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CELL BIOLOGY AND MORPHOGENESIS

Expression of the gene encoding transcription factor PaVP1 differs in *Picea abies* embryogenic lines depending on their ability to develop somatic embryos

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Abstract Maturation of Norway spruce (*Picea abies* L.) somatic embryos is induced by abscisic acid (ABA). Several proteins were proven to be involved in ABA sensing including ABI3/VP1 transcription factors and their orthologue PaVP1 was characterized in spruce. To evaluate the role of PaVP1 both in embryogenic potential and in the process of embryo maturation, we studied *PaVP1* expression in lines with contrast embryogenic capacities in parallel with detailed anatomical characterization. *PaVP1* expression was determined by northern blot hybridisation, which revealed presence of two differentially regulated VP1-like B3-domain transcripts. Full-length *PaVP1* transcript level was negligible in all lines on the proliferation media, but it differed strongly on the maturation media containing ABA. In non-embryogenic line, lacking any differentiated structures, the transcript remained undetectable. In contrast, in embryogenic lines with meristematic centres attached to suspensor cells, *PaVP1* expression increased strongly after transition onto the maturation media. In highly embryogenic lines, it kept on a high level until the embryos reached cotyledonary stage, while in developmentally arrested line incapable to form mature embryos, the expression dropped down in connection with advanced disintegration of the meristematic centres. Removal of ABA from the maturation media after 2 weeks of maturation resulted in aberrant embryo development and rapid decrease in *PaVP1* expression, indicating the impact of exogenously supplemented ABA on both initiation and maintenance of *PaVP1* expression and proper embryo development. Since permanently high or increasing *PaVP1* transcript levels accompanied proper embryo development in all experiments, it could be regarded as a good marker of this process.

Keywords ABI3/VP1 transcription factor · Alternative splicing · Anatomy · Embryogenic capacity · Norway spruce (*Picea abies*) somatic embryogenesis

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Introduction

Somatic embryogenesis of Norway spruce [*Picea abies* (L.) Karst.] belongs to the best-characterized systems in conifers. Embryogenic cultures are predominantly induced from immature zygotic embryos and maintained on proliferation media containing auxins and cytokinins. Proliferating cultures consist of embryogenic-suspensor mass, i.e. proembryos attached to the suspensor cells. Significant portion of primarily induced lines are either completely non-embryogenic or incapable of producing mature somatic embryos. The early embryos stop their development before reaching the cotyledonary stage in so-called developmentally arrested lines (Filonova et al. 2000). Since maturation of somatic embryos is induced on the medium containing abscisic acid (ABA, Tautorus et al. 1991), we analysed the role of ABA sensing in the determination of embryogenic capacity of the lines.

Abscisic acid plays a key role in the process of somatic embryo maturation. It corresponds to the situation in

zygotic embryos, where initiation of seed maturation correlates with a peak of internal ABA content. This increase in ABA is maternally derived, while following peak of ABA accumulation (as documented in wild-type *Arabidopsis thaliana* seeds) depends on synthesis in the embryo itself (Karssen et al. 1983). This second peak is essential for the induction of dormancy, which is maintained despite a substantial decrease in ABA by seed maturity (Finkelstein et al. 2002). In embryogenic cultures of *P. abies*, ABA blocks proliferation of embryogenic-suspensor mass and induces further development of early proembryos (Bozhkov et al. 2002). Abscisic acid also stimulates deposition of storage compounds within the somatic embryo and induces embryo dormancy (Gutmann et al. 1996).

Though the precise mechanism of ABA action in embryo/seed development remains unknown, it is clear that ABA directly or indirectly regulates the expression of many genes. There are several genes in *A. thaliana*: *ABI3*, *ABI4* and *ABI5*, which were proven to be involved in ABA signal transduction pathway during seed development. *ABI3*, an orthologue of maize *VIVIPAROUS1* (*VP1*, Giraudat et al. 1992) encodes a B3-domain transcription factor (Gazzarrini and McCourt 2001). ABI3 acts in combination with ABI4 and ABI5 to regulate seed ABA response and late embryogenesis in *Arabidopsis* (Soderman et al. 2000). *PaVP1*, an orthologue of *ABI3* gene in *P. abies*, was isolated by Footitt et al. (2003), who demonstrated its expression by semi-quantitative RT-PCR in highly embryogenic line.

