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RESEARCH PAPER

# Two *SERK* genes are markers of pluripotency in *Cyclamen persicum* Mill.

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## Abstract

**The genetic basis of stem cell specification in somatic embryogenesis and organogenesis is still obscure. *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK)* genes are involved in embryogenesis and organogenesis in numerous species. *In vitro* culture of *Cyclamen persicum* immature ovules provides a system for investigating stem cell formation and maintenance, because lines forming either organs or embryos or callus without organs/embryos are available for the same cultivar and plant growth regulator conditions. The present aim was to exploit this property of cyclamen cultures to understand the role of SERK(s) in stem cell formation and maintenance in somatic embryogenesis and organogenesis *in vitro,* in comparison with expression *in planta*. *CpSERK1* and *CpSERK2* were isolated from embryogenic callus. *CpSERK1* and *CpSERK2* levels by RT-PCR showed that expression is high in embryogenic, moderate in organogenic, and null in recalcitrant calli. *in situ* hybridizations showed that the expression of both genes started in clumps of pluripotent stem cells, from which both pre-embryogenic aggregates and organ meristemoids derived, and continued in their trans-amplifying, meristem-like, derivatives. Expression declined in organ meristemoids, in parallel with a partial loss of meristematization. In mature somatic embryos, and in shoot and root primordia, *CpSERK1* and *CpSERK2* were expressed in meristems, and similar patterns occurred in zygotic embryo and primary meristems *in planta*. The results point to *SERK1* and *SERK2* as markers of pluripotency in cyclamen*.* It is proposed that the high expression of these genes in the trans-amplifying derivatives of the stem cells maintains a pluripotent condition leading to totipotency and, consequently, somatic embryogenesis.**

**Key words:** *Cyclamen persicum*, meristems, pluripotency, stem cells, *SOMATIC EMBYROGENESIS RECEPTOR-LIKE KINASE (SERK)*, totipotency, trans-amplifying cells.

## Introduction

Niches of stem cells are present in the plant shoot (SAM) and root (RAM) apical meristems. Stem cells are undifferentiated cells that have the unique characteristics of both self-renewal and to develop into precursors that can form different cell types and tissues. Based on these capabilities, the stem cells are considered pluripotent (Verdeil *et al.*, 2007). The plant also possesses stem cells out of the apical meristems, for example, the procambium, providing the stem

Abbreviations: 2 iP, 6-γ-γ-(dimethylallylamino) purine; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; CDS, coding sequence; CTAB, cetyltrimethylammonium bromide; EST, expressed sequence tag; iPCR, inverse PCR; LRR, leucine-rich repeat; MW, molecular weight; PEA, pre-embryogenic aggregate; PEM, pro-embryogenic mass; PGR, plant growth regulator; pI, isoelectric point; RAM, root apical meristem; RKL, receptor-like kinase; Rp, root pole; SAM, shoot apical meristem; SERK, *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE*; Sp, shoot pole; SPP, serine-proline-proline; TA, trans-amplifying cell; ZIP, leucine zipper.

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cell pool for primary xylem and phloem (Gray *et al.*, 2008), and the meristemoids, i.e. single cells or small cell groups leading to stomata and hairs after de-differentiation and re-programming events (Jakoby and Schnittger, 2004). Meristemoids are also formed in the callus produced *in vitro,* under specific hormone-inductive treatments, and give rise to various types of *de novo* formed organs (i.e., roots, shoots, and flowers), and to xylogenesis (i.e. *de novo* formation of vascular elements surrounded by a neo-cambial ring) (Altamura *et al.*, 1994; Dhaliwal *et al.*, 2003; Fattorini *et al.*, 2009). The presence of pluripotent stem cells has been also supposed for *in vitro* organogenesis (Zhao *et al.*, 2008; Sena *et al.*, 2009). Nevertheless, the molecular mechanisms underlying stem cell definition and pluripotency in the meristemoids formed *in vitro* remain unknown. Little is also known about the mechanisms that change somatic cells into embryogenic cells, i.e. cells able to give rise to a somatic embryo following a developmental pattern quite similar to that by which the fertilized egg cell produces the zygotic embryo (Zimmerman, 1993). Somatic embryogenic cells have been proposed to be included in the plant stem cell concept, as totipotent stem cells (Verdeil *et al.*, 2007; Su and Zhang, 2009). As for pluripotency in meristemoids, totipotency in somatic embryogenic cells involves cellular reprogramming following de-differentiation (Zeng *et al.*, 2007; Wang *et al.*, 2011), thus organogenesis and somatic embryogenesis might share early molecular components related to pluripotency/totipotency in stem cells.

Auxin, but also other plant growth regulators (PGRs), for example, cytokinin, are involved in the specification and maintenance of stem cells in the zygotic embryo and in meristems *in planta* (Aida *et al.*, 2004; Müller and Scheen, 2008; Iyer-Pascuzzi and Benfey, 2009; Zhao *et al.*, 2010). Usually, auxin is required to induce somatic embryogenesis (Feher *et al.*, 2003; Mahalakshmi *et al.*, 2007), and auxin gradients to trigger the formation of stem cells (Su and Zhang, 2009). However, there are also species in which cytokinin alone induces somatic embryogenesis, for example, in sunflower (Thomas *et al.*, 2004), and species in which a cytokinin must be combined with auxin to induce somatic embryogenesis, for example, in *Medicago truncatula* and *Vitis vinifera* (Nolan *et al.*, 2003; Schellenbaum *et al.*, 2008).

Exogenous auxin, alone or combined with cytokinin in a specific balance, is also required for the induction of roots and shoots, and for xylogenesis, for example, in tobacco thin cell layers and pith explants (Altamura *et al.*, 1995; Fattorini *et al.*, 2009). In *Arabidopsis*, auxin is the inductive PGR for organogenesis *in vitro*, whereas cytokinin modulates auxin output (Pernisová *et al.*, 2009). In the same plant, transcriptional regulatory networks controlling stem cell population and maintenance have been demonstrated in SAM and RAM and in the procambium, *in planta*, and homologous transcription factors have been found to be involved (Stahl and Simon, 2010; Sablowski, 2011, and references therein).

In addition to the control by PGRs and transcriptional networks, ligand-receptor-like kinase signalling pathways have been revealed as potentially crucial regulators in stem cell specification (De Smet *et al.*, 2009, and references therein), and an intercellular leucine-rich repeat receptor-mediated pathway has been proposed for the maintenance of plant stem cells, and meristematic cell fate decisions (Gray *et al.*, 2008, and references therein). *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK)* genes encode leucine-rich repeat receptor-like kinases. They form a subgroup among leucine-rich repeat-receptor-like kinases in plants (Shiu and Bleecker, 2001). Their subcellular localization as a membrane protein (Shah *et al.*, 2001*a*) and phosphorylation/dephosphorylation activities with kinase associated protein phosphatase has been reported (Shah *et al.*, 2001*b*). *SERK* genes are up-regulated by auxin in some species, and by auxin and cytokinin in others (Zhang *et al.*, 2011, and references therein). The positive involvement of some *SERK* genes in the induction of somatic embryogenesis has been widely demonstrated in monocots and dicots (Schmidt *et al.*, 1997; Somleva *et al.*, 2000; Hecht *et al.*, 2001; Nolan *et al.*, 2003; Santa-Catarina *et al.*, 2004; Thomas *et al.*, 2004; de Oliveira Santos *et al.* 2005; Hu *et al.*, 2005; Shimada *et al.*, 2005; Schellenbaum *et al.*, 2008; Sharma *et al.*, 2008; Singla *et al.*, 2008; Pérez-Núñez *et al.*, 2009; Huang *et al.*, 2010; Zhang *et al.*, 2011). Moreover, *SERK* genes are also positively related to zygotic embryogenesis in various species (Schmidt *et al.*, 1997; Hecht *et al.*, 2001; de Oliveira Santos *et al.* 2005; Mantiri *et al.*, 2008; Singla *et al.*, 2008), and to apomixis (Tucker *et al.*, 2003; Albertini *et al.*, 2005). However, numerous studies in *in vitro* culture and *in planta,* suggest a role in development, at least for specific *SERK*s, broader than in somatic and zygotic embryogenesis. For example, in maize, *ZmSERK1* and *ZmSERK2* are expressed in both embryogenic and non-embryogenic *in vitro* cultures, whereas *in planta ZmSERK1* exhibits a broader expression in the reproductive tissues and *ZmSERK2* a constitutive expression in all tissues analysed, including the vegetative ones (Baudino *et al.*, 2001). In sunflower, a *SERK* gene is expressed during induction of somatic embryogenesis and shoot organogenesis (Thomas *et al.*, 2004). In *Medicago truncatula, MtSERK1* expression is associated with somatic embryogenesis and *in vitro* rhizogenesis, but also with zygotic embryogenesis and primary meristems *in planta*, including procambium (Nolan *et al.*, 2003, 2009; Wang *et al.*, 2011). Moreover, AtSERK1 and AtSERK2 function redundantly in the control of male sporogenesis in *Arabidopsis* (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005), and *AtSERK1* is also expressed in the procambium (Kwaaitaal and De Vries, 2007).

*Cyclamen persicum* is an important ornamental plant with high economic relevance, commercially propagated by $F_1$ hybrid seeds. Because of technical and economic difficulties caused by inbreeding depression and expensive seed production, *in vitro* somatic embryogenesis is nowadays a promising alternative for cyclamen propagation (Winkelmann *et al.*, 2006, and references therein). The protocol for somatic embryogenesis starts from immature ovules (Winkelmann *et al.*, 1998). Embryogenic calli are formed in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-γ-γ-(dimethylallylamino) purine (2iP) as PGRs. This is the induction phase, during which no macroscopic morphogenesis occurs. The differentiation of macroscopic embryos

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occurs by transfer of callus to a PGR-free medium (i.e. in the PGR-free expression phase; Rensing *et al.*, 2005; Lyngved *et al.*, 2008). Moreover, starting from the same cultivar and PGR conditions, cell lines can form callus with organs (shoots and roots) instead of embryos, callus with embryos and organs, or callus with neither organs nor embryos (i.e. recalcitrant callus) (Savona *et al.*, 2007; Lyngved *et al.*, 2008).

Thus, cyclamen provides a useful *in vitro* system to investigate the genetic mechanisms regulating embryogenic and organogenic competence under the same PGR input and in the same cultivar. Numerous ESTs annoted as *SERK*s have been found in the embryogenic cultures of *Cyclamen persicum* (Rensing *et al.*, 2005), and one of these putative *SERK* ESTs has recently been shown to be up-regulated in an embryogenic line (Hoenemann *et al.*, 2010).

In this study, *Cyclamen persicum* callus culture system was used to understand the involvement of *SERK*(s) in stem cell formation and maintenance in somatic embryogenesis and organogenesis *in vitro.* A further aim was to compare *SERK*(s) expression in somatic embryos and organs *in vitro* with their expression in zygotic embryos and organs *in planta*.

The overall results show that *CpSERK1* and *CpSERK2* are always expressed in stem cells and may be regarded as markers of pluripotency *in planta* and *in vitro*.

Moreover, a tight correlation between *CpSERK1*/*CpSERK2* expression and embryogenic potential *in vitro* was found, suggesting that high expression of the two genes maintains the trans-amplifying derivatives of the stem cells in a pluripotent condition, and this leads to totipotency and consequent somatic embryo formation.

