Expansins in the bryophyte Physcomitrella patens

Plant Molecular Biology **50:** 789–802, 2002.

Expansins in the bryophyte *Physcomitrella patens*

Ori Schipper[1], Didier Schaefer[2], Ralf Reski[3] and Andrew Fleming[1,*]
[1]*Institute of Plant Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 2, 8092 Zurich, Switzerland (*author for correspondence; e-mail: andrew.fleming@ipw.biol.ethz.ch)*
[2]*Institute of Ecology, University of Lausanne, 1015 Lausanne, Switzerland;*
[3]*Plant Biotechnology, Albert-Ludwigs University, 79104 Freiburg, Germany*

Received 22 January 2002; accepted in revised form 24 April, 2002

Key words: abiotic stress, auxin, expansin, gene targeting, Physcomitrella patens

Abstract

Expansins are cell wall proteins which play a key function in basic processes of plant growth and differentiation. It has been proposed that expansins are likely to be present in all land plants and, to date, they have been reported in angiosperms, gymnosperms and pteridophytes. In this paper, we provide the first report and analysis of genes encoding expansin-like proteins in the bryophyte, *Physcomitrella patens*. Our analysis indicates that both α- and β-expansins are present as gene families in this plant and expression analysis indicates that these genes are subject to a complex regulation by both hormonal and environmental factors. In particular, the expression of many expansin genes in *P. patens* is upregulated by stress conditions, suggesting that they play a role in the specific cellular differentiation displayed by *P. patens* in response to such stress. Finally, we provide the first report on the generation and analysis of a series of knockout mutants for individual expansin genes.

Abbreviations: IAA, indole-acetic acid; BAP, 6-benzylaminopurine; ABA, abscisic acid; npt, neomycin phosphotransferase; KO, knockout

Introduction

Expansins are extracellular proteins involved in cell wall loosening (reviewed in Cosgrove, 2000; Lee *et al.*, 2001) and have been implicated in hypocotyl growth (McQueen-Mason *et al.*, 1992), rice internode expansion (Cho and Kende, 1997) and fern rachis extension (Kim *et al.*, 2000), as well as specific events of morphogenesis (Cho and Cosgrove, 2000; Pien *et al.*, 2001) and differentiation (Rose *et al.*, 1997). Since their first characterisation in cucumber (McQueen-Mason *et al.*, 1992), genes encoding expansin-like proteins have been identified in a wide range of plants including angiosperms (Shcherban *et al.*, 1995), gymnosperms (Hutchison *et al.*, 1999) and ferns (Kim *et al.*, 2000) and it has been proposed that expansins are likely to be present in all land plants (Shcherban *et al.*, 1995). However, until now reports on expansins in bryophytes have been limited to two sequence reports, with no analysis of expression pattern or function (Li *et al.*, 2002).

Bryophytes (consisting of mosses, liverworts and hornworts) are considered to be amongst the simplest land plants (reviewed in Cove *et al.*, 1997). They display an alternation of generations with the haploid gametophyte forming the predominant phase. In mosses, the gametophyte consists of a branching system of cells (termed the protonema) from which leafy gametophores arise. As with all plants, the form of the moss plant is determined by the pattern of growth and division. However, in contrast to morphologically more complex plants (such as angiosperms), these changes in cell growth and division can be followed continuously and non-invasively in moss under the microscope. Coupled with the well-characterised regulation of developmental transitions by hormonal and environmental triggers, the moss gametophore is an attractive system not only to test the prediction

that expansin genes are present in bryophytes, but it also allows testing of the purported role of expansins in modulating plant growth and form via modulation of cell wall extensibility. In particular, the moss *Physcomitrella patens* offers the potential of tackling this problem using a molecular genetic approach due to the facility of generating knockout mutants via gene targeting (Schaefer and Zrijd, 1997; Strepp *et al.*, 1998; Schaefer, 2001).

We set out to use *P. patens* as a system to identify and characterise expansin genes in a bryophyte. Our results show that *P. patens* contains a multigene family encoding expansins. Although our analysis indicates that the encoded proteins generally contain all the motifs characteristic of expansins, the sequence divergence indicates an evolutionary separation of bryophyte α-expansins from those found both in seed plants and ferns. Our data also show that expansin gene expression in moss is subject to a complex regulation by hormonal and environmental factors and, in particular, highlight the role of salt and osmotic stress in inducing expansin gene expression. Finally, we have created and analysed a series of gene knockouts for a number of the expansin genes in *P. patens* via homologous recombination.

Materials and methods

Plant material, tissue growth and treatments

The Gransden wild-type strain (Ashton and Cove, 1977) of the moss *Physcomitrella patens*, recently renamed *Aphanoregma patens* (Hedw.) Lindb., was used in this study. The plant was grown axenically in 9 cm Petri dishes on cellophane disks (W. E. Canning, Bristol, UK) overlaid over extended medium (derived from Ashton *et al.*, 1979) containing 500 mg/l (di-)ammonium tartrate and 5 g/l glucose. Plants were grown in a phytochamber (20 hours light at 25 °C/4 hours dark at 23 °C and a quantum irradiance of ca. 120 μmol m^{-2} s^{-1}). Protonemal tissue was propagated by fragmenting it once a week with a blender (Ultraturrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) in sterile water and dispensing in 2 ml aliquots to fresh petri dishes. For experiments, tissue was grown for 5 to 6 days and then transferred to the same medium supplemented with phytohormones or stress agents, as indicated in the results section.

RNA analysis

Tissue was frozen and total RNA extracted using an RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions and quantified by UV spectroscopy. Northern blot analysis was performed using MOPS/formaldehyde gels and Hybond-N nylon membranes (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland) according to the manufacturer's instructions. Size standards (Invitrogen, Basel, Switzerland) were also loaded and blotted. Equal loading of RNA was visualized by methylene blue staining (Herrin and Schmidt, 1988). cDNAs for the various exapnsins were used as substrates for probe synthesis incorporating [^{2}P]dATP using a PrimeIt II kit (Stratagene, La Jolla, California) according to the manufacturer's instructions. Unincorporated nucleotides were removed using ProbeQuant columns (Amersham Pharmacia Biotech). Hybridizations were conducted at 65 °C and membranes washed to a final stringency of 0.5 × SSC, 0.1% SDS at 65 °C before exposure to X-ray films (X-omat AR from Kodak, Lausanne, Switzerland) at −80 °C with intensifying screens.

Identification of expansin sequences

The clone *PpExp1* was identified as follows. 1 μg of total RNA was reverse transcribed using AMV reverse transcriptase (Promega, Wallisellen, Switzerland) according to the manufacturer's instructions. The resulting cDNA served as a template in a PCR reaction using degenerate primers (derived from aligned rice α-expansin sequences; a gift from B. Reidy, ETH Zürich) with the following sequences: α-monocot-ff (5′ atgggyggggcgktgcygg 3′ and α-monocot-rv (5′ ccccarttsckssawcat 3′). Two bands were isolated from a preparative gel and reamplified using a nested α-monocot-ff2 primer (5′ tgcggitacggnaacytntac) with the same α-monocot-rv primer. The nested bands were cloned into the pPCR-Script Amp vector (Stratagene) and positive clones selected by southern blotting with a DIG-labeled full-length cDNA of *OsExp2* (a gift from H. Kende, Michigan State University). The hybridising plasmid insert was sequenced (Microsynth, Balgach, Switzerland). The 3′end of the cDNA was then obtained using a Marathon cDNA amplification kit (Clontech, Allschwil, Switzerland) and the following primers: 3′RACE1 (5′ aggcgatgtgcacgcagtagacatcaagg 3′) and 3′RACE2 (nested; 5′ tcaagggatccaatacagaatggattccc 3′). The 5′-end was

