A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos

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SUMMARY

The first somatic single cells of carrot hypocotyl explants having the competence to form embryos in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) were identified using semi-automatic cell tracking. These competent cells are present as a small subpopulation of enlarged and vacuolated cells derived from cytoplasm-rich and rapidly proliferating non-embryogenic cells that originate from the provascular elements of the hypocotyl. A search for marker genes to monitor the transition of somatic into competent and embryogenic cells in established suspension cell cultures resulted in the identification of a gene transiently expressed in a small subpopulation of the same enlarged single cells that are formed during the initiation of the embryogenic cultures from hypocotyl explants. The predicted amino acid sequence and in vitro kinase assays show that this gene encodes a leucine-rich-repeat containing receptor-like kinase protein, designated Somatic Embryogenesis Receptor-like Kinase (SERK). Somatic embryos formed from cells expressing a SERK promoter-luciferase reporter gene. During somatic embryogenesis, SERK expression ceased after the globular stage. In plants, SERK mRNA could only be detected transiently in the zygotic embryo up to the early globular stage but not in unpollinated flowers nor in any other plant tissue. These results suggest that somatic cells competent to form embryos and early globular somatic embryos share a highly specific signal transduction chain with the zygotic embryo from shortly after fertilization to the early globular embryo.

Key words: *Daucus carota* L., somatic embryo, embryogenic cell, leucine-rich repeat receptor-like kinase, zygotic embryo, SERK

INTRODUCTION

Somatic or asexual embryogenesis is the process whereby somatic cells develop into plants via characteristic morphological stages. The later stages, in particular, closely resemble zygotic embryo development, and in dicots pass through the globular, heart and torpedo stage. Somatic embryos have been obtained in many different plant species and from a wide variety of starting materials such as microspores, protoplasts, immature embryos, tissue explants and in vitro cultured cells (see for recent reviews Dudits et al., 1995; Mordhorst et al., 1997). The events that take place during the period in which plant cells undergo the transition from somatic to embryogenic cell are poorly understood (reviewed by De Jong et al., 1993). In tissue explants, the first response is often noted to be the rapid replacement of the vacuole with cytoplasm, followed by the first division. In *Dactylis glomerata*, leaf mesophyll cells respond this way (Trigiano et al., 1989), while in *Sorghum bicolor* also vascular tissue, close to the wound surface, responds (Wernicke and Brettel 1980). The same responses are observed in *Cichorium* (Dubois et al., 1991), but in contrast to these studies, in *Medicago sativa* (Dos Santos et al., 1983) and in *Ranunculus sceleratus* (Konar et al., 1972), particular types of epidermal cells were noted to be the responsive ones. In all of these examples, in vitro regenerated plantlets were the source of the explants used, and it appears that there is quite some variability in the tissue that responds first. A further complicating factor in unraveling the early stages of somatic embryogenesis is the fact that there is almost no evidence to show that the cellular changes observed in particular cells are indeed directly responsible or even necessary for embryo formation.

In *Daucus carota* (carrot), the formation of embryogenic cell cultures usually commences with the incubation of seed-derived seedling hypocotyl explants in auxin-containing medium (De Vries et al., 1988a). As in other species, following an increase in cytoplasmic content, cell division is resumed in provascular cells, but not in cortical or in epidermal cells (Guzzo et al., 1994). Continued cell divisions then result in the formation of masses of small isodiametric cells. These cells can then enlarge and finally detach into the culture medium to contribute to the developing embryogenic cell culture (Guzzo et al., 1994). Such an embryogenic culture contains morphologically and biochemically different cell types, that can be present as single cells or cell clusters (Van Engelen and De Vries, 1992), of which only 1-2% of the cells are actually embryogenic (De Vries et al., 1988a). The term ‘embryogenic’ is defined as the ability to form somatic embryos without further exogeneous application of growth regulators (De Jong et al., 1993). The origin of embryogenic cells, that are usually present as clusters of small cytoplasm-rich cells (Komamine et al., 1990) is not clear and is...
thought to involve an auxin dependent transition stage occurring in single cells. Cells in this transition between the somatic and embryogenic cell state are defined as competent cells (Komamine et al., 1990; Toonen et al., 1994), which is an operational definition based on the requirement for exogenous auxin. Recording of the developmental fate of many thousands of individual carrot cells from established embryogenic suspension cultures by cell tracking revealed that competent cells have a highly variable appearance that prevents their identification on the basis of morphology (Toonen et al., 1994). Using this system with cells from an activated carrot hypocotyl explant revealed that a small subset of a particular type of elongated single cells are the ones that first acquire the competence to form embryogenic cells. Apart from the ability to form embryogenic cells, these cells are indistinguishable from the majority of the elongated single cells.

