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Differential expression of α- and β-expansin genes in the elongating leaf of *Festuca pratensis*

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Abstract

Grasses contain a number of genes encoding both α- and β-expansins. These cell wall proteins are predicted to play a role in cell wall modifications, particularly during tissue elongation. We report here on the characterisation of five α- and three vegetative β-expansins expressed in the leaf elongation zone (LEZ) of the forage grass, *Festuca pratensis* Huds. The expression of the predominant α-expansin (*FpExp2*) was localised to the vascular tissue, as was the β-expansin *FpExpB3*. Expression of another β-expansin (*FpExpB2*) was not localised to vascular tissue but was highly expressed in roots and initiating tillers. This is the first description of vegetative β-expansin gene expression at the organ and tissue level and also the first evidence of differential expression between members of this gene family. In addition, an analysis of both α- and β-expansin expression along the LEZ revealed no correlation with growth rate distribution, whereas we were able to identify a novel xyloglucan endotransglycosylase (*FpXET1*) whose expression profile closely mimicked leaf growth rate. These data suggest that α- and β-expansin activities in the grass leaf are associated with tissue differentiation, that expansins involved in leaf growth may represent more minor components of the spectrum of expansin genes expressed in this tissue, and that XETs may be useful markers for the analysis of grass leaf growth.

Abbreviations: LER, leaf elongation rate; LEZ, leaf elongation zone; SER, segmental elongation rate; XET, xyloglucan endotransglycosylase

Introduction

Expansins are a family of cell wall proteins proposed to play a key role in the regulation of tissue elongation, as well as cell wall differentiation (reviewed by McQueen-Mason and Rochange, 1999; Cosgrove, 2000). Since their initial characterisation in cucumber (McQueen-Mason *et al.*, 1992), genes encoding expansins have been identified in a wide variety of plants including dicotyledons, monocotyledons and gymnosperms (Shcherban *et al.*, 1995; Rose *et al.*, 1997; Hutchison *et al.*, 1999). One surprising finding arising from the rice genome project was the identification of a novel but related class of genes encoding expansin-like proteins. This family was termed β-expansins to distinguish it from the original family of α-expansins (Cosgrove *et al.*, 1997).

α-expansins are encoded by large gene families whose members often show tissue specific expression patterns (Rose *et al.*, 1997; Cho and Kende, 1997; Reinhardt *et al.*, 1998; Brummell *et al.*, 1999). Although a correlation of α-expansin gene expression and high rates of elongation can often be made in

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ 276006 (*FpExp1*), AJ276007 (*FpExp2*), AJ276008 (*FpExp3*), AJ276009 (*FpExp4*), AJ276010 (*FpExp5*), AJ275940 (*FpExpB1*), AJ295941 (*FpExpB2*), AJ295942 (*FpExpB3*) and AJ295943 (*FpXET1*)

tissues such as hypocotyls, coleoptiles and internodes (Cosgrove and Li, 1993; Cho and Kende, 1997; Caderas *et al.*, 2000), expression of some α-expansins in fruit (Civello *et al.*, 1999; Rose *et al.*, 2000), meristems (Fleming *et al.*, 1997, 1999) and abscission zones (Cho and Cosgrove, 2000) indicates a role for α-expansin in tissue differentiation and morphogenesis. With respect to β-expansins the situation is less clear. The originally described β-expansin was identified in pollen where it acts as an allergen (Cosgrove *et al.*, 1997). Its endogenous function in the pollen cell wall is still unclear, although *in vitro* assays indicate that it can function to loosen grass cell walls in an analogous fashion to α-expansins (Cosgrove *et al.*, 1997). However, β-expansins are also found in vegetative tissues in grasses, although as yet they have only been described as sequence reports with no data on possible tissue or organ-specific expression (Cosgrove, 2000). Moreover, although originally described in grasses (where they are probably prevalent, based on the number of sequence submissions), β-expansin sequences have also been reported in non-grass species, although again with no description of any tissue specificity (Downes and Crowell, 1998). The existence of both α- and β-expansins in both grass and non-grass species raises the question of what specific roles these related but distinct cell wall proteins might play.

We are interested in understanding the molecular processes underlying leaf growth in the forage grass *Festuca pratensis* Huds. This forage grass provides many ideal characteristics, both in terms of the quantity and quality of the forage produced as well as the ability to withstand harsh climatic conditions (Meister and Lehmann, 1990). However, *F. pratensis* lacks persistence when grown in intensively managed grasslands with other competitive companion grasses (Mott and Lennartz, 1977). This limited competitive ability, especially under frequent cutting or intensive grazing, appears to be linked to leaf growth characteristics (Nösberger *et al.*, 1998).

A better understanding of the molecular basis of leaf growth in *F. pratensis* would thus not only give an insight into the regulation of a basic process of plant physiology, but might also provide tools for marker assisted breeding. Although grass leaf growth has been well characterised at the physiological level (reviewed by Skinner and Nelson, 1995), the molecular biology underlying this process has been neglected.

Our data provide the first insight into the organ- and tissue-specific expression of vegetative β-expansins and describe a careful quantitative comparison of leaf growth rates along the leaf with α- and β-expansin genes expressed in that region. They indicate a poor correlation of both α- and β-expansin gene expression with growth rate (as opposed to a xyloglucan endotransglycosylase) but do suggest a role for both α- and β-expansins in vascular tissue differentiation in the leaf. Moreover, we show that two vegetative β-expansins are differentially expressed, one accumulating predominantly in the vascular tissue, the other in the initiating tiller.

Materials and methods

Plant material and growth conditions

A *F. pratensis* genotype (*Festuca pratensis* Huds.) was selected from the cv. Prefest (RAC, Changins, Switzerland). Clonal replicates were produced by cutting individual tillers to 5 cm tiller and root length and growing them in a hydroponical cultivation system under non-limited nutrient supply (Hammer *et al.*, 1978). The growth medium was continuously aerated and replaced every seventh day. The plants were cultivated in a growth chamber (PGV36, Conviron Instruments, Winnipeg, Canada) at a temperature of 20 °C, 80% relative humidity and 400 μmol m^{-2} s^{-1} photosynthetic photon flux density.

cDNA cloning and sequence analysis

Total RNA (5 μg) was extracted with a RNeasy Plant Kit (Qiagen, Basel, Switzerland) from the 40 mm LEZ, coleoptiles, 4 mm basal section of the LEZ including the apex and the 30 mm apical region of the growing root tip, and used as template for reverse transcription with an oligo d(T) primer. The products were subjected to PCR amplification by using degenerate sense 5′-GCAGGXTXNGNCATGTCGAAGTG-3′ (α-expansin), 5′-GGCMRGGCSACCTGGTACGG-3′ (β-expansin), 5′-CGAGATCGACNTCGAGTTC-3′ (XET) and antisense 5′-TGAGCNCGGNGCTGTTCA ACG-3′ (α-expansin), 5′-CCRCAGCCCYKGCCGXC CTTG-3′ (β-expansin), 5′-SGTCNGRGCAGTAGTN-GTAG-3′ (XET) primers, respectively. PCR products of the predicted size were subcloned into pPCR-Script (Stratagene, Basel, Switzerland). The DNA inserts were sequenced with universal and specific internal primers on an automated ABI 373A DNA Stretch Sequencer. Signal peptides were predicted with the program PSORT (Nakai and Kanehisa, 1992). Den-

drograms were obtained by using the clustal procedure of Megalign (DNASTAR, Madison, WI).