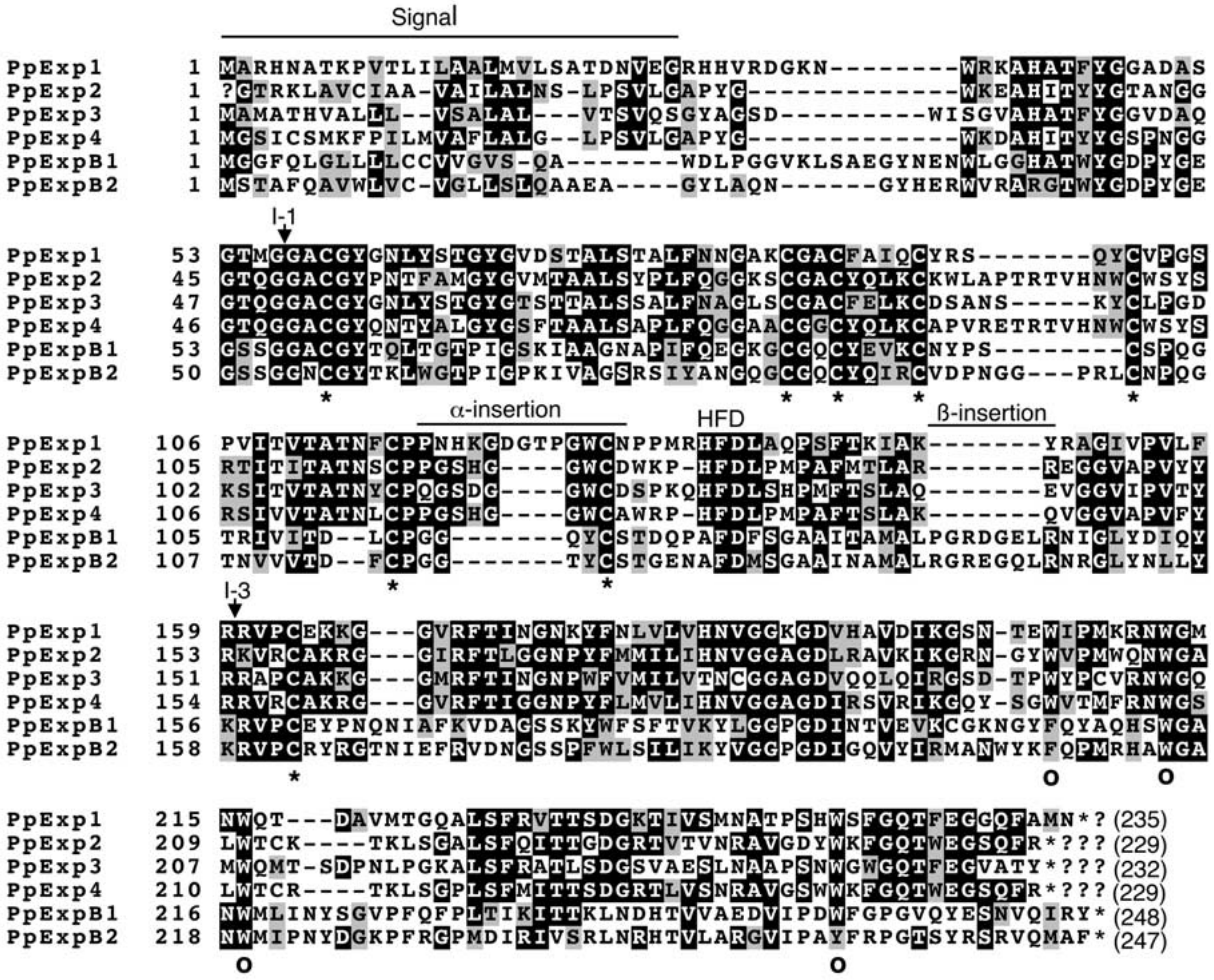

Figure 1. Alignment of amino acid sequences predicted from *P. patens* cDNAs. Sequences were aligned with the ClustalW 1.8 program using the PAM250 residue weight table and default parameters. The predicted signal peptides are indicated as 'signal'; the strictly conserved residues are highlighted with black boxes, conserved residues are highlighted with grey boxes. The 8 conserved cysteine residues are indicated by (∗); the four conserved tryptophan residues by (o). The positions of the α- and βinsertion sequences are indicated, as are the positions of the introns 1-1 and I-3 and the HFD motif. Amino acid positions after the stop codon were not taken into consideration for the alignment and are indicated by (?).

identified using a SMART RACE cDNA Amplification kit (Clontech) with 0.5 μg isolated mRNA (PolyATtract kit from Promega) as template and the following gene specific primers: 5fullRACE1 (5′ atctcgggatatctgggtaaagcgtgg 3′) and 5fullRACE2 (nested; 5′ caattcatagcgaactgacctccctcg 3′). The amplified products were cloned into the pPCR-Script Amp vector (Stratagene) and sequenced. The full-length sequence of *PpExp1* was submitted to GenBank and has the accession number AY028634. The cDNA sequences of the other *P. patens* expansin genes (PpExp2 to PpExp132) were kindly provided by Jens Lerchl and Elke Duwenig (BASF Plant Science, Ludwigshafen, Germany) upon BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1990) of an EST library generated by Freiburg University and BASF Plant Science. The expansin cDNAs and genomic sequences have been given the following accession numbers: *PpExp2* (bankit448883), *PpExp3* (bankit442138), *PpExp4* (bankit448901), *PpExpB1* (bankit448903) and *PpExpB2* (bankit448909).

Molecular cloning and PCR analysis

The following PCR primers were used for the amplification of genomic fragments of the different expansin genes of *P. patens*: Exp1KO-ff (5′

gacactcatTCT**A**G**A**tgcactgatggttc 3′; in uppercase the restriction site used for cloning and linearization, in bold mutated nucleotides), Exp1KO-rv (5′ ctgacctccCTCGA**G**ggtctggccgaag 3′), Exp2KO-ff (5′ ctgaaCTC**GAG**gccttcggtgcttg 3′), Exp2KO-rv (5′ gaactg**G**GA**T**CCctcccacgtttg 3′), Exp3KO-ff (5′ gatcC**T**C**GA**Gctggcagcgaaactg 3′), Exp3KO-rv (5′ ctcagGG**ATC**Ctctgaaggagag 3′), Exp4KO-ff (5′ gcttacCT **cgA**Gtgcttggag 3′), Exp4KO-rv (5′ tgaactgG**GA**TCCctcccagg 3′), ExpB2KO-ff (5′ acctggtacggGGATCCttatggcg 3′), ExpB2KO-rv (5′ gccatttgcacTCTAGAcctgtaag 3′). The fragments lacked in particular the start codon (at the 5′end) and the stop codon (at the 3′end) to allow for disruption even when the construct was to be inserted by a single crossing-over event. The reactions were carried out with the HiFi Expand system (Boehringer, Roche Molecular Biochemicals, Rotkreuz, Switzerland) and 50 to 100 ng genomic DNA as template. The amplified fragments were then cloned into pBluescriptII (KS-) (Stratagene).

Site-directed mutagenesis (QuikChange kit from Stratagene) was employed to create an *Eco*RI restriction site in the middle of the *PpExp3* and *PpExp4* fragments with the primers 3mut1 (5′ gtcttccagggga**G**AA**T**TCcatcacagttac 3′; nucleotides in bold are mutagenised, in uppercase the *Eco*RI restriction site) and 3mut2 (which is the reverse complement of 3mut1), as well as 4mut1 (5′ ggtgttgctcttG**AA**TTCtacagaaggtgtg 3′) and 4mut2 (the reverse complement of 4mut1).

