

Overexpression of *PaHAP3A* stimulates differentiation of ectopic embryos from maturing somatic embryos of Norway spruce

Daniel Uddenberg^{1,2} · Malin Abrahamsson² · Sara von Arnold²

Received: 2 November 2015 / Revised: 26 January 2016 / Accepted: 9 February 2016 / Published online: 26 February 2016
© The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract Somatic embryogenesis can be used for large-scale propagation of plants. In most conifers, it is only possible to establish embryogenic cultures from zygotic embryos or young seedlings. There is, however, a great interest to propagate selected trees with valuable traits via somatic embryos. To be able to establish embryogenic cultures from adult tissues, more knowledge about the molecular regulation of totipotency and embryogenic potential is needed. In *Arabidopsis thaliana*, *LEAFY COTYLEDON1 (LEC1)* is required for somatic embryogenesis, and overexpression of *LEC1* can stimulate the formation of embryo-like structures from vegetative tissues. We have previously characterized a conifer LEC1-type gene, *PaHAP3A*, which is preferentially active during embryo development in Norway spruce (*Picea abies*). In this work, we show, by using histochemical GUS assays, that *PaHAP3A* is expressed in germinated embryos at presumptive sites from which embryogenic tissue differentiate during initiation of embryogenic cultures. Furthermore, we have overexpressed *PaHAP3A*, using both constitutive and inducible promoters, in order to elucidate whether elevated

transcript levels of *PaHAP3A* are sufficient to induce embryonic properties after germination. In contrast to its angiosperm counterpart, *PaHAP3A* does not stimulate embryonic features in vegetative tissues. However, overexpression of *PaHAP3A* during the maturation stage leads to the differentiation of ectopic embryos from maturing somatic embryos. Our results not only indicate at least partial conservation between the conifer and angiosperm LEC1-type genes but also suggest that *PaHAP3A* is dependent of the cellular status to confer its presumptive role as an important factor influencing the competence of the tissue to initiate somatic embryos.

Keywords Ectopic embryos · Conifer · LEC1-type gene · *PaHAP3A* · *Picea abies* · Somatic embryogenesis

Introduction

During their life cycle, higher plants undergo distinct transitions through embryonal, vegetative, and reproductive developmental stages. The life cycle starts with the fertilization of egg cells by sperm cells to produce zygotic embryos. The embryonal stage can be divided into two distinct phases, an early morphogenic phase and a late maturation phase (West 1993; Goldberg et al. 1994). Seed germination marks the end of the embryonal development, and rapid repression of embryonic genes is observed with seed imbibition (Tai et al. 2005). Developmental transitions require the precise temporal and spatial expression of regulatory genes, modulated by epigenetic mechanisms. Histone deacetylases (HDACs) are involved in the repression of embryonic properties upon germination, and inhibition of HDAC activity results in growth arrest and the formation of embryo-like structures after germination, possibly through spurious expression of embryo-specific factors (Tanaka et al. 2007). A variety of plant cells

Communicated by A. Brunner

Electronic supplementary material The online version of this article (doi:10.1007/s11295-016-0974-2) contains supplementary material, which is available to authorized users.

✉ Daniel Uddenberg
daniel.uddenberg@ebc.uu.se

¹ Physiological Botany, Department of Organismal Biology and Linnean Centre for Plant Biology, Uppsala BioCenter, Uppsala University, PO Box 7080, 75007 Uppsala, Sweden

² Department of Plant Biology and Linnean Centre for Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences, PO Box 7080, 75007 Uppsala, Sweden

have been demonstrated to possess the capacity to redirect development and regain their totipotent state, a characteristic that can be manifested in the capacity to differentiate into somatic embryos. Initiation of somatic embryogenesis requires an induction signal that causes a somatic cell to change its identity and become embryogenic and also that the cell is competent to respond to the signal. Phytohormones play an important role during zygotic and somatic embryogenesis, and auxin is suggested to be the universal induction signal for somatic embryogenesis (Braybrook and Harada 2008). In addition to auxin being necessary for the initiation of somatic embryogenesis in most plant species, it also plays a central role during embryo development (reviewed in e.g., ten Hove et al. 2015). High levels of abscisic acid (ABA) and low levels of gibberellic acid (GA) are necessary for embryo maturation, whereas the ratio is reversed during embryo germination (reviewed in Gutierrez et al. 2007).

Knowledge of the genetic regulation of plant embryogenesis has to a large extent been obtained by studying gain- and loss-of-function mutants in the angiosperm model plant *Arabidopsis* (*Arabidopsis thaliana*). Several genes have been shown to stimulate the formation of embryogenic callus or somatic embryos from vegetative cells. Expression of *Somatic Embryogenesis Receptor-like Kinase1* (*SERK1*) marks embryogenic competence, but the gene can also be expressed in non-embryogenic cells (Schmidt et al. 1997; Hecht et al. 2001). Ectopic expression of *BABY BOOM* (*BBM*) primarily induces spontaneous somatic embryo formation in seedlings (Boutillier et al. 2002). Overexpression of *WUSCHEL* (*WUS*) promotes the formation of somatic embryos from various vegetative tissues without an extra supply of plant hormones (Zuo et al. 2002). Ectopic expression of *AGAMOUS-Like 15* (*AGL15*) affects the embryonic program and enhances the competence of a shoot apical meristem to undergo somatic embryogenesis (Harding et al. 2003). *LEAFY COTYLEDON1* (*LEC1*) is an important regulator of both early and late embryogenesis and is also required for somatic embryogenesis (Gaj et al. 2005; Braybrook and Harada 2008). *LEC1*, which encodes a HAP3 subunit of the CCAAT-BOX BINDING FACTOR (CBF), is specifically expressed in the seed, both in the embryo and the endosperm, and its expression becomes repressed during the switch from embryonic to vegetative growth (Lotan et al. 1998). Ectopic expression of *LEC1* in *Arabidopsis* induces a range of abnormalities. Root and hypocotyl growth of seedlings are impaired, secondary cotyledons develop instead of leaves, and embryo-like structures emerge from vegetative tissue and express embryo-specific genes that encode seed storage proteins (Lotan et al. 1998). Another gene encoding a LEC1-type HAP3 subunit in *Arabidopsis*, *LEC1-like* (*LIL*), is required for embryogenesis but with distinct roles from *LEC1* (Kwong et al. 2003). Nevertheless, ectopic expression of *LIL* can rescue the defects of the *lec1* mutant. *PICKLE* (*PKL*), which encodes a CHD3

chromatin-remodeling factor, is required for the repression of *LEC1* and other embryo master regulators during and after germination (Ogas et al. 1997; Ogas et al. 1999; Rider et al. 2003).