Generation of full-length cDNAs

Full-length α-expansin cDNA clones were obtained in a two-step procedure by RACE-PCR. Poly(A)$^+$ RNA (1 μg) extracted from the LEZ, coleoptiles, 4 mm basal sections of the LEZ including the apex and the apical region of the growing root tips was converted into double-stranded cDNA and ligated to adapters according to the manufacturer's instructions (Marathon cDNA Amplification Kit, Clontech, Basel, Switzerland). 3′ and 5′ RACE clones were made by using *FpExp*-specific degenerate sense primers with the RACE adapter primer. Based on the 3′ sequence data, gene-specific antisense primers were designed and used with the 3′ RACE adapter primer to obtain the full-length cDNAs. The full-length *FpExp2* clone was generated by using a gene-specific antisense primer from the 5′ end with the 3′ RACE adapter primer. Full-length β-expansins and the *FpXET1* clone were obtained by a similar procedure. Products were subcloned and sequenced as described above.

Preparation of gene-specific probes

Gene-specific probes were generated mainly from the 3′-untranslated region of the respective clones. For *FpExp1*, *FpExp2* and *FpExp3*, gene-specific probes were produced with the internal primer 5′-CTACCTCAACGGGCAGGGCCTATCCTTC-3′ together with the gene-specific primer from the 3′ end used in RACE PCR or the 3′ RACE adapter primer for *FpExp2*, respectively. For the detection of *FpExp4* and *FpExp5*, a fragment between the *Sma*I and a *Sac*I restriction site was used as gene-specific probe. The generated gene-specific probes were tested for their specificity by DNA gel blot analysis. Gene-specific β-expansin probes were generated with internal sense primers 5′-GCCATTGTCCTTGCGCATCACC-3′ (*FpExpB1*) and 5′-CCCTTCTTCATGCGCATCACCA-3′ (*FpExpB2* and *FpExpB3*) together with the RACE primer. A gene-specific probe for *FpXET1* was obtained with the internal sense primer 5′-AGGAGCTCGGCGACAT GAGCTACC-3′ and the RACE primer.

RNA gel blot analysis

Total RNA was extracted using a FastRNA Green Kit (Bio101, Luzerna Chem, Luzern, Switzerland) from the LEZ (40 mm basal end of the growing leaf), coleoptiles, 4 mm basal sections of the LEZ including the apex and 3 cm apical region of growing root tips including root hairs. For comparison of the transcript abundance along the LEZ, total RNA was extracted from five 8 mm long successive tissue segments of the LEZ. RNA samples (10 μg) were separated on gels of 1.1% agarose and 0.65 M formaldehyde. Blotting and hybridisation were performed under standard conditions (Sambrook *et al.*, 1989). The blots were hybridised at 65 °C with the corresponding ^{32}P-labelled probes generated with a random labelling kit (Stratagene). Membranes were washed to a final stringency of 0.2 × SSC, 0.5% SDS at 50 °C before autoradiography at −80 °C on intensifying screens.

In situ hybridisation

In situ hybridisation was performed according to Coen *et al.* (1990). Briefly, leaf segments taken from the LEZ were fixed in 4% w/v formaldehyde in PBS (Sigma, Buchs, Switzerland), dehydrated with ethanol, then exchanged with Histoclear (National Diagnostics, Chemie Brunschwig, Basel, Switzerland), before embedding in paraffin. Sections (8 μm) were mounted on Polysine slides (BDH, Merck, Dietlikon, Switzerland), digested with proteinase K for 30 min at 37 °C, treated with acetic anhydride, dried in ethanol, then hybridised with appropriate DIG-labelled probes overnight at 50 °C. After washing with 0.2 × SSC at 55 °C, the slides were treated with RNaseA for 30 min at 37 °C, washed again at 55 °C with 0.2 × SSC, then processed for revealing the DIG antigen. This involved blocking with DIG-blocking reagent and BSA, followed by incubation with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics, Rotkreuz, Switzerland), washing with blocking reagent, then colour revealed by incubation in NBT and X-phosphate for periods of 12–48 h. Reactions were stopped with 10 mM Tris pH 7.0, slides air-dried, then mounted in Euparal (TAAB Laboratories, Berkshire, UK) before viewing. Antisense and sense probes were used in parallel hybridisations.

Immunoblot analysis of F. pratensis α-expansins

Proteins were extracted from corresponding tissue samples as used for the analysis of the transcript abundance along the LEZ. The tissue was homogenised in protein extraction buffer (0.6 M Tris-HCl, 20% glycerol, 18% SDS, 0.2 M DTT, 0.05% bromophenol blue), incubated for 10 min at 65 °C and in-

soluble material removed by centrifugation. Proteins were quantified colorimetrically according to Minamide and Bamburg (1990). For SDS-PAGE, 40 μg proteins from each sample were separated on 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes (BioRad, Glattbrugg, Switzerland). The rabbit polyclonal antiserum against cucumber α-expansin (S. M.-M., unpublished data) was used at a dilution of 1:2000. The immunoblot was developed with goat anti-rabbit IgG-conjugated alkaline phosphatase (1:3000 dilution) (BioRad).

Measurement of α-expansin activity

α-expansin activity was measured by the method described by Whitney *et al.* (2000). Briefly, tissue from either the basal 0–15 mm or the distal 25–40 mm end of the LEZ was frozen then homogenised in 25 mM Hepes, 1% polyvinylpyrolidone (40 000), 0.1% Triton X-100 and the cell wall material collected. Proteins were eluted from the cell walls by extracting for 1 h at 20 °C in 25 mM Hepes, 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulfite, 5 mM dithiothreitol pH 6.8. Extractable wall proteins were precipitated with 60% w/v ammonium sulfate and collected by centrifugation. The proteins were desalted on a Sephadex G-25 colum (Pharmacia Biotech, St. Albans, UK) into 50 mM sodium acetate pH 4.5 and assayed for expansin activity with a cellulose/xyloglucan composite. Protein concentrations were calculated colorimetrically by the micro-Bradford method (Pierce, Rockford, IL).

RT-PCR analysis of FpExp4

For the detection of the transcript of *FpExp4* total RNA was extracted using a RNeasy Plant Kit (Qiagen, Basel, Switzerland) from the corresponding segments and used as template for reverse transcription with an oligo-d(T) primer. Care was taken to use for each sample the same amount of RNA by measuring OD_{260}. For PCR amplification the *FpExp4* gene-specific primer pair 5′-GACGAAATTACAATGGTGTTTG-3′ and 5′-TAGGCTCTACTCAAACGATCG-3′ was used, giving a 150 bp product.

Determination of LER, SER and epidermal cell sizes

All growth measurements were performed during the linear phase of leaf elongation on the second intact leaf that developed on the main tiller after clipping. Leaf elongation rate (LER) was calculated from daily increments in leaf length. Segmental elongation rates (SER) were determined by measuring the short-term displacement of holes within the LEZ as described by Schnyder *et al.* (1987). Length of abaxial epidermal cells within the LEZ was determined by the preparation of leaf replicas modified according to Meister *et al.* (1999). The length of 7–10 epidermal cells was measured at different positions along the LEZ under a microscope.

