The role of cell wall-related proteins during leaf growth of Festuca pratensis Huds.

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The Role of Cell Wall-related Proteins during Leaf Growth of Festuca pratensis Huds.

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SUMMARY

Festuca pratensis Huds. is a valuable forage grass in semi-natural and sown grasslands. In contrast to other forage grasses, it has the advantage of withstanding harsh climatic conditions. However, F. pratensis lacks persistence when grown with other competitive companion grasses. This limited competitive ability, especially in intensively managed grasslands, has been shown to be tightly linked to leaf growth, mainly after defoliation. Leaf growth is determined by cell division and cell expansion, two related processes that are spatially separated within the leaf elongation zone (LEZ) at the basal end of the growing grass leaf. Turgor-driven cell expansion has a large impact on leaf growth and has been shown to depend on the biophysical relaxation of the cell wall. Expansins and xyloglucan endotransglycosylases (XETs) are two gene families that encode proteins, which have been proposed as candidates important in the molecular regulation of wall relaxation. In order to improve our understanding of the molecular mechanisms involved in the control of leaf growth in F. pratensis, we aimed to elucidate the role of expansins and XET-related proteins in the control of leaf growth in F. pratensis.

Although α- and β-expansins were primary gene candidates responsible for the control of cell expansion and leaf growth, a detailed analysis of the spatial distribution of growth along the LEZ revealed no correlation between tissue elongation and the expression of different members of this gene family. However, as demonstrated by in situ mRNA hybridisation, the expression of the predominant α-expansin (FpExp2) and β-expansin (FpExpB3) was strictly localised to the differentiating vascular tissue at the distal end of the LEZ. In addition, another β-expansin (FpExpB2) was highly expressed in roots and tiller primordia. These results demonstrate that the activity of the predominantly leaf
expressed α- and β-expansins in *F. pratensis* is associated primarily with tissue differentiation rather than tissue elongation.

In contrast to the findings for expansins, the characterisation of three different cDNAs encoding XET-related proteins revealed a strictly leaf-specific gene expression pattern. Transcript accumulation for *FpXET1* and *FpXET2* was linked to the onset of the dark period, slightly delayed with respect to the recorded peak of the leaf elongation rate. A detailed expression analysis of the three genes in two *F. pratensis* genotypes with contrasting leaf growth characteristics showed a specific correlation between *FpXET1* expression and tissue elongation that was maintained under the different growth conditions, while the two other *FpXET* s showed different transcript dynamics. *In situ* mRNA localisation of *FpXET1* and *FpXET2* indicated an accumulation in young tissue. The results indicate that the encoded XET-related proteins may play an important role in cell wall modification processes during leaf growth. However, the contrasting spatial expression pattern within the LEZ of *FpXET1* and *FpXET2* suggests different physiological functions for the encoded proteins.

The results presented support the idea that the control of cell expansion is an important checkpoint for the control of leaf growth in *F. pratensis*. However, an analysis of the cellular growth dynamics in the LEZ of *F. pratensis* revealed that low nitrogen supply had a greater impact on cell division than on cell expansion, emphasizing also the significance of cell division in the control of leaf growth. Future studies focusing on the molecular regulation of leaf growth in forage grasses should therefore also incorporate an analysis of key enzymes involved in the control of the cell cycle.


Die hier präsentierten Ergebnisse belegen deutlich, dass die Regulation der Zellstreckung einen wichtigen Faktor für die Regulation des Blattwachstums von *F. pratensis* darstellt. Eine Analyse der zellulären Wachstumsdynamik zeigte aber auch, dass sich die relative Bedeutung der Zellstreckung und der Zellteilung für das Blattwachstum mit den Wachstumsbedingungen änderte. Bei einer niedrigen Stickstoffversorgung limitierte die Zellteilung das Blattwachstum signifikant stärker als die Zellstreckung. Um einen tieferen Einblick in die molekulare Regulation des Blattwachstums von Futtergräsern zu gewinnen, sind deshalb auch Schlüsselenzyme zu berücksichtigen, die massgeblich an der Regulation des Zellzyklus beteiligt sind.
In most crop species, dry matter production is directly related to the amount of intercepted solar radiation by the canopy (Monteith 1977). Growth and development of leaves is therefore a major determinant of crop productivity. This is of specific significance for forage grasses and legumes, where the greatest proportion of the shoot is harvested and used as feed for ruminants. Leaves of forage plants are therefore not only the photosynthetic active tissue, but also the harvestable product. In managed grassland systems forage plants are regularly subjected to a more or less severe defoliation by grazing or mowing, resulting in a complete or partial loss of the leaf area. To avoid prolonged periods with a reduced interception of the solar radiation, a rapid leaf growth is a prerequisite. Factors associated with the growth of leaves are thus key factors for the productivity as well as for the quality of the produced forage of managed grassland.

Forage grasses are members of the Poaceae plant family, which comprises also the three main cereal crops (rice, maize and wheat) that account for about half of the total world food production (Bennetzen and Freeling 1997). One of the distinct characteristics of forage grasses is that they are extremely well adapted to defoliation. This is mainly because during the vegetative phase of their life cycle the meristematic zones are located close to the soil surface, beyond the reach of animals or machines. Even after a partial loss of the meristematic zones, new shoots can emerge by the development of new tillers from axillary buds. Few other plants have developed such efficient mechanisms to recover from defoliation.

Despite the significance of leaf growth for the forage yield and its nutritive value, little information is available on the molecular mechanisms controlling these processes. A vast number of studies have focused on physiological
aspects of leaf growth, such as the photosynthetic capacity, light interception and responses of leaf growth to environmental stresses. Investigations that have specifically analysed the molecular mechanisms underlying leaf growth have mainly concentrated on the characterisation of mutants with altered leaf development in Arabidopsis, tobacco or maize (e.g. Hemerly et al. 1995; Sato et al. 1996; Smith et al. 1996a; Tsuge et al. 1996; Veit et al. 1998).

Due to the specific significance of leaf growth for forage grasses, the aim of this study was to improve our understanding of the molecular mechanisms involved in the regulation of this important trait. Festuca pratensis Huds. (meadow fescue), a widely cultivated forage grass and important component of semi-natural and artificial grasslands in cooler regions, has been chosen as model system. Firstly because of its agronomic importance as a valuable forage grass, and, secondly, due to its inherently limited ability to recover from defoliation (Nösberger et al. 1998).

1 THE GROWING GRASS LEAF: MORPHOLOGICAL AND PHYSIOLOGICAL ASPECTS

The significance of leaf growth and processes contributing to leaf growth in forage grasses have been widely discussed (e.g. Volenec and Nelson 1981; Schnyder et al. 1987; Schnyder and Nelson 1989). The leaf elongation rate (LER) and the leaf elongation duration are determining the development of the leaf area per grass tiller. The size of the growth zone and the associated leaf growth rate are strongly affected by the genetic background and development stage as well as environmental conditions (e.g. Kemp 1980; Volenec and Nelson 1981; Schnyder et al. 1990; Andrews et al. 1991).

