ORIGINAL ARTICLE

Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor

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Abstract Somatic embryogenesis is used for vegetative propagation of conifers. Embryogenic cultures can be established from zygotic embryos; however, the embryogenic potential decreases during germination. In *Arabidopsis*, *LEAFY COTYLEDON* (*LEC*) genes are expressed during the embryonic stage, and must be repressed to allow germination. Treatment with the histone deacetylase inhibitor trichostatin A (TSA) causes de-repression of *LEC* genes. *ABSCISIC ACID3* (*ABI3*) and its *Zea mays* ortholog *VIVIPAROUS1* (*VP1*) act together with the *LEC* genes to promote embryo maturation. In this study, we have asked the question whether TSA treatment in a conifer affects the embryogenic potential and the expression of embryogenesis-related genes. We isolated two conifer LEC1-type *HAP3* genes, *HAP3A* and *HAP3B*, from *Picea abies* and *Pinus sylvestris*. A comparative phylogenetic analysis of plant *HAP3* genes suggests that *HAP3A* and *HAP3B* are paralogous genes originating from a duplication event in the conifer lineage. The expression of *HAP3A* is high, in both somatic and zygotic embryos, during early embryo development, but decreases during late embryogeny. In contrast, the expression of *VP1* is initially low but increases during late embryogeny. After exposure to TSA, germinating somatic embryos of *P. abies* maintain the competence to differentiate embryogenic tissue, and simultaneously the germination progression is partially inhibited. Furthermore, when embryogenic cultures of *P. abies* are exposed to TSA during embryo maturation, the maturation process is arrested and the expression levels of *PaHAP3A* and *PaVP1* are maintained, suggesting a possible link between chromatin structure and expression of embryogenesis-related genes in conifers.

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Abbreviations

ABI3	*ABSCISIC ACID INSENSITIVE3*
HDAC	Histone deacetylase
LEC1	*LEAFY COTYLEDON1*
TSA	Trichostatin A
VP1	*VIVIPAROUS1*

Introduction

Somatic embryogenesis is the process of differentiation of embryos from somatic cells. This requires a signal that induces a somatic cell to dedifferentiate and gain

embryogenic competence as well as the expression of an appropriate cellular environment for the response of the inductive signal (Braybrook and Harada 2008). The molecular mechanisms involved in this transition, from a differentiated vegetative cell to a cell with embryogenic competence, have been best described in model angiosperm species, but are largely uncharacterized in gymnosperms. This process is, however, the foundation for propagation of conifers through somatic embryos.

Seed development can be divided into distinct phases, an early morphogenesis phase and a late maturation phase followed by desiccation (West and Harada 1993; Goldberg et al. 1994). During morphogenesis, most cell divisions and differentiation occur and the basic body plan of the embryo is established. During the maturation phase, embryo morphogenesis is arrested and the embryo increases in size by cell elongation. Furthermore, storage compounds are synthesized and at the end of the maturation phase the embryo becomes desiccation tolerant. Seed germination marks the end of the embryonic development, and rapid repression of embryonic genes has been observed with seed imbibition (Tai et al. 2005). Several transcriptional regulators that play critical roles in promoting expression of seed transcriptional programs have been identified in *Arabidopsis thaliana* (*Arabidopsis*) (Zhang and Ogas 2009; Le et al. 2010). The *LEAFY COTYLEDON* (*LEC*) genes *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*) and *FUSCA3* (*FUS3*) are transcription factors that act as master regulators of embryogenesis and they have been used as tools to define the mechanisms that underlie the initiation of somatic embryogenesis (Braybrook and Harada 2008). All three *LEC* genes encode transcriptional activators that are primarily expressed in the seed (Santos-Mendoza et al. 2008). *LEC1* encodes a HAP3 subunit of the CCAAT-box binding factor (CBF) (Lotan et al. 1998; Lee et al. 2003). *LEC2* and *FUS3* encode transcription factors of the plant-specific B3 family (Luerssen et al. 1998; Stone et al. 2001). During early embryogenesis, the *LEC* genes are required to maintain the embryonic fate and to specify cotyledon identity (reviewed by Santos-Mendoza et al. 2008). The activity of the *LEC* genes must be repressed post-embryonically to allow vegetative development to proceed (Braybrook and Harada 2008). Ectopic expression of all three *LEC* genes causes cells in vegetative and reproductive tissues to adopt characteristics of maturation phase embryos (reviewed by Braybrook and Harada 2008). *ABSCISIC ACID3* (*ABI3*) is another master regulator that together with the *LEC* genes promotes maturation (Giraudat et al. 1992; Parcy et al. 1997; To et al. 2006). *ABI3* is orthologous to *VP1* from maize (*Zea mays*) (McCarty et al. 1991) and belongs to the same subfamily as *FUS3* and *LEC2,* commonly jointly referred to with the acronym *AFL* genes (Suzuki and McCarty 2008 with

references). The role of B3 domain transcription factors in the regulation of embryo maturation and ABA-regulated gene expression in seeds has been studied extensively (Gutierrez et al. 2007). Regulators of *LEC1* and *AFL* genes include the *VP1/ABI3-LIKE* (*VAL*) family of B3 domain transcription factors, which forms a sister clade to the AFL family (Suzuki et al. 2007). Furthermore, *LEC1* seems to act earlier and as a regulator of *AFL* genes, since ectopic expression of *LEC1* also activates the expression of the *AFL* genes (Kagaya et al. 2005).

The intricate control of regulatory genes during development of the seed has in several studies been shown to involve altered histone modifications and epigenetic regulation (reviewed by Zhang and Ogas 2009). Inhibition of histone deacetylases (HDACs), by mutant analysis or treatment with a chemical inhibitor, affects development of the embryo as well as the expression of seed associated genes including transcription factors (Tai et al. 2005; Tanaka et al. 2008). In addition, it has been postulated that the CHD3-chromatin-remodeling factor *PICKLE* (*PKL*) is a regulator of the *LEC* genes in *Arabidopsis* acting to repress embryonic identity during germination (Dean Rider et al. 2003).

Mutant analysis has been used, most extensively in *Arabidopsis*, to elucidate the genetic regulation of embryo development in angiosperms (Laux et al. 2004). In gymnosperms, however, knowledge about the regulation of embryo development is limited. Molecular and fossil data suggest that extant seed plants (gymnosperms and angiosperms) share a common ancestor approximately 300 million years ago (Smith et al. 2010). Despite this, the complement of genes expressed during embryogenesis in both groups shares striking sequence similarity (Cairney and Pullman 2007). Furthermore, it has been shown that certain regulatory pathways controlling seed- and spore-specific gene expression are conserved across phylogenetically distant species, ranging from ferns through cycads, and gymnosperms to angiosperms (Schallau et al. 2008). This suggests that genes central to embryogenesis may exhibit a high degree of conservation between angiosperms and gymnosperms, despite the fact that patterning during embryo development is very different between gymnosperms and angiosperms (von Arnold et al. 2002).

