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RESEARCH PAPER

Suppression of *SERK* gene expression affects fungus tolerance and somatic embryogenesis in transgenic lettuce

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ABSTRACT

The *Somatic embryogenesis receptor-like kinase (SERK)* gene plays an important role in plant somatic and zygotic embryogenesis induction. The gene encodes an LRR-containing receptor-like kinase protein. Studies have been carried out focusing on different aspects of its function, but definitive conclusions on its role are far from being reached. *SERK* expression is generally detected in cells in which somatic or zygotic embryogenesis has been triggered. Transgenic lettuce lines were produced to silence the endogenous *SERK* gene using antisense RNA. The average number of seeds per flower in the R_1 and R_2 generations was similar for both transgenic and non-transgenic lines. However, a reduction in the number of viable grained seeds was observed in four studied transgenic lines. Endogenous *SERK* expression analysis revealed the absence of detectable *LsSERK* gene transcripts in three transgenic lines, which presented a reduction in their ability to form *in vitro* somatic embryonic structures. In addition, transgenic lines showed enhanced susceptibility to the pathogenic fungus *Sclerotinia sclerotiorum*, when compared to control plants. The results support the idea that *SERK* genes might not only be involved in plant growth and development, but probably also in a general mechanism of biotic and abiotic stress perception.

INTRODUCTION

The *Somatic embryogenesis receptor-like kinase (SERK)* gene is hypothesized to play an important role in plant embryogenesis induction (Hecht *et al.* 2001). The gene encodes a protein that belongs to the receptor-like kinase (RLK) protein family (Schmidt *et al.* 1997). This gene is highly conserved among plant species and has been grouped into a small family in different plant species, highlighting its importance in plant development (Santos *et al.* 2005). The *SERK* gene contains 10–11 exons coding five to six domains of a transmembrane protein (Hecht *et al.* 2001). The SERK protein is responsible for signal transduction, carrying an external leucine-rich repeat (LRR) domain that is typically found in this class of proteins (Shah *et al.* 2001). Studies have been carried out focusing on different aspects of its function, but conclusions about its role are far from being reached. The functional redundancy between receptors in this class of proteins constrains the determination of the exact role of a specific protein (Diévart & Clark 2004).

SERK expression is generally detected in cells in which somatic or zygotic embryogenesis has been triggered. In *Daucus carota* L. (carrot) and *Dactylis glomerata* L. it has been observed that single cells overexpressing the *SERK* gene are among those that are differentiated into somatic embryos (Schmidt *et al.* 1997; Somleva *et al.* 2000). Thus, this gene has been named as a molecular marker for both somatic and zygotic embryogenesis (Schmidt *et al.* 1997). In *Arabidopsis thaliana* (L.) Heynh., the promoter of the *SERK* gene is functional during the early stages of zygotic embryogenesis. In addition, transgenic plants that have expressed the *SERK* gene showed a slight increase in somatic embryogenesis ability (Hecht *et al.* 2001). However, *SERK* expression has not been strictly associated with somatic embryogenesis in maize, since *ZmSERK1* and *ZmSERK2* were expressed in different tissues (Baudino *et al.* 2001). Similar results were found in *Hieracium*

pilosella L. (Tucker *et al.* 2003), *Medicago truncatula* Gaertn. (Nolan *et al.* 2003), *Helianthus annuus* L. (Thomas *et al.* 2004) and *Theobroma cacao* L. (Santos *et al.* 2005). Constitutive expression of the *SERK* gene slightly increased somatic embryogenesis induction in *Arabidopsis thaliana* (Hecht *et al.* 2001). In *Arabidopsis*, elimination of the tapetum cell layer induced male sterility in double mutants (Colcombet *et al.* 2005), and single mutants do not show alterations in somatic embryo development in plants where the expression of *SERK1* or *SERK2* genes has been suppressed (Albrecht *et al.* 2005). In rice, a reduction in shoot number has been observed in transgenic embryogenic callus with RNA interference for *OsSERK1*, while increased host defence against the fungus *Magnaporthe grisea* was observed in *OsSERK1* overexpressing calli (Hu *et al.* 2005). The promoter analyses showed broad expression of *OsSERK1* and *OsSERK2* in different tissues (Ito *et al.* 2005). The difficulties associated with 'null' phenotypes are due to the multiple interactions of LRR domains with signalling molecules (Diévart & Clark 2004).