The EcoRI fragment carrying a 35S-*nptII*-resistance cassette from pHP23 ΔBam ΔSal (Bonnema *et al.*, 1992) was then inserted into the *Eco*RI site using standard molecular biology techniques (Sambrook, Fritsch *et al.*, 1989). This led to constructs (KO1, KO2, KO3, KO4 and KOB2), where the resistance cassette was flanked at either end by homologous genomic sequences of a few hundred bp (cf. Table 1 for further details).

For PCR analysis of the KO2B lines, the 5′ junction was amplified using primers 5′- ccgagagctcacatc gatactgc-3′ and 5′- gtaggagccaccttccttttcc-3′ leading to an expected amplification product of 920 bp. The 3′ junction was amplified using primers 5′- tggctggaggactatcgatgagg-3′ and 5′- gcatcgccttctatcgccttcttg-3′ leading to an expected amplification product of 650 bp.

Plant transformation and selection of stable regenerants

Protoplasts were isolated from 5 to 6 days old *P. patens* protonemal cultures and transformed using PEG-mediated direct uptake of linearised DNA (Schaefer and Zrÿd, 1997). After overnight incubation in darkness, the protoplasts were embedded directly in top layer and plated on extended medium containing 0.36 M (66 g/l) mannitol.

After 6 days the cellophane disks with the regenerating protoplasts on top were transferred to Petri dishes containing extended medium supplemented with 40 mg/l G-418 (geneticin sulphate; Gibco Europe Ltd., Paisley, UK). After one to two weeks the regenerating colonies were transferred back to non-selective extended medium. One to two weeks later the colonies were subjected to a second selection with G-418 to eliminate the non-stable transformants. The colonies surviving the second selection were picked and subcultured as individual lines.

Sequence analysis

The deduced amino acid sequences were aligned without the predicted signal peptides (http://www.cbs.dtu.dk/services/SignalP/; Nielsen *et al.*, 1997) to other known expansins using the ClustalW 1.8 program available on the web (http://searchlauncher.bcm.tmc.edu/multi-align/ multi-align.html; Thompson *et al.*, 1994). These data included all Arabidopsis expansin sequences, which were kindly provided by Dr Catherine Darley, University of York, UK. The alignment was further adjusted by eye and used to infer phylogenetic relationships by applying the parsimony method with the Protpars program of the PHYLIP package version 3.573c (http://evolution.genetics.washington.edu/phylip.html; Felsenstein, 1993). Bootstrapping was performed using the Seqboot program of the same package. Protein distances were estimated with the programs Protdist and Fitch and then scaled to the output of Protpars as suggested by the programmer (J. Felsenstein, University of Washington, personal communication). The resulting tree was plotted using NJPlot (http://pbil.univ-lyon1.fr/software/njplot.html; Perrière and Gouy, 1996)

Table 1. Efficiency of *PpExp* knockout strategy. For each knockout construct, the length of 5′ and 3′ genomic sequences flanking the selectable marker is given. The relative positions of the genomic fragments used is shown in Fig. 3. The number of stable transformants obtained is given and how many of these lines were analysed. The confirmed knockout lines (as assayed by lack of relevant transcript accumulation) are indicated in absolute numbers, as well as in percentages.

construct	5′ flanking region (bp)	3′ flanking region (bp)	stable transformants	lines analysed	KO	% (KO lines (in lines analysed)
KO1	412	841	21	10	0	0
KO2	432	263	14	10	1	10
KO3	430	494	14	5	4	80
KO4	387	408	22	4	4	100
KOB2	616	282	26	5	4	80

Results

Physcomitrella patens contains a family of genes encoding expansin proteins

To identify genes encoding α-expansins in *P. patens* we used a combination of RT-PCR using degenerate primers and sequence analysis of an EST database. This approach led to the identification of six cDNAs encoding expansin-like proteins. Four sequences encode α-expansin-like proteins (termed *PpExp1* to *PpExp4*), whereas two encode β-expansin-like proteins (*PpExpB1* and *PpExpB2*). An alignment of the encoded proteins is shown in Fig. 1. All six sequences contain a putative signal peptide of 20 to 28 amino acids consistent with an expected extracellular localisation of the translated proteins. After cleavage of the signal peptide, the theoretically mature α-expansins consist of between 229 to 235 amino acids whereas the mature βexpansins contain 247 and 248 amino acids. The amino acid identity between the two predicted *P. patens* βexpansin proteins is 56%. Between the α-expansins it ranges from 44 to 70%, with the highest identity to an angiosperm expansin being 70% shared between PpExp1 and LeExp5 from tomato. All six *P. patens* expansins contain 8 cysteines at conserved positions throughout the protein characteristic of α-expansins (Cosgrove, 2000). Four tryptophan residues conserved within the C-terminal portion of expansins are found in PpExp1, PpExp2, PpExp3 and PpExp4. In PpExpB1 and PpExpB2 a phenylalanine substitution is found at the position of the first tryptophan and a tyrosine substitution at the position of the fourth tryptophan for PpExpB2. The conserved HFD motif characteristic of expansins is found in PpExp1, PpExp2, PpExp3 and PpExp4, but in PpExpB1 and PpExpB2 the histidine residue is substituted by an alanine.

α- and β-expansins can be distinguished by the presence (or absence) of short stretches of amino acids at conserved positions within the consensus expansin sequence (termed α-insertion and β-insertion) (Li *et al.*, 2002: Wu *et al.*, 2001). According to this definition, PpExp1, PpExp2, PpExp3 and PpExp4 contain an α-insertion whereas PpExpB1 and PpExpB2 possess a β-insertion. PpExpB1 and PpExpB2 do not contain a classical α-insertion sequence, although the two cysteine residues normally found flanking an α-insertion are present. Taken together with the altered HFD motif found in these two genes, it is likely that PpExpB1 and PpExpB2 encode novel expansins which we have assigned to the class of β-expansins.

The analysis presented in Figure 1 indicates that *P. patens* expansin sequences show some divergence from those characterised in angiosperms. This divergence was corroborated by a phylogenetic comparison of *P. patens* expansin sequences with those present in the databases from other plants, as shown in Figure 2. This analysis included all annotated expansins from Arabidopsis (Li *et al.*, 2002), plus representatives from a variety of other species. The novel family of γ-expansins (Li *et al.*, 2002) was used as the outgroup. PpExpB1 and PpExpB2 grouped within the family of β-expansins, whereas three of the four *P. patens* α-expansins (PpExp2, PpExp3 and PpExp4) came to lie outside of the identified α-expansin subfamilies

