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Differential expression of XET-related genes in the leaf elongation zone of *F. pratensis*

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Abstract

***Festuca pratensis* Huds. is a forage grass with the ability to withstand harsh climatic conditions. However, its potential agronomic use is limited by its poor competitive ability, which can be traced to limitations in leaf growth. In order to characterize this process and to identify genes which might function as markers for leaf growth, three XET-related genes in the leaf elongation zone (LEZ) of *F. pratensis* are reported. A detailed expression analysis is presented of the three genes in two *F. pratensis* genotypes with contrasting leaf growth characteristics grown under two nitrogen levels. By means of a detailed spatial analysis of growth and XET encoding transcript pattern along the LEZ, a specific correlation is shown between *FpXET1* expression and tissue elongation that is maintained under the different growth conditions, while the two other XETs expressed in the LEZ show different transcript dynamics. Tissue localization of *FpXET1* and *FpXET2* transcripts indicate an accumulation throughout young tissue, which is consistent with the encoded proteins playing roles in cell wall modification processes during growth. It is proposed that *FpXET1* is a potential marker for tissue elongation and leaf growth in *F. pratensis*.**

Key words: Xyloglucan endotransglycosylase, leaf elongation, *Festuca pratensis*, nitrogen, gene expression.

Introduction

Festuca pratensis Huds. is a member of the Poaceae, a family of plants of fundamental agronomic importance. While some species are primarily important for grain yield (e.g. rice, wheat), many others are cultivated for forage production or are important components of semi-natural grasslands. *F. pratensis* is an ideal forage grass, both in terms of the quantity and quality of the produced forage 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). There was therefore considerable interest in a better understanding of the molecular basis of leaf growth in *F. pratensis*. Understanding the genetic regulation of the leaf growth process would not only provide insight into the regulation of grass leaf growth in general, but could also provide tools for marker-assisted breeding.

Recent experiments have highlighted the biochemical regulation of cell wall extensibility as a key process in controlling growth in plants and have led to the identification of a number of proteins which are potentially involved in this process (Cosgrove, 1999). A candidate gene approach was taken in an attempt to identify genes whose expression pattern might function as markers of tissue elongation and leaf growth. In previous work (Reidy *et al.*, 2001) a detailed analysis of the spatial expression of α- and β-expansin genes in the leaf elongation zone (LEZ) of *F. pratensis* was reported. These data indicated that none of the expansin genes analysed showed an expression pattern which correlated with leaf growth. However, it was possible to identify an XET-related gene (*FpXET1*) which on initial analysis showed a pattern of transcript accumulation that closely matched the tissue elongation pattern within the LEZ.

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

EMBL accession numbers: *FpXET1* (AJ295943), *FpXET2* (AJ295944), *FpXET3* (AJ295945).

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XET catalyses the transglycosylation of xyloglucan, the major hemicellulose polymer that is thought to mediate the cross-linking of cellulose microfibrils in the cell wall (Fry *et al.*, 1992; Nishitani and Tominaga, 1992) and it has been proposed to be involved in the control of cell wall relaxation. XET proteins are encoded by relatively large gene families in various plant species (Xu *et al.*, 1996; Schünmann *et al.*, 1997; Uozu *et al.*, 2000) and several of these members have been shown to be specifically up-regulated by various environmental stimuli and growth-promoting hormones (Potter and Fry, 1994; Xu *et al.*, 1995; Palmer and Davies, 1996; Smith *et al.*, 1996). Additionally, in many cases significant correlations between high levels of XET activity and tissue elongation have been described (Potter and Fry, 1993; Schünmann *et al.*, 1997; Burstin, 2000; Uozu *et al.*, 2000). Although the causal role of XET in growth remains debatable (McQueen-Mason *et al.*, 1993), the reported data have been interpreted as indicating an important function for XET in either reversibly loosening the cellulose–xyloglucan network or altering the cell wall architecture during growth.

In this paper a detailed analysis of three XET-related genes expressed in the growing leaf of two *F. pratensis* genotypes with contrasting leaf growth characteristics is reported. By supplying two different levels of nitrogen to modify the spatial distribution of tissue elongation within the LEZ (Fricke *et al.*, 1997), the contribution that the three XET-related genes could make to regulate leaf growth in *F. pratensis* was assessed. By means of a detailed spatial analysis of growth and the XET encoding transcripts along the LEZ, a specific correlation was shown between *FpXET1* expression and leaf growth that is maintained under the different growth conditions, while *FpXET2* and *FpXET3* show different transcript dynamics. *In situ* localization of *FpXET1* and *FpXET2* transcripts indicate an accumulation in young tissue, consistent with the encoded proteins playing a role in cell wall modification processes during growth.

Materials and methods

Plant material and growth conditions

Two *F. pratensis* (*Festuca pratensis* Huds.) genotypes differing in leaf growth characteristics were selected from the cultivar 'Prefest' (RAC, Changins, Switzerland). Clonal replicates of each genotype were produced by growing individual tillers of the two genotypes. After 28 d, plants were cut to 5 cm tiller and root length and individual tillers of similar weight transferred to hydroponics filled with a nitrogen-free nutrient solution (Hammer *et al.*, 1978). The medium was continuously aerated and replaced every seventh day. The plants were cultivated in a growth chamber (PGV36, Conviron Instruments, Winnipeg, MB, Canada) at a temperature of 20 °C, 80% relative humidity and 400 μmol m^{-2} s^{-1} photosynthetic photon flux density.

Nitrate was added daily at a rate of either 5% (N) or 500% (NN) of the amount that plants would have bound daily under non-limiting growth conditions using a modified version of the *addition-rate* approach described by (Ingestad and Lund, 1986).