In this study, we provide insights into the molecular regulation of the transition from the embryonic to the vegetative stage during embryo development in conifers. Both Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) are included, since the developmental pattern of the zygotic embryos differs between the two species. We have isolated two LEC1-type conifer *HAP3A* genes, *Pa-HAP3A* and *PsHAP3A*, as well as the Scots pine *ABI3* homolog, *PsVP1* (*PaVP1* has been reported earlier by Footitt et al. 2003). A comparative phylogenetic analysis of

plant *HAP3* genes suggests that *HAP3A* and *HAP3B* are paralogous genes originating from a duplication event in the conifer lineage. The expression levels of both *PsHAP3A* and *PsVP1* are similar during development of zygotic and somatic embryos. In addition, changes in the expression levels of the *HAP3A* and *VP1* genes during somatic embryogenesis show similar trends in the two conifer species and resemble that of homologous genes in angiosperms. Treatment of germinating embryos of Norway spruce with an HDAC inhibitor partially inhibits the progression of germination and maintains the embryogenic potential. Furthermore, we show that HDAC inhibitor treatment during embryo maturation arrests maturation and affects the expression of *PaHAP3A* and *PaVP1*, suggesting a possible link between chromatin structure and expression of embryogenesis-related genes in conifers.

Materials and methods

Plant material and growth conditions

Embryogenic cell lines 06.21.00 and 06.28.05 of Norway spruce (*Picea abies* L. Karst.) and 12:12 of Scots pine (*Pinus sylvestris* L.) were used in this study. The embryogenic cultures were treated as described previously for Norway spruce (von Arnold and Clapham 2008) and Scots pine (Burg et al. 2007), except that the cultures of Norway spruce were proliferated on solidified medium. Briefly, the cultures proliferated as proembryogenic masses (PEMs) on proliferation medium, i.e., medium supplemented with the plant growth regulators (PGRs), auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (N^6-benzyladenine). To stimulate differentiation of early somatic embryos from PEMs, the cultures were transferred to pre-maturation medium, i.e., medium lacking PGRs for 1 week (Norway spruce) or 2 weeks (Scots pine). Maturation of embryos was stimulated as cultures were transferred to maturation medium containing abscisic acid (ABA). Cotyledonary somatic embryos were formed after 4–8 weeks of maturation treatment. Before germination, the embryos were partially desiccated (von Arnold and Clapham 2008).

New embryogenic cultures of Norway spruce were initiated from mature cotyledonary somatic embryos and from germinating somatic embryos incubated on proliferation medium containing PGRs for 4–5 weeks.

Zygotic embryos of Scots pine representing eight developmental stages were isolated from seeds collected every second to the third day during late June to late July 2009, from trees growing around Uppsala, Sweden. Somatic embryos of Scots pine at corresponding developmental stages as the zygotic embryos were isolated from embryogenic cultures during proliferation, pre-maturation

and maturation. PEMs, early-, late- and cotyledonary embryos were collected from embryogenic cultures of Norway spruce after 2 weeks on proliferation medium, 1 week on pre-maturation medium, 1 week on maturation medium and 5 weeks on maturation medium. After sampling, the embryos were stored at −70°C until future use.

TSA treatments

The inhibitor of histone deacetylases, trichostatin A (TSA) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to obtain a stock solution of 10 mM. Initially mature zygotic embryos of Norway spruce were screened for their potential of initiating embryogenic cultures on media containing 0.01, 0.1, 1 and 10 μM TSA. The highest initiation frequency was obtained on media containing 1 and 10 μM TSA (data not shown). In this study, we have used 10 μM TSA. A medium lacking TSA was used as control in each experiment. Since no effect of DMSO alone was observed, we did not include data from DMSO controls.

To assess the effects of TSA on embryo maturation and gene expression, the cultures were exposed to TSA during the whole maturation phase. Tissues for gene expression analyses were sampled weekly. The presented data are based on three biological replicates.

To elucidate if the embryogenic potential in germinated embryos was influenced by inhibition of histone deacetylases, TSA was added to the germination medium for 10 days. Thereafter, the germinated embryos were transferred to proliferation medium containing PGRs but lacking TSA. The embryogenic potential of germinated embryos was determined 4–5 weeks after transfer to the proliferation medium. The data presented are based on three biological replicates with each replicate including at least 100 embryos per treatment. To examine the possibility of recovering the embryogenic potential after germination, embryos were first germinated for 10 days under standard germination conditions and then transferred to germination medium supplemented with TSA for an additional 5 days, before incubation on proliferation medium.

RNA isolation

Total RNA was isolated from zygotic embryos of Scots pine using the RNAqueous Micro Kit (Ambion-Applied Biosystems, Austin, TX, USA) and somatic embryos of Scots pine and Norway spruce with the Spectrum Plant Total RNA kit (Sigma-Aldrich). Total RNA was subjected to DNase digestion, according to the manufacturer's instruction (DNA-free, Ambion). cDNA was prepared from 0.5 to 1 μg total RNA using the Quanta cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA).

Isolation and sequencing of *PaHAP3A, PsHAP3A* and *PsVP1*

Putative *LEC1* homologs from the *HAP3* gene family in Norway spruce and Scots pine as well as the *VP1/ABI3* gene from the B3 gene superfamily in Scots pine were isolated from cDNA derived from embryogenic cultures. *PaVP1* has previously been isolated in Norway spruce (Footitt et al. 2003). Primers for isolating conifer homologs of *LEC1* and *ABI3* were designed from loblolly pine (*Pinus taeda*) and white spruce (*Picea glauca*) expressed sequence tags (ESTs) and the *PaVP1* gene (Supplementary Table 1). PCR amplifications were performed in 50 μl reactions using the Phusion enzyme (Finnzymes Oy, Espoo, Finland) according to the manufacturer's protocol. The products were separated on a 1% TBE-gel and bands were excised and purified using the Gel purification kit (Fermentas-Thermo Fischer Scientific, Burlington, Canada). Purified products were sent to GATC Biotech, Konstanz, Germany, for sequence verification.

Sequence alignment and analysis

Additional *HAP3* and *VP1/ABI3* sequences were selected based on previously published data and BLAST searches (Altschul et al. 1997; for references and accession numbers, see Supplementary Table 2). In cases where the database contained identical or nearly identical sequences from the same taxon, only one representative was included.