It is generally assumed that the formation of plant embryos requires the activation of specific sets of genes (reviewed by Goldberg et al., 1994; Thomas et al., 1993) and many studies have employed differential screening techniques to identify such genes (eg. Wilde et al., 1988; Aleith and Richter, 1990; Wurtele et al., 1993; Heck et al., 1995). The corresponding expression pattern of these genes during zygotic embryogenesis allowed classification into several groups (Goldberg et al., 1989; Sterk and de Vries, 1992). While many of the genes found to be expressed in early somatic embryos appeared to encode genes normally expressed late in zygotic embryogenesis or throughout plant development (reviewed by Zimmerman, 1993), others such as LTP (Sterk et al., 1991) and EMB-1 (Wurtele et al., 1993) have been shown to be expressed at the corresponding, globular, stage in zygotic embryogenesis. Embryo-expressed MADS box-containing regulatory proteins have been identified in both Brassica napus (Heck et al., 1995) and in Arabidopsis thaliana (Rounsley et al., 1995). The Arabidopsis gene, AGL15, is expressed as early as the 8-celled zygotic embryo, and is also present uniformly in torpedo stage embryos, in seedlings and to a lower level in leaves (Rounsley et al., 1995). Several genes have been reported (reviewed by Zimmerman, 1993) that are putative markers for embryogenic cell clusters, but none have been described to date that are reliable markers for the preceding stage of competent cells. There may be several reasons for this, such as the bias in many screening procedures towards more abundantly expressed genes, the low number of competent cells present in embryogenic cultures and the unavailability of rapid and reliable procedures to detect gene expression in single cells. To overcome these difficulties screens were carried out employing a series of carrot cell cultures with widely differing numbers of single competent cells as the starting material. A small number of genes were found for which expression is detectable in single competent cells in embryogenic cell cultures. One of these genes was investigated in more detail and found to encode a receptor-like kinase that appears to mark competent and embryogenic cells.

**MATERIALS AND METHODS**

**Plant material, cell cultures, hypocotyl explant activation and cell tracking**

Seedlings of Daucus carota cv. Flakkese were grown for 2-3 weeks in vermiculite, while adult plants of this cultivar were obtained from S&G Seeds (Enkhuizen). Controlled pollination was performed by hand, and complete umbels removed at various days after pollination (DAP). Flower RNA was obtained from three complete umbels for each time point and contained all flower organs including residual pollen grains. Cell cultures were derived from Daucus carota cv. Flakkese and maintained as previously described (De Vries et al., 1988a). Cell suspension culture was carried out at high cell density in B5 medium (Gamborg et al., 1968) supplemented with 2 μM 2,4-D (2,4-dichlorophenoxy-acetic acid; B5-2 medium). Embryo cultures with globular, heart and torpedo-stage somatic embryos were derived from <30 μm sieved cell cultures cultured at low cell density (100,000 cells/ml) in B5 medium without 2,4-D (B5-0). For hypocotyl explant induction experiments, plantlets were obtained from seed of Daucus carota cv. S. Valery as described previously (Guzzo et al., 1994). The hypocotyls of 1-week old plantlets were divided in segments of 3-5 mm, incubated for various periods of time in B5-2 medium and returned to B5-0 medium. Seven days after explantation and exposure to 2,4-D, the hypocotyl segments were washed in B5-2 medium and subsequently fragmented on a 170 μm sieve and the resulting cells collected to form a fine cell suspension. Immobilization of these cells in B5-0.2 medium was performed in a thin layer of phytagel (Toonen et al., 1994). After 1 week of further culture, 2,4-D was removed by washing the plates with B5-0 medium. This allowed embryos to develop beyond the globular stage. Development of the immobilized cells was recorded using a modified procedure described by Toonen et al. (1994). The main change involved a new MicroScan program for automatic 3-axis movement to scan all cells in the phytagel (Toonen and de Vries, 1997).

**Nucleic acid isolation and analysis**

RNA was isolated from cultured cells and plant tissues as described by De Vries et al. (1988b). Poly(A)* RNA was obtained by purification by oligo(dT) cellulose (Biolabs). For RNA gel blot analysis samples of 10 μg total RNA were electrophoresed on formaldehyde gel, and transferred to Nytran-plus membranes (Schleicher & Schuell). For spot-dot northern analysis, 5 μg of total RNA was denatured and spotted onto nytran-plus filters using a hybridot manifold (BRL). Hybridization of RNA blots took place at 42°C in hybridization buffer containing 50% formamide, 6·SSC, 0.5% SDS and 0.1 mg/ml salmon sperm DNA. Genomic DNA was isolated according to the method of Sterk et al. (1991). Samples of 10 μg genomic DNA were digested with different restriction enzymes and separated on agarose gel, and transferred to Nytran-plus membranes. Hybridization of DNA blots was performed as previously described (Sterk et al., 1991). Following hybridization, filters were washed under stringent conditions (3× 20 minutes in 0.1% SSC, 1% SDS, at 65°C). Filters were exposed to Kodak XOMat AR film. Nucleotide sequence analysis was performed on an ABI 373A automated DNA sequencer (Applied Biosystems). The sequences reported here have been deposited in the GenBank database, accession number U93048.

**SERK promoter-luciferase expression**

A 2200 bp HindIII/Drai genomic DNA fragment, with the Drai site 42 bp upstream of the predicted translational start codon of SERK, was cloned into the binary vector pMTS500 (Toonen et al., 1996b) containing the firefly luciferase reporter gene (Millar et al., 1992). The resulting construct was transformed into carrot cells as described for the AtLTP1 promoter-luciferase constructs (Toonen et al., 1996b). A subpopulation of <50 μm diameter cells from a primary transformed suspension culture was embedded in phytagel with B5-0 medium at a concentration of 100,000 cells/ml. In single suspension cells, luciferase activity was not detectable with the CCD camera system described (Toonen and de Vries, 1997), even after a one hour exposure. It was only possible to measure luciferase activity in single cells by using a CCD camera (Photometrics) without a microscope, directly over the cells. Since luciferase is quickly deactivated in the presence of luciferin, measurements were made immediately after the addition of luciferin (Promega) to a final concentration of 20 μM in order to obtain the signal from the accumulated luciferase. The spatial resolution in the digitized images of the CCD camera is restricted to a pixel size of 30 by 30 μm. To dis-
tinction in the expression pattern of the corresponding genes.

