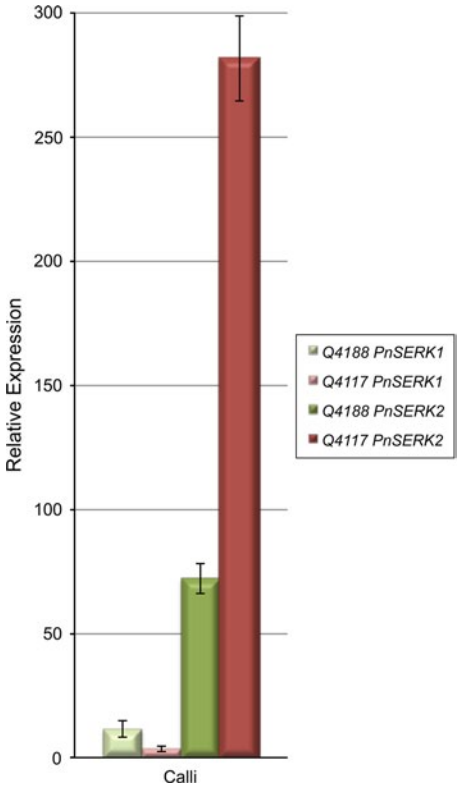


Fig. 5 Relative expression of *PnSERK1* and *PnSERK2* transcripts in calli induced from mature seeds of genotypes Q4188 (sexual) and Q4117 (apomictic). Amplification efficiencies were equivalent for primers amplifying both genes. *PnSERK1* showed lower expression compared to *PnSERK2* in both sexual and apomictic plants. High levels of expression were observed for *PnSERK2*, especially in calli originating from the apomictic plant

genotypes showed that *PnSERK1* expression at anthesis was significantly higher ($p < 0.002$) in Q4188 with respect to Q4117. On the other hand, *PnSERK2* showed significantly higher expression at premeiosis and postmeiosis in Q4117 with respect to Q4188 ($p < 0.020$), but decreased expression at anthesis ($p < 0.002$). No differences between genotypes were detected for either gene at meiosis (Fig. 4).

Similar assays were carried out to quantify the expression of *PnSERK1* and *PnSERK2* in embryogenic calli of the Q4188 and Q4117 genotypes (Fig. 5). Again, *PnSERK2* activity was significantly higher ($p < 0.05$) with respect to *PnSERK1*. In addition, highly significant differences ($p < 0.004$) in *PnSERK2* expression were detected in calli originating from the apomictic plant in comparison with the sexual one, which correlated with an increase in the plant regeneration ratio of calli originating from genotype Q4117 (data not shown). *PnSERK1* also showed significantly different expression between the apomictic and sexual genotypes ($p < 0.005$), but in this case the expression increase was detected in the sexual genotype (Fig. 5).