We have previously identified two LEC1-type genes in Norway spruce (*Picea abies*), *PaHAP3A* and *PaHAP3B*, and a comparative phylogenetic analysis of HAP3 genes suggested that *PaHAP3A* and *PaHAP3B* are paralogous genes originating from a duplication event in the conifer lineage (Uddenberg et al. 2011). *PaHAP3A* is a conifer homolog to *LEC1* and *LIL*. The expression of *PaHAP3A* is high during early embryo development but decreases during late embryogenesis. Chemical treatment of maturing somatic embryos of Norway spruce with the HDAC inhibitor trichostatin A (TSA) arrests maturation simultaneously as the expression of *PaHAP3A* remains high. TSA treatment also alters the expression of another embryogenesis-related gene, the Norway spruce homologue to *ABSCISIC ACID INSENSITIVE3/VIVIPAROUS1*, *PaVP1*. In addition, TSA treatment of germinating embryos of Norway spruce partially inhibits the progression of germination and maintains the embryogenic potential (Uddenberg et al. 2011).

In this work, we have asked the questions if overexpression of *PaHAP3A* stimulates the embryogenic potential and if it affects embryo maturation and germination in a similar way as TSA treatment. Our results indicate that the *PaHAP3A* expression coincides with early embryogenesis and localizes to foci on germinated embryo explants from which new embryogenic tissue differentiate. Overexpression of *PaHAP3A* is neither sufficient to affect normal progression of embryo maturation or germination nor sufficient to alter the embryogenic potential after germination. This shows that TSA has a wider effect on gene expression patterns than that governed by *PaHAP3A*. Nevertheless, increased activity of the conifer LEC1-type gene during embryo maturation is sufficient for ectopic embryo formation.

Materials and methods

Plant material and growth conditions

Embryogenic cell lines 88.22 and 61.21 of Norway spruce (*Picea abies* L. Karst.) were used in this study. Cell line 88.22 was chosen since this cell line was used in our previous study of the expression of *PaHAP3A* during embryo development and germination (Uddenberg et al. 2011), and cell line 61.21 because this is the cell line that we routinely use for studying gene function in Norway spruce (e.g., Alvarez et al. 2015). Embryogenic cultures were treated as previously described (von Arnold and Clapham 2008), except that the cultures were proliferated on solidified medium. Briefly, the cultures were proliferated as proembryogenic masses (PEMs)

on proliferation medium containing 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 prematuration medium, i.e., medium lacking PGRs, for 1 week. Further development of the embryos was stimulated by transferring the cultures to maturation medium containing abscisic acid (ABA). Late embryos developed within 2 weeks on maturation medium, and mature embryos were formed after 4–8 weeks of maturation treatment. Mature embryos were desiccated for 1 week before they were transferred to germination medium (von Arnold and Clapham 2008).

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA from embryogenic cultures was isolated using the Spectrum Plant Total RNA kit (Sigma-Aldrich), according to the manufacturer's instruction. Isolated RNA was subjected to DNase digestion using the accessory on-column DNase digestion kit (Sigma-Aldrich). Complementary DNA (cDNA) was prepared from 1- μ g total RNA using the Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Scientific).

Quantitative real-time PCR (qPCR) was performed using an iQ5 Real-Time Detection System in iCycler iQ 96-well PCR plates with adhesive seals (Bio-Rad). Primers used to quantify expression levels are presented in Table S1. Target gene expression values were normalized against the reference genes *PHOSPHOGLUCOMUTASE* (*PaPHOS*), *ELONGATION FACTOR1- α* (*PaEF1- α*), and *CELL DIVISION CYCLE 2* (*PaCDC2*), 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 (Thermo Scientific). 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. Samples were analyzed in triplicates. 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).

Vector construction and transformation

The full-length coding sequence of *PaHAP3A* was amplified from cDNA of proliferating cultures using primers with attB adapters for compliance with the Invitrogen Gateway system (Invitrogen). Full-length coding sequence of *PaHAP3A* has previously been submitted to GenBank with accession number JF280794 (Uddenberg et al. 2011). All primers are listed

in Table S2. To produce an entry clone, the amplified sequence was cloned into the pDONR/Zeo vector, according to the manufacturer's protocol. All subsequent introductions of the pDONR/Zeo entry clone, containing *PaHAP3A*, into destination vectors were performed using the Gateway technology according to the manufacturer's protocol (Invitrogen). An inducible *PaHAP3A* construct was made by inserting the entry vector into the estradiol-inducible destination vector pMDC7 (Curtis and Grossniklaus 2003). The pMDC7 vector contains the estrogen receptor-based transactivator XVE, which is regulated by the addition of exogenous β -estradiol (Zuo et al. 2000). A constitutive *PaHAP3A* construct was created by introducing the entry clone into the pMDC32 destination vector. The pMDC32 vector harbors a dual cauliflower mosaic virus (CaMV) 35S promoter for constitutive expression (Curtis and Grossniklaus 2003).

For reporter gene constructs, putative promoter constructs of *PaHAP3A* were isolated from embryogenic cultures using primers amplifying a 3–4-kb region upstream the ORF based on the scaffold (212005) from the Norway spruce genome 0.8 (www.congenie.org; Nystedt et al. 2013). Primers amplifying the putative promoters contained attB adapters for compliance with the Invitrogen Gateway technology (Invitrogen). Amplified promoter products were cloned into entry clones (pENTR), and *PaHAP3A* promoter-reporter constructs were created by inserting entry clones into the pGWB3 destination vector (Nakagawa et al. 2007) according to the manufacturer's protocol (Invitrogen). The pGWB3 vector lack promoter site but contains a N-terminal β -glucuronidase (*GUS*) sequence after the 3' recombination site, thus producing the reporter gene construct *pPaHAP3A:GUS*.

The destination vectors were introduced into the *Agrobacterium tumefaciens* strain C58:C1, which contains an extra virulence plasmid (pTOK47), through freeze-thawing (Wenck et al. 1999). Cell line 88.22 was used as target for inducible induction and cell line 61.21 for constitutive expression and promoter studies. Embryogenic cultures were transformed by cocultivation with *A. tumefaciens* according to the protocol of Wenck et al. (1999), with slight modifications (Wenck et al. 1999). Briefly, after initial cocultivation in liquid proliferation medium for 2–7 h, the cultures were collected on filter papers and placed on solidified proliferation medium. After 1 week, the cultures were transferred to proliferation medium supplemented with 10 μ g/ml hygromycin, 250 μ g/ml cefotaxime, and 250 μ g/ml ticarcillin (Ticarcillin and Clavulanate). Thereafter, the cultures were subcultured to fresh culture medium of the same composition every week. Stable transformants were selected after 4 weeks.

Transgenic sublines

Transgenic sublines harboring the *GUS* gene (*35S:GUS*, and *XVE-GUS*), which were used as transformed controls (T-

controls), were kindly provided by Alyona Minina (unpublished data). The sublines were derived from cell line 61.21.

Selection of overexpressing transgenic sublines, both constitutive (*35S:PaHAP3A*) and inducible (*XVE-PaHAP3A*), for further studies, was done based on transgene expression levels in proliferating PEMs analyzed by qPCR. Three sublines constitutively overexpressing *PaHAP3A* under the dual strong 35S promoter (*35S:PaHAP3A.1*, *35S:PaHAP3A.2*, and *35S:PaHAP3A.3*) (Table S3), and three estradiol-inducible sublines (*XVE-PaHAP3A.1*, *XVE-PaHAP3A.2*, and *XVE-PaHAP3A.3*) (Table S4) showing high transgene expression levels were selected for further analyses.