In this study, the expression of *PaVP1* was determined in four *P. abies* lines with contrast embryogenic capacities to evaluate the impact of *PaVP1* expression on embryo maturation, and to assess the potential use of *PaVP1* as a marker of embryogenic capacity. The expression of *PaVP1*, determined by northern blot hybridisation, was supplemented with anatomical analysis of the cultures. The changes of *PaVP1* transcript levels during maturation of individual lines and the effect of ABA removal on both morphology of the culture and *PaVP1* expression are discussed in the respect of PaVP1 role in embryo development.

Materials and methods

Plant material

Embryogenic line of Norway spruce [P. abies L. (Karst.)] AFO 541 was obtained as a gift from AFOCEL (France). The embryogenic lines C203 and C110 and non-embryogenic line C101N were induced from matured zygotic embryos as described by Vágner et al. (2005).

Cultivation

The cultures were grown on media according to Gupta and Durzan (1986) solidified with 0.75% (w/v) agar, and adjusted to pH 5.8 prior to autoclaving. Cultures were kept in darkness at 24°C. Proliferation medium was supplemented with 5 μM 2,4-dichlorophenoxyacetic acid, 2 μM kinetin, 2 μM benzylaminopurine, and 30 g/l sucrose. The maturation medium for somatic embryo development lacked auxins and cytokinins but contained 20 μM ABA and 3.75% (w/v) polyethylene glycol (PEG) 4000. PEG solution was autoclaved separately and mixed with the remaining medium after autoclaving. The cultures were subcultivated weekly onto fresh media in Magenta vessels. From the proliferation medium the cultures were transferred directly to the maturation medium.

Anatomical analysis

Paraffin sections (12 μm thickness) of maturing cultures were prepared according to Johansen (1940). The sections were stained by the two-step procedure using alcian blue and nuclear fast red as described by Svobodova et al. (1999). Alcian blue is specific to polysaccharides of the cell wall and nuclear fast red counterstains chromatin structures.

PaVP1 expression analysis

RNA was isolated from 150 mg of culture fresh weight according to Stiekema et al. (1988). The total RNA (20 μg/line) was separated on agarose gel electrophoresis (Logemann et al. 1987) blotted onto the Nylon+membrane (Roche, Mannheim, Germany) and cross-linked by UV radiation (1,200 kJ/cm^2). The whole hybridisation and detection procedure was done according to The DIG System Users Guide for Filter Hybridisation (Roche). The membranes were hybridised with digoxygenin (DIG) labelled probe overnight in high salt buffer and extensively washed to reach maximum stringency. Thereafter, the blots were incubated with anti-DIG antibody (Fab fragments, Roche) conjugated with alkaline phosphatase diluted 1:15,000 in blocking solution. After extensive washes of non-specifically bound antibody, the blots were equilibrated in detection buffer and incubated with chemiluminiscent substrate (CDP-Star CL-AP Substrate, 1 ml per 100 cm^2 of membrane area; Novage-Merck, Darmstadt, Germany) in a plastic wrap. RTG films attached to the wrapped blots were exposed for 3–15 min to reach equal intensity of internal hybridisation standard present on all membranes.

For probe preparation, the fragment of *PaVP1* gene (containing the sequence encoding B3 conservative domain) was amplified with specific primers VP12US and VP12DS (Footitt et al. 2003) from cDNA prepared with anchored oligo-T_{23} primer and RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas, Burlington, Canada). The identity of the fragment was confirmed by sequencing. The DIG labelled probe was prepared by PCR in the presence of dUTP-DIG according to the manufacturer instructions (Roche).