In plants, gene silencing may occur during transcription (TGS) or after transcription (PTGS). Sense RNA, double-stranded RNA, aberrant RNA and antisense RNA transcript arrangement may induce silencing of endogenous genes (Fagard & Vaucheret 2000). Similarly, heterologous antisense RNA can generate post-transcriptional gene suppression (Bolitho *et al.* 1997). In the present work, we have used a heterologous *SERK* gene fragment in antisense orientation to generate transgenic plants of lettuce (*Lactuca sativa* L.) to evaluate its influence on somatic and zygotic embryogenesis from cotyledons during *in vitro* culture and to evaluate its effect on plant fungus colonization. The use of somatic embryogenesis associated with zygotic embryogenesis is based on the hypothesis that both processes have a similar signalling pathway (Dodeman *et al.* 1997), although the mechanisms that induce somatic embryogenesis are far from being clear (von Arnold *et al.* 2002).

MATERIALS AND METHODS

Antisense RNA construct

The pC1390UBQ3SERKAS plasmid (Fig. 1) used to produce transgenic lettuce plants, which contains a *SERK* gene fragment in antisense orientation, was isolated from *Coffea canephora* Pierre ex Froehn. (*CcSERK*) embryogenic calli, and amplified by RT-PCR using primers SERK3 (5′-TGGAGCTTTACAGCAATAACAT-3′) and SERK4 (5′-ACACTTCCATTACGCATGTATGG-3′). The reaction was carried out as described by Santos *et al.* (2005), and the isolated sequence was entered into the NCBI GenBank database with the accession number EF370120. The 859-bp fragment from *CcSERK* was inserted into the pGEMTeasy vector (Promega, Madison, WI, USA) and cloned in antisense orientation into pC1390UBQ3 (using the *Eco*RI site), under control of the ubiquitin gene promoter from *A. thaliana*. The final vector, called pC1390UBQ3SERKAS (Fig. 1) was used to transform lettuce plants. The antisense cassette will hereafter be called *SERKAS*.

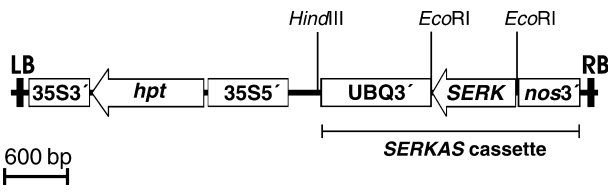

Fig. 1. Map of the T-DNA region of the vector pC1390UBQ3SERKAS used for lettuce transformation.

Plant transformation

Commercial seeds of lettuce (cv. Verônica) were treated with 70% ethanol for 30 s, surface sterilized with 2% sodium hypochlorite (w/v) solution containing two drops of Tween 20 for 15 min and rinsed five times in sterile distilled water. For germination, seeds were cultured on filter paper containing MS liquid medium (Murashige & Skoog 1962) for 2 days under a 16 h photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After germination, the cotyledons were excised and co-cultivated for 15 min with *Agrobacterium* strain EHA105 ($OD_{600nm} = 0.6$) harbouring the pC1390UBQ3SERKAS vector. The explants were transferred to Petri dishes containing regeneration medium (RM; MS medium supplemented with 0.1 $mg \cdot l^{-1}$ BA and 0.1 $mg \cdot l^{-1}$ IBA) and cultured for 2 days. Transformed plants were recovered after selection on RM containing 10 $mg \cdot l^{-1}$ hygromycin. Explants were subcultured every 14 days during the selection period. Shoots chosen after 30–40 days and measuring 4–6 cm in length were separated from callus and transferred to 0.5× MS medium for rooting. Elongated plants were screened by PCR analysis for the presence of the transgenes. As a control group, non-transgenic plants were regenerated.