Results

Cloning expansin cDNAs from F. pratensis

To identify α- and β-expansins from *F. pratensis*, we took an RT-PCR-based screening strategy. This resulted in the cloning of five α-expansin and three β-expansin cDNA clones (Figure 1). The ORFs ranged from 756 bp for *FpExp1, 4* and *5*, 759 bp for *FpExp2* to 765 bp for *FpExp3*. The ORF for the three β-expansins were all slightly longer and ranged from 789 bp for *FpExpB1*, 801 for *FpExpB3* to 807 bp for *FpExpB2*. The cDNA clones contained 5′-untranslated regions of 45 bp (*FpExp1*), 64 bp (*FpExp2*), 28 bp (*FpExp3*), 55 bp (*FpExp4*), 50 bp (*FpExp5*) and of 65 bp (*FpExpB1*), 82 bp (*FpExpB2*) and 102 bp (*FpExpB3*). The 3′-untranslated regions consisted of 341 bp (*FpExp1*), 428 bp (*FpExp2*), 324 bp (*FpExp3*), 200 bp (*FpExp4*), 194 bp (*FpExp5*) for the α-expansins, and of 370 bp (*FpExpB1*), 305 (*FpExpB2*) and 316 bp (*FpExpB3*) for the β-expansins. The presence of signal peptides, predicted with the program PSORT (Nakai and Kanehisa, 1992), suggests that the encoded proteins are targeted to the endoplasmatic reticulum, as is assumed for other members of the expansin gene family (Cosgrove, 2000). The sequences of *FpExp4* and *FpExp5* are highly conserved, with an amino acid sequence identity of 89% and only limited variation in the 3′-untranslated region, while the amino acid identity of the other three *FpExp* clones ranged from 59% to 64%. All five deduced α-expansin polypeptides contained the eight conserved cysteines within the cysteine-rich region at the N-terminal half characteristic of α-expansins and the four conserved tryptophans at the C-terminal end with similar spacing to that found in some cellulose binding domains (Figure 1a; Shcherban *et al.*, 1995).

The deduced polypeptides of *FpExpB2* and *FpExpB3* were highly similar to each other (80% identity), showing significant variation only at the 5′- and

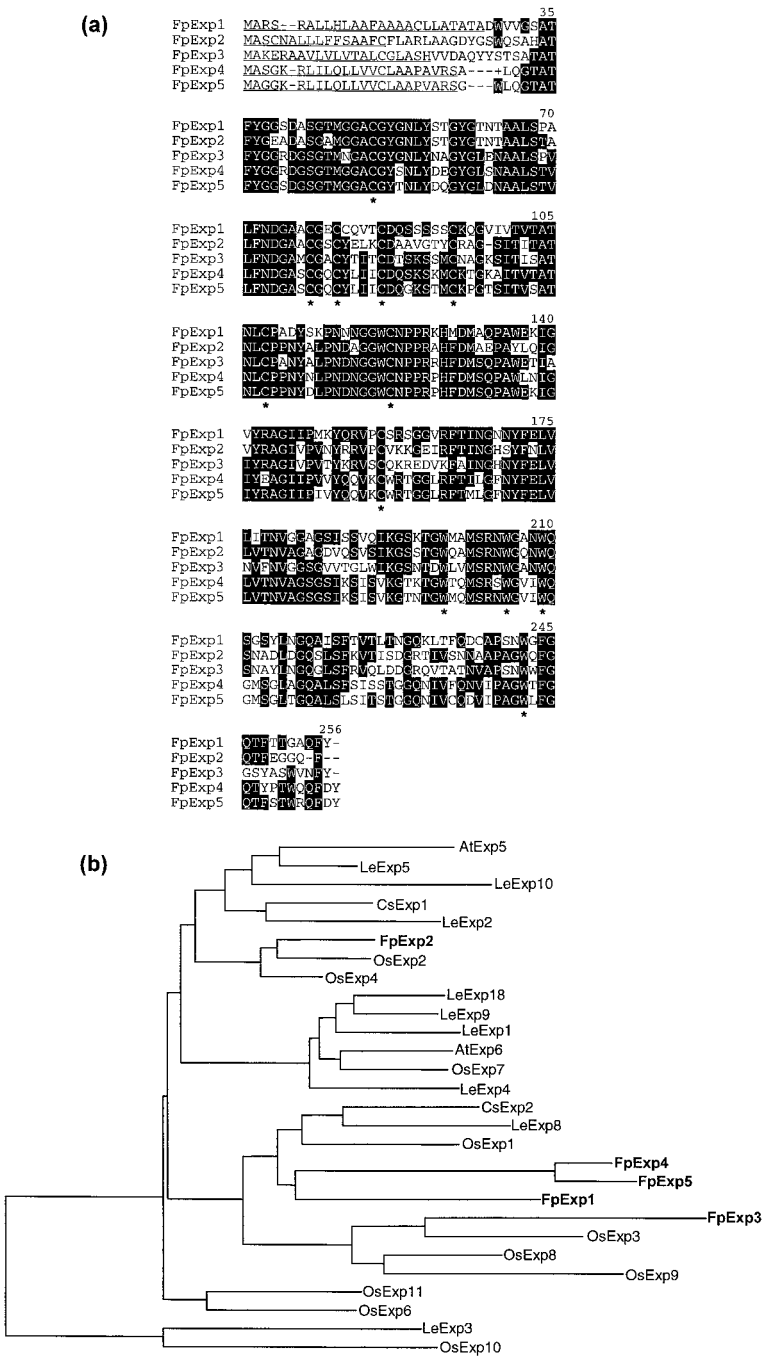

Figure 1. Sequence comparison of the deduced amino acid sequences of *F. pratensis* expansin proteins. a. Alignment of the deduced amino acid sequences of α-expansins. Signal peptides (underlined) were predicted using the program PSORT (Nakai and Kanehisa, 1992). Identical amino acids are indicated by asterisks. The α-expansin characteristic conserved cysteines and tryptophans are indicated (Shcherban *et al.*, 1995; Cosgrove, 2000). The amino acid at position 29 of *FpExp4* (+) could not be determined. b. Dendrogram of α-expansins from different plant species. Full-length proteins were clustered using the Clustal procedure of Megalign (DNASTAR, Madison, WI). Sequences and GenBank accession numbers: *OsExp1* (Y07782), *OsExp2* (U30477), *OsExp3* (U30479), *OsExp4* (U85246), *OsExp6* (AF247163), *OsExp7* (AF247165), *OsExp8* (AAD38296), *OsExp9* (AAD38297), *OsExp10* (AF247165), *OsExp11* (BAA88200), *LeExp1* (U82123), *LeExp2* (AF096776), *LeExp3* (AF059487), *LeExp4* (AF059488), *LeExp5* (AF059489), *LeExp8* (AF184232), *LeExp9* (AJ243340), *LeExp10* (AF184233), *LeExp18* (AJ004997), *AtExp5* (U30478), *CsExp1* (U30382), *CsExp2* (U30460), *FpExp1* (AJ276006), *FpExp2* (AJ276007), *FpExp3* (AJ276008), *FpExp4* (AJ276009), *FpExp5* (AJ276010).