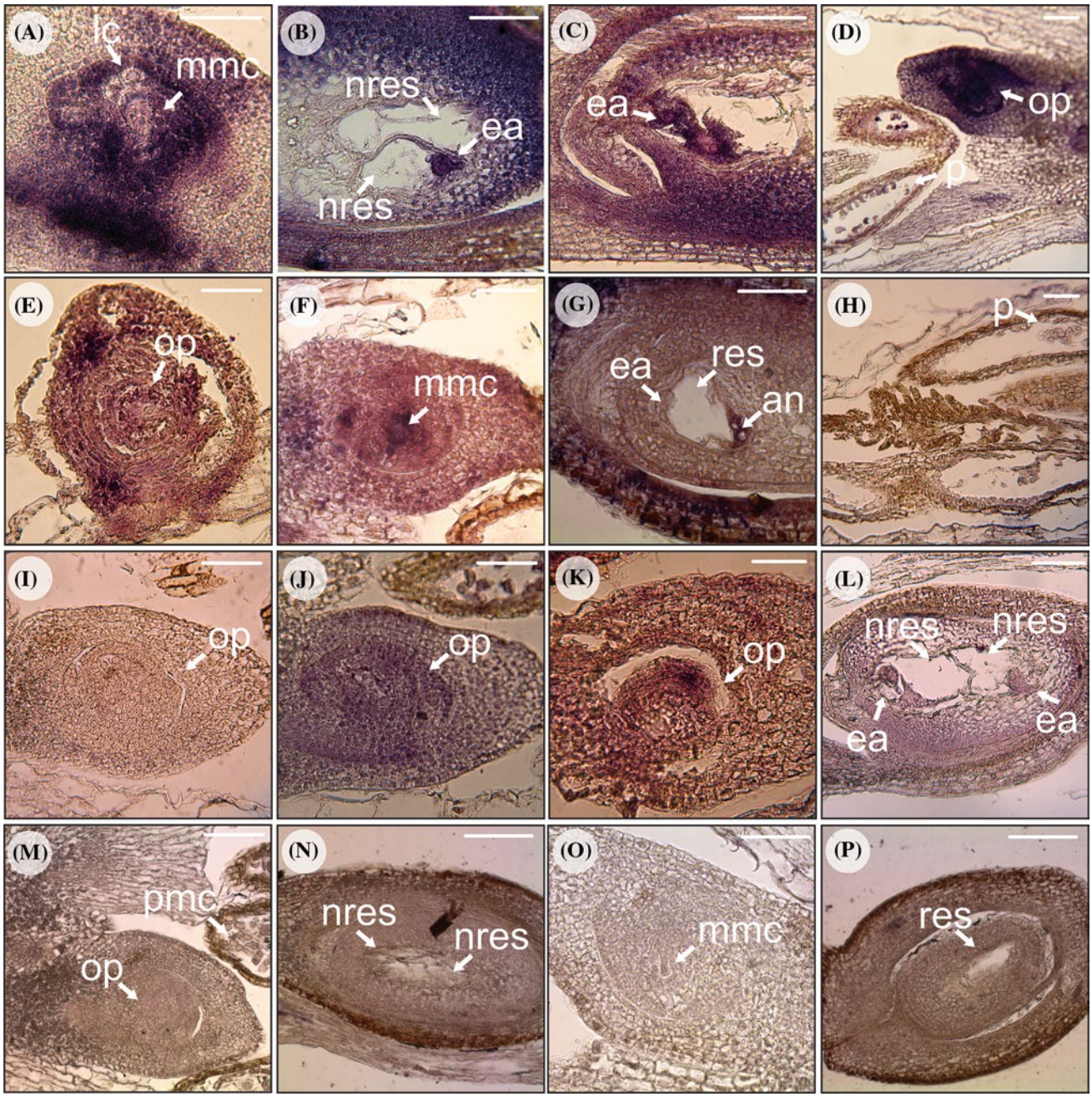
In situ hybridization studies

In situ hybridization analyses were performed at two different developmental stages: late premeiosis/meiosis and anthesis. These particular stages were selected because of the striking differences detected between the sexual and aposporous developmental pathways. In aposporous plants, immediately prior to the onset of meiosis, several nucellar cells named "apospory initials" differentiate and start an ordered pattern of mitotic divisions to produce non-reduced megagametophytes. At anthesis, the non-reduced egg-cells, characteristic of apomeiotic sacs, carry out parthenogenesis in the absence of fertilization. Specific probes were used to detect the representation of *PnSERK1* and *PnSERK2* transcripts independently. For *PnSERK1*, the probe consisted of a fragment of 550 nt (corresponding to positions 750–1,300), which shared no significant homology with *PnSERK2*. To reveal *PnSERK2*, a probe of 600 nt (corresponding to positions 1,330–1,930) presenting no significant homology to *PnSERK1* was used. The results of hybridization experiments with both sense and antisense *PnSERK1* and *PnSERK2* probes are shown in Fig. 6. *PnSERK1* showed no hybridization signals with either sense (not shown) or antisense probes in both Q4117 and Q4188 at meiosis (Fig. 6m, o, respectively) and anthesis (Fig. 6n, p, respectively). This outcome agreed with the results obtained in qRT-PCR experiments, which showed a low level of expression for this gene. Hybridization with the *PnSERK2* probe antisense strand revealed a strong signal in both sexual and apomictic genotypes at late premeiosis/meiosis (Fig. 6a, e and f; and Supplemental Material 2). However, the spatial distribution of the signal was contrasting between genotypes. In apomictic individuals, hybridization was detected in nucellar cells surrounding the MMC and part of the integuments, but was absent from the MMC and lip cells (two cells adjacent to the MMC) (Fig. 6a). Conversely, in sexual genotypes the signal was restricted to the megaspore mother cell and lip cells and was absent in nucellar cells (Fig. 6f). These results indicate that, at meiosis, *PnSERK2* is active in the MMC and lip cells of the sexual genotype, but is repressed in the nucellus. On the contrary, in the apomictic genotype *PnSERK2* is active in nucellar cells, but is repressed in the MMC and lip cells. At anthesis, the *PnSERK2* antisense probe revealed a strong signal in the embryo sac and ovule integuments of the apomictic genotype (Fig. 6c). In the sexual genotype, hybridization with this probe showed weak signals in the ovary walls and antipodal cells (Fig. 6g). Curiously, in the sexual genotype (at late premeiosis), the *PnSERK2* sense probe hybridized with a group of cells located at the nucellus (Fig. 6k) while in the apomictic genotype (in the same stage), this probe produced a weak signal evenly distributed throughout the ovule (Fig. 6J). These results suggest that at premeiosis, the antisense strand of *PnSERK2* gene is expressed with a different spatial distribution in sexual and apomictic ovules. In order to prove this assumption the expression level of the antisense strand of *PnSERK2* in both apomictic and sexual flowers at late premeiosis was investigated. qRT-PCR reactions targeting only this specific strand were carried out in cDNA samples of flowers from the hybrids JS-90 (sexual) and JS-40 (apomictic). Antisense transcript expression was detected in both samples but no significant differences were detected between them (expression ratio: $1.429 \pm 1.055 - 2.043$; p: 0.074) (Fig. 7).

