RESEARCH PAPER

Expression of the viviparous 1 (Pavp1) and p34cdc2 protein kinase (cdc2Pa) genes during somatic embryogenesis in Norway spruce (Picea abies [L.] Karst)

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Abstract

Detailed expression analysis of the Norway spruce (Picea abies [L.] Karst) Viviparous 1 (Pavp1) and p34cdc2 (cdc2Pa) genes was carried out during somatic embryogenesis. Pavp1, a gene associated with embryo development, was expressed in proliferating embryogenic suspension cultures in the absence of exogenous ABA. When somatic embryo formation was promoting by blocking proliferation, Pavp1 expression was reduced. During maturation, exogenous ABA induced increased Pavp1 expression, which peaked at the early cotyledonary stage of somatic embryogenesis. Following partial desiccation of mature somatic embryos at high relative humidity, Pavp1 expression persisted under germination conditions. Pavp1 expression was also detected in non-dormant immature male strobili and dormant terminal buds. These data confirm the functional conservation of Pavp1 during the evolution of seed plants and extend its function beyond the embryo.

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Introduction

A plant’s progression through embryogenesis requires the expression of a complex array of genes, some of which are specific to discrete phases of embryo development. Their expression is in turn, co-ordinately controlled in response to an array of developmental signals. In angiosperm embryogenesis a number of genes have roles in the control of meristematic activity and differentiation (Laux and Jürgens, 1997; Wobus and Weber, 1999). Many of these are putative transcription factor genes which profoundly influence embryo development by inducing (Li and Thomas, 1998; McCarty, 1995) and/or repressing (Hoecker et al., 1995; Paek et al., 1998) a potentially diverse complement of genes.

In gymnosperms, understanding of the molecular regulation of embryogenesis is limited. However, advances in conifer somatic embryogenesis now allow a more detailed investigation of gymnosperm embryogenesis. This is especially so in Norway spruce, where somatic embryo production is well characterized, as are the growth and morphology of distinct cell lines (Bozhkov and von Arnold, 1998; Filonova et al., 2000a, and references therein).

Gymnosperm embryogenesis classically has three phases: (1) pro-embryogeny, those events prior to suspensor elongation; (2) early embryogeny, which includes elongation of the suspensor and events prior to root meristem formation; and (3) late embryogeny, the formation of the root and shoot meristems and development leading to embryo maturity (Misra, 1994). During Norway spruce somatic embryogenesis, the early and late
embryo stages are well characterized, however no proembryo stage has been identified (Filonova et al., 2000a, and references therein). Hence, Norway spruce somatic embryogenesis provides an opportunity to study the biochemical and molecular processes of gymnosperm embryogenesis in the absence of maternal tissues.

At the molecular level the embryo-specific conifer genes identified are predominantly homologous to angiosperm seed storage protein and Lea (Late embryogenesis abundant) genes (Dunstan et al., 1998; Misra, 1994). This suggests that genes central to embryogenesis will exhibit a high degree of conservation with their angiosperm cousins despite the passage of approximately 300 million years. Hence, Norway spruce somatic embryogenesis system for the homeobox gene PaHB1 (Ingouff et al., 2001).

In this study, the potential has been demonstrated of the Norway spruce somatic embryogenesis system for detailed investigations of the molecular events of gymnosperm embryogenesis as development proceeds from proliferating pro-embryogenic masses (PEMs), to differentiating somatic embryos and finally to germinable embryos. To this end the expression patterns of two genes were examined, Pavp1, a Norway spruce homologue of the angiosperm VP1/ABI3 gene family and cdc2PA a Norway spruce p34cdc2 protein kinase homologue (Kvarnheden et al., 1995). The VP1/ABI3 gene family controls the expression of embryo maturation genes, the acquisition of desiccation tolerance and dormancy (Wobus and Weber, 1999, and references therein). The p34cdc2 protein kinase genes are involved in cell cycle progression and apoptosis (Shi et al., 1994; Fowler et al., 1998).