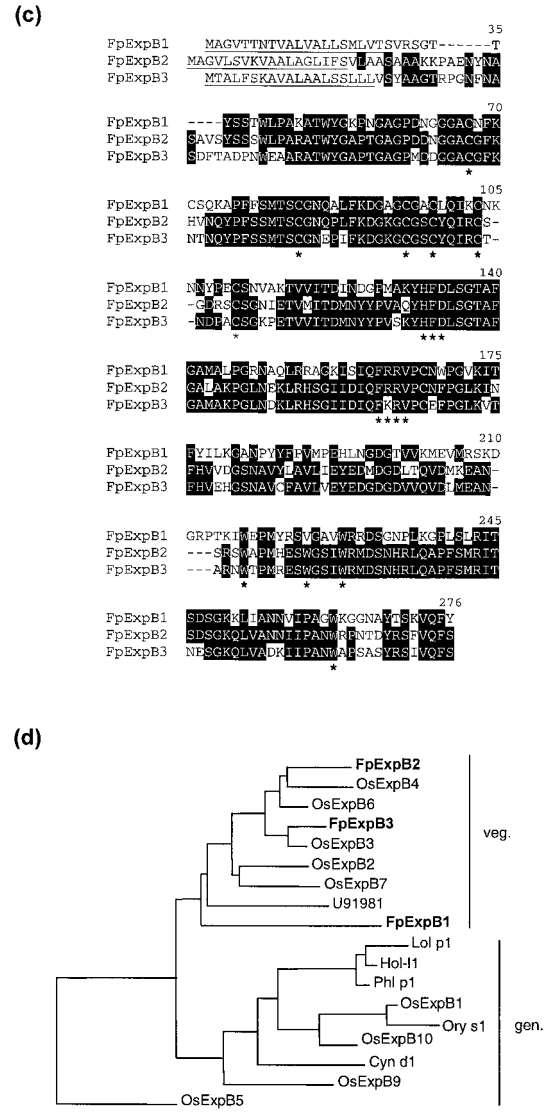

Figure 1. (continued) c. Alignment of the deduced amino acid sequences of β-expansins. Signal peptides (underlined) were predicted using the program PSORT (Nakai and Kanehisa, 1992). The β-expansin characteristic residues (cysteines, tryptophans, HFD and FRRV motifs) are indicated by asterisks (Shcherban *et al.*, 1995; Cosgrove, 2000). d. Dendrogram of vegetative (veg.) and generative (gen.) β-expansins from different grass species. Deduced polypeptides were analysed as described above. Sequences and GenBank accession numbers: *OsExpB1* (AF261270), *OsExpB2* (OSU95968), *OsExpB3* (AF261271), *OsExpB4* (AF261272), *OsExpB5* (AF261273), *OsExpB6* (AF261274), *OsExpB7* (AF261275), *OsExpB9* (AF261277), *OsExpB10* (AF261278), *U91981* (*Triticum aestivum*), *Lol p1* (M57476), *Hol-l1* (Z27084), *Phl p1* (X78813), *Ory s1* (U31771), *Cyn d1* (S83343), *FpExpB1* (AJ295940), *FpExpB2* (AJ295941), *FpExpB3* (AJ295942).

3′-untranslated region. With 60% amino acid identity to *FpExpB2* and *FpExpB3*, respectively, the mature polypeptide of *FpExpB1* is more distinct. The three β-expansin clones contained the characteristic conserved amino acid residues thought to be important for the structural and catalytic roles of α- and β-expansins. We identified six conserved cysteines at the N-terminal half and the conserved tryptophans at the C-terminal end, as well as the HFD and FRRV motifs (Figure 1c; Shcherban *et al.*, 1995; Cosgrove, 2000). It is noticeable that *FpExpB1* contained only three of the four conserved tryptophans at the C-terminal end.

A comparison of the α-expansin clones with their putative orthologues in rice and α-expansins from other species, revealed a high similarity of *FpExp2* and *FpExp3* to *OsExp2* and *OsExp3*, respectively (Figure 1b). *FpExp2* shared 86% amino acid identity with *OsExp2* and was placed together with *OsExp4* and *OsExp2* in a separate sub-group on the dendrogram. The amino acid identity of *FpExp3* to *OsExp3* was somewhat lower (75%) and both sequences were placed together with *OsExp8* and *OsExp9* in an additional sub-branch. *FpExp1*, *FpExp4* and *FpExp5* showed highest amino acid similarity to *OsExp1* and formed together with *CsExp2* (cucumber), *LeExp8* (tomato) and *OsExp1* a separate sub-branch on the dendrogram. To obtain further information on the relationship of the *FpExpB* clones, we compared the mature proteins with generative (group I allergens, Cosgrove *et al.*, 1997) and vegetative β-expansins from other grass species. The cluster analysis clearly distinguished between β-expansins originating from vegetative and β-expansins originating from generative tissue, as indicated by the two major branches of the dendrogram (Figure 1d). *FpExpB2* and *FpExpB3* were highly similar to their putative orthologues in rice (Figure 1d). After removal of the predicted signal peptide, we found that *FpExpB2* and *OsExpB6* had an amino acid identity of 81%. While the amino acid identity of *FpExpB3* and *OsExpB3* was surprisingly high (90%), *FpExpB1* shared only 65% identity to its closest rice β-expansin (*OsExpB2*).

Tissue-specific expression of expansin genes

To compare the expression pattern of the α- and β-expansin genes in various tissues, we performed Northern blot analyses with probes derived from the 3′-untranslated region of the corresponding cDNAs. Because of the high sequence identity of *FpExp4* and *FpExp5*, we used gene-specific RT-PCR to distinguish

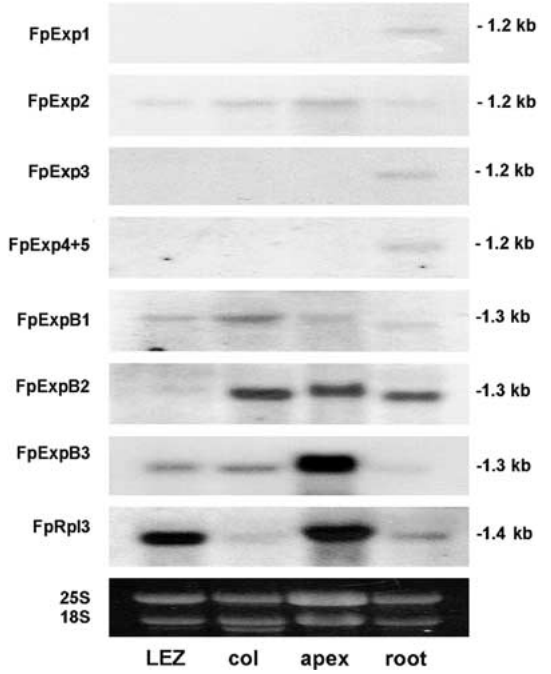

Figure 2. Tissue-specific expression of *F. pratensis* α- and β-expansin genes. Total RNA (10 μg) was extracted from the LEZ (40 mm basal end of the growing leaf) (LEZ), coleoptiles (col), 4 mm basal sections of the LEZ including the apex (apex), 3 cm apical region of growing root tips including root hairs (root). Northern blots were hybridised with probes prepared from the 3′-untranslated region of the corresponding clone indicated at the side of each blot. Transcript levels of *FpRpl3*, encoding a ribosomal L3 protein and ethidium bromide-stained 2SS and 18S rRNA served as controls.

the two transcripts. The results, shown in Figure 2, show that the different expansin clones analysed are expressed in a variety of organ-specific patterns.

With respect to the α-expansins, *FpExp1*, *FpExp3*, *FpExp4* and *FpExp5* were only expressed in the 3 cm apical region of growing adventitious root tips. In contrast, *FpExp2* mRNA was detectable at comparable abundance in all four tissues analysed (LEZ, coleoptile, apex and root).