Fig. 6 Reproductive tissue in situ hybridization with *PnSERK2* (**a–l**) and *PnSERK1* (**m–p**) probes. **a** Q4117 (apomictic) ovule at meiosis hybridized with the antisense *PnSERK2* probe. Strong hybridization signal was observed in the nucellus, around the megaspore mother cell, and also in part of the integuments. No hybridization signals were observed in either the megaspore mother cell or the lip cells (indicated by arrows). **b** Q4117 (apomictic) ovule at anthesis hybridized with the antisense *PnSERK2* probe. Strong hybridization was observed in both the integuments and the egg apparatus. **c** A closer view of a different Q4117 (apomictic) ovule at anthesis hybridized with the antisense *PnSERK2* probe. Strong hybridization was observed in both the integuments and the egg apparatus. **d** Q4117 (apomictic) ovule at premeiosis and anther primordia hybridized with the antisense *PnSERK2* probe. A strong signal was observed in ovule primordium and pollen mother cells. **e** Q4188 (sexual) ovule at premeiosis hybridized with the antisense *PnSERK2* probe. Hybridization was detected around the vascular system. **f** Q4188 (sexual) ovule at premeiosis/meiosis hybridized with the antisense *PnSERK2* probe. Hybridization was detected in the MMC region. **g** Q4188 (sexual) ovule at anthesis hybridized with the antisense *PnSERK2* probe. A weak hybridization signal was detected in the antipodal cells. **h** Q4188 (sexual) anthers at anthesis hybridized with the antisense *PnSERK2* probe. No signal was observed in pollen cells. **i** Q4117 (apomictic) ovule at meiosis hybridized with a control buffer without probe. No signal was observed. **j** Q4117 (apomictic) ovule at meiosis hybridized with a sense *PnSERK2* probe. A weak signal on the whole ovule was observed. **k** Q4188 (sexual) ovule at meiosis hybridized with a *PnSERK2* sense probe. A hybridization signal was observed in part of the nucellus, in a cell layer close to the MMC toward the chalazal pole. **l** Q4117 (apomictic) ovule at anthesis hybridized with a sense *PnSERK2* probe. A faint signal was observed in the integuments and the egg apparatus. **m** Q4117 (apomictic) ovule at meiosis hybridized with the antisense *PnSERK1* probe, no signal was detected. **n** Q4117 (apomictic) ovule at anthesis hybridized with the antisense *PnSERK1* probe, no signal was detected. **o** Q4188 (sexual) ovule at meiosis hybridized with the antisense *PnSERK1* probe, no signal was detected. **p** Q4188 (sexual) ovule at anthesis hybridized with the antisense *PnSERK1* probe, no signal was detected. *ea* egg apparatus, *lc* lip cells, *mmc* megaspore mother cell, *nres* non-reduced embryo sac, *op* ovule primordium, *pmc* pollen mother cells, *res* reduced embryo sac. Scale bars represent 100 μm