Determination of LER and SER

All growth measurements were performed 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 using linear voltage displacement transducers connected to a data logger. Segmental elongation rates (SER) were determined by measuring the short-term displacement of holes within the LEZ as described by (Schnyder *et al.*, 1987).

The length of abaxial epidermal cells within the LEZ was determined by the preparation of leaf replicas modified according to Meister *et al.* (Meister *et al.*, 1999). The length of 7–10 epidermal cells was measured at different positions along the LEZ under a microscope.

cDNA cloning and sequence analysis

Total RNA (5 μg) was extracted using an RNeasy Plant Kit (Qiagen, Basel, Switzerland) from the 40 mm LEZ and used as the template for reverse transcription with an oligo d(T) primer. The products were subjected to PCR amplification using degenerated sense 5′-CGAGATCGACNTCGAGTTC-3′ and antisense 5′-SGTCNGRGCAGTAGTNGTAG-3′ primers based on alignments of XET-related sequences from other Graminaceae species. PCR products of the predicted size (520 bp) 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 using the program PSORT (Nakai and Kanehisa, 1992). To determine the relatedness to XET-related sequences from other species, the deduced polypeptides were clustered using the Clustal procedure of DNASTAR Megalign (DNASTAR Inc., Madison, WI).

Generation of full-length cDNAs

Full-length cDNA clones were obtained in a two-step procedure using RACE-PCR. Poly$(A)^{+}$ RNA (1 μg) extracted from the LEZ, coleoptiles (obtained by germinating seeds of the cultivar 'Prefest'), apical meristems, and the apical region of the growing root tips was converted into cDNA according to the manufacturer's instructions (Smart™ RACE cDNA Amplification Kit, Clontech, Basel, Switzerland). 5′ and 3′ RACE fragments were produced by using gene-specific antisense 5′-AGCCTGAACTGCTGCTCCCGCTTG-3′ (*FpXET1*), 5′-GTGCTCCTTCTTGCCGTCGCCGC-3′ (*FpXET2*), 5′-TGTAGGGCTGTCCGGTCTCGTTCC-3′ (*FpXET3*), and gene-specific sense 5′-AGGAGCTCGGCGACATGAGCTACC-3′ (*FpXET1*), 5′-CGTCGCCTGGACGGAGCGCAAC-3′ (*FpXET2*), and 5′-GGAAGATGCAGTGGGTACAGGAGAG-3′ (*FpXET3*) primers together with the corresponding supplied RACE primers. Obtained PCR products were cloned and sequenced as described above.

DNA gel blot analysis

High molecular weight genomic DNA was extracted using the DNeasy Plant Kit (Qiagen, Basel, Switzerland). 8 μg DNA was digested with *Pst*I, *Eco*RI or *Hind*III, separated on a 0.8% agarose gel and transferred to Nytran membranes (Schleicher & Schuell, Dassel, Germany). Blotting and hybridization were performed under standard conditions (Sambrook *et al.*, 1989). The blot was hybridized at 65 °C with the corresponding randomly ^{32}P-labelled gene-specific *FpXET* 3′ fragments obtained in the RACE procedure. Membranes were washed to a final stringency of 0.2×SSC, 0.5% SDS at 50 °C before autoradiography at −80 °C using intensifying screens.

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 apical meristems, 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 of 10 μg were separated on gels of 1.1% agarose and 0.65 M formaldehyde. Blotting, hybridization, washing, and autoradiography was performed as described for DNA gel blot analysis.

RT-PCR analysis of FpXET3

For the detection of *FpXET3*, total RNA was extracted using a RNeasy Plant Kit (Qiagen, Basel, Switzerland) from the corresponding segments and used as the template for reverse transcription with an oligo d(T) primer. Care was taken to use the same amount of RNA for each sample by measuring OD_{260}. For PCR amplification (30 cycles) the *FpXET3* gene-specific primer pair 5′-GGAAGATGCAGTGGGTACAGGAGAG-3′ and 5′-TGCATGAGCGGCGATCACGAATGC-3′ resulting in a 145 bp product, was used. Either 2 μl of the RT-PCR reaction (tissue specific expression) or 1 μl of the 1 : 10 diluted RT-PCR reaction (expression along the LEZ) were used as substrates.

In situ mRNA hybridization

In situ hybridization 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 hybridized with appropriate gene specific 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 hybridizations.

Results

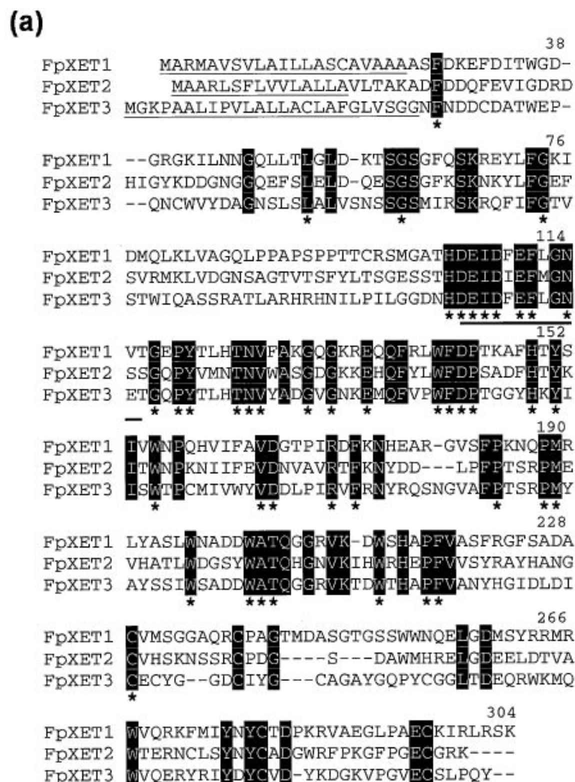
Cloning XET-related cDNAs from F. pratensis