Full-length amino acid alignments of 28 *HAP3* genes were initially compiled using the MUSCLE and ClustalX algorithms in the program Geneious 5.0 (Drummond et al. 2010). Both alignments were then compared for discrepancies using the AltaVist alternative alignment visualization tool v. 1.0 (Morgenstern et al. 2003) before manual refinement and back-translation using Geneious. The nucleotide alignment was used for phylogenetic analyses, while the equivalent amino acid alignment was used only to identify shared sequence characters and sequence motifs. Bayesian phylogenetic analyses of the *HAP3* genes were based on the conserved B-domain of the sequences, using the program MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Owing to the long evolutionary distances of the *HAP3* genes, a test for substitution saturation was performed using the software Dambe v4.5.56 (Xia et al. 2003). The variable third position was found to be saturated and was omitted in subsequent analyses. The model of evolution selected was GTR + I + G, which assumes a general time reversibility (GTR), a certain proportion of invariable sites (I) and a gamma approximation of the rate variation among sites (G). We ran four heated chains of the Markov chain Monte Carlo in parallel, sampling one tree every 500 generations for 1,500,000 generations starting with a random tree. The search reached stationarity after ~110,000 generations. The first 110,000 generations were considered the "burn-in" period and were discarded from generating the consensus phylogeny. Parsimony analysis was performed using PAUP* 4.0 (Swofford, 2001). Trees were generated using heuristic search replicates with 1,000 random stepwise taxon additions with a Tree Bisection–Reconnection branch-swapping algorithm and MulTrees ON. Gaps were treated as missing data and third position nucleotides were assigned zero weight. Bootstrap support for nodes (Felsenstein 1985) was estimated with 1,000 heuristic search replicates, using the same settings as the original search, with 1,000 random stepwise additions for each bootstrap replicate.

The analyses of *PaVP1* and *PsVP1* were made using all B3 family genes from *Arabidopsis* and the *VP1* gene from maize. The conserved B3 region was used for alignment and phylogenetic analyses. *Arabidopsis* B3 protein sequences were collected using domain search (http://www.sanger.ac.uk/). The amino acids were then back-translated to nucleotide sequences using the Protogene server (Moretti et al. 2006). Duplicate sequences were omitted before sequence alignment. Alignments and Bayesian phylogenetic analyses were performed as described for the *HAP3* genes. Starting with a random tree, one tree was sampled every 500 generations for 11,500,000 generations. The search reached stationarity after about 1,150,050 generations, and the "burn-in" period was set to the first 1,150,050 generations. The consensus tree showed a clear separation of known subgroups in the phylogeny of B3 genes, i.e., the AFL (*ABI3/FUS3/LEC2*) genes and the VAL (*VP1/ABI3-LIKE*) embryonic repressor genes (*VAL1, VAL2* and *VAL3*), as suggested in Swaminathan et al. (2008); Romanel et al. (2009). From the consensus tree, a subsample of 35 sequences were selected for the final analysis, which was run for 2,000,000 generations. The search reached stationarity after 200,000 generations and the "burn-in" period was set to 200,000 generations.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the iQ5 Real-Time Detection System in iCycler iQ 96-well PCR plates with adhesive seals (Bio-Rad Laboratories, Hercules, CA, USA). Primers used to quantify expression levels are presented in Supplementary Table 3. The expression data of each gene was normalized against the expression of three reference genes, *PHOSPHOGLU-COMUTASE, CELL DIVISION CYCLE 2* (*CDC2*) and *ELONGATION FACTOR1-α* (*EF1α*), previously selected based on their stability during embryo development (Vestman et al. 2011) using the geNorm software (Vandesompele et al. 2002). Amplifications were carried out using the

DyNAmo Flash Sybr Green qPCR kit (Finnzymes). PCR cycling conditions were as advised by the manufacturer, with annealing and extension at 60°C for 30 s. The reactions were run for 40 cycles and at the end of each run melt curves were generated to ensure product uniformity. Three independent biological replicate samples were assessed and samples were added to the plates in triplicates. In all studies, inter-run connector samples were included to correct for the use of multiple plates. All calculations and normalizations were done using the iQ5 software (Bio-Rad).

Statistical methods

Data from the qRT-PCR were analyzed using a mixed model approach, see e.g., Littell et al. (2007), as implemented in the mixed procedure of the SAS (2008) system. The data from the zygotic and somatic embryo comparison in Scots pine were assessed with one-way ANOVA analyses, followed by post hoc Tukey's HSD comparison tests. For the data from TSA-treated Norway spruce embryos, the model included time, treatment and the interaction between time and treatment. An unstructured covariance matrix was used to model the within-treatment covariances over time. Different genes were analyzed separately. Differences of $P < 0.05$ were regarded as significant.

Results

The embryogenic potential decreases during germination, but can be maintained with a histone deacetylase inhibitor

Embryogenic cultures of Norway spruce are routinely established from mature non-desiccated zygotic embryos (von Arnold and Clapham 2008). The potential to initiate embryogenic cultures in conifers decreases successively as the embryos germinate (Klimaszewska et al. 2010b). Initially, we analyzed this decrease in embryogenic potential during germination in Norway spruce (Fig. 1a). About 70% of the cotyledonary embryos and embryos germinated for 1 day differentiated embryogenic tissue within 5 weeks on proliferation medium. When embryogenic tissue was induced on cotyledonary embryos, usually the whole explant developed protruding embryogenic tissue (Fig. 1b). The embryogenic potential decreased drastically during germination (Fig. 1a). Furthermore, when germinating embryos were used as explants, embryogenic tissue differentiated from fewer more localized regions on the explant (Fig. 1c).

To establish whether treatment with an HDAC inhibitor affects the embryogenic potential, cotyledonary somatic embryos were germinated for 10 days on medium supplemented with TSA and thereafter transferred to proliferation

medium lacking TSA to stimulate differentiation of embryogenic tissue. The germination progression was partially inhibited when the embryos were exposed to TSA (Fig. 2a, b). Among untreated embryos, the frequency of initiating embryogenic tissue was on average only 35%. However, when embryos were TSA treated during germination, the average frequency of initiation of embryogenic tissue was high, 85% (Fig. 2c). This is similar to the initiation frequency from cotyledonary embryos (Fig. 1a). This shows that the embryogenic potential of embryos (both cotyledonary embryos and embryos germinated for 10 days) was maintained when they were transferred to medium containing TSA. To test if treatment with TSA could also affect the embryogenic potential of already germinated embryos, we first germinated embryos for 10 days and then exposed them to TSA for 5 days before transfer to the proliferation medium. The initiation frequency was only 5% in untreated control embryos germinated for 15 days. In contrast, this 5-day treatment of already germinated embryos resulted in an initiation frequency of 22%, which was significantly higher ($P < 0.05$, Fisher's exact test) than for untreated control embryos. The embryogenic tissue from TSA-treated germinating embryos differentiated from localized regions on the explant, similar to the patterns seen on germinated control embryos (Fig. 1c). Embryogenic cultures derived from TSA-treated embryos proliferated and differentiated into cotyledonary embryos as in the original embryogenic culture (data not shown).