Whole-mount in situ hybridization
Whole-mount in situ hybridizations were performed essentially as previously described (Engler et al., 1994). Cell cultures and somatic embryos were immobilized on poly-L-lysine coated slides during fixation to improve handling. Whole-mount in situ hybridization on explants took place by embedding hypocotyls from 7-day-old plantlets in 3% Seaplague agarose (Duchefa) and processing them in Eppendorf tubes. Transverse as well as longitudinal sections were made with a vibratome (Biorad Microcut). Sections of 50-170 μm thick were incubated in B5-2 medium for a minimum of 3 days to induce formation of embryo-forming cells. Optimal induction was achieved with longitudinal hypocotyl sections with a thickness of at least 90 μm. To obtain proliferating, non-embryogenic cell cultures, hypocotyl sections were exposed to 2,4-D for only 1 day, and subsequently transferred to B5-0 medium (Guzzo et al., 1994). Whole-mount in situ hybridization on developing seeds was performed by removing the chalazal end of the seeds to allow easier probe penetration. After hybridization, the enveloping layers of integuments and endosperm were carefully removed to expose the developing embryos. All samples were fixed for 60 minutes in PBS containing 70 mM EGTA, 4% paraformaldehyde, 0.25% glutaraldehyde, 0.1% Tween 20, and 10% DMSO. Samples were then washed, treated with protease K for 10 minutes, again washed and fixed a second time. Hybridization solution consisted of PBS containing 0.1% Tween 20, 330 mM NaCl, 50 μg/ml heparin, and 0.5% deionized formamide. Hybridization took place for 16 hours at 42°C using digoxigenin-labeled sense or antisense riboprobes (Boehringer Mannheim). After washing, the cells were treated with RNase A, and incubated with anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) which had been preabsorbed with a plant protein extract. Excess antibody was removed by washing, followed by rinsing in staining buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM levamisole) and the staining reaction was performed for 16 hours in a buffer containing 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate. Observations were performed using a Nikon Optiphot microscope equipped with Nomarski optics or brightfield optics.

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the open reading frame was cloned into the pGEX expression vector (Pharmacia). A fusion protein consisting of SERK and the glutathione S-transferase (GST) gene product was isolated and purified as described previously (Horn and Walker, 1994). Purified fusion protein was coupled to glutathione agarose beads (Sigma) and incubated for 20 minutes at 20°C in a volume of 10 μl buffer: 50 mM Hepes (pH 7.6), 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 1 μCi [γ-³²P] (3,000 Ci/mmol). Excess label was removed by washing the fusion protein/glutathione agarose beads three times for 5 minutes in 50 mM Tris-HCl (pH 7.3), 10 mM MgCl₂ at 4°C. Protein was removed from the beads by cooking in SDS-PAGE loading buffer. Equal amounts of protein were separated by SDS-PAGE and protein autophosphorylation was visualized by autoradiography.

RESULTS

Isolation of cDNA clones that are preferentially expressed in embryogenic cell cultures of carrot

In order to increase the chance of obtaining genes expressed in carrot suspension cells competent to form embryos, the number of such cells in a series of established cell cultures was determined. A subpopulation of cells that had passed through a 30 μm nylon sieve was isolated from eight different cultures ranging in age between 2 months and 4 years. In these <30 μm populations, the number of embryos formed from the single cells and small cell clusters was determined and expressed as a percentage of the total number of cells present at the start of embryogenesis. Three sieved <30 μm cultures able to form somatic embryos at a frequency of more than 100 embryos per 10,000 cells were then used as a source for competent cells, and three other cultures that produced less than 1 embryo per 10,000 cells were used as non-embryogenic controls. The assumptions made were that a 100-fold difference in the embryogenic capacity would indicate a similar difference in the number of competent cells, and that this would be sufficient to detect the mRNA of a hypothetical gene only expressed in competent single cells with the screening methods employed. As main selection strategies, cold plaque screening (Hodge et al., 1992) and differential display (dd) RT-PCR (Li et al., 1994), cloning proved to be essential prior to undertaking further characterization of the PCR fragments obtained. All clones obtained were subjected to a second screen that consisted of spot-dot northern hybridization performed under conditions of high stringency. This method, which used RNA from entire unsieved embryogenic and non-embryogenic suspension cultures, proved to be a fast and reliable additional selection method. Only one clone (22-28) of the 30 clones obtained after differential screening, proved to be restricted to embryogenic cell cultures while the majority was constitutively expressed. The 26 clones obtained from the cold plaque screening required very long exposure times in the spot-dot northern analysis. Six of these clones failed to show any hybridization signal and 19 proved to be expressed in both embryogenic and non-embryogenic cell cultures (results not shown). One clone (31-50) showed low expression in all embryogenic cultures and in one non-embryogenic culture, but not in the others (Fig. 1). Of the six cloned fragments obtained by ddRT-PCR display, four showed hybridization more or less restricted to transcripts present in embryogenic cultures (6-11, 7-13, 10-25, 11-21; Fig. 1). All clones that passed through the second screening were sequenced. The clones 6-11 and 7-13 were identical to the carrot Lipid Transfer Protein (LTP) gene, previously identified as a marker for embryogenic carrot cell cultures. LTP expression is restricted to embryogenic cell clusters and the protoderm of somatic and zygotic embryos from the early globular stage onwards (Sterk et al., 1991). Therefore, while the LTP gene is not a marker for competent cells, its appearance in the screening confirms the validity of our methods with respect to the cloning of genes expressed early during somatic embryogenesis.