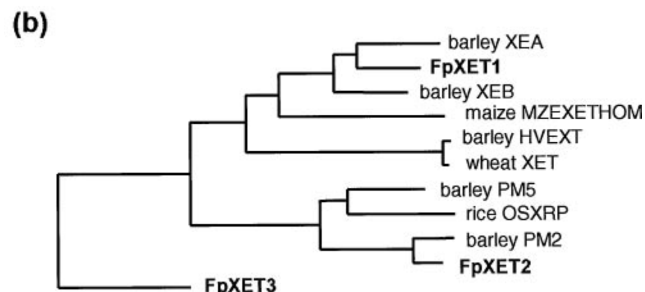
Three full-length XET-related cDNA clones were obtained using an RT-PCR coupled RACE procedure (Fig. 1a). The cDNAs contained 5′-untranslated regions of 123 bp (*FpXET1*), 63 bp (*FpXET2*), 120 bp (*FpXET3*), and 3′-untranslated regions of 188 bp (*FpXET1*), 280 (*FpXET2*) and 215 bp (*FpXET3*). The ORF ranged from 876 bp for *FpXET1*, 846 for *FpXET2* to 864 bp for *FpXET3* and encoded polypeptides of 292, 282 and 288 amino acids. All three sequences contained a hydrophobic signal peptide as predicted by the program PSORT (Nakai and Kanehisa, 1992). The *FpXET1* sequence is from Reidy *et al.* (Reidy *et al.*, 2001).

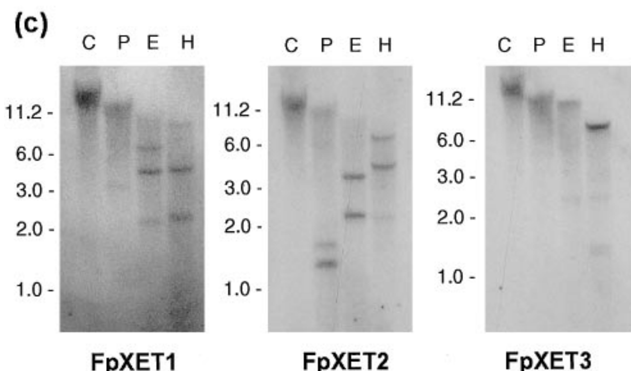
Although the three deduced *FpXET* polypeptides showed relatively little identity to each other (40% between *FpXET1* and *FpXET2*, 36% between *FpXET1* and *FpXET3* and 29% between *FpXET2* and *FpXET3*), a sequence alignment revealed several stretches of conserved amino acid residues between the three clones (Fig. 1a). The three deduced polypeptides also contained the XET conserved DEIDFEFLG motif and the putative *N*-glycosylation signal previously described from other XET-related sequences (Campbell and Braam, 1999).

To obtain further information about the relationship of the *FpXET* clones, the mature proteins were compared by means of cluster analysis to XET-related sequences reported from other grass species (Fig. 1b). The compared sequences fall into three main groups: (i) *FpXET1* showed 74% identity to *XEB* and forms a distinct branch on the tree together with XET-related sequences from barley, maize and wheat. (ii) *FpXET2* shares a surprisingly high amino acid identity to *PM2* (83%), an XET-related gene from barley and together with related sequences from rice and barley forms another distinct group. (iii) *FpXET3* forms a separate branch on the tree and shows the lowest level of identity to the compared sequences. However, a Blast search revealed 44% identity at the amino acid level to the accession number BAB08789, a putative XET clone from *Arabidopsis*.

To estimate the number of genes potentially encoding the RNAs from which the *FpXETs* were cloned, gene specific probes were used prepared from the 3′ end of the three cDNAs to probe digested genomic DNA in a Southern blot analysis (Fig. 1c). Membranes probed with *FpXET1* and *FpXET2* yielded two restriction bands, indicating that these sequences are likely to be encoded by multiple genes. In contrast, hybridization with *FpXET3* resulted in a single hybridization band, consistent it with being encoded by a single gene.







Gene and tissue-specific expression of FpXET

Gene-specific probes with little sequence homology were designed from the 3′-untranslated region of the corresponding clones to analyse the tissue-specific expression pattern of the *FpXET* genes. The transcript levels were examined by Northern blot analysis in the LEZ (40 mm basal end of the growing leaf), coleoptiles, the 4 mm basal end of the growing tiller including the apical meristem, and in the 3 cm apical region of growing root tips (Fig. 2a). The expression pattern of *FpXET3* was determined by RT-PCR, since the transcript was not detectable by Northern blot analysis (Fig. 2b).

The expression of all three *FpXET* genes was strictly restricted to leaf tissues, none of the genes was expressed in the apical region of the growing root tip. However, the three genes showed considerable variation at the level of their expression depending on leaf tissue and gene analysed. *FpXET1* was expressed at the highest levels in the basal end of the growing tiller, the abundance was slightly lower in the LEZ and only barely detectable in coleoptiles. In contrast, the abundance of *FpXET2* was highest in the LEZ while the transcript was found at similar levels in coleoptiles or in the basal end of the growing tiller. As detected by RT-PCR, the expression of *FpXET3* was limited to the LEZ and the basal end of the growing tiller.