Results

Morphological characterization of the lines with contrast embryogenic capacity

Non-embryogenic line C101N

Callus-like C101N culture propagated on the proliferation medium was white-yellow and consisted of uniform, more or less isodiametric cells, which did not form organized structures (Fig. 1a). After the transfer to the maturation medium no structural changes were observed in the culture, whose proliferation gradually ceased. Prolonged cultivation under maturation conditions induced browning connected with the accumulation of phenolic compounds and extensive cell death (not shown).

Developmentally arrested line C203

Proliferating C203 culture was characterized by the presence of properly developed somatic proembryos attached to the suspensor cells (Fig. 1b). More than 99% of these proembryos failed to undergo further development under maturation conditions. During 3 weeks of maturation, the majority of proembryonic regions expanded into meristematic clusters (Fig. 1c) that further disintegrated during prolonged cultivation. The cells in the clusters successively lost their meristematic activity.

Line AFO 541 with high embryogenic capacity

Proliferating culture AFO 541 consisted of meristematic centres connected with suspensor cells (Fig. 1d). The majority of proembryos developed into mature cotyledonary

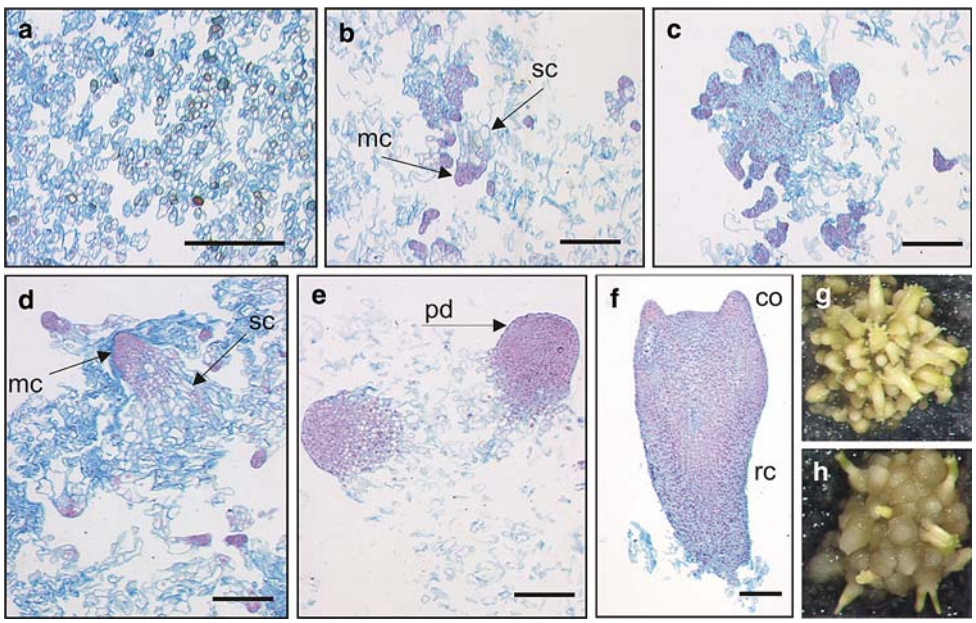

Fig. 1 Anatomy and macroscopical view of Norway spruce (*Picea abies*) lines growing on proliferation and maturation media. Staining with alcian blue and nuclear fast red; microscopy in bright field: proembryos and embryos in longitudinal sections. **a** Non-embryogenic line C101N during cultivation on proliferation media. **b** Developmentally arrested line C203 during cultivation on proliferation media; *mc* meristematic cells (*red stained*), *sc* suspensor cells (*blue stained*). **c** Developmentally arrested line C203 after 3 weeks on maturation media. **d** Line AFO 541 with high embryogenic capacity during cultivation on proliferation media: *mc* meristematic cells (*red stained*), *sc* suspensor cells (*blue stained*). **e** Developing somatic embryos of AFO 541 line after 3 weeks on maturation media—early somatic embryo: *pd* protoderm. **f** Early cotyledonary somatic embryo of AFO 541 line after 5 weeks on maturation media: *co* cotyledons, *rc* root cap. **g** Macroscopical view of line C110 with high embryogenic capacity after 5 weeks on maturation media. **h** Macroscopical view of line C110 with high embryogenic capacity after 5 weeks of cultivation: 2 weeks on maturation media with ABA followed by 3 weeks on maturation media without ABA. **a–f** *Bars* represent 200 μm

somatic embryos capable to germinate. Typical stages of zygotic embryo development, from proembryos to early cotyledonary embryos, could be observed during 5 weeks under maturation conditions (Figs. 1e, f). During the sixth week of maturation, only the elongation of cotyledons was observed with no further changes in embryo anatomy (data not shown).

