

Expression analysis of somatic embryogenesis-related *SERK*, *LEC1*, *VPI* and *NiR* orthologues in rye (*Secale cereale* L.)

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Received: 20 September 2009 / Revised: 6 August 2010 / Accepted: 6 August 2010 / Published online: 12 January 2011
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Abstract The genetic basis of the regeneration process in cultured immature embryos of rye (*Secale cereale* L.) was analyzed. The experiments were designed to reveal differences between the in vitro culture responses of two inbred lines: L318 (a high regeneration ability) and L9 (a low potential for regeneration). The rye orthologues of plant genes previously recognized as crucial for somatic embryogenesis and morphogenesis in vitro were identified. Using oligonucleotide primers designed to conserved regions of the genes *Somatic Embryogenesis Receptor-like Kinase* (*SERK*), *Leafy Cotyledon 1* (*LEC1*), *Viviparous 1* (*VPI*) and *NiR* (encoding ferredoxin-nitrite reductase), it was possible to amplify specific homologous sequences from rye RNA by RT-PCR. The transcript levels of these genes were then measured during the in vitro culture of zygotic embryos, and the sites of expression localized. The expression profiles of these genes indicate that their function is likely to be correlated with the in vitro response of rye. In line L9, increased expression of the rye *SERK* orthologue was observed at most stages during the culture of immature embryos. The suppression of *ScSERK* expression appears to start after the induction of somatic embryogenesis and lasts up to plant regeneration. The rye orthologues of the *LEC1* and *VPI* genes may function in a complimentary manner

and have a negative effect on the production of the embryogenic callus. The expression of the rye *NiR* orthologue during in vitro culture reveals its importance in the process of plant regeneration.

Keywords Expression profile · Immature embryos · In situ RT-PCR · Real Time RT-PCR · Regeneration ability

Introduction

Efficient regeneration from cultured cells is a prerequisite for most aspects of plant biotechnology, especially for transformation experiments. Rye is known to be one of the most recalcitrant cereals with regard to its regeneration ability (Ma et al. 2003), which is highly dependent on genotype (Linacero and Vasquez 1990; Rakoczy-Trojanowska and Malepszy 1993, 1995). Immature embryos are the most widely used explants and have been used successfully to regenerate rye (Ward and Jordan 2001).

In vitro plant regeneration depends on numerous factors including the physiological status of the donor plant and the organ used as the explant (Rakoczy-Trojanowska and Malepszy 1990). However, the genotype is the factor that has the most influence on this process. Rakoczy-Trojanowska and Malepszy (1993, 1995) found significant differences between the tissue response (TCR) of several inbred rye lines, both in the case of immature inflorescences and immature embryos cultured. Genetic analysis at the Mendelian level showed that the in vitro response of immature inflorescences is controlled by a polygenic system with different gene interactions, and that the ability to regenerate plants is a recessive trait (Rakoczy-Trojanowska and Malepszy 1993). Results obtained in a study of immature embryos demonstrated that the production of embryogenic callus and the plant and

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root regeneration are determined by recessive genes, whereas the reduced ability to produce non-embryogenic callus most probably depends on dominant genes. The lack of response was found to be controlled by at least two interacting genes (Rakoczy-Trojanowska and Malepszy 1995).

Recently, we have identified nine putative QTLs for rye TCR: two loci (*eci-1*, *eci-2*) for percentage of immature embryos producing callus, four loci for percentage of immature embryos producing embryogenic callus (*ece-1*, *ese-2*, *ese-3*, *ese-4*), two loci for percentage of immature inflorescences producing callus (*ici-1*, *ici2*) and one locus for percentage of immature inflorescences producing embryogenic callus (*ise-1*) (Bolibok et al. 2007). These loci were mapped to chromosomes 1R, 4R, 5R, 6R and 7R, respectively. Further insight into the molecular mechanisms controlling rye TCR was gained by the application of the GDDSC (genetically directed differential subtraction chain) method. We have isolated several GDDSC-products that are potentially connected with callus induction and somatic embryo formation (Hromada et al. 2007).