Comparison of *PnSERK*s and two sequences with retrotransposon elements including *SERK* transduplicated segments

In a previous paper, our group reported the identification of two different non-coding RNAs (A43 and A46)



expressed in the ovules of aposporous *P. notatum* plants, which consisted of retrotransposon-like sequences of the gypsy type associated with transduplicated *SERK* segments (Ochogavía et al. 2011). BLAST2seq analyses were carried out with the A43 and A46 sequences to compare them with the *PnSERK1* and *PnSERK2* sequences isolated here. The first 164 nucleotides of A43 showed 86 % identity to *PnSERK1* between nucleotides 1,067–1,231 (E-val: 2×10^{-51}), but no homology to *PnSERK2*. These first 164 nucleotides were mapped in silico onto the rice genome using tBLASTx and matched only with locus Os02g14120.1 (*BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 PRECURSOR*, putatively expressed), which was localized on chromosome 2: 7,710,511–7,715,738 reverse strand. Therefore, we concluded that A43 is a gypsy element carrying a transduplicated gene segment highly similar to a fragment of *PnSERK1*. Likewise, sequence analysis of A46 showed a fragment (nt 78–258) with 92 % homology to *PnSERK1* between nucleotides 1,052–1,231 (E-val 3×10^{-73}), while another segment (nt 159–218) showed 73 % homology to *PnSERK2* between nucleotides 1,275–1,333 (E-val: 9×10^{-05}). The fragment 78–258 was mapped in silico onto the rice genome using tBLASTx

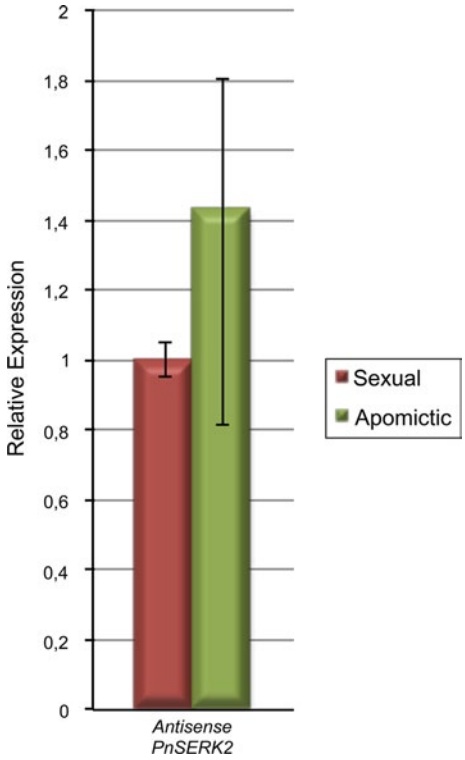


Fig. 7 Relative expression of *PnSERK2* antisense transcripts in flowers of genotypes JS-90 (sexual) and JS-40 (apomictic) at premeiosis/meiosis stages. No significant differences were detected between genotypes ($p = 0.074$)

and matched only with locus Os02g14120.1, the same locus described above. However, no significant similarity was detected at Gramene with segment 159–218. Therefore, A46 was determined to be a gypsy element carrying a transduplicated segment highly similar to a fragment of *PnSERK1*, while an association with *PnSERK2* could not be confirmed.