Rooted plants were transferred to soil and cultivated in a growth chamber (Conviron, Winnipeg, Canada) at 28 ± 2 °C, light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under a 16 h photoperiod. After 1 week, plants were transferred to a greenhouse to set seeds.

Polymerase chain reaction analysis

For PCR analysis of transgenic plants, total DNA was extracted from leaves as described by Aragão *et al.* (1996). Primers hyg 268 (5′-TCCGGAAGTGCTTGACATTGG-3′) and hyg 672 (5′-ATGTTGGCGACCTCGGTATTGG-3′) were used to amplify a 404-bp fragment within the *hpt* gene coding sequence to identify transgenic lineages. Primers SERK3 and SERK4 were used to amplify an 859-bp fragment within the SERKAS cassette. Each PCR

reaction was carried out in the PTC-100 thermocycler (MJ Researcher). A total of 25 μl of solution containing 40 ng of total DNA, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$, 200 nM of each dNTP, 200 nM of each primer, 2 U of *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used in each reaction. The mixture was overlaid with mineral oil, denatured at 95 °C (5 min) and subjected to 35 cycles of amplification (95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) with a final elongation cycle of 5 min at 72 °C.

Seed analysis

Mature seeds were harvested and immediately analysed under a stereomicroscope to evaluate their morphology. The number of seeds per flower was also recorded. Seeds were classified according to their morphology as non-developed (ND), non-grained (NG) or completely grained (CG) (Fig. 2). The seeds were submitted to the tetrazolium test to evaluate their viability. Basically, seeds were incubated in a solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium at 1% (w/v) for 16 h at 30 °C.

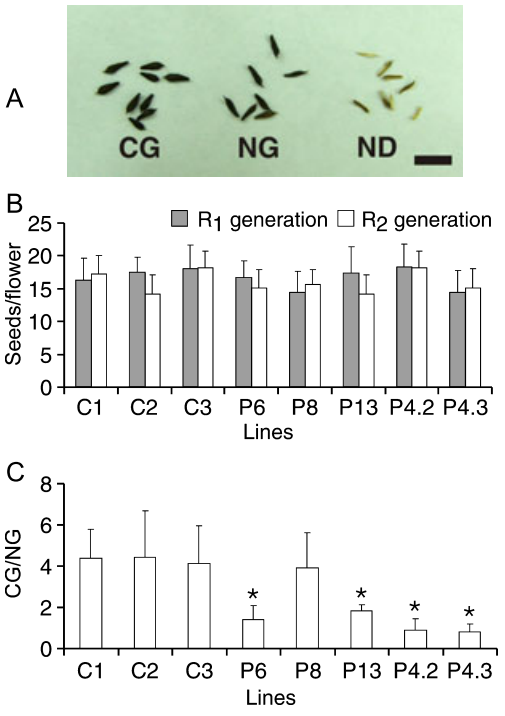

Fig. 2. A: Types of seeds found in lettuce progenies: CG, completely grained seeds; NG: non-grained seeds; ND: non-developed seeds. B: Number of seeds per flower observed in the R_1 and R_2 generations of transgenic lettuce lines. C: Proportion of grained seeds to non-grained seeds (CG/NG) in the R_2 generation. C1, C2 and C3 are non-transgenic lines (controls). Bars represent the mean ± SD of n = 29 (Student's *t*-test: significantly different means were labelled with asterisks (*), $P < 0.01$ *versus* controls). Bar in A represents 1 cm.

Somatic embryogenesis induction

CG segregant F_2 seeds from transgenic lineages were germinated on MS liquid medium as described above. Cotyledons (2–4 days after germination) were transferred to MS medium supplemented with NAA (2 $mg \cdot l^{-1}$) and BA (0.2 $mg \cdot l^{-1}$) and cultivated for 15 days in the dark, as described by Zhou *et al.* (1992), to induce somatic embryos. Cotyledons were cultivated on embryo induction medium containing 10 $mg \cdot l^{-1}$ hygromycin or without this selective agent.