Molecular mechanisms of somatic embryogenesis have also been investigated in somatic embryo induction systems and classical tissue culture systems of immature embryos of carrot and *Arabidopsis*. The employment of various molecular techniques has led to the identification of several embryogenesis-related genes such as *LEA* (*Late Embryogenesis Abundant*), *SERK* (*Somatic Embryogenesis Receptor-like Kinase*), *AGL15* (*Agamous-like15*), *BBM* (*Baby Boom*), *LEC1*, *FUS3* (*Fusca3*) and *ABI3* (*ABA-Insensitive 3*) (Ikeda et al. 2006).

In carrot cell suspension cultures, presence of the *SERK* mRNA was found to indicate the capability of single cells to develop into somatic embryos (Schmidt et al. 1997). Similar results were obtained for *Dactylis glomerata* (Somleva et al. 2000) and *Arabidopsis thaliana* (Hecht et al. 2001). Moreover, transgenic *A. thaliana* overexpressing the *AtSERK1* gene exhibited an enhanced capacity for somatic embryogenesis in comparison with non-transformed plants (Hecht et al. 2001).

The results obtained by Gaj et al. (2005) indicate that *LEC* genes have a key role in somatic embryogenesis of *A. thaliana*. The authors shown that the embryogenic potential of the *lec1*, *lec2* and *fus3* mutants was much worse in comparison with a wild type (0.0–3.9% of explants producing somatic embryos via callus stage vs 65–94% explants forming somatic embryos directly). The double (*lec1 lec2*, *lec1 fus3* and *lec2 fus3*) and triple (*fus3 lec1 lec2*) mutants did not formed somatic embryos at all. Ectopic expression of *LEC1* (Lotan et al. 1998), *LEC2* (Stone et al. 2001) and *BBM* (Boutilier et al. 2002) in *Arabidopsis* caused the spontaneous formation of somatic embryos on intact plants or explants. The *LEC* and *BBM* genes encode transcription factors. Furthermore, *LEC2*

shares greatest similarity with the B3 domain of transcription factors VIVIPAROUS1 and FUSCA3 (Stone et al. 2001).

The *NiR* gene, encoding ferredoxin-nitrite reductase, was found to determine regeneration ability in rice (Nishimura et al. 2005). Molecular analysis revealed that the poor regeneration ability of rice is strictly correlated with TCR, especially with plant regeneration (Taguchi-Shiobara et al. 1997).

The aim of the present study was to identify ortologues of *SERK*, *LEC1*, *VPI* and *NiR* in rye and analyze their expression in immature embryos during in vitro culture.

Materials and methods

Tissue culture procedure

The tissue culture protocol used for immature embryos was similar to that described previously (Rakoczy-Trojanowska and Malepszy 1995) except that the auxin added to the induction medium was DICAMBA (dichloro-2-metoxo-3, 6-benzoic acid) instead of 2,4-D. Briefly, the immature embryos (19–21 days after pollination) were cultured on MS medium (Murashige and Skoog 1962) supplemented with DICAMBA (3 mg/dm³), sucrose (20 g/dm³) and Difco agar (7.5 g/dm³). The calli were subcultured every four weeks. After three subcultures, the explants were transferred to regeneration medium without growth regulators.

RNA isolation

Total RNA was isolated from immature and mature zygotic embryos, leaves and callus of rye from the highly embryogenic line L318 and the non-responding line L9 by the single-step procedure of Chomczynski and Sacchi (1987). The samples of callus were collected from induction medium after two, four, eight or twelve weeks incubation and after two days, two weeks and four weeks growth on the regeneration medium. Tissue samples were homogenized with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The quality and quantity of the isolated RNA was verified on the agarose gel.