Diurnal cycle of XET gene expression

Preliminary Northern blot analysis revealed a high variation of the *FpXET* transcript levels within the LEZ, depending on the time of tissue collection. To standardize time of tissue sampling and growth conditions for further experiments, the time-course of the *FpXET* mRNA levels within the LEZ was analysed with gene specific probes

Fig. 1. Sequence comparison of the deduced amino acid sequences of *F. pratensis* xyloglucan endotransglycosylase (XET) related proteins. (a) Alignment of the deduced amino acid sequences. Signal peptides (underlined) were predicted using the program PSORT (Nakai and Kanehisa, 1992). The XET conserved DEIDFEFLG motif and the putative *N*-glycosylation site (Campbell and Braam, 1999) (underlined, bold), as well the conserved motifs between XETs from other grass species (asterisks). (b) Comparison of the deduced amino acid sequences of XETs. Dendrogram showing the relationship of XETs from different grass species. Deduced polypeptides (without signal peptides) were clustered using the Clustal procedure of DNASTAR Megalign (DNASTAR Inc., Madison, WI). Sequences and GeneBank accession numbers: barley *XEB* (T06202), barley *XEA* (T06201), maize *MZEXETHOM* (T02090), barley *EXT* (CAA62847), wheat *EXT* (E49539), barley *PM5* (T06200), rice *OSXRP* (JE0156), barley *PM2* (T06166), *FpXET1* (AJ295943), *FpXET2* (AJ295944), and *FpXET3* (AJ295945). (c) Southern Blot Analysis of *FpXET*. Genomic DNA (8 μg) was digested with either *Pst*I (P), *Eco*RI (E) or *Hind*III (H), separated by gel electrophoresis and hybridized with ^{32}P-radiolabelled gene-specific probes of the corresponding XET-related cDNAs. Blots are shown for high (0.2×SSC, 50 °C) stringency washes. Lane C shows undigested DNA as control. DNA size standards (kb) are given on the left of each blot.

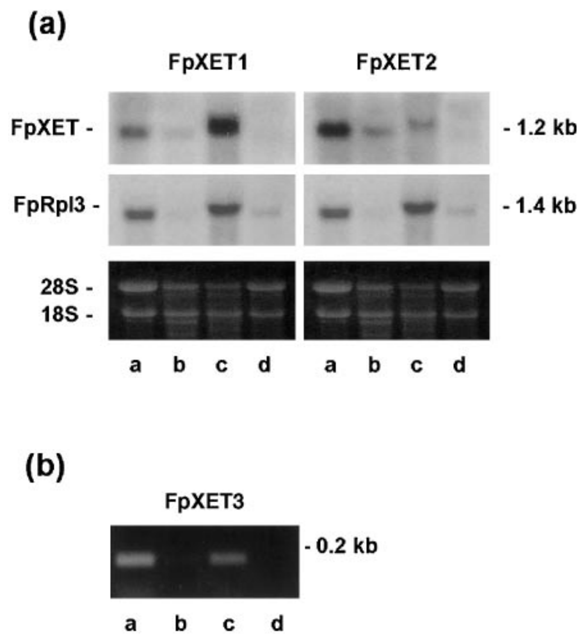


Fig. 2. Northern blot analysis reveals tissue specific expression of *FpXET* genes. (a) Gene and tissue specific expression of *F. pratensis* XET-related genes. Total RNA was extracted from the LEZ (40 mm basal end of the growing leaf) (a), coleoptiles (b), apical meristems (c), 3 cm apical region of growing root tips (d), and 10 µg of each sample separated by gel electrophoresis. Northern blots were hybridized with gene-specific probes prepared from the 3′-untranslated region of the corresponding clone indicated above each blot. Transcript levels of *FpRpl3*, encoding a ribosomal L3 protein and ethidium bromide stained 28S and 18S rRNA served as controls. (b) RT-PCR analysis of *FpXET3*. RT-PCR was performed with the same RNA samples used for tissue specific Northern blot analysis. RNA concentration was determined by OD260 and 2.5 µg subjected to reverse transcription using an oligo d(T) primer. PCR amplification (30 cycles) was done using a *FpXET3* gene-specific primer pair and 2 µl of the RT reaction.

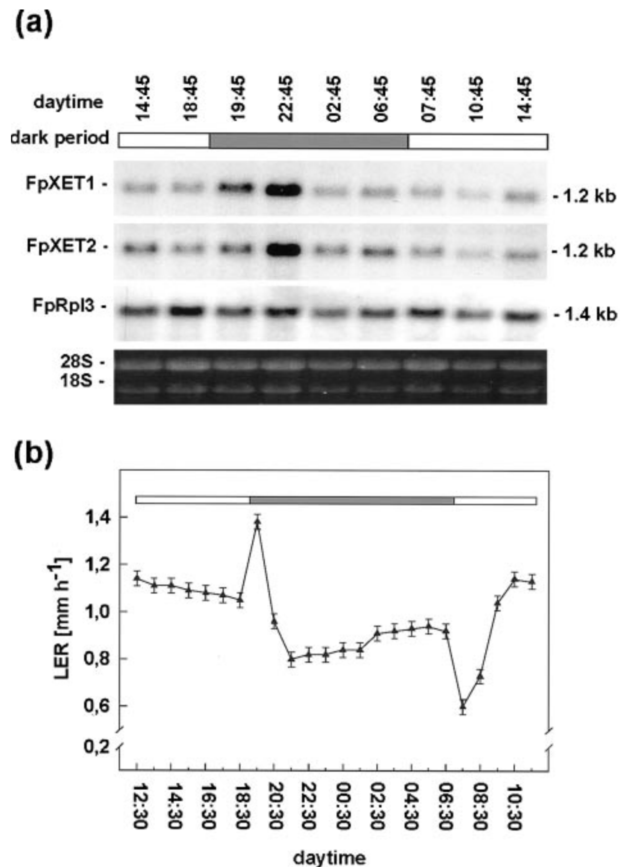


Fig. 3. Northern blot analysis reveals diurnal variation of XET-related gene expression in *F. pratensis*. (a) Influence of light and dark period on the transcript level of two *FpXET* genes. Samples were taken at the times indicated. The bar above the blot indicates the dark period. Total RNA was isolated from the 40 mm basal end of the growing leaf consisting of the LEZ and 20 µg of each sample subjected to gel blot analysis. ^{32}P-radiolabelled gene-specific *FpXET1* and *FpXET2* probes were used. Stripped blots were rehybridized to a control probe encoding ribosomal L3 protein (*FpRpl3*) to ensure integrity and equal loading of the samples. Ethidium bromide stained 28S and 18S rRNA served as additional controls. (b) Diurnal fluctuation of the leaf elongation rate (LER). LER was determined using linear displacement transducers connected to a data logger. Data are means (±SE) of 13 measurements.