Induction of the introduced *PaHAP3A* transcript (*XVE-GUS*) was effective with both 1 and 10 μM β -estradiol; however, since the expression level in average was five times higher after treatment with 10 μM β -estradiol, this concentration was chosen as standard (data not shown). The induced *PaHAP3A* expression remained high for at least 10 days (Table S5). Therefore, the cultures were routinely transferred to fresh medium every 10th day.

PaHAP3A transcriptional reporter gene constructs (*pPaHAP3A:GUS*) were selected based on GUS activity. The transgenic reporter sublines displayed similar staining patterns across multiple tissue samples. Three sublines (*pPaHAP3A:GUS.1*, *pPaHAP3A:GUS.2*, and *pPaHAP3A:GUS.3*) were selected for subsequent studies.

Development of mature somatic embryos

The effect of overexpressing *PaHAP3A* during embryo maturation was assessed by careful examination of embryo progression in three *35S:PaHAP3A* sublines (*35S:PaHAP3A.1*, *35S:PaHAP3A.2*, and *35S:PaHAP3A.3*) and in the untransformed control. Randomly collected tissue samples from three biological replicates were analyzed weekly, starting from the differentiation of early somatic embryos ($-PGR$) to 7 weeks on maturation medium. Collected samples were mounted in 1.2% (*w/v*) low-melt agarose and assessed for phenotypes and maturation progression under a light microscope (Zeiss Axioplan). The developmental stages of at least 50 embryos per sample were classified as those described by Larsson et al. (2012): stage 1, PEMs; stage 2, early embryos; stage 3, developing late embryos; stage 4, late embryos; and stage 5, early maturing embryos.

Embryogenic potential in germinated somatic embryos

The decrease in embryogenic potential during somatic embryo germination was analyzed in cell line 88.22. Embryos were germinated for 0 to 30 days on germination medium before they were transferred to medium containing PGRs (proliferation medium). The frequency of germinated embryos differentiating new embryogenic tissue as well as the position of the

protruding embryogenic tissue was estimated after 5-week incubation on proliferation medium. At least 60 embryos per time point were assessed.

The effect of overexpressing *PaHAP3A* on the embryogenic potential in germinated somatic embryos was tested in four experimental series:

1. Mature embryos from 37 *XVE-PaHAP3A* sublines were germinated for 10 days, with or without 10 μM β -estradiol. The germinated embryos were then transferred to medium containing PGRs (proliferation medium). Samples were assessed both for phenotypic deviations during germination and for the ability to stimulate differentiation of embryogenic tissue, which was scored after 5 weeks on proliferation medium. More than 50 germinated embryos per treatment and subline were analyzed.
2. Mature embryos from three *XVE-PaHAP3A* sublines (*XVE-PaHAP3A.1*, *XVE-PaHAP3A.3*, and *XVE-PaHAP3A.4*) were first germinated for 14 days and then subjected to inducible or non-inducible conditions for an additional 5 days on germination medium, before transferred to medium containing PGRs (proliferation medium). The ability to stimulate differentiation of embryogenic tissue was estimated after 5 weeks on proliferation medium. More than 30 germinated embryos per treatment and subline were analyzed.
3. Mature embryos from three *35S:PaHAP3A* sublines (*35S:PaHAP3A.1*, *35S:PaHAP3A.2*, and *35S:PaHAP3A.3*) and the untransformed control were germinated for 10 days. Thereafter, the germinated embryos were transferred to medium containing PGRs (proliferation medium). The ability to stimulate differentiation of embryogenic tissue was estimated after 5 weeks on proliferation medium. More than 50 germinated embryos per subline were assessed, and the experiment was repeated three times.
4. The effect of ABA treatment on the embryogenic potential was estimated for 10-day-old germinated embryos from *XVE-PaHAP3A* sublines (*XVE-PaHAP3A.1*, *XVE-PaHAP3A.2*, and *PaHAP3A.3*). The germinated embryos were then treated in liquid germination medium supplemented with (i) 10 μM β -estradiol, (ii) 30 μM ABA, (iii) 10 μM β -estradiol and 30 μM ABA, or (iv) control medium lacking supplements for another 10 days. Thereafter, the germinated embryos were transferred to medium containing PGRs (proliferation medium). The ability to stimulate differentiation of embryogenic tissue was estimated after 5 weeks on proliferation medium. At least 30 germinated embryos per treatment and subline were analyzed.

GUS assay

To analyze GUS activity, the samples were first collected in ice-cold 90 % acetic acid and vacuum infiltrated for 10 min at room temperature. Thereafter, the samples were placed on ice and vacuum infiltrated in GUS buffer (50 mM Na₂PO₄, 0.25 % Triton X-100, 1 mM Fe^{+III}/Fe^{+II}CN, 1 mM Na₂EDTA) for 30 min; subsequently, the samples were transferred to fresh GUS buffer supplemented with 1-mM 5-bromo-4-chloro-3-indole β-glucuronic acid for an additional 30 min. The samples were finally released from vacuum and incubated at 37 °C for 2–12 h in darkness.

Histochemical and histological analyses

Mature embryos from subline *XVE-PaHAP3A.1* were used to investigate if increased *PaHAP3A* expression would stimulate accumulation of storage compounds during germination. Mature embryos were germinated on germination medium supplemented with (i) 10 μM β-estradiol, (ii) 30 μM ABA, (iii) 10 μM β-estradiol and 30 μM ABA, or (iv) control medium lacking supplements. After 10 days, the germinating embryos were collected, hand-dissected, and stained for 5 min with Fat Red 7B (Sigma-Aldrich) for visualizing lipid bodies or Lugol (Sigma-Aldrich) for visualizing starch granules. The samples were analyzed in a light microscope (Leica, Leitz DM RX).

To assess the origin of ectopic embryos, mature somatic embryos from *XVE-PaHAP3A* sublines and the untransformed control were fixed and embedded in histowax (following the embedding method described by Karlgren et al. (2009)). The embryos were serially sectioned at 8 μM using a microtome (Microm, HM 350), and sectioned samples were stained with Toluidine blue O to visualize general anatomical features (Sigma-Aldrich) before analysis in a light microscope (Leica, DMI 4000).