RT-PCR, cloning and sequence analysis

The SuperScript one-Step RT-PCR with Platinum *Taq* System (Invitrogen) was used for cDNA synthesis and PCR. All reaction components were mixed in a single tube. To design oligonucleotide primers to amplify rye ortologues (Sc) of embryogenesis-related genes, the nucleotide sequences of the selected genes were obtained from GenBank:

SERK – mRNA sequences of wheat *SERK* (GenBank accession no. BT009426), rice *SERK* (AY652735) and maize *SERK1* (NM_001111662); *LEC1* – mRNA sequences of rice *LEC1* (AY062184, AU088581) and maize *LEC1* (AF410176); *VPI* – mRNA sequence of maize *VPI* (M60214); *NiR* – mRNA sequence of rice *NiR* (D50556). Where multiple sequences were available for a gene, they were aligned using the on-line software Primer3 (<http://frodo.wi.mit.edu/primer3/>) and the region of highest homology was chosen for the selection of gene-specific primers. The primers used in RT-PCR are listed in Table 1.

The amplified RT-PCR products were cloned into the pCRII-TOPO vector, using an Invitrogen TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions and sequenced by the Sequencing Service at the Institute of Biochemistry and Biophysics Polish Academy of Sciences (<http://www.ibb.waw.pl/services/>). The RT-PCR fragment sequences were analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/>); alignments and comparisons were performed using ClustalW on Bioedit.

Real Time and in situ RT-PCR

The relative expression of putative orthologues of the genes *SERK*, *LEC1*, *VPI* and *NiR* was measured by Real Time RT-PCR analysis using a Roche system (LightCycler with the Software v. 3.5) and reagents (LightCycler-RNA Amplification Kit SYBR Green I, LightCycler-Control Kit RNA). Expression of the rye gene coding for 18 S RNA was used to normalize the expression data for the other genes. RNA concentration in reaction mixture was 10 ng/μl. The three samples were used for q-PCR and each experiment was repeated three times. The sample with RNA isolated from immature embryos of line L318 was used as a calibrator.

In situ RT-PCR analysis of sections prepared from immature zygotic embryos was performed according to the protocol described by Przybecki et al. (2006). Stained sections were visualized and recorded using the Olympus Provis AX70 light microscope with analysis software.

Results

Amplification and sequence analysis of rye orthologues

SERK

Using primers designed to a conserved region of the *SERK* gene (Table 1), a 234-bp fragment was amplified by RT-PCR (*ScSERK*, GenBank accession no. EG999307) as presented in Fig. 1. The nucleotide sequence of this fragment showed the highest similarity to the *SERK1* mRNAs of rice, *Oryza sativa* (90%, AY652735) and wheat, *Triticum aestivum* (100%, AK333001).

LEC1

Primers based on *LEC1* sequences of monocotyledonous species (Table 1) were used in RT-PCR to amplify a fragment of 180 bp (*ScLEC1*, GenBank accession no. ES351494), which showed a high degree of sequence similarity to the *Oryza sativa HAP3* mRNA (98%, AB288036), *OsLEC1* mRNA (98%, AY264284) and *Zea mays LEC1* mRNA (96%, NM_001112048) as shown in Fig. 1.

VPI

The 203-bp fragment amplified by RT-PCR using VP primers (Table 1), (*ScVPI*, GenBank accession no. ES584596) shared a high degree of sequence similarity with the *VPI* mRNAs of *Hordeum vulgare* (97%, AY150678), *Triticum aestivum* (97%, FJ640559) and *Zea mays* (91%, NM_001112070) as demonstrated in Fig. 1.

NiR

Use of the *NiR_zyt* primers (Table 1) in an RT-PCR amplified a DNA fragment of 235 bp (*ScNiR*, GenBank accession no. EG999306) which showed highest sequence similarity to the *Triticum aestivum* ferredoxin-nitrite reductase mRNA (97%, FJ527909) (Fig. 2a), and to the *NiR* mRNA of rice (91%, D50556) (Fig. 2b).

