Cloning and expression of an ABSCISIC ACID-INSENSITIVE 3 (ABI3) gene homologue of yellow-cedar (Chamaecyparis nootkatensis)

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Received 15 November 2001; Accepted 21 December 2001

Abstract
A homologue of the ABI3 gene was isolated from the conifer species, Chamaecyparis nootkatensis. The deduced protein of 794 amino acids exhibited sequence similarity to other VP1 ABI3 proteins within four regions. Expression occurs exclusively in seeds, with no detectable mRNA in leaves and roots. Unlike the homologues of angiosperms, CnABI3 may be encoded by more than one gene.

Key words: Abscisic acid, ABI3, seed dormancy, yellow-cedar.

ABI3/VP1 proteins are members of a large group of transcription factors which act as intermediaries in regulating abscisic acid (ABA)-responsive genes during seed development. Lesions in the genes affect the later stages of seed development and have adverse effects on many important processes such as reserve deposition, dormancy imposition and the acquisition of a tolerance of seed tissues to desiccation. ABI3 does not act in isolation to control gene expression central to seed maturation programmes, but rather acts in concert with various other transcription factors such as LEC1, LEC2 and FUS3 (Harada, 2001); these interactions prevent the precocious activation of genes associated with germination and growth (Nambara et al., 2000). Homologues of VP1/ABI3 genes have been isolated from several angiosperm species, and recently from a woody angiosperm (Rohde et al., 1998).

Following dispersal from the parent tree, seeds of yellow-cedar (Chamaecyparis nootkatensis D. Don Spach) are dormant and require several months of moist chilling before they will germinate. The dormancy mechanism of this conifer species is complex and ABA has been implicated as a key regulator (Schmitz et al., 2002). The isolation of the ABI3 gene homologue from yellow-cedar (CnABI3) is reported here and its expression at the mRNA level characterized.

Yellow-cedar seeds (clone 108) at mid-maturation were collected from the Mount Newton Seed Orchard (Saanichton, BC, Canada). Embryos and megagametophytes were excised from the developing seeds, flash frozen in liquid nitrogen and stored at −80 °C prior to use. Mature seeds of seedlot 30156 were obtained from the Tree Seed Centre in Surrey, BC, Canada. Analyses were also conducted on leaves and roots of 15–20 cm seedlings; these were immediately ground to a fine powder in liquid nitrogen and stored at −80 °C.

Yellow-cedar seeds at different stages (mid-maturation, maturity and mature seed subjected to different durations of a dormancy-breaking treatment) (Ren and Kermode, 1999) were pooled together for extraction of total RNA. Poly(A)⁺ RNA was purified from total RNA using the PolyATtract magnetic-bead system (Promega, Madison, WI, USA) and the poly(A)⁺ RNA was used for cDNA library construction with the ZAP express cDNA synthesis kit from Stratagene (La Jolla, CA, USA) using the primer 5’-(GA)₁₀ACTAGTCTCGAG(dT)₁₅-3’ for reverse transcription. PCR was used to clone a partial ABI3/VP1-like gene fragment from this cDNA library. Based on the sequences of highly conserved regions of ABI3/VP1 genes from other species, a degenerate primer was designed (5’-GTNTGG-AAYATGMGNTAY-3’ (fwd)) and used for PCR together with an anchor primer, 5’-GAGGAGACTAGTCTCGAG-3’ (rev). Products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and sequence analysis identified a clone having high homology to other ABI3/VP1 genes. A 3²P-labelled probe using this cDNA as a template was synthesized for cDNA library screening. In total, six positive clones were obtained from library screening and pBK-CMV phagemids containing the inserts were excised by the in vivo method from the ZAP express vectors. All six clones were sequenced and compared with ABI3/VP1 genes from other species. One of the clones, B11 (designated CnABI3) contained the entire coding region for an ABI3/VP1-like protein, as well as 516 bp of 5’-untranslated sequence and 300 bp of a 3’-flanking region. Based on sequences of clone B11 cDNA and VP1/ABI3 genes of other species, PCR primers, 5’-ATGGACCAACATGAATTGCG-3’ (fwd) and 5’-CGAGTCACCCAGAATCTGAGAA-3’ (rev) were designed and PCR amplifications were carried out using genomic DNA. A single band product was obtained and directly sequenced for analysis of intron positions and their sizes.