Materials and methods

Plant material

The following Norway spruce organs were used. Hypocotyls, roots and shoot apical meristems were collected from 6-week-old seedlings, grown in continuous light at 20 °C. Needles were from 1-year-old plants. All were derived from seeds of the Saleby seed orchard, central Sweden. The Emmaboda (56° 38’ N) seed population provided terminal buds from plants grown in continuous light at 20 °C (non-dormant) or under 8/16 h light/dark cycles at 20 °C for 28 d (dormant). Immature non-dormant male and female strobili were harvested 2 weeks prior to pollen release and pollination from a local 40-year-old tree. Mature pollen was collected from a seed orchard.

Embryogenic cell lines of Norway spruce were initiated from zygotic embryos and maintained as described in Bozhkov and von Arnold (1998). The Norway spruce cell lines used were the A type lines, A66 and 95:88:22. A66 was the source material for the original cDNA library (Sabala et al., 1997); cell line 95:88:22 was the source material for the rapid amplification of cDNA ends (RACE) and expression analysis.

Embryo culture

Proliferating embryogenic suspension cultures were maintained in 0.5 l Erlenmeyer flasks containing 100 ml of half-strength LP (0.5 LP) at pH 5.8 (pH 6.6 prior to autoclaving) in the dark at 22±1 °C on a gyratory shaker at 100 rpm and subcultured weekly. Prior to transfer to maturation media, cells were collected on a 100 µm nylon mesh, and washed in 100 ml plant-growth-regulator-free 0.5 LP minus NH4NO3 and sucrose. Washed cells (1 g) were inoculated into 100 ml pre-maturation media (PGR-free 0.5 LP, pH 5.8) supplemented with 1 mM NH4NO3, and 20 mM 2-(N-morpholino)-ethane sulphonic acid (MES) (pH 6.4), cultured for 7 d then re-washed as above. Pre-matured cells were then inoculated on to the maturation media, BMS-1 at pH 6.0 (pH 6.4 prior to autoclaving), as amended in Bozhkov and von Arnold (1998) and including 7.5% (w/v) polyethylene glycol 4000 (PEG 4000), 24 μM ABA and 20 mM MES (pH 6.4). In liquid maturation media, PEG 4000 was omitted and 0.5 g of cells inoculated into 50 ml of media in 0.25 l Erlenmeyer flasks and incubated as above for up to 48 h. With solid media, 0.5–1 g of cells was spread in a thin layer over two Whatman No. 2 filter papers held on BMI-S1 media in 6 cm Petri plates. Cells were cultured in the dark at 22±1 °C and the filter papers transferred to fresh media every 14 d for 56 d.

Embryo partial desiccation and germination

At the end of maturation, fully developed mature somatic embryos were partially desiccated under high relative humidity (HRH) conditions for 28 d. Embryos were then transferred to germination conditions (Bozhkov and von Arnold, 1998).

Gene isolation

Degenerate primers based on the sequence of the conserved B3 domain of the Zea mays L. vp1 (M60214) and its homologues from Arabidopsis thaliana (L.) Heynh. (X68141), Oryza sativa L. cv. Nipponbare (D16640), Phaseolus vulgaris L. cv. Tendergreen (U28645), and Hordeum vulgare L. var. distichum cv. Bomi (Y09939) were used (VP3US, nt 1850–1868, 5¢-A(C/T)/C(T)/TGTCC(A/G)GACCT(A/C/G)(A/G)AG(A/G)-3¢; VP3DS, nt 2009–2034, 5¢-GTA(G/T)A(G/T)/G/AC(A/G)/ATGAAATA(C/A)/CC(C/T)/TC(T)/T-3¢; VP4US, nt 1853–1871, 5¢-TGCC(A/G)GAGCTC(A/C/G)(A/G)AG(A/G)-3¢; VP5DS, nt 2005–2027, 5¢-AC(A/C)/ATGAAAATA(C/A)/CC(C/T)/TC(T)/TGC(A/G)-3¢).

