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ORIGINAL ARTICLE

Identification of molecular processes that differ among Scots pine somatic embryogenic cell lines leading to the development of normal or abnormal cotyledonary embryos

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Abstract

Several coniferous species can today be propagated through somatic embryogenesis, but for species belonging to the *Pinus* genus, there are still problems related to the small number of genotypes from which embryogenic cultures can be established and the low yield of high-quality cotyledonary embryos. In order to pinpoint molecular processes that might be disturbed during somatic embryogenesis in Scots pine, we have analyzed the expression pattern of selected transcripts during development of somatic embryos in a normal and an abnormal cell line of Scots pine. The selected transcripts have been previously shown to be differentially expressed during early zygotic embryogenesis in Scots pine (Merino et al. 2016). Based on the transcripts that accumulated differentially between the two cell lines, we conclude that the apical–basal polarization in early somatic embryos and the transition from the morphogenic phase to the maturation phase are disturbed in the abnormal cell line. A comparison between transcript accumulation during somatic and zygotic embryogenesis highlighted problems with the continuous embryo degeneration processes in embryogenic cultures of Scots pine. Transcripts that were highly abundant during the cleavage process in zygotic embryos showed different accumulation patterns during somatic embryogenesis in Scots pine compared to those in Norway spruce. Furthermore, altering the expression of these transcripts in embryogenic cultures of Norway spruce resulted in a changed proliferation pattern of the embryos so that they became more similar to proliferating somatic embryos of Scots pine. Taken together, our results provide a deeper understanding of the deviations in abnormal cell lines and indicate that embryogenic cultures of Scots pine are proliferating by a cleavage-like process.

Keywords Cleavage polyembryony · Conifers · Molecular regulation · *Pinus sylvestris*

Introduction

Somatic embryogenesis is a powerful tool to propagate conifers vegetatively. The possibilities to scale-up the multiplication rate in bioreactors and to cryostore the embryogenic cultures confer advantages over propagation via cuttings. However, it is important that the quality of the somatic embryo plants is comparable to that of seedlings. Several coniferous species can today be propagated via somatic embryos, but most species belonging to *Pinus* are recalcitrant to develop somatic embryos. A number of experimental approaches, mainly based on trial and error strategies, have been taken to improve the protocols for initiation of embryogenic tissue and subsequent development of cotyledonary embryos (Klimazewska et al. 2007; Lelu-Walter et al. 2013). Although improvements have been made, further studies and new approaches are required to gain a better knowledge of the somatic embryogenesis process in species belonging to the *Pinus* genus. For example, comparative studies of somatic and zygotic embryogenesis will be important for making it possible to produce refined protocols and to determine the quality of the somatic embryos.

Irene Merino and Malin Abrahamsson contributed equally to this work.

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Polyembryony is a common feature in coniferous species (Buchholtz 1926). There are two types of polyembryony in conifers with respect to the origin of the embryos: (a) simple polyembryony, owing to fertilization of eggs in more than one archegonium, and (b) cleavage polyembryony, due to the splitting of the proembryo. During cleavage polyembryony, the proembryo goes through a cleavage process usually resulting in four equal-sized embryos. Successively, one of the embryos becomes dominant, while the remaining subordinate embryos are degraded by programmed cell death (PCD) (Filonova et al. 2002). Embryogenic tissue, from which high-quality mature somatic embryos can be regenerated, can be established from a high frequency of cotyledonary zygotic embryos in, for example, Nordmann's fir (*Abies nordmanniana*) (Find 2016), larch species (*Larix* sp.) (Lelu-Walter et al. 2016), and Norway spruce (*Picea abies*) (von Arnold and Clapham 2008), species having simple polyembryony. However, in most *Pinus* species, having cleavage polyembryony, embryogenic cultures have to be initiated from immature zygotic embryos during the cleavage process (Klimazewska et al. 2007). The main limitations with *Pinus* species are that proliferating embryogenic tissue only can be established from a limited number of immature zygotic embryos and that only few cell lines give rise to high-quality mature somatic embryos. We have previously shown that early embryos in Scots pine (*Pinus sylvestris*) cell lines that give rise to abnormal cotyledonary embryos carry supernumerary suspensor cells (Abrahamsson et al. 2012) and that the degeneration pattern of early and late embryos differs between normal and abnormal cell lines (Abrahamsson et al. 2017). Furthermore, the degeneration pattern observed in abnormal cell lines seems to start already during the initiation of embryogenic tissue. It has been suggested that the low initiation frequency of embryogenic tissue and the poor regeneration rate in *Pinus* species are directly or indirectly connected to cleavage polyembryony (Klimazewska et al. 2007; Abrahamsson et al. 2017).

Most of our knowledge about molecular regulation of embryonic patterning has been derived from the angiosperm model plant Arabidopsis (*Arabidopsis thaliana*), while our knowledge about molecular regulation of embryo development in conifers is limited. Molecular data suggest that angiosperms and gymnosperms shared a final common ancestor about 300 million years ago (Smith et al. 2010) and later evolved different embryo development pathways. Despite the different patterning during embryo development, significant homologies between conifers and angiosperms have been shown, for example, in apical–basal embryo patterning driven by polar auxin transport (Larsson et al. 2008a, b, 2012a, b) and in radial patterning (Zhu et al. 2016). In addition, out of 295 Arabidopsis genes related to embryogenesis, 85% had a strong sequence similarity to an expressed sequence tag in the loblolly pine (*Pinus taeda*) database (Cairney and Pullman 2007). However, it is important to gain knowledge about the temporal and spatial expression pattern for different genes in order to understand the fundamentals of embryogenesis in conifers.

Studies have been performed to gain information about the molecular processes regulating different steps of somatic and zygotic embryogenesis in conifers. In white spruce (*Picea glauca*), an association between biotic defense elicitation and suppression of embryogenic competence was detected (Rutledge et al. 2013). However, in Norway spruce, both stress-related processes and auxin-mediated processes were shown to be associated with early somatic embryo development (Vestman et al. 2011). In maritime pine (*Pinus pinaster*), it was found that epigenetic regulation and transcriptional control related to auxin transport and response are critical during the differentiation of dominant zygotic embryos (Vega-Bartol et al. 2013). Furthermore, low global DNA methylation in embryogenic cultures of black spruce (*Picea nigra*) was shown to be associated with higher maturation ability (Noceda et al. 2009).

We recently performed a transcriptome analysis during early zygotic embryo development in Scots pine (Merino et al. 2016). A number of different transcripts were identified as potential regulators of the cleavage process and for the development of dominant embryos. In order to pinpoint molecular processes that might be disturbed during somatic embryogenesis, we have here analyzed the expression of these transcripts during somatic embryo development in a normal and an abnormal cell line of Scots pine. In addition, we analyzed their expression pattern during somatic embryogenesis in Norway spruce, which has simple polyembryony and not cleavage polyembryony as in Scots pine. Transcripts that were differentially expressed in the two Scots pine cell lines were related to apical–basal polarization of the somatic embryo and transition from the morphogenic phase to the maturation phase. Differences in transcript accumulation during somatic and zygotic embryogenesis were mainly related to the degeneration processes in somatic embryos. Transcripts that were highly abundant during the cleavage process in zygotic embryos were differentially expressed during somatic embryogenesis in Scots pine as compared to Norway spruce. Altering the expression of these transcripts in embryogenic cultures of Norway spruce resulted in a changed proliferation pattern of the embryos so that it became more similar to the cleavage-like proliferation process occurring in embryogenic cultures of Scots pine.

Taken together, our results provide a deeper understanding of the deviations in abnormal cell lines of Scots pine. This in turn provides clues on how to improve the culture conditions

in order to regenerate high-quality mature somatic embryos in *Pinus* species.

Material and methods

Plant material

Two embryogenic cell lines of Scots pine (*P. sylvestris* L.) were used in this study: cell line 12:12 which mainly gives rise to cotyledonary embryos with normal morphology and cell line 3:10 which mainly gives rise to cotyledonary embryos with abnormal morphology (Abrahamsson et al. 2012). Both cell lines were established in the year 2000 (Burg et al. 2007). For qRT-PCR analyses, the Norway spruce (*P. abies* L. Karst) embryogenic cell line 61:21 (established in 1995; Högberg et al. 1998) was used. The same cell line as well as cell line 11:18 (established in 2011, unpublished data and kindly provided by the Swedish Forestry Research Institute) was used as background to generate transgenic sub lines. All cell lines were stored in liquid nitrogen and thawed before starting the experiments. The Scots pine cell lines were cultured on solidified medium (see Abrahamsson et al. 2017), and the Norway spruce cell lines were cultured in liquid and on solidified medium (see von Arnold and Clapham 2008). Briefly, the cultures were proliferated on proliferation media containing 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) as plant growth regulators (PGRs). To stimulate differentiation of early embryos, the cultures were transferred to pre-maturation media lacking PGRs. Thereafter, the cultures were transferred to maturation media containing abscisic acid (ABA) for the development of late and mature embryos.

Sample collection for expression analyses

Somatic embryos from the Scots pine cell lines 3:10 and 12:12 were collected at six consecutive developmental stages during embryo development (Abrahamsson et al. 2012, 2017). Stage 1a, embryogenic cell aggregates proliferating on proliferation medium. Stage 1b, embryogenic cell aggregates, with globular structures, collected one week after transfer to pre-maturation medium. Stage 1c, differentiating early embryos, collected 1–2 weeks after transfer to maturation medium. Stage 2, early embryos, collected 2–3 weeks after transfer to maturation medium. Stage 3, late embryos, collected 2–5 weeks after transfer to maturation medium. Stage 4, late embryos before cotyledon differentiation, collected 5–7 weeks after transfer to maturation medium.