As in other graminaceous species, leaf growth in F. pratensis occurs primarily along one axis and is restricted to the leaf elongation zone (LEZ), consisting of the basal end of the elongating leaf (Schnyder et al. 1990). The life
history of a leaf starts with the initiation of a leaf primordium at the apical meristem (Langer 1979). This early phase of leaf development is largely accomplished by the production of new cells that become committed to form organised tissues creating a dorsiventral structure. As in other growing plant organs (e.g. roots or internodes), cell division and cell expansion in leaves occur in clearly defined zones (e.g. Granier and Tardieu 1998). While in dicotyledonous leaves these zones may overlap temporally as well as spatially to a considerable extent, cell division and cell expansion are much more separated in monocotyledonous leaves (e.g. Ben Haj Salah and Tardieu 1995). Soon after the initiation of a leaf primordium, cell division becomes restricted to a basal meristem at the end of the growing leaf, which produces parallel files of cells. With ongoing development, these cell files are continuously displaced along the growing leaf, mainly through the production and subsequent expansion of new cells at the basal meristem. The distance between a cell and the leaf base is therefore a function of its developmental stage and age (Schnyder et al. 1990). Total length of the basal meristem is under strong genetic control and influenced by environmental conditions. In contrast to the epidermal cells, mesophyll cells keep dividing for a longer time period, resulting in the production of 10 to 14 mesophyll cells per epidermal cell (MacAdam et al. 1989). Epidermal cells displaced into the adjacent expansion zone undergo a tremendous expansion process, characterised by a significant increase in the mean epidermal cell length. Depending on the species and environmental conditions, an over 50-fold increase in the mean epidermal cell length has frequently been observed (e.g. Ben Haj Salah and Tardieu 1995; Bacon et al. 1997; Schäufele and Schnyder 2000). With the cessation of cell expansion, cells are displaced into a maturation zone which is characterised by secondary cell wall deposition (MacAdam et al. 1992), formation of the photosynthetic apparatus (Skinner and Nelson 1995) and differentiation of the vascular tissue (Martre et al. 2000).
The elongation zone represents a strong sink for assimilates and nutrients (e.g. Schnyder and Nelson 1989; Gastal and Nelson 1994; Skinner and Nelson 1995). While nitrogen deposition seems to be greatest in the cell division and early cell expansion zone (Gastal and Nelson 1994), highest sucrose deposition rates are found in the zone of most rapid cell expansion (Schnyder and Nelson 1987). Much of the imported sucrose is used for biosynthesis of structural components and for associated respiration, but carbohydrate deposition seems to exceed the demand for biosynthesis and respiration (Allard and Nelson 1991). Conversion to fructans can therefore act as a balance between sucrose import and utilisation (Schnyder et al. 1988).

Although single-leaf net photosynthesis and herbage yield are not correlated in forage grasses (Nelson et al. 1975), the leaf growth rate strongly reflects the productivity of forage grasses. Several studies have thus focused on the relationship between the rate of leaf growth and subsequent forage yield. The LER is considered to influence leaf area expansion more than leaf width (Nelson et al. 1977) and a high LER in combination with larger leaves has been shown to be directly associated with higher forage yield (Nelson et al. 1978; Jones et al. 1979; Clifton-Brown and Jones 1997).

2 ENVIRONMENTAL FACTORS AFFECTING GRASS LEAF GROWTH

Forage production is frequently delimited to climatic regions unfavourable for the cultivation of arable crops such as, e.g. wheat, maize or rice. For the development of economically viable and sustainable management practices under very variable plant growth conditions, it is of great interest to understand and predict the influence of environmental conditions on the growth and development of forage plants. Consequently, a great number of studies have
been conducted to explore the impact of various environmental factors on the leaf growth parameters.

Fertilisation, especially nitrogen supply, does strongly influence leaf growth. Volenec and Nelson (1983) found a 90% increase in the rate of leaf growth with increased nitrogen supply. Temperature has a strong and immediate effect on leaf growth. In *Lolium temulentum* L. this has been shown to be the result of changes in the mechanical properties of the cell wall (Thomas *et al.* 1989). Also the different growth responses of barley genotypes to temperature correlated well with differences in cell wall extensibility (Pollock *et al.* 1990). The often described increased leaf growth rate under reduced radiation or during the dark period (e.g. Volenec and Nelson 1982; Schnyder and Nelson 1989) is most likely an indirect effect of a reduced evaporative demand (Ben Haj Salah and Tardieu 1996). Changes in light quality greatly affect leaf growth of forage grasses. In *Festuca arundinacea* Schreb. a lower irradiance of blue light has been shown to increase the leaf growth rate (Gautier and Varlet-Grancher 1996). Drought has a very strong impact on the growth of leaves. Depending on the severity of water restriction, reduced leaf growth under drought has been attributed to reduced cell division rates as well as cell expansion rates (Dale 1988; Ben Haj Salah and Tardieu 1996; Bacon *et al.* 1997; Bacon *et al.* 1998; Bacon 1999; Granier *et al.* 2000).

### 2.1 Leaf growth following defoliation

Although it has been clearly demonstrated that the LER is reduced after defoliation (Schäufele and Schnyder 2000), there is still some controversy about the mechanisms responsible for a fast regrowth after defoliation. Water soluble carbohydrates (WSC) are often considered to be the primary source of carbon for regrowth under sub-optimal conditions (e.g. Deregibus *et al.* 1978). However, although WSC may play an important role during regrowth, there is striking evidence that current photosynthesis of the residual and the newly expanded leaf area may become a major carbon source for leaf growth within a
few days after defoliation (e.g. Richards 1993; De Visser et al. 1997; Schäufele and Schnyder 2000).

3 CELL DIVISION AND CELL EXPANSION

Regardless of the diversity of shapes and sizes of plant leaves, leaf growth is determined by two processes: cell division and cell expansion (Dale and Milthrome 1983; Tardieu et al. 1999). A tight co-ordination of both processes is therefore necessary for controlled leaf development. Both processes have been considered to be independent (Green 1976). However, newer data indicate that exceptions exist (e.g. Ben Haj Salah and Tardieu 1995; Fleming et al. 1997). Tardieu and Granier (2000) therefore suggested that although cell division and cell expansion are apparently independent processes in the short term, they might be tightly linked on a longer-term basis.

Good correlations between epidermal cell number and leaf area are often observed (e.g. Tardieu and Granier 2000) and have led to the conclusion that cell division is the main mechanism determining growth and development of leaves. However, results of several studies clearly indicate that an alteration in cell division does not necessarily have a significant effect on the final size of leaves (Smith 1996). It is thus assumed that reduced cell division rates can be compensated to some extent by an increase in cell expansion. Early evidence for this view came from experiments performed with wheat seedlings, where grains have been irradiated to block cell division (Haber 1962). Leaves of irradiated wheat seedlings attained the same size and shape as control plants but had significant larger cells. More recent experiments with transgenic tobacco plants expressing a dominant negative allele of the Arabidopsis gene encoding Cdc2 kinase, a key positive regulator of cell division, support the findings of Haber (Hemerly et al. 1995). Leaves of the tobacco plants were slightly smaller with considerably fewer but larger cells, which underwent normal
differentiation. These findings clearly demonstrate the fundamental role of cell division for leaf growth, but they also unequivocally point out the significance of cell expansion for leaf morphogenesis. Although cell division produces the units for future growth, the actual volumetric growth depends on the expansion process of each individual cell.