Although all three *FpExpB* genes were expressed in all four different tissues analysed, we observed significant variation in their relative abundance. The transcripts detected by the *FpExpB1* probe were expressed at low levels in all four tissues analysed, with a slightly higher abundance in the elongating coleoptiles. In contrast, while the transcripts of *FpExpB2* were relatively abundant in coleoptiles, in the 4 mm basal end of the growing leaf and in elongating root tips, the transcripts were only barely detectable in the LEZ. The abundance of *FpExpB3* transcripts was low in the LEZ, coleoptiles and root tips, but substantial accumulation was detected in the apex.

In situ localisation of the FpExp2 transcript

The function of a gene is delimited by its pattern of expression. Therefore, we performed a series of *in situ* hybridisations to characterise the expression patterns of the α- and β-expansin cDNAs described above (Figure 3). In particular, we concentrated on genes expressed in the LEZ and apical tissue (*FpExp2, B2, B3*). *FpExp2* mRNA accumulated only in a few cells associated with differentiating vascular tissue (Figure 3a). Examination of the vascular bundles (Figure 3b) and comparison with hybridisations performed with the relevant sense probe (Figure 3c) indicates that expression of the *FpExp2* gene occurs only in the xylem parenchyma. This pattern is distinct from that observed for a control probe encoding a ribosomal protein, *FpRpl3*, which showed a high accumulation of transcript in the palisade mesophyll and only limited signal in the vascular tissue (Figure 3d). The pattern observed with the probe for *FpExp2* thus does not simply reflect the general pattern of RNA within the tissue. In the more proximal regions of the leaf (where vascular differentiation is less advanced), no accumulation of *FpExp2* RNA could be detected (Figure 3e). Although some weak signal was apparent in the walls of differentiating xylem vessels (Figure 3e and f), a similar signal was observed in sections hybridised with a sense probe (Figure 3g), indicating non-specific binding of the probe. In contrast, hybridisations with a probe for the ribosomal protein *FpRpl3* showed a high signal intensity in all tissues, especially in the differentiating vascular bundles (Figure 3h).

Hybridisation with the gene-specific *FpExpB3* probe of longitudinal sections of the apex and cross-sections at the distal end of the LEZ also revealed a specific accumulation of *FpExpB3* mRNA in cells of the vascular tissue adjacent to xylem vessels (Figure 3i, k, l). Comparison with hybridisations performed with the sense probe (data not shown) and a control probe encoding a ribosomal protein, *FpRpl3*, which showed a high accumulation of transcript in young developing leaves and only limited signal in the vascular tissue (Figure 3j), supports the specificity of this expression pattern. We did not observe accumulation of *FpExpB3* mRNA in cells located in the proximal part of the LEZ (data not shown).

Hybridisations with an antisense probe for *FpExpB2* revealed no specific localisation of transcript

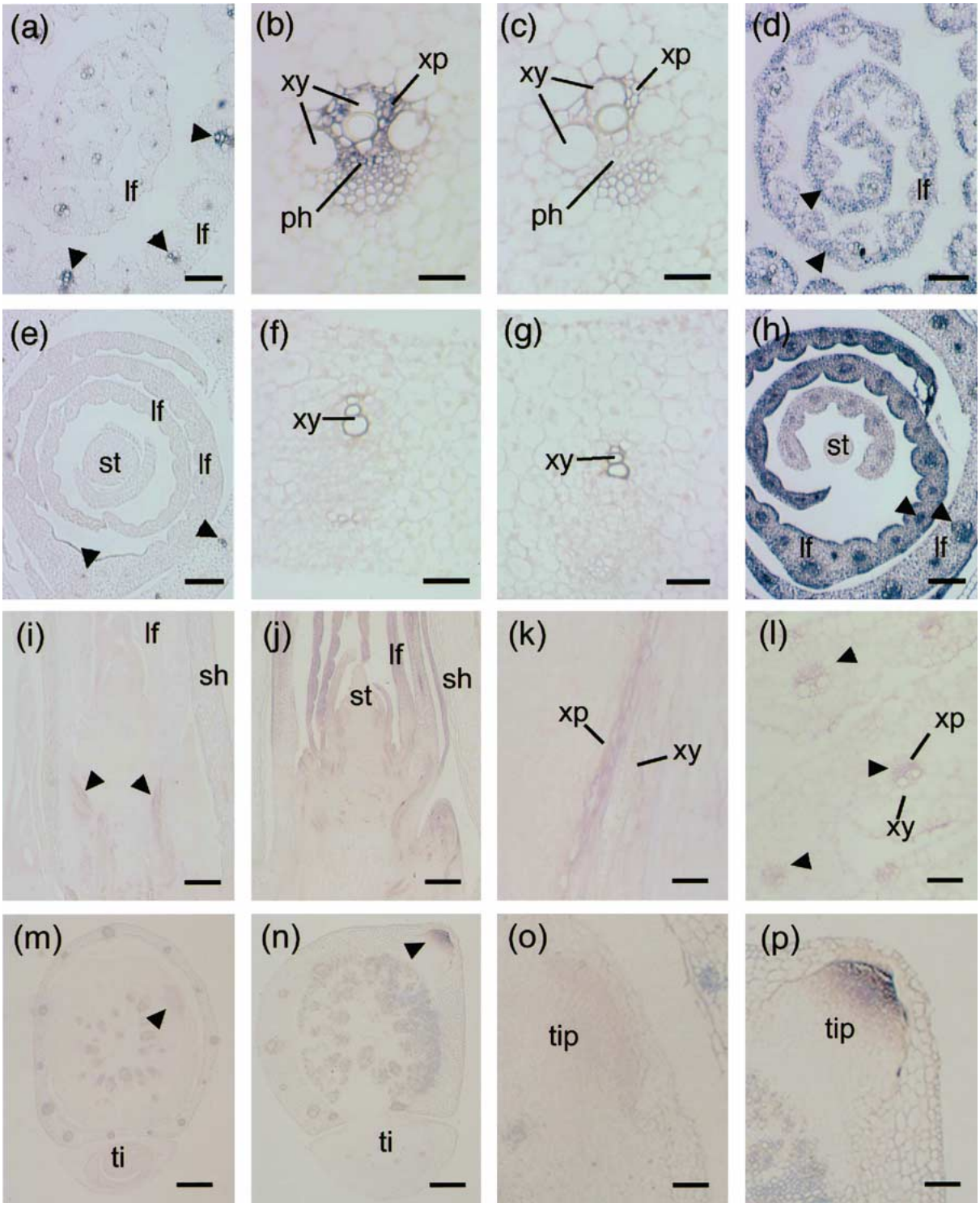
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Figure 3. In situ localisation of α- and β-expansins within the LEZ of a *F. pratensis* genotype. a–d. Cross-sections taken at 30 mm distance from the base of the growing leaf (distal end of the LEZ). e–h. Cross-sections taken at 10 mm distance from the base of the growing leaf (proximal end of the LEZ). a, b, e. f. Hybridisation with a gene specific *FpExp2* antisense probe. c, g. Hybridisation with an *FpExp2* sense probe. d, h.Hybridisation with an *FpRpl3* antisense probe. In a, e and f, the arrows identify cells showing hybridisation signals, enlargements of which are shown in c, g and h, respectively. In a and e, the arrows identify vascular bundles, enlargements of which are shown in b and f, respectively. Parts c and g show tissue sections similar to b and f, respectively, hybridised with a sense probe. In d the arrows identify palisade mesophyll tissue of the leaf, and in h the arrows identify vascular bundles. i–k. Longitudinal sections taken from the basal zone of the LEZ. l. Cross-section taken at 40 mm distance from the base of the growing leaf (proximal end of the LEZ). m–o. Cross-section prepared from the basal zone of the LEZ below the apex. i, k, l. Hybridisation with the gene-specific *FpExpB3* antisense probe. m, o. Hybridisation with the *FpExpB2* antisense probe. j, n, p. Hybridisation with an *FpRpl3* antisense probe. In i, arrows identify vascular bundles, enlargements of which are shown in k. In l, arrows refer to the xylem parenchyma. In m and n, arrows identify tiller primordia, enlargements of which are shown in o and p. Bars: 500 μm in a, d, e, i, j, m and n, 250 μm in h, 150 μm in b and c, 160 μm in o and p, 100 μm in f and g, and 62 μm in k and l. lf, leaf; sh, sheath; st, stem; xy, xylem; xp, xylem parenchyma; tip, tiller primordium; ti, tiller.