## Materials and methods

### In vitro *culture*

Eight $F_1$ plants of *Cyclamen persicum* Mill. cv. Halios (S.A.S. MOREL® Diffusion, France), cultured in the greenhouse, were chosen as explant donors. Ovules of immature ovaries from flowers in pre-anthesis (Winkelmann *et al.*, 1998) were used as starting material for the production of callus lines. The explants were surface-sterilized in 70% ethanol solution (30 sec) followed by sodium hypochlorite solution (1.2% active chlorine) for 20 min, and then rinsed twice (10 min) in distilled and sterile water. The aseptic explants were cut longitudinally and plated in Petri dishes (two explants per dish) on a medium composed by half-strength macro- and micro-elements MS medium (Murashige and Skoog, 1962), 87 mM sucrose, 25 mM D-glucose monohydrate, 0.01% (w/v) casein-hydrolysate, 9 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 3.94 μM 2iP [6-γ-γ-(dimethylallylamino) purine], agarized with 0.4% (w/v) Gelrite™ (inductive medium; Savona *et al.*, 2007). pH was set at 5.6 before autoclaving and the callus was subcultured three times at 30 d intervals (24±1 °C in the dark). At the end of the third subculture, 100 fragments of callus per line (about 50 mg each, 10 per Petri dish) were transferred onto the same medium without PGRs (24±1 °C in the dark) for 2 months (expression medium; Savona *et al.*, 2007), and then scored under the stereomicroscope (MZ8, Leica, Wetzlar, Germany) to determine the productivity in terms of somatic embryos and/or organs. Eight callus lines were scored for the macroscopic response at the end of the expression phase. Four lines showed an embryogenic response, two lines showed an organogenic response, and two exhibited only callus formation.

One line per developmental behaviour was randomly selected, as follows: forming only somatic embryos, named 'embryogenic', forming shoots and roots, named 'organogenic', and forming only callus, named 'recalcitrant', and then used for further analyses.

### *Histological analysis*

Ten callus fragments (about 5 mg in fresh weight each) per selected line were randomly chosen and histologically analysed at 0, 1, 3, 7, 14, and 30 d of the third subculture on the induction medium, and after the first and the second month on the expression medium. The explants were fixed in 70% ethanol, dehydrated, embedded in paraffin (melting point 52–54 °C; BDH laboratory, Lutterworth, Leicestershire, UK) and sectioned at 8 μm intervals with a Top-Super S-150 microtome (Pabisch, Milano, Italy). Sections were stained with eosin and Carazzi's haemalum (Carlo Erba, Milano, Italy) as previously described (Altamura *et al.*, 1991). Images were acquired by a DC 500 digital camera applied to a DMRB microscope (Leica, Germany).

Two callus fragments for each of the other three embryogenic callus lines were randomly chosen and histologically analysed at 0, 1, 3, and 7 d of the third subculture on the induction medium.

### *DNA and RNA isolation and cDNA synthesis*

Callus tissue was grinded with liquid nitrogen and genomic DNA was extracted with a CTAB method slightly modified from Doyle and Doyle (1990). Plasmid DNA for cloning or sequencing purposes was extracted from bacterial cells using a plasmid DNA mini kit (Quiagen, Milan, Italy). Total RNA was extracted from callus material with a modified Kiefer method (Kiefer *et al.*, 2000), according to Pilotti *et al.* (2008). Reverse transcription was performed from 1 μg of total RNA using oligo $(dT)_{20}$ as primer and SuperScript II™ (Invitrogen, Carlsberg, USA), as reverse transcriptase, according to manufacturer's instructions.

### *Cloning and sequence analysis*

To amplify *SERK*-like genes from *Cyclamen persicum* calli, according to the CODEHOP strategy (COnsensus-DEgenerate Hybrid Oligonucleotide Primers; Rose *et al.*, 2003), degenerate primers were designed (see Supplementary Table S1 at *JXB* online). Amplification products were purified from agarose gel with Quiagen purification spin columns (Quiagen), subcloned in pGEM-Teasy vectors (Promega, Madison, USA), and introduced into XL1blue *E. coli* strain by electroporation, using standard methods. DNA sequences were determined by the dideoxy-chain terminator method using an ABI 3730 DNA sequencer (Applied Biosystem, Foster City, USA). DNA sequences were aligned with SerialCloner (SerialBasics, version 2.1). Similarity search was made with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and FASTA (http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html), multiple alignments with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and protein predictions with SignalP (http://www.cbs.dtu.dk/services/SignalP/) and PROSITE programs (http://www.expasy.org/prosite/). ORFs were found with FGENESH-M (fgenesh-m; http://linux1.softberry.com/berry.phtml?topic= fgenesh- m&group =programs&subgroup =gfind). Phylogenetic analysis was performed by sequential use of Seqboot, Protdist, neighbor, Consense and drawtree programs of the Phylip Package v. 3.69 (Felsenstein, 1989), using 100 bootstrap replicates.

### *5′ and 3′ RACE, genome walking, and iPCR*

The 3′ end of the putative *SERK*-like fragment of 750 bp was obtained from embryogenic calli using the BD SMART™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) as recommended by the manufacturer. Primers used for the first RACE PCR reaction were the 3′-RACE CDS Primer A (Clontech) and the gene-specific primers 5′-CGC TTC ACC GCC ACT AAC GA-3′ for the primary PCR, and 5′-TAT TCC TGT GGC TTC CTG CGT CGT-3' for the secondary nested PCR. The 5′ ends

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of *CpSERK1* and *CpSERK2* were obtained using the 5′-RACE system version 2 (Invitrogen) according to manufacturer's instructions, using 5′-TTG CCC AAA GTG TCC GGA AT-3′ and 5′-CGG CCC GGT TAT GCT GTT AC-3′ as *CpSERK1*-specific primers and 5′-CCC GTC AAG CTG GTA TTG TT-3′ and 5′-AAC CAA GTG CAG GGA TTG AC-3′ as *CpSERK2*-specific primers, for primary and secondary PCRs, respectively.

DNA walking was performed on genomic DNA template using a Genome walker™ universal kit (Clontech) following the protocols provided with the kit. iPCR was performed according to standard methods (Silver, 1991) using inverse primers derived from walking primers. A list of gene-specific primers for either genome walking or iPCR is reported in Supplementary Table S1 at *JXB* online. All primers were designed with the aid of primer 3 software (http://frodo.wi.mit.edu/primer3/).

### *Quantitative real-time RT-PCR*

Quantitative RT-PCR amplifications (qRT-PCR) measurements were performed, with a Bio-Rad iCycler iQ (Bio-Rad, Milan, Italy) using an *ATPase*-like EST isolated from *Cyclamen persicum* (see Supplementary Fig. S1 at *JXB* online) as normalizing control. RNA isolation and single strand cDNA synthesis was performed, as already mentioned, from embryogenic and organogenic calli at 7, 15, and 30 d from the third subculture on the induction medium, and from recalcitrant calli (at day 15 from the third subculture). Amplifications were monitored using the SYBR Green fluorescent stain. The presence of a single PCR product was verified by dissociation analysis in all amplifications. The comparative threshold cycle (ΔΔCT) method was used to calculate the relative amount of gene expression, normalized using the CT values derived for the *ATPase* gene. All quantifications were made in triplicate. Because *CpSERK1* and *CpSERK2* sequences share extensive nucleotide identity (88%), to distinguish the two genes the following primers were designed in their highly divergent, 3′-untranslated regions (UTRs): qSERK1 for: 5′-CGG GTT ATT TTG GGA GTA TTT CTC TA T-3′; qSERK1 rev: 5′-TCC ACT GCA CAA ATT TCA CAA TAA A-3′; qSERK2 for: 5′-GTG CAG AGG AAA TTG TAA TGA CAG AT-3′; qSERK2 rev: 5′-GCA ATT CCT TTT CAG GCC AA-3′; ATPase for: 5′-GGG CCA GAA ATT GCA GAG G-3′; ATPase rev: 5′-TTC TTG TAG CAG CCA CCG C-3′.

### In situ *hybridization*

Ten randomly chosen callus fragments per selected embryogenic, organogenic, and recalcitrant line (about 5 mg in fresh weight each), and two fragments per unselected line, were sampled, fixed, and sectioned as for the histological analysis, and used for *in situ* hybridization (Lopez-Dee *et al.*, 1999). Roots, vegetative shoots, flowers, and ovules were excised from specimens of *Cyclamen persicum* Mill. cv. Halios cultured in the greenhouse and processed in the same way. The sections were mounted on glass slides pretreated with bind-silane (Amersham Biosciences). Digoxigenin-labelled RNA probes were synthesized by *in vitro* transcription using the DIG RNA Labelling kit according to the supplier's specifications (Roche, Basel, Switzerland). In order to distinguish between *CpSERK1* and *CpSERK2*, specific sequences were amplified from the 3′-UTR regions of the two genes (i.e. the regions with the lowest level of identity; see Supplementary Fig. S2 at *JXB* online), and used as templates to generate RNA probes. The primer pairs used were: UT3-1 for: 5′-CGG GTT ATT TTG GGA GTA TTT CTC T-3′; UT3-1 rev 5′-AAC CCA TAG CTC AAA TAC ACA TCC A-3′, and UT3-2 for: 5′-GGG TTA TTT GCT GAT TTT CGG TGT-3′; UT3-2 rev: 5′-GGG CTC GAG TAA AAT TTG ACA AAA C-3′ to amplify *CpSERK1* and *CpSERK2* probes, respectively. The microscopic images were acquired in digital form with a Leica DC 5003 camera applied to the DMRB microscope, and analyzed with a Leica IM1000 image analysis software.

## Results

### *Both embryogenesis and organogenesis start from meristem-like cell clumps*

Samples from the three callus lines selected for the different macroscopic response at the end of the expression phase were investigated histologically at different times both during the PGR-activated induction phase and during the PGR-free expression phase, to determine the events characterizing embryogenesis and organogenesis, respectively, and to verify the absence of any embryonic/organogenic structure in the recalcitrant line.

In the embryogenic line, groups of 4–6 cells with small size, dense cytoplasm, and a large nucleus, showing an intense staining of the dye, became clearly distinguishable from the highly-vacuolated, expanded, and less stained, callus cells at the onset of the third subculture on the induction medium (Fig. 1A). No single cell with these features was ever observed. The few-celled groups continued to appear in the following days of the first week of this subculture and the most superficial ones, in particular, proliferated into multicellular clumps (Fig. 1B, C), showing the same meristem-like features of the first few-celled groups (Fig. 1B, Inset, compared with Fig. 1A). At day 7, large superficial meristem-like zones (Fig. 1D, arrows), and globular aggregates of meristem-like cells (Fig. 1E), collectively named pre-embryogenic aggregates (PEAs), were present in the callus. Easy detachment of PEAs from the callus surface was observed at the end of the following week (Fig. 1F). At this time (day 14), most of the PEAs had developed into irregularly-sized pro-embryogenic masses (PEMs). The PEMs contained a wide portion of small-sized, but vacuolated and parenchyma-like cells, and a reduced portion of meristem-like cells (Fig. 1G). The PEMs appeared frequently organized in clusters, which either remained attached to the callus or were detached (Fig. 1H). A few PEMs gave rise to single globular-shaped (Fig. 1I) and pre-torpedo embryos (Fig. 1J, arrows), or to embryo clusters up to the end of the induction phase (day 30). Further embryo development occurred during the first month of culture on the expression medium. At the torpedo stage, the embryo showed a procambial central strand connecting the shoot pole to the root pole (Fig. 1K). At the cotyledonary stage, the meristematic cell populations of the embryo were restricted to the root pole, the shoot pole, the forming cotyledon, the primordium of the second cotyledon, and the procambium (Fig. 1L). The primordium of the second cotyledon only occasionally exhibited further development. At the end of the second month of culture on the expression medium, the hypocotyledonary axis of the embryo tuberized (Fig. 1M). Macroscopically, the somatic embryos acquired the bottle shape with one cotyledon (Fig. 1N), similarly to the mature zygotic embryo *in planta*.