4 REGULATION OF PLANT CELL EXPANSION

The cell wall, consisting of a heterogeneous polymeric network in which cellulose microfibrils are embedded in a complex matrix of hemicellulose, pectins and proteins, plays a central role in the control of plant cell expansion (Carpita and Gibeaut 1993; Carpita 1996). It determines cell shape and permits the development of a hydrostatic turgor pressure within the cell protoplast. Cell expansion is thus the result of two coherent processes: The development of turgor pressure and the subsequent controlled relaxation of the cell wall. Turgor pressure in plant cells is usually in the order of 0.5 - 1.0 MPa. However, Nobel (1983) calculated that this could translate into a tangential tension of up to 25 - 50 MPa in the wall, due to the relative thinness of plant cell walls. This implies that wall relaxation and subsequent cell enlargement through the influx of water have to be highly co-ordinated. Hydraulic conductance has long been regarded as limiting factor for cell expansion. However, studies that are more recent suggest that water transport is too rapid to be a major growth limitation (reviewed in Cosgrove 1993). During the last years, attention has thus shifted to the cell wall and its resident enzymes controlling the regulation of cell wall extensibility and thus cell expansion.

4.1 Wall-loosening enzymes

Plant cell walls contain numerous enzymes, of which some have been considered as catalysts capable of weakening the cell wall to permit turgor-
driven wall extension (reviewed by Cosgrove 1999). During the last few years, research has mainly concentrated on two different gene families, both supposed to be involved in cell wall relaxation, expansins and xyloglucan endotransglycosylases (XET).

4.1.1 The α-expansin gene family

McQueen-Mason et al. (1992) were able by means of a reconstitution approach to isolate two similar proteins from growing cucumber seedlings that possessed the ability to induce extension of heat inactivated cell walls. The activity of both proteins strongly depended on acidic conditions and was restricted to walls of rapidly growing cells of the stem. The subsequent cloning of the cDNAs encoding two α-expansins, as the proteins were termed to reflect their activity, revealed them to be a novel class of wall proteins, without any strong sequence similarity to other previously described wall-related enzymes (Shcherban et al. 1995). The deduced polypeptides were about 25 kDa in size, contained a hydrophobic signal peptide and were highly conserved among each other. Characteristic were six conserved cysteine residues at the amino terminus and four conserved tryptophans at the C-terminal end whose spacing resembled that of tryptophans in the cellulose binding domains of bacterial cellulases (Cosgrove 1999).

Although genes encoding α-expansins have been cloned from a wide range of plant species including dicots and monocots, as well as gymnosperms and ferns (Shcherban et al. 1995; Rose et al. 1997; Hutchison et al. 1999; Kim et al. 2000), there is still controversy about how α-expansins biochemically mediate wall extension. The proteins contain a central domain that displays some homology to family-45 glycosyl hydrolases (Cosgrove 2000b), but there is evidence that α-expansin activity is not based on hydrolysis of wall polysaccharides (McQueen-Mason and Cosgrove 1994, 1995). It has thus been suggested that α-expansins act, analogous to DNA helicases, by the disruption
of hydrogen bonds between cellulose fibres and the matrix hemicelluloses (McQueen-Mason and Cosgrove 1994; McQueen-Mason 1997).

α-Expansins are encoded by relatively large gene families (e.g. at least 24 in Arabidopsis (Cosgrove 2000c)), with some members showing specific patterns of expression (e.g. Cho and Kende 1997; Rose et al. 1997; Reinhardt et al. 1998; Huang et al. 2000; Vriezen et al. 2000). Significant correlations between high levels of α-expansin activity and high rates of tissue elongation have been described in many plant species and organs, supporting the idea that regulation of tissue extensibility is a primary function of these proteins (e.g. Wu et al. 1996; Cho and Kende 1997; Huang et al. 2000). In vivo functional data on the role of α-expansins have come from experiments in which purified expansin protein was locally ectopically applied to the meristems of tomato leading to the induction of leaf morphogenesis (Fleming et al. 1997; Fleming et al. 1999). These data are consistent with α-expansin acting to locally loosen the outer cell wall of the epidermis and thus directly influencing cell wall extensibility. Similarly, experiments in which tobacco BY2 cells showed a change in cell shape and size after treatment with purified α-expansins are consistent with the protein acting directly to alter cell wall architecture to allow growth (Link and Cosgrove 1998).

With respect to leaf growth, analysis of tomato leaves showed that α-expansin activity was high in growing tissue (Keller and Cosgrove 1995), and it has been demonstrated that in rice at least one specific α-expansin gene was expressed in the leaf tissue (Cho and Kende 1997). However, although a link between leaf growth and α-expansins seems to be likely, the relationship between in vivo growth rate of leaves and α-expansin gene expression and activity has not been documented.

4.1.2 The β-expansin gene family

Upon the discovery of α-expansins, database comparisons of the cDNA sequences revealed a distant sequence similarity to a group of grass pollen
allergens, known as group-1 allergens (Cosgrove et al. 1997). Despite the relatively poor sequence conservation of the two protein groups, experiments showed that protein extracts of group-1 allergens also possessed an α-expansin like activity and were able to restore the extension capacity of heat inactivated cell walls. Consequently, Cosgrove et al. (1997) proposed to define the new protein group and their homologues expressed in vegetative tissues as β-expansins. Further database searches revealed that β-expansins are highly represented in grasses such as maize or rice while only one single Arabidopsis expressed sequence tag has been considered to be a β-expansin (Cosgrove 2000b). Consistent with these findings, the activity of β-expansins has been shown to be strictly limited to cell walls of graminaceous species (Cosgrove et al. 1997). Based on the α-expansin-like activity and the almost exclusive existence of β-expansins in grasses it has been postulated that the evolutionary emergence of β-expansins is tightly linked to the specific polymer composition of the grass cell wall, which differs strongly from that of dicotyledonous species (Carpita 1996). However, although it seems likely that β-expansins may play an important role in the control of cell wall relaxation in grasses, data on tissue and gene-specific expression of β-expansins are still lacking. So far, evidence of the existence of β-expansins has come only from the rice and maize genome sequencing projects.

4.1.3 Xyloglucan endotransglycosylase (XET)

Xyloglucan endotransglycosylase (XET) is an other family of cell wall-related enzymes proposed to play a key role in cell wall loosening (reviewed in Campbell and Braam 1999). This enzyme has been shown to catalyse the transglycosylation of xyloglucans by internally cleaving xyloglucan polymers and ligating the newly generated reducing ends to other xyloglucan chains. Because xyloglucans mediate the cross-linking of cellulose microfibrils in the cell wall, it has been proposed that XET controls cell expansion through either reversibly loosening the cellulose-xyloglucan network or by anchoring newly synthesised
xyloglucan polymers into the cell wall (Fry et al. 1992; Nishitani and Tominaga 1992). However, because experiments to induce extension of heat inactivated plant cell walls via an exogenous application of XET proteins analogous to expansins failed (McQueen-Mason et al. 1993), causal evidence for the role of XET as wall loosening agent is still lacking. In various plant species, including dicots as well as monocots, XET has been shown to be encoded by multigene families (e.g. Xu et al. 1996; Schünmann et al. 1997; Burstin 2000; Uozu et al. 2000). Several of these genes are specifically up-regulated in response to different environmental stimuli as well as by growth enhancing hormones, indicating an important role in plant development and stress response (e.g. Potter and Fry 1994; Xu et al. 1995; Xu et al. 1996). Additionally, striking correlations of XET gene expression or protein activity and cell expansion have been demonstrated (e.g. Hetherington and Fry 1993; Smith et al. 1996b; Schünmann et al. 1997), supporting thus the idea that XETs are likely to be involved in the control of cell wall relaxation.