Results and discussion

Expression of PaHAP3A during embryo development

We have previously shown that the expression of *PaHAP3A* is high during early embryo development; thereafter, the expression decreases to become low in mature embryos and not detectable in 10-day-old germinated embryos (Uddenberg et al. 2011). To gain more insight into the spatial expression of *PaHAP3A*, histochemical GUS assays were performed with developing and germinated embryos from three selected *pPAHAP3A:GUS* sublines. In the *pPaHAP3A-GUS* sublines, strong GUS signals were detected in PEMs (Fig. 1a) and in late embryos (Fig. 1b), both in the embryonal mass and the suspensor. Similar GUS assay results were obtained in PEMs

and late embryos from the *35S:GUS* subline (Fig. 1f, g). In mature embryos, the GUS signal was stronger in embryos from the *35S:GUS* subline (Fig. 1h) than in embryos from the *pPaHAP3A:GUS* sublines (Fig. 1c). During germination, the GUS signal successively decreased in embryos from the *pPaHAP3A:GUS* sublines. After 10 days, the GUS staining was localized to an area in the upper hypocotyl surrounding the SAM (Fig. 1d), and sometimes also in the cotyledons, while no GUS signal could be detected in 30-day-old germinated embryos (Fig. 1e). In contrast, the GUS staining was strong throughout the germination in embryos from the *35S:GUS* subline (Fig. 1i, j). GUS staining was not detected in embryos from the untransformed control (Fig. 1k–o).

The GUS assay results are in accordance to our previous results based on qPCR analyses (Uddenberg et al. 2011). However, by using the GUS assay, we could also show a weak *PaHAP3A* expression in the SAM region in 10-day-old germinated embryos. In angiosperms, *LEC1* and its ortholog *LIL* demonstrate overlapping embryo-specific expression patterns (Lotan et al. 1998; Kwong et al. 2003; Yazawa et al. 2004; Fambrini et al. 2006; Le et al. 2010). Thus, our data show that the expression pattern of *PaHAP3A* is similar to the expression pattern of *LEC1*-type genes in angiosperms.

Overexpression of PaHAP3A during embryo maturation stimulates differentiation of ectopic embryos

In order to elucidate if overexpression of *PaHAP3A* affects embryo maturation and the embryogenic potential, we established transgenic cultures of Norway spruce that overexpressed *PaHAP3A*, using both constitutive (*35S:PaHAP3A* sublines) and inducible (*XVE-PaHAP3A* sublines) promoters (Tables S3 and S4).

The effect of overexpressing *PaHAP3A* on embryo development was analyzed weekly during the maturation process. The developmental pattern of embryos in the *35S:PaHAP3A* sublines was similar to the developmental pattern of embryos in the untransformed control (Fig. 2a), as well as in the *XVE-PaHAP3A* sublines (data not shown). The endogenous *PaHAP3A* and *PaVP1* genes were expressed in mature embryos both from the *PaHAP3A* overexpressing sublines and from the control (Fig. S1a, c), while the introduced *PaHAP3A* gene (transgenic *PaHAP3A*) was only expressed in mature embryos from the overexpressing sublines (Fig. S1b). These results suggest that overexpression of *PaHAP3A* does not affect the expression of the endogenous *PaHAP3A* gene and that the transgenic *PaHAP3A* gene is expressed during the whole maturation process. Thus, together, our data show that overexpression of *PaHAP3A* is not affecting the maturation process and that the arrested maturation process caused by TSA treatment (Uddenberg et al. 2011) cannot be explained, at least not solely, by a retained high expression of *PaHAP3A*. More likely, the retained high expression of *PaHAP3A* during TSA-

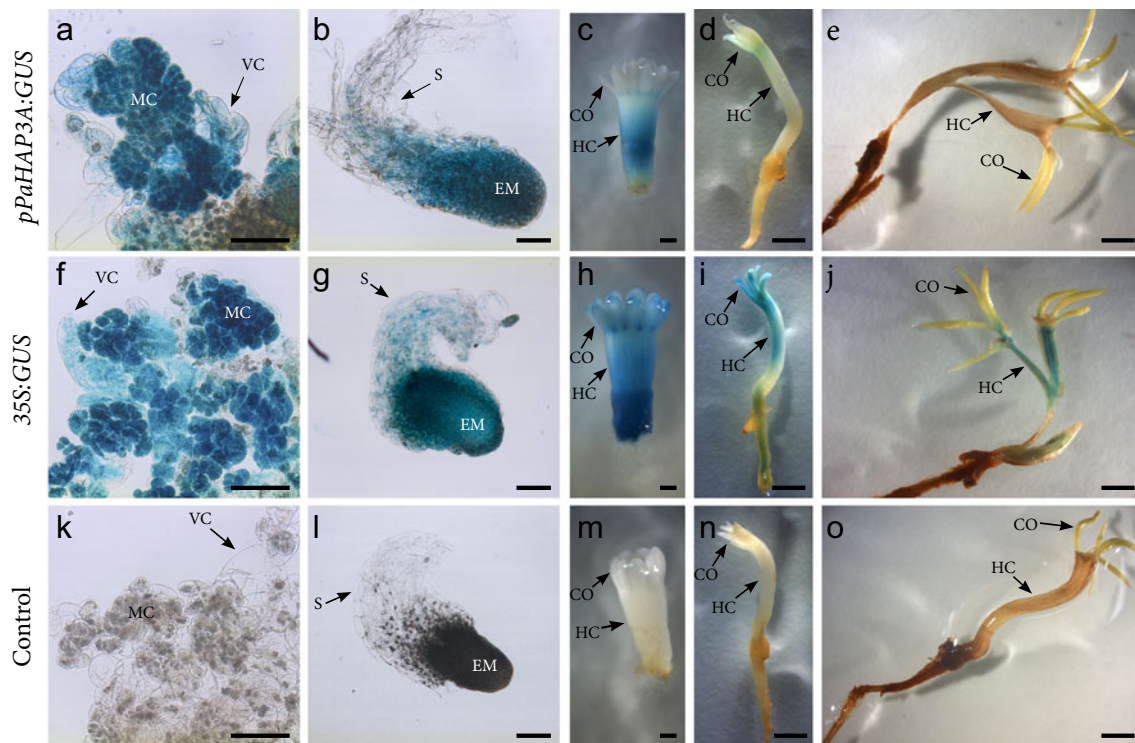


Fig. 1 GUS reporter gene expression during embryo development and germination. GUS signal was developed after incubation in GUS solution. Embryos were collected from: **a–e** a *pPaHAP3A:GUS* subline, **f–j** a *35S:GUS* subline, and **k–o** an untransformed control. Developmental stages: **a, f, k** PEMS, **b, g, l** late embryos, **c, h, m** mature embryos, **d, i, n** embryos germinated for 10 days, and **e, j, o**

embryos germinated for 30 days. Embryos germinated for 30 days were sectioned prior to GUS staining to allow for complete buffer penetrance. *MC* meristematic cell, *VC* vacuolated cell, *EM* embryonic mass, *S* suspensor, *CO* cotyledon, *HC* hypocotyl. Bars at **a–c, f–h, k–m** 200 μ M and **d, e, i, j, n, o** 2 mm

treatment is an indirect effect of the continued proliferation of embryogenic tissue and the blocking of the maturation process.