Somatic embryos from the Norway spruce cell line 61:21 were collected at eight consecutive developmental stages during embryo development (Larsson et al. 2012a). Stage 1, proliferating proembryogenic masses (PEMs) in proliferation medium. Stage 2, early embryos, collected one week after transfer to pre-maturation medium. Stage 3, late embryos, collected 1–2 weeks after transfer to maturation medium. Stage 4, late embryos before cotyledon differentiation, collected 2–3 weeks after transfer to maturation medium. Stages 5, 6, and 7, maturing embryos, collected 3–7 weeks after transfer to maturation medium. Stage 8, cotyledonary embryos, collected 6–7 weeks after transfer to maturation medium.

Three biological replicates were collected from each respective developmental stage. Whole mount of tissue (100–150 mg) was collected from stages 1a and 1b in Scots pine and from stages 1 and 2 in Norway spruce. From later stages, each sample included 20 to 30 embryos collected individually under a stereomicroscope. The samples were snap frozen in liquid nitrogen and stored at − 80 °C until use.

RNA extraction and cDNA synthesis

The frozen embryo samples were ground to powder in a Tissue lyser II (Qiagen) using a single stainless steel bead. Total RNA was isolated from the samples using the Spectrum Plant Total RNA kit (Sigma-Aldrich), including the additional On-Column DNAse I Digestion protocol, according to the manufacturer's instructions. The quantity of the extracted RNA was measured using a NanoDrop-1000 spectrophotometer (Nanodrop Technologies), and the integrity was verified by 1% agarose gel electrophoresis. cDNA was synthesized from 500 ng total RNA using the PrimeScript™ RT reagent Kit (Takara), according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative RT-PCR was performed in a Bio-Rad CFX Connect™ Real-Time PCR Detection System Cycler (Bio-Rad Laboratories) starting from 5 ng cDNA. Three biological replicates, each with two technical replicates, were analyzed for each transcript. Transcript expression values were normalized against two reference genes, *ELONGATION FACTOR 1* (*EF1*) and *PHOSPHOGLUCOMUTASE* (*PHOS*) (Merino et al. 2016). The relative expression profile for each transcript was estimated by the Livak method ($2^{-\Delta\Delta C}$), by referring to the developmental stage showing the lowest accumulation level. The primer sequences used to analyze the expression level of the transcripts in Scots pine embryogenic cultures were shown in Merino et al. (2016), except for the primers used to analyze *NAC DOMAIN CONTAINING PROTEIN 47* (*NAC47*), which are presented in Table S1. All primers used for Norway spruce are listed in Table S1. Significant differences in transcript accumulation between different embryo developmental stages were estimated by a *t* test mean comparison analysis ($P \leq 0.05$) using the JMP software (v11).

Vector construction and transformation

To generate overexpression constructs, full-length coding sequences for the beta-expansin gene *EXPB1*, and for *ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 3* (*RIC3*), and *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1* (*SERK1*) were amplified from cDNA of needles of Scots pine. Total RNA was extracted from 100 mg of powdered needles following the protocol described by Canales et al. (2012). Traces of genomic DNA were removed using the DNA-free™ Kit (Ambion, Life Technologies), and the corresponding cDNA was synthesized using the PrimeScript™ RT reagent Kit (Takara), according to the manufacturer's instructions. To produce an entry clone, specific primers containing the cloning adapter CACC were designed by using the Scots pine zygotic transcriptome output sequences as templates (Merino et al. 2016). The obtained PCR products, amplified with the Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific), were gel-purified with the GeneJET Gel Extraction Kit (Thermo Scientific) and introduced into the pENTR™/D-TOPO® vector by using the pENTR™ Directional TOPO® Cloning Kit (Invitrogen), following the manufacturer's instructions. The primer sequences used to generate overexpression constructs are listed in Table S2.

To obtain RNA interference constructs, total RNA was extracted from 100 mg of powdered Norway spruce needles and the corresponding cDNA was synthesized as described for overexpression constructs. From non-conserved regions of *TRANSPARENT TESTA7* (*TT7*) and the cytochrome P450 gene *CYP78A7* coding sequences, two fragments around 300 and 400 bp long, with 100-bp sequence overlap, were amplified with primers including *BamH*I or *EcoR*I restriction endonuclease sites. The resulting PCR products (F1 and F2 fragments, respectively) were digested, purified with the GeneJET PCR Purification Kit (Thermo Scientific) and ligated to form a hairpin construct (Fig. S1). The desired fused fragment (F1 + F2), around 700 bp long, was purified from a 1.2% agarose gel and introduced into the pENTR/D-TOPO vector, as described for overexpression constructs. The primers designed for this purpose are listed in Table S3.

Both overexpression and RNA interference constructs were inserted into the pMDC32 (2x35S promoter) destination vector (Curtis and Grossniklaus 2003) using the *att* site LR recombination reaction (Life Technologies) and cloned into *Escherichia coli* DH5α™-competent cells (Thermo Scientific) following the manufacturer's instructions. Plasmid DNA from transformed cells was isolated with the GeneJET Plasmid Miniprep Kit (Thermo Scientific), and the final overexpression or RNA interference constructs were confirmed by sequencing. The constructs were introduced into the *Agrobacterium tumefaciens* C58C1 strain carrying the additional virulence plasmid pTOK47 through the freeze/thaw method (Holsters et al. 1978; Höfgen and Willmitzer 1998). The GUS:pMDC32 vector was used as transformation control.

Norway spruce embryogenic cell lines 61:21 and 11:18 were transformed by co-cultivation with *A. tumefaciens* according to the protocol described by Zhu et al. (2014), with some modifications. Briefly, cell cultures, which had been proliferating for 1 week in liquid medium, were co-cultivated with *A. tumefaciens* for 5 h. The cultures were then poured onto filter papers placed on solidified proliferation medium and incubated in darkness for 48 h. The filter papers were thereafter transferred to proliferation medium supplemented with 400 mg ml^{-1} timentin (Duchefa), 250 mg ml^{-1} cefotaxime (Sandoz A/S), and 5 mg ml^{-1} hygromycin B (Duchefa). After one week, the filter papers were transferred to a medium of the same composition except that the hygromycin supply was increased to 7.5 mg ml^{-1}. Two weeks later, the hygromycin supply was increased to 10 mg ml^{-1}, and after another two weeks, to 15 mg ml^{-1}.

Transgenic sub lines

Cell line 61:21 was used as background to generate *35S:GUS*, *35S:CYP78A7i*, *35S:SERK1*, and *35S:TT7i* transgenic sub-lines, and cell line 11:18 was used as background to generate *35S:GUS*, *35S:EXPB1*, and *35S:RIC3* transgenic sub-lines. *35S:GUS* sub-lines were used as transformed controls (T-controls) in both cell lines. Furthermore, untransformed cell lines were included as U-controls.

Putative transformants, proliferating after about two months on selection medium, were analyzed for the presence of the transgene by PCR. Genomic DNA was extracted from proliferating embryogenic tissue by using the CTAB method (Doyle and Doyle 1987) in combination with the Nucleo Spin gel DNA and RNA purification kit (Machery-Nagel). Briefly, frozen and pulverized tissue, 150 mg per sample, was extracted with CTAB extraction buffer, supplemented with 20 mM β-mercaptoethanol and 10 μg ml^{-1} RNase A, and washed twice with 500 μl chloroform/isoamylalcohol (24:1). The upper phase was mixed with the same volume of binding buffer and purified with the Nucleo Spin gel DNA and RNA purification kit (Machery-Nagel) following the manufacturer's instructions. One hundred nanograms of the isolated DNA was used as template to perform the PCR reaction, using one single primer pair designed over the pMDC32 vector sequence (Table S1). The corresponding transgene size, different depending on the sub-line analyzed, was confirmed on 1% agarose gels.

Quantitative RT-PCR analysis of the relative transcript level of each gene was performed with the PCR-confirmed sub-lines. For each construct, eight different sub-lines were tested, each in three biological replicates. For *35S:CYP78Ai*, *35S:EXPB1*, *35S:RIC3*, and *35S:SERK1* sub-lines, the

expression level of the transcripts was analyzed at stage 1. Since the transcript accumulation of *TT7* was very low at stage 1, selection of *35S:TT7i* sub-lines was based on the transcript abundance at stage 4. The primers used for analyzing transcript abundance in overexpressing sub-lines have been presented before (Merino et al. 2016). The primers used to quantify *CYP78A* and *TT7* in *35S:CYP78Ai* and *35S:TT7i* sub-lines are shown in Table S1. The primers used for analyzing transcript abundance in the control lines (U-controls and T-controls) are shown in Table S1. The sub-lines showing the highest differences in transcript accumulation compared to U-control and T-controls were selected for morphological analysis (Fig. S2). Significant differences in transcript accumulation between different sub-lines were estimated by a *t* test mean comparison analysis ($P < 0.05$) using the JMP software (v11).

T-controls were selected by incubating approximately 100 mg tissue from stage 1 in GUS buffer containing 1 mM 5-bromo-4-chloro-3-indole β-D-glucuronic acid in the darkness at 37 °C for up to 5 h (Uddenberg et al. 2016). Based on high GUS activity, sub-lines *35S:GUS.1-1*, *35S:GUS.1-2*, and *35S:GUS.1-3* derived from cell line 61:21, and sub lines *35S:GUS.1-4* and *35S:GUS.1-5* derived from cell line 11:18, were selected.