LsSERK expression analysis

Total RNA was extracted, using the RNeasy kit (Qiagen, Valencia, CA, USA), from 200 mg of fresh tissue (clumps of 15-day-old somatic embryos arising from transgenic and non-transgenic cotyledons). Remaining genomic DNA was eliminated by DNase digestion of the RNA samples. Two micrograms of total RNA were used to produce total cDNA using the Superscript II kit (Invitrogen, Carlsbad, CA, USA). PCR reactions were carried out as described above, except that 25 ng of cDNA [quantified using the DyNA Quant 200 fluorimeter (Amersham Pharmacia Biotech, Buckinghamshire, UK)] were used as a template with 24 cycles of amplification. Primers SERK3 and SERK4 were utilized to amplify a fragment from the *LsSERK* sequences. The fragments of the SERK-encoding sequence amplified from expressing lettuce somatic embryos were cloned into the pTOPO 2.1 vector for PCR products (Invitrogen, Carlsbad, CA) and sequenced using universal M13 and T7 primers on an automatic sequencer (ABI Prism1 3700). The sequence revealed 100% similarity when compared to the *LsSERK* gene fragment (GenBank accession number EU122227). As an internal control, primers rRNA1 (5′-AACGGCTACCACATCCAAGG-3′) and rRNA2C (5′-TCATTACTCCGATCCCGAAG-3′) were used to amplify a sequence from the *L. sativa 18S rRNA* gene.

Inoculation with *Sclerotinia sclerotiorum*

An isolate of *S. sclerotiorum* obtained from tomato plants grown in Guaíra, SP, Brazil and kept at Embrapa Hortaliças (Brasília, DF, Brazil) was used in the experiments. Inoculation was carried out as described by Dias *et al.* (2006) using leaves detached from a 9-week-old plant (12 leaves from the transgenic line P6, nine leaves from transgenic lines P4.2, P4.3 and P13, and 10 leaves from non-transgenic plants). Symptoms were observed every 12 h and lesion area was determined using the ImageJ software (Abramoff *et al.* 2004). Experiments were repeated three times.

RESULTS

Plant transformation

We obtained 14 transgenic independent lines, and all lines showed the expected 404-bp *hpt* gene fragment in the

PCR analysis. Primary transformants (R_0) presented a normal phenotype (roots, plant height, leaves and flowers) and were cultivated to set seeds in the greenhouse. In addition, the life cycle was not altered and plants produced fruits within 3–4 months.

Seed analysis

Seeds classified as completely grained (CG), non-developed (ND) or non-grained (NG) seeds (Fig. 2) appeared in all lines, including the control. The average number of seeds per flower that included these three seed types in the R_1 and R_2 generations was similar for both transgenic and non-transgenic lines (Fig. 2). However, we observed a reduction in the number of CG seeds in transgenic lines P4.2, P4.3, P6 and P13 (Fig. 2). The transgenic line P8, in which the presence of *LsSERK* transcripts was detected in RT-PCR analyses, presented the same number of CG observed in the control (non-transgenic line) (Fig. 2), with a Mendelian ratio of three seeds carrying the transgene to one seed not carrying the transgene (data not shown). The number of non-developed seeds (about 10% out of the total seeds per flower) was similar for both transgenic and non-transgenic lines (data not shown). In addition, tetrazolium analysis revealed that all NG seeds were not viable in both the transgenic and non-transgenic lines, while the CG seeds had viability of over 90%. The CG seeds were germinated on MS medium and showed a frequency of germination ranging from 85% to 92%.