5 F. PRATENSIS, AN IMPORTANT FORAGE GRASS OF COOLER REGIONS

F. pratensis is closely related to Lolium spp. (Charmet and Balfourier 1994; Stammers et al. 1995). Although comparable to Lolium multiflorum Lam. or L. perenne L. for its yield and quality parameters, it shows several advantages under rough climatic conditions (Meister and Lehmann 1990). Tolerance to low temperature and winterhardiness make F. pratensis an important component in grasslands of cooler regions (Büring-Stucki 1990). However, growth of this species with other competitive companion grasses in intensively managed grasslands results very often in a low persistence of F. pratensis (Mott and Lennartz 1977). It is assumed that this characteristic is mainly due to the low competitive ability of F. pratensis after frequent cutting or
intensive grazing. However, intensive management per se cannot be the main cause for its low persistence, because it performed very well in intensively managed pure stands (Gügler 1993). The low persistence is therefore more likely the result of its inherently lower shoot competitive ability. During a two-year field experiment, the shoot competitive ability decreased steadily while the root competition was relatively high and only influenced by seasonal fluctuations (Carlen 1994). Focusing closer on the parameters influencing the low shoot competitive ability, Messerli (1997) concluded that the significantly lower leaf elongation rate, mainly during the second half of the re-growth cycle, seems to be a key element in determining the low shoot competitive ability of F. pratensis and thus its persistence in a multi-species sward.

Beside these leaf growth characteristics, other factors may contribute to the low persistence of F. pratensis within a sward. Zimmermann (1995) reported that the propagation through seeds is important for F. pratensis in extensively managed semi-natural grassland. Frequent defoliation of such grasslands may result in the cutting of reproductive tillers before anthesis and thus not allow reproduction by seed. Finally Kölliker et al. (1998, 1999) reported that the genetic variability of F. pratensis cultivars and intensively managed natural populations was, compared to other species, relatively low which could contribute additionally to the low persistence of F. pratensis.

6 OBJECTIVES OF THE STUDY

Growth and development of leaves, particularly after defoliation, are key elements contributing to the productivity and persistence of natural and sown grasslands. The aim of this study was to investigate the molecular mechanisms controlling this specific agronomic trait. Elucidating the role of specific wall-related enzymes in the control of tissue elongation during leaf growth was of particular interest. F. pratensis was chosen as a model system because of its
agronomic importance as a valuable forage grass, and due to its limited ability to recover from defoliation. A better understanding of the molecular basis of leaf growth would not only give insight into the regulation of a fundamental plant growth process, but might also provide tools for marker assisted breeding purposes to improve *F. pratensis* as a forage grass. Using a candidate gene approach based on the results of the genome sequencing projects of *Arabidopsis*, maize and rice, we defined our objectives as follows:

I. Identification and cloning of genes encoding expansin and XET proteins in *F. pratensis*.

II. Characterisation of expansin and XET gene expression within the LEZ during leaf growth, and, particularly in response to low nitrogen supply.

III. Identification and characterisation of leaf-specific expansin and XET genes which play a key role in the control of tissue elongation and thus for the control of leaf growth.
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IV DIFFERENTIAL EXPRESSION OF EXPANSIN GENES DURING LEAF GROWTH OF FESTUCA PRATENSIS HUDS.

1 ABSTRACT

Grasses contain a number of genes encoding both $\alpha$- and $\beta$-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 $\alpha$- and three vegetative $\beta$-expansins expressed in the leaf elongation zone (LEZ) of the forage grass, Festuca pratensis Huds.. The expression of the predominant $\alpha$-expansin ($FpExp2$) was localised to the vascular tissue, as was the $\beta$-expansin, $FpExpB3$. Expression of another $\beta$-expansin ($FpExpB2$) was not localised to vascular tissue but was highly expressed in roots and initiating tillers. This is the first description of vegetative $\beta$-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 $\alpha$- and $\beta$-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 $\alpha$- and $\beta$-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.
2 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 in McQueen-Mason and Rochange 1999; Cosgrove 2000b). 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. 1999b). Although a correlation of α-expansin gene expression and high rates of tissue elongation can often be made in 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, Fleming et al. 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 2000c). 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 in 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.
3 MATERIALS AND METHODS

3.1 Plant material and growth conditions

A *F. pratensis* genotype (*Festuca pratensis* Huds.) was selected from the cultivar '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, MB, Canada) at a constant temperature of 20°C, 80% relative humidity and 400 µmol m⁻² s⁻¹ photosynthetic photon flux density during a 16 h photoperiod.

3.2 cDNA cloning and sequence analysis

Total RNA (5 µg) was extracted using 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 using degenerate sense 5'-GCAGGXTXNGNCATGTCGAGTG-3' (α-expansin), 5'-GGCMRGGCSACCTGGTACGG-3' (β-expansin), 5'-CGAGATCGACNTCGAGTTC-3' (XET) and antisense 5'-TGAGCNCGGNGCTGTTCAACG-3' (α-expansin) 5'-CCRCAGCCCYKGCCGXCCTTG-3' (β-expansin), 5'-SGTCNGRGCAGTNGTAG-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 using the program PSORT (Nakai and Kanehisa, 1992). Dendrograms were obtained by using the clustal procedure of DNASTAR Megalign (DNASTAR Inc., Madison, WI).
3.3 Generation of full length cDNAs

Full length α-expansin cDNA clones were obtained in a two step procedure using 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 ds 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 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 an 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.

3.4 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'-CTACCTCAACGGGCAAGGGCCTATCCTTC-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 SmaI and a SacI 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 using internal sense primers 5'-GCCATTGTCTTTGCGCATCACC-3' (FpExpB1) and 5'-CCCTTCTTTCATGCGCATCACC-3' (FpExpB2 and FpExpB3) together with the RACE primer. A gene-specific probe for FpXET1 was obtained with the internal sense primer 5'-AGGAGCTCGGCGACATGAGCTACC-3' and the RACE primer.
3.5 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 ³²P labelled probes generated using a random labelling kit (Stratagene, Basel, Switzerland). Membranes were washed to a final stringency of 0.2 x SSC, 0.5% SDS at 50°C before autoradiography at -80°C using intensifying screens.

3.6 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-labelised probes overnight at 50°C. After washing with 0.2 x SSC at 55°C, the slides were treated with RNaseA for 30 min at 37°C, washed again at 55°C with 0.2 x 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 to 48 hours. 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.

3.7 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 insoluble material removed by centrifugation. Proteins were quantified colorimetrically according to Minamide and Bamburg (1990). For SDS-PAGE, 40 μg of proteins from each sample were separated on 12% polyacrylamide gels and electroblotted to 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 using goat anti-rabbit IgG-conjugated alkaline phosphatase (1:3000 dilution) (BioRad, Glattbrugg, Switzerland).