cDNA clone 31-50 encodes a leucine-rich repeat containing receptor-like kinase

The predicted amino-acid sequence of the cDNA clone 31-50 (Fig. 2A) shows homology with the structural features of plant and animal receptor kinase genes. Because clone 31-50 is...
Comparison between the SERK amino acid sequence and related sequences. (A) The predicted amino acid sequence of SERK is presented in the single letter code. Asterisks denote potential N-linked glycosylation sites and open circles indicate potential O-glycosylation sites. The putative membrane-spanning region is underlined. The nucleotides are numbered, starting at 1 from the translational start site. Capital marks the open reading frame (B) The conserved sequences of SERK LRRs compared with other LRR-containing proteins. Non-conserved amino acids are indicated by an asterisk. The other LRR-containing sequences are from the following proteins: Arabidopsis RLK5 (Walker, 1993), Arabidopsis ERECTA (Torii et al., 1996), Petunia PRK1 (Mu et al., 1994), Lycopersicon Cf-9 (Jones et al., 1994) and Drosophila Toll (Hashimoto et al., 1998). (C) Sequence alignment of the predicted kinase domain and the remaining C-terminal part of the carrot seed protein with protein kinases from Osyr 9306 OsPK10 (Zhang et al., 1994), Osyr 9276 representing a partial cDNA, Petunia PRK1 and Drosophila Pelle (Shelton and Wasserman, 1993). The 11 conserved protein kinase subdomains are indicated 1 to XI (Hanks and Hunter, 1995).
expressed in embryogenic cell cultures it was renamed Somatic Embryogenesis Receptor Kinase (SERK). The SERK protein contains an N-terminal domain with five leucine-rich repeats (LRRs; Fig. 2B) that is proposed to act as a protein-binding region in LRR receptor kinases (Kobe and Deisenhofer, 1994).

Several potential N-glycosylation sites are present in the LRR motifs and one in the intracellular kinase domain (Fig. 2A). Between the extracellular LRR domain of SERK and the membrane-spanning region there is a 32 amino acid region rich in prolines (13), that is unique for the SERK protein. Of particular interest is the sequence SPPPP, which is conserved in extensins, a class of universal plant cell wall proteins (Varner and Lin, 1989). The significance of this proline-rich box (Fig. 2A) is not clear, it might act as a hinge region by providing flexibility to the extracellular part of the receptor, or act as a region for interaction with the cell wall. In extensins, usually all prolines in the SPPPP repeat are hydroxylated and are considered to be used as targets for O-linked glycosylation. Although a transmembrane domain is present in the protein, the N-terminal amino acids do not clearly display characteristics of a

![DNA blot hybridization analysis](image1)

![Autophosphorylation assays](image2)

![Embryo formation](image3)
The proposed intracellular domain of the protein contains the 11 subdomains characteristic of the catalytic core of protein kinases (Hanks et al., 1988; Fig. 2C). The core sequences HRDVKAAN and GTLYIAPE in respectively the kinase subdomains VI and VIII suggest a function as a serine/threonine kinase (Hanks et al., 1988). Another interesting feature of the intracellular part of the SERK protein is that the second half of the C-terminal motif resembles an LRR and is also present in two other plant proteins that resemble protein kinases (Fig. 2C). This domain may be involved in mediating the protein-protein interaction necessary for transmission of an intracellular phosphorylation cascade.

Hybridization of the SERK cDNA clone to the carrot genome revealed the presence of only a single main hybridizing band, indicating a single SERK gene in the carrot genome (Fig. 3A). No signal was observed after northern blotting of mRNA from embryogenic cell cultures and hybridization with labeled SERK probes (results not shown), reflecting the

**Table 1. Correspondence between number and cell type of embryo-forming cells and cells expressing the SERK gene**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell tracking</th>
<th>In situ hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of cells</td>
<td>No. of cells forming embryos (% of total)</td>
</tr>
<tr>
<td>Small cytoplasm rich (±16x16 μm)</td>
<td>4976</td>
<td>0</td>
</tr>
<tr>
<td>Enlarging (±16x40 μm)</td>
<td>10764</td>
<td>0</td>
</tr>
<tr>
<td>Enlarged (±35x90 μm)</td>
<td>3511</td>
<td>20 (0.56%)</td>
</tr>
<tr>
<td>Large (&gt;60x140 μm)</td>
<td>5471</td>
<td>0</td>
</tr>
</tbody>
</table>
low levels of transcript present in these cultures. Detection of the SERK transcript on the original spot-dot northerns was only possible after long exposure times compared with other probes (Fig. 1).

Recently a number of LRR receptor-like kinases have been isolated from other plant species (Walker, 1993, 1994). Homology between the SERK protein and receptor-like kinases from Petunia (Mu et al., 1994) and Oryza (Zhao et al., 1994) could be observed (Fig. 2C), as well as with some of the recently identified pathogen resistance gene products (Fig. 2B) like tomato Cf-9 (Jones et al., 1994). BLAST database searches resulted in the identification of the dbEST clone R2976. This partial rice clone shows a 74% identity on the amino acid level with SERK, suggesting that this gene is highly conserved between monocots and dicots. The ability of the SERK protein to autophosphorylate was investigated in vitro, using a previously described autophosphorylation assay (Mu et al., 1994), with a bacterial fusion protein that contained the complete intracellular region of the SERK protein. The bacterially expressed SERK fusion protein indeed proved to be able to autophosphorylate (Fig. 3B), indicating that the SERK protein is able to fulfill a role as a protein kinase.