and RT-PCR over a period of 36 h (Fig. 3a). Depending on the time of tissue collection, the *FpXET1* and *FpXET2* mRNA levels varied considerably, with the most striking differences shortly after the light/dark transition. The *FpXET1* and *FpXET2* mRNAs started to accumulate shortly after the onset of the dark period and increased considerably within a period of 2.5 h. In contrast, the beginning of the light period resulted in slightly decreased *FpXET1* and *FpXET2* mRNA levels, with a daily minimum at approximately 2.5 h after the onset of the light period. This pattern of transcript accumulation can be compared with the measured diurnal fluctuation in the LER (Fig. 3b). RT-PCR analysis revealed no significant diurnal fluctuations in the mRNA level of *FpXET3* (data not shown).

Epidermal cell size and SER within the LEZ

To test for any correlation between XET gene expression and growth rate, the spatial distribution of growth within the LEZ of elongating *F. pratensis* leaves of two different genotypes under different nitrogen supply was characterized first. In particular, to determine the size of the LEZ and regions of maximum tissue elongation within the LEZ, the lengths of abaxial epidermal cells and the segmental elongation rates (SER) of 4 mm segments of a long-leafed (LL) and a short-leafed (SL) *F. pratensis* genotype grown under two levels of nitrogen supply were measured (Fig. 4).

When the plants were grown under high nitrogen supply, both genotypes showed a comparable distribution of epidermal cell lengths along the LEZ (Fig. 4a, LL NN versus SL NN). Epidermal cell elongation stopped in both genotypes at 25–35 mm distance from the leaf base, indicating the distal end of the LEZ. Growing the LL genotype under low nitrogen supply reduced the zone in which epidermal cell elongation occurred considerably (Fig. 4a, LL N). Compared to plants grown under high