LcSERK expression analysis and somatic embryogenesis induction

The embryogenic calli were analysed by RT-PCR to detect endogenous *LsSERK* transcripts (Fig. 3). Cotyledons were cultivated on MS medium supplemented with NAA to induce somatic embryos. Results showed *LsSERK* gene transcripts in the P8 line, while the lines P4.2, P4.3, P6 and P13 lacked detectable *LsSERK* gene transcripts (Fig. 3). Transgenic and non-transgenic lines presented the same level of the *L. sativa 18S rRNA* housekeeping gene. The fragment corresponding to the *L. sativa* SERK-encoding sequence, amplified from expressing explants, was partially sequenced. The sequence revealed low similarity compared to the *CcSERK* sequence present in the *SERKAS* cassette, although the region of primer annealing was similar. Analysis of five independent clones generated from each transgenic line showed the same SERK-encoding sequence.

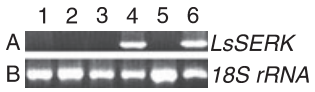

Fig. 3. Expression of the endogenous *LsSERK* gene in transgenic lettuce plants. A: RT-PCR analysis for the presence *LsSERK* transcripts in callus from the transgenic lines P4.2 (lane 1), P4.3 (lane 2), P6 (lane 3), P8 (lane 4) and P13 (lane 5). Lane 6 represents the non-transgenic explant (control). B: RT-PCR analysis for the presence of transcripts from the lettuce *18S rRNA* gene (housekeeping gene used as internal control).

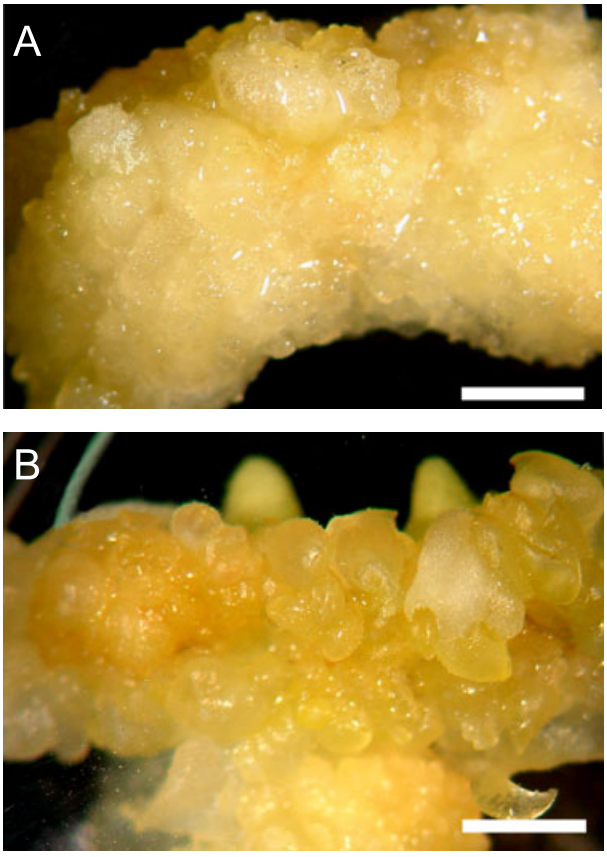

Fig. 4. Effect of *LsSERK*-silencing on somatic embryogenesis. Calli generated from transgenic (A) and non-transgenic (B) cotyledons were cultivated on MS medium supplemented with NAA and BA to induce somatic embryos. All *LsSERK*-silenced transgenic lines (P4.2, P4.3, P6 and P13) presented similar phenotype. Bars represent 3 mm.

Cotyledons from transgenic and non-transgenic plants were cultivated on MS medium supplemented with NAA and BA to induce somatic embryos. Both transgenic and non-transgenic explants were able to generate embryogenic calli. However, calli derived from *SERK*-silenced transgenic cotyledons showed a drastic reduction (statistically significant at $P < 0.01$ *versus* control) in their ability to form embryonic structures (mean 0.15 embryos per explant ± 0.38; n = 16) (Fig. 4) when compared to explants from non-transgenic lines (mean 9.5 embryos per explant ± 3.3; n = 19).