Expression of the SERK gene corresponds to the first appearance of competent cells during hypocotyl activation

The formation of competent cells was determined after exposing seed-derived carrot hypocotyl explants to 2,4-D (Guzzo et al., 1994). When carrot hypocotyls are induced with
2,4-D, only the cells of the provascular tissue proliferate. This suggests that the cells derived from this tissue form all the different cells, including the embryogenic ones, that are present in a newly initiated suspension culture. Explant cells of cortical and epidermal origin only expand, and are quickly lost upon subculture. The duration of 2,4-D treatment is important in the formation of embryogenic cells in activated hypocotyls (Guzzo et al., 1994), and has to be at least 3 days, with an optimal period of around 7 days. In the presence of 2,4-D, the formation of competent cells and the transition towards embryogenic cells is initiated. After removal of 2,4-D, the formation of somatic embryos from embryogenic cells occurs after 2-3 weeks. The first appearance of single competent cells in the explant was determined experimentally by semi-automatic cell tracking (Toonen et al., 1994) and was performed on large populations of immobilized cells. Hypocotyl explants activated with 2,4-D for 7 days were first washed to remove previously released cells and to ensure that only cells still in the explant were included in the analysis. After mechanical fragmentation of the explants, samples of the resulting population of mainly single cells were immobilized to allow recording of their development by cell tracking. A typical example of the recordings made is shown in Fig. 4. In the immobilized cell populations obtained in this way all the morphologically discernible cell types were present that were also seen in the unfragmented activated hypocotyls. Fig. 5A-E shows the different types of cells, which can be divided into four groups according to mean size and cytoplasmic content (Table 1). Because the same cell types were observed in sections of activated hypocotyl explants (Guzzo et al., 1995), it was possible to predict the position of each type of cell in the explant. Small cytoplasm-rich cells (16×16 μm) are found as the proliferating cells that surround the vascular elements. Enlarging vacuolated cells (16×40 μm) are encountered on the surface of the mass of proliferating cells and these can detach from the surface when fully enlarged (35×90 μm). Large vacuolated cells (more than 60×140 μm) are the non-proliferating remnants of the hypocotyl epidermis and cortical parenchyma. The shape of the enlarging and fully enlarged cells ranged from oval to elongate or triangular. Cell tracking on a total of 24,722 cells released from 7-day activated hypocotyls showed that only 20 single cells formed a somatic embryo. Because of their dependance on continued 2,4-D treatment, the embryo-forming single cells are still in the competent cell stage. All of the embryo-forming single cells belonged to the category of 3,511 enlarged cells (Fig. 5B-E) that therefore contained competent cells in a frequency of 0.56%. The cell tracking experiment clearly reveals that none of the highly cytoplasmic and rapidly proliferating cells has reached the competent cell state, but that only after elongation the first competent cells form. It is also evident that only a very limited number of the cells that make up the newly initiated embryogenic suspension culture are actually competent to form embryogenic cells. The expression of the SERK gene was determined by whole-mount in situ hybridization in a population of cells similar to the one used for the cell tracking experiment. Examples of the results obtained are shown in Fig. 6A-E, and the results of 3,531 cells hybridized with the SERK antisense riboprobe are included in Table 1. Expression of the SERK gene was found to be restricted to only 0.44% of the enlarged cell type. Therefore, the expression of the SERK gene appears to be closely corre-

Fig. 8. SERK gene expression in established embryogenic cell cultures and in zygotic embryos. Gene expression is visible as a purple precipitate. (A–D) Very few cells of all morphologically recognizable types of single cells in embryogenic suspension cultures show SERK expression. (E–I) Embryo cell culture. SERK transcripts are not detectable in large clusters. (E) SERK transcripts are present in small clusters. (F,G) SERK expression is detected in small globular embryos. No signal could be detected using a sense SERK riboprobe (H) or during later stages of somatic embryogenesis (I). (J) SERK transcript is detectable in an approximately 8-celled embryo from a seed collected at 14 DAP. Note the absence of any hybridization in the other tissues of the seed. (K) An early globular embryo at 17 DAP of approximately 100 cells. (L) An embryo at the globular-heart transition stage at 17 DAP. Bar: A–I, 50 μm; J–L, 100 μm.
reaction, was hybridized with an 18S ribosomal probe. northern blot with similar amounts of RNA as used for the RT-PCR probe containing the kinase domain of SERK. As a control, a resulting 680 bp PCR fragment was blotted and hybridized with a m. Video cell-tracking of the cells was performed for a period of 13 m camera (left image). The single pixel in the left image measures 30 by 30 and seedling roots (lane eight). The electrophoresis pattern of the obtained from flower/seed RNA at 0 to 20 days after pollination (lanes one to five), from leaves (lane six), seedling stems (lane seven) and seedling roots (lane eight). The electrophoresis pattern of the resulting 680 bp PCR fragment was blotted and hybridized with a probe containing the kinase domain of SERK. As a control, a northern blot with similar amounts of RNA as used for the RT-PCR reaction, was hybridized with an 18S ribosomal probe.

lateral, both qualitatively and quantitatively, with the presence of competent single cells.