Isolation and phylogenetic analysis of LEC1 and VP1/ABI3 homologs from Norway spruce and Scots pine

We isolated two HAP3 genes from both Norway spruce and Scots pine using primers designed from publicly available homologous conifer sequences (Supplementary Table 1). The genes were annotated PaHAP3A (accession number JF280794), PaHAP3B, PsHAP3A (accession number JF280795) and PsHAP3B. Complete putative coding sequences were obtained for both HAP3A genes comprising 540 bp of coding sequence corresponding to translated regions of 180 amino acids. Both peptides, generated from the nucleotide sequences, are highly similar to those of the two LEC1-type Arabidopsis genes LEC1 and LEAFY COTYLEDON1-LIKE (L1L). The conserved B-domain of PaHAP3A shares 83 and 89% of the amino acids with LEC1 and L1L, respectively, and PsHAP3A shares 80 and 86% of translated amino acid identity. Both PaHAP3A and PsHAP3A contain all characteristic amino acids that define the LEC1-type HAP3 genes (Kwong et al. 2003), except that the glutamic acid (77) is replaced by asparagine in PsHAP3A. So far, only partial transcripts of the HAP3B genes have been sequenced, including the conserved B-domain.
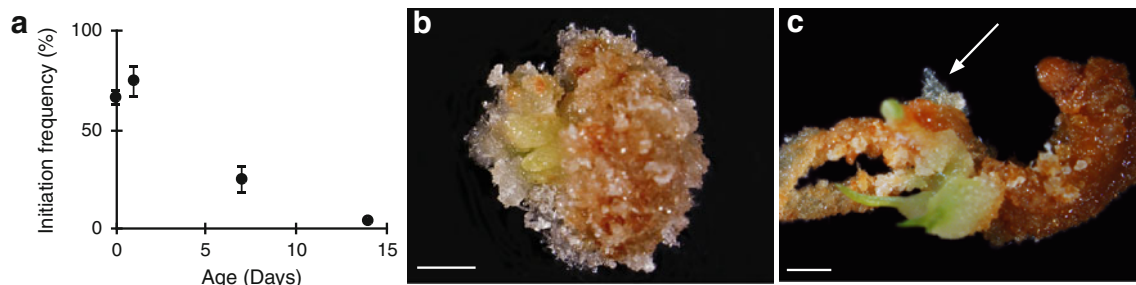
Fig. 1 The embryogenic potential decreases during germination of Norway spruce. **a** Cotyledonary somatic embryos germinated for 0–14 days were stimulated to differentiate embryogenic tissue. The proportion of embryos that had initiated embryogenic tissue within 5 weeks is presented. Frequencies are based on three biological replicates with 30 embryos per treatment. **b** Embryogenic tissue differentiated from a mature cotyledonary embryo. **c** Embryogenic (*arrow*) and non-embryogenic tissue differentiated from a 2 week-old germinated embryo. *Bars*, 2 mm
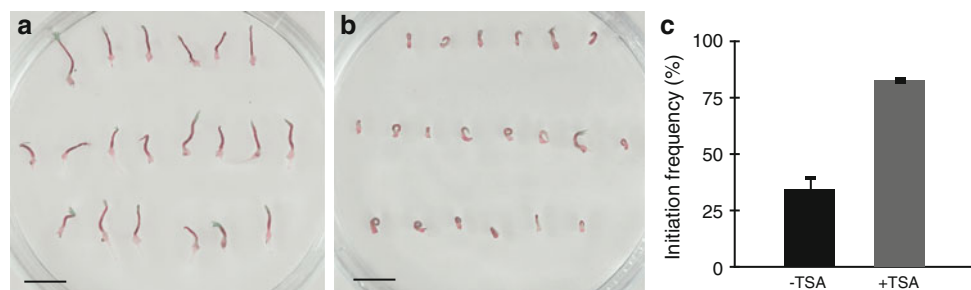

Fig. 2 The HDAC inhibitor TSA inhibits post-germination growth and maintains the embryogenic potential in germinating somatic embryos of Norway spruce. **a** Control embryos germinated for 10 days and **b** embryos germinated on medium containing 10 μM TSA for 10 days. *Bars*, 10 mm. **c** After 10 days germination on medium lacking (−TSA) or supplemented (+TSA) with TSA, the embryos were incubated on proliferation medium for 5 weeks to stimulate initiation of embryogenic tissue. The frequency of initiation is based on three biological replicates with 100 embryos per treatment. The difference in initiation frequency between −TSA and +TSA was significant, $P < 0.001$, Fisher's exact test

To analyze the relationship between the conifer *HAP3* genes and *HAP3* genes from other species, phylogenetic analyses based on nucleotide sequences from the conserved B-domain were made (Fig. 3). The phylogenetic analyses of *HAP3* genes using Bayesian inference or heuristic searches of most parsimonious trees are in general agreement with Kwong et al. (2003) and Xie et al. (2008), supporting that plant *HAP3* genes can be subdivided into LEC1-type and non-LEC1-type *HAP3* genes (Fig. 3). Lycophyte and fern LEC1-type genes group at the base of the seed plant LEC1-type *HAP3* genes. Both seed plant lineages harbor paralogous LEC1-type *HAP3* genes, as exemplified by the presence of two LEC1-type *HAP3* genes in rice (*Oryza sativa*) and *Arabidopsis*, as well as in conifers (*HAP3A* and *HAP3B*).

ABI3 belongs to the large B3 family of plant-specific transcription factors (Giraudat et al. 1992; Suzuki and McCarty 2008) The full-length *PaVP1* cDNA (accession number AF175576), which has been reported earlier (Footitt et al. 2003), contains all described conserved regions (A1, B1–B3) reported for *ABI3* and *VP1* (McCarty et al. 1991; Giraudat et al. 1992). The putative Scots pine *VP1/ABI3* homolog *PsVP1* was isolated using primers designed from a loblolly pine EST and *PaVP1* (Supplementary Table 1). The partial *PsVP1* cDNA, starting in the A1 region and extending beyond the conserved B3 region, included 1,939 nucleotides and shares 78% similarity with *PaVP1*.