Morphological analysis of somatic embryos in transgenic sub-lines

To assess the effect of *EXPB1*, *RIC3*, and *SERK1* overexpression, as well as of *CYP78A7* and *TT7* down-regulation, on embryo morphology at the beginning of late embryogeny (stage 3), samples from two to four biological replicates were collected after two to three weeks on maturation medium from *35S:CYP78A7i*, *35S:EXPB1*, *35S:RIC3*, *35S:SERK1*, and *35S:TT7i* sub-lines, as well as from T-controls. The samples were examined under a light microscope (Zeiss Axioplan or Leica DMI 4000). Based on the aberrant embryo morphologies observed in the transgenic sub-lines, all stage 3 embryos were classified into four main categories: normal morphology, degenerating embryos, embryos carrying supernumerary suspensor cells, and embryogenic complexes. Significant differences in embryo morphology among lines were estimated by a *t* test mean comparison analysis ($P \leq 0.05$) using the JMP software (v11).

Results

Zygotic and somatic embryo development in Scots pine and Norway spruce

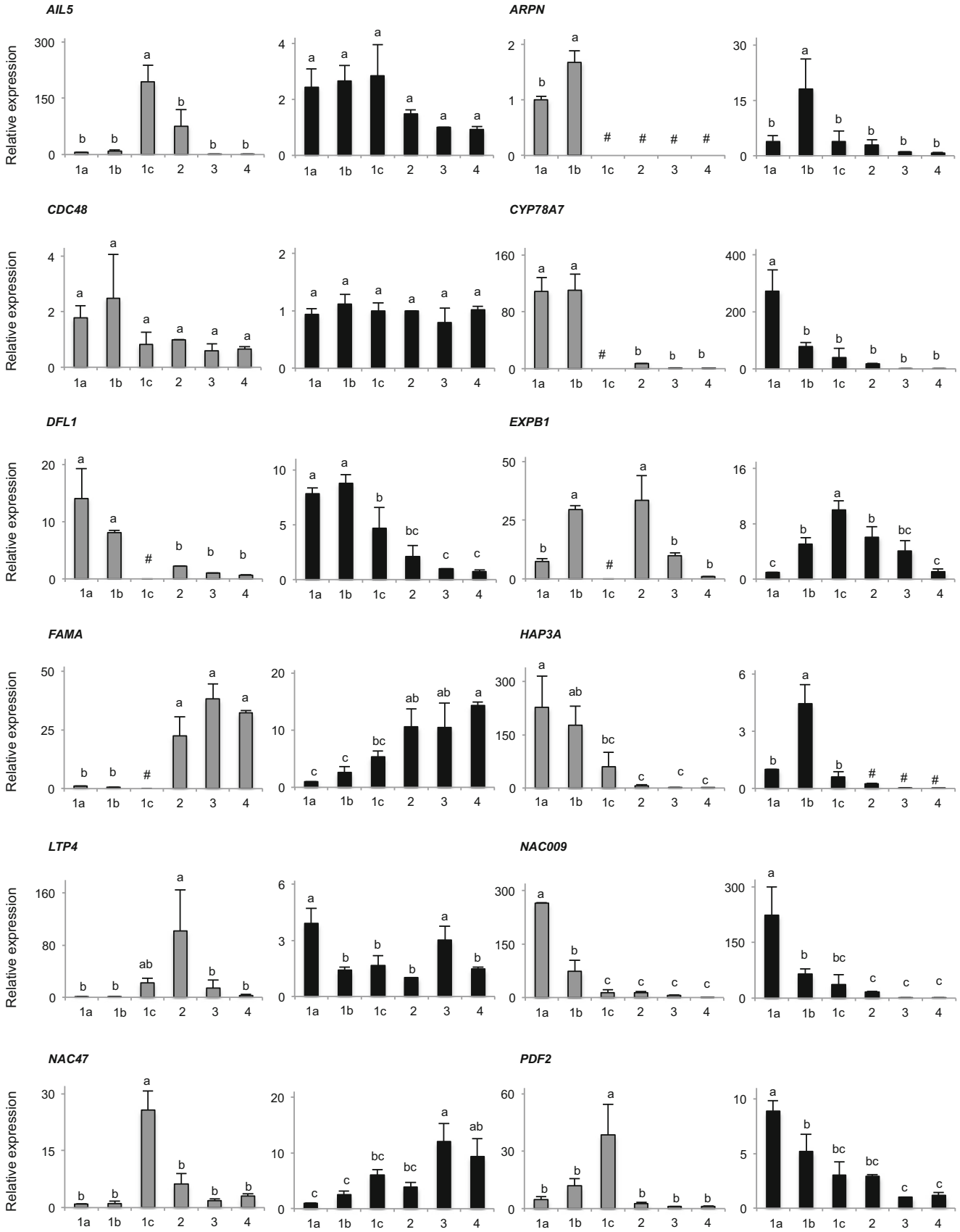
Zygotic embryo development in Scots pine starts with a single zygotic-derived embryo (stage E1) that goes through a cleavage process, giving rise to four equal-sized embryos (stage E2), of which one becomes dominant (stage E3DO) and develops into a cotyledonary embryo (Merino et al. 2016, summarized in Table S4). In Norway spruce, the single zygotic-derived embryo directly starts to develop further, without going through a cleavage process.

The developmental stages of Scots pine and Norway spruce somatic embryogenesis have been described before in Abrahamsson et al. (2012) and Larsson et al. (2008a), respectively, and are schematically compared in Table S4. Embryogenic cultures of both species proliferate as different types of cell aggregates composed of small rounded cells with dense cytoplasm in combination with elongated cells harboring large vacuoles. In Scots pine, the globular structures in stage 1b aggregates successively develop into early somatic embryos, which frequently develop lobes (Abrahamsson et al. 2012). In zygotic embryos of Scots pine, lobing is a consequence of unequal growth rates between different domains in the embryonal mass and can result in cleavage polyembryony if the lobes develop their own independent embryonal tube cells (Dogra 1967). In contrast, in Norway spruce, only a low frequency of the early embryos, which differentiate from large clusters of proembryogenic masses (PEM III), develop lobes (Filonova et al. 2000; Larsson et al. 2008a). However, in both species, the early somatic embryos are polarized with a compact embryonal mass in the apical part and vacuolated suspensor cells in the basal part.

Transcript abundance during development of somatic embryos of Scots pine

To identify deviations during somatic embryo development in recalcitrant cell lines, we have previously compared the developmental pattern of somatic embryos in Scots pine cell lines giving rise to normal cotyledonary embryos to that of cell lines giving rise to abnormal cotyledonary embryos (Abrahamsson et al. 2012, 2017). No differences in the developmental pattern were observed before stage 3 embryos had developed. However, in stage 3 embryos, the ratios of the embryonal mass to the suspensor are different and the embryos degenerate by different processes.

In order to get a better understanding of the molecular differences regulating embryo development in normal and abnormal cell lines of Scots pine, we compared the expression pattern of selected transcripts during development of somatic embryos between cell line 12:12, giving rise to normal cotyledonary embryos, and cell line 3:10, giving rise to abnormal cotyledonary embryos. The transcripts were selected based on our previous transcriptome analysis of zygotic embryogenesis in Scots pine (Merino et al. 2016, Table S5). The transcript abundance of all selected genes, except for *CELL DIVISION CYCLE 48* (*CDC48*), differed significantly during the cleavage process or the development of a dominant embryo during


AIL5
ARPN
CDC48
CYP78A7
DFL1
EXPB1
FAMA
HAP3A
LTP4
NAC009
NAC47
PDF2
Relative expression
1a 1b 1c 2 3 4

◀ **Fig. 1** Quantitative real-time PCR analysis of the relative transcript level of selected genes during somatic embryo development in Scots pine. The transcript level of 18 selected genes was analyzed in six consecutive stages (Table S4) during embryo development in cell line 12:12 (gray bars), giving rise to normal embryos and cell line 3:10 (black bars), giving rise to abnormal embryos. Relative expression levels, estimated by the Livak method ($2^{-\Delta\Delta Ci}$), are referred to the developmental stage showing the lowest accumulation for each transcript. Expression values are normalized against two reference genes (*EF1* and *PHOS*). The expression levels are means ± SD of three biological replicates, analyzed with two technical replicates each, except for *ARNP* which is based on two biological replicates. Different letters indicate significant differences in the transcript level between different developmental stages (Student's *t* test, $P \leq 0.05$). # indicates no detectable expression

zygotic embryogenesis. In addition to those transcripts, we also included the *ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 47* (*NAC47*) transcription factor, which is associated with senescence in Arabidopsis (Kim et al. 2014), and could potentially be involved in embryo degeneration.

For most transcripts, the general expression pattern was relatively similar in both cell lines (Fig. 1, Fig. S3). Transcripts related to *PLANTACYANIN* (*ARPN*), *CYP78A7*, *DWARF IN LIGHT 1* (*DFL1*), and *NAC009* showed the highest accumulation at early developmental stages, while transcripts related to *FAMA* and *VIVIPAROUS 1* (*VP1*) were more abundant from stage 2. A set of transcripts represented by putative homologs to *EXPB1*, *LIPID TRANSFER PROTEIN 4* (*LTP4*), *RIC3*, *TT7*, and *WUSCHEL-RELATED HOMEOBOX 2* (*WOX2*) accumulated transiently at specific stages during embryo development. No significant difference in the accumulation level of transcripts related to *CDC48* was detected during embryo development. However, putative homologs of *AINTEGUMENTA-like* (*AIL5*), *HAP3A* (encoding for a LEC1-type HAP3 subunit of the CCAAT-box binding factor), *NAC47*, *PROTODERMAL FACTOR 2* (*PDF2*), *SERK1*, and *WOX8/9* were differentially expressed in the normal cell line (12:12) compared to the abnormal cell line (3:10).