Table 1 Primers used to amplify rye homologues of embryogenesis-related genes

| Gene | Primer | Sequence 5'-3' | Size of product [bp] |
|-------------------|---------|---|----------------------|
| <i>SERK</i> | SERK_z | F: TTGCTGGAGGTGTTGCTG R: TACACCTTCCAAAGCCAC | 234 |
| <i>LEC1</i> | AU088 | F: CAAGGAGACGATCCAGGAGT R: GGTAGCGGTGGAGGTAGACG | 180 |
| <i>VPI</i> | VP | F: AGAAGGTGCTGAAGCAGAGC R: CCCTGTGTTTTCCAGCAGAT | 203 |
| <i>NiR</i> | NiR_zyt | F: GAGAAGAGGATGCCGAACG R: ATGTTCTGCTCCACGGTGA | 235 |
| Control 18 S rRNA | 18 S | F: CATCCCAAAGTCCAACCTA R: GCTACCACATCCAAGGAA | 250 |

Fig. 1 Sequence alignments of *SERK* (EG999307 – rye, AK333001 – wheat, AY652735 – rice), *LECI* (ES351494 – rye, AB288036 and AY264284 – rice, NM_001112048 – maize) and *VPI* (ES584596 – rye, AY150678 – barley, FJ640559 – wheat, NM_001112070 maize) homologues. The nucleotides differing from the consensus are shaded

SERK

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EG999307 | ATTGGATTTGCACTGTGGCGGCGACGTA AACCTGAAGAGCA|TTCTTTGA
AK333001 | ATTGGATTTGCACTGTGGCGGCGACGTA AACCTGAAGAGCA|TTCTTTGA
AY652735 | ATTGGATTTGCATGGTGGCGGCGCGTAAACCTGAAGAA|CACTTCTTTGA

EG999307 | TGTCCCTGCTGAGGAGGATCCAGAAGTGCACCTTGGCCAGCTGAAGAGGT
AK333001 | TGTCCCTGCTGAGGAGGATCCAGAAGTGCACCTTGGCCAGCTGAAGAGGT
AY652735 | TGTCCCTGCTGAGGAGGATCCAGAAGTGCACCTTGGCCAACTTAAGAGAT

EG999307 | TCTCACTAAGGGAGCTTCAAGTTGCTAGCGATAACTTCAGCAATAAGAAC
AK333001 | TCTCACTAAGGGAGCTTCAAGTTGCTAGCGATAACTTCAGCAATAAGAAC
AY652735 | TCTCACTCCGGGAGCTTCAAGTTGCTACTGATAACTTTAGCAATAAGAAAT

EG999307 | ATTCTAGGAAGAGGTGGCTTTGGAAAGGTGTAA
AK333001 | ATTCTAGGAAGAGGTGGCTTTGGAAAGGTGTAC
AY652735 | ATTCTGGGAAGAGGTGGCTTTGGAAAGGTGTAC

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LECI

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ES351494 | CAAGGAGACGATCCAGGAGTGCCTGTCGGAGTACATCAGCTTCATCACGG
AB288036 | CAAGGAGACGATCCAGGAGTGCCTGTCGGAGTACATCAGCTTCATCACCG
AY264284 | CAAGGAGACGATCCAGGAGTGCCTGTCGGAGTACATCAGCTTCATCACCG
NM_001112048 | CAAGGAGACGATCCAGGAGTGCCTGTCGGAGTACATCAGCTTCATCACCG

ES351494 | GGGAGGCCAACGAGCGGTGCCAGCGCGAGCAGCGCAAGACCATCACCGCC
AB288036 | GGGAGGCCAACGAGCGGTGCCAGCGCGAGCAGCGCAAGACCATCACCGCC
AY264284 | GGGAGGCCAACGAGCGGTGCCAGCGCGAGCAGCGCAAGACCATCACCGCC
NM_001112048 | GGGAGGCCAACGAGCGGTGCCAGCGCGAGCAGCGCAAGACCATCACCGCC