CnABI3 encodes an ABI3/VP1-like protein of 794 amino acids with a predicted molecular mass of 88 kDa and isoelectric point of 5.08. The protein from yellow-cedar is the largest so far of the ABI3/VP1 family. Like other homologues, the CnABI3 gene contains six exons and five introns, the latter having sizes of 105, 113, 110, approximately 1000, and 142 bp, respectively. All introns of the CnABI3 gene contain splice sites consistent with the consensus sequence 5’GT ... AG3’. Comparison of the deduced amino acid sequence of CnABI3 to other ABI3/VP1 proteins by multiple-alignment (Fig. 1) indicates that the homologue of yellow-cedar has all four regions that are typically conserved. These include the three highly conserved basic regions: B1 (aa 261–327), B2 (aa 459–490) and B3 (aa 533–651). The core of B2, RKRR, is cited by several authors as a putative nuclear targeting motif and is invariably in all angiosperm...
Fig. 1. Alignment of the deduced amino acid sequence of *Chamaecyparis nootkatensis* ABI3 protein (CnABI3) with that of other VP1 ABI3 proteins. VP1/ABI3 proteins (CnABI3 [a]13113; PtABI3, *Populus trichocarpa* ABI3 [a]003165; PvAlf, *Phaseolus vulgaris* ABI3-like factor [u]28645; ABI3, *Arabidopsis thaliana* ABI3 [x]68141; VP1, *Zea mays* VP1 [m]60214) were aligned and compared with the computer programs Clustal W and Boxshade. The four darkly shaded boxes correspond to the previously described regions of highest sequence homology: the acidic A1 region and the three basic regions, B1, B2 and B3. The putative nuclear targeting signals (RKNR located in the B2 region, and RKKK of CnABI3, located in the B1 region) are indicated by the solid dots above the sequence.
homologues characterized so far. In the yellow-cedar protein, this sequence of the B2 region is RKNR. However, a putative nuclear targeting motif (RRKR) is found in CnABI3 at position 325–328, within the B1 region. In the B3 region, the sequence identity is over 90% within a stretch of 119 amino acids. There is a substitution of glycine (which is conserved in all other homologues) with valine at position 593. The N-terminal region contains the acidic domain (A1) that plays an important role in the function of these proteins as transcription activators; this region shares a much lower degree of homology, which is typical of other ABI3/VP1 proteins (Fig. 1).

Southern blot hybridization conducted under high stringency conditions exhibited multiple bands of varying intensities for DNA samples digested with BamHI, EcoRI or HindIII (Fig. 2A). Two BamHI sites occur at 1222 and 1236 bp in B11 (Fig. 2B); if CnABI3 is encoded by a single gene, there should be two bands only, instead of the three detected (Fig. 2C, predicted numbers of bands on Southern blot). Similarly, EcoRI digestion should yield a single band (due to a site at 687 bp in B11). There is no HindIII site, therefore only one band is expected instead of the two (or possibly three) that are evident. Figure 2C also shows predicted numbers of bands that would be consistent with a gene copy number of two and three copies. Sequence analysis revealed no BamHI, EcoRI or HindIII sites within the introns of the CnABI3 gene; the numbers of fragments on the Southern blot are inconsistent with a single copy or multiple copies (2 or 3 copies) of a single gene. Although inconclusive, the results are indicative of a gene family for CnABI3 in this coniferous species, or perhaps some other gene(s) with high homology to CnABI3 (e.g. FUSCA3; Leurssen et al., 1998). This is in contrast to the homologues of angiosperms, in which ABI3/VP1 is encoded by a single gene. A possible exception may be the homologue of Phaseolus vulgaris, in which an additional band is revealed on Southern blots, although only under lower stringency conditions (Bobb et al., 1995).

Under normal growth conditions, expression of the CnABI3 gene occurs exclusively in seeds of yellow-cedar, with no detectable mRNA in leaves and roots of young seedlings (Fig. 3). Expression in seeds is not confined to mid-maturation (Fig. 3), but is also detected in both the embryo and mega-gametophyte of the mature (dormant) seed (data not shown). In Avena fatua, expression of the VP1 gene is correlated with the degree of embryo dormancy and may be important for maintaining ABA-controlled metabolism in the imbibed seed (Jones et al., 1997). The role of CnABI3 in maintaining dormancy of yellow-cedar seeds is presently being examined.

Acknowledgements
This research was supported by a Forest Renewal BC grant and a Natural Sciences and Engineering Research Council of Canada Strategic grant awarded to ARK.

References