To obtain insight into the temporal regulation of SERK expression during explant activation, whole mount in situ hybridization was performed on entire intact or hand-sectioned explants treated for different periods with 2,4-D (Fig. 6F-J). Representative samples were collected from explants that were untreated (Fig. 6F) and treated for 7 days (Fig. 6G, H) or 10 days (Fig. 6I) with 2,4-D. While enlarged cells became present after the first 5 days of culture, the first few SERK-expressing enlarged cells were found after 7 days of culture in the presence of 2,4-D (Fig. 6H). These cells were present at the surface of the mass of proliferating cells originating from the provascular tissue. In the hypocotyls treated for 10 days with 2,4-D, the number of SERK-positive cells had increased to about 3% and included at this stage cells also present in small clusters (Fig. 6I). No SERK transcript was ever detected in small cytoplasm-rich cells or large vacuolated cells. Hypocotyls treated for only 1 day with 2,4-D and subsequently cultured in hormone free medium for 10 days showed proliferating explant cells that gave rise to roots and non embryogenic cell cultures. SERK expression could never be detected in such explants (Fig. 6J). The in situ hybridization results described above were obtained from a relatively small number of explants, so RT-PCR followed by Southern hybridization was performed to obtain more quantitative results. These are shown in Fig. 7 and confirm the close temporal correlation between the expression of the SERK gene and the first appearance of competent cells in explants treated for at least 3 days with 2,4-D before being returned to basal medium. Hybridization of northern blots, derived from RNA electrophoresis patterns on formamide gels, never gave any signal after hybridization with SERK cDNA probes, not even after prolonged exposure in a PhosphorImager (results not shown), in line with the very restricted expression pattern of the SERK gene.

The SERK gene is expressed in established embryogenic cell cultures and transiently during somatic and zygotic embryogenesis

While the results described so far indicate that competent and embryogenic cell formation is restricted to a particular class of enlarged cells during explant activation, the situation in an established embryogenic cell culture is more complex. Competent single cells in such cultures do not appear to belong to one cell type in particular, but have been shown to originate from all morphologically different cell types. Embryogenic cells, that do not require exogenous auxin treatment, are thought to be present only in the form of clusters of at least 3-4 cells and not as single cells (Toonen et al., 1994). SERK expression was found in all morphologically discernible single cell types that were present in an embryogenic cell culture (Fig. 8A-D) at a frequency between 0.1 and 0.5% (results not shown) depending on the cell type. In non-embryogenic cultures, SERK-expressing cells were never encountered. Unfortunately, the non-embryogenic culture in which the original spot-dot northern showed SERK expression was lost and could not be included in this analysis. We expect that in this particular cell culture the competent cell stage is initiated but not completed. As was observed in the activated explants, SERK expression was not restricted to single cells, but also occurred in small clusters of 2 to 8 cells (Fig. 8E). Since clusters of this size are known to consist of embryogenic cells, these data show that SERK expression is not restricted to competent single cells, but may persist in small clusters of embryogenic cells. SERK expression was also followed during the course of somatic embryo development. While, in small globular somatic embryos of up to about 100 cells, there is a high level of expression (Fig. 8F,G), no SERK expression was encountered during the mid to late globular, heart (Fig. 8I) and torpedo-stages of somatic embryogenesis.

Whole-mount in situ hybridization on partially dissected carrot seeds showed that the SERK gene was only expressed in early embryos up to the globular stage (Fig. 8J,K). No expression

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**Fig. 9.** SERK gene expression in plant organs. RT-PCR products obtained from flower/seed RNA at 0 to 20 days after pollination (lanes one to five), from leaves (lane six), seedling stems (lane seven) and seedling roots (lane eight). The electrophoresis pattern of the resulting 680 bp PCR fragment was blotted and hybridized with a probe containing the kinase domain of SERK. As a control, a northern blot with similar amounts of RNA as used for the RT-PCR reaction, was hybridized with an 18S ribosomal probe.

**Fig. 10.** Luciferase expression under control of the SERK promoter. Luciferase activity of immobilised cells was recorded at day 1 with a CCD camera (left image). The single pixel in the left image measures 30 by 30 μm. Video cell-tracking of the cells was performed for a period of 13 days (light microscopic images). The light microscope images were sized to match the CCD image.
was seen in seedlings, roots, stems, leaves, developing and mature flower organs, pollen grains and stigmas before and after fertilization (results not shown). Tissues in the developing seed such as seed coat, integuments, all embryo sac constituents before fertilization, as well as the endosperm at all stages of development investigated, did not show any SERK expression. Later stages of carrot zygotic embryos (Fig. 8L) were also completely devoid of SERK mRNA. These results were confirmed using RT-PCR (Fig. 9) and indicate that no SERK mRNA accumulates in any of the adult plant organs nor in flowers prior to pollination. The first occasion when SERK expression can be detected is in flowers at 3 days after pollination (DAP), at which stage fertilization has taken place and endosperm development has commenced. SERK mRNA remains present in flowers up to 20 DAP, corresponding to the early globular stage of the zygotic embryo (Lackie and Yeung, 1996). Therefore, the SERK mRNA as detected by RT-PCR in flowers at 3 and 7 DAP is likely to come from SERK gene expression in the zygotes, because in carrot the zygote remains undivided up to 1 week after pollination (Lackie and Yeung, 1996). Attempts to perform whole-mount in situ hybridisation on seeds containing only zygotes have so far been unsuccessful.