Interestingly, a subset of the mature embryos from *PaHAP3A* overexpressing sublines differentiated ectopic embryos from the hypocotyl subtending the cotyledons (Fig. 2c–h). Ectopic embryos were never observed on mature embryos from the controls (more than 1000 mature control embryos were assessed) (Fig. 2b). On average, 5.5 % of the mature embryos from induced *XVE-PaHAP3A* sublines had differentiated ectopic embryos when transgene expression had been induced from the beginning of the maturation treatment (*XVE-PaHAP3A.1* 5.4 %, *XVE-PaHAP3A.2* 5.5 %). In addition, 2.5 % of the non-induced embryos differentiated ectopic embryos (*XVE-PaHAP3A.1* 2.9 %, *XVE-PaHAP3A.2* 1.9 %), which most likely is explained by the fact that the *XVE* promoter is partially activated in Norway spruce even in the absence of β -estradiol (Table S4, Zhu et al. 2014). Ectopic embryos were also observed on mature embryos from *35S:PaHAP3A* sublines. The developmental stages of the observed ectopic embryos varied from protruding embryogenic tissue (Fig. 2c, f), to late somatic embryos (Fig. 2d, g), and mature somatic embryos (Fig. 2e, h). Mature embryos with ectopic embryos were sectioned to analyze the origin of the

ectopic embryos. Embryos carrying ectopic embryos displayed a wider hypocotyl circumference than control embryos, mainly owing to enlarged cortical cells outside the provascular tissue (Fig. 3c, d). Furthermore, the radial symmetry was lost just below the cotyledons at the position from which the ectopic embryo had differentiated (Fig. 3). This shows that the ectopic embryos had differentiated from the mature embryo explant and not only superficially adhered to the surface.

In *Arabidopsis*, overexpression of *LEC1* induces embryonic processes postembryonically even in the absence of plant growth regulators (Lotan et al. 1998; Casson and Lindsey 2006; Junker et al. 2012). Likewise, overexpression of *LEC1* in tobacco plants (*Nicotiana tabacum*) is sufficient to induce embryonic properties in vegetative tissues (Guo et al. 2013). Furthermore, in rare cases, the *LEC1* overexpressing phenotype in the *Arabidopsis* mutants lacking proper *LEC1* function has been manifested as differentiation of ectopic embryo-like structures from vegetative tissues (Lotan et al. 1998). Thus, in angiosperms, overexpression of *LEC1* can stimulate differentiation of ectopic embryo-like structures. In this work, we show that overexpression of *PaHAP3A* can stimulate differentiation of ectopic embryos in Norway spruce, which suggests that the conifer *LEC1*-type gene shares functional similarities with its angiosperm homologue.

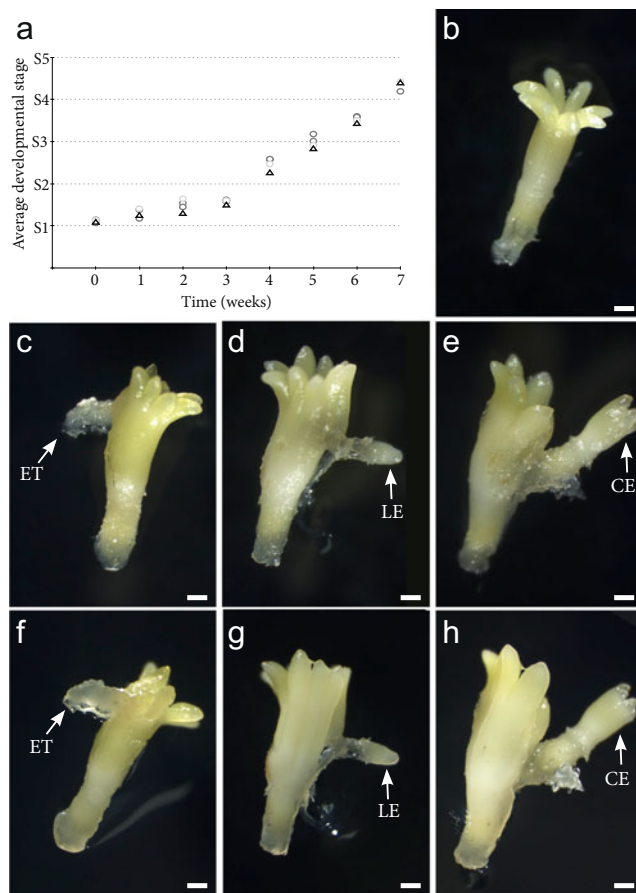


Fig. 2 Overexpression of *PaHAP3A* during embryo maturation. **a** Proliferating embryogenic tissue from three *35S:PaHAP3A* sublines (light gray circles) and the untransformed control (black triangles) were transferred to maturation medium. The developmental stage of the embryos was examined each week from the beginning of maturation treatment (week 0) until 7 weeks on maturation medium. Time points (x-axis) correspond to the number of weeks on maturation medium. Developmental stages (y-axis) are presented as the average developmental stage of more than 50 embryos in each randomly assessed sample and annotated as in Larsson et al. (2012): S1, PEMs; S2, early embryos; S3, developing late embryos; S4, late embryos; S5, early maturing embryos. **b–h** Mature embryos after 6 weeks on maturation medium. **b** Mature embryo from the untransformed control. **c–h** Mature embryo from subline *XVE-PaHAP3A.2*. The culture had been treated with β -estradiol during the whole maturation process. **f–h** The same embryos as in **c–e**, but images are captured from underneath. Images show embryos with differentiating ectopic embryos at different developmental stages: **c, f** embryogenic tissue (ET), **d, g** late embryo (LE), **e, h** early cotyledonary embryo (CE). Ectopic embryos typically protruded from the hypocotyl region subtending the cotyledons. Bars = 200 μ m

We do not know when during the maturation process, the ectopic embryos on mature somatic embryos of Norway spruce are initiated, but they differentiate just below the cotyledons and seem to originate from cells neighboring provascular tissues. It has been shown that pericycle cells in the hypocotyl of both angiosperms (reviewed in Verstraeten et al. 2014) and gymnosperms (Grönroos and von Arnold 1988) have the potential to differentiate adventitious roots.

In Arabidopsis, stimulation of calli from various tissues, including aerial postembryonic tissues, leads to ectopic activation of a pericycle reporter, and the proliferating callus cells differentiate and express genes with characteristics of the root meristem (Sugimoto et al. 2010). It is therefore possible that similar processes occur in gymnosperms, although the establishment of reporter genes for different cell fates is required before comparisons and firm conclusions can be made. Recent advances in single cell *omics* and the release of the Norway spruce genome (Nystedt et al. 2013; Junker and van Oudenaarden 2014) may now supply possibilities to further investigate the origin of the ectopic embryos.