DNA (200 ng) from an A66 cell line cDNA library was amplified by the polymerase chain reaction (PCR) using the primer pair VP3US/VP3DS. The products were re-amplified with nested primers (VP4US and VP5DS). The resulting fragments were cloned into pGEM-T Easy (Promega Corp. Madison, WI, USA), and transformed into the host JM109. Positive clones were sequenced using the Thermo Sequenase dye terminator cycle sequencing kit (Amersham, Little Chalfont, UK). One clone contained a 344 bp fragment with sequence homology to the B3 domain of previously reported vp1 homologues.

This sequence information was used to design the specific primers; VP7US, nt 1850–1868, 5¢-ATCTTTCTGAAGCTGGGACG-3¢; VP7DS, nt 1897–1917, 5¢-TACTCGAGACGTTACAATGCTC-3¢; VP8US, nt 1787–1804, 5¢-TCAAGCAGGACACGGCTTG-3¢; VP8DS, nt 1955–1978, 5¢-TATTTTCAAGCAGATACATCCTGC-3¢; and VP9DS, nt 1887–1914, 5¢-CGAGACGTTACAATGCTCCTCCATCG-3¢. These primers were then used in conjunction with the dT17 anchor primer (5¢-GACTCGAGTCGACATCGA(T)17-3¢) and an adapter primer (5¢-GACTCGAGTCGACATCGA(T)17-3¢) to isolate the entire Pavp1 sequence by RACE (Zhang and Frohman, 1997) using DNase-treated total RNA (5 μg) extracted from mature somatic embryos after Chang et al. (1993). The resulting 3¢ and 5¢ ends were cloned and sequenced as above.
Sequences analysis used the MACVECTOR™ program (Oxford Molecular Group plc, UK).

Southern analysis

Genomic DNA was isolated from proliferating 95:88:22 cells (Aljanabi and Martinez, 1997) and 16 µg cleaved with BamHI and EcoRI. Digests and a copy number reconstruction were run out on a 1% agarose gel and blotted to Hybond-N+ and probed with a PCR product containing the genomic B3 domain. For calculating copy number reconstructions, the nuclear DNA content of the diploid Norway spruce genome was taken as 32 pg (Mo et al., 1989).

Expression pattern and specificity

The Pavp1 expression pattern was determined in proliferating and pre-maturing embryogenic cultures and during somatic embryogenesis, partial desiccation and germination. During maturation on solid media, individual embryos were picked at distinct stages of development based on the classification of Dong et al. (1997b) (Fig. 1). Samples of 50 embryos were taken during the partial desiccation and germination treatments. The specificity of expression was determined in a range of Norway spruce vegetative and reproductive organs. Samples from liquid culture were recovered on nylon mesh (100 µm) and weighed. All samples were frozen in liquid nitrogen and stored at –80 °C.