To analyze the correspondence and evolution of *PaVP1* and *PsVP1*, a Bayesian phylogenetic analysis was performed using nucleotide sequences from the B3 domains of the two conifer genes, the maize *VP1* gene and all 118 B3 genes of *Arabidopsis*. The consensus tree showed a clear separation of known subgroups in the phylogeny of B3 genes, i.e., the *AFL* genes and the embryonic repressor genes (*VAL1*, *VAL2* and *VAL3*), as suggested previously (Swaminathan et al. 2008; Romanel et al. 2009). From the consensus tree, a subsample of 35 genes was selected for a final analysis (Supplementary Fig. 1) The conifer genes were positioned closest to the maize *VP1* gene and *ABI3* from *Arabidopsis*, confirming a close relationship between the conifer and angiosperm *ABI3/VP1* genes.
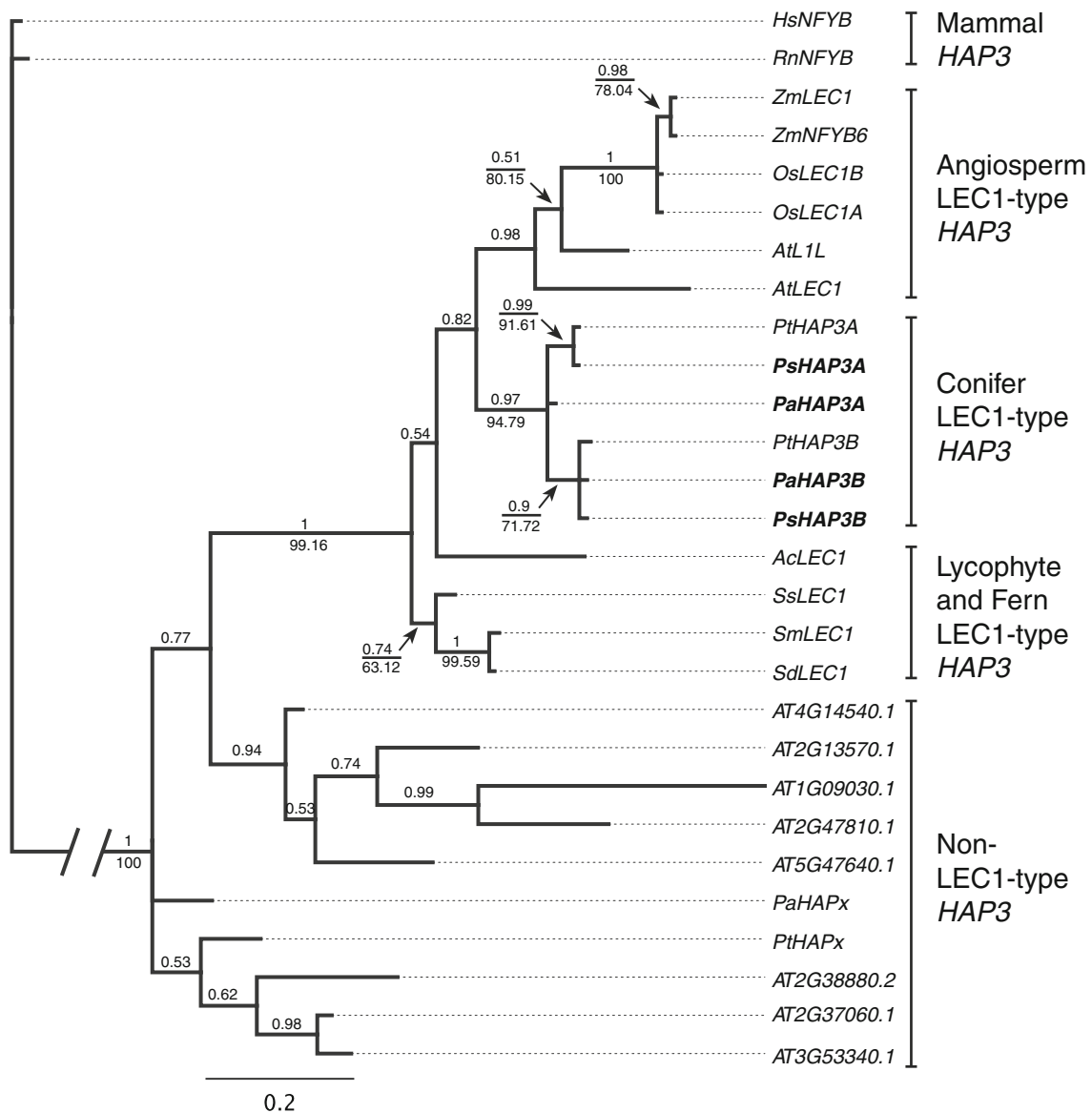
Fig. 3 The conifer *HAP3A* and *HAP3B* genes are grouped within the plant-specific LEC1-type *HAP3* genes. The figure shows a 50% majority rule tree derived from trees sampled after "burn-in" using Bayesian inference. *Numbers above branches* represent the posterior probabilities and *numbers below branches* represent parsimony bootstrap support values for branches that agreed between the estimates. Major clades are indicated to the right of the tree. Genes in boldface type were identified in this study. The root branch has been decreased to save space as indicated with the two leaning vertical lines. Names and accession numbers are found in Supplementary Table 2

Expression levels of the conifer *HAP3A* and *VP1* genes during development of zygotic and somatic embryos

The expression of *PsHAP3A* and *PsVP1* during development of zygotic and somatic embryos in Scots pine was analyzed by quantitative RT-PCR. The differences in expression levels between various developmental stages (Fig. 4a) were significant (Supplementary Table 4). *PsHAP3A* was highly expressed during early zygotic embryogenesis and a significant decrease in expression occurred at stage 4 (when the subordinate embryos were degraded), whereafter the expression remained low (Fig. 4b). In contrast, the expression of *PsVP1* was initially low, but increased significantly at stage 3 (after cleavage polyembryogeny), whereafter it remained high (Fig. 4c). We also analyzed the expression of *PsHAP3A* and *PsVP1* during development of somatic embryos of Scots pine at developmental stages corresponding to stages 2–8 in zygotic embryos. Stage 1 zygotic and somatic embryos are not comparable. Stage 1 zygotic embryos represent single, zygote-derived embryos, while stage 1 somatic embryos represent proliferating PEMs. *PsHAP3A* was highly

expressed during proliferation (stage 1), but decreased significantly during embryo development (Fig. 4d). The expression of *PsVP1* was low during proliferation (stage 1) and increased significantly at stage 3 (Fig. 4e). Our results show a similar trend in expression levels of both *PsHAP3A* and *PsVP1* during the development of zygotic and somatic embryos.

Expression of *PaHAP3A* and *PaVP1* in Norway spruce was assessed at four different time points during the development of somatic embryos, i.e., proliferating PEMs