Overexpression of *PaHAP3A* is not sufficient to increase the embryogenic potential in germinated embryos

It has previously been shown that the frequency of germinated conifer embryos differentiating embryogenic tissue decreases successively as the age of the germinated embryos increases (Klimaszewska et al. 2010; Uddenberg et al. 2011). In Norway spruce, the decrease of the embryogenic potential includes both a decrease in the frequency of germinated embryos that initiate embryogenic tissue and a decrease in the number of sites on the embryo explants from which embryogenic tissue protrude (Fig. S2). To test whether overexpression of *PaHAP3A* affects the embryogenic potential, germinated embryos from *35S:PaHAP3A* and *XVE-PaHAP3A* sublines were transferred to proliferation medium to stimulate differentiation of embryogenic tissue. Overexpression of *PaHAP3A* did not affect the germination process (data not shown). No significant change in the embryogenic potential could be observed between germinated embryos from transgenic sublines overexpressing *PaHAP3A* and controls, (Supplementary Note). Our results that overexpression of *PaHAP3A* is not sufficient to postembryonically stimulate an increase of the embryogenic potential are in line with previous reports. In angiosperms, it has been shown that overexpression of *AtLECI* in tobacco seedlings is not sufficient to stimulate differentiation of embryogenic tissue (Guo et al. 2013). Accordingly, it was not possible to stimulate differentiation of embryogenic tissue in germinated somatic embryos of white spruce (*Picea glauca*) derived from a transgenic cell line overexpressing a *HAP3A* gene (Klimaszewska et al. 2010). Based on the results from Klimaszewska et al. (2010) and observations of the expression levels of a *HAP3A* gene in *Pinus radiata* callus lines with varying degrees of embryogenic potential, Garcia-Mendiguren et al. (2015) suggested that high expression levels of *HAP3A* alone is not enough for establishing embryogenic cultures and that additional embryonic regulators are likely required to confer embryogenic potential in conifers (Garcia-Mendiguren et al. 2015). In Arabidopsis, Junker et al. (2012) showed that overexpression of *LECI* alone causes minor effects on seedlings germinated

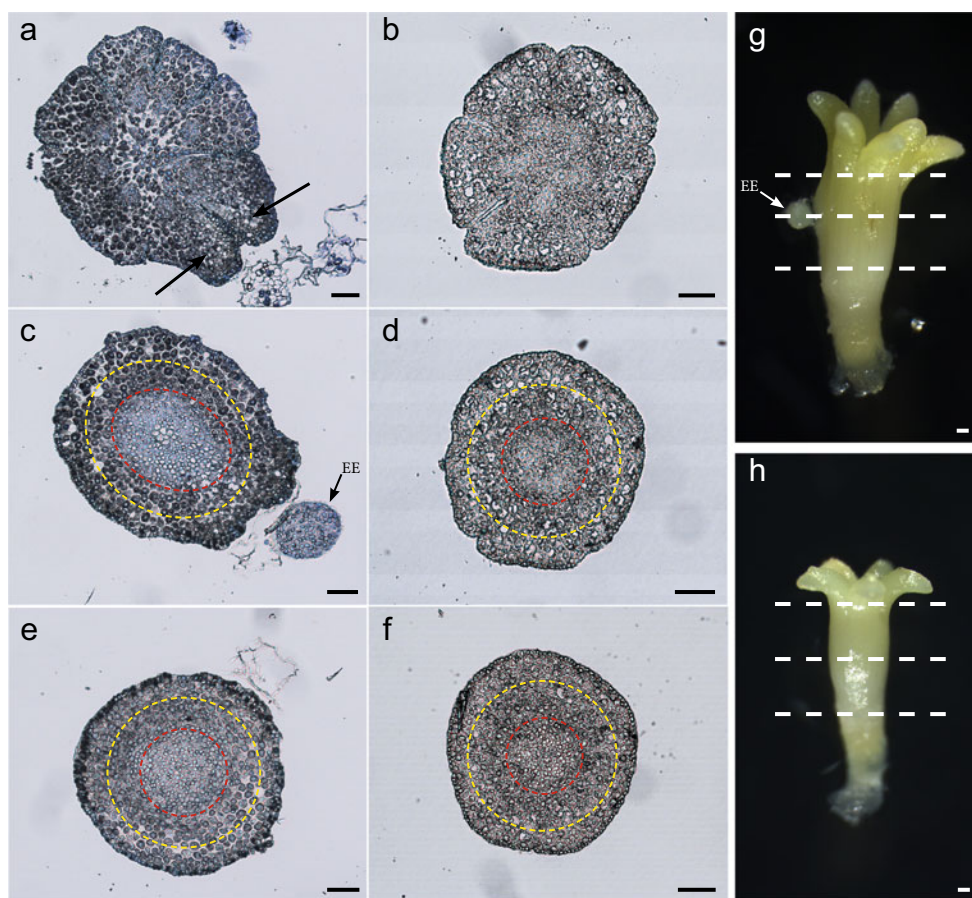


Fig. 3 Transverse sections of a mature somatic embryo displaying a protruding ectopic embryo. **a, c, e, g** Embryos from the estradiol-induced *XVE-PaHAP3A.2* subline display protrusions of ectopic embryos (*EE*) subtending the base of the cotyledons. **b, d, f, h** Untransformed control embryos. *Dashed lines in g, h* indicate the plane of which the radial sections are displayed (**a, b** upper line, **c, d** middle line, and **e, h** lower line). The *area inside the red circles* in the sections includes provascular tissue and pith, and the *area between the yellow and*

red circles include the inner part of the cortex. **a, b** Note the aberrant differentiation of cotyledons in the *XVE-PaHAP3A* embryo (**a**), indicated by *black arrows*, compared to the symmetric arrangement of cotyledons in the control embryo (**b**). **c, d** Note the enlarged cortical cells and disturbed radial symmetry of the vascular tissue in the *XVE-PaHAP3A* embryo (**c**), compared to the control embryo (**d**). **e, f** Note that the radial symmetry is similar in **e, f**. *Bars* = 100 μ m. Transverse sections are stained with Toluidine Blue O

for 10 days, followed by 10 days of induced *LEC1* expression. However, overexpression of *LEC1* together with addition of exogenous ABA causes seedlings to inhibit vegetative development and adopt maturation characteristics, such as an accumulation of seed storage compounds (Junker et al. 2012). Overexpression of *PaHAP3A* in the presence of ABA did not affect the embryogenic potential in germinated embryos of Norway spruce (data not shown), neither could we observe any obvious effect on the accumulation of fatty acids and starch (Fig. S3). Thus, our results suggest that overexpression of *PaHAP3A*, with or without exogenous ABA, does not cause any postembryonic maturation properties in germinated Norway spruce embryos, in contrast to what has been shown for *LEC1*-type genes in angiosperms.