From each sample, 5 µg of total RNA was DNase-treated and reverse transcribed using the dT17 anchor primer then purified using the High Pure PCR product purification kit (Boehringer Mannheim GmbH, Mannheim, Germany). The expression patterns of Pavp1 and cdc2Pa were determined by PCR. The Pavp1 (460 bp) and cdc2Pa (638 bp) cDNA fragments amplified both spanned introns. A Pavp1 fragment was amplified from a 0.01 dilution of cDNA template using the gene-specific primers VP12US (nt 1639–1661, 5'-TTGCTCTCATCCACAGCAATGCCC-3') and VP12DS (nt 2076–2098, 5'-ATGTTGTTGCTCGACCCTGGGCACC-3'). A cdc2Pa fragment was amplified from the same dilution using the primers p9 and p11 (Kvarnheden et al., 1995). Expression analysis of Pavp1 in cDNAs from proliferation and pre-maturation treatments used a 0.1 dilution owing to the low expression level. All PCR reactions used five replicates. The expression patterns of the genes were determined by running out each reaction on 1% (w/v) agarose gels. Gels were then Southern blotted to Hybond-N+ and hybridized with radiolabelled probes. The Pavp1 probe was internal to the Pavp1 fragment and the cdc2Pa probe was the p9/p11 fragment. The hybridized bands were of the expected sizes (Fig. 4). Southern blots were exposed on PhosphorImager screens (Molecular Dynamics Inc., Sunnyvale, CA, USA). Expression was quantified by integration of peak areas for each hybridized probe. Data are expressed as the mean number of counts per peak area ± the standard error of the mean. The signal intensity was calibrated to be within the linear range of the PhosphorImager screens by probing Pavp1 and cdc2Pa fragments amplified from increasing volumes of cDNA dilutions from proliferating and ABA-treated cells. Consequently, 4 µl of cDNA template was used in a 20 µl reaction for expression analysis.

To determine the specificity of Pavp1 expression RT-PCR was carried out as above on total RNA from a series of vegetative and reproductive organs. The specific Pavp1 primers VP17US (nt 1018–1041, 5'-TCTCATCACAGGGCTTTGAAAGG-3') and VP12DS were used to amplify a 1080 bp region encompassing the B3 domain. The cdc2Pa primers were used as above. PCR reactions were analysed as above.

Results

Isolation and characterization of Pavp1

The full length Pavp1 cDNA (accession number AF175576) was 2726 bp to the start of the poly-A tail. The first start codon occurred at nucleotide 49, and the first termination codon (TAA) at nucleotide 2515. This gave an open reading frame of 2469 nucleotides, translating to a protein of 823 amino acids with a predicted pI of 4.89 and molecular weight of 90 558. Sequence alignment of the deduced protein with other vp1 homologues, identified four conserved domains (A1, and B1–B3) within Pavp1 (Fig. 2). These occur at nucleotides 301–417, 805–1005, 1534–1620 (with a highly conserved core sequence at 1552–1602), and 1729–2082. The percentage sequence identity of these protein domains with the gymnosperm, yellow cedar VP1 sequence was; A1, 67%; B1, 95%; B2, 90%; and B3, 98%. And in the angiosperm VP1 sequences was; A1, 23–36%; B1, 55–72%; B2, 47–63%; and B3, 77–91%. The putative nuclear localization signal (NLS) within the B2 domain was RKNR (Fig. 2C) and was confirmed by sequencing the relevant genomic region. The sequence downstream of the B3 domain to the first termination codon was longer than in other VP1 sequences.

The genomic sequence contained five introns (105, 176, 241, 126, and 171 bp long) 3-prime to nucleotides 1741, 1833, 1934, 1981, and 2058, all within the B3 domain (Fig. 2D). Southern analysis indicated that Pavp1 is a single copy gene (Fig. 3).

Organ specificity of Pavp1 expression

Pavp1 transcripts were detected in non-dormant immature male strobili, dormant terminal buds and the embryogenic cell line 95:88:22 (A-cell line) (Fig. 4). No Pavp1 expression was detected in the hypocotyls and shoot apical meristems of 6-week-old seedlings, needles and roots from 1-year-old plantlets, non-dormant immature unfertilized female strobili, non-dormant terminal buds (Fig. 4) or in mature pollen from mature dehiscing male strobili (data not shown). Expression of cdc2Pa, was detected in all tissues (Fig. 4).