An *AIL5*-related transcript accumulated at stage 1c in cell line 12:12; thereafter, the transcript level decreased, while no significant decrease in the accumulation of *AIL5*-related transcripts was detected during embryo development in cell line 3:10 (Fig. 1). The highest accumulation of *HAP3A*-related transcripts was detected at stage 1a in cell line 12:12 and at stage 1b in cell line 3:10. However, in both cell lines, the transcript level of *HAP3A* decreased from stage 1c (Fig. 1). In accordance, we have previously shown that the level of *HAP3A*-related transcripts decreased during the transition from the morphogenic phase to the maturation phase in both zygotic and somatic embryos of Scots pine (Uddenberg et al. 2011). The level of a transcript related to a putative homolog to *NAC47* increased from stage 3 in cell line 3:10, but it showed a transient expression at stage 1c in cell line 12:12

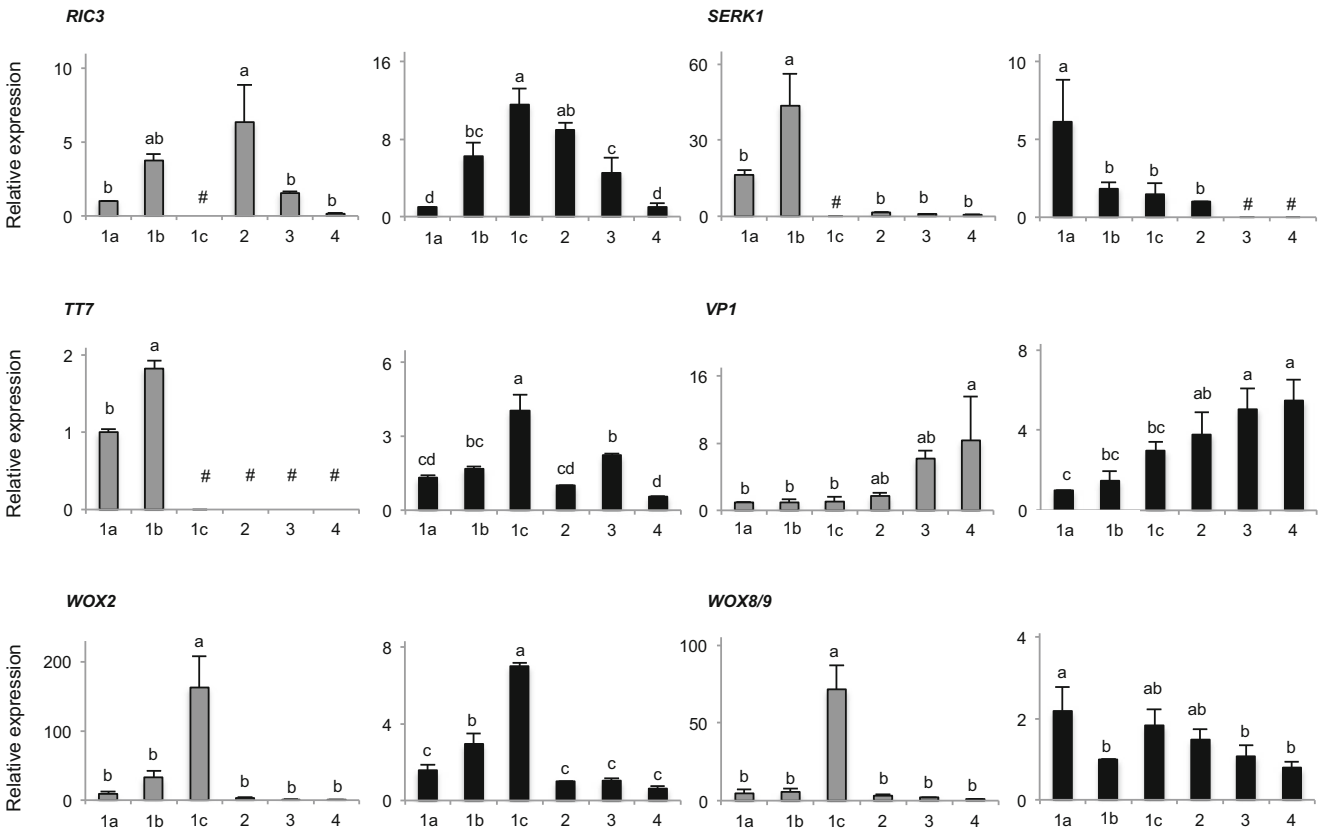


Fig. 1 continued.

(Fig. 1). The abundance of a transcript related to *PDF2* differed between cell lines 12:12 and 3:10 at the earliest developmental stages, but in both cell lines, there was a significant decrease in the transcript level at stages 2 to 3. The accumulation of a transcript was related to *SERK1* increased from stage 1a to stage 1b in the cell line 12:12 but decreased in cell line 3:10 (Fig. 1). In cell line 12:12, but not in cell line 3:10, a transient accumulation of a *WOX8/9*-related transcript was detected at stage 1c (Fig. 1).

Transcript abundance during development of somatic embryos of Norway spruce

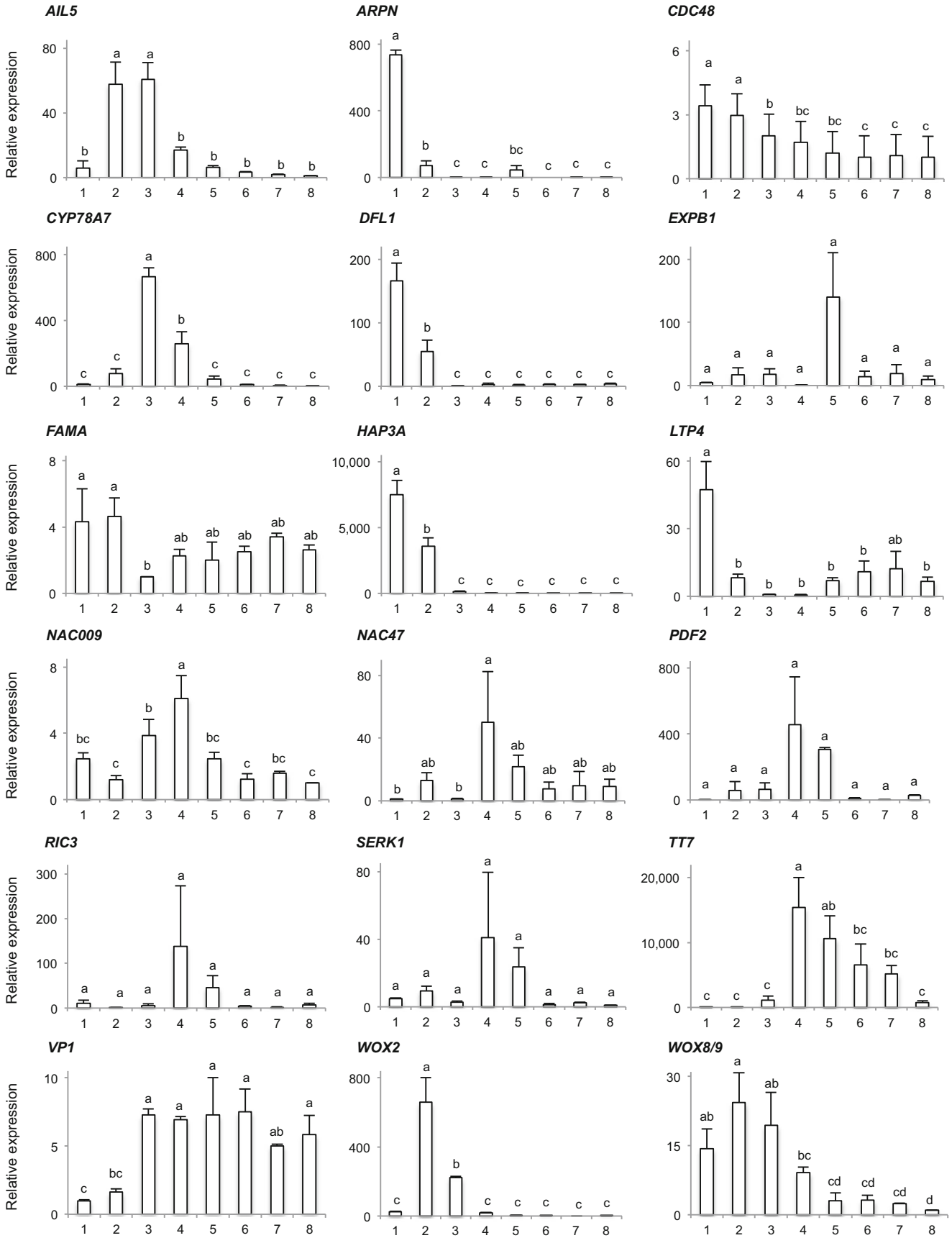
To identify putative differences in the regulation of somatic embryogenesis in species having different forms of polyembryony, we compared the accumulation of the analyzed transcripts in Scots pine with that at eight consecutive stages during somatic embryo development in Norway spruce (Fig. 2, Fig. S4). The expression profiles detected for transcripts related to *HAP3A*, *VP1*, *WOX8/9*, and *WOX2* in Norway spruce confirmed what has been reported before (Uddenberg et al. 2011; Zhu et al. 2014, 2016). For several transcripts like *ARPN*, *DFL1*, *HAP3A*, *VP1*, and *WOX2*, the general expression profile was similar during somatic embryogenesis in Scots pine and Norway spruce (cf. Figs. 1 and 2, Figs. S3 and S4). A transient accumulation was observed at stage 4 for transcripts related to *NAC009* and *NAC47* in Norway spruce, but not in Scots pine, except for a small increase at stage 3 for a *NAC47*-related transcript in cell line 3:10. Differences in the expression level during somatic embryo development in Norway spruce and Scots pine were also detected for transcripts related to *AIL5*, *FAMA*, *LTP4*, and *WOX8/9* (cf. Figs. 1 and 2). Interestingly, transcripts homologous to *CYP78A7*, *EXPB1*, *RIC3*, *SERK1*, and *TT7*, which were all highly abundant at stage E1 in zygotic embryos and were suggested to be involved in the cleavage process (Merino et al. 2016, Table S5), were expressed differently between somatic embryos of Norway spruce and those of Scots pine. Because of a large variation among biological replicates in the abundance of transcripts related to *EXPB1*, *RIC3*, and *SERK1*, at the developmental stage having the highest transcript accumulation, it was not possible to detect significant differences in the accumulation of the transcripts between the different developmental stages in Norway spruce. Despite this limitation, it seems that transcripts related to *EXPB1*, *RIC3*, and *SERK1* were highly accumulated at stage 4 and stage 5 in somatic embryos of Norway spruce, while in Scots pine, the highest accumulation of *EXPB1* and *RIC3* was at stages 1b to 2 and for *SERK1* at stages 1a and 1b. Transcripts related to *CYP78A7* and *TT7* accumulated at stages 3 and 4 in Norway spruce, while they were mainly expressed before stage 2 in Scots pine.