Discussion

The *SERK* gene family has been partially characterized at the functional level in several plant species. In *A. thaliana*, the family consists of five members that are involved in at least five different signaling pathways. *AtSERK1* and *AtSERK2* are essential for male microsporogenesis, while *AtSERK3* functions in brassinosteroid-controlled responses and is partially redundant with *AtSERK1*. *AtSERK3* alone also controls innate immunity (Kemmerling et al. 2007) and is involved in flagellin perception (Chinchilla et al. 2007, Heese et al. 2007). Together with *AtSERK4*/*BKK1*, *AtSERK3* also controls plant cell death (He et al. 2007, Kemmerling et al. 2007). The function of *AtSERK5* remains uncharacterized.

The *SERK* gene family seems to be a classic example of redundancy between members, because no phenotypes were recorded for the single loss-of-function mutants, except *serk3*/*bak1* (Albrecht et al. 2008). A systematic genetic approach was used to analyze double, triple and quadruple Arabidopsis mutants for BR-related phenotypes and response to pathogen treatment. The results suggested that only *AtSERK1* and *AtSERK3* participate in BRI1-mediated signaling. Triple and quadruple *serk* mutants only showed additive phenotypes, suggesting that the different *SERK*s have no further redundancy. Moreover, *AtSERK4* was partially redundant with *AtSERK3* in mediating a pathogen-induced response (Albrecht et al. 2008). It was proposed that the different *SERK* members are only redundant in pairs, and that members that are redundant within one pathway are not redundant in another (Albrecht et al. 2008).

In this work, two different members of the *P. notatum SERK* family (*PnSERK1* and *PnSERK2*) were identified, and their expression in vegetative and reproductive tissues of sexual and apomictic genotypes was characterized. Phylogenetic analysis grouped the translated sequences of *PnSERK1* and *PnSERK2* in different branches of the SERK protein family. Specifically, PnSERK2 appeared to be closely related to the DcSERK, AtSERK1, AtSERK2 and ZmSERK2 proteins, which have been associated with the somatic embryogenesis process (Schmidt et al. 1997, Hecht et al. 2001, Baudino et al. 2001). Moreover, genomic hybridization analysis indicated that at least three copies of *PnSERK* are present in the *P. notatum* genome. The same copy number was reported in maize by Baudino et al. (2001), but 11 copies, including two *OsSERK*s and nine *OsSERK*-like genes, were detected in rice (Singla et al. 2009).

One of the candidates analyzed here (*PnSERK2*) showed high expression levels in leaves and reproductive tissues, but low levels in roots. Moreover, quantitative expression analysis showed that *PnSERK2* is expressed significantly higher in the apomictic genotype than in the sexual one at premeiosis and postmeiosis, but decrease at anthesis. No differences were found between genotypes at meiosis. However, in situ hybridization experiments showed that *PnSERK2* displayed a contrasting spatial expression pattern in immature ovules at late premeiosis/meiosis between sexual and apomictic plants. In sexual individuals, the candidate sense strand was expressed only in the MMC and adjacent lip cells. In aposporous individuals, strong expression was observed in a large portion of the nucellus and part of the integuments, precisely where apospory initials differentiate, but no mRNA was detected in either the MMC or lip cells. Interestingly, the *PnSERK2* sense probe repeatedly produced hybridization signal in a small group of nucellar cells in the sexual genotype at late premeiosis. Meanwhile,

in the apomictic genotype a faint signal was detected throughout the whole nucellus at the same developmental stage. Antisense strand expression quantitation revealed no differences between the sexual and the apomictic genotypes. Therefore, the signal detected by the *PnSERK2* sense probe in sexual plants could be reflecting an unequal spatial distribution of the antisense strand in reproductive tissues of both plant types (apomictic and sexual).