**Fig. 1.** Stages of somatic embryogenesis in Norway spruce. Numbers in parenthesis are those used in the classification of Dong et al. (1997b). For a detailed description of the trans-differentiation of PEMs to somatic embryos see Filanova et al. (2000a).
Pavp1 and cdc2Pa expression during somatic embryogenesis

Expression in proliferating and pre-maturing cells: Proliferation media promoted rapid PEM growth with cell mass increasing from 1.0 to 7.3 g over 7 d, acidifying the cultures from pH 5.8 to 4.8. Removal of PGRs and transfer to pre-maturation media promotes the transition from PEMs to somatic embryos. Here, cell mass did not increase, remaining constant at 1 g over 7 d. During this time the culture pH decreased from 5.8 to 5.4. Pavp1 was expressed relatively strongly during culture in proliferation media (Fig. 5). However, on transfer to pre-maturation media Pavp1 expression declined dramatically.
on the first day and remained low (Fig. 5). cdc2Pa expression was lower in proliferation media than in prematuration media, where its expression increased more than 3-fold before decreasing to its original level. As different cDNA concentrations were used direct comparisons cannot be made between Pavp1 and cdc2Pa expression levels.

Expression in maturing somatic embryos: Pavp1 expression was initially indistinguishable from background. After only 1 h in maturation medium Pavp1 expression increased, reaching a plateau after 8 h (Fig. 6) where it remained for the first 48 h (data not shown). The expression of cdc2Pa also increased in early maturation before declining after 2 d. During ABA treatment in liquid culture the pH remained unchanged. As embryo maturation progressed a dramatic increase in Pavp1 expression occurred between day two and the formation of globular/late polar stage embryos (stage 2) which occurs at approximately 14 d (Fig. 7). Expression peaked in early cotyledonary stage embryos (stage 3a, approximately 21 d) but, in embryos with fully formed cotyledons (approximately 25 d) expression had declined to that seen after 2 d, where it remained until the end of maturation (day 56, stage 3b).

Expression during partial desiccation and germination

During HRH partial desiccation, Pavp1 expression initially declined, after which it increased to the end of the partial desiccation period (Fig. 8). During the latter phase of HRH partial desiccation, some embryos became translucent and appeared to rehydrate. On transfer of embryos to germination media, Pavp1 expression declined rapidly over the first 2 d then remained constant before declining from days 36 to 47 (Fig. 8). During this period, germination, as defined by radicle growth, was not observed, although the root tip region of some embryos exhibited anthocyanin accumulation, swelling and root hair formation. The hypocotyls also accumulated anthocyanin and elongated, while the cotyledons turned green and ex-
panded. Isolated zygotic embryos exhibited the same characteristics after 16 d on similar medium where germination was 22 ± 7% after 28 d.

In the case of cdc2Pa, expression remained fairly constant during partial desiccation. On transfer to germination media, expression increased greatly after 4 d before declining (Fig. 8).

Discussion

Structure of the Pavp1 gene

The Pavp1 gene isolated from Norway spruce somatic embryos encodes a predicted protein of 823 amino acids making it longer than the reported gymnosperm [Chamaecyparis nootkatensis D. Don Spach (Lazarova et al., 2002)] and angiosperm VP1 homologues [e.g. Z. mays (McCarty et al., 1991)]. It also possesses a long sequence downstream of the B3 domain to the termination codon. At the protein level, homology is highest within the four conserved domains identified in angiosperm VP1 homologues suggesting a high degree of functional conservation.

The conserved domains of the Pavp1-encoded protein and angiosperm VP1 homologues mainly differ with respect to the putative NLS within the B2 domain. The Pavp1 putative NLS, RKNR fits the non-typical (R/K)2X(R/K) consensus which is also found in the yellow cedar homologue, while the angiosperm homologues predominantly have the RKKR motif [C-ABI3 is an exception (Shiota et al., 1998)] fitting the typical NLS consensus (K/R)4 (LaCasse and Lefebvre, 1995).