In the organogenic line, groups of few cells with small size, dense cytoplasm, and a large nucleus became clearly distinguishable from the highly-vacuolated, expanded, and less stained callus cells at the onset of the third subculture on the induction medium (Fig. 2A). They were similar to

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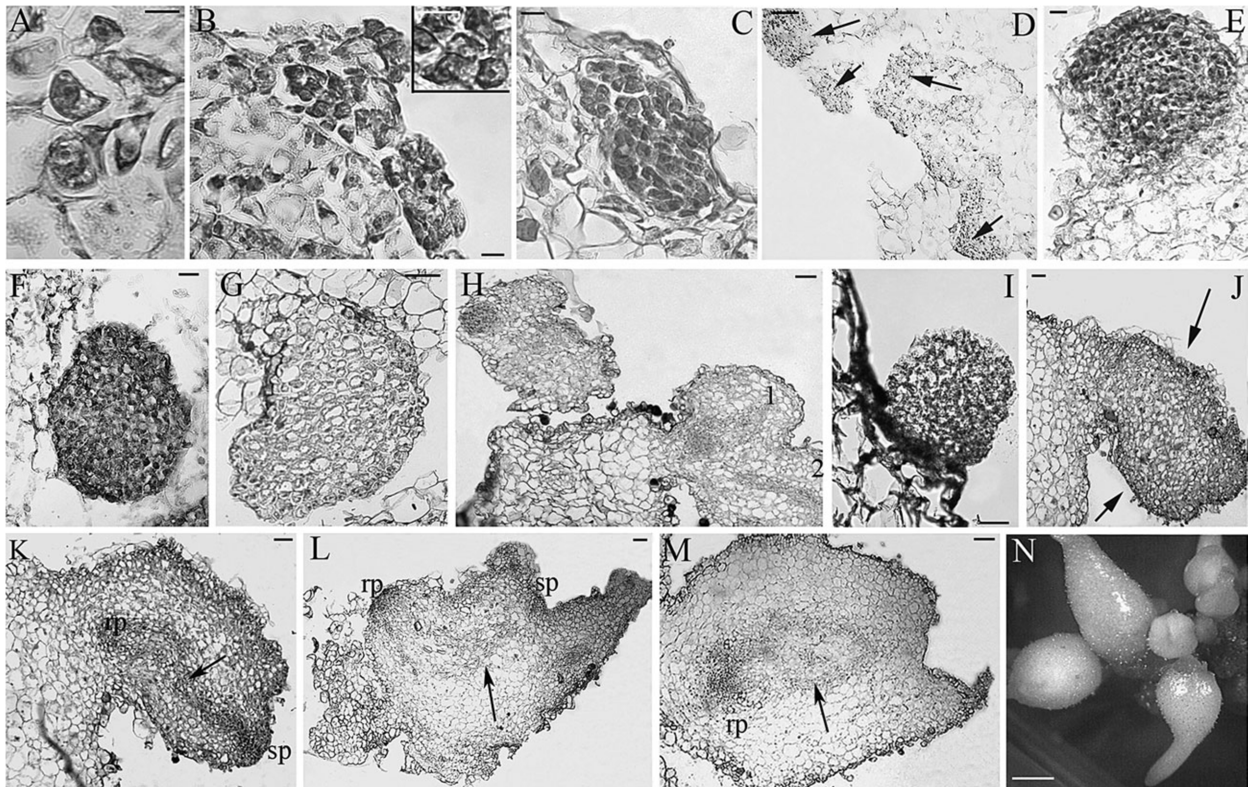


**Fig. 1.** Histological sequence of *Cyclamen* somatic embryogenesis [A–J, culture on the induction medium (third subculture), K–N, culture on the expression medium]. (A) Group of few cells with dense cytoplasm and a large nucleus within the embryogenic callus at the onset of the third subculture on the induction medium (day 1). (B, C) Differently-sized clumps of meristem-like cells at the periphery of the embryogenic callus. The meristem-like features of the cells are magnified in the Inset (day 3). (D, E) View of the two types of PEAs present at day 7, i.e. large meristem-like zones at the embryogenic callus periphery (D, arrows), and globular aggregates of meristem-like cells (E). (F) Globular PEA at callus periphery (day 14). (G) Pro-embryogenic mass (PEM) (day 14). (H) PEMs isolated and in clusters (as shown by 1 and 2) (day 30). (I) Globular shaped embryo detaching from PEM surface (day 30). Note the irregularly defined surface lacking a true protoderm, according to Hoenemann *et al*. (2010). (J) Longitudinal section of a somatic embryo at the pre-torpedo stage (day 30). (K) Longitudinal section of a somatic embryo at the torpedo stage. Procambium is shown by the arrow (rp, root pole and sp, shoot pole) (end of the first month on the expression medium). (L) Longitudinal section of a somatic embryo at the cotyledonary stage. Procambium is shown by the arrow (rp, root pole and sp, shoot pole) (end of the first month on the expression medium). (M) Longitudinal section of a tuberized somatic embryo. The arrow shows procambium in the hypocotyledonary axis (rp, root pole) (end of the second month on the expression medium). (N) Image under the stereo-microscope of tuberized embryos (end of the second month on the expression medium). Sections stained with eosin and Carazzi's haemalum. Bars: 10 μm (A, B and Inset in B); 20 μm (E, F, I); 50 μm (C, G, H, J, K); 100 μm (D, L, M); 1.5 mm (N).

those *de novo* formed in the embryogenic calli at the same time (compare Figs 1A, 2A). They grew into meristemoids into about a week (Fig. 2B–E). The meristemoids were randomly located within the callus, and their presence was many fold lower than that of the meristem-like multicellular clumps and of the superficial meristem-like zones occurring in the embryogenic callus (compare Figs 1D, 2B). The cells of the meristemoids were smaller than those of the surrounding callus (Fig. 2B, C), and showed a prominent nucleus (Fig. 2D), similar to those of the meristem-like clump/superficial zone cells of the embryogenic callus (Fig. 1C, D). However, different from the latter, not all of the cells in the meristemoid exhibited meristematic features, because of a higher cell size and vacuole presence (Fig. 2C, E). At day 7, a few meristemoids had grown into polarized structures, containing a reduced population of cells with meristematic features, located at one pole only, and an extended population of parenchyma-like cells (Fig. 2F). At day 14, the polarized meristemoids had developed either into shoot dome primordia (Fig. 2G), exhibiting meristematic tunica and corpus (Fig. 2G, arrow), or into root primordia, exhibiting a meristematic root dome (Fig. 2H). The development of other meristemoids into either shoot or root primordia occurred sporadically in the second half of the third subculture on the induction medium. During the first month of culture on the expression medium, shoot dome primordia developed into complete adventitious shoot buds (Fig. 2I), and root primordia developed into young adventitious roots (Fig. 2J). At the end of the expression phase, rhizogenesis was the prevalent organogenic response, and even roots in primary structure were observed in the callus (Fig. 2K, arrow). All over the culture on the expression

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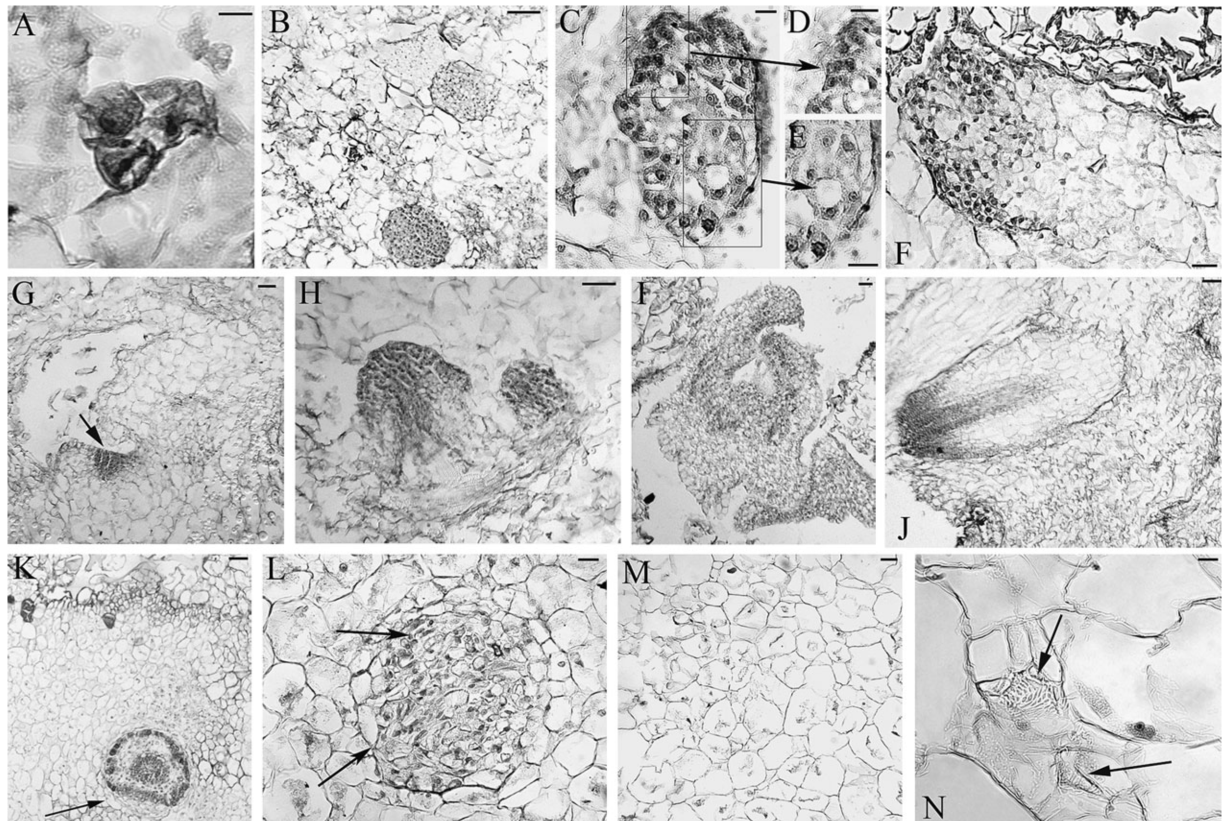


**Fig. 2.** Histological sequence of organogenesis (A–K) and xylogenesis (L) in *Cyclamen* organogenic line, and details of the callus in the recalcitrant line (M, N). (A–H, M, N, induction medium; I–L, expression medium). (A) Group of few cells with dense cytoplasm and a large nucleus within the organogenic callus at the onset of the third subculture on the induction medium (day 1). (B) Meristemoids within the callus (day 7). (C) Meristemoid at higher magnification. (D, E) Cells with typical meristematic features (D) and vacuolated cells (E) magnified from the rectangles of (C). (F) Polarized meristemoid showing meristematic cells at one pole only (day 7). (G) Shoot dome showing meristematic tunica and corpus (arrow) (day 14). (H) Root primordia at different developmental stages (day 14). (I) Longitudinal section of an adventitious vegetative bud (end of the first month on the expression medium). (J) Longitudinal section of an adventitious root (end of the first month on the expression medium). (K) Transection of an adventitious root (arrow) within the callus (end of the second month on the expression medium). (L) Xylogenic nodule in the callus. The arrows show neo-cambial cells at the border of the nodule (end of the first month on the expression medium). (M) Morphology of the callus in the recalcitrant line (day 30). (N) Tracheary elements (arrows) formed by trans-differentiation of callus cells in the recalcitrant line (day 30). Sections stained with eosin and Carazzi's haemalum. Bars: 10 μm (A, C–F, L, N); 20 μm (M); 50 μm (B, H–K); 100 μm (G).

medium, some xylogenic nodules, arising from small meristematic clumps, randomly located deep in the callus, were also formed. They exhibited a vascular core of tracheary elements, in part or totally surrounded by a sheath of neocambial cells (Fig. 2L).