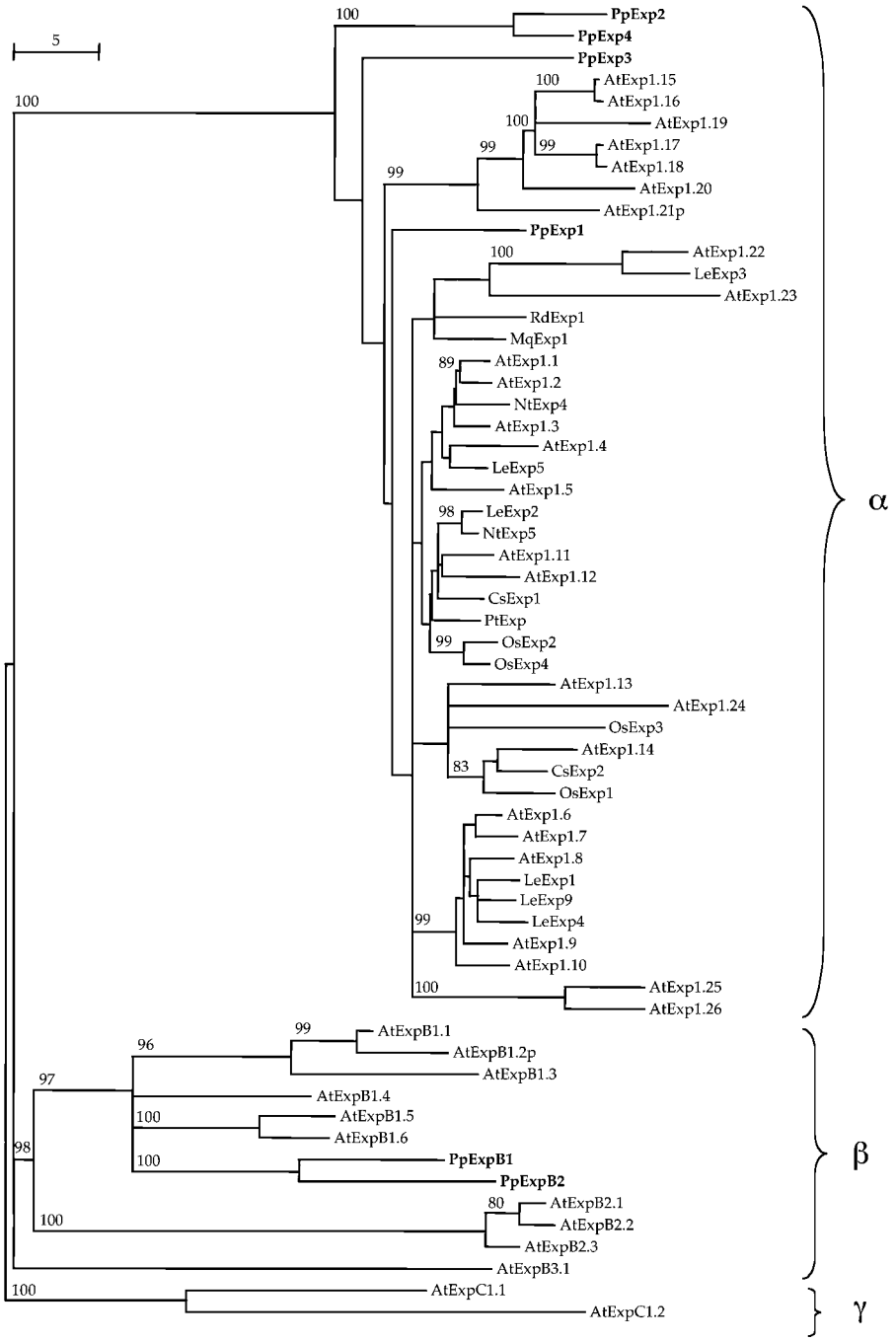

Figure 2. Phylogenetic analysis of *P. patens* expansins and those from other plant species. Phylogenetic analysis of six *P. patens* expansin sequences and 55 other expansin sequences in the database representing examples of dicotlydeon, monocotyledon, gymnosperm and pteridophyte plants. The tree was derived by parsimony analysis (cf. methods), using the γ-expansin AtExpC1.2 as the outgroup. Bootstrap numbers (from 500 replicates) above 80% are indicated above the relevant branches and groupings into α, β-, and γ-expansins shown. Distances are approximated and given in number of changes of state of the sequences. In addition to the six *PpExp* cDNAs reported here, the following protein sequences (the accession numbers are given in parentheses) were used in this analysis: CsExp1 (U30382) and CsExp2 (U30460) from *Cucumis sativa*; LeExp1 (U82123), LeExp2 (AF096776), LeExp3 (AF059487), LeExp4 (AF059488), LeExp5 (AF059489) and LeExp9 (AJ243340) from *Lycopersicon esculentum*; OsExp1 (Y07782), OsExp2 (U30477), OsExp3 (U30479) and OsExp4 (U85246) from *Oryza sativa*; NtExp4 (AF049352) and NtExp5 (AF049354) from *Nicotiana tabacum*; PtExp (AF085330) from *Pinus taeda*; MqExp1 (AF202119) from *Marsilea quadrifolia*; RdExp1 (AF202120) from *Regnellidium diphyllum*. The *Arabidopsis* sequences and accession numbers are as described in Li *et al.* (2002).

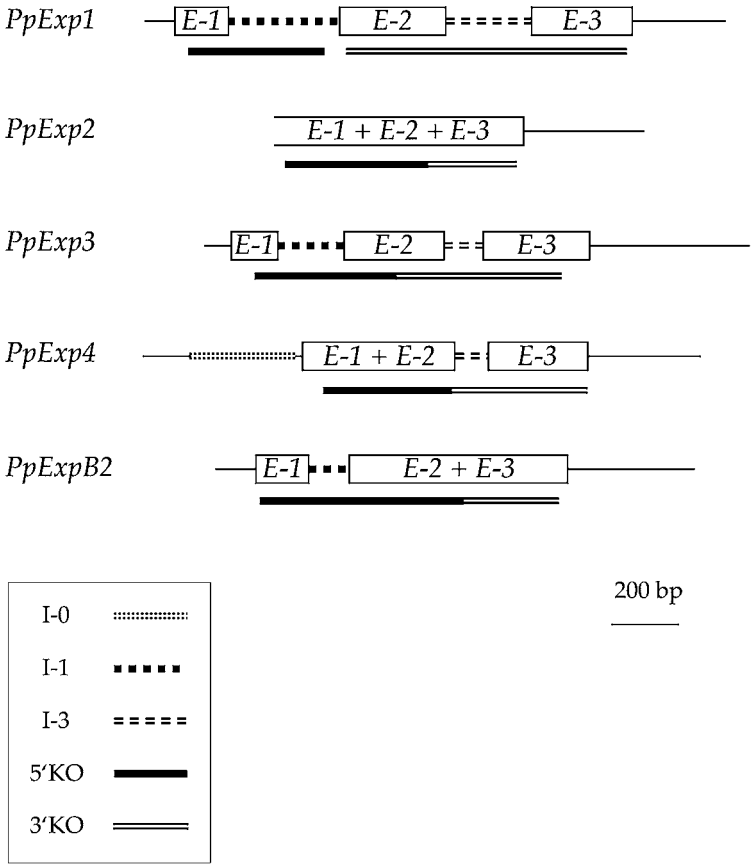

Figure 3. Organisation of *P. patens* expansin genomic loci. The *PpExp1* and *PpExp3* genes consist of exons E-1, E-2 and E-3 separated by introns I-1 and I-3, as indicated. The organisation of the other *PpExp* genes can be interpreted as being derived from this structure by progressive loss of intron and consequent exon fusion, as indicated. *PpExp4* contains a novel intron (I-0). The genomic fragments used for the construction of the knockout constructs (Table 1) are indicated below the relevant genomic diagram.

of seed plants and ferns. The remaining expansin sequences from Arabidopsis and other species grouped into sub-families of α, β- and γ-expansins essentially as previously described (Li *et al.*, 2002), although the relatively low bootstrap numbers for some of the branches of the tree leads to some variability. The potential uniqueness of moss α-expansin sequences was further indicated by our inability to detect expansin activity in cell wall extracts using an *in vitro* assay or to detect cross-reacting proteins in western blots using an antibody raised against cucumber α-expansin (data not shown). Parallel analysis of tissue from higher plant tissue indicated that the assays detected expansin protein and activity (Pien *et al.*, 2001; Reidy *et al.*, 2001).