Evaluation of fungus infection on leaves

Detached leaves from transgenic line P6 were inoculated with 2-mm diameter agar plugs from growing margins of 2-day-old *S. sclerotiorum* cultures and lesion area was recorded. The progression of disease development in the control (non-transgenic plant) and transgenic lettuce lines P4.2, P4.3, P6, P8 and P13 over a 48-h period is illus-

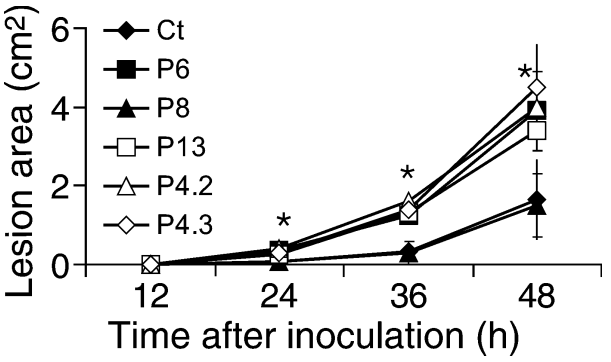

Fig. 5. Lesion area progression following inoculation of detached leaves of transgenic lettuce lines P4.2, P4.3, P6, P8, P13 and control (Ct, non-transgenic line) with mycelial agar plugs containing *Sclerotinia sclerotiorum*. Bars represent the mean ± SD (n = 12 for line P6; n = 9 for lines P4.2, P4.3, P8 and P13; n = 10 for control; Student's *t*-test: significantly different means were labelled with asterisks (*), $P < 0.01$ *versus* control).

trated (Fig. 5). Transgenic lines P4.2, P4.3, P6 and P13 showed enhanced symptom development compared with the non-transgenic line (control). The transgenic line P8 presented symptom development similar to that observed in the control (Fig. 5).

DISCUSSION

SERK is an LRR-containing receptor-like kinase (RLK). The RLK class of proteins is a monophyletic family that seems to have originated before separation of plants and animals (Shiu & Bleecker 2001). Despite the presence of more than 400 genes that encode RLKs in the *A. thaliana* genome, very little is known about the range of biological processes that they control, or the mechanisms by which they function (Diévart & Clark 2004). In *Arabidopsis*, protein–protein interaction studies using mass spectrometry (MALDI-TOF) analysis suggest that the SERK-1 protein is involved in the brassinosteroid pathway (Karlova *et al.* 2006). It has been suggested that the *SERK* gene activates embryogenesis because its expression is generally observed in cells in which somatic or zygotic embryogenesis has been triggered (Karlova *et al.* 2006). Nevertheless, the *SERK* gene is fairly broadly expressed in different tissues of several plant species, such as in leaves, roots, microspores and ovules in maize (Baudino *et al.* 2001), in roots, leaves and somatic and zygotic embryos in cacao (Santos *et al.* 2005) or in ovules, anthers, embryos and seedlings in *Arabidopsis* (Kwaaitaal *et al.* 2005). These findings suggest additional roles for *SERK* genes other than only somatic embryogenesis (Albrecht *et al.* 2005; Colcombet *et al.* 2005). In this work, it was observed that a transgenic lettuce line presenting suppression of endogenous *SERK* gene transcription showed normal vegetative development, architecture and rooting. However, in the transgenic lines P4.2, P4.3, P6 and P13 that showed *LcSERK* suppression, we observed a reduction in the number of viable seeds and somatic embryogenesis ability. Similar results were found in transgenic rice explants, with a reduction in shoot regeneration rates after suppression of the *OsSERK* gene (Hu *et al.* 2005). However, the *OsSERK* genes were highly expressed in the scutellum, which is sensitive to auxins that are essential for embryogenic callus formation, but not for embryogenesis development (Ito *et al.* 2005).