on proliferation medium, early embryos 1 week after withdrawal of PGRs, late embryos after 1 week on maturation medium and cotyledonary embryos after 5 weeks on maturation medium (Fig. 5). The selected stages corresponded to somatic embryo stages 1, 3, 4 and 8 of Scots pine (Fig. 4a). *PaHAP3A* expression was high in proliferating PEMs and low in cotyledonary embryos (Fig. 5a). In contrast, the expression of *PaVP1* was low in PEMs and high in cotyledonary embryos (Fig. 5b). The expression of *PaVP1* correlates well with our earlier studies, where the
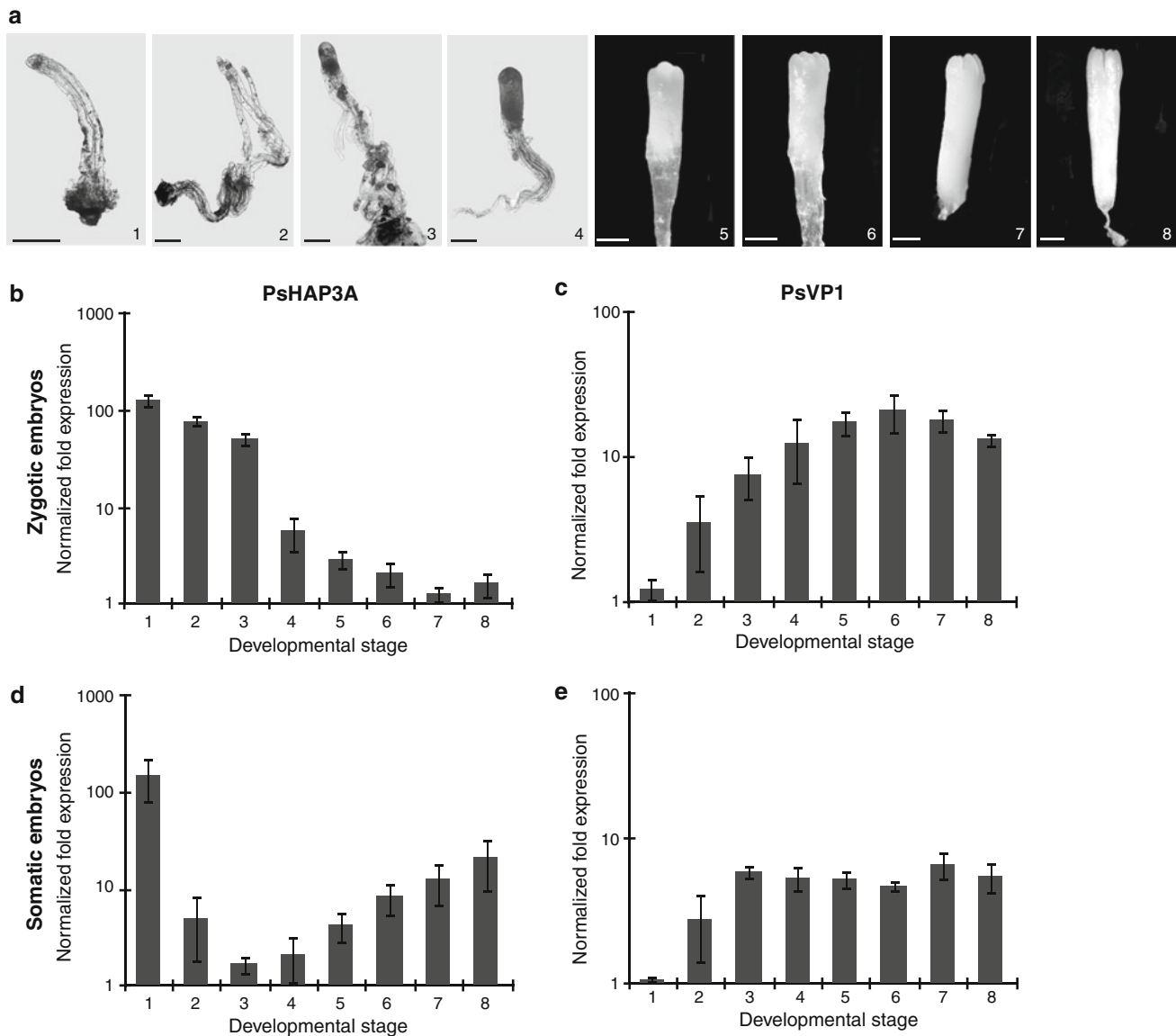

Fig. 4 Development of embryos in Scots pine and the expression of *PsHAP3A* and *PsVP1* at sequential developmental stages. **a** Stages during development of a zygotic embryo; *1* a single zygote-derived early embryo, *2* four equal-sized embryos formed after cleavage, *3* a dominant embryo and several subordinate embryos, *4* a dominant embryo, and the subordinate embryos have been eliminated, *5* and *6* maturing embryos with developing cotyledon primordia, *7* and *8* fully mature cotyledonary embryos. *Bars*, 0.5 mm. Quantitative real-time

PCR analysis of *PsHAP3A* (**b**, **d**) and *PsVP1* (**c**, **e**) during the development of zygotic (**b**, **c**) and somatic (**d**, **e**) embryos. Samples were taken at the stages presented in **a**. Expression values are presented as relative values to the sample with lowest expression in each replicate. Expression levels are mean values of three biological replicates. *Error bars* indicate ± SE of biological replicates. The significance of the differences in expression levels between various developmental stages are presented in Supplementary Table 4
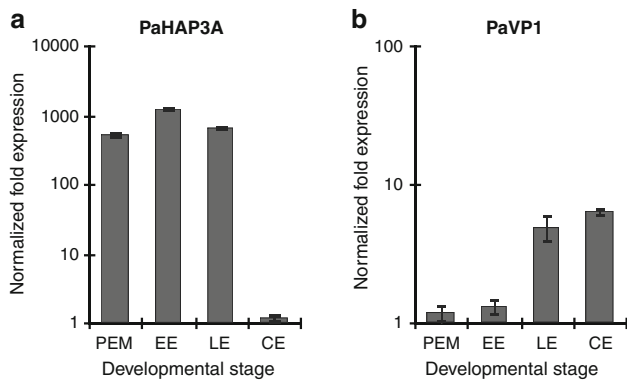
Fig. 5 Expression of *PaHAP3A* and *PaVP1* during the development of somatic embryos in Norway spruce. Somatic embryos collected from embryogenic cultures of Norway spruce were taken at four developmental stages, i.e., proliferating PEMs on proliferation medium, early embryos (EE) 1 week after withdrawal of PGRs, late embryos (LE) after 1 week on maturation medium and cotyledonary embryos (CE) after 5 weeks on maturation medium. Quantitative real-time PCR analysis of *PaHAP3A* (**a**) and *PaVP1* (**b**). Expression values are presented as relative values to the sample with lowest expression in each replicate. Expression levels are mean values of two biological replicates assessed three times each. *Error bars* indicate ±SE of biological replicates

expression of *PaVP1* is highest around the early cotyledonary stage (Footitt et al. 2003).

TSA affects the expression of *PaVP1* and *PaHAP3A* during embryo development

To test whether HDAC inhibition affects the expression of *PaHAP3A* and *PaVP1*, expression analyses were initially carried out on 10 day-old germinated somatic embryos of Norway spruce treated with TSA. However, the expression levels of both genes were very low, under a reliable detection limit, in both control and TSA-treated embryos.

The effect of TSA treatment on the expression of *PaHAP3A* and *PaVP1* was therefore analyzed during the maturation phase of somatic embryos. In control cultures, many cotyledonary embryos had developed after 4 weeks on maturation medium (Fig. 6a). In contrast, cultures developing on maturation medium supplemented with TSA continued to proliferate and maturation was arrested (Fig. 6b). However, if TSA was excluded from the maturation medium after 4 weeks, the embryos continued to mature and develop into cotyledonary embryos. In control cultures, *PaHAP3A* expression decreased during maturation. This decrease in *PaHAP3A* expression was significantly inhibited in TSA-treated cultures (Fig. 6c). The expression of *PaVP1* increased in control cultures during maturation. The increase in expression of *PaVP1* was significantly inhibited in TSA-treated cultures (Fig. 6d).