3.8 Measurement of α-expansin activity

α-expansin activity was measured using the method described by Whitney et al. (2000). Briefly, tissue from either the basal 0-15 mm or distal 25-40 mm end of the LEZ was frozen then homogenised in 25 mM Hepes, 1% polyvinylpyrrolidone (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 sulphate and collected by centrifugation. The proteins were desalted on a Sephadex G-25 column (Pharmacia Biotech, St. Albans, UK) into 50 mM sodium acetate, pH 4.5 and assayed for expansin activity using a cellulose/xyloglucan composite. Protein concentrations were calculated colorimetrically by the Microbradford method (Pierce, Rockford, Ill., USA).
3.9 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.

3.10 Determination of LER, SER and epidermal cell sizes

All growth measurements were performed during the linear phase of leaf growth 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 to 10 epidermal cells was measured at different positions along the LEZ under a microscope.
4 RESULTS

4.1 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. The ORF’s ranged from 756 bp for *FpExp1, 4* and 5, 759 bp for *FpExp2* to 765 bp for *FpExp3*. In contrast, 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*), respectively. 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 using 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 2000b).

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 1, (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 3'-untranslated region.
Figure 1. 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 2000c). The amino acid at position 29 of FpExp4 (+) could not be determined.

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FpExp1: MARS--RAHHLAAPAAAACLATATAPVW-AT
FpExp2: MARSCNILLPFSASAAPHCPLAALACGYYG-ASAAT
FpExp3: MAKFRAVLLVTLALGCLASHVYDDTYG-SSAT
FpExp4: MAGK-RLIQVLCVCLAAAPVRSAD---C-LAATA
FpExp5: MAGKK-RLIQLLLVCLAAAPVRS---LAAT

FpExp1: FYGGQDGTNGACGYGLIHYTAYTNLALSAP
FpExp2: FYGGQDGTNGACGYGLIHYTAYTNLALST
FpExp3: FYGGQDGTNGACGYGLIHYTAYTNLALSST
FpExp4: FYGGQDGTNGACGYGLIHYTAYTNLALSTD
FpExp5: FYGGQDGTNGACGYGLIHYTAYTNLALSTV

FpExp1: LFNDGQSCC-CCVTDDGSSSSTCVTVVTAT
FpExp2: LFNDGQSCC-CCVTDDGSSSSTCVTVVTAT
FpExp3: LFNDGQSCC-CCVTDDGSSSSTCVTVVTAT
FpExp4: LFNDGQSCC-CCVTDDGSSSSTCVTVVTAT
FpExp5: LFNDGQSCC-CCVTDDGSSSSTCVTVVTAT

FpExp1: NLCPGTPNFECPGNCNPPHPPFDSQPAWLT
FpExp2: NLCPGTPNFECPGNCNPPHPPFDSQPAWLT
FpExp3: NLCPGTPNFECPGNCNPPHPPFDSQPAWLT
FpExp4: NLCPGTPNFECPGNCNPPHPPFDSQPAWLT
FpExp5: NLCPGTPNFECPGNCNPPHPPFDSQPAWLT

FpExp1: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp2: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp3: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp4: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp5: VYRAGIHPRTVRHELTIKTFTYFEL

FpExp1: LTIGNASL-SLVLYITKCGYGTGAMSRPHMX
FpExp2: LTIGNASL-SLVLYITKCGYGTGAMSRPHMX
FpExp3: LTIGNASL-SLVLYITKCGYGTGAMSRPHMX
FpExp4: LTIGNASL-SLVLYITKCGYGTGAMSRPHMX
FpExp5: LTIGNASL-SLVLYITKCGYGTGAMSRPHMX

FpExp1: RNLDAQGALS-TPMFLDEPSCSNGFC
FpExp2: RNLDAQGALS-TPMFLDEPSCSNGFC
FpExp3: RNLDAQGALS-TPMFLDEPSCSNGFC
FpExp4: RNLDAQGALS-TPMFLDEPSCSNGFC
FpExp5: RNLDAQGALS-TPMFLDEPSCSNGFC

FpExp1: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp2: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp3: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp4: VYRAGIHPRTVRHELTIKTFTYFEL
FpExp5: VYRAGIHPRTVRHELTIKTFTYFEL

FpExp1: 28
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 2, (Shcherban et al. 1995; Cosgrove 2000c)). It is noticeable that FpExpB1 contained only three of the four conserved tryptophans at the C-terminal end.

**Figure 2.** 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 2000c).
A comparison of the α-expansin clones with their putative orthologs in rice and α-expansins from other species, revealed a high similarity of FpExp2 and FpExp3 to OsExp2 and OsExp3, respectively (Figure 3). 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.

Figure 3. Dendrogram of α-expansins from different plant species. Full length proteins were clustered using the clustal procedure of DNASTAR Megalign (DNASTAR Inc., Madison, WI). Sequences and GeneBank accession numbers: OsExp1 (Y07782), OsExp2 (U30477), OsExp3 (U30479), OsExp4 (U85246), OsExp6 (AF247163), OsExp7 (AF247165), OsExp8 (AAD38926), OsExp9 (AAD389297), 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).
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 \( \beta \)-expansins from other grass species. The cluster analysis clearly distinguished between \( \beta \)-expansins originating from vegetative and \( \beta \)-expansins originating from generative tissue, as indicated by the two major branches of the dendrogram (Figure 4). \( FpExpB2 \) and \( FpExpB3 \) were highly similar to their putative orthologs in rice. 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 \( \beta \)-expansin (\( OsExpB2 \)).

**Figure 4.** Dendrogram of vegetative (veg.) and generative (gen.) \( \beta \)-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-11 (Z27084), Phi p1 (X78813), Ory s1 (U31771), Cyn d1 (S83343), \( FpExpB1 \) (AJ295940), \( FpExpB2 \) (AJ295941), \( FpExpB3 \) (AJ295942).
4.2 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 using 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 the two transcripts. The results, shown in Figure 5, show that the different expansin clones analysed are expressed in a variety of organ-specific patterns.

Figure 5. 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 28S and 18S rRNA served as controls.
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 analysed tissues (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 analysed tissues, 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.

4.3 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 5). In particular, we concentrated on genes expressed in the LEZ and apical tissue (FpExp2, FpExpB2, FpExpB3). FpExp2 mRNA accumulated only in a few cells associated with differentiating vascular tissue (Figure 6a). Examination of the vascular bundles (Figure 6b) and comparison with hybridisations performed with the relevant sense probe (Figure 6c) 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 6d). 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 6e). Although some weak signal was apparent in the walls of differentiating xylem vessels (Figure 6e and f), a similar signal was observed in sections hybridised with a sense probe (Figure 6g), 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 6h).