ES351494 | GAGGACGTGCTCTGGGCCATGAGCCGCCTCGGCTTCGACGACTACGTGCA
AB288036 | GAGGACGTGCTCTGGGCCATGAGCCGCCTCGGCTTCGACGACTACGTGCA
AY264284 | GAGGACGTGCTCTGGGCCATGAGCCGCCTCGGCTTCGACGACTACGTGCA
NM_001112048 | GAGGACGTGCTGTGGGCCATGAGCCGCCTCGGCTTCGACGACTACGTGCA

ES351494 | GCCCCTCAGCGTCTACCTCCACCGCTACCA
AB288036 | GCCCCTCAGCGTCTACCTCCACCGCTACCG
AY264284 | GCCCCTCAGCGTCTACCTCCACCGCTACCG
NM_001112048 | GCCGCTCAGCGCTACCTCCACCGCTACCG

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VPI

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ES584596 | GGTGCTGAAGCAGAGCGACGTCGGAAACCCTCGGCCGCATCGTGCTCCCCA
AY150678 | GGTGCTGAAGCAGAGCGACGTCGGAAACCCTCGGCCGCATCGTGCTCCCCA
FJ640559 | GGTGCTCAAGCAGAGCGACGTCGGAAACCCTCGGCCGCATCGTGCTCCCCA
NM_001112070 | GGTGCTGAAGCAGAGCGACGTCGGAGCCTCGGCCGCATCGTGCTCCCCA

ES584596 | AAAAGGAAGCGGAGACTCACCTGCCGGAGCTCAAGACGGGGACGGCATC
AY150678 | AAAAGGAAGCGGAGACTCACCTGCCGGAGCTCAAGACGGGGACGGCATC
FJ640559 | AAAAGGAAGCGGAGACTCACCTGCCGGAGCTCAAGACGGGGACGGCATC
NM_001112070 | AAAAGGAAGCGGAGGTTTACCTGCCGGAGCTGAAGACGAGGGATGGCATC

ES584596 | TCGATCCCCATTGAGGACATCGGCACATCTCAGGTGTGGAGCATGCGGTA
AY150678 | TCGATCCCCATTGAGGACATCGGCACATCTCAGGTGTGGAGCATGCGGTA
FJ640559 | TCGATCCCCATTGAGGACATCGGCACATCTCAGGTGTGGAGCATGCGGTA
NM_001112070 | TCCATCCCCATTGAGGACATCGGAACGTCCGCGGTGTGGAAATGCGGTA

ES584596 | CCGATTTTGGCCCAACAACAAGAGCAGAATGTATCTCTGCTGGAAACACAG
AY150678 | CCGATTTTGGCCCAACAACAAGAGCAGAATGTATCTCTAGAGAACACTG
FJ640559 | CCGATTTTGGCCCAACAACAAGAGCAGAATGTATCTCTAGAGAACACTG
NM_001112070 | CAGGTTTTTGGCCCAACAACAAGAGCAGAATGTATCTCTGCTGGAAACACAG

ES584596 | GGGG
AY150678 | GGTG
FJ640559 | GGTG
NM_001112070 | GGGG

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Expression analysis of *ScSERK*, *ScLECI*, *ScVPI* and *ScNiR*

Real Time RT-PCR analysis demonstrated that the rye orthologues of the *SERK*, *LECI*, *VPI* and *NiR* genes were expressed in all investigated tissues in both rye lines

(Fig. 3). The expression level of *ScSERK* and *ScNiR* was higher in line L9 in most stages of tissue culture except for two: after two weeks on induction medium and four weeks on regeneration medium. After two weeks on regeneration medium, the expression of these genes was