The callus of the recalcitrant line appeared to be constituted by proliferating cells, similar in size and features to the callus cells of the embryogenic and organogenic lines, but without any embryogenic or organogenic event, on both the induction (Fig. 2M) and the expression media. The only morphogenic event observed was the occasional trans-differentiation of some callus cells into tracheary elements (Fig. 2N, arrows).

### *Cloning of* CpSERK1 *and* CpSERK2

*SERK* homologues were searched in *Cyclamen persicum* to investigate their possible role(s) in the processes of embryogenesis and organogenesis *in vitro*. A partial cDNA of 750 bp was isolated by RT-PCR from the embryogenic callus line using degenerate primers derived from *SERK* conserved domains (Baudino *et al.*, 2001). The putative SERK-like fragment was elongated by 3′-RACE and gave a partial cDNA of 1158 bp, encompassing a putative 3′ untranslated sequence (3′-UTR). DNA sequencing of the cloned cDNA fragment revealed the presence of two different clones of similar size, showing high nucleotide identity to each other (92%), and to *SERK* genes from different species (ranging from 79% to 82% identity), consistent with the presence in the cyclamen genome of two genes belonging to the *SERK* family. Accordingly, the two genes were named *CpSERK1* and *CpSERK2*.

To distinguish unambiguously these two cDNA fragments, the *SERK*-like genes were isolated from genomic DNA using a combination of genome walking and iPCR techniques. Two full-length genomic sequences belonging to

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*CpSERK1* and *CpSERK2* were assembled from overlapping contigs and deposited in GenBank under the Accession Number JF511659 and GU189408, respectively. The contiguity and identity of the genomic sequences were verified by PCR amplification and re-sequencing of overlapping regions lying between introns and covering the whole genomic sequences. *CpSERK1* and *CpSERK2* turned out to be 10360 bp and 8132 bp in length, respectively, with a conserved exon/intron structure, typical of the *SERK* family, made up of 11 exons and 10 introns (Fig. 3). Unlike intron sequences and UTR regions (see Fig. 3 and Supplementary Figs S2 at *JXB* online), all exons are highly conserved resulting in exons of similar size and sequence, with an overall predicted coding sequence (CDS) of 1887 bp for both genes. In particular, the nucleotide identity between *CpSERK1* and *CpSERK2* is 90% in exon regions, 40% in intron regions, 75% in the 5′-UTR region, and 32% in the 3′-UTR region (see Supplementary Fig. S2 at *JXB* online). The coding sequences of both genes were verified by a combination of *in silico* analysis, pair-wise comparison with other *SERK* genes, and RT-PCR (from embryogenic calli) with primers designed to amplify exon regions. Full-length cDNA sequences, including the 5′- and 3′-UTR regions, were eventually determined for both genes by means of 5′-RACE. As a control, the RT-PCR fragments, amplified with primers specific for the 5′- and 3′-UTR portions of *CpSERK1* and *CpSERK2*, respectively, were re-sequenced.

A Blast search, queried with *CpSERK1* and *CpSERK2* coding sequences, returned several high-identity matches to *SERK* genes from a number of plant species, confirming that *CpSERK1* and *CpSERK2* can be regarded as genuine *SERK* genes.

### *Structure of CpSERK1 and CpSERK2*

Both *CpSERK1* and *CpSERK2* encode a putative SERK-like protein of 628 aa, with a deduced amino acid sequence highly similar (up to 96%) to a number of SERK-like proteins from different species. As shown in the ClustalW alignment of Fig. 4, the similarity between the two cyclamen proteins and other SERK proteins is striking and it is spread all over the sequence, with the notable exception of the first exon, where only a weak similarity is found.

A bioinformatic analysis of both amino acid sequences evidenced all the typical features of the SERK-like proteins (Fig. 3), such as putative signal peptide (SP), with possible cleavage sites between position 29–30 of CpSERK1 and 23–24 of CpSERK2 (see Supplementary Fig. S3 at *JXB* online), a leucine zipper (ZIP) domain, with a canonical $Lx_6Lx_6Lx_6L$ signature, and five leucine-rich repeats domains (LRRs). A proline-rich domain (SPP) was found in the seventh exon, immediately preceding a single transmembrane domain (TM) in the eighth exon. A typical serine/threonine kinase domain, made up of 11 canonical subdomains and including a putative activation loop region (a SERK-specific A-loop catalytic domain, spanning residues 450–478) was detected from exons 9 to 11, just before a leucine-rich terminal region (C domain).

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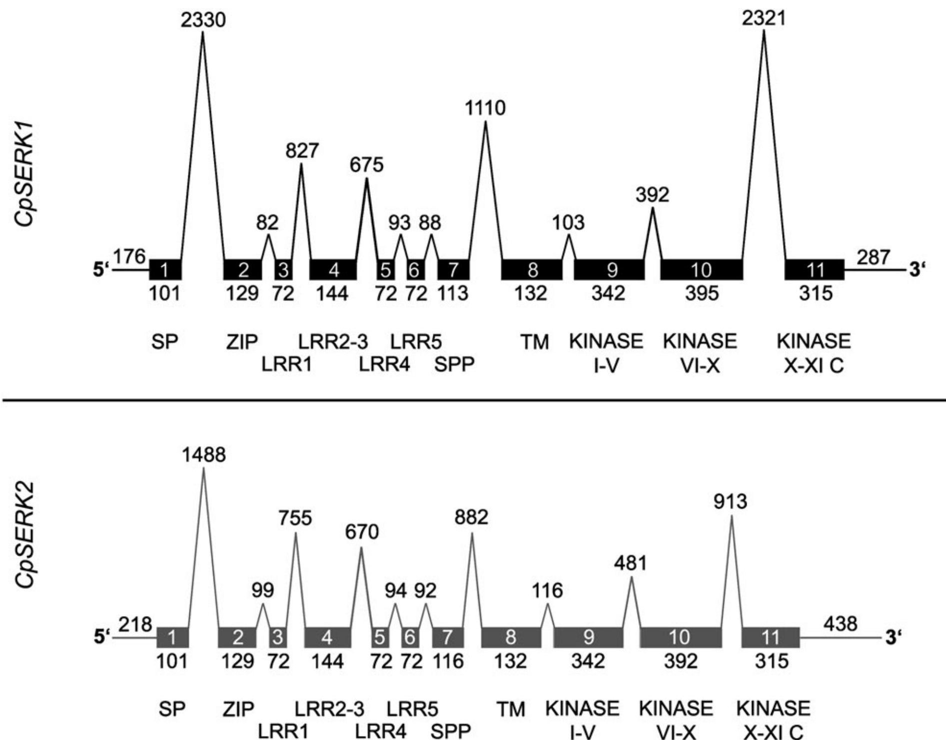


**Fig. 3.** Structure of *CpSERK1* and *CpSERK2*. A schematic drawing of *CpSERK1* and *CpSERK2* genomic sequences shows the conserved exon/intron structure, typical of the SERK family, made up of 11 exons and 10 introns. Open boxes represent exons and lines represent introns. Numbers represent bp length. SP, signal peptide; ZIP, leucine zipper; LRR, leucine rich repeats; SPP, serine-proline-proline domain; TM, transmembrane region; Kinase, kinase catalytic domains; C, C-terminal domain.

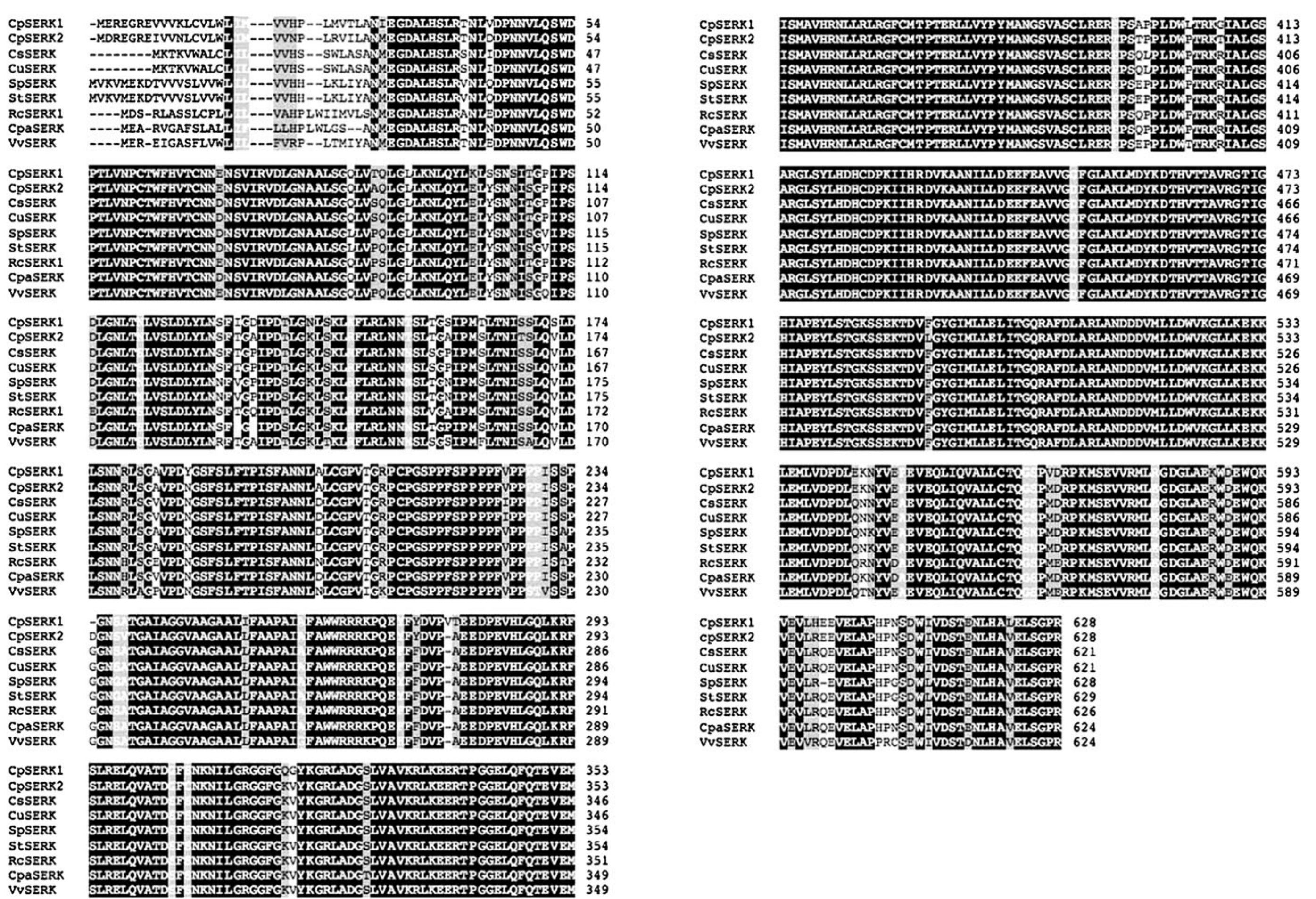

**Fig. 4.** ClustalW alignment of the deduced amino acid sequence of CpSERK1 (JF511659) and CpSERK2 (GU189408) with SERK sequences from *Rosa canina* (ADM94278), *Solanum peruvianum* (ABR18800), *Citrus sinensis* (ACP20180) and *C. unshiu* (AB115767), *Carica papaya* (ABS32228), *Solanum tuberosum* (ABS32228), and *Vitis vinifera* (XP_002270847). The SERK proteins chosen for the multi-alignment were those found most similar to CPSERK1 and CpSERK2 by mean of a BLAST analysis.