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 6i, 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 6j), 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 accumulation in leaf tissue, but analysis of sections just below the apex showed a distinct, localised signal in developing tiller primordia (Figure 6m, o). A similar pattern was observed in hybridisations performed with the *FpRpl3* control probe (Figure 6n, 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 (Figure 5).
Figure 6. 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) and (e), (f), hybridisation with a gene-specific FpExp2 antisense probe; (c) and (g), hybridisation with an FpExp2 sense probe; (d) and (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 similar tissue sections 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) and (l), hybridisation with the gene-specific FpExpB3 antisense probe; (m) and (o) hybridisation with the FpExpB2 antisense probe; (j), (n) and (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 in (a), (d), (e), (i), (j), (m) and (n) = 500 μm; Bars in (h) = 250 μm; Bars in (b) and (c) = 150 μm; Bars in (o) and (p) = 160 μm; Bars in (f) and (g) = 100 μm; Bars in (k) and (l) = 62 μm. If, leaf; sh, sheath; st, stem; xy, xylem; xp, xylem parenchyma; tip, tiller primordium; ti, tiller.
4.4 $\alpha$- and $\beta$-expansin gene expression along the LEZ

Our initial hypothesis was that the $\alpha$- and $\beta$-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 expansion stopped at about 35 mm distance from the leaf base, indicating the distal end of the LEZ (Figure 7a). Within the LEZ, epidermal cells underwent a ten-fold expansion, resulting in a mean length of about 550 $\mu$m 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 7b).

**Figure 7.** 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. Third order sigmoidal regression curves were fitted to the data points. Data are means (± SE) of seven to ten measurements. (b) Segmental elongation rates (SER) of the same genotype. Data are means (± SE) of eight to ten measurements.
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 \(\alpha\)-expansins, a high accumulation of \(FpExp2\) transcripts was detected in the tissue at 24-40 mm distance from the leaf base (Figure 8). As shown in the bar chart, these tissue segments showed relatively low rates of elongation. 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. 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 8, immunoblot analysis revealed accumulation of \(\alpha\)-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 \(\beta\)-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 9). In contrast, \(FpExpB2\) mRNA was expressed at significantly higher levels. A strong signal, with increasing intensity from tissue at 24 mm to 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 8.** Comparison of tissue elongation and α-expansin gene expression along the LEZ.

The bar chart shows the SER of the relevant tissue segments along the axis of the leaf. 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 32P-labelled probe for *FpExp2* and washed at high (0.2 x 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.
Figure 9. Comparison of SER and β-expansin gene expression along the LEZ.
The bar chart shows the SER of the relevant tissue segments along the axis of the leaf. These
data are the means (± SE) of eight to ten measurements. Total RNA (10 μg) was extracted from
the indicated segments (0-8, 8-16, 16-24, 24-32, 32-40 mm) and hybridised with 32P-labelled
gene-specific β-expansin probes indicated at the left of the blot. Blots were washed at high
stringency (0.2 x 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.
4.5 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 8) we did not detect a significant level of α-expansin activity (Figure 10). 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 8), showed a high capacity to induce wall extension (Figure 10).

Figure 10. 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 (± 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 (± SE) of 3 measurements.
Since $\alpha$-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 10, 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 10 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 8.

4.6 Expression pattern of FpExp4 and FpXET1 along the LEZ

Although our analysis using FpExp2 showed a very poor correlation between $\alpha$-expansin expression and leaf growth rate, we could not exclude the possibility that other $\alpha$-expansins not detectable by the Northern blot, 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 1). 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 11). 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 using 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 12).
Figure 11. Expression of FpExp4 and FpXET1 along the LEZ. 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 32P-labelled FpXET1 gene-specific probe was used. Final washes were at 0.2 x 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.

This sequence showed 73% identity with the most closely related sequence from barley, HvXEB and HvXEA, which has 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 parameters occurring between 8-24 mm from the leaf base (Figure 11).
Figure 12. Amino acid sequence alignment of three XET-related proteins. The deduced amino acid sequence of FpXETI is compared to HvXEA and HvXEB, two XET-related proteins from barley (Schümman et al. 1997). Signal peptides (underlined) were predicted using the program PSORT (Nakai and Kanehisa, 1992). Sequences and GeneBank accession numbers: FpXETI (AJ295943), HvXEA (X93174), HvXEB (X93175).

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<th>FpXETI</th>
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<td>MARKAVSILLAILSSAVAAATCRAEETF-NQD</td>
<td>MRTVELGIVAMACVVVWRR-CNFDSTESVMDK</td>
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FpXETI: MARMAVSLAILSSAVAAATCRAEETF-NQD
HvXEB: MRTVELGIVAMACVVVWRR-CNFDSTESVMDK
HvXEB: MRTVELGIVAMACVVVWRR-CNFDSTESVMDK

DISCUSSION

5.1 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 already reported for α-expansins. The functional significance of such specific patterns is (as with α-expansins) unclear. On the one hand, they could reflect the distribution of specific substrates for the different β-expansins, or, alternatively, they might reflect genetic redundancy. The 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 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 as to how it can be controlled.

Our data also show an association of both an α- (FpExp2) and a β- (FpExpB2) expansin 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 recent published data on xylem differentiation of F. arundinacea 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 contained many β-expansins whereas
dicotyledons did not (Cosgrove 2000b), 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.

5.2 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 growth. 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 (vascular 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 supports the finding of 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 be focussed on testing this hypothesis.

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V DIFFERENTIAL EXPRESSION OF XET-RELATED GENES IN THE LEAF ELONGATION ZONE OF F. PRATENSIS

1 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 characterise this process and to identify genes, which might function as markers for leaf growth, we report on three XET-related genes in the leaf elongation zone (LEZ) of F. pratensis. We present a detailed expression analysis 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, we show a specific correlation 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 localisation 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. We propose that FpXET1 is a potential marker for tissue elongation and leaf growth in F. pratensis.
2 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). We are therefore interested 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). We therefore took a candidate gene approach 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) we reported on a detailed analysis of the spatial expression of α- and β-expansin genes in the leaf elongation zone (LEZ) of *F. pratensis*. These data indicated that none of the expansin genes analysed showed an expression pattern, which correlated with leaf growth. However, we were able 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.
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 (e.g. 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 (e.g. Potter and Fry 1994; Xu et al. 1995; Palmer and Davies 1996; Smith et al. 1996b). 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 we report on a detailed analysis of three XET-related genes expressed in the growing leaf of two F. pratensis genotypes with contrasting leaf growth characteristics. By supplying two different levels of nitrogen to modify the spatial distribution of tissue elongation within the LEZ (Fricke et al. 1997), we assessed the contribution that the three XET-related genes could make to regulate leaf growth in F. pratensis. By means of a detailed spatial analysis of growth and the XET encoding transcripts along the LEZ, we show a specific correlation between FpXET1 expression and leaf growth that is maintained under the different growth conditions, while FpXET2 and FpXET3 show different transcript dynamics. In situ localisation 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.
3 MATERIALS AND METHODS

3.1 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 days, 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 constant temperature of 20°C, 80% relative humidity and 400 μmol m⁻² s⁻¹ photosynthetic photon flux density during a 16 h photoperiod (12 h for diurnal gene expression experiment).

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).

3.2 Determination of LER and SER and epidermal cell length

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). 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 to 10 epidermal cells was measured at different positions along the LEZ under a microscope.
3.3 cDNA cloning and sequence analysis

Total RNA (5 μg) was extracted using a RNeasy Plant Kit (Qiagen, Basel, Switzerland) from the 40 mm LEZ and used as 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).