Up- and down-regulation of selected transcripts in transgenic sub-lines

To further understand the role of *CYP78A7*, *EXPB1*, *SERK1*, *RIC3*, and *TT7*, we established transgenic lines in which the genes were either over-expressed or down-regulated. It has so far been difficult to transform embryogenic cultures of Scots pine (Abrahamsson, unpublished), while embryogenic cultures of Norway spruce are routinely transformed (Zhu et al. 2014; Uddenberg et al. 2016). We therefore performed these studies in Norway spruce. Based on the accumulation level of the different transcripts during somatic embryo development in Norway spruce, we chose to down-regulate *CYP78A7* and *TT7*, of which the transcript abundances varied significantly at different developmental stages, and to up-regulate *EXPB1*, *RIC3*, and *SERK1*, of which the transcript abundances varied among biological replicates. Three sub-lines containing *35S:CYP78A7i*, *35S:EXPB1*, *35S:RIC3*, or *35S:SERK1* and two sub lines containing *35S:TT7i* as well as four T-controls were selected for further studies (Fig. S2).

Initially, the selected transgenic sub-lines were screened for their ability to develop cotyledonary embryos, and samples were collected after two and four weeks for analyzing the morphology of the developing embryos. No altered phenotype of cotyledonary embryos was detected in any of the transgenic sub-lines. However, aberrant phenotypes of early and late embryos were detected in all transgenic lines, including the T-control. The aberrant phenotypes were most obvious at developmental stage 3, which corresponds to the developmental stage when the dominant embryo develops in Scots pine (Table S4). The stage 3 embryo morphologies were classified into four different morphology groups: (N) Embryos with normal morphology, characterized by a well-delineated and rounded embryonal mass in the apical part and vacuolated suspensor cells in the basal part (Fig. 3a). (D) Degenerating embryos, characterized by a disintegrated embryonal mass and differentiated vacuolated cells in the embryonal mass (Fig. 3b). (SU) Embryos carrying supernumerary suspensor cells, ranging from embryos with a cone-shaped embryonal mass to embryos where the embryonal mass was almost completely surrounded by suspensor cells (Fig. 3c). (EC) Embryogenic complexes, characterized by embryos that developed lobes (Fig. 3d), differentiated meristematic cells from the apical or basal part of the embryonal mass, or by several fused embryos.

The frequency of stage 3 embryos with aberrant morphologies varied among the transgenic lines. In the T-control lines, 45% of the embryos had a normal morphology, 8% were degenerating, 26% carried supernumerary suspensor cells, and 21% had formed embryogenic complexes (Fig. 3e). In most *35S:SERK1*, *35S:CYP78A7i*, and


AIL5
ARPN
CDC48
CYP78A7
DFL1
EXPB1
FAMA
HAP3A
LTP4
NAC009
NAC47
PDF2
RIC3
SERK1
TT7
VP1
WOX2
WOX8/9
Relative expression

◀ **Fig. 2** Quantitative real-time PCR analysis of the relative transcript abundance of selected genes during somatic embryo development in Norway spruce. The transcript level of 18 selected genes was analyzed in eight consecutive stages during embryo development in cell line 61:21. Relative expression levels, estimated by the Livak method ($2^{-\Delta\Delta Ct}$), are referred to the stage showing the lowest accumulation level for each transcript, and are normalized against two reference genes (*EF1* and *PHOS*). The expression levels are means ± SD of three biological replicates analyzed with two technical replicates each, except for *PDF2* which is based on two biological replicates. Different letters indicate significant differences in the transcript level between different developmental stages (Student's *t* test, $P \leq 0.05$)

35STT7i sub-lines, the frequency of stage 3 embryos which had formed embryogenic complexes was significantly higher than in the T-control lines (Fig. 4a–d, Table S6a). It has to be noted that the morphology of stage 3 embryos that had formed embryogenic complexes varied in the different transgenic lines (discussed subsequently). In *35S:RIC3* sub-lines, there was a significant increase in the frequency of embryos carrying supernumerary suspensor cells, and in two out of three *35S:EXPB1* sub-lines, the frequency of degenerating embryos was significantly higher than that in the T-control lines (Fig. 4e–f, Table S6b).

Characteristic for stage 3 embryos in the *35S:SERK1* sub-lines that had formed embryogenic complexes was that meristematic cells proliferated from the apical part of the embryonal mass (Fig. 5a,b) or that lobes developed from the apical or the basal part of the embryonal mass (Fig. 5c,d). After transfer to maturation medium, the frequency of early, less developed somatic embryos was higher in *35S:SERK1* sub-lines than that in the T-control (Fig. S3a and b). This suggests that SERK1 stimulates differentiation of early embryos. Since no differences in the frequency and morphology of cotyledonary embryos were observed between the control and the *35S:SERK1* sub-lines at later developmental stages, we assume that the stage 3 embryos with normal morphology in *35S:SERK1* sub-lines (Fig. 4) developed normally. Characteristic for the *35S:CYP78A7i* sub-lines, within the group of embryogenic complexes, was the presence of stage 3 embryos with a disturbed apical–basal polarity in which a distinct border between the embryonal mass and the suspensor was missing (Fig. 5e). Furthermore, the frequency of embryos carrying supernumerary suspensor cells was significantly lower than that in the T-control (Fig. 4c). Typically for stage 3 embryos in the *35S:TT7i* sub-lines, within the group of embryogenic complexes, were embryos with a disturbed apical–basal polarity (Fig. 5f) and embryos developing lobes from the basal part of the embryonal mass (Fig. 5g).

The frequency of stage 3 embryos in the *35S:RIC3* sub-lines that carried supernumerary suspensor cells (Fig. 5h) was significantly higher than that in the T-control (Fig. 4e). In two out of three *35S:EXPB1* sub-lines, the frequency of degenerating stage 3 embryos (Fig. 5i) was significantly higher than that in the T-control (Fig. 4f).

Discussion

Differentially expressed transcripts during embryo development in a normal and an abnormal cell line of Scots pine

Embryo development can be divided into two phases, the early morphogenic phase when most cell divisions and differentiation occur and the late maturation phase when the embryo increases in size by cell expansion and storage compounds are synthesized. We have previously shown that the level of *HAP3A*-related transcripts decreases and the level of *VIVIPAROUS1* (*VP1*)-related transcripts increases during the transition from the morphogenic phase to the maturation phase in both zygotic and somatic embryos of Scots pine (Uddenberg et al. 2011). Furthermore, when embryogenic cultures of Norway spruce were treated with the HDAC inhibitor trichostatin A (TSA) during maturation, the process was arrested and the expression level of *PaHAP3A* remained high while the expression of *PaVP1* remained low. The development of cotyledonary embryos was not affected when overexpressing *PaHAP3A* in somatic embryos of Norway spruce, but overexpression stimulated differentiation of ectopic embryos on cotyledonary somatic embryos (Uddenberg et al. 2016). In this study, we show that the expression level of a *HAP3A*-related transcript decreased after stage 1b in both the normal and the abnormal cell lines of Scots pine (Table S7). However, the accumulation of a transcript related to *AIL5*, which in Arabidopsis is important for maintaining the embryonic identity (Tsuwamoto et al. 2010), decreased after stage 1c in the normal cell line 12:12, while no significant decrease in the accumulation of *AIL5*-related transcripts was detected during embryo development in the abnormal cell line 3:10 (Fig. 1, Table S7). Furthermore, the level of an *AIL5*-related transcript decreased in the dominant embryos at stage 3 during zygotic embryo development in Scots pine. Assuming that AIL5 has a similar function in Scots pine as in Arabidopsis, a lower accumulation of *AIL5* during later developmental stages in cell line 12:12 and in the dominant embryo during zygotic embryogenesis might reflect the transition to the maturation phase. In accordance, we have previously shown that the embryogenic potential in cotyledonary embryos from cell line 12:12 is lost while it is maintained in cell line 3:10 (Abrahamsson et al. 2017). Together, these results indicate that the transition from the morphogenic phase to the maturation phase is disturbed in embryos from cell line 3:10.