accumulation in leaf tissue, but analysis of sections just below the apex showed a distinct, localised signal in developing tiller primordia (Figure 3m, o). A similar pattern was observed in hybridisations performed with the *FpRpl3* control probe (Figure 3n, p), but hybridisations with a probe for *FpExpB3* did not reveal any accumulation of transcript in tiller primordia. Repeated attempts to obtain hybridisation signals on sections probed with *FpExpB1* failed, most likely due to the low expression of the mRNA (Figures 2 and 5b).

α- and β-expansin gene expression along the LEZ

Our initial hypothesis was that α- and β-expansin gene expression correlates with leaf elongation growth. To test this hypothesis, we first characterised the spatial distribution of growth within the LEZ of elongating *F. pratensis* leaves to determine the size of the LEZ and regions of maximum tissue elongation.

As revealed by the spatial analysis of growth within the LEZ, epidermal cell elongation stopped at about 35 mm distance from the leaf base, indicating the distal end of the LEZ (Figure 4). Within the LEZ, epidermal cells underwent a ten-fold elongation, resulting in a mean length of about 550 μm at the distal end of the LEZ. Comparison of cell length data and data on the spatial distribution of SER agreed well in the estimation of the size of the LEZ (Figure 4b). Tissue elongation occurred with increasing rates up to 26 mm distance from the leaf base, where it achieved a maximum of about 0.076 mm mm^{-1} h^{-1}. After this position, SER started to decrease until it was reduced to almost zero, at about 40 mm distance from the leaf base. Thus, the zone within which 80% of tissue elongation occurred consisted of the first 26 mm of the basal end of the LEZ.

With respect to α-expansins, a high accumulation of *FpExp2* transcripts was detected in the tissue at 24–40 mm distance from the leaf base (Figure 5). As shown in the bar chart, these tissue segments showed relatively low rates of elongation (Figure 5a). At points more proximal to the base (in which the highest rates of tissue elongation were measured), the transcript was not detectable under high stringency washing conditions (Figure 5b). At lower stringency, a weak signal was observed in this tissue, but the gradient of increasing transcript accumulation from leaf base to more distal regions was maintained (data not shown). This pattern of transcripts can be compared with that for a ribosomal protein (*FpRpl3*) which is a marker of general cellular metabolism. The *FpRpl3* transcript shows an opposite gradient to that observed with *FpExp2*, i.e., high levels of transcript at the base of the LEZ and low levels at more distal regions from the leaf base. The patterns observed for *FpExp2* and *FpRpl3* do not reflect loading differences on the blot, as revealed by ethidium bromide staining for total RNA. In addition, the pattern of *FpExp2* transcript accumulation was also observed at the protein level. As shown in Figure 5b, immunoblot analysis revealed accumulation of α-expansin protein at a distance of 16–40 mm from the leaf base, with no detectable protein in the lowest 8 mm of the LEZ.

Characterisation of the transcript levels of the three β-expansins within the LEZ revealed a similar pattern as observed for *FpExp2*. *FpExpB1* mRNA was detectable only at very low abundance in the tissue at 24–40 mm distance from the leaf base (Figure 5c). In contrast, *FpExpB2* mRNA was expressed at significantly higher levels. A strong signal, with increasing intensity from tissue at 24–40 mm distance from the leaf base, was detectable. However, also for this clone, no signal was detectable in tissue close to the leaf base. As for *FpExpB1*, we detected the *FpExpB3* transcript only in tissue at 24–40 mm distance from the leaf base but the mRNA expression level was significantly higher than that of *FpExpB1*.

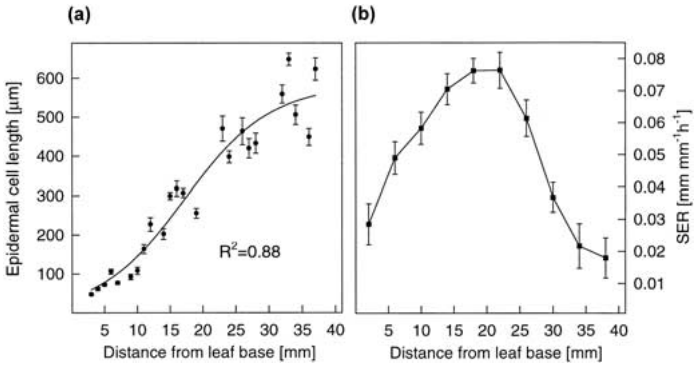

Figure 4. Spatial distribution of tissue elongation along the LEZ of a *F. pratensis* genotype. a. Spatial distribution of the epidermal cell lengths of a *F. pratensis* genotype along the LEZ. Epidermal cell length was determined using a replica technique according to Meister *et al.* (1999). Third-order sigmoidal regression curves were fitted to the data points. Data are means (± SE) of 7–10 measurements. b. Segmental elongation rates (SER) of the same genotype. Data on SER were obtained by the method described by Schnyder *et al.* (1987). Data are means (± SE) of 8–10 measurements.

α-expansin activity within the LEZ

To determine whether the expression pattern of the *FpExp2* transcript and protein were reflected at the level of α-expansin activity, we measured the ability of crude cell wall protein extracts to induce elongation of heat-inactivated plant cell walls (McQueen-Mason *et al.*, 1992; Whitney *et al.*, 2000) using protein extracted from two different tissue segments along the LEZ. In wall protein extracts from the proximal 15 mm end of the LEZ, where we measured high rates of tissue elongation but low or not detectable levels of *FpExp2* transcript and α-expansin protein (Figure 5b), we did not detect a significant level of α-expansin activity (Figure 6). In contrast, protein extracts from the 15 mm distal end of the LEZ, where tissue elongation was low but high *FpExp2* transcript and α-expansin protein levels were measured (Figure 5b), showed a high capacity to induce wall extension (Figure 6).

Since α-expansin is a cell wall protein, its physiological level of activity can be expressed relative to the mass of the extracellular matrix (Cosgrove and Li, 1993). To estimate any change in this parameter, we measured the dry weight distribution within the LEZ. As also shown in Figure 6, there is indeed a gradient of dry mass along the LEZ with the highest mass being at the proximal end of the LEZ. If the expansin activities described in Figure 6 are expressed on the basis of dry weight, then the difference in activity calculated between the two tissue samples analysed is even more exaggerated. This is also true for the northern and immunoblot data shown in Figure 5b.