3.4 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’-GTGCTCCTTCTTGGCGCTCAGGC-3’ (FpXET2), 5’-TGTAGGGGCTGTCCGGTCTGCGGTCTCGTTCC-3’ (FpXET3) and gene-specific sense 5’-AGGAGCTCGCGACATGACGTCCTGGAC-3’ (FpXET1), 5’-CGTCGCCGGACGAGGCAC-3’ (FpXET2) and 5’-GGAAGATGCGGTGGGTTACAGGAGAG-3’ (FpXET3) primers together with the corresponding supplied RACE primers. Obtained PCR products were cloned and sequenced as described above.
3.5 DNA gel blot analysis

High molecular weight genomic DNA was extracted using DNeasy Plant Kit (Qiagen, Basel, Switzerland). 8 µg DNA was digested with PstI, EcoRI or HindIII, separated on a 0.8% agarose gel and transferred to Nytran membranes (Schleicher&Suell, Dassel, Germany). Blotting and hybridisation were performed under standard conditions (Sambrook et al. 1989). The blot was hybridised at 65°C with the corresponding randomly 32P labelled gene-specific FpXET 3' fragments obtained in the RACE procedure. Membranes were washed to a final stringency of 0.2 x SSC, 0.5% SDS at 50°C before autoradiography at -80°C using intensifying screens.

3.6 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, hybridisation, washing and autoradiography were performed as described for DNA gel blot analysis.

3.7 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 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 OD260. For PCR amplification (30 cycles) the FpXET3 gene-specific primer pair 5'-GGAAGATGCAGTGGGTACAGGAGAG-3' and 5'-TGCATGAGCGGCGATCAGTACGAG-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) was used as substrates.

3.8 In situ mRNA 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 gene specific DIG-labelled probes overnight at 50°C. After washing with 0.2 x SSC at 55°C, the slides were treated with RNaseA for 30 min at 37°C, washed again at 55°C with 0.2 x 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 to 48 hours. 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.

4 RESULTS

4.1 Cloning XET-related cDNAs from F. pratensis

Three full length XET-related cDNA clones were obtained using an RT-PCR coupled RACE procedure (Figure 13). 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) 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. (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 (Figure 13). The three deduced polypeptides contained also 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, we compared the mature proteins by means of cluster analysis to XET-related sequences reported from other grass species (Figure 14). 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 forms together
Figure 13. Alignment of the deduced amino acid sequences of *F. pratensis* xyloglucan endotransglycosylase (XET) related proteins. 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) (Figure 14).

with related sequences from rice and barley 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*. 

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Figure 14. 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), FpXET3 (AJ295945).

To estimate the number of genes potentially encoding the RNAs from which the FpXETs were cloned, we used gene specific probes prepared from the 3’ end of the three cDNAs to probe digested genomic DNA in a Southern blot analysis (Figure 15). Membranes probed with FpXET1 and FpXET2 yielded two restriction bands, indicating that these sequences are likely to be encoded by multiple genes. In contrast, hybridisation with FpXET3 resulted in a single hybridisation band, consistent it with being encoded by a single gene.

4.2 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. We examined the transcript levels 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 (Figure 16a,b). The expression pattern of FpXET3 was determined by RT-PCR, since the transcript was not detectable by Northern blot analysis (Figure 16b).
Figure 15. Southern Blot Analysis of FpXET.
Genomic DNA (8 µg) was digested with either PstI (P), EcoRI (E) or HindIII (H), separated by gel electrophoresis and hybridised with 32P radiolabelled gene-specific probes of the corresponding XET-related cDNAs. Blots are shown for high (0.2 x SSC, 50°C) stringency washes. Lane C shows undigested DNA as control. DNA size standards (kb) are given on the left of each blot.

Figure 16. 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 hybridised 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 (Figure 16a). 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.
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 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.

4.3 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 standardise time of tissue sampling and growth conditions for further experiments, we analysed the time course of the *FpXET* mRNA levels within the LEZ with gene specific probes and RT-PCR over a period of 36 hours (Figure 17). 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 two and a half hours. In contrast, the beginning of the light period resulted in slightly decreased *FpXET1* and *FpXET2* mRNA levels, with a daily minimum at approximately two and a half hours after the onset of the light period. This pattern of transcript accumulation can be compared with the measured diurnal fluctuation in the LER (Figure 18). RT-PCR analysis revealed no significant diurnal fluctuations in the mRNA level of *FpXET3* (data not shown).
Figure 17. Influence of light and dark period on the transcript level of two *FpXET* genes. Samples were taken at the times indicated. The hatched 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 rehybridised 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.

![Figure 17](image17)

Figure 18. 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. The hatched bar indicates the dark period.

![Figure 18](image18)
4.4 Epidermal cell size and SER within the LEZ

To test for any correlation between \textit{XET} gene expression and growth rate, we first characterised the spatial distribution of growth within the LEZ of elongating \textit{F. pratensis} leaves of two different genotypes under different nitrogen supply. In particular, to determine the size of the LEZ and regions of maximum tissue elongation within the LEZ, we measured 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) \textit{F. pratensis} genotype grown under two levels of nitrogen supply (Figure 19).

When the plants were grown under high nitrogen supply, both genotypes showed a comparable distribution of epidermal cell lengths along the LEZ (Figure 19a, LL NN vs. SL NN). Epidermal cell expansion stopped in both genotypes at 25 mm to 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 (Figure 19a, LL N). Compared to plants grown under high nitrogen supply, epidermal cells stopped elongating at 15 mm to 20 mm distance from the leaf base, resulting in a strongly reduced length of the LEZ.

Comparison of cell length data (Figure 19a) and data on the spatial distribution of SER (Figure 19b) 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 (Figure 19b, 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 (Figure 19b, LL N).
Figure 19. 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 according to Meister et al. (1999). Third order sigmoidal regression curves were fitted to the data points. Data are means (± SE) of seven to ten 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 by Schnyder and Nelson (1987). Data are means (± SE) of eight to ten measurements.
4.5 \textit{FpXET} gene expression within the LEZ

To test if the expression of the \textit{FpXET} genes can be correlated with the spatial distribution of tissue elongation along the LEZ, we characterised the transcript abundance of the three \textit{FpXET} genes in successive 8 mm long segments along the LEZ (Figure 20).

The expression of \textit{FpXET1} correlated closely with the measured spatial distribution of growth along the LEZ. In LL plants grown under high nitrogen supply (Figure 20a), the \textit{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 we measured maximum rates of tissue elongation (Figure 19). 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 \textit{FpXET1}, the \textit{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 \textit{FpXET1} and \textit{FpXET2} transcript accumulation was maintained in the other treatments, e.g. in the SL genotype grown under high nitrogen supply and in the LL genotype grown under low nitrogen supply (Figure 20b, 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 \textit{FpXET1} in both treatments always peaked in the segments where maximum rates of tissue elongation occurred, whereas the accumulation of the \textit{FpXET2} transcript was always maintained at a high level in segments distal to the points where we observed maximum rates of tissue elongation. Semi-quantitative RT-PCR detection of \textit{FpXET3} yielded an additional pattern (Figure 20a) 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 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 (Figure 20).