In addition, for both CpSERK1 and CpSERK2, six putative *N*-glycosylation [five in the LRR domains (from LRR2 to LRR5) and one in the fifth kinase domain] were detected by Scan Prosite. Moreover, ten putative *N*-myristoylation sites [four in the LRR domains, from LRR1 to LRR5, four in the TM domain, and two in the kinase domains (V and VI) for CpSERK1, whereas five in the LRR domains, from LRR1 to LRR5, four in the TM domain, and one in the fifth kinase domain for CpSERK2] were also detected.

To infer evolutionary relationships among SERK proteins, a phylogenetic tree was built by multi-aligning the amino acid sequences of CpSERK1 and CpSERK2 to the most similar SERKs, as identified by the BLAST search (Fig. 4). In addition, DcSERK and AtSERK amino acid sequences were added to the multi-aligment.

The resulting unrooted tree (Fig. 5) confirmed that the two cyclamen SERKs are closely related to each other. SERK proteins from *Solanum* species, such as *S. tuberosum* and *S. peruvianum,* appear the SERKs most evolutionary related to *Cyclamen persicum* SERKs, which are also close to SERK proteins from *Vitis vinifera* and, in a distinct branch, *Citrus sinensis* and *C. unshiu, Carica papaya*, and *Rosa canina.* DcSERK and AtSERKs appear well separated in a distinct cluster, with AtSERK1 and AtSERK2 relatively closer to, and DcSERK, AtSERK3, AtSERK4, and AtSERK5 more distantly related to CpSERK1 and CpSERK2.

### CpSERK1 *and* CpSERK2 *are expressed during embryogenesis and organogenesis both* in vitro *and* in planta

To investigate the expression of *CpSERK1* and *CpSERK2* in somatic embryogenesis and organogenesis, a qRT-PCR analysis was performed in calli of the embryogenic, organogenic, and recalcitrant lines collected at different times (i.e. from 7–30 d during the third subculture on the induction medium). As shown in Fig. 6, *CpSERK1* (upper panel) and *CpSERK2* (lower panel*)* are mainly expressed in the early phases (i.e. at day 7) of somatic embryogenesis. A strong correlation between the expression of *CpSERK1* and *CpSERK2* and the embryogenic potential of the tissues was found, as expression was high in embryogenic, moderate in organogenic, and absent in recalcitrant calli. However, the observation that *CpSERK1* and *CpSERK2* are also expressed

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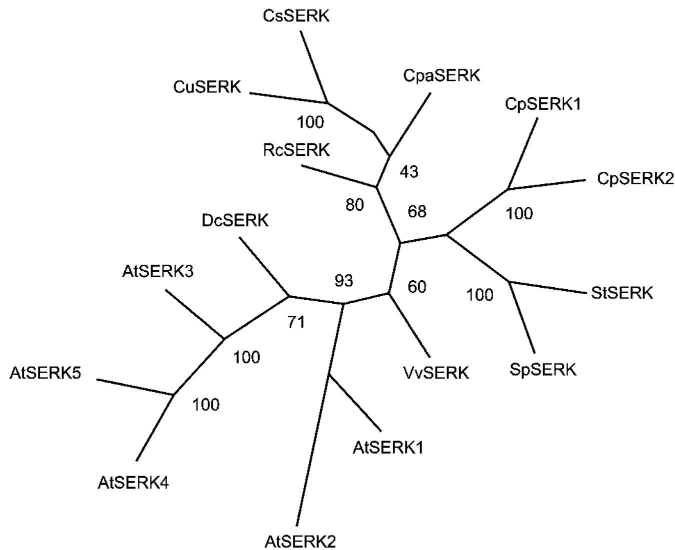


**Fig. 5.** Unrooted phylogenetic tree of SERK proteins built from significant BLASTP alignments to CpSERK1 and CpSERK2. The evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei, 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. At, *Arabidopsis thaliana*; Cpa, Carica *papaya;* Cs, *Citrus sinensis*; Cu, *Citrus unshiu*; Cp, *Cyclamen persicum*; Dc, *Daucus carota*; Rc, *Rosa canina*; Sp, *Solanum peruvianum*; St, *Solanum tuberosum;* Vv, *Vitis vinifera*.

during the early phases of organogenesis, athough at a lower level than in embryogenesis, suggests that both genes are needed for, or involved in, the early control of various developmental programmes *in vitro* (i.e. embryogenesis, rhizogenesis, and shoot formation).

*In situ* hybridization analyses were carried out for determining *CpSERK1* and *CpSERK2* expression at tissue level during the different programmes. The analyses were carried out at the same times of the histological analysis (Figs 1, 2) using RNA probes designed in regions discriminating between the two genes (see Supplementary Fig. S2 at *JXB* online). The results are reported in Figs 7 and 8. The absence of hybridization signal using the sense probes is shown in Supplementary Fig. S4A–J at *JXB* online. The same results were obtained by *in situ* hybridizations with the same probes on sections of calli from all the unselected embryogenic, organogenic, and recalcitrant lines (data not shown).

In the groups of few cells with meristem-like features (Figs 1A, 2A) present in the embryogenic and organogenic calli at the onset of the third subculture on the induction medium, both *SERK*s were expressed (Figs 7A, 8A, B). In the embryogenic line, the meristematic multicellular clumps derived by such groups (Fig. 1B, C) continued to show the expression of both genes, as exemplified for *CpSERK2* in Fig. 7B, C. In the organogenic programme, the same event occurred, because also the meristemoids, derived by division activity from similar few-celled groups, showed uniform expression, as exemplified for *CpSERK1* in Fig. 8C. However, when meristemoids grew further by cell divisions, becoming larger and heterogeneous in cell types (Fig. 2C–E), expression was still present in them (Fig. 8D), but localized in their smaller and not yet vacuolated cells (Fig. 8D, E).

On day 7, the majority of the cells in both types of PEAs (Fig. 1D, E) of the embryogenic calli showed the expression of both genes (Fig. 7D–F). Only in the largest globular aggregates was the expression of *SERK* genes less uniform (Fig. 7G, H).

In the polarized meristemoids of the organogenic calli (Fig. 2F), gene expression remained confined to the meristemoid pole exhibiting meristematic cells (Fig. 8F).

The comparison between the embryogenic and organogenic calli at day 7 showed that the number of cells expressing the two genes was many fold higher in the former than in the latter calli, in accordance with the qRT-PCR results (Fig. 6), and with the higher meristematization of the embryogenic callus at the same day (compare Figs 1D, 2B).

On day 14, PEMs (Fig. 1G) showed a weak expression (Fig. 7I), independently on the gene, and expression became weaker in the biggest (clustered) ones (Fig. 7J). By contrast, expression was high in the PEM-derived globular shaped embryos (Fig. 7K). On the same day, the shoot and root primordia, present in the organogenic callus (Fig. 2G, H), showed the expression of both genes restricted to the meristematic domes (Fig. 8G, H).

During the culture on the expression medium, both *CpSERK1* and *CpSERK2* were expressed in all the meristematic cell populations of the embryo at the torpedo stage (Fig. 7L), the same as in the few meristematic cells of the mature embryo, i.e. in the shoot pole (Fig. 7M), the procambium in the hypocotyl, and in the root pole (Fig. 7N). In the shoot primordium, the expression of both genes was observed in the apical dome, in the procambium and in the inception sites of the leaf primordia (Fig. 8I). Expression strongly declined in the rare, and high callused, shoot buds (Fig. 8J). By contrast, the adventitious roots showed expression in the apex (Fig. 8K), in some pericycle cells (Fig. 8L), possibly those involved in lateral root initiation, and in lateral root primordia (Fig. 8M).

Moreover, during culture on the expression medium, small meristematic clumps with signal were also randomly observed in the organogenic callus, the same as rare deep meristemoids with a central core of unexpressed cells surrounded by a sheath of cells with expression. The xylogenic nodules arising from such meristemoids were flanked, in longitudinal section, and surrounded, in transection, by neocambial cells maintaining expression (Fig. 8N, O).

The genes were not expressed in the callus cells and in the trans-differentiated tracheary elements of the recalcitrant line (Fig. 8P, Q). The results were confirmed by the sense probes (see Supplementary Fig. S4K at *JXB* online).

The expression of *CpSERK1* and *CpSERK2* was also investigated *in planta* by *in situ* hybridizations with the same probes used for the analysis on *in vitro* embryogenic and organogenic responses (Fig. 9).

In the SAM, the expression of both genes was detected in the leaf primordia, in the inception sites of the axillary buds, in the rib meristem, and in the tunica; moreover, it

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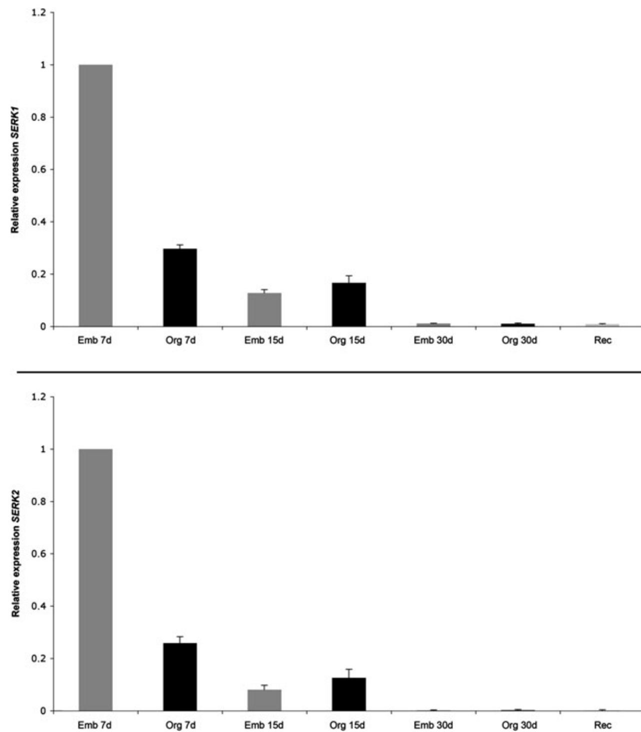


**Fig. 6.** Real-time RT-PCR of *SERK1* (upper panel) and *SERK2* (lower panel) expression in embryogenic (grey bars) and organogenic (black bars) calli analysed from 7 d to 30 d from the onset of the third subculture on the induction medium. The recalcitrant callus line (light grey bars) (Rec) was analysed at day 15. The two genes appear much more expressed in embryogenic calli than in organogenic calli in the early phases (day 7) rather than in the more advanced phases (days 15 and 30) of the culture. No significant expression is observed in recalcitrant calli.

was observed in the procambial strands of stem and leaves (Fig. 9A). In the RAM, the expression of both *SERK*s was also evident (Fig. 9B) and, in the differentiating root, it was shown in the still meristematic rhizodermis and in the central procambial core from which the vascular system of the root develops (Fig. 9C). In the floral apex the expression of both genes was shown by the primordia of each whorl but, in particular, by the forming pistil (Fig. 9D). In the unopened flower, the two genes showed expression in the differentiating stamens, with a conspicuous signal in the initial archesporial (sporogenous) cells and in the surrounding tapetum within the anther thecae (Fig. 9E, G, H). The signal was also in the connective tissue and procambium of the central strand connecting the anther to the filament (Fig. 9G). In the pistil, the expression of both genes characterized the forming ovules (Fig. 9F), the receptacle (Fig. 9G), and the procambial strands (Fig. 9E). No expression was observed with the sense probes (see Supplementary Fig. S4L–N at *JXB* online). In the pollinated flowers, a strong expression was observed in the developing seeds in the ovary, i.e. in the early divisions of the zygote within the embryo sac (globular-like stage included), in some cells of the nucellus, and in the outer ovular layers (Fig. 9I, J). In seeds still enclosed in the fruit, and containing embryos at the torpedo stage, the expression of both genes was still high, and located in the embryo (Fig. 9K, L). In the mature embryo, showing only one fully developed cotyledon, according to Wicart *et al.* (1984), the expression of both genes was highly reduced and present only at the shoot and root poles, and in the procambium (not shown). No expression was observed with the sense probes neither in the embryo nor in the primary root (see Supplementary Fig. S4O–Q at *JXB* online).