Expression of *PaVP1*

The probe prepared from the sequence encoding the B3 domain of PaVP1 hybridised on northern blots with two distinct bands in the majority of samples (Fig. 2). The changes in the intensity of these two signals had an equal trend, but with more pronounced extremes in the case of the longer (upper) one. The size of this longer band corresponded to the *PaVP1* transcript (2,760 nucleotides, Footitt et al. 2003) being situated between the band of 18S and 25S rRNAs (1,808 and 3,374 nucleotides long, respectively; Fig. 2a). Therefore, in the following text, the upper band is referred as *PaVP1* transcript, and the "lower band" of unknown origin is commented separately.

Non-embryogenic line C101N

The level of *PaVP1* transcript in the non-embryogenic line C101N was under the detection limit in all samples (Fig. 2b). The *PaVP1* probe weakly hybridised with the "lower band" with no pronounced changes in response to the transfer onto the maturation medium containing ABA (Fig. 2b).

Line AFO 541 with high embryogenic capacity

Very low, almost undetectable levels of *PaVP1* transcript in proliferating culture gradually increased by at least two orders of magnitude during standard 5 weeks of maturation, and then the expression almost completely disappeared when cultivation continued for the sixth week (Fig. 2c). The intensity of the "lower band" was significantly higher compared to the *PaVP1* transcript on the proliferation medium, while the increase during maturation was more pronounced in case of the *PaVP1* transcript. Gradual increase in the intensity of the lower band during cultivation on the maturation medium was