Previous investigations have revealed the loss and acquisition of introns in expansin genes in *Arabidopsis* and rice and have led to a proposed evolutionary sequence of gene arrangements (Lee *et al.*, 2001, 2002). These analyses indicate that the ancestral expansin gene probably contained introns at conserved positions termed I-1 (or A) and I-3 (or B), as indicated on Figure 1. We cloned genomic sequences corresponding to five *PpExp* cDNAs and examined the intron/exon organisation. As shown in Figure 3, *PpExp1* and *PpExp3* both show a typical organisation for an α-expansin gene with introns at positions I-1 and I-3 separating the open reading frame into 3 exons. In the other *P. patens* genomic sequences analysed, loss of either intron I-1 (*PpExp4*) or I-3 (*PpExpB2*) or both (*PpExp2*) has occurred. In β-expansins from both *Arabidopsis* and rice an evolutionary linked acquisition of introns has been proposed (Lee *et al.*, 2001, 2002) which does not appear to be the case for the putative β-expansin in *P. patens* (*PpExpB2*). In this respect, the genome organisation of *PpExp4* is distinguished by

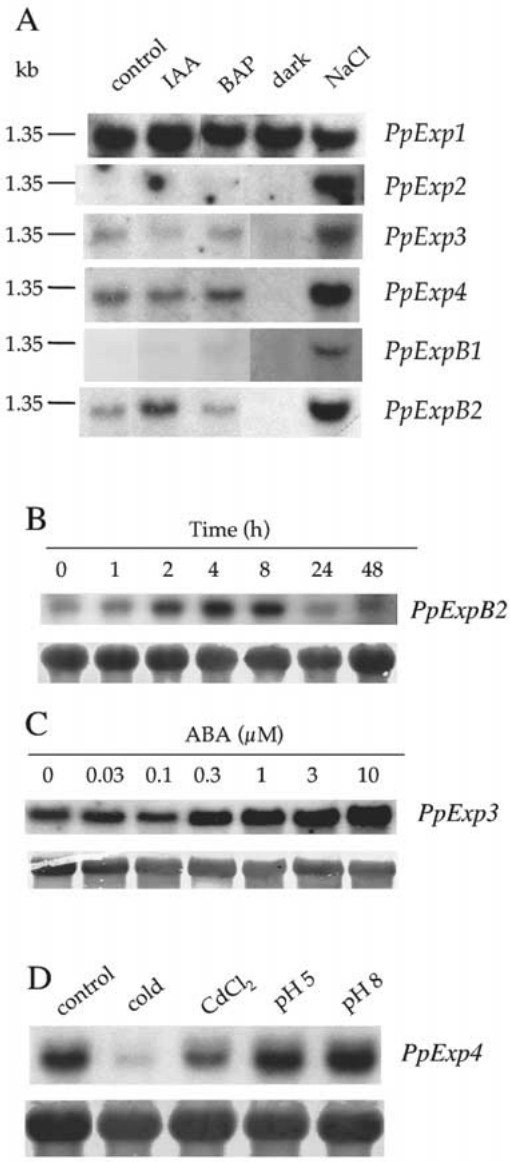

Figure 4. Hormonal and environmental regulation of *P. patens* expansin gene expression

A) RNA was extracted from protonemal tissue subjected to various treatments and hybridised with ^{32}P-labelled probes for each of the *PpExp* cDNAs, as indicated. Control = tissue grown for 5 days on control medium before transfer to fresh control medium for 24 hours in a light (20 h)/dark (4 h) cycle; IAA = as control except the fresh medium contained 1 μm IAA; BAP = as control except the fresh medium contained 1 μm BAP; dark = as control except the tissue was maintained in darkness after transfer to fresh medium; NaCl = as control except the fresh medium contained 100 mM NaCl. 15 μg RNA was loaded per lane. Methylene blue staining (data not shown) confirmed equal loading of lanes for each blot.

B) Time dependence of auxin induction of *PpExpB2* transcript accumulation. Tissue was grown for 5 days on control medium before transfer to fresh medium containing 1 μM IAA. Tissue was collected at various time points (as indicated) and RNA analysed by northern blot hybridisation using a ^{32}P-labelled probe for *PpExpB2*. 15 μg RNA was loaded per lane and transfer of RNA visualised by methylene blue staining.

C) ABA concentration dependence of *PpExp3* transcript accumulation. Tissue was grown and analysed as in A except that the fresh medium contained ABA at various concentrations (as indicated). The blot was hybridised with a ^{32}P-labelled probe for *PpExp3*. 15 μg RNA was loaded per lane and transfer of RNA visualised by methylene blue staining.

D) Cold repression of *PpExp4* transcript accumulation. Tissue was grown and analysed as in A except that the fresh medium contained either 100 μM CdCl2, the medium pH was adjusted to either pH5 or pH8, or the tissue was grown for 24 hours at 4 °C after transfer to fresh control medium (cold). The blot was hybridised with a ^{32}P-labelled probe for *PpExp4*. 15 μg RNA was loaded per lane and transfer of RNA visualised by methylene blue staining.

the presence of an intron within the 5′ UTR. We have defined this novel intron position as I-0 (Figure 3). This seems to represent a novel evolutionary event in *P. patens* since no intron at this position has been reported for any α-expansin gene in the database, but has been identified for a β-expansin in rice (*OsExpB12*, intron D) (Lee *et al.*, 2001).

P. patens *expansin transcript levels are responsive to environmental and hormonal factors*

To investigate the expression patterns of the six *PpExp* genes, a northern blot analysis was performed using protonemal tissue treated with various hormones and grown under various conditions. The results of this analysis are shown in Fig. 4A. Under standard growth conditions, *PpExp1* was expressed at a relatively high level whereas transcript levels for the other expansin genes were lower or undetectable. After growth in the dark, transcript levels for all the expansin genes analysed became undetectable, with the exception of those for *PpExp1*, which remained high. In contrast, treatment with a high salt concentration led to an accumulation of transcripts for all the expansin genes tested.

In addition to these generalised responses of expansin transcript level to specific factors, some individual expansin genes showed specific responses at the transcript level in response to specific factors. Thus, as shown in Fig. 4A, *PpExpB2* transcript levels were elevated following auxin treatment. To further investigate the response of *PpExpB2* to auxin we performed a time course analysis of transcript accumulation. As shown in Fig. 4B, elevated levels of *PpExpB2* mRNA were detected within 2 hours of treatment with auxin at 1μM, with a maximum being reached after 8 hours. Transcript levels subsequently declined. Induction of

elevated transcript level occurred within 24 hours following treatment with auxin at concentrations of 0.3 μm and higher (data not shown). A hormone response was also observed in the transcript levels for *PpExp3* which were elevated after treatment of tissue with ABA at concentrations of 0.3 to 10 μM, as shown in Fig. 4C. The influence of IAA and ABA on transcript level were only observed for *PpExpB2* and *PpExp3*, respectively, and no change in transcript level for any *PpExp* gene was observed after treatment of protonemal tissue with gibberellic acid, cytokinin, brassinolide or ethylene (data not shown). With respect to environmental factors, a response to cold treatment was observed for *PpExp4*, with transcripts decreasing to undetectable levels after incubation at 4 °C (Fig. 4D). Other stress conditions, such as heavy metal or medium pH, did not affect *PpExp4* transcript level. No influence on the transcript level of other *PpExp* genes was observed after treatment of tissue with these factors (data not shown).

The general response of expansin transcript levels to high salt concentration led us to further investigate this phenomenon. As shown in Figure 5A, a triggering of transcript accumulation occurred for all of the *PpExp* genes analysed at a salt concentration of approximately 100 mM. Comparison with tissue treated with osmotic equivalents of mannitol (Figure 5A) indicated that for some *PpExp* genes (e.g., *PpExp3, PpExp4*) the salt response was likely linked to an osmotic response. However, for other *PpExp* genes (e.g., *PpExpB2*) the responsive of transcript accumulation to osmotic stress was very limited, suggesting a link predominantly to salt stress after incubation of tissue on high concentrations of NaCl. It is noticeable that for *PpExp4* a rapid decline in transcript level occurred under conditions of extreme salt and osmotic stress. This was not observed with other *PpExp* transcripts. A time course of *PpExp* transcript accumulation after treatment of tissue with 100 mM salt indicated that induction occurred within 2 hours and was generally maintained over the subsequent 48 hours. The reason for the low signal corresponding to *PpExpB2* transcripts observed after 24 hours incubation is unclear since in all other analyses (e.g., Figure 4A, Figure 5A) a high signal was observed after 24 hours incubation on 100 mM NaCl.