Discussion

Stimulation of somatic embryogenesis in germinating embryos by TSA

Embryogenic cultures of conifers are routinely established from immature or mature zygotic embryos. The initiation frequency deceases dramatically when the embryos start germinating (Bonga et al. 2010; Klimaszewska et al. 2010b, and this work). Here, we demonstrate that after exposure to the HDAC inhibitor TSA, germinating embryos of Norway spruce maintain the competence to differentiate into embryogenic tissue at the same time as the germination progression is partially inhibited. It has previously been demonstrated that seed germination in *Arabidopsis* is inhibited by TSA. Simultaneously, the expression of embryogenesis-related genes, including the *LEC* genes and *ABI3*, are activated and embryo-like structures differentiate on the true leaves after withdrawal of TSA (Tanaka et al. 2008). Our results imply that TSA affects the embryogenic potential and germination in a similar way in conifers. To analyze if TSA also affects gene expression in conifers, we isolated embryogenesis-related genes from Norway spruce and Scots pine.

Conifer homologs of *AtLEC1* and *AtABI3* are expressed during embryo development

To isolate putative conifer homologs of *LEC1*, *LEC2*, *FUS3* and *ABI3*, we initially blasted the *Arabidopsis* sequences against conifer EST databases. Only genes highly similar to *LEC1* and *ABI3* were found. At present, no full genome sequence of any gymnosperm is available and therefore it is too early to exclude the possibility that there are conifer homologs to *LEC2* and *FUS3* as well.

We isolated two conifer LEC1-type *HAP3* genes in both Norway spruce and Scots pine, *PaHAP3A*, *PaHAP3B*, *PsHAP3A* and *PsHAP3B*. Both conifer *HAP3A* and *HAP3B* genes encode proteins that are highly similar to LEC1-type HAP3 subunits of the CCAAT binding transcription factor (CBF, or NF-Y) (Lotan et al. 1998; Lee et al. 2003; Kwong et al. 2003). Furthermore, they all contain nearly all amino acids characteristic for LEC1-type HAP3 subunits in *Arabidopsis*, including the important amino acid asp 55, which confer DNA binding specificity to the CBF complex (Lee et al. 2003). Phylogenetic analyses position both conifer *HAP3A* and *HAP3B* genes among LEC1-type genes. Within the LEC1-type genes there are well-supported clades separating angiosperms, gymnosperms and lycophytes (Fig. 4). Lycophyte LEC1-type genes group basal to the angiosperm and gymnosperm clade suggesting that an ancestral LEC1-type gene was present in the last common ancestor of all extant seed plants. Both angiosperms and gymnosperms
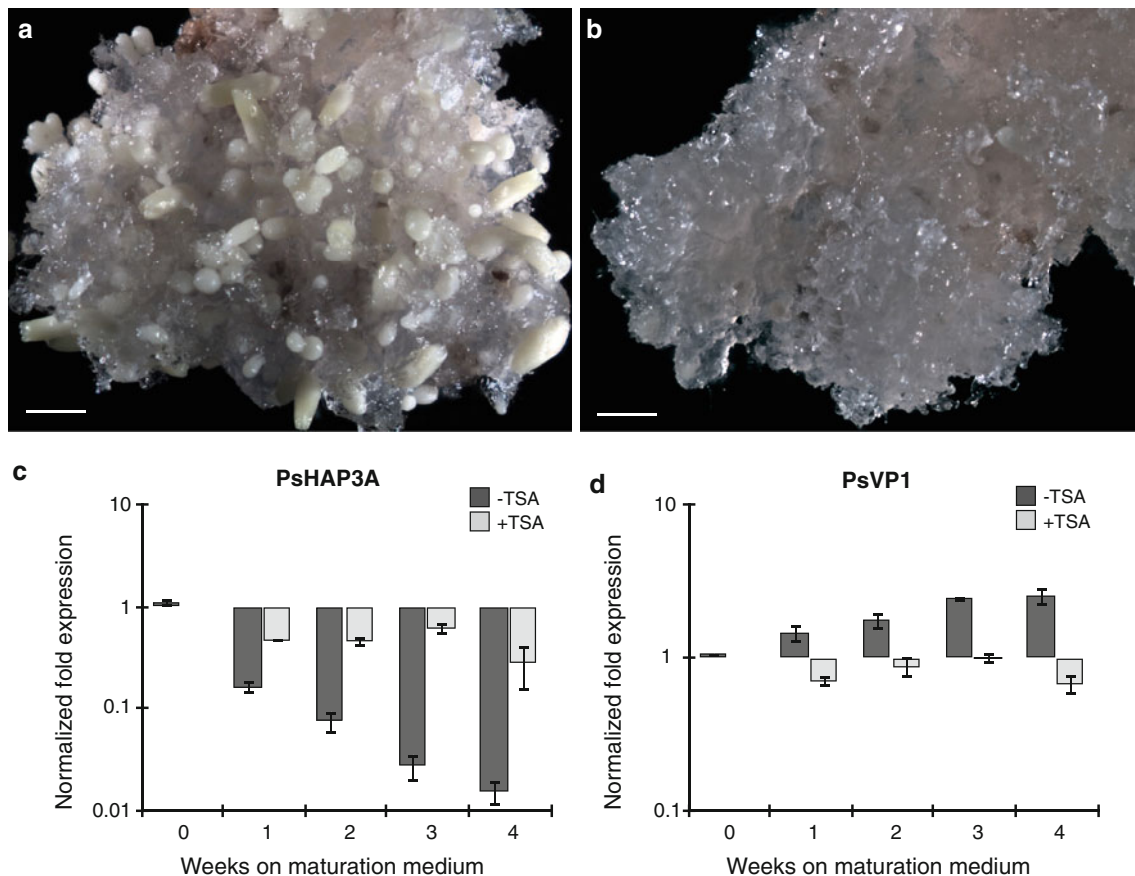
Fig. 6 The HDAC inhibitor TSA blocks maturation and affects the expression of *PaHAP3A* and *PaVP1* during the development of somatic embryos in Norway spruce. Embryogenic cultures incubated for 4 weeks on maturation medium lacking TSA (**a**) or containing 10 μM TSA (**b**). *Bars*, 2 mm. Quantitative real-time PCR analysis of *PaHAP3A* (**c**) and *PaVP1* (**d**) in embryogenic cultures treated with TSA (*light bars*) and control cultures without TSA (*dark bars*). Samples were collected during differentiation of early somatic embryos (0 weeks) and after 1–4 weeks on maturation medium. Expression values are presented as relative values to one of the samples from untreated early embryogenic cultures (0 weeks). Expression levels are shown as mean values of three biological replicates assessed three times each. *Error bars* indicate ±SE of biological replicates. The expression levels of TSA-treated samples (+TSA) significantly differed from the untreated control samples (−TSA) at each developmental stage for both genes, $P < 0.05$

seem to have undergone separate duplications within the LEC1-type lineage. Maize has been suggested to harbor up to three copies of LEC1-type *HAP3* genes, although the accessions are not publicly available (Suzuki et al. 2008). Similarly, the conifer LEC1-type *HAP3A* and *HAP3B* genes are most likely the result of a separate duplication event within the gymnosperm lineage.