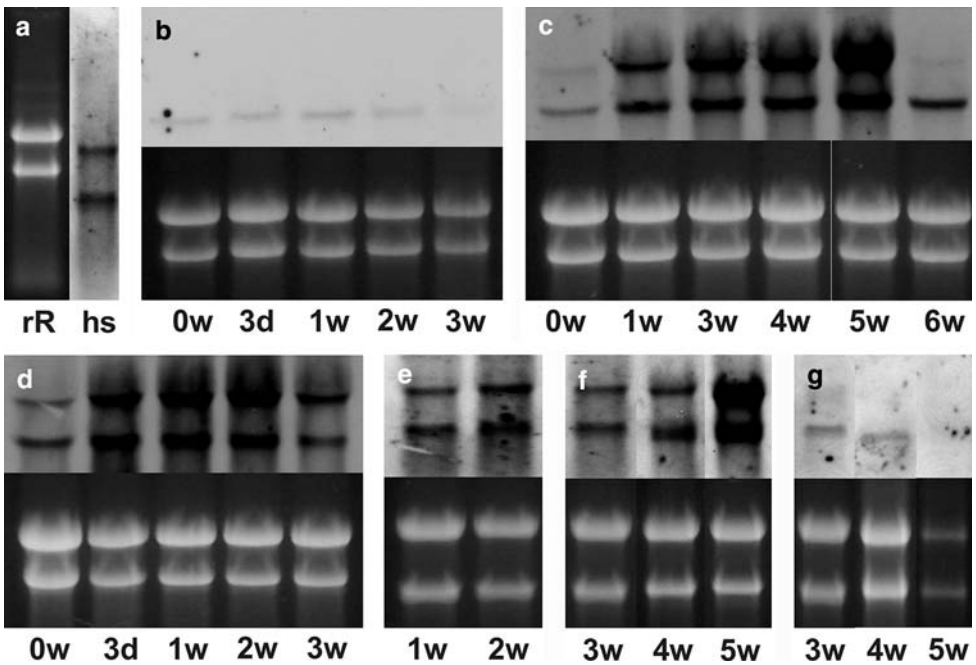

Fig. 2 Expression of *PaVP1* in Norway spruce (*Picea abies*) lines growing on proliferation and maturation media. Northern blots with the total RNA (20 μg per line) isolated from non-fractionated culture (**a**-**f**) were hybridised with digoxygenin labelled fragment (450 bp) of *PaVP1* gene encoding B3 domain. RTG films (*the upper part* of the pictures on bright background) were exposed to reach equal intensity of internal hybridisation standard present on all membranes (not shown). Comparable loading of samples was confirmed by fluorescence of ethidium bromide stained rRNA (*the bottom part* of the pictures on the dark background). **a** Comparison of the position of hybridising signals (*hs*) with the ribosomal RNA bands (*rR*); 25S rRNA (*the upper band:* approximately 3400 nucleotides) and 18S rRNA (*the lower band:* approximately 1800 nucleotides). **b** Non-embryogenic line C101N. **c** Line AFO 541 with high embryogenic capacity. **d** Developmentally arrested line C203. **e–g** Line C110 with high embryogenic capacity. After initial 2 weeks of maturation with ABA (**e**) the culture either continued cultivation on ABA containing medium (**f**) or was further cultivated on the medium without ABA [RNA was isolated from separated embryos (**g**)]. Days (*d*) or weeks (*w*) of cultivation on the maturation medium are indicated

followed by a drop in the sixth week as in case of the *PaVP1* transcript, but only to the level detected in the first week of maturation (Fig. 2c).

Developmentally arrested line C203

The level of both *PaVP1* transcript and the "lower band" quickly increased after the transition of the culture onto the maturation medium (Fig. 2d). The signal intensity detected after 3 days remained more or less unchanged for 2 weeks and then substantially decreased during the third week on the maturation medium. Later on, isolation of intact RNA from the culture was not successful.

The effects of ABA removal on embryo development and expression of *PaVP1*

In order to study the impact of ABA on *PaVP1* expression and embryo development and to compare *PaVP1* expression in another line with high embryogenic capacity, the line C110 was cultured on the maturation medium with ABA for 2 weeks. Thereafter, the cultures either continued further in maturation on the medium containing ABA for additional 3 weeks, or were transferred, in parallel, onto the maturation medium without ABA. Five weeks long maturation of C110 line on the medium with ABA resulted in fully developed embryos comparable with those of AFO 541 line. The majority of early embryos transferred onto the ABA-free medium after 2 weeks of maturation became vitrified and underwent callus formation. Only few partially malformed embryos (Fig. 1h) developed under these conditions, but later on, they were not able to germinate.

The expression of *PaVP1* in C110 line kept on a high level on the maturation medium containing ABA, reaching its maximum after 5 weeks (Figs. 2e, f). Removal of ABA resulted in disappearing of *PaVP1* transcript in both the whole culture (not shown) and even in separated embryos. The "lower band" remained on relatively high level even after ABA removal (Fig. 2g).

Discussion

Is the transcript of *PaVP1* alternatively spliced?

Expression of *PaVP1* was on a high level during maturation in both lines with high embryogenic capacity, C110 and AFO541, culminating after 5 weeks, at the time when the embryos developed into the early cotyledonary stage. Similar trend in *PaVP1* expression was demonstrated by Footitt et al. (2003) in 95:88:22 line. Though embryos in that line developed by 2 weeks earlier, the peak in *PaVP1* expression still correlated with the early cotyledonary embryo stage. Despite the similar trend, the relative transcript levels detected in our study by northern blot hybridisation differed strongly from that determined by semi-quantitative PCR by Footitt et al. (2003). In our study, the maximum level of *PaVP1* transcript (in cotyledonary embryos) was by about two orders of magnitude (!) higher as compared to the level detected on the proliferation media. In contrast, Footitt et al. (2003) demonstrated relatively high levels of *PaVP1* transcript already in the proliferation phase, and the expression increased only approximately three times (!) towards the peak at the end of maturation. Though the difference might be explained by dissimilar character of the lines, used in the two studies, and by differences in the cultivation conditions, there is another possibility related to the nature of *PaVP1* probe hybridisation on northern blots. The "lower band" levels resembled those detected by Footitt et al. (2003) much better than the levels of the longer *PaVP1* band. Since the method of RT-PCR could not distinguish between transcripts of variable length outside the amplified region, both RNAs hybridising with *PaVP1* probe might have been amplified by RT-PCR at once and products of identical size were obtained.