P. patens displays a specific morphogenic response to stress conditions leading to the formation of 'brood' or tmema cells (Goode *et al.*, 1993). As shown in Figure 6, following treatment with either NaCl or ABA, normally filamentous cells (Fig. 6A) form spherical brood cells (Figure 6B). These provide structures which allow the plant to survive environmental conditions adverse to growth, for example, dessiccation (Figure 6C). Brood cell formation involves the reshaping of specific cells within the moss protonema and, thus, presumably controlled changes in cell wall extensibility. To test the potential role of expansin genes in *P. patens* development and, in particular, the morphogenic response to stress conditions, we created a number of transgenic plants in which specific expansin genes were knocked out.

Creation of single gene knockouts for expansin genes

Knockout constructs (KO) were made consisting of the NptII selectable marker flanked by genomic sequences for the individual *PpExp* genes (Schaefer, 2001). These genomic flanking sequences ranged in size from 263 bp to 841 bp (Table 1) and are indicated in Figure 3. The constructs were transformed into protoplasts of *P. patens* and stable regenerants analysed. Table 1 summarises the analysis of these regenerants.

Putative knockout lines were analysed at the RNA level for the accumulation of the respective *PpExp* transcript in tissue which had been treated with NaCl (a treatment which leads to the accumulation of transcripts for all expansin genes analysed). As shown in Figure 7A, several independent lines were obtained showing no accumulation of transcripts for the targeted expansin genes *PpExpB2* (lines KOB2A, D, E and G), *PpExp2* (line KO2D), *PpExp3* (lines KO3A, B, D and G) and *PpExp4* (lines KO4B, C, D and F). To test the specificity of the knockout events, northern blot analyses were performed using RNA from various knockout lines hybridised with probes for expansin transcripts not targeted in the individual knockout lines. For example, transcripts for *PpExpB2* were still detectable in the knockout lines KO3D and KO4F in which *PpExp3* and *PpExp4*, respectively, had successfully been knocked out. Further corroboration of the specificity of the knockout events came from molecular analysis of the site of knockout construct integration. Thus, as shown in Figure 7B, PCR using primers designed to generate products across the 5′ and 3′ borders of a successful integration event for the *PpExpB2* knock-out construct led to the amplification of specific products of the expected size for lines KOB2A, D and E but not for line KOB2H. Comparison with the northern blot data in Figure 7A shows a 100% correlation between loss of *PpExpB2* RNA accumulation and successful PCR product identification.

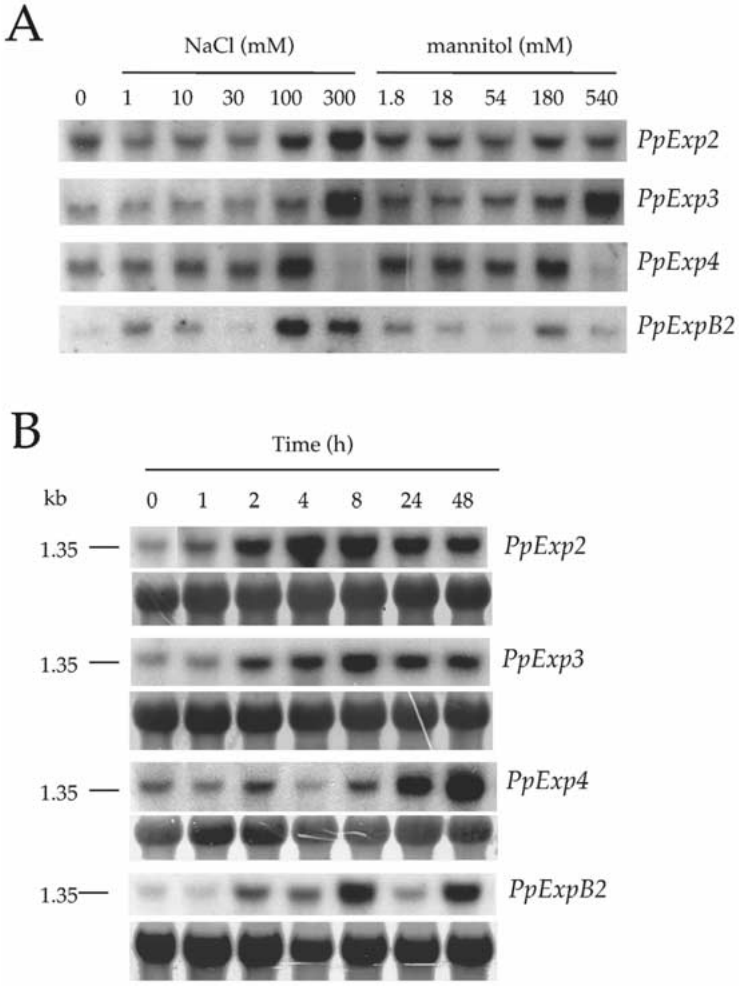

Figure 5. Response of *PpExp* transcript accumulation to salt and osmotic stress
A) Protonemal tissue was grown for 5 days on control medium before transfer to fresh medium containing NaCl or mannitol at various concentrations (as indicated). After further growth for 24 h, RNA was extracted and analysed by northern blot hybridisation using ^{32}P-labelled probes for the *PpExp* clones indicated. 15 μg total RNA was loaded per lane and equal loading and transfer to the membrane was visualised by methylene blue staining (data not shown).
B) Protonemal tissue was grown for 5 days on control medium before transfer to fresh medium containing 100 mM NaCl. At various time points, RNA was extracted and analysed as described in A.

Taken together, these data are consistent with a double recombination event occurring at the *PpExpB2* locus in these lines to generate a knockout at this site.

Several verified regenerant lines corresponding to each individual expansin gene knockout (KO2, KO3, KO4 and KOB2) were subjected to an analysis to identify any phenotype associated with the induced mutation. This analysis involved the observation of protonemal growth under standard conditions, as well as a range of hormonal and environmental factors previously shown to influence moss development. Particular attention was paid to the ability of regenerants to form brood cells in response to both salt stress and ABA and their ability to withstand subsequent dessiccation. No overt change in development or stress response was observed in any of the mutant lines tested (data not shown).

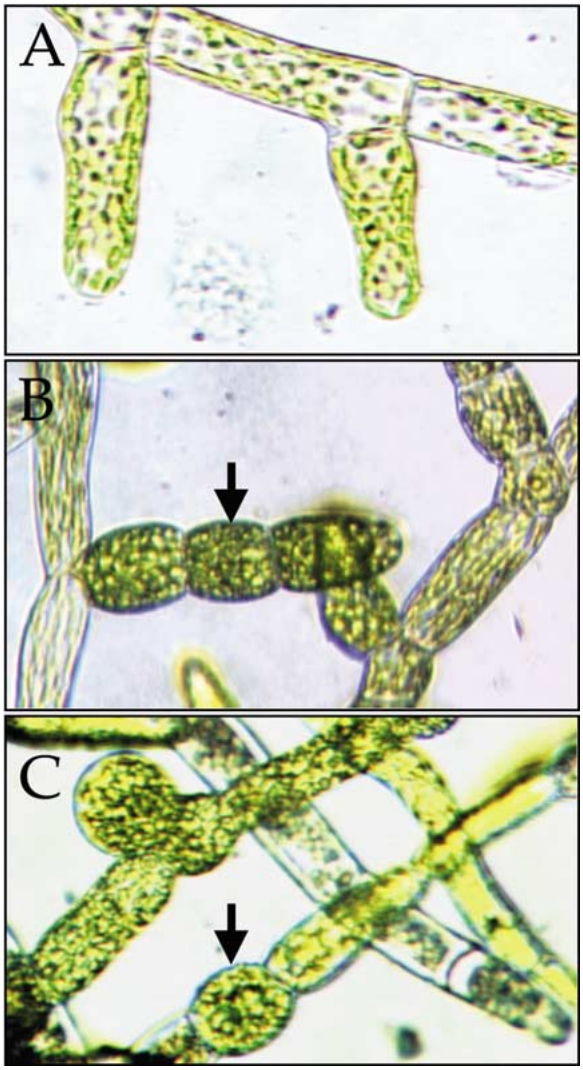

Figure 6. Salt and ABA induction of brood cells
A) Micrograph of protonemal tissue grown under control conditions. The cells are arranged in filaments.
B) Protonemal tissue grown on 1 μM ABA for 48 h. Brood cell formation is apparent (arrow).
C) As in B but after ABA treatment the tissue was subjected to rapid drying (3 hours in empty petri dish with open lid on flowbench). Brood cells (arrow) remain viable whereas surrounding filament cells become plasmolysed.