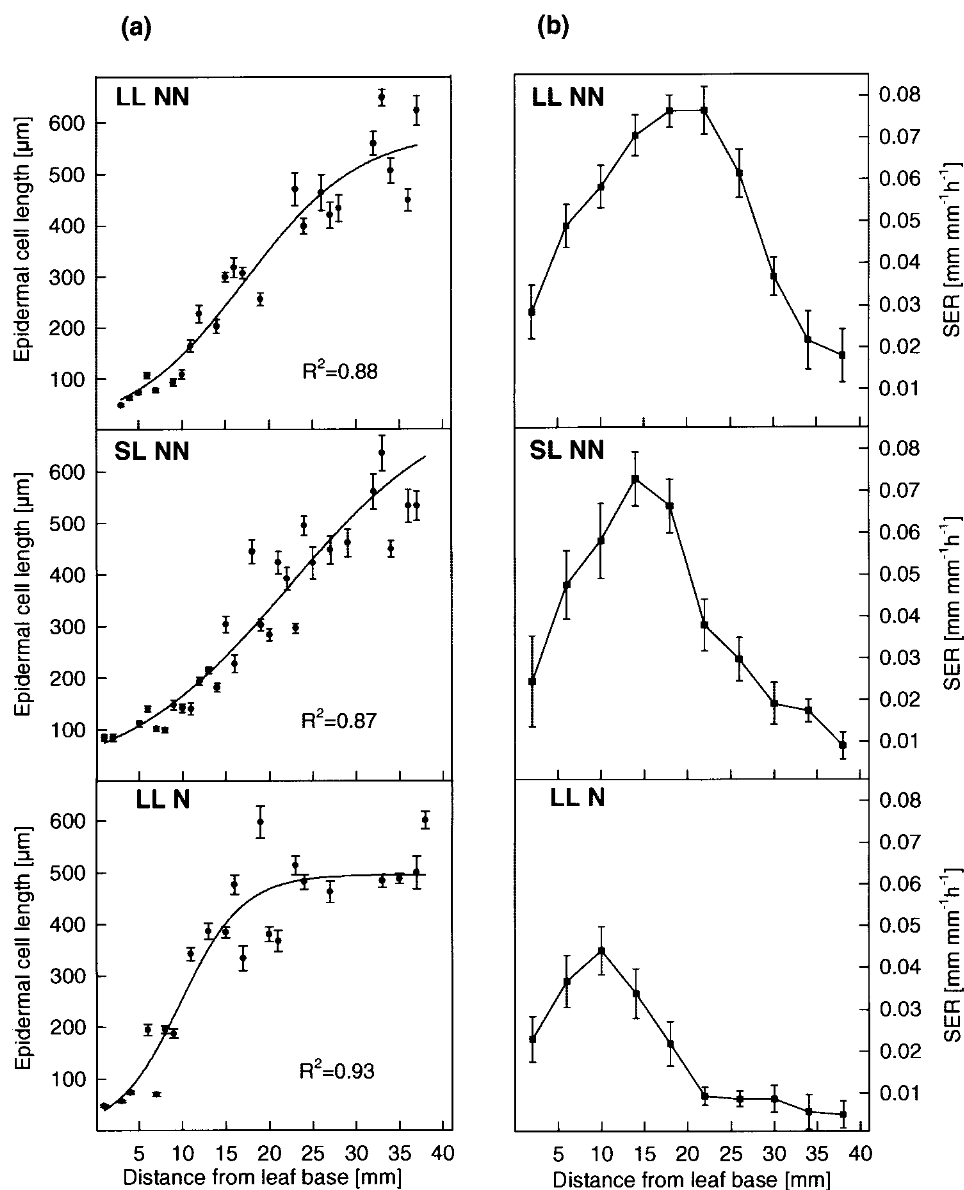


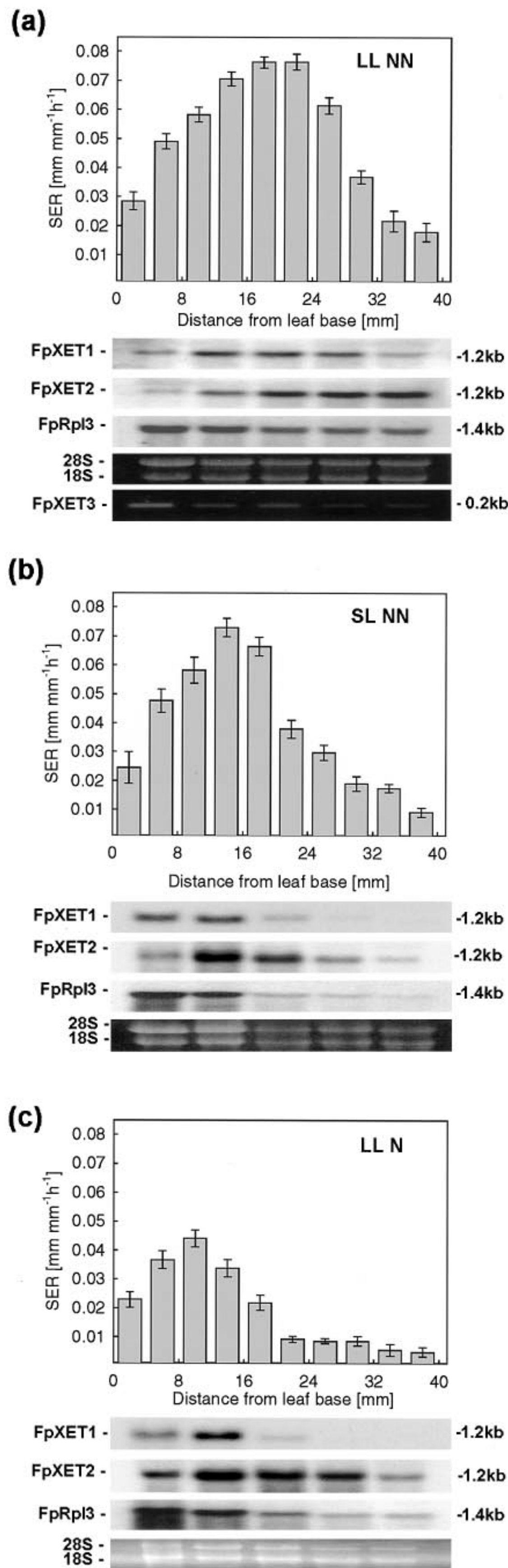
Fig. 4. Distribution of epidermal cell length and tissue elongation along the LEZ. (a) Spatial distribution of the epidermal cell lengths of a short-leafed (SL) and a long-leafed (LL) genotype along the LEZ grown under high (NN) or low (N) nitrogen supply. Epidermal cell length was determined using a replica technique (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 two different genotypes (SL or LL) grown under high (NN) and low (N) nitrogen supply. Data on SER were obtained by using the method described previously (Schnyder and Nelson, 1987). Data are means (±SE) of 8–10 measurements.

nitrogen supply, epidermal cells stopped elongating at 15–20 mm distance from the leaf base, resulting in a strongly reduced length of the LEZ.

Comparison of cell length data (Fig. 4a) and data on the spatial distribution of SER (Fig. 4b) agreed well in the estimation of the size of the LEZ. Under high nitrogen supply, the maximum SER occurred in the SL genotype closer to the leaf base (16 mm distance from the leaf base) than in the LL genotype (Fig. 4b, SL NN and LL NN). Thus, the zone within which 80% of tissue elongation occurred consisted of 26 mm for the LL and 22 mm for the SL genotype, respectively. Under low nitrogen supply, the maximum SER was strongly decreased (−42%) and was located close to the leaf base (10 mm distance from the leaf base), resulting in a significant shorter zone within which 80% of tissue elongation occurred (Fig. 4b, LL N).

FpXET *gene expression within the LEZ*

To test if the expression of the *FpXET* genes can be correlated with the spatial distribution of tissue elongation along the LEZ, the transcript abundance of the three *FpXET* genes was characterized in successive 8 mm long segments along the LEZ (Fig. 5).



The expression of *FpXET1* correlated closely with the measured spatial distribution of growth along the LEZ. In LL plants grown under high nitrogen supply (Fig. 5a), the *FpXET1* transcript showed a strong accumulation up to approximately 24 mm distance from the leaf base where it reached its maximum. This was also the region where maximum rates of tissue elongation were measured (Fig. 4). After this position, the transcript abundance decreased steadily in the following two leaf segments, matching closely the decreasing rates of tissue elongation. In contrast to *FpXET1*, the *FpXET2* transcript accumulated towards the end of the LEZ, with highest levels in segments where tissue elongation had already decreased to low levels. This different pattern of *FpXET1* and *FpXET2* transcript accumulation was maintained in the other treatments, for example, in the SL genotype grown under high nitrogen supply and in the LL genotype grown under low nitrogen supply (Fig. 5b, c). Since in both treatments the LEZ was significantly shorter and maximum rates of tissue elongation occurred closer to the leaf base, the distribution of the transcript was shifted towards the leaf base. However, the abundance of *FpXET1* in both treatments always peaked in the segments where maximum rates of tissue elongation occurred, whereas the accumulation of the *FpXET2* transcript was always maintained at a high level in segments distal to the points where maximum rates of tissue elongation were observed. Semi-quantitative RT-PCR detection of *FpXET3* yielded an additional pattern (Fig. 5a) in which transcript accumulation was maximal in the segment close to the leaf base and slightly less abundant in segments more distal to the leaf insertion point. A similar pattern of *FpXET3* transcripts was observed for the SL genotype grown under high nitrogen supply and the LL genotype grown under low nitrogen supply (data not shown). The distinct pattern of transcript accumulation for *FpRpl3*, a ribosomal protein encoding a marker of general cellular metabolism, and ethidium bromide