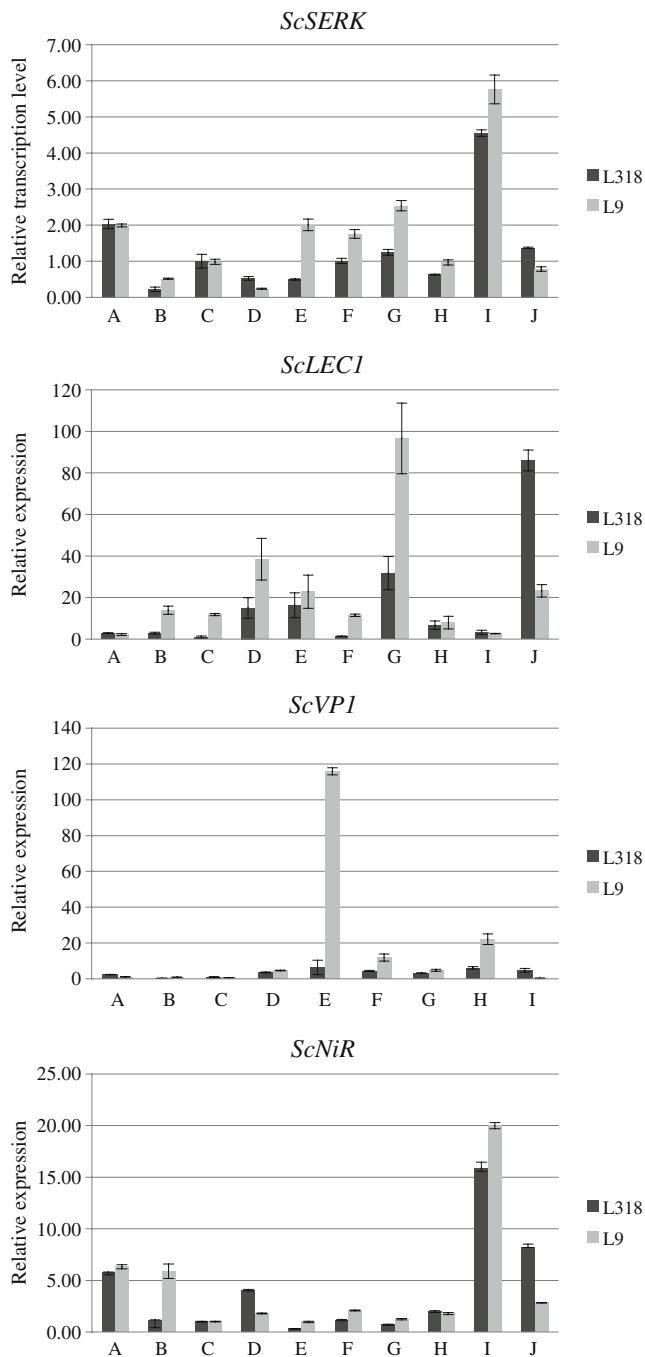


Fig. 3 Relative expression levels (Y axis) of rye *SERK*, *LEC1*, *VP1* and *NiR* orthologues in two lines L318 and L9 with different regeneration ability. **a** – leaves; **b** – mature zygotic embryos; **c** – immature zygotic embryos; **d** – **g** (callus derived from induction medium after two, four, eight or twelve weeks incubation, respectively); **h** – **j** (callus collected from regeneration medium after two days, two weeks and four weeks, respectively). The expression level of immature embryos of line L318 was used as a calibrator

was found in the embryogenic and non-embryogenic cultures (Baudino et al. 2001). Similar levels of the *MtSERK1* transcript were found in leaf explants of two lines of *Medicago truncatula* that differ in their regenera-

tion potential (Nolan et al. 2003). Although the expression of *ScSERK* was detected at all tissue culture stages, its level in line L9 was lightly higher. Comparable levels of *ScSERK* transcripts in immature embryos of L318 and L9 may indicate their similar embryogenic potential. During the first 2 weeks of culture the expression of *ScSERK* decreased by half in line L318 and by about 75% in L9, in comparison to the explants. We suppose that at this culture stage the SERK protein is activated in callus of line L318. SERK is a receptor kinase that can participate in signal transduction pathways and in the initiation of processes leading to somatic embryogenesis (Schmidt et al. 1997). However, SERK protein seems to exert a negative influence on plant regeneration as the transcript level was decreased in line L9 after 4 weeks on regeneration medium.