**SERK promoter-luciferase expression during somatic embryogenesis**

To determine directly whether SERK-expressing cells indeed develop into somatic embryos, transformed carrot suspension cultures containing a SERK promoter-luciferase construct were analysed for luciferase expression in cell cultures sieved through a 50 μm mesh to enrich for single cells and small cell clusters. Development of the immobilized cells after recording the luciferase images was determined using automated cell tracking (Toonen et al., 1994, 1997). The origin of nine torpedo stage somatic embryos was determined this way. Of these, three developed from a single cell that showed luciferase activity at day 1, four developed from cell clusters consisting of 2-6 luciferase-expressing cells while two embryos developed from single cells that failed to show a detectable level of luciferase activity at day 1. The somatic embryo shown in Fig. 10 originated from a luciferase-expressing two-celled cluster. These results demonstrate that most somatic embryos develop from single cells and small cell clusters expressing SERK at day 1. Somatic embryos beyond the globular stage did not show luciferase expression, confirming the transient SERK gene expression pattern (results not shown).

**DISCUSSION**

**The SERK gene as a marker for the competent cell stage in somatic embryogenesis**

In plants, embryo formation in the absence of the fusion of two gametes is a widespread phenomenon. It occurs naturally in certain species in the developing ovule, as exemplified by apomictic embryogenesis (Koltunow, 1993), or on the surface of leaves as in *Malaxis* (Taylor, 1967). More commonly, embryogenesis can be induced experimentally with a wide variety of tissue explants as the starting material after treatment of explants with synthetic growth regulators. It is generally accepted that the genetic and physiological constitution of the donor plant as well as the age and type of the explant are important parameters in successfully inducing somatic embryogenesis. However, knowledge about the first events that take place during the transition of somatic cells into embryogenic cells is largely lacking. There appear to be two main reasons for this. The first is that cellular changes observed in somatic explant cells that have responded to the inducing treatment in general have not been proved to be essential for the formation of embryogenic cells. The second is that no specific markers have been described so far that reliably predict which explant cells will become embryogenic.

The process of cellular reactivation and the subsequent formation of embryogenic cells in carrot explants has been described by Guzzo et al. (1994, 1995). That work showed that a particular elongated cell type appeared in culture, derived from small rapidly proliferating cytoplasmic cells that themselves derived from reactivated provascular cells. It was further shown cytologically, that some of the elongated cells underwent an asymmetrical division. After continued culture, small clusters of dividing cytoplasmic cells appeared that resembled the proembryogenic masses seen in established embryogenic suspension cultures (Guzzo et al., 1994, De Vries et al., 1988a). The fact that only a limited number of cells actually undergoes the transition of somatic into embryogenic cell is postulated to be the result of the presence of different sets of auxin receptors (Filippini et al., 1992). One of the goals of the work presented here was to determine which cells of a carrot hypocotyl explant have completed the transition of a somatic cell into an embryogenic cell. The results revealed that the first cells to become competent belong to a type of enlarged cells that are detaching from the surface of the mass of proliferating cells, confirming the previous predictions (Guzzo et al., 1994). All other cells, including the majority of the enlarged cells present, were completely unresponsive. This result is in contrast to the generally accepted idea that a population of small rapidly dividing meristematic cells are the ones that are competent to become embryogenic. Instead, the results presented here, together with the cytological observations (Guzzo et al., 1994), demonstrate that competent cells arise first as elongating cells, when still attached to the explant. This is a situation that is strikingly similar to the rapid increase in cell volume observed in plant egg cells after fertilization (Mansfield and Briarty, 1991). Unlike the zygote, it was so far not possible to predict which elongated cell on the surface of the explant will become competent. This raises the question whether the observed correspondence between elongation and competent cell formation is causal or merely reflects a particular state common to most cells present on the surface of the explant. To answer this question, it was essential to achieve another goal of this work: to obtain markers that are able to distinguish precisely between competent and non-competent cells. The expression of the SERK gene described here was found to be very tightly correlated with the ability of cells of the correct morphology to attain the competent cell state. In line with the much less defined morphology of competent single cells in established suspension cultures (Toonen et al., 1994), no clear cell type specificity was apparent in cells expressing the SERK gene in those cultures. The difference between these findings and the
results obtained employing activated explants is at present not explained. In comparison with other markers that have been suggested to distinguish individual embryo-forming cells, such as callose (Dubois et al., 1991) and the monoclonal antibody JIM8 (Pennell et al., 1992), the SERK gene appears to be quite specific under culture conditions. While the presence of the JIM8 epitope was restricted to embryogenic cell cultures, cell tracking of cells labeled with this antibody failed to show a correlation with the ability of these cells to develop into somatic embryos (Toonen et al., 1996a). As shown in this work, such a correlation was established for the SERK gene by cell tracking of cells expressing luciferase under the control of the SERK promoter. The availability of a vital marker for competent cells offers the possibility of following, with great precision, the events that take place during formation of such cells. It could for instance be a useful tool to help determine the frequency of pseudomeiotic segregation events proposed to accompany embryogenic cell formation (Giorgetti et al., 1995).

The identification of the SERK gene and other markers for competent cells was facilitated by the availability of a range of suspension cultures differing strongly in the number of competent single cells present. This strategy avoided the potential problem that a non embryogenic cell line would be employed in which competent cell formation had occurred, but in which a later stage in embryogenic cell formation would have been inhibited, thus reducing the chance of finding genes expressed during the early competent cell states. In addition, the availability of in situ hybridization methods that allowed the visualization of gene expression in single suspension cells while preserving cell morphology, together with cloning methods aimed at avoiding selection towards abundantly expressed genes, explains why similar experiments carried out previously only yielded genes expressed in later stages, such as proembryogenic masses (Choi and Sung, 1984; Wilde et al., 1988; Aleith and Richter, 1990).