The pattern of embryogenic tissue differentiation changes when the embryos germinate (Fig. S2). When mature zygotic embryos of Norway spruce were used as explants, usually,

embryogenic tissue was protruding from the whole embryo (Fig. S2b). In contrast, when germinated embryos were used as explants, embryogenic tissue differentiated from more localized regions (Fig. S2c–e). Before the primary explants are overgrown by calli, it is evident that embryogenic tissue preferentially differentiates from the upper part of the hypocotyl or from the cotyledons (Uddenberg et al. 2011). Simultaneously, as the embryogenic potential decreased, the formation of non-embryogenic callus increased (Fig. S2d, e, and f). To elucidate a putative colocalization of the expression of *PaHAP3A* and the differentiation of embryogenic tissue, germinated embryos from *pPaHAP3A:GUS* sublines were transferred to proliferation medium to stimulate initiation of embryogenic tissue. Explants were assessed for GUS expression patterns after 4 weeks on proliferation medium, a time point corresponding to just before embryogenic tissue could be visualized. The expression of *PaHAP3A*, according to GUS assay results,

was mainly localized to the upper part of embryos germinated for 10 days (Fig. 4a, d). Patchy patterns of GUS signals were detected along the cotyledons. In embryos germinated for 30 days, only a weak diffuse GUS staining was observed with occasional GUS foci in the upper hypocotyl bordering the cotyledons (Fig. 4g, j). In contrast, strong GUS staining was detected in germinated embryos from the *35S:GUS* subline (Fig. 4b, e, h, k), while no GUS staining was observed in germinated embryos from the untransformed control (Fig. 4c, f, i, l). The patchy expression pattern of *PaHAP3A* in the cotyledons and upper hypocotyl of germinated *pPaHAP3A:GUS* embryos, before the embryogenic tissue protrude, coincide with the place at which embryogenic tissue normally differentiate. It is thus tempting to speculate that *PaHAP3A* may be important for the embryogenic potential, and although it might not by itself be enough to stimulate embryogenic tissue formation, it may very well be an early genetic maker of tissues harboring embryogenic potential.

Conclusions

In this study, we show that *PaHAP3A* is expressed at presumptive sites of new embryogenic tissue, suggesting that the gene might be an early genetic maker of tissues harboring embryogenic potential. However, in contrast to what has been shown for other LEC1-type genes in angiosperms, overexpression of *PaHAP3A* is not sufficient to promote embryonic properties postembryonically. Overexpression of *PaHAP3A* during the maturation stage does not affect the maturation process, but a subset of the mature embryos differentiate ectopic embryos. The ectopic embryos most likely differentiate from cells neighboring provascular tissues just below the cotyledons, which indicates that the hormonal and or cellular status in these cells may be required to augment the effect of *PaHAP3A* overexpression. Future studies using novel gene identification methods, e.g., cell/tissue-specific transcriptome analyses, will become useful to unravel the molecular processes underlying the capacity of somatic cells to differentiate somatic embryos.

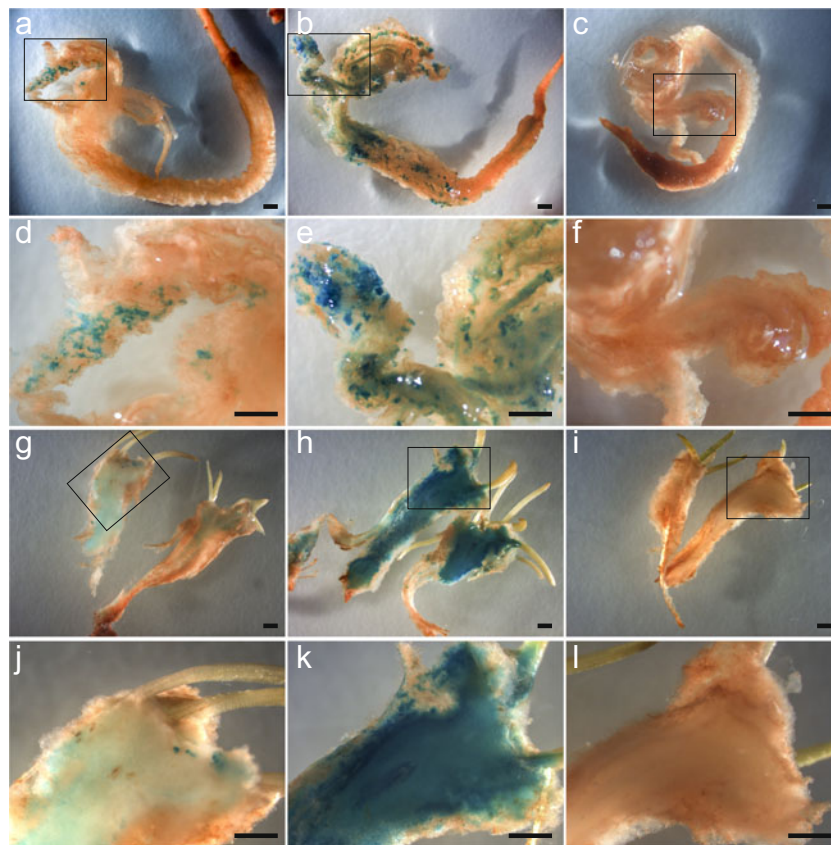


Fig. 4 Expression pattern of *PaHAP3A* in germinated embryos according to GUS reporter assays. Germinated embryos were generated from **a, d, g, j** a *pPaHAP3A:GUS* subline, **b, e, h, k** a *35S:GUS* subline, and **c, f, i, l** an untransformed control. **a–c** Embryos were germinated for 10 days or **g–i** 30 days, before transferred to proliferation medium to stimulate differentiation of embryogenic tissue. Images are captured after 4 weeks on proliferation medium, which is just before the

embryogenic tissue protrude from the explant. Rectangles in **a–c** and **g–i** indicate approximate areas of images in **d–f** and **j–l**, respectively. **a, d, g, j** Note that the fragmentary GUS signals in germinated embryos from the *pPaHAP3A:GUS* subline mainly localize to the upper part of the explant, whereas in **b, e, h, k**, the GUS signals are more evenly distributed in the germinated embryos from *35S:GUS* subline. No GUS expression was detected in the control embryos (**c, f, i, l**). Bars = 1 mm

Acknowledgments The authors would like to thank Alyona Minina for GUS control constructs and Iva Mozgova for critical reading of the manuscript. This work was supported by the Swedish Research Council Formas.