A similar pattern of expression was observed in the *ScNiR* gene. The expression profile of *ScNiR* during the in vitro culture of rye confirmed its relation in the regeneration process. The 4-fold higher levels of the *ScNiR* transcript in calli of line L318 collected after two weeks of culturing on medium containing auxins may indicate the positive effect of its gene product on embryogenic callus formation. Nitrate is added as the nitrogen source for in vitro culture, but its metabolite, nitrite, has a toxic effect on the plant cell growth. The rapid metabolism of nitrite is therefore essential for callus growth and so the *NiR*-encoded ferredoxin-nitrite reductase, which catalyzes the reduction of nitrite to ammonium ions, plays a key role. The increased expression of *ScNiR* in the embryogenic calli of line L318 subcultured on medium without hormones is probably due to the elevated level of metabolic activity connected with the intensive regeneration process. Metabolic products, including the toxic NO_2^- , are released into the medium. Presumably *ScNiR* expression is enhanced to counter the accumulation of nitrite ions.

The *LEC1* gene is an important regulator of somatic embryogenesis (Lotan et al. 1998; Yazawa et al. 2004; Zhang et al. 2002). *LEC1* encodes a protein related to the heme-activated protein 3 (HAP3) subunit of the CCAAT box-binding factor (CBF), a eukaryotic transcriptional regulator (Lotan et al. 1998). Because *LEC1* is a component of a plant CBF, it may regulate embryonic processes by activating the transcription of specific genes (Lotan et al. 1998). Ectopic expression of the *LEC1* gene in post-embryonic *Arabidopsis* plants induces embryonic processes and a set of specific genes required for embryo development (Lotan et al. 1998).

The expression of *ScLEC1* was detected in all tissues and at all culture stages, but was always higher in line L9 than in L318, except during the 4 weeks on regeneration medium. It is possible that *ScLEC1*, as an element of a CBF complex, is connected with the suppression of genes regulating somatic