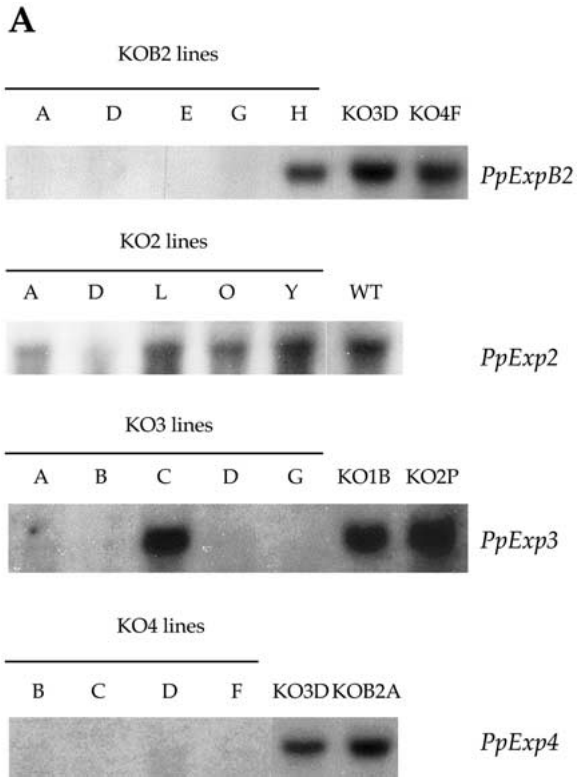

Figure 7. Identification of *PpExp* knockout mutants
A) Protonema of various transgenic lines were grown for 5 days on control medium before transfer to fresh medium containing 100 mm NaCl and further growth for 24 h. RNA was then extracted and analysed by northern hybridisation using ^{32}P-labelled probes for *PpExp* clones, as indicated. For each construct, several putative knockout lines were analysed (KO lines). As controls, hybridisations were also performed using RNA from lines in which a separate *PpExp* gene had been successfully mutated (KO3D, KO4F, KOB2A), lines which had resulted from the transformation procedure but without successful knockout of a particular *PpExp* gene (KO1B, KO2P), or wild-type tissue (WT).
B) PCR analysis of KOB2 lines. DNA extracted from several putative knockout lines for PpExpB2 was analysed by PCR using primers designed to amplify a DNA fragment either across the 5′ or 3′ hybrid junction between the genomic DNA and knockout construct, as described in materials and methods. DNA from wild-type (WT) tissue was used as a control. Size markers (kb) are shown for each gel.

Discussion

P. patens *contains a gene family encoding expansin-like proteins*

A significant body of evidence now supports the proposal that expansins play a key role in modulating plant cell wall architecture and, as a consequence, can influence growth, development and differentiation (Cosgrove, 2000). It has been suggested that expansin-mediated modulation of cell wall extensibility plays a fundamental role in plant physiology and that, as such, expansins are likely to be present in all multicellular land plants. The data reported here provide the first characterisation of expansins in a bryophyte, *P. patens*, and indicate that, indeed, expansins are likely to play a role in cell wall processes in this multicellular lower plant. The expansins identified in *P. patens* show

a number of similarities to those described in higher plants. Firstly, they generally contain the sequence motifs characteristic of α- or β-expansins. Secondly, it is apparent that expansins in *P. patens* are encoded by a multigene family. RT-PCR screening and analysis of an EST library containing more than 110 000 clones has revealed the presence of 6 expansin encoding cDNAs. The Arabidopsis genome sequencing project has led to the identification of 26 α- expansins and 10 β-expansins, approximately half of which are present in the EST database. It is thus likely that the 6 cDNAs reported here represent an underestimate of the total number of expansin genes in the *P. patens* genome.

Within the large gene family encoding expansins in higher plants it is clear that a number of gene family members show specific patterns of expression linked to developmental, hormonal and environmental triggers (e.g., Rose *et al.*, 1997; Cho and Kende, 1997; Fleming *et al.*, 1997). A similar complexity in the regulation of expansin gene expression is apparent in *P. patens* with specific gene members showing altered transcript levels in response to diverse signals including light, ABA, auxin, salt and osmotic pressure. The response of *PpExpB2* transcript levels to auxin is of potential interest since this hormone is involved in the developmental transition from chloronema to caulonema formation, a transition associated with a change in the dynamics of cell elongation (Cove *et al.*, 1997). A potential role for expansin in modulating the changes in cell wall architecture which must be associated with this response is intriguing. A decrease of expansin transcript level in response to darkness has been reported previously in higher plants (Caderas *et al.*, 2000). However, in *P. patens* a differential response with respect to transcript level in response to darkness was observed. Thus, *PpExp1* transcript level was unaffected whereas the mRNAs for all other expansin genes analysed decreased to undetectable levels after transfer of the plants to darkness. The functional significance of this differential response is unclear, but the continued expression of *PpExp1* in the dark suggests a specific and possibly essential function for this gene product, as will be discussed later.

Despite these similarities, *P. patens* expansins show some differences from those previously described. Thus, at the level of primary sequence several of the the *P. patens* α-expansin proteins show a divergence from those described in higher plants and ferns. In the absence of an understanding of the biochemical mode of action of expansin, the functional significance of these variations is difficult to predict. It is noticeable that we were unable to detect expansin activity using an *in vitro* assay with cell wall extracts from *P. patens*, nor were we able to detect cross-reacting proteins using an antibody raised against cucumber expansin. These data might simply reflect the mode of growth of moss protonema in which extension is limited to only the tip of filaments, i.e. the majority of cells within a protonema is not growing and so the level of active protein may be very low. However, at the RNA level at least some of the *PpExp* genes are moderately expressed (detectable in northern blot analysis of total RNA). An alternative possibility is that *P. patens* expansin sequence diversity reflects a specialisation of expansin protein function for substrates specific to moss cell walls. The development of an *in vitro* assay to measure *P. patens* cell wall extensibility would help to distinguish these possibilities.

Expansins and response to stress

It has recently been reported that drought stress increases the expression of expansin genes in maize roots and that this might be part of a mechanism to maintain growth under adverse conditions (Wu *et al.*, 2001). It has also been shown that altered expansin expression affects abscission, a programmed developmental response to stress conditions involving altered cell wall structure (Cho and Cosgrove, 2000). An emerging theme is thus the involvement of expansins in plant stress response. Our data add to this body of evidence by showing that 5 of the 6 expansin genes analysed in *P. patens* showed increased transcript levels in response to salt and osmotic stress, and that one gene (*PpExp3*) showed elevated mRNA accumulation following tissue treatment with ABA, a stress-associated hormone also shown to influence gene expression in *P. patens* (Knight *et al.*, 1995). It is tempting to speculate that the increase in expansin transcript levels during abiotic stress treatment is required for the specific changes in cell morphology associated with brood cell formation and, thus, for the acquisition of dessiccation tolerance. Dessiccation tolerance is a feature of many mosses which has attracted only limited attention, and then generally with a view to changes in cytoplasmic proteins and osmolytes (Oliver, 1991). The role of the cell wall in facilitating the drastic volume changes associated with drying and rehydration has attracted little attention and needs to be addressed. Our data are consistent with a role for expansins in mediating the changes in cell wall structure required during stress response.