In the Pavp1 genomic sequence, the intron positions were conserved between this conifer and the angiosperm VP1 genes and for introns 2–5 in the B3 domain identified within FUSCA3 (Leurssen et al., 1998). Southern analysis also showed Pavp1 to be a single copy gene as found in angiosperms (McCarty et al., 1989). This further highlights the highly conserved nature of this gene over

Fig. 6. Expression analysis by RT-PCR of Pavp1 and cdc2Pa in Norway spruce embryogenic 95:88:22 cells post-pre-maturation treatment during the first 8 h exposure to 24 μM ABA in liquid culture. The cDNA dilutions used were 0.1 in each case and 4 μl of each dilution was amplified. Each point represents the mean ±SE of five independent PCR reactions.

Fig. 7. Expression analysis by RT-PCR of Pavp1 and cdc2Pa in Norway spruce somatic embryos of cell line 95:88:22 during maturation over 56 d at 20 °C in the dark on solid media (pH 6.0) containing 24 μM ABA. The cDNA dilutions used were 0.1 in each case and 4 μl of each dilution was amplified. Each point represents the mean ±SE of five independent PCR reactions.

Fig. 8. Expression analysis by RT-PCR of Pavp1 and cdc2Pa in Norway spruce somatic embryos of cell line 95:88:22 during high relative humidity partial desiccation and culture on germination media at 20 °C in the dark. The cDNA dilutions used were 0.1 in each case and 4 μl of each dilution was amplified. Each point represents the mean ±SE of five independent PCR reactions.
evolutionary time since the gymnosperms diverged from the line leading to the angiosperms. The conservation of these structural features indicates \textit{Pavp1} to be a conifer relative of the angiosperm VP1 genes.

**Organ specificity of Pavp1 expression**

When diverse Norway spruce organs were screened, \textit{Pavp1} expression was detected in non-dormant immature male strobili approximately 2 weeks prior to pollen release. It is proposed that this signal originated from developing and/or dormant pollen within the male strobili. This is the first report of \textit{vp1} expression in male reproductive organs. Pollen from dehiscing male strobili showed no signal. More recently, GUS staining of immature pollen was seen in floral buds of \textit{Arabidopsis} containing the ABI3::GUS construct (S Footitt and MJ Holdsworth, unpublished results). The absence of a signal in non-dormant immature unfertilized female strobili suggests \textit{Pavp1} is only expressed in ovules following fertilization. The expression of \textit{Pavp1} in dormant terminal buds is consistent with the detection of \textit{vp1} homologous transcripts in dormant poplar buds and in growth-arrested (quiescent) vegetative tissues of \textit{Arabidopsis} (Rohde et al., 1999, 2002). This indicates a general role for \textit{Pavp1} in developmental arrest and quiescence as well as embryogenesis.

**Somatic embryogenesis and Pavp1 gene expression**

The expression of \textit{Pavp1} in proliferating cultures of embryogenic cells is consistent with that seen in similar angiosperm cultures (Shiota et al., 1998). This indicates the presence of low levels of endogenous ABA as found in embryogenic cultures of white spruce (Dong et al., 1997a) and carrot where endogenous ABA was higher in embryogenic cells than in somatic embryos (Shiota et al., 1998). The subsequent decrease in \textit{Pavp1} expression during pre-maturation reflects the removal of factors stimulating cell proliferation, and the onset of programmed cell death (PCD) (Filonova et al., 2000b; Dyachok et al., 2002).

Exogenous ABA induced a rapid increase in \textit{Pavp1} expression during the first 24 h of maturation. As maturation progressed, \textit{Pavp1} expression peaked at the early cotyledonal stage of embryogenesis (stage 3a) which represents the end of embryogenesis \textit{sensu stricto}. This stage is characterized by the emergence of cotyledon initials, which develop rapidly into cotyledons over the next few days. Once the early cotyledonal stage of development was attained, expression declined until the end of maturation (stage 3b).

This pattern of \textit{Pavp1} expression during the maturation of Norway spruce somatic embryos is similar to that of angiosperm VP1 homologues during zygotic and somatic embryogenesis (McCarty et al., 1991; Shiota et al., 1998).