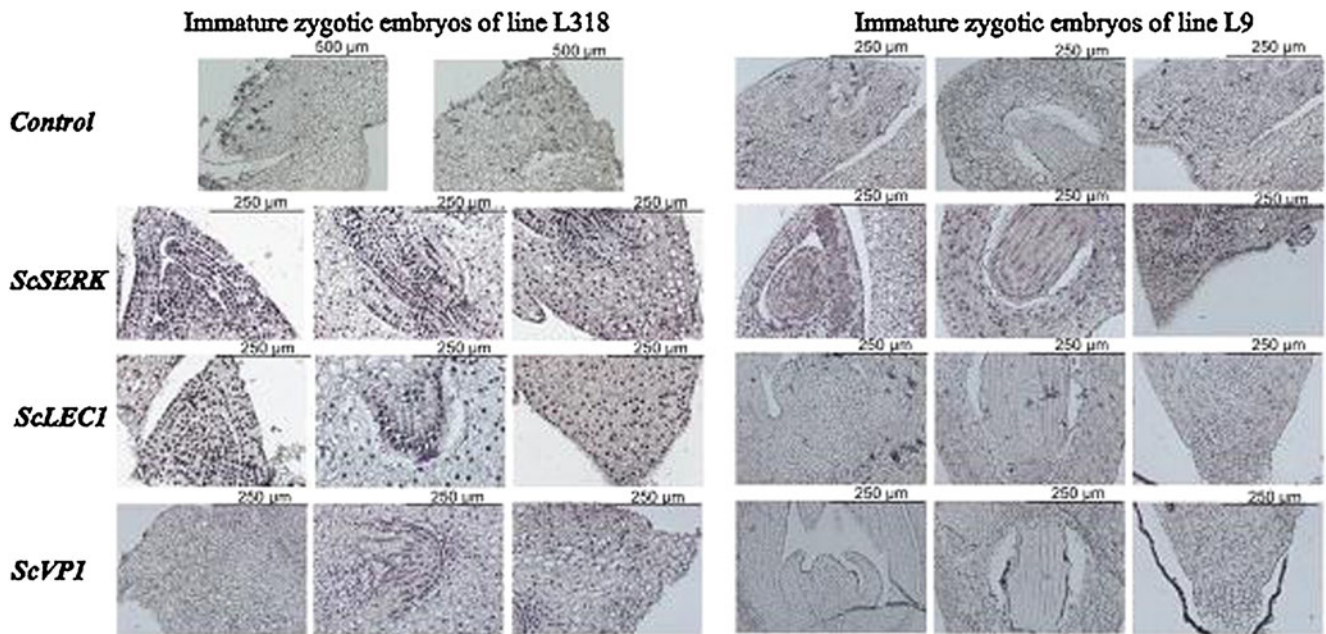


Fig. 4 In situ RT-PCR detection of *ScSERK*, *ScLECI*, *ScVPI* mRNA in immature zygotic embryos of rye. The negative control was a specimen without specific primers added to the amplification mixture. The blue-purple staining indicates the location of transcript accumulation

embryogenesis. A significant increase in the expression of *LECI* in 4-week-old callus of line L318 may indicate that this gene is crucial for the induction of plant regeneration. However, further studies are required to precisely characterize its function. In this respect it would be informative to identify the other components of the CBF complex and to examine the expression of the *PKL* (*Pickle*) gene, which acts as the repressor of *LECI* (Lotan et al. 1998).

The *ScVPI* transcript was barely detectable in zygotic embryos and leaves of both rye lines. Only callus of line L9 collected after 4 weeks of in vitro culture showed definite expression of this gene. Similarly in wheat, expression of the *VPI* gene was not detected in leaves, but some of the transcript was present in mature zygotic embryos (Nakamura and Toyama 2001). *VPI* has been shown to be a major regulator of late embryo development in wheat and ancestral species (McKibbin et al. 2002). Inactivation of *VPI* leads to the vivipary (McKibbin et al. 2002). Due to the good regeneration ability of L318 it might have been expected that *ScVPI* would be expressed in this line. However, the very low *ScVPI* expression in 4-week-old L318 callus compared to a significant increase in a transcript level in L9, suggests that it functions as a suppressor of somatic embryogenesis and plant regeneration. It is possible that in its role as a transcription factor, *VPI* may act both as activator and a repressor for different genes (Suzuki et al. 1997). Among the genes that are regulated by *VPI* are two kinases (Suzuki et al. 2003, supplemental data 1, www.plantphysiol.org), implying a potential function for this factor in the suppression of

ScSERK during the culture of immature embryos of rye. The findings of the present study are in agreement with those of a previous study employing classical genetic analysis (Rakoczy-Trojanowska and Malepszy 1995). This analysis showed that the lack of response is determined by at least two interacting genes of a suppressive character. Three of the genes examined here – *ScSERK*, *ScLECI* and *ScVPI*, may be appropriate candidates.

While further research is essential to gain a greater understanding of the molecular mechanisms controlling the tissue culture response of rye, it is hoped that most of the presented data will find application in plant biotechnology and molecular breeding.

Conclusions

1. Expression of rye orthologue of the *SERK* gene is likely to be involved in the induction of somatic embryogenesis initiated from immature embryos and its expression is suppressed in the later stages of in vitro culture.
2. The expression profiles of the genes *ScLECI* and *ScVPI* in line L9 suggest that their complementary interaction negatively influences somatic tissue development.
3. Enhanced expression of the *ScNiR* gene in line L318 at the start of tissue culture and in callus in medium without hormones (4th week of culture), may indicate its important double role: in callus induction and in plant regeneration.

Acknowledgements We would like to thank Grzegorz Koczyk (Institute of Plant Genetics Polish Academy of Sciences, Poznań) for bioinformatics instruction when designing the primers.

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