The shorter hybridising band could be either a product of alternatively spliced mRNA of *PaVP1* gene or might represent a transcript of some other B3 domain transcription factor. Since no other genes encoding B3-domain proteins were found in spruce, we tested the hypothesis of cross-hybridisation among *Arabidopsis* genes encoding proteins containing B3 domains. The best Blastn (Altschul et al. 1997) matches in *Arabidopsis* transcript database were genes encoding transcription factors LEC2 (acc. no. *AT1G28300.1*) and FUS3 (acc. no. *AT3G26790.1*), which shared 85% and 83% identity with the *ABI3* gene, but just in a very short region of 65 and 56 nucleotides, respectively (data not shown). Since the whole length of the *PaVP1* probe used for hybridisation was about 450 nucleotides, cross-hybridisation could be hardly expected with these genes. It is still possible, that there is an additional differentially regulated gene in spruce, which encodes a short VP1-like B3-domain transcription factor. Presence of three paralogues *VP1*-like genes, slightly differing in length, was demonstrated in *Physcomitrella patens*. Though it was suggested that two out of them had been lost in the seed plant lineage, genomic data confirming this view are available only for angiosperms (Marella et al. 2006). Therefore, the presence of a truncated *VP1* paralogue in gymnosperms cannot be excluded.

The "lower band" might also represent a product of alternative splicing, demonstrated in wheat embryos, where

the majority of *VP1* transcripts were spliced incorrectly, contained insertions of intron sequences or deletions in the coding region (McKibbin et al. 2002). In contrast to the situation in wheat, the ratio between the *PaVP1* transcript and the "lower band" correlated with the culture stage, suggesting that the supposed alternative splicing might have participated in the regulation of full-length PaVP1 protein synthesis.

Proper embryo development was connected with high *PaVP1* expression

Removal of ABA during maturation resulted in dramatic decrease in *PaVP1* expression accompanied by strongly impaired embryo development. However, few malformed embryos developed under these conditions, suggesting that once initiated, the maturation (of impaired and germination incapable embryos) can continue even in the absence of ABA and *PaVP1* expression. Exogenously supplemented ABA was necessary for both induction and maintenance of high *PaVP1* expression in agreement with the situation in *Arabidopsis*, where either maternally derived ABA, necessary for the induction of embryo maturation, or embryo-synthesized ABA accompanied the whole process of embryo development (Karssen et al. 1983).

PaVP1 expression was detectable only in the embryogenic lines, corresponding with predominantly embryonic tissue specific expression of *VP1* orthologues (Holdsworth et al. 1999). In any embryogenic line, application of ABA resulted in strong increase in *PaVP1* transcript level. In developmentally arrested C203 line, the rise in *PaVP1* transcription was similar to that observed in high embryogenic line a preceded disintegration of proembryonic centres. It indicates that the incapability of the line to develop mature embryos in response to application of ABA was unlikely connected with *PaVP1* signal transduction pathway. Our results also indicated that high initial expression of *PaVP1* was not sufficient for embryo maturation, though *ABI3* (*VP1* orthologue) expression was demonstrated to be intimately connected with the complex regulatory network controlling embryo development (To et al. 2006). Differences in *PaVP1* expression between the developmentally arrested line and the lines with high embryogenic capacity were not significant until the line had exhibited macroscopic and anatomical developmental alterations. Therefore, the expression of *PaVP1* cannot be used as a marker for pre-evaluation of the embryogenic potential of the lines. On the other hand, stable or increasing expression of *PaVP1* accompanied proper maturation of somatic embryos in all experiments, so it could be regarded as a good marker of this process.

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