During HRH partial desiccation the rapid decline and subsequent increase in \textit{Pavp1} expression paralleled that of moisture content (Bozhkov and von Arnold, 1998). At the end of this treatment, despite signs of hypocotyl extension in some embryos, there was no germination as expressed by radicle growth. Partial hydration under HRH conditions is not permissive for germination, as endogenous ABA is unable to diffuse from the embryos. The resulting increase in \textit{Pavp1} expression reflects its function as a germination repressor (Hoecker et al., 1995).

Following transfer to germination medium, \textit{Pavp1} expression decreased, suggesting a decline in ABA levels. However, despite hypocotyl extension and expansion of the cotyledons, germination as denoted by radicle extension was not seen. This was also observed in isolated zygotic embryos. Maintenance of \textit{Pavp1} expression under germination conditions again reflects its function as a germination repressor (Hoecker et al., 1995). However, the observed hypocotyl and cotyledon growth and persistence of the \textit{Pavp1} signal suggests that prolonged \textit{Pavp1} expression may reflect the residual embryonic nature of some tissues in the transition from embryo to embling during germination.

**Somatic embryogenesis and up-regulated cdc2Pa expression**

Activity of the p34\textsuperscript{G2/M} cyclin-dependent kinase (CDC2) indicates a competence for cell division as it functions in the G2/M transition of the cell cycle initiating nuclear membrane breakdown and chromosome condensation (Shi et al., 1994; Fowler et al., 1998). However, up-regulated p34\textsuperscript{G2/M} transcription, increased CDC2 kinase activity and the above G2/M transition events are also associated with the form of PCD known as apoptosis (Shi et al., 1994; Furukawa et al., 1996; Ye et al., 2001). Therefore, up-regulated expression of the single copy \textit{cdc2Pa} gene during Norway spruce somatic embryogenesis and germination may reflect the latter aspect of cell growth.

During Norway spruce somatic embryogenesis and germination three peaks of \textit{cdc2Pa} expression were seen. These coincide with periods of PCD. The first and most intense peak occurred during pre-maturation. This treatment enhances somatic embryo development by removing factors that stimulate cellular proliferation and PEM formation (Dyachok et al., 2002). The resulting transition from PEM proliferation to somatic embryo formation is marked by a wave of PCD in PEMs (Filonova et al., 2000a, b). The pre-maturation conditions used in this study are now known to block PCD as measured by cell death (Bozhkov et al., 2002). This may explain the prolonged high level of \textit{cdc2Pa} expression if cells destined to undergo PCD were unable to initiate PCD in the absence of an active \textit{cdc2Pa} protein as seen by Ye et al. (2001). This block on PCD may occur at the level of \textit{cdc2Pa} translation, a phenomenon seen in \textit{COMATOSE} gene expression when
germination is blocked (Footitt et al., 2002). The second peak occurred in early embryo maturation coincident with a second wave of PCD that eliminates embryo suspensor cells (Filonova et al., 2000b). The final peak was during germination where PCD is associated with the formation of tracheary and sieve elements, abscission of root cap cells, and cotyledon senescence (Beers, 1997; Filonova et al., 2000b).

If these peaks in cdc2Pa expression are analogous to the transcriptional activation of cdc2 seen during apoptosis (Furukawa et al., 1996), the resulting increase in CDC2 kinase activity may initiate apoptosis by phosphorylation of a plant equivalent of the pro-apoptotic protein, BAD, one of a family of proteins that act as gatekeepers to the apoptotic pathway (Konishi et al., 2002). This implicates cdc2Pa not only as a controlling factor in the progression of cell division but also in the initial stages of an apoptosis-like pathway of PCD during somatic embryogenesis in Norway spruce. Other features of apoptosis as found in the animal kingdom have also been identified during PCD in Norway spruce somatic embryogenesis (Filonova et al., 2000b). Together, these data indicate the kingdom wide conservation of aspects of the apoptosis pathway during evolution.

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