## Discussion

In *Cyclamen persicum* callus, embryogenesis and organogenesis originate from small groups of cells exhibiting the same meristematic features. These cells produce derivatives, i.e. pre-embryogenic aggregates (PEAs) and meristemoids, respectively. The meristematic features of the stem cells persist in the PEAs, whereas a rapid loss of meristematization occurs in the meristemoids.

The initial cells and their derivatives in both embryogenesis and organogenesis show the activity of two *SERK* genes, *CpSERK1* and *CpSERK2*. The expression of both genes declines in the *de novo* formed organs and in somatic embryos, remaining confined to their meristematic cell populations. The same occurs in the SAM, RAM, and procambium during vegetative and reproductive growth *in planta*, and in the zygotic embryo. Both genes are not expressed during tracheary differentiation.

### *Embryogenesis and organogenesis in cyclamen callus derive from clumps of pluripotent stem cells, and, the switch to totipotency occurs in PEAs*

In a previous histological study, shoots, roots, and embryo-like structures were obtained in cyclamen callus changing the concentration and the type of auxin and cytokinin in the medium, however, in some cases, all the types of the regenerated structures were observed at the same time independently on the exogenous hormonal input, suggesting that a single organogenic pattern was active, and that it started from a meristemoid, both in organogenesis and in embryogenesis (Wicart *et al.*, 1984). According to these authors, a complete dedifferentiation event in the meristemoid allows pluricellular-in-origin somatic embryogenesis to occur instead of organogenesis.

Here the pluricellular origin of somatic embryos is confirmed because, in no case, were single cells found to originate the embryos, neither in the selected embryogenic line (see Results), nor in all the other embryogenic lines produced (M Savona, unpublished data). Moreover, present histological analysis shows that the initiating clumps leading to either embryos or organs are composed of a very low number of cells. This number is many fold smaller than in a meristemoid, even if the cells exhibit the meristematic features of a meristemoid (Capitani *et al.*, 2005; Fattorini *et al.*, 2009; present results). On this basis, the origin of both embryogenesis and organogenesis in cyclamen was re-interpreted

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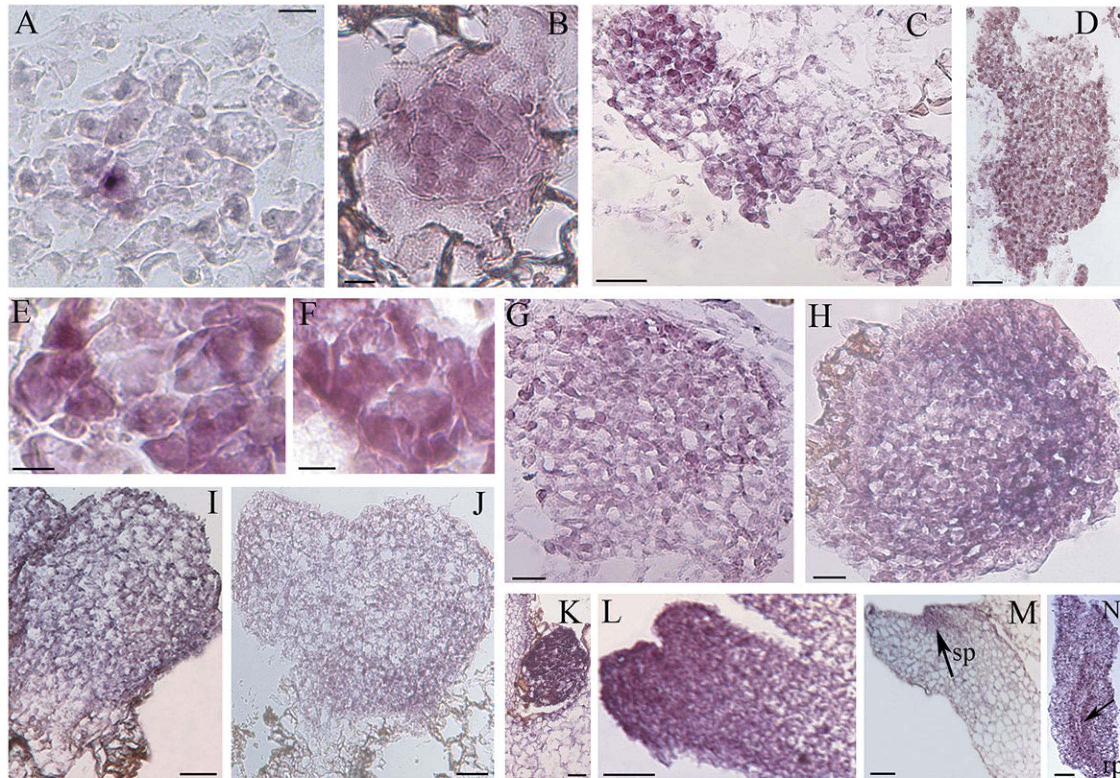


**Fig. 7.** Analysis of the expression profiles of *CpSERK1* and *CpSERK2* by RNA *in situ* hybridization during somatic embryogenesis in *Cyclamen persicum* (A–K, induction medium; L–N, expression medium). (A) Small group of cells showing the appearance of *CpSERK1* expression (day 1). (B) Meristematic cell clump showing *CpSERK2* expression (day 1). (C) Strong signal of *CpSERK2* in a meristematic multicellular clump at callus surface (day 3). (D) Large PEA showing uniform *CpSERK1* expression (day 7). (E, F) Magnifications of meristem-like cells within a PEA with a strong signal of *CpSERK1* (E) and *CpSERK2* (F) (day 7). (G, H) Large globular PEAs with uneven expression of either *CpSERK1* (G) or *CpSERK2* (H) (day 7). (I) PEM showing a weak *CpSERK2* expression (day 14). (J) Clustered PEM with a very weak *CpSERK1* signal (day 14). (K) Globular shaped embryo showing an uniform and high expression of *CpSERK1* (day 14). (L) Somatic embryo at torpedo stage showing strong *CpSERK2* signal in the meristematic cells (end of the first month on the expression medium). (M, N) Mature embryo with expression of *CpSERK1* (M) and *CpSERK2* (N) (end of the first month on the expression medium). Arrows show expression in the shoot pole (sp, M), in the procambium (N), and in the root pole (rp, N). Bars: 10 μm (A–C, E, F); 20 μm (G, H); 50 μm (D, I–K); 100 μm (L–N).

by us as pluricellular, starting from founder clumps (and not meristemoids) of similarly pluripotent cells.

The mechanism of stem cell formation in the callus is still unclear. However, in *Arabidopsis* and *Medicago truncatula* the presence of stem cells has been hypothesized for both organogenesis *in vitro*, and somatic embryogenesis (Zhao *et al.*, 2008; Su and Zhang, 2009; Wang *et al.*, 2011).

*In planta*, the precursors of stomata are considered to be cells with a transient stem cell activity (Gray *et al.*, 2008). Similar to the precursors of stomata, the cells in the founder clumps of cyclamen callus seem to exhibit features of transient stem cells, because they are formed *de novo* and because they exhibit the characteristics both of self-renewing by division activity and of producing derivative cells with a different developmental fate (embryonic/caulogenic/rhizogenic/xylogenic fate).

The present results also show that the stem cell clumps grew into multi-celled PEAs in the embryogenic callus, and into meristemoids in the organogenic one. According to the classical concept of trans-amplifying cells (TAs) in animals and plants (Singh and Bhalla, 2006, and references therein), both PEAs and meristemoids seem to exhibit characteristics of TAs, because both derive from stem cells, are formed by cells with meristem-like features and division capabilities, and give rise to organized structures with a specific development, i.e. somatic embryos from PEAs, and shoots/roots/vascular nodules from the meristemoids.

*In planta*, TAs amplify the number of cells produced by division from stem cells. Initially, they maintain many of the characteristics of the stem cells, but gradually lose these properties, and restrict differentiation potential (Singh and Bhalla, 2006; Kwaaitaal and de Vries, 2007). This seems exactly the case of cyclamen meristemoids, in which a rapid restriction of meristematic features, resulting in a canalization towards either caulogenesis (i.e. shoot formation) or rhizogenesis or xylogenesis, is observed.

By contrast, the case of PEAs seems different. In fact, their TAs maintain the meristematic features of their stem cells for a much longer time than those of the meristemoids.

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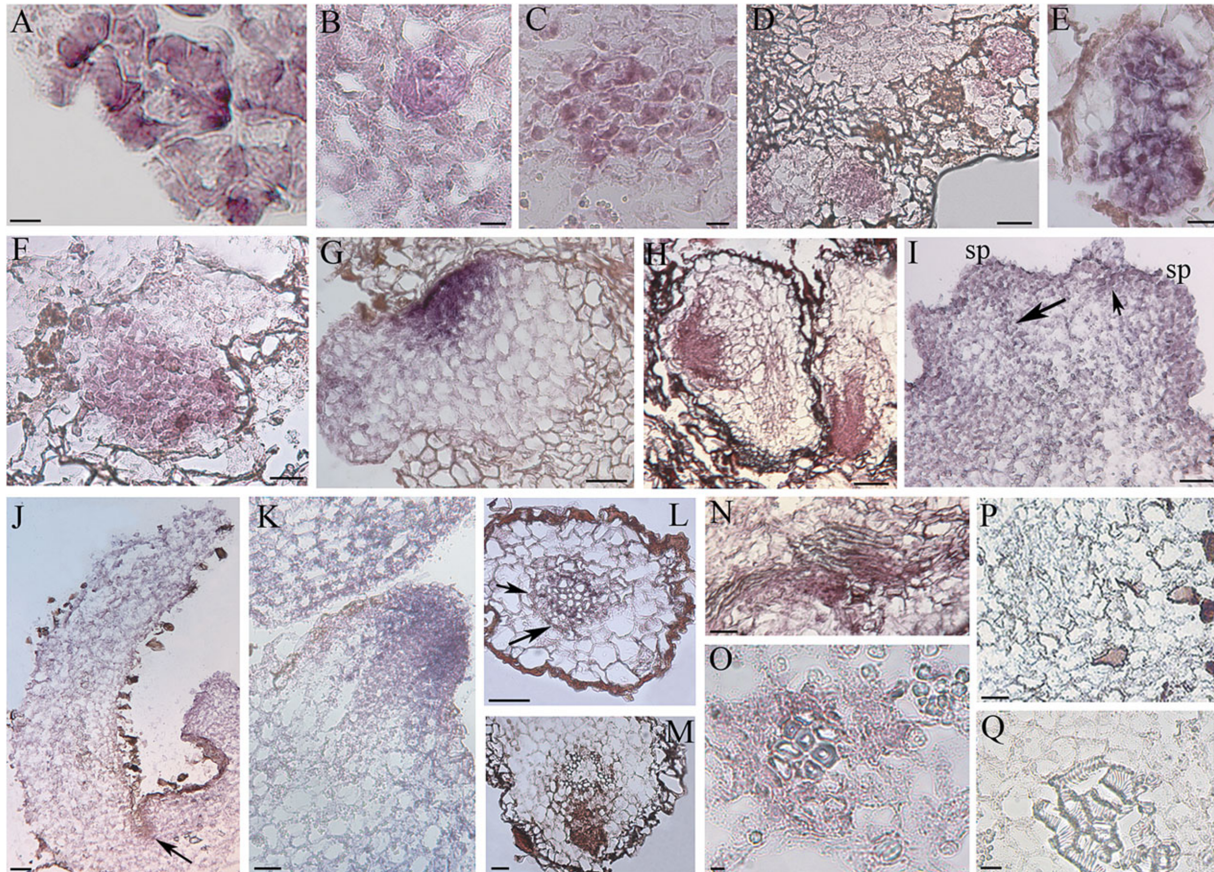