Expression pattern of FpExp4 and FpXET1 along the LEZ

Although our analysis of *FpExp2* showed a very poor correlation between α-expansin expression and leaf growth rate, we could not exclude the possibility that other α-expansins not detectable by northern blots, immunoblot or activity assays used were expressed in the basal region of the leaf showing high rates of growth. Therefore, we re-screened by RT-PCR a cDNA library prepared exclusively with RNA extracted from the basal part of the LEZ. This resulted in the repeated cloning only of *FpExp4* (Figure 1a). Although not detectable by northern blot analysis, RT-PCR analysis revealed the presence of transcripts encoding *FpExp4* in all regions of the LEZ with no apparent difference in mRNA accumulation in the various segments analysed (Figure 7a).

In an effort to identify any gene encoding a cell wall protein whose expression might correlate with the measured growth pattern, we also screened the LEZ cDNA library with primers designed to amplify clones encoding xyloglucan endotransglycosylase (e.g. Fry *et al.*, 1992; Palmer and Davies, 1996; Uozu *et al.*, 2000). The first cDNA characterised from this screen encoded a novel XET, *FpXET1* (Figure 7b). This sequence showed 73% identity with the most closely related sequences from barley, *HvXEB* and *HvXEA*, which have been well characterised as XETs (Schünmann *et al.*, 1997). Analysis of the expression pattern shown by *FpXET1* within the LEZ revealed a very close correlation between transcript accumulation and growth rate distribution, with a peak of both

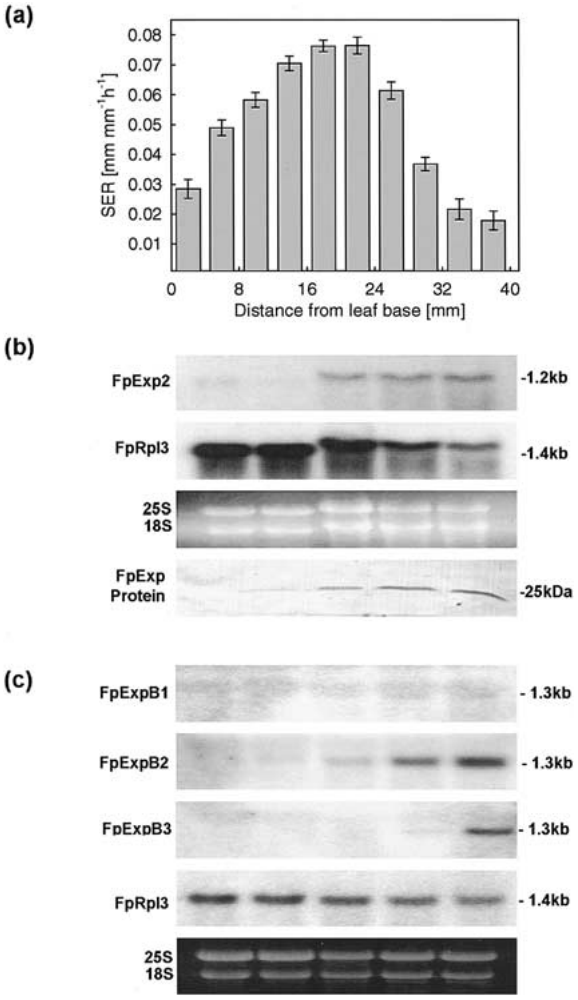

Figure 5. Comparison of tissue elongation and the expression of α- and β-expansins along the LEZ. a. The bar chart shows the SER of the relevant tissue segments along the axis of the leaf. These data are the means ($\pm$ SE) of 8–10 measurements. b. Expression of *FpExp2* along the LEZ. RNA (10 μg) was extracted from the indicated segments (0–8, 8–16, 16–24, 24–32, 32–40 mm) and hybridised with a ^{32}P-labelled probe for *FpExp2* and washed at high (0.2 $\times$ SSC, 50 °C) stringency. After stripping, blots were re-hybridised with a probe for *FpRpl3*. Loading and integrity of RNA in the gels is indicated by ethidium bromide staining of ribosomal RNA. The lowest panel (*FpExp* Protein) shows an immunoblot analysis of proteins extracted from the relevant tissue samples using an antibody against α-expansin. 40 μg protein was loaded per lane. b. Comparison of SER and β-expansin gene expression along the LEZ. Total RNA (10 μg) was extracted from the indicated segments (0–8, 8–16, 16–24, 24–32, 32–40 mm) and hybridised with ^{32}P-labelled gene-specific β-expansin probes indicated at the left of the blot. Blots were washed at high stringency (0.2 $\times$ SSC, 50 °C). After stripping, blots were re-hybridised with a probe for *FpRpl3*. Loading and integrity of RNA in the gels is indicated by ethidium bromide staining of ribosomal RNA.

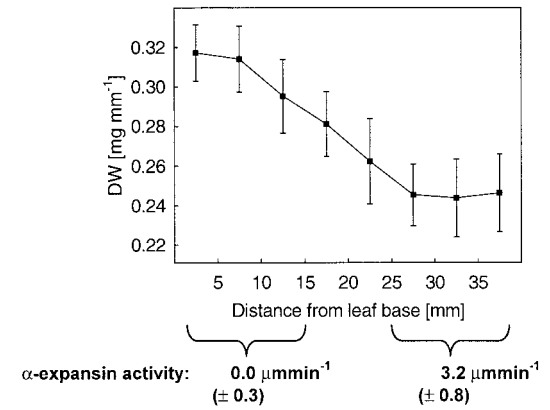

Figure 6. Spatial distribution of dry weight and α-expansin activity from two different sections within the LEZ. Spatial distribution of dry weight (DW) along the LEZ of the long leafed genotype grown under high nitrogen supply. Data are means ($\pm$ SE) of ten measurements. Cell wall proteins were extracted from 15 mm long sections consisting of the proximal (0–15 mm) and distal (25–40 mm) end of the LEZ. α-expansin activity was determined as described in the experimental procedures. Data are means ($\pm$ SE) of 3 measurements.

parameters occurring 8–24 mm from the leaf base (Figure 7a).

Discussion

Patterns of β-expansin expression in the elongating F. pratensis leaf

Our data provide the first description of organ- and tissue-specific expression of vegetative β-expansins, a novel class of cell wall proteins associated with (but not exclusive to) grasses. In particular, we show a differential tissue specific-expression of the *FpExpB3* gene (predominantly expressed in differentiating vascular tissue) and the *FpExpB2* gene (predominantly expressed in root tissue and initiating tillers). These data indicate that β-expansins are likely to display a spectrum of expression patterns as broad as that reported for α-expansins. The functional significance of such specific patterns is unclear (as with α-expansins). On the one hand, they could reflect the distribution of specific substrates for the different β-expansins, or, alternatively, they might reflect genetic redundancy. Testing of such alternatives will require both a better understanding of the endogenous substrates for β-expansins as well as the analysis of mutants lacking specific members of the gene family.