Compliance with ethical standards

Data archiving statement The full-length CDS of *PaHAP3A* (accession number JF280794) has been submitted to GenBank.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Alvarez JM, Sohlberg J, Engström P et al (2015) The *WUSCHEL-RELATED HOMEODOMAIN 3* gene *PaWOX3* regulates lateral organ formation in Norway spruce. *New Phytol* 208:1078–1088. doi:10.1111/nph.13536
- Boutillier K, Offringa R, Sharma VK et al (2002) Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryonic growth. *Plant Cell* 14:1737–1749. doi:10.1105/tpc.001941
- Braybrook S, Harada J (2008) LECs go crazy in embryo development. *Trends Plant Sci* 13:624–630. doi:10.1016/j.tplants.2008.09.008
- Casson SA, Lindsey K (2006) The *turnip* mutant of *Arabidopsis* reveals that *LEAFY COTYLEDON1* expression mediates the effects of auxin and sugars to promote embryonic cell identity. *Plant Physiol* 142:526–541. doi:10.1104/pp.106.080895
- Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* 133:462–469. doi:10.1104/pp.103.027979
- Fambrini M, Durante C, Cionini G et al (2006) Characterization of *LEAFY COTYLEDON1-LIKE* gene in *Helianthus annuus* and its relationship with zygotic and somatic embryogenesis. *Dev Genes Evol* 216:253–264. doi:10.1007/s00427-005-0050-7
- Gaj MD, Zhang S, Harada JJ, Lemaux PG (2005) Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*. *Planta* 222:977–988. doi:10.1007/s00425-005-0041-y
- Garcia-Mendiguren O, Montalbán IA, Stewart D et al (2015) Gene expression profiling of shoot-derived calli from adult radiata pine and zygotic embryo-derived embryonal masses. *PLoS ONE* 10, e0128679. doi:10.1371/journal.pone.0128679
- Goldberg RB, de Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. *Science* 266:605–614. doi:10.1126/science.266.5185.605
- Grönroos R, von Arnold S (1988) Initiation of roots on hypocotyl cuttings of *Pinus sylvestris*, with emphasis on direct rooting, root elongation, and auxin uptake. *Can J For Res* 18:1457–1462
- Guo F, Liu C, Xia H et al (2013) Induced expression of *AtLECI* and *AtLEC2* differentially promotes somatic embryogenesis in transgenic tobacco plants. *PLoS ONE* 8, e71714. doi:10.1371/journal.pone.0071714
- Gutierrez L, Van Wuytswinkel O, Castelain M, Bellini C (2007) Combined networks regulating seed maturation. *Trends Plant Sci* 12:294–300. doi:10.1016/j.tplants.2007.06.003
- Harding EW, Tang W, Nichols KW et al (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of *AGAMOUS-Like 15*. *Plant Physiol* 133:653–663. doi:10.1104/pp.103.023499
- Hecht V, Vielle-Calzada JP, Hartog MV et al (2001) The *Arabidopsis* *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol* 127:803–816
- Junker JP, van Oudenaarden A (2014) Every cell is special: genome-wide studies add a new dimension to single-cell biology. *Cell* 157:8–11. doi:10.1016/j.cell.2014.02.010
- Junker A, Mönke G, Ruten T et al (2012) Elongation-related functions of *LEAFY COTYLEDON1* during the development of *Arabidopsis thaliana*. *Plant J* 71:427–442. doi:10.1111/j.1365-3113X.2012.04999.x
- Karlgrén A, Carlsson J, Gyllenstrand N et al (2009) Non-radioactive in situ hybridization protocol applicable for Norway spruce and a range of plant species. *J Vis Exp*. doi:10.3791/1205
- Klimaszewska K, Pelletier G, Overton C et al (2010) Hormonally regulated overexpression of *Arabidopsis* *WUS* and conifer *LECI (CHAP3A)* in transgenic white spruce: implications for somatic embryo development and somatic seedling growth. *Plant Cell Rep* 29:723–734. doi:10.1007/s00299-010-0859-z
- Kwong R, Bui A, Lee H et al (2003) *LEAFY COTYLEDON1-LIKE* defines a class of regulators essential for embryo development. *Plant Cell* 15:5–18. doi:10.1105/tpc.006973
- Larsson E, Sundström JF, Sitbon F, von Arnold S (2012) Expression of *PaNAC01*, a *Picea abies* *CUP-SHAPED COTYLEDON* orthologue, is regulated by polar auxin transport and associated with differentiation of the shoot apical meristem and formation of separated cotyledons. *Ann Bot* 110:923–934. doi:10.1093/aob/mcs151
- Le BH, Cheng C, Bui AQ et al (2010) Inaugural article: global analysis of gene activity during *Arabidopsis* seed development and identification of seed-specific transcription factors. *Proc Natl Acad Sci* 107:8063–8070. doi:10.1073/pnas.1003530107
- Lotan T, Ohto M, Yee K et al (1998) *Arabidopsis* *LEAFY COTYLEDON1* is sufficient to induce embryo development in vegetative cells. *Cell* 93:1195–1205
- Nakagawa T, Kurose T, Hino T et al (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 104:34–41. doi:10.1263/jbb.104.34
- Nystedt B, Street NR, Wetterbom A et al (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* 497:579–584. doi:10.1038/nature12211
- Ogas J, Cheng JC, Sung ZR, Somerville C (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana* *pickle* mutant. *Science* 277:91–94
- Ogas J, Kaufmann S, Henderson J, Somerville C (1999) *PICKLE* is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. *Proc Natl Acad Sci U S A* 96:13839–13844
- Rider S, Henderson J, Jerome R et al (2003) Coordinate repression of regulators of embryonic identity by *PICKLE* during germination in *Arabidopsis*. *Plant J* 35:33–43. doi:10.1046/j.1365-3113X.2003.01783.x
- Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* 124:2049–2062
- Sugimoto K, Jiao Y, Meyerowitz EM (2010) *Arabidopsis* regeneration from multiple tissues occurs via a root development pathway. *Dev Cell* 18:463–471. doi:10.1016/j.devcel.2010.02.004
- Tai HH, Tai GCC, Beardmore T (2005) Dynamic histone acetylation of late embryonic genes during seed germination. *Plant Mol Biol* 59:909–925. doi:10.1007/s11103-005-2081-x

- Tanaka M, Kikuchi A, Kamada H (2007) The *Arabidopsis* histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. *Plant Physiol* 146:149–161. doi:10.1104/pp.107.111674
- ten Hove CA, Lu K-J, Weijers D (2015) Building a plant: cell fate specification in the early *Arabidopsis* embryo. *Development* 142:420–430. doi:10.1242/dev.111500
- Uddenberg D, Valladares S, Abrahamsson M et al (2011) Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. *Planta* 234:527–539. doi:10.1007/s00425-011-1418-8
- Vandesompele J, De Preter K, Pattyn F, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:–. doi: 10.1186/gb-2002-3-7-research0034
- Verstraeten I, Schotte S, Geelen D (2014) Hypocotyl adventitious root organogenesis differs from lateral root development. *Front Plant Sci*. doi:10.3389/fpls.2014.00495
- Vestman D, Larsson E, Uddenberg D et al (2011) Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. *Tree Genet Genomes* 7:347–362. doi:10.1007/s11295-010-0336-4
- von Arnold S, Clapham D (2008) Spruce embryogenesis. *Methods in Molecular Biology*. Humana Press, Totowa, NJ. 427:31–47
- Wenck AR, Quinn M, Whetten RW et al (1999) High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Mol Biol* 39:407–416. doi:10.1023/A:1006126609534
- West M (1993) Embryogenesis in higher plants: an overview. *Plant Cell Online* 5:1361–1369. doi:10.1105/tpc.5.10.1361
- Yazawa K, Takahata K, Kamada H (2004) Isolation of the gene encoding Carrot leafy cotyledon1 and expression analysis during somatic and zygotic embryogenesis. *Plant Physiol Biochem* 42:215–223. doi:10.1016/j.plaphy.2003.12.003
- Zhu T, Moschou PN, Alvarez JM, et al (2014) Wuschel-related homeobox 8/9 is important for proper embryo patterning in the gymnosperm Norway spruce. *J Exp Bot* 65:6543–6552. doi:10.1093/jxb/eru371
- Zuo J, Niu QW, Chua NH (2000) Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J* 24: 265–273
- Zuo J, Niu QW, Frugis G, Chua NH (2002) The *WUSCHEL* gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *Plant J* 30: 349–359