**Fig. 8.** Analysis of the expression profiles of *CpSERK1* and *CpSERK2* by RNA *in situ* hybridization during organogenesis (A–M) and xylogenesis (N, O) in the organogenic line, and in the recalcitrant callus (P, Q) of *Cyclamen persicum*. (A–H, P, Q, induction medium; I–O, expression medium). (A, B) Small initial groups of meristematic cells in which *CpSERK1* (A) and *CpSERK2* (B) expression appears (day 1). (C) Early meristemoid showing *CpSERK1* uniform expression (day 3). (D) Meristemoids in the callus with *CpSERK1* expression (day 7). (E) Strong signal of *CpSERK2* only in the still meristematic cells of the meristemoid (day 7). (F) Polarized meristemoid showing *CpSERK1* signal only in the part with meristematic cells (day 7). (G) Meristematic shoot dome with a strong *CpSERK2* expression (day 14). (H) Root primordia showing the signal of *CpSERK2* in the root domes (day 14). (I) Twin shoot primordia showing *CpSERK1* expression in the apical domes (sp), procambium (large arrow), and in the inception sites of the leaf primordia (small arrow) (end of the first month on the expression medium). (J) Vegetative bud showing a very weak *CpSERK1* expression which is mainly localized in the inception site of an axillary bud (arrow) (end of the second month on the expression medium). (K) Apex of an adventitious root with *CpSERK2* signal (end of the first month on the expression medium). (L, M) Transections of mature roots showing *CpSERK1* expression in the pericycle (L, arrows) and in a lateral root primordium (M) (end of the second month on the expression medium). (N, O) Longitudinal (N) and transverse (O) sections of xylogenic nodules. The signal of *CpSERK1* is localized in the neo-cambial cells (end of the first month on the expression medium). (P) Callus proliferation from the recalcitrant line, without any *SERK* signal (note that the isolated stained cells contained inclusions of terpenoid compounds (Mihci-Gaidi *et al*., 2010) (day 14). (Q) Trans-differentiated tracheary elements without expression of *CpSERK2* (day 30). Bars: 10 μm (A–C, E, O, P, Q) and 50 μm (D, F–N).

The possible interpretation of this difference is that a cell fate switch from the pluripotency to the totipotency, known to be necessary for embryogenesis, occurs specifically in PEAs. Thus, totipotency in cyclamen callus might result from a maintenance over time of the pluripotent fate in the TAs of PEAs.

### *Isolation of* CpSERK1 *and* CpSERK2 *as a tool to mark founding events in somatic embryogenesis and organogenesis*

To study the role of SERK(s) in the founding events underpinning somatic embryogenesis and organogenesis in cyclamen, two *SERK* genes were isolated from embryogenic calli during the induction phase. The two genes were named *CpSERK1* and *CpSERK2* because they encoded proteins resembling receptor-like kinases belonging to the LRR superfamily and exhibiting extensive similarity to *SERK*s of other species.

The isolation of two *SERK* genes in cyclamen is not surprising as, in other species such as *Arabidopsis thaliana* (Hecht *et al.*, 2001), *Zea mais* (Baudino *et al.*, 2001; Zhang *et al.*, 2011), *Triticum aestivum* (Singla *et al.*, 2008), *Poa pratensis* (Albertini *et al.*, 2005), *Oryza sativa* (Ito *et al.*, 2005), *Vitis vinifera* (Schellenbaum *et al.*, 2008), and *Medicago truncatula* (Nolan *et al.*, 2011), the presence of small

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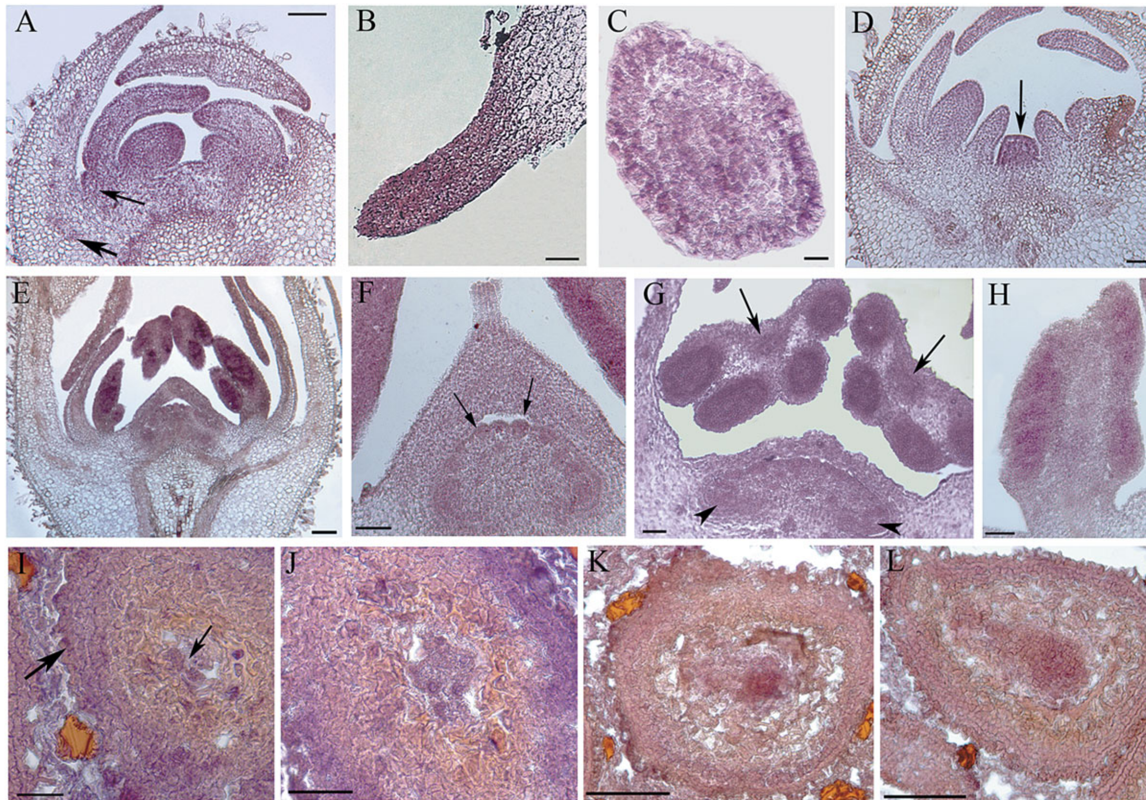


**Fig. 9.** Analysis of the expression profiles of *CpSERK1* and *CpSERK2* by RNA *in situ* hybridization during organ formation and zygotic embryogenesis *in planta*. (A) Shoot apical meristem (SAM) showing strong *CpSERK2* expression in the youngest leaf primordia, in their procambium (large arrow), and in an initiating axillary bud (small arrow). (B) Longitudinal section of a primary root showing *CpSERK1* expression in the apex. (C) Transection of a root differentiating the primary structure. A strong *CpSERK2* expression is evident in the protoderm and central procambium. (D) Longitudinal section of the floral apex showing the expression of *CpSERK1* in the primordia of petals and stamens, and a stronger one in the just forming pistil (arrow). (E) Longitudinal section of a flower showing strong *CpSERK2* expression in stamen primordia and procambia. (F) Magnification of the immature pistil showing the expression of *CpSERK2* in the ovule primordia (arrows) (longitudinal section). (G, H) Transection (G) and longitudinal section (H) of anthers (G, H) and forming pistil (G). A strong signal of *CpSERK2* (G) and *CpSERK1* (H) is present in the archesporium of the thecae (G), in the procambium connecting the anther to the filament (G, arrows), and in the receptacle (arrowheads). (I) Ovule after zygote formation in the embryo sac. *CpSERK2* is expressed in the dividing zygote (small arrow), in the nucellus and in the outer ovular layers (large arrow). (J) Ovule containing the globular embryo. A strong *CpSERK1* expression is shown by the embryo and the outer ovular layers. (K, L) Immature seeds containing embryos at torpedo stage showing *CpSERK1* (K) and *CpSERK2* (L) expression. Bars: 10 μm (C); 50 μm (B, E–K); 100 μm (A, D).

families of *SERK* genes has been described. Furthermore, seven SERK-like ESTs have been isolated by Rensing *et al.* (2005) from a cDNA library derived from a *Cyclamen persicum* embryogenic line, although an expression profiling study based on the same ESTs could only detect one gene belonging to the *SERK* family (Hoenemann *et al.*, 2010). Importantly, in the present study, a large cDNA fragment similar to *AtSERK3* (deposited in GenBank under the Accession Number EF661828), and a number of smaller fragments similar to *AtSERK4* and *AtSERK5* (data not shown), were isolated from the embryogenic calli in addition to *CpSERK1* and *CpSERK2*, strongly suggesting the existence of a *SERK* family also in cyclamen.

Pairwise alignment of the amino acid sequences of CpSERK1 and CpSERK2 shows high sequence identity (90%) between the two proteins, suggesting that, by similarity with other SERKs, they form a functional redundant pair. Indeed, it has been shown that SERK proteins tend to function as pairs of redundant proteins evolutionarily organized in clades related either to *AtSERK1/2* or *AtSERK3/4/5* (Albrecht *et al.*, 2008; Nolan *et al*, 2011). A phylogenetic analysis, carried out at the protein level, confirmed that CpSERK1 and CpSERK2 are tightly evolutionarily related, probably being derived by a recent gene duplication, and are relatively close to AtSERK1 and AtSERK2, but more distant to AtSERK3, AtSERK4, and AtSERK5.

Interestingly, in addition to a structural and phylogenetic similarity, these proteins seem to share a common localization since, as presently shown for *CpSERK1* and *CpSERK2* in cyclamen *in planta, AtSERK1* and *AtSERK2* are expressed in the same cells in *Arabidopsis in planta* (Hecht *et al.*, 2001; Albrecht *et al.*, 2005; Colcombet *et al.*, 2005; Kwaaitaal *et al.*, 2005). In addition*, CpSERK1* and *CpSERK2* also express in *Arabidopsis* and an *AtSERK1* promoter-*AtSERK1*-YFP construct [p*AtSERK1:AtSERK1:YFP*

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(Kwaaitaal *et al.*, 2005)], introduced into cyclamen embryogenic calli, expresses correctly the fusion protein and localizes in the PEAs and PEMs, as *CpSERK1* and *CpSERK2* (M Savona *et al.*, unpublished results).