A high proportion of the developing somatic embryos of Scots pine degenerate (Abrahamsson et al. 2017). In the

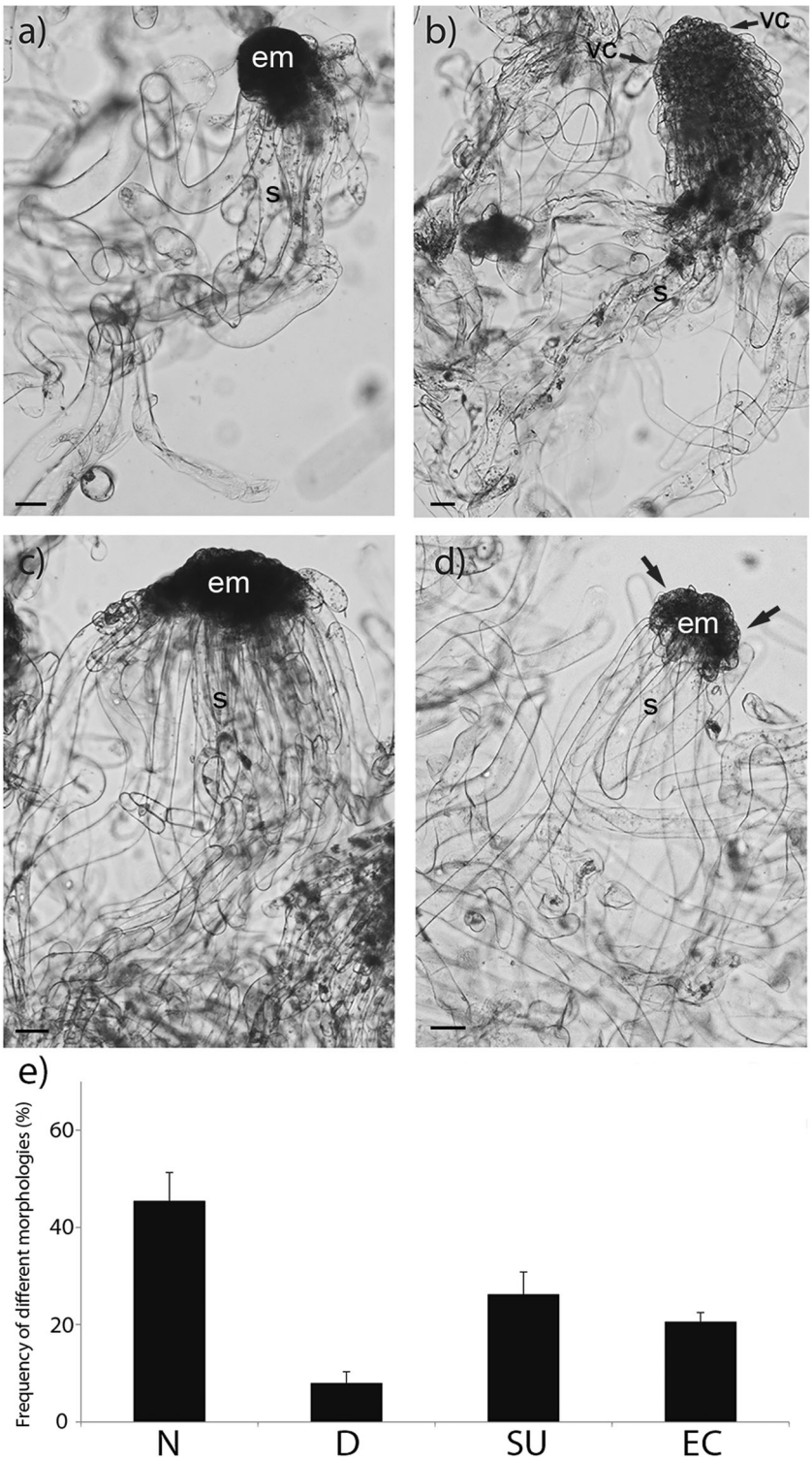


Fig. 3 Different morphologies of stage 3 somatic embryos in transgenic control lines of Norway spruce. **a** Embryo with normal morphology (*N*). Note the well-delineated, rounded embryonal mass in the apical part and vacuolated suspensor cells at the basal part. **b** Degenerating embryo (*D*). Note the disintegration of the embryonal mass and the presence of vacuolated cells in the embryonal mass. **c** Embryo carrying supernumerary suspensor cells (*SU*). Note the increased ratio of width to length of the embryonal mass. **d** Embryogenic complex (*EC*). Note the irregular shape of the embryonal mass, where the embryo depicted has developed lobes denoted by *arrows*. **e** The frequency of different embryo morphologies in line *35S:GUS.1-4* and line *35S:GUS.1-5*. Presented data show the mean frequency (± SE) of embryos with different morphologies. The data are based on three biological replicates for each line, which includes close to 600 stage 3 embryos. *em*, embryonal mass; *s*, suspensor; *vc*, vacuolated cells. *Bars*, 100 μm

normal cell line 12:12, the embryos degrade in a similar way as the subordinate embryos in the seed, while the degeneration of embryos in the abnormal cell line 3:10 results in a continuous loop of embryo degeneration and differentiation of new embryos. In Arabidopsis, *ARNP* is involved in PCD (Dong et al. 2005), and the expression of *NAC47* has been associated with senescence (Kim et al. 2014). A transcript related to *ARNP* accumulated at early developmental stages in both cell lines 12:12 and 3:10 (Fig. 1, Table S7). This is in contrast to zygotic embryogenesis in which an *ARNP*-related transcript was transiently up-regulated at stage E3DO coinciding with the degeneration of the suspensor. The level of a transcript