Researchers working in the aposporous apomixis field have long tried to elucidate how nucellar cells of aposporous individuals could detect signals governing cellular reprogramming of the gametophytic fate. They have speculated on the existence of signal transduction pathways operating in the MMC while concurrently repressed in the nucellar surrounding cells of sexual individuals, which would be de-repressed in the nucellar cells of aposporous individuals. Evidence for the existence of repressed molecular pathways in the nucellus of sexual plants was provided by Olmedo-Monfil et al. (2010), who showed that loss-of-function *argonaute9 (ago9)* Arabidopsis mutants display elongation of several cells in the nucellus, which adopt a gametophytic fate. Interestingly, AGO9 is involved in the generation of siRNAs, and is a major factor in the transcriptional and posttranscriptional gene silencing pathway. Therefore, AGO9 might control a repression mechanism, acting in the nucellus to inhibit re-programming to a gametic fate. Curiously, the main AGO9 targets appear to be retrotransposons. Olmedo-Monfil et al. (2010) proposed that AGO9 produces tasiRNAs, which could move as signal molecules beyond their cellular initiation sites, triggering RNA-directed DNA methylation and gene silencing.

The candidate *PnSERK2* seems to be active in the MMC of sexual plants, but repressed in the nucellus. Conversely, it is strongly expressed in the nucellus of aposporous plants. Moreover, *PnSERK2* is a putative orthologue of *AtSERK1*, which was shown to be essential for tapetum specification and pollen development during male sporogenesis (Albrecht et al. 2005; Colcombet et al. 2005). In *M. truncatula*, the expression of *MtSERK1* (the orthologue of *AtSERK1*) was associated with developmental change, possibly reflecting cellular reprogramming (Nolan et al. 2009). Cloning of the *MtSERK1* promoter into a GUS-containing vector was used to analyze its expression in embryogenic cultures and *in planta* (Nolan et al. 2009). Its expression was associated with meristems, transition zones between one type of tissue or organ and another, and vascular tissue procambial cells. The authors proposed a role for *MtSERK1* in the acquisition of pluripotency and cellular reprogramming in new developmental directions (Nolan et al. 2009). These conclusions are in agreement with our observations of *PnSERK2* expression in the nucellar cells of aposporous individuals, since they are being re-programmed to acquire a female gametophytic fate.

PnSERK1 is highly similar to *PpSERK1*, which was reported by Albertini et al. (2005) to be differentially expressed in the ovules of sexual and aposporous *P. pratensis*. However, in *P. notatum* no significant hybridization signals were detected in reproductive tissues in the in situ hybridization studies. Moreover, real-time PCR quantitation showed lower *PnSERK1* expression compared to *PnSERK2*. Interestingly, *PnSERK2* displayed a spatial expression pattern similar to that reported for *PpSERKs*; sense expression in the MMC of sexual genotypes and in the nucellar cells of aposporous genotypes. These results raise the question "does *PpSERK* play a role in *P. pratensis* analogous to that of *PnSERK2* in *P. notatum*?" Functional analysis as well as identification and characterization of additional *SERK* sequences in *Paspalum* and *Poa* will provide complementary evidence to help answer this question.

In a recent paper, our group reported the expression in *P. notatum* reproductive tissues of two retrotransposon sequences of the *gypsy* subfamily, which carried transduplicated segments of *SERK* (Ochogavia et al. 2011). We proposed that these elements might have a role in regulating *SERK* gene expression in this species. Here, we determined that *SERK*-like segments within both elements are highly homologous to *PnSERK1*, but have little or no similarity to *PnSERK2*. The detection of reduced *PnSERK1* activity in the ovule compared to *PnSERK2* supports the hypothesis that these elements have a role in controlling *SERK* expression in *P. notatum*. Further functional analysis aimed at overexpressing and/or repressing these elements should provide evidence to clarify their role in the regulation of *SERK* expression.

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