Fig. 5. Comparison of SER and expansin gene expression along the LEZ of two *F. pratensis* genotypes grown under different nitrogen supply. Data are shown for (a) the long-leafed genotype (LL) under high nitrogen supply (NN), (b) the short-leafed genotype (SL) under high nitrogen supply (NN), (c) the long-leafed genotype (LL) under low nitrogen supply (N). (a–c) In each part the bar chart shows the SER of the relevant tissue segments along the axis of the leaf. These data are the means (±SE) of 8–10 measurements. RNA was extracted from the indicated segments (0–8, 8–16, 16–24, 24–32, and 32–40 mm) and 10 μg separated on a formaldehyde gel, blotted, then hybridized with the corresponding ^{32}P-labelled gene-specific *FpXET* probe and washed at high (0.2×SSC, 50 °C) stringency. After stripping, blots were rehybridized 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 in (a) (*FpXET3*) shows the result of a semi-quantitative RT-PCR for *FpXET3*. RNA concentration was determined by OD260 and 2 μg subjected to reverse transcription using an oligo d(T) primer. PCR amplification (30 cycles) was done using a *FpXET3* gene-specific primer pair and 1 μl of the 1 : 10 diluted RT-reaction.

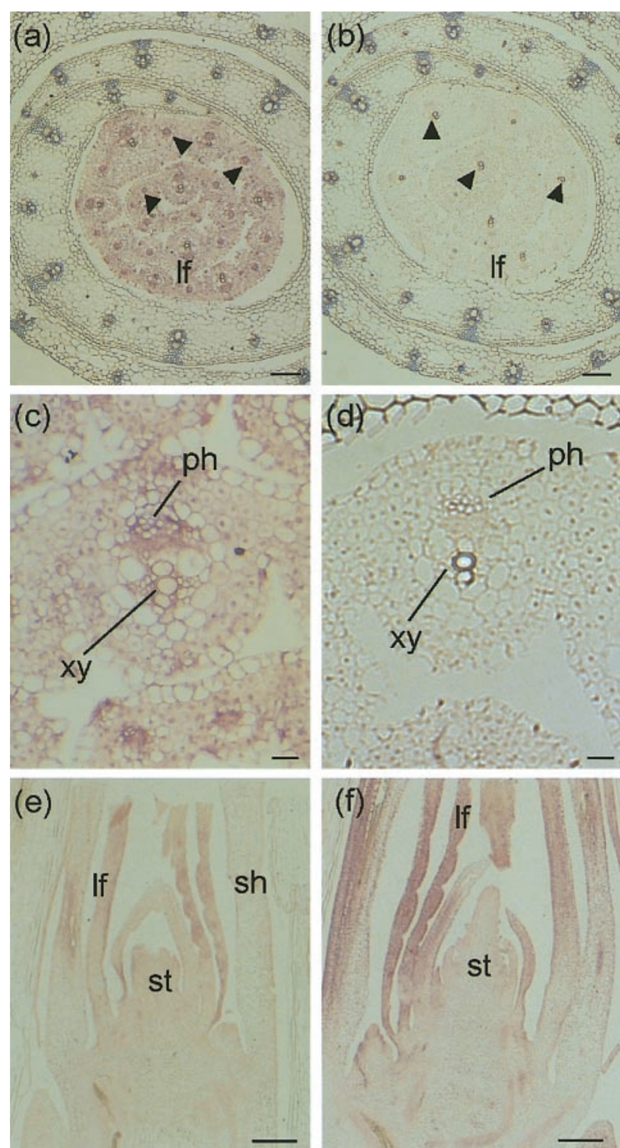


Fig. 6. *In situ* localization of *FpXET* mRNA in the leaf elongation zone. (a–d) Cross-sections taken from the basal zone of the LEZ; (e, f) longitudinal sections prepared from the basal part of the LEZ including the apical meristem; (a, c, e) hybridization with the gene specific *FpXET1* antisense probe; (b, d) hybridization with the *FpXET1* sense probe; (f) hybridization with the *FpRpl3* antisense probe. In (a) and (b) the arrows identify vascular tissue, enlargements of which are shown in (c) and (d). Bars in (a) and (b) = 210 μm; Bars in (c) and (d) = 42 μm; Bars in (e) and (f) = 420 μm; lf, leaf; sh, sheath; st, stem; xy, xylem; ph, phloem.

staining for total RNA revealed that the differential gene expression patterns observed for *FpXETs* do not reflect differences in general RNA accumulation or quantity of the loaded RNA (Fig. 5).