The SERK gene is transiently expressed in embryogenesis

The analysis of the expression pattern of the SERK gene during embryogenic cell formation and during somatic embryogenesis revealed that SERK expression continues during proembryogenic mass formation and also during somatic embryogenesis up to about the 100-celled globular stage. Because after this stage expression in somatic embryos is completely abolished, the SERK gene shows a transient expression pattern, one that was also found in zygotic embryogenesis, perhaps as early as the zygote. This transient expression pattern, following the classification system for embryo-expressed genes would place the SERK gene in class 2, comprising very few genes exclusively expressed during early embryogenesis (Sterk and De Vries, 1992). So far, none of the genes identified using embryo mutational analysis, such as Bio-1 (Schneider et al., 1989), Prolifera (Springer et al., 1995), EMB30/Gnom (Shevell et al., 1994), Fusca-1 (Castle and Meinke, 1994), Knolle (Lukowitz et al., 1996) and STM (Long et al., 1996) exhibit an expression pattern that is restricted to the embryo. Thus, the expression pattern of the SERK gene points to a function in a signal transduction cascade only required for the first seven or so cell divisions of the plant embryo. While the nature of the signal, its transduction and its importance are not clear yet, it is clear that this cascade is reproduced with great fidelity in somatic embryogenesis.

The possible function of the SERK gene in early plant embryogenesis

The predicted SERK protein sequence resembles a leucine-rich repeat (LRR) receptor kinase protein, a class of plant proteins that was originally described by Chang et al. (1992). Some members of this class of plant receptor-like kinases are known regulators of developmental processes, like the Arabidopsis Clavata protein (Meyerowitz, 1995) and the Arabidopsis Erecta protein (Torii et al., 1996). Others, like the Petunia PRK1 protein seem to be involved in signal transduction during pollen development or pollination (Mu et al., 1994). Expression of this particular Petunia gene is restricted to pollen and pollen tubes prior to fertilization in contrast to the carrot SERK gene of which no expression can be detected in pollen. PRK1 and SERK therefore regulate non-overlapping processes, separated from each other by the process of fertilization. Other plant receptor-like proteins with LRRs are involved in pathogen resistance, presumably by the specific binding to elicitors (reviewed by Dangl, 1995). The specificity of protein-protein interactions mediated by LRR-containing proteins are most probably due to the composition of the non-consensus residues within the LRRs (Kobe and Deisenhofer, 1994).

The presence of the perfect consensus sequence SPQQQQ found in extensins and some types of arabinogalactan proteins or AGPs suggests an interaction of the extracellular part of the SERK protein with components of the cell wall. The SPQQQQ domain is considered to be a target sequence for arabinosylation onto hydroxylated prolines (reviewed by Carpita and Gibeaut, 1993). All prolines in the SPQQQQ consensus are normally hydroxylated. Whether a glycosylated SPQQQQ sequence is mediating a possible anchoring to specific regions of the cell wall remains to be determined. If so, it would be an elegant mechanism to prevent free movement of receptor molecules and yet prevent inflexibility preventing dimerization, which would be difficult to reconcile with covalent attachment to a cell wall polymer. While the average primary cell wall has a thickness of approximately 50 nm (Pruitt et al., 1993) and the maximum size of the entire extracellular domain can only be about 15 nm when present as an α helix, the extracellular ligand binding domain is likely to be completely embedded within the cell wall. The most likely type of ligand for SERK will therefore consist of a cell wall-diffusible peptide. Peptides effective in inducing plant responses, such as systemin (McGurl et al., 1992) and ENOD40 (Van de Sande et al., 1996) have been described.

Thus, it appears that while LRR containing receptor-like protein kinases play several roles in plant development, intercellular peptides are now being uncovered that are likely signal molecules that can activate developmental processes mediated through such receptors.

Clues about the function of SERK might be found in the homology between SERK and two proteins in Drosophila that are required for the establishment of the dorsoventral polarity in the embryo. The kinase domain of SERK shows homology with the Drosophila Pelle protein, a serine/threonine kinase
involved in formation of the dorsoventral axis during embryogenesis (Shelton and Wasserman, 1993). The Drosophila Pelle protein itself is activated by the Toll transmembrane receptor (Hashimoto et al., 1988; Govind and Steward, 1991), of which the ligand-binding domain, as in SERK, consists of LRRs. In the plant embryo sac and in the activated explant a situation may exist whereby an unknown inducer is present uniformly, but embryo formation awaits the presence of the SERK protein. Such a model may fit with the restricted expression pattern found for the SERK gene both in vivo and in vitro. It is also in line with the hypothesis that in plants inductive interactions mediated by diffusible signal molecules are an important regulatory mechanism (reviewed by Schmidt et al., 1994). Direct evidence for the existence of cell inductive processes in plants was recently presented by Van Den Berg et al. (1995) for the Arabidopsis root. While most of the elements concerning the origin and targets of processes of cell to cell communication in early plant embryogenesis are lacking, the SERK gene described here may represent a significant part of a mechanism that is essential for the formation of plant cells destined to become embryos.

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2062 E. D. L. Schmidt and others


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