The Norway spruce homolog to *ABI3*, *PaVP1*, has previously been characterized (Footitt et al. 2003). In this study we isolated its Scots pine homolog, *PsVP1*. The protein is highly similar to that of Norway spruce and sequence analysis revealed close relationship of both conifer genes to *ABI3* and its maize homolog *VP1*, within the group of plant-specific B3 transcription factors.

During early conifer embryogenesis, the expression of *PaHAP3A* and *PsHAP3A* is initially high, in agreement with recently reported expression of a putative LEC1-type

HAP3 gene in lodgepole pine (*Pinus contorta*) (Park et al. 2010). Later, during development of the embryos, the expression of *PaHAP3A* and *PsHAP3A* decreases dramatically and remains low throughout maturation (Figs. 4, 5). In contrast, the expression of the conifer *PaVP1* and *PsVP1* genes increases early during embryo development and peaks at the time point when the cotyledons emerge (Figs. 4, 5). These results are analogous with what has been reported in *Arabidopsis*, where *LEC1* is expressed at higher levels during early embryo development than in maturing embryos (Lotan et al. 1998; Baumbusch et al. 2004). Furthermore, the expression of *ABI3* can first be detected at the globular stage and persists throughout maturation of the embryo (Parcy et al. 1997; To et al. 2006).

Preliminary expression studies of *PaHAP3B* indicate a comparable low overall expression, and in contrast to *PaHAP3A* the expression increases at later maturation

stages (data not shown). Duplication within a gene line-age could be indicative of functional divergence leading to subfunctionalization, and/or neofunctionalization according to Irish and Litt 2005, which may be reflected by differential expression during development. Hence, the data suggest that the conifer *HAP3* genes might have gone through subfunctionalization, and/or neofunctional-ization, with *PaHAP3A* showing the highest similarity in activity to extant angiosperm *LEC1* genes. However, we cannot exclude that the *HAP3B* gene might have impor-tant roles and partly overlapping functions with *HAP3A* during embryogenesis in conifers, as suggested for *LEC1* and *L1L* in *Arabidopsis* (Kwong et al. 2003; Yamamoto et al. 2009).

The *LEC* genes (*LEC1*, *LEC2* and *FUS3)* have been shown necessary to confer embryonic cell fate and specification of cotyledon identity early during embryo-genesis (Meinke 1992; Bäumlein et al. 1994; Keith et al. 1994; Meinke et al. 1994; West et al. 1994; Lotan et al. 1998; Luerssen et al. 1998; Stone et al. 2001; Gazzarrini et al. 2004). Mutant analysis has indicated that *LEC1* might act upstream of *LEC2* and *FUS3* (Meinke et al. 1994). The expression of *FUS3* and *ABI3* is regulated by *LEC1* and *LEC2* as well as by themselves. Furthermore, the spatial expression seems to be very important, i.e., it has been demonstrated that *ABI3* is regulated by a com-plex network that involves *LEC1* in the cotyledons, but not in the embryo axis (To et al. 2006). Molecular data has also shown that ectopic *LEC1* expression induces expression of *ABI3* (Kagaya et al. 2005). Later during embryogenesis, *ABI3*, together with the *LEC* genes, is responsible for the initiation and maintenance of the maturation phase. To what extent the conifer *HAP3A a*nd *VP1* genes interact is presently not known, but the fact that the expression profiles of the genes are each other's opposite during embryo development might indicate a direct or indirect feedback regulation or at least a com-mon regulatory machinery.

Ectopic expression of *CHAP3A* (a putative LEC1-type *HAP3* gene from *Picea mariana*) under an inducible pro-moter was recently reported in germinating somatic embryos of white spruce (Klimaszewska et al. 2010b). However, the ectopic expression of *CHAP3A* did not induce the expression of other embryogenesis-related genes, neither was the development of the embryos affec-ted. This is in contrast to that previously shown in *Ara-bidopsis*, where ectopic post-embryonic expression of the *LEC1* gene in vegetative cells can induce the expression of embryo-specific genes and stimulate formation of embryo-like structures (Lotan et al. 1998). Further studies on the functions of the LEC1-type *HAP3A* and *HAP3B* genes are necessary to understand their roles during conifer embryogenesis.

Expression of embryo-specific transcription factors is affected by TSA

We have shown that treatment of Norway spruce embryos with TSA during germination partially inhibits germination and maintains the embryogenic potential (Figs. 2, 3). However, we could not show whether the TSA treatment during germination affects the expression of *PaHAP3A* or *PaVP1*, since both genes were expressed at very low levels. Of importance is that inhibition of HDAC activity during embryo germination stimulates the initiation of embryo-genic tissues in a localized non-uniform pattern. Recently, Klimaszewska et al. (2010a) reported initiation of embryogenic tissue from buds of 10 year-old trees regen-erated from somatic embryos of white spruce. The induc-tion of embryogenic tissue was shown to be associated with formation of nodules on the surface of the needle. The nodules differentiate in a similar pattern as we have shown for protruding embryogenic tissue on germinating embryos (Fig. 1c). Interestingly, Klimaszewska et al. (2010a) showed a high expression of *CHAP3A* in embryogenic tissue, but could not detect any expression in the needles. The results from the Klimaszewska group and results presented in this study suggest that the expression of genes stimulating the initiation of embryogenic competence are localized to a limited number of cells in the explant, not detectable in total RNA from the whole explant. We assume that some cells in the germinating embryo still possess the intracellular environment needed for embryo-genic competence and that application of the HDAC inhibitor TSA enables transcription of embryogenesis-related genes and transcription factors needed for gener-ating embryogenic tissue from these cells.

To examine if TSA affects the expression of *PaHAP3A* and *PaVP1,* we analyzed the expression of the genes during the maturation phase. The expression of *PaHAP3A* decreases during maturation, while that of *PaVP1* increa-ses. When embryogenic cultures of Norway spruce are exposed to TSA during the maturation phase, the cultures continue to proliferate and maturation is arrested (Fig. 6a, b). Simultaneously, the expression of *PaVP1* remains low while the expression of *PaHAP3A* remains high throughout the maturation treatment (Fig. 6c, d). Assuming that TSA affects histone acetylation in conifers, our results indicate a connection between changes in acetylation patterns and the levels of embryogenesis-related gene expression in Norway spruce.

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