The expression of *FpExB2* in initiating tillers is especially intriguing. Tillering is a developmentally

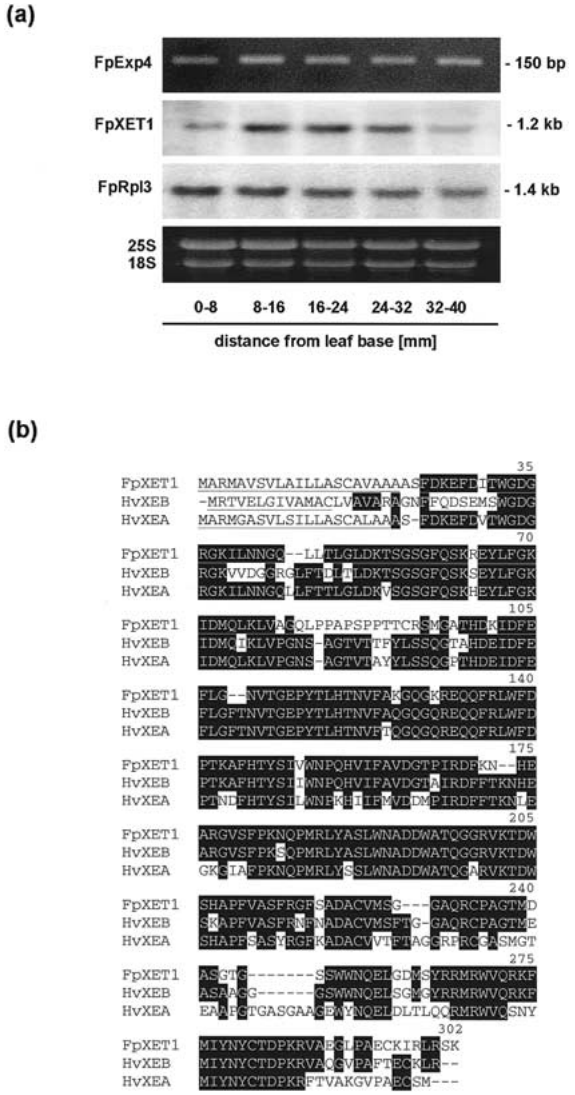

Figure 7. Expression of *FpExp4* and *FpXET1* along the LEZ. a. RT-PCR analysis of *FpExp4* was performed using gene-specific primers with cDNA from the indicated segments (0–8, 8–16, 16–24, 24–32, 32–40 mm) along the LEZ. Northern blot analysis of *FpXET1* was performed with RNA (10 μg) extracted from the indicated segments (0–8; 8–16; 16–24; 24–32; 32–40 mm) along the LEZ. A ^{32}P-labelled *FpXET1* gene-specific probe was used. Final washes were at 0.2 × SSC, 50 °C. After stripping, blots were re-hybridised with a probe for *FpRpl3*. Loading and integrity of RNA in the gels is indicated by ethidium bromide staining of ribosomal RNA. b. Amino acid sequence alignment of three XET-like proteins. The deduced amino acid sequence of *FpXET1* is compared to *HvXEA* and *HvXEB*, two XET-like proteins from barley (Schünmann *et al.*, 1997). Signal peptides (underlined) were predicted with the program PSORT (Nakai and Kanehisa, 1992). Sequences and GenBank accession numbers: *FpXET1* (AJ295943), *HvXEA* (X93174), *HvXEB* (X93175).

controlled process for the generation of stems originating from axillary meristems. As such, it has great influence on the architecture of the mature plant and also, in agronomic terms, on the yield and vegetative propagation (Hill and Pearson, 1985; Frank and Hofmann, 1994). However, the molecular processes underlying tillering are not well understood. Our data on the differential expression of a vegetative β-expansin in the young tiller provide the first step of a molecular description of this process which might provide insights into how it can be controlled.

Our data also show an association of both an α-expansin (*FpExp2*) and a β-expansin (*FpExpB2*) with differentiating vascular tissue, although with a different time course of expression. Previous investigations have also revealed an association of α-expansin gene expression with differentiation events (Cho and Kende, 1998; Kyung-Hoan *et al.*, 2000) but this is the first intimation that β-expansins might also be involved. The exact molecular processes facilitated by α- and β-expansins in the vasculature is unclear, but recently published data on xylem differentiation of *Festuca arundinacea* Schreb. indicate that at around 30–40 mm from the leaf base, where in our experiments transcript accumulation of *FpExp2* and *FpExpB2* was highest, metaxylem differentiation occurs (Martre *et al.*, 2000). A role for α- and β-expansins in cell wall re-arrangements occurring during this process seems likely.

The initial finding that grasses contain many β-expansins whereas dicotyledons do not (Cosgrove, 2000), coupled with the well characterised differences in the molecular composition of the respective cell walls (Carpita and Gibeaut, 1993), led to the suggestion that β-expansins might have supplanted or replaced α-expansin function. Our data indicate that in at least one tissue (leaf vasculature), α- and β-expansins are both expressed, i.e., α- and β-expansin expression is not exclusive at the tissue level. This implies that the substrates for the two expansins are present in the same tissue, although the precise endogenous molecular substrate for any expansin remains unclear.

Expansins, XETs and leaf growth

The initial aim of our investigation was to identify expansin genes (α or β) expressed in the growing *F. pratensis* leaf. Although this aim was achieved, our hypothesis that the expression of at least some of the expansin genes might correlate with leaf growth was

not substantiated by our results. None of the α- or β-expansin cDNAs analysed showed an expression profile consistent with a function in grass leaf elongation. There are several potential reasons for this finding.

It is clear that both α- and β-expansins are encoded by relatively large gene families. It is thus possible that our screening process missed specific members of the families involved in leaf growth. Indeed, it was only by screening a cDNA library generated from the basal 16 mm of the LEZ that enabled us to identify *FpExp4* in the LEZ. This α-expansin is certainly expressed throughout the LEZ, but the pattern of expression (at the transcript level) does not seem to vary with growth rate. Moreover, the level of transcript accumulation for *FpExp4* throughout the LEZ is significantly lower than for *FpExp2* (vascularly localised). This relatively high accumulation of *FpExp2* was reflected by assays for α-expansin protein and activity along the leaf. Although we cannot exclude the possibility that the antibody used did not detect all α-expansins in the tissue extracts or that the activity assay did not detect all α-expansin activity, the parallel profiles of *FpExp2* mRNA levels, protein levels detected by the antibody and the activity assayed make it reasonable to suggest that the most abundant α-expansins in the LEZ have been detected. A lack of correlation between α-expansin gene expression and tissue growth rates has also recently been reported in tomato, a dicotyledon (Caderas *et al.*, 2000). Our data thus add to a body of data suggesting that a simple link between α-expansin gene expression and elongation growth does not always occur.

As an alternative to α-expansin, β-expansins are obvious candidates in grass tissue as a potential mediator of elongation growth processes. However, using the cDNAs described here we could not detect any correlation between vegetative β-expansin gene expression and leaf growth rate. Although it is likely that we have characterised only a few β-expansins expressed in the elongating leaf, any other β-expansins must either be relatively lowly expressed or be so dissimilar to the conservative probes used in preliminary experiments of our study that even at low stringency they could not be detected in northern blots.

The ease of identification of *FpXET1* whose expression profile correlated very closely with the measured leaf growth rate stands in a surprising contrast to the repeated attempts of identifying α- or β-expansins displaying a correlation with leaf elongation growth. Although causal evidence linking XET expression and growth has still not been established and there is also data against such a function (McQueen-Mason *et al.*, 1993), our data support the finding in several other studies, reporting a close correlation between tissue elongation and XET expression (e.g. Pritchard *et al.*, 1993; Palmer and Davies, 1996; Schünmann *et al.*, 1997; Vissenberg *et al.*, 2000).

An explanation for these findings could therefore be that XETs and expansins may function together to modulate grass leaf growth. In such a scenario, relatively lowly expressed expansins (such as *FpExp4*) might act as keys to transiently unlock or relax cell wall architecture, allowing access to other modifiers which might modulate cell wall structure and function. Irrespective of the lack of functional data supporting XET activity in growth, it does seem that the XET reported here might serve as a useful marker for leaf growth in *F. pratensis*. Our future work will focus on testing this hypothesis.

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