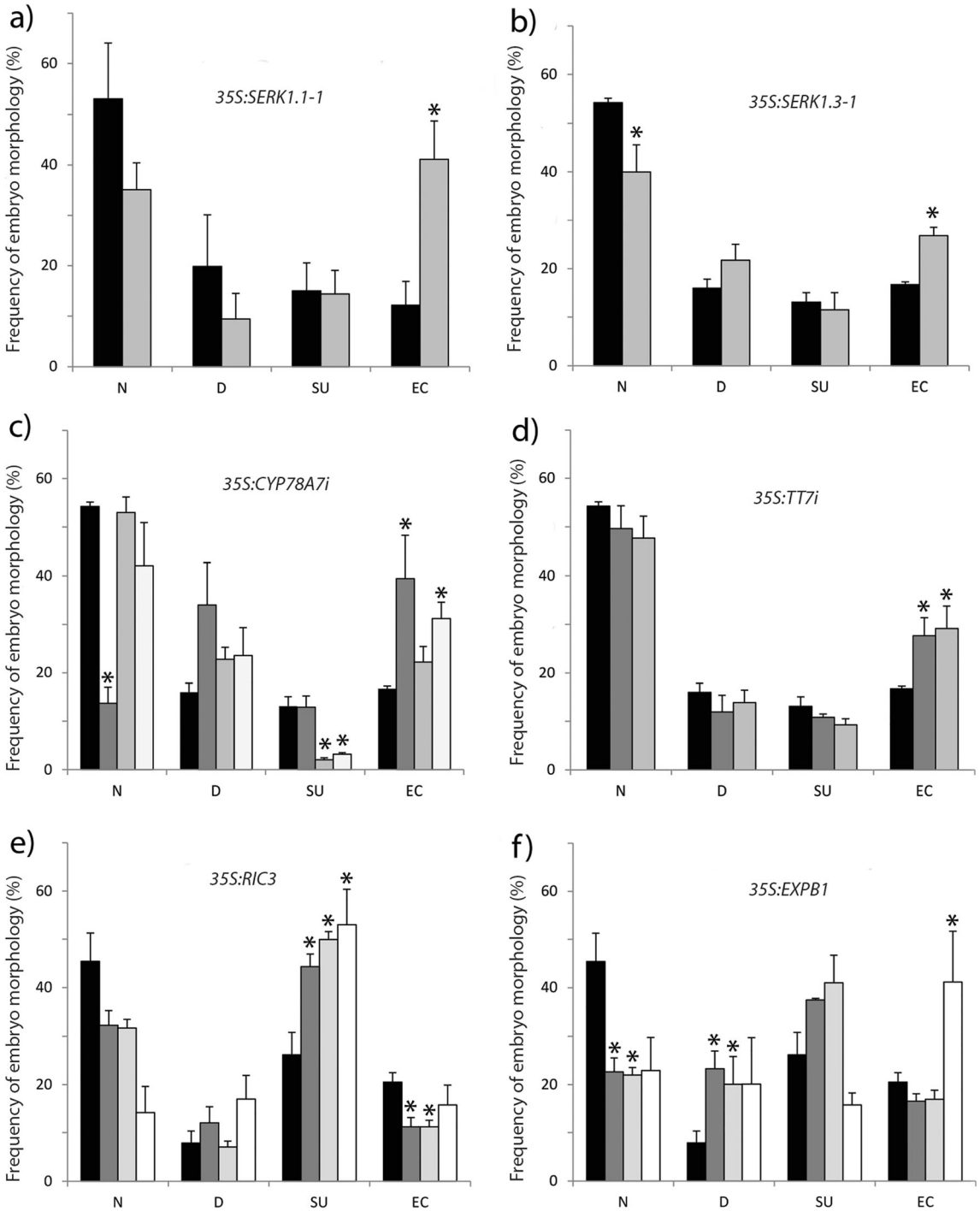


Fig. 4 Frequency of different morphologies of stage 3 somatic embryos in transgenic lines of Norway spruce. Sub-lines *35S:SERK1*, *35S:CYP78A7i*, and *35S:TT7i* were derived from cell line 61:21 (**a**–**d**), and sub-lines *35S:RIC3* and *35S:EXPB1* were derived from cell line 11:18 (**e**, **f**). The stage 3 embryos were classified as embryos with normal morphology (*N*), degenerating embryos (*D*), embryos carrying supernumerary suspensor cells (*SU*), and embryogenic complexes (*EC*), as presented in Fig. 3. (**a**–**f**) The frequency of embryos with different morphologies in T-control lines (*black bars*). **a** Embryos from sub-line *35S:SERK1.1-1* (*light gray bars*). **b** Embryos from sub-line *35S:SERK1.3-1* (*light gray bars*). The experiments with sub-line *35S:SERK1.1-1* shown in (**a**) and with sub-line *35S:SERK1.3-1* shown in (**b**) were performed at different time-points. **c** Embryos from sub-lines 35S*:CYP78A7i.1-3* (*dark gray bars*), 35S*:CYP78A7i.2-1* (*light gray bars*), and *35S:CYP78A7i.2-2* (*white bars*). **d** Embryos from sub-lines *35S:TT7i.1-1* (*dark gray bars*) and 35S*:PaTT7i.1-2* (*light gray bars*). **e** Embryos from sub-lines *35S:RIC3.1* (*dark gray bars*), *35S:RIC3.5* (*light gray bars*), and *35S:RIC3.10* (*white bars*). **f** Embryos from sub-lines *35S:EXPB1.5* (*dark gray bars*), *35S:EXPB1.8* (*light gray bars*), and *35S:PsEXPB1.14* (*white bars*). Data presented show the mean (± SE) frequency of stage 3 embryos with different morphologies in three to four biological replicates (Tables S6a and S6b). Asterisks (*) indicate significant differences, in embryo morphology, between the T-control and the transgenic sub-line ($P \leq 0.05$)

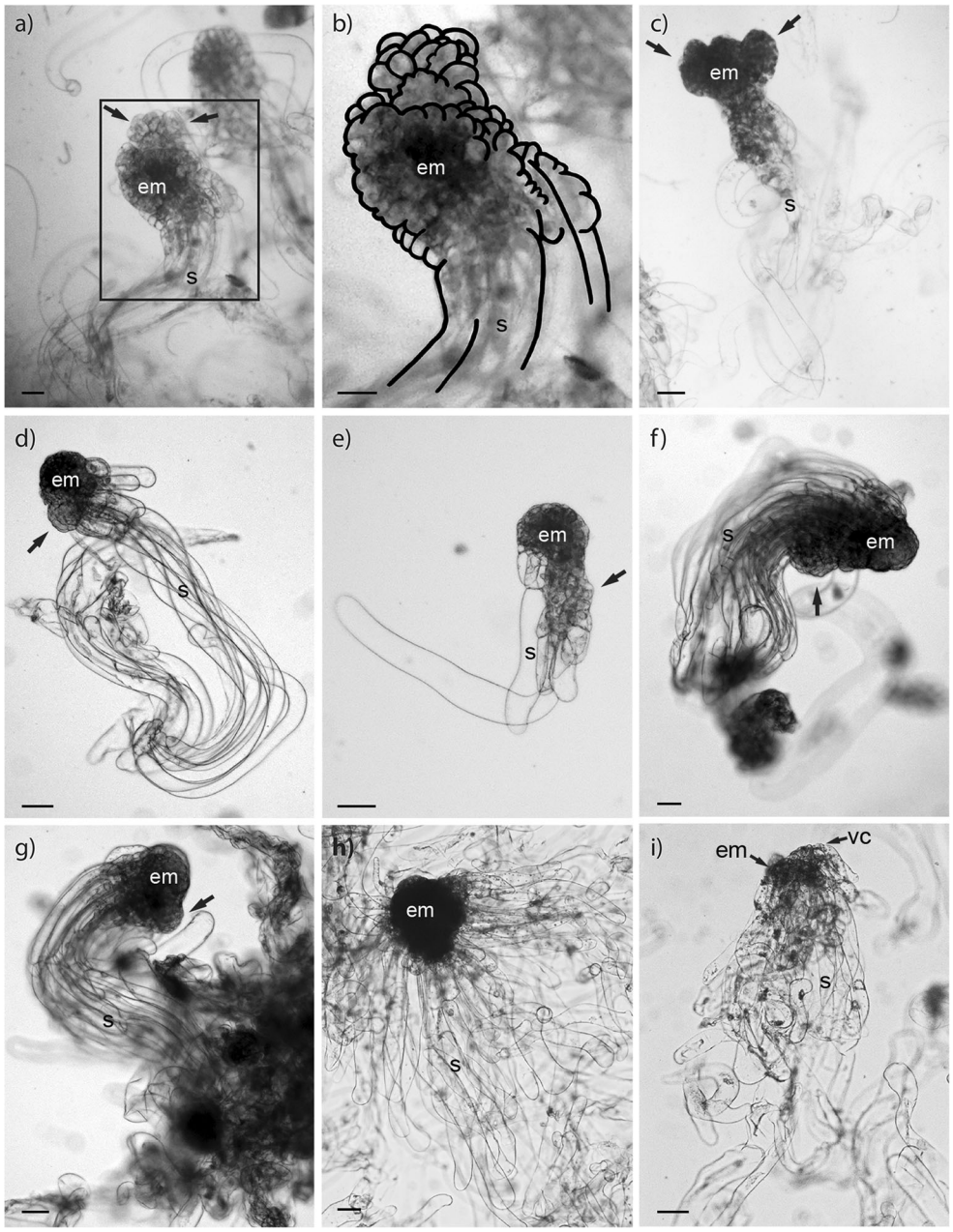


Fig. 5 Different morphologies of stage 3 somatic embryos in transgenic lines of Norway spruce. Sub-lines *35S:SERK1*, *35S:CYP78A7i*, and *35S:TT7i* were derived from cell line 61:21 (**a**–**g**), and sub-lines *35S:RIC3* and *35S:EXPB1* were derived from cell line 11:18 (**h**, **i**). **a**–**d** Embryos from *35S:SERK1* sub-line. **a** Embryogenic complex. Note the cluster of cells differentiating from the apical part of the embryonal mass denoted by *arrows*. **b** A higher magnification of the embryo shown in (**a**), where the outline of the cells has been manually marked. **c** Embryogenic complex. Note the developing lobes from the embryonal mass, denoted by *arrows*. **d** Embryogenic complex. Note the developing lobe from the basal part of the embryonal mass, denoted by an *arrow*. **e** Embryogenic complex from a *35S:CYP78A7i* sub-line. Note that the embryo has a disturbed apical–basal polarity, lacks a strict border between the embryonal mass and the suspensor, and meristematic cells are present in the suspensor region, denoted by an *arrow*. **f**–**g** Embryos from *35S:TT7i* sub-line. **f** Embryogenic complex. Note that the embryo has a disturbed apical–basal polarity, lacks a strict border between the embryonal mass and the suspensor, and meristematic nodules have developed in the suspensor region, denoted by an *arrow*. **g** Embryogenic complex. Note the developing lobe from the basal part of the embryonal mass, denoted by an *arrow*. **h** Embryo carrying supernumerary suspensor cells from a *35S:RIC3* sub-line. Note the increased ratio between the width and length of the embryonal mass. **i** Degenerating embryo from a *35S:EXPB1* sub-line. Note the disintegration of the embryonal mass. *em*, embryonal mass; *s*, suspensor; *vc*, vacuolated cells. *Bars*, 100 μm

related to a putative homolog to *NAC47* increased from stage 3 in the abnormal cell line 3:10, but it showed a transient expression at stage 1c in the normal cell line 12:12 (Fig. 1, Table S7). It is tempting to assume that the accumulation of transcripts related to *ARNP* during early somatic embryo development directly or indirectly is associated with the

degeneration process of somatic embryos. Furthermore, the different accumulation patterns of the *NAC47*-related transcript might reflect the different degeneration patterns of embryos in the two cell lines.

The orientation of the cell division plane determines the position and the fate of the daughter cells (van den Berg et al. 1995). During early somatic embryo development in Norway spruce, *PaWOX8/9* regulates the establishment of the apical–basal embryo pattern by controlling the cell division orientation and cell fate determination of the basal cells in the embryonal mass (Zhu et al. 2014). In the normal cell line 12:12, but not in the abnormal cell line 3:10, a transient accumulation of a transcript related to *WOX8/9* was detected at stage 1c (Fig. 1, Table S7). Two other transcripts, *FAMA* and *NAC009*, which are both related to regulators of cell division patterns in Arabidopsis (Ohashi-Ito and Bergmann 2006; Willemsen et al. 2008), were expressed similarly in the two cell lines (Fig. 1, Table S7). The abundance of a *FAMA*-related transcript increased from stage 2, while a transcript related to *NAC009* was highly abundant at the earliest developmental stages and then decreased. In contrast, both *FAMA*- and *NAC009*-related transcripts increased significantly in stage 4 embryos during zygotic embryo development (Fig. 1, Table S7). The differences in expression pattern of transcripts related to *WOX8/9* and *NAC009* indicate that the establishment of apical–basal patterning during early embryogenesis is regulated differently in the two cell lines and also between somatic and zygotic embryos.