Taken together, these results are consistent with the idea that different SERKs function in pairs in a particular developmental pathway, and that they cannot be substituted by other SERKs. The possibility of (homo/hetero)-dimerization has been suggested for AtSERK1 and AtSERK2 in tapetum specification and male sporogenesis and gametogenesis (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005), and for AtSERK1 and AtSERK3 in mediating brassinosteroid responses (Albrecht *et al.*, 2008).

Based on the present results, it is not possible to state whether CpSERK1 and CpSERK2 form homo/heterodimers in the developmental pathways in which they are involved. However, the high amino acid similarity between the two proteins, their phylogenetic relationships, the similar expression profile, and the overlapping expression pattern, discussed later, might suggest that they act together.

### CpSERK1 *and* CpSERK2 *are expressed in the stem cells for somatic embryogenesis and organogenesis* in vitro

Present results show that, in the embryogenic callus of cyclamen, the expression of *CpSERK1* and *CpSERK2* was initially confined to the small clumps of initial cells.

A similar expression has been observed for *DgSERK* in *Dactylis glomerata* and for *DcSERK* in *Daucus carota* (Schmidt *et al.*, 1997; Somleva *et al.*, 2000). Thus, independently on the multicellular (*Dactylis glomerata* and *Cyclamen persicum*) or unicellular (*Daucus carota*) origin of somatic embryos, present and past results are in accordance in showing an early expression of specific *SERK*s in the initial cells.

However, in cyclamen, the same *CpSERK1* and *CpSERK2* positive clumps were also present in the organogenic callus.

In sunflower, caulogenesis and somatic embryogenesis originate from the same groups of cells under the same inductive PGR condition, and the transcripts of *HaSERK* accumulate at the beginning of both processes (Thomas et *al.*, 2004). In *Medicago truncatula,* rhizogenesis and somatic embryogenesis are induced by different PGR conditions, in contrast to cyclamen and sunflower, however, *MtSERK1* is also expressed in the early phases of both programmes (Nolan et *al.*, 2003; Wang *et al.*, 2011). As discussed above, the cells of the initial small clumps of embryogenesis and organogenesis in cyclamen callus seem to exhibit features of transient stem cells. Thus, independently of the species and the PGR input necessary for the expression of (one/more) *SERK*(s) in one/more developmental programme(s) in *in vitro* culture, the proteins coded by such *SERK*s, e.g. CpSERK1 and CpSERK2 in cyclamen embryogenic/organogenic calli, may be involved in the specification of pluripotency of the initiating stem cells.

Accordingly, *StSERK* has been hypothesized to mark the generation of pluripotent cells, capable of developing into several different cell types and pathways, i.e. organogenesis, embryogenesis, and apoptosis, in potato (Sharma *et al.*, 2008), and *MtSERK1* expression has been proposed to be associated with developmental change, reflecting cellular reprogramming *in planta* and in the embryogenic callus, in *Medicago truncatula* (Nolan *et al.*, 2009; Wang *et al.*, 2011).

### CpSERK1 *and* CpSERK2 *are expressed in TAs involved in the formation of somatic embryos and organs, and expression persists in the meristems of embryos and organs,* in vitro *and* in planta

Present *in situ* hybridization analysis reveals that the majority of the cells in the PEAs of the embryogenic calli exhibited *CpSERK1* and *CpSERK2* expression. By contrast, in the polarized meristemoids of the organogenic calli, the expression of both genes was restricted to the meristemoid pole only. The analysis by qRT-PCR parallels these quantitative differences between the programmes and not between the two genes.

In accordance with *CpSERK1* and *CpSERK2* expression patterns, real-time PCR results in sunflower show that a rise in *HaSERK* expression, many fold higher in the embryogenic than in the shoot-forming programme, occurs only few days after induction (Thomas *et al.*, 2004).

As discussed above, in a PEA, TAs are numerous and totipotent, with this fate resulting from the original pluripotent fate of the initiating clump. By contrast, in a meristemoid, due to its early canalization towards differentiation, only a few pluripotent TAs remain present from the original pluripotent initiating clump. Accordingly, the more extended expression by *in situ* hybridization analysis of *CpSERK1/2* in the TAs of PEAs compared with the TAs of the meristemoids might suggest that CpSERK1/2 are responsible of, or involved in, keeping the TAs of PEAs in the pluripotent state of the original stem cells, in agreement with the hypothesis advanced for AtSERK1 in *Arabidopsis* embryogenesis from procambial cells and derived TAs (Kwaaitaal and de Vries, 2007).

The present results showed that *CpSERK1* and *CpSERK2* expression decreased and, in a similar way, during the following developmental phases of both somatic embryogenesis and organogenesis, remaining confined in the globular embryos and in the meristems of mature embryos and organs. Also in sunflower, *HaSERK* returns to the basal level in both embryogenic and shoot-forming media, but the signal persists in the meristematic tissues of the few developing structures (Thomas *et al.*, 2004), and the same occurs in somatic embryos of *Dactylis glomerata*, *Theobroma cacao*, and coconut (Somleva *et al.*, 2000; de Oliveira Santos *et al.*, 2005; Pérez-Núñez *et al.*, 2009).

Moreover, *CpSERK1* and *CpSERK2* were also expressed, without any difference in the localization sites between the two, in all the types of apical and primary lateral meristems of the vegetative and reproductive plant.

These observations are in general accordance with the expression pattern of *MtSERK1* in *Medicago truncatula in planta* (Nolan *et al.*, 2009). However, *MtSERK1* is not

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expressed in the anther thecae, excluding a role for this gene in male sporogenesis (Nolan *et al.*, 2009). By contrast, a strong expression of *CpSERK1* and *CpSERK2* was present in the sporogenous tissue and in the tapetum of the developing anthers of cyclamen. The same localization has been observed for *AtSERK1* and *AtSERK2* (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005, Kwaaitaal *et al.*, 2005). Moreover, *AtSERK1* and *AtSERK2* are expressed in the ovule primordium and embryo sac up to fertilization in *Arabidopsis* (Colcombet *et al.*, 2005; Hecht *et al.*, 2001; Kwaaitaal *et al.*, 2005), the same as *CpSERK1* and *CpSERK2*, in cyclamen (present results). This overlapping expression in male and female reproductive organs further contribute to the hypothesis that CpSERK1 and CpSERK2 may act as a functional pair evolutionary related to AtSERK1 and AtSERK2.

The two genes were highly expressed during early zygotic embryogenesis, and remained expressed in the meristems of the mature embryo, confirming the expression patterns of *AtSERK1* in *Arabidopsis* (Hecht *et al.*, 2001; Kwaaitaal *et al.*, 2005), and *MtSERK1* in *Medicago truncatula* (Nolan *et al.*, 2009).

In mammalian embryogenesis, the totipotent derivatives of the zygote provide early formative divisions, and their pluripotent derivatives subsequent divisions up to the construction of the mature embryo (Burdon *et al.*, 2002). Consistently, *CpSERK*s, as *SERK*s with similar patterns in other species, might mark the totipotent and pluripotent TAs in the early phases, and the pluripotent meristematic cells deriving from them, in the mature phases of zygotic embryogenesis.

### CpSERK1 *and* CpSERK2 *are not related to apoptosis*

In cyclamen, the organogenic calli showed xylogenesis as a late morphogenic event. Apoptosis is known to occur in the differentiating tracheary cells of the xylogenic nodule (Falasca *et al.*, 2008, and references therein). Interestingly, in the organogenic callus both *CpSERK1* and *CpSERK2* were expressed in the initiating clumps of xylogenesis, formed by stem cells, and in the neo-cambial (TA) cells at the periphery of the mature xylogenic nodules. The expression, instead, disappeared in the central core of the nodule, where the cells differentiated into tracheary elements. This expression pattern further sustains CpSERK1 and CpSERK2 roles in stem cell pluripotency and in the definition and maintenance of the meristem condition.

Moreover, direct tracheary trans-differentation was observed in the recalcitrant line, but no signal of *CpSERK*s occurred in the trans-differentiating cells. Similar to tracheary differentiation during xylogenesis *in vitro* and xylem formation *in planta* (Gray, 2004), apoptosis is required for the trans-differentiation into a tracheary cell, as demonstrated in the *Zinnia elegans* culture system (Obara and Fukuda, 2004, and references therein).

It is interesting that during both direct tracheary trans-differentiation and indirect tracheary element formation in xylogenesis, cells undergoing apoptosis do not express either *CpSERK1* or *CpSERK2*. The lack of expression of the two genes strongly suggests that they are not related to apoptosis. Similarly, *AtSERK1* and *AtSERK2* do not seem to be involved in apoptosis in *Arabidopsis* (He *et al.*, 2007; Kemmerling *et al.*, 2007). This further sustains the evolutionary relationships between CpSERK1/2 and AtSERK1/2.

In conclusion, in somatic embryogenesis, organogenesis, and xylogenesis *in vitro*, *CpSERK1* and *CpSERK2* are markers of the pluripotent initiating stem cells, further becoming markers of totipotency for somatic embryo formation. CpSERK1 and CpSERK2 are also involved in defining and maintaining meristems in both somatic and zygotic embryos, and in vegetative and reproductive organs.

Thus, *in planta* and *in vitro*, their action seems to characterize developmental pathways starting from pluripotent and totipotent cells, and the conversion between the two fates.

## Supplementary data

Fig. S1 *ATPase*-like EST isolated from *Cyclamen persicum* embryogenic calli. A blast analysis revealed high nucleotide identity with ATPase alpha subunit (atp1) genes from a number of species. The expression of this putative *ATPase* gene has been found at similar levels in all tissues analyzed, as expected for a housekeeping gene.

Fig. S2- Clustal W alignment of *CpSERK1* and *CpSERK2* cDNAs, showing high level of identity in the CDS region (90%) but low level of identity in the 5′-(75% ) and 3′-(32%) UTR, respectively. The 3′-UTR region appears as the most dissimilar region between the two genes and was chosen to design highly specific primers and probes capable to distinguish *CpSERK1* from *CpSERK2*.

Fig. S3 - Prediction of signal peptide. The presence of a N-terminal signal peptide is predicted at high probability by SignalP (http://www.cbs.dtu.dk/services/SignalP/) for both CpSERK1 (upper panel) and CpSERK2 (lower panel). Putative cleavage sites are predicted between position 22 and 23 (TLA-NI) and position 23 and 24 (VNP-LR) for CpSERK1 and CpSERK2, respectively. Signal peptide probability is 0.994 for CpSERK1 and 0.207 for CpSERK2, while anchor probability is 0.003 and 0.563 for CpSERK1 and CpSERK2, respectively.

Fig. S4 RNA *in situ* hybridizations using sense probes of *CpSERK1* (A, E, G, H, I, J, N, O) and *CpSERK2* (B, C, D, F, K, L, M, P, Q) genes.

A-E, Developmental stages of *Cyclamen persicum* somatic embryogenesis showing the absence of any signal with *CpSERK* sense probes. F-J, Developmental stages of *cyclamen rhizogenesis* and shoot formation *in vitro*. No specific signal with *CpSERK* sense probes is present at any stage. K, Trans-differentiated tracheary elements in the recalcitrant callus without any signal after hybridization with the sense probe of *CpSERK*. L-N, Various phases of flower development *in planta* showing no signal with *CpSERK* sense probes. O-P, Detail of fertilized ovules at early (O) and late (P) stages of zygotic embryogenesis. No specific signal with *CpSERK*

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sense probes is present. Q, Transection of a primary root without signal after hybridization with *CpSERK* sense probe. Bars: 10 μm (A-B, F-G, K); 20 μm (C, O-P); 50 μm (D, H-J, M, Q); 100 μm (E, L, N).

Table S1 List of primers used in this study.

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