In situ *localization of the* FpXET *transcripts*

By means of *in situ* hybridization on sections prepared from different positions of the LEZ, the cell specific expression pattern of the three XET-related genes was analysed. Hybridization with the gene-specific *FpXET1* antisense probe of cross-sections from the basal zone of the LEZ (Fig. 6a, c) and of longitudinal sections (Fig. 6e) revealed an accumulation of the *FpXET1* mRNA in young elongating leaves. Although the expression did not seem to be delimited to specific cell types, significant hybridization was seen in cells between the phloem and xylem vessels (Fig. 6c). No signal was observed in the non-growing sheaths and leaves surrounding the young leaf (Fig. 6a, e). Comparison with hybridizations performed with the sense probe on similar sections revealed no signal, supporting the specific expression pattern (Fig. 6b, d). Hybridizations with an antisense control probe encoding a ribosomal protein (*FpRpl3*) of longitudinal sections indicate general distribution of mRNA in the young elongating leaves (Fig. 6f). Antisense hybridizations of cross-sections with the gene-specific probe for *FpXET2* showed a similar expression pattern as observed for *FpXET1* (data not shown). Repeated attempts to obtain hybridization signals on sections probed with *FpXET3* failed, most likely due to the low expression of the mRNA (Figs 2b, 5a).

Discussion

The aim of this study was to test the potential of specific XET-related genes as markers for tissue elongation and leaf growth in *F. pratensis*. XET in *F. pratensis* is encoded by a multigene family, as has been shown for other plants. In particular, sequence comparison shows that the cDNAs of *FpXET1* and *FpXET2* are very similar to XET-related genes previously described in barley (Schünmann *et al.*, 1997), whereas *FpXET3* showed little sequence identity to previously described XET-related genes from various other species (including dicots and monocots). *FpXET3* was also not grouped into one of the four XET-related subfamilies that have been previously described (Campbell and Braam, 1999) and its transcript was present at an abundance not detectable by Northern blot or *in situ* hybridization analysis. However, the *FpXET3* protein does share the commonly observed XET characteristic motifs and showed significant homology to a putative XET-related gene described in *Arabidopsis*. It is thus likely that *FpXET3* represents a member of a novel XET-related subfamily.

Despite the high degree of the amino acid identity between *FpXET2* to *PM2* from barley, this homology was not reflected at the level of gene expression. While *PM2* is expressed in barley in the basal part of the LEZ and has therefore been considered as a potential candidate for a direct involvement in the control of tissue elongation (Schünmann *et al.*, 1997), *FpXET2* mRNA accumulation occurs in the LEZ of *F. pratensis* more distally, in tissue

where elongation ceases. This suggests that *FpXET2* does not simply represent an orthologue of *PM2* but most likely an additional member of the XET-related gene family in grasses. None of the three *FpXET* genes was expressed in the apical region of the growing root tip. However, it is also conceivable that root-expressed XET-related genes exist in *F. pratensis*, as has been reported in maize (Palmer and Davies, 1996) or rice (Uozu *et al.*, 2000).

The present results provide a novel insight into the environmental regulation of *FpXET* gene expression, particularly in response to the onset of the dark period when a significant increase was measured in the transcript abundance of *FpXET1* and *FpXET2*. This altered expression occurred with a slight delay relative to a distinct maximal and minimal short-term peak of the LER measured after the off- or onset of the light period, respectively. As reported by other authors, such short time changes in the LER can be interpreted as the result of an altered leaf water status (Parrish and Wolf, 1982) and do not therefore necessarily reflect enzymatically altered rheological properties of the cell wall. Despite the temporal relationship between *FpXET* transcript accumulation and LER this suggests therefore that the transcript accumulation is more likely effected indirectly through the leaf water status or the light/dark transition.

Since the *FpXET* genes are potential markers for leaf growth, the contribution that these genes could make to the regulation of tissue elongation and leaf growth was analysed using two different genotypes with characteristic leaf growth dynamics under two nitrogen supply levels. This approach made it possible to modulate the growth distribution within comparable tissues and so to test the correlation of *FpXET* expression and this trait. This detailed quantitative analysis of tissue elongation within the LEZ showed very clearly significant differences in the gradient of tissue elongation along the axis of the leaf and in the position of the characteristic maximal growth peak depending on the genotype and nitrogen level (Volenec and Nelson, 1981; Fricke *et al.*, 1997).

Under all three growth conditions tested, the expression pattern of *FpXET1* correlated closely with the measured spatial distribution of the SER, suggesting that the function of this gene is associated with cell wall modification processes during tissue elongation. This is further supported by the results of the *in situ* hybridization studies, which revealed a highly specific accumulation in rapidly elongating tissue. The specific expression characteristics of *FpXET1* therefore make it a potential candidate as a marker gene for tissue elongation and leaf growth in *F. pratensis*.

The specificity of the *FpXET1* expression is further supported by the contrasting pattern of accumulation of *FpXET2* transcripts in segments at the distal end of the LEZ, after the occurrence of the maximal SER peak. The high expression of *FpXET2* in tissue where elongation ceases and differentiation events occur (MacAdam *et al.*, 1992; Martre *et al.*, 2000) suggests alternative biological functions to that of *FpXET1*. One possibility is that *FpXET2*, together with other proteins (such as expansins (Reidy *et al.*, 2001) or peroxidases (MacAdam *et al.*, 1992) which have been shown to be expressed in similar spatial gradients) is involved in differentiation events occurring in relation to the cessation of elongation growth. In such a scenario *FpXET2* could catalyse the integration of newly synthesized xyloglucan polymers into the differentiating secondary cell wall.

In conclusion, this study shows that several XET-related genes are differentially expressed in the elongating leaf of *F. pratensis*. Based on the quantitative analysis of the spatial growth distribution, and the specific transcript accumulation of *FpXET1* in young elongating tissue, it is proposed that *FpXET1* is a potential marker for tissue elongation and leaf growth in *F. pratensis*. Although the precise function of the XET-related proteins remain unclear, based on the contrasting expression of *FpXET1* and *FpXET2* it is proposed that *FpXET1* plays an important role in cell wall modification processes during tissue elongation, while the function of *FpXET2* is more likely to be associated with cell wall differentiation processes upon the cessation of tissue elongation.

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