Early during embryogenesis, the differentiation of a protoderm is important for further development of a viable embryo (Dodeman et al. 1997). We have previously shown that the expression of *P. abies HOMEOBOX 1* (*PaHB1*, a homolog of *PDF2* in Arabidopsis) must be restricted to the outer cell layer in early somatic embryos of Norway spruce to allow differentiation of the protoderm (Ingouff et al. 2001). Although the abundance of transcripts related to *PDF2* differed between cell lines 12:12 and 3:10 at the earliest developmental stages, there was a significant decrease in the transcript level at stages 2 to 3 in both cell lines (Fig. 1). Since the early embryos in both cell lines had differentiated a protoderm (Abrahamsson et al. 2017), it is reasonable to assume that the expression of *PDF2* is localized to the outer cell layer of early embryos in both the normal and the abnormal cell lines. Furthermore, a transcript homologous to *WOX2*, which is crucial for protoderm specification in early embryos of Norway spruce (Zhu et al. 2016), was transiently up-regulated at stage 1c in both cell lines (Fig. 1).

Functional analyses of candidate genes

The different expression patterns of transcripts related to *CYP78A7*, *EXPB1*, *RIC3*, *SERK1*, and *TT7*, genes suggested to be involved in the cleavage process (Merino et al. 2016), between somatic embryogenesis in Norway spruce and in Scots pine, indicate that embryogenic tissues of the two species might proliferate in different ways. In order to test this assumption, we over-expressed or down-regulated the corresponding genes and analyzed how the changed expression level affected the development of somatic embryos in Norway spruce. We have mainly analyzed the phenotypes of stage 3 embryos, since the changed phenotype in the transgenic lines was most obvious at that stage.

In Arabidopsis, *SERK1* marks cells that are competent to form embryos, and it also influences the competence of the cells to differentiate into embryos (Hecht et al. 2001). Typical for stage 3 embryos overexpressing *SERK1* was that lobes developed from the apical or the basal part of the embryonal mass (Fig. 5c,d). A similar lobing process occurs in early somatic embryos of Scots pine (Abrahamsson et al. 2017) in which a *SERK1*-related transcript accumulated at the earliest developmental stages (Fig. 1, Table S8). In several coniferous species, the embryonal mass in early zygotic embryos can differentiate lobes, which results in cleavage polyembryony if each lobe develops independent embryonal tube cells (Dogra 1967). During zygotic embryogenesis in Scots pine, a *SERK1*-related transcript was abundant at stage 1 and thereafter the transcript level decreased. It was suggested that *SERK1* stimulates the initiation of the cleavage process by stimulating the four apical cells in the proembryo to differentiate into separate embryos and that down-regulation of *SERK1* is important for allowing the four embryos to develop further. In addition to stimulating lobing of early somatic embryos, overexpression of *SERK1* also stimulated differentiation of new embryos (Fig. S5), possibly by a process resembling cleavage polyembryony. Thus, our results indicate that overexpression of *SERK1* changes the proliferation pattern of embryogenic tissue in Norway spruce to become more similar to the proliferation pattern in Scots pine.

Early embryogenesis is a critical developmental phase when the apical–basal polarity is established through directional auxin transport giving rise to local auxin maxima and gradients. The transport is flexible and needs to change direction at an early embryonal stage in order for the development to proceed (Friml et al. 2003). In addition to the intracellular transport, mainly mediated by auxin influx and efflux carriers (Petrásek and Friml 2009), flavonols (plant-specific polyphenolic compounds) have been proposed to act as endogenous auxin transport regulators (Lewis et al. 2011). In Arabidopsis, *TT7* encodes a flavonoid 3′hydroxylase, a flavonol biosynthetic enzyme that contributes to the synthesis of quercetin, an endogenous negative regulator of auxin transport (Jacobs and Rubery 1988). Typically for stage 3 embryos in the *35S:TT7i* sub-lines were embryos with a disturbed apical–basal polarity (Fig. 5f) and embryos developing lobes from the basal part of the embryonal mass (Fig. 5g). These phenotypes are frequently observed in somatic embryos of

Scots pine (Abrahamsson et al. 2017) and might correlate to the flavonol-mediated-disturbed PAT caused by the expression of a transcript related to *TT7* at early stages of somatic embryogenesis in Scots pine (Fig. 1, Table S8). Since a *TT7*-related transcript also accumulates in zygotic embryos at stage 1, it is tempting to assume that PAT is involved in the cleavage process, but more research is required for understanding how auxin regulates the cleavage process.

The basal cells in the embryonal mass of early somatic embryos of Norway spruce divide asymmetrically and give rise to both apical meristematic daughter cells in the embryonal mass and basal vacuolated suspensor cells (Zhu et al. 2016). Down-regulation of *CYP78A7* resulted in embryos, which lacked a distinct border between the embryonal mass and the suspensor, carried fewer suspensor cells, and meristematic cells were present in the suspensor region (Fig. 5e). This suggests that *CYP78A7* directly or indirectly is important for controlling the division of the cells in the basal part of the embryonal mass and for the specification of the daughter cells. *CYP78A7*-related transcripts accumulated specifically at stages 1 and 2 during zygotic embryogenesis in Scots pine, when four new polarized embryos are formed after a cleavage process, and at stages 1a and 1b in the normal cell line 12:12, when the globular structures successively will differentiate into early embryos (Fig. 1, Table S8). Interestingly, *CYP78A7* is, redundantly with its close homolog *CYP78A5*, regulating shoot apical meristem size in Arabidopsis (Wang et al. 2008). Thus, together, our results suggest that *CYP78A7* is important for cell fate determination during differentiation of early embryos.

Stage 3 embryos overexpressing *RIC3* carried supernumerary suspensor cells (Fig. 5h). In Arabidopsis, *RIC3* promotes actin disassembly, which is required for exocytosis (Lee et al. 2008). De-polymerization of actin, by treating embryogenic cultures of Norway spruce with actin de-polymerization drugs, disrupted correct embryo pattern formation through the inhibition of suspensor differentiation (Smertenko et al. 2003). Therefore, overexpression of *RIC3* affects embryogenesis in a different way than treatment with actin de-polymerization drugs. During somatic and zygotic embryo development in Scots pine, a *RIC3*-related transcript accumulated during stages 1 and 2, concomitantly with the differentiation of suspensor cells, and thereafter decreased (Fig. 1, Table S8). Together, our results indicate that *RIC3* directly or indirectly is involved in the differentiation of suspensor cells.

Expansins are involved in loosening cell walls at cell expansion as well as during several morphogenic and destructive processes (Sharova 2007). During zygotic embryogenesis, in Scots pine, an *EXPB1*-related transcript accumulated at stage 1 and thereafter decreased (Merino et al. 2016). It was suggested that *EXPB1* is involved in loosening the cell walls to allow separation of the four early embryos during the cleavage process. During somatic embryogenesis, in Scots pine, the transcript level of an *EXPB1*-related transcript decreased at stages 2 to 3 (Fig. 1, Table S8). In Scots pine, a high frequency of the early somatic embryos degenerate (Abrahamsson et al. 2017). Although the degeneration pattern differs between cell lines 3:10 and 12:12, in both cell lines, vacuolated cells differentiate in the embryonal mass, in a similar way as in somatic embryos of Norway spruce overexpressing *EXPB1* (Fig. 5i), which indicates that *EXPB1* is involved in the degeneration of early somatic embryos.

Conclusions

Together, our results point out some important differences in transcript expression profiles during development of normal and abnormal somatic embryos. The differentially expressed transcripts were related to the following: (a) the transition from the morphogenic phase to the maturation phase; (b) embryo degeneration; and (c) apical–basal polarization. The comparison between somatic and zygotic embryos suggested differences in the cell division pattern and highlighted our previous findings of continuous degeneration processes taking place in early somatic embryos of Scots pine.

The general expression profile for several transcripts was similar during somatic embryo development in Scots pine and Norway spruce. However, transcripts homologous to *CYP78A7*, *EXPB1*, *RIC3*, *SERK1*, and *TT7*, which have been suggested to be involved in the cleavage process during zygotic embryogenesis, were expressed differently during somatic embryo development in Scots pine and Norway spruce. Down-regulation of *CYP78A7* or *TT7* resulted in early embryos with disturbed apical–basal polarization. Up-regulation of *EXPB1* caused degeneration of the embryonal mass, while up-regulation of *RIC3* stimulated differentiation of supernumerary suspensor cells. Up-regulation of *SERK1* stimulated lobing of the embryonal mass. This feature can be a first step in the cleavage process and is common in early and late somatic embryos of Scots pine, but not in Norway spruce. Therefore, by increasing the expression of *SERK1*, it was possible to stimulate initiation of a cleavage-like process in somatic embryos of Norway spruce, a species normally having simple polyembryony.

Taken together, we here contribute to a better understanding of the molecular regulation of somatic embryo development in normal and abnormal cell lines of Scots pine, which can help to identify processes that are disturbed in abnormal cell lines. Based on this knowledge, processes that are disturbed in abnormal cell lines can be identified and new approaches can be taken to improve the culture conditions in order to propagate *Pinus* species via somatic embryos.

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Data Archiving Statement Transcripts and data from Scots pine zygotic transcriptome mentioned in this study have been already published before, and they are available in the ArrayExpress database of the European Bioinformatics Institute (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-5193 (Merino et al. 2016). The list of accession number of Norway spruce genes and of Scots pine transcripts selected for this study has been included in Table S1 and Table S5, respectively.

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