The *SERK* gene is highly expressed in embryogenic tissues, being considered a molecular marker for somatic embryogenesis (Schmidt *et al.* 1997). Consequently, suppression of *SERK* gene expression is expected to preferentially affect the embryogenic pathway rather than plant development or plant architecture, although its expression is up-regulated by auxin, a general plant growth regulator (Nolan *et al.* 2003). In *SERK*-silenced transgenic lettuce, the total number of seeds was not significantly altered, but the number of viable grained seeds was drastically reduced. In anthers of *Arabidopsis*, *AtSERK1* and *AtSERK2* double mutants caused male sterility by tapetum elimination (Albrecht *et al.* 2005; Colcombet *et al.* 2005). The results presented here could be explained by a similar phenomenon, leading to pollination disturbance and absence of embryo development or maturation. However, further studies are needed in order to confirm this conjecture.

Experiments were carried out to evaluate somatic embryogenesis in the transgenic line P8 that showed *LsSERK* gene expression and transgenic lines P4.2, P4.3, P6 and P13, which lacked detectable *LsSERK* gene expression. Results showed that transgenic lines retained their regeneration capacity, but presented a drastic reduction in ability to form embryonic structures when compared to the P8 line and non-transgenic line. The results seem to confirm the hypothesis that *SERK* has a role in somatic embryogenesis.

SERK-encoding sequences represent gene families with a high degree of similarity in some plant species. Indeed, five copies were found in *Arabidopsis*, three in maize, and three in *H. pilosella* (Tucker *et al.* 2003). The transgenic lines P4.2, P4.3, P6 and P13 presented undetectable transcription levels of an endogenous *SERK* gene (*LsSERK*). The strategy of antisense RNA has been shown to be capable of generating post-transcriptional silencing for related sequences representing gene families in some plant species. Although only one *LsSERK* sequence was obtained from RT-PCR expression analysis, we cannot definitively conclude that only one *LsSERK* gene was suppressed.

Transgenic *SERK*-silenced lines were tested for their tolerance to *S. sclerotiorum* (a natural pathogen for lettuce), and it was found that plants become more sensitive to this biological stress, when compared to control plants. Since the work carried out by Walker & Zhang (1990), when the first plant RLK was identified in maize, many RLKs have been identified, demonstrating that, like other eukaryotes, plant cells are able to perceive external signals

at the plasma membrane. In animals, numerous studies have reported functions for LRR-containing receptors in neuronal development, pattern formation, differentiation and growth of gonads and thyroid glands and in antifungal responses.

In plants, phenotypes associated with mutations in LRR-containing RLKs showed that they play roles in diverse processes during growth and development and the recognition of microbial pathogens (reviewed by Diévart & Clark 2004). In *A. thaliana*, the *FLS2* gene (which codes for an LRR-RLK) was found to be responsible for sensitivity to flagellin (the major antigen of several bacteria) (Gomez-Gomez & Boller 2000). Asai *et al.* (2002) showed that FLS2 activates a cascade of phosphorylation, implicating the MAPK pathway proteins that target the well-described defence genes. The activation of the MAPK cascade confers resistance to bacteria and fungi. Recently, Heese *et al.* (2007) have identified SERK3 (also named BAK1) as a component of the plant pathogen-associated molecular pattern. In addition, Chinchilla *et al.* (2007) showed that SERK3 is involved in signalling by FLS2 and EFR, and has a functional role in pattern recognition receptor-dependent signalling, which initiates innate immunity.

The *SERK* gene family has been shown to be up-regulated by auxin, which is the main plant growth regulator used to induce *in vitro* somatic embryogenesis (Nolan *et al.* 2003) and which could be considered a stress factor. In addition, it was recently demonstrated that the *SERK* gene is induced by different stress signalling molecules, such as benzothiadiazole, jasmonic acid and abscisic acid (Hu *et al.* 2005). Thus, the results presented here support the idea that plant *SERK* genes might not only be involved in growth and development, but probably in a general mechanism of biotic and abiotic stress perception, which includes somatic embryogenesis, probably a stress response of plants to desiccation and other factors that stimulate embryogenesis using auxin as a signalling molecule.

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