We attempted to approach this problem, and the general function of expansins, using a molecular genetic strategy of targeted mutation of individual expansin genes.

Expansin gene knockouts

Previous investigations of expansin gene function have utilised overexpression and antisense strategies (e.g., Cho and Cosgrove, 2000; Pien *et al.*, 2001). Our data provide the first reported identification and characterisation of knockout mutations for a series of expansin genes. They corroborate the utility of this approach in *P. patens* in that we were able to generate a number of complete knockouts for a number of expansin genes, as indicated both by lack of transcript accumulation and molecular analysis of the targeted loci. However, no overt phenotype could be identified for 4 of the 5 loci targeted. Such lack of phenotype is a common occurrence using reverse genetic approaches (Bouché and Bouchez, 2001). Two explanations are possible. Firstly, it is possible that our screening procedure (based on qualitative visual assessment of tissue response to single variables) did not encompass conditions in which a phenotype became scorable. Secondly, since expansins in Physcomitrella are encoded by a multigene family, it may be necessary to create double or multiple knockouts before a phenotype is observed, i.e, there is functional redundancy. In this context, it is interesting to note that the only expansin gene for which we failed to obtain any knockout lines was *PpExp1*. This was also the only expansin gene found to be constitutively expressed, consistent with it playing an essential role in plant growth. If *PpExp1* does encode an essential gene, the possibility in *P. patens* of replacing the endogenous *PpExp1* gene with versions mutated in residues proposed to be essential for expansin activity would provide a powerful tool to investigate structure/function relationships in this important cell wall protein.

Acknowledgements

We thank Jens Lerchl and Elke Duwenig (BASF Plant Science, Ludwigshafen, Germany) for performing the initial analysis of the EST library; Prof. Nikolaus Amrhein for providing laboratory space and support throughout the project; Prof. Simon McQueen-Mason (University of York, UK) for help in assaying moss expansin activity; Dr Catherine Darley (University of York, UK) for providing the *Arabidopsis* sequence data used in the phylogenetic analysis and members of the Fleming group for critical discussions. The research was supported by a grant from the Swiss Federal Institute of Technology to A.J.F. who is supported by a START Fellowship from the Swiss National Science Foundation.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215: 403–410.

Ashton, N. W. and Cove, D. J. 1977. The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens*. Mol. Gen. Genet. 154: 87–95.

Ashton, N. W., Grimsley, N. H. and Cove, D. 1979. Analysis of gametophytic developoment in the moss Physcomitrella patens using auxin and cytokinin resistant mutants. Planta 144: 427–435.

Bonnema, A. B., Peytavi, R., van Daelen, R. A. J., Zabel, P. and Grimsley, N. 1993. Development of an *in vivo* complemention system for identification of plant genes using yeast artificial chromosomes (YACS). In: Bacterial Wilt. (ACIAR proceedings) Eds. Hartmann, G. L and Hayward, A. C.

Bouché, N. and Bouchez, D. 2001. *Arabidopsis* gene knockout: phenotypes wanted. Curr. Opin. Plant Biol. 4: 111–117.

Caderas, D., Muster, M., Vogler, H., Mandel, T., Rose, J.K.C., McQueen-Mason, S. and Kuhlemeier, C. 2000. Limited correlation between expansin gene expression and elongation growth rate. Plant Physiol. 123: 1399–1413.

Cho, H-T. and Cosgrove, D. J. 2000. Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 97: 9783–9788.

Cho, H-T. and Kende, H. 1997. Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell 9: 1661–1671.

Cosgrove, D. J. 2000. Loosening of plant cell walls by expansins. Nature 407: 321– 326.

Cove, D. J., Knight, C. D. and Lamparter, T. 1997. Mosses as model systems. Trends Plant Sci. 2: 99–105.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Fleming, A. J., McQueen-Mason, S., Mandel, T. and Kuhlemeier, C. 1997. Induction of leaf primordia by the cell wall protein expansin. Science 276: 1415–1418.

Goode, J. A., Stead, A. D. and Duckett, J. G. 1993. Redifferentiation of moss protonemata: an experimental and immunofluorescence study of brood cell formation. Can. J. Bot. 71: 1510–1519.

Herrin, D. L. and Schmidt, G. W. 1988. Rapid, reversible staining of northern blots prior to hybridization. Biotechniques 6: 196–200.

Hutchison, K. W., Singer, P. B., Diaz-Sala, C. and Greenwood, M. S. 1999. Expansins are conserved in conifers and expressed in response to exogenous auxin. Plant Physiol. 120, 827–832.

Kim, J. H., Cho, H.-T. and Kende, H. 2000. α-expansins in the semiaquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum*: evolutionary aspects and physiological role in rachis elongation. Planta 212: 85–92.

Knight, C. D., Sehgal, A., Atwal, K., Wallace, J. C., Cove, D. J., Coates, D., Quatrano, R. S., Bahadur, S., Stockley, P. G. and

Cuming, A. C. (1995) Molecular response to abscisic acid and stress conserved between mosses and cereal. Plant Cell 7: 499–506.

Lee, Y., Choi, D. and Kende, H. 2001. Expansins: ever-expanding numbers and functions. Curr. Opin. Plant Biol. 4: 527–532.

Li, Y., Darley, C. P., Ongaro, V., Fleming, A., Schipper, O., Baldauf, S. L. and McQueen-Mason, S. J. 2002. Plant expansins are a complex multigene family with an ancient evolutionary origin. Plant Physiol. 128, 854–864.

McQueen-Mason, S., Durachko, D. M. and Cosgrove, D. J. 1992. Two endogenous proteins that induce cell wall expansion in plants. Plant Cell 4: 1425–1433.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Prot. Engineer. 10: 1–6.

Oliver, M. J. 1991. Influence of protoplasmic water loss on the control of protein synthesis in the desiccation-tolerant moss *Tortula ruralis*. Plant Physiol. 97: 1501–1511.

Perrière, G. and Gouy, M. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 78: 364–369.

Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C. and Fleming, A.J. 2001. Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proc. Natl. Acad. Sci. USA 98: 11812–11817.

Reidy, B., McQueen-Mason, S., Nosberger, J. and Fleming, A. J. 2001. Differential expression of alpha and beta expansin genes in the elongating leaf of *Festuca pratensis*. Plant Mol. Biol. 46: 491–504.

Rose, J. K., Lee, H. H. and Bennett, A. B. 1997. Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proc. Natl. Acad. Sci. USA 94: 5955– 5960.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1992. Molecular Cloning: a laboratory manual. (Cold Spring Harbor, New York).

Schaefer, D. 2001. Gene targeting in *Physcomitrella patens*. Curr. Opin. Plant Biol. 4: 143–150.

Schaefer, D. and Zrÿd, J.-P. 1997. Efficient gene targeting in the moss *Physcomitrella patens*. Plant J. 11: 1195–1206.

Shcherban, T. Y., Shi, J., Durachko, D. M., Guiltinan, M. J., McQueen-Mason, S. J., Shieh, M., and Cosgrove, D. J. 1995. Molecular cloning and sequence analysis of expansins – a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc. Natl. Acad. Sci. USA 92: 9245–9249.

Strepp, R., Scholz, S., Kruse, S., Speth, V. and Reski, R. 1998. Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. Proc. Natl. Acd. Sci. USA 95: 4368–4373.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22: 4673–4680.

Yu, W., Meeley, R. B. and Cosgrove, D. J. 2001. Analysis and expression of the α-expansin and β-expansin gene families in maize. Plant Physiol. 126: 222–232.