**Figure 20.** 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 leaved genotype (LL) under high nitrogen supply (NN), (b) the short leaved genotype (SL) under high nitrogen supply (NN), (c) the long leaved genotype (LL) under low nitrogen supply (N). In each part a–c, the bar chart shows the SER of the relevant tissue segments along the axis of the leaf. These data are the means (± SE) of eight to ten measurements. RNA was extracted from the indicated segments (0-8, 8-16, 16-24, 24-32, 32-40 mm) and 10 μg separated on a formaldehyde gel, blotted, then hybridised with the corresponding 32P-labelled gene-specific FpXET probe and washed at high (0.2 x 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 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.
4.6 \textit{In situ} localisation of the \textit{FpXET} transcripts

By means of \textit{in situ} hybridisation on sections prepared from different positions of the LEZ we analysed the cell specific expression pattern of the three \textit{XET}-related genes. Hybridisation with the gene-specific \textit{FpXET1} antisense probe of cross sections from the basal zone of the LEZ (Figure 21a, c) and of longitudinal sections (Figure 21e) revealed an accumulation of the \textit{FpXET1} mRNA in young elongating leaves. Although the expression did not seem to be delimited to specific cell types, significant hybridisation was seen in cells between the phloem and xylem vessels (Figure 21c). No Signal was observed in the non growing sheaths and leaves surrounding the young leaf (Figure 21a, e). Comparison with hybridisations performed with the sense probe on similar sections revealed no signal, supporting the specific expression pattern (Figure 21b, d). Hybridisations with an antisense control probe encoding a ribosomal protein (\textit{FpRpl3}) of longitudinal sections indicate general distribution of mRNA in the young elongating leaves (Figure 21f). Antisense hybridisations of cross sections with the gene-specific probe for \textit{FpXET2} showed a similar expression pattern as observed for \textit{FpXET1} (data not shown). Repeated attempts to obtain hybridisation signals on sections probed with \textit{FpXET3} failed, most likely due to the low expression of the mRNA (Figure 17, 20a).

\textbf{Figure 21.} \textit{In situ} localisation of \textit{FpXET} mRNA in the leaf elongation zone (LEZ). \textbf{(a)-(d)} Cross sections taken from the basal zone of the LEZ; \textbf{(e), (f)} longitudinal sections prepared from the basal part of the LEZ including the apical meristem; \textbf{(a), (c), (e)} hybridisation with the gene specific \textit{FpXET1} antisense probe; \textbf{(b), (d)} hybridisation with the \textit{FpXET1} sense probe; \textbf{(f)} hybridisation with the \textit{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 \, \mu m; Bars in (c) and (d) = 42 \, \mu m; Bars in (e) and (f) = 420 \, \mu m. If, leaf; sh, sheath; st, stem; xy, xylem; ph, phloem.
5 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 hybridisation 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 ortholog 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 conceivable that also root expressed XET-related genes exist in F. pratensis, as it has been demonstrated in maize (Palmer and Davies 1996) or rice (Uozu et al. 2000).
Our results provide a novel insight into the environmental regulation of *FpXET* gene expression, particularly in response to the onset of the dark period when we measured a significant increase 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 therefore not 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, we analysed the contribution that these genes could make to the regulation of tissue elongation and leaf growth using two different genotypes with characteristic leaf growth dynamics under two nitrogen supply levels. This approach allowed us to modulate the growth distribution within comparable tissues and so to test the correlation of *FpXET* expression and this trait. Our 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 (Volenc 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* hybridisation studies, which revealed a highly specific accumulation in rapidly elongating tissue. The specific expression characteristics of *FpXET1*
make it therefore 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* is, 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) involved in differentiation events occurring in relation to the cessation of elongation growth. In such a scenario *FpXET2* could catalyse the integration of newly synthesised 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, we propose 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* we propose 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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VI DISCUSSION

The goal of this study was to elucidate the molecular processes associated with the control of tissue elongation and leaf growth in *F. pratensis*. Of special interest was to determine of the role of specific cell wall-related proteins (expansins and XETs), which have been proposed to be involved in the control of tissue elongation. Despite the significance of leaf growth for forage grasses, the molecular processes controlling their leaf growth have rarely been investigated. Such experiments are of specific significance for the ecophysiological understanding of the behaviour of a species such as *F. pratensis*. Leaf growth after defoliation has been demonstrated to be a major factor limiting the competitive ability of this species within the sward (Nösberger *et al.* 1998). *F. pratensis* provides therefore a relevant model system to explore the molecular regulation of a plant growth process, which influences productivity and competitive ability. In a candidate gene approach, we have used this system to assess the contribution that expansins and XET-related proteins could make to the regulation of leaf growth.

1 CONTROL OF CELL EXPANSION IN THE LEAF ELONGATION ZONE OF *F. PRATENSIS*

Leaf growth is, as any other plant growth process, delimited by two coherent cellular components: cell division and cell expansion (Green 1976). While cell division in the meristem produces the units for future growth, it is the expansion process of each cell that determines the growth of a plant organ.
1.1 The role of the expansin gene family during leaf growth

During the last few years, many different experiments have provided apparently compelling evidence that expansins are directly involved in the control of cell expansion. The most important findings have been: (i), expansin proteins can induce the extension of heat inactivated plant tissue (McQueen-Mason et al. 1992); (ii), application of expansins to living cells stimulates cell expansion (Link and Cosgrove 1998); (iii), several expansin genes are expressed in organs and at time points consistent with a role in growth control (e.g. Cho and Kende 1997, Vriezen et al. 2000) and, (iv), antisense-mediated reduction in expansin gene expression in Arabidopsis plants led to less growth (Cho and Cosgrove 2000). All of these data are consistent with the hypothesis that expansins are directly involved in the control of cell expansion in many plant species. Our results from the spatial analysis of α- and β-expansins during leaf growth in F. pratensis, however, provide evidence contrary to a simplistic view of expansin function being narrowly confined to cell wall loosening and growth. Indeed, they showed a negative correlation of α- and β-expansins with elongation growth. Instead of controlling tissue elongation, a major function of expansins in this tissue appears to be in differentiation processes. Taken in conjunction with recent data in the literature (Caderas et al. 2000), our results require a re-assessment of the direct functioning of expansins in tissue growth and raise the possibility of expansin-independent processes controlling tissue elongation during leaf growth.

1.1.1 α-Expansins are involved in differentiation processes

α-Expansins are generally encoded by multigene families, an observation that has been interpreted to reflect the fundamental role that these proteins play for plant growth. It is estimated that Arabidopsis contains at least 24 different α-expansin genes (Cosgrove 2000c) and that the rice α-expansin gene family consists of a comparable number of members (H. Kende, pers. communication, 2000). Although we do not know how many members the α-
expansin gene family comprises in *F. pratensis*, it is reasonable to assume that the five α-expansins analysed in our experiments represent only a part of the complete gene family. This is supported by results obtained from a Southern blot analysis. The probing of differentially digested genomic DNA with the full-length *FpExp2* clone results in a complex banding pattern (Figure 22). This indicates several additional related α-expansin genes to which the probe cross-hybridised. Moreover, in our initial RT-PCR experiments in which we isolated the conserved regions of the five described α-expansins, we also identified fragments of several other α-expansin sequences.

It is therefore plausible that, in addition to *FpExp2* and *FpExp4*, yet unidentified α-expansin genes are expressed in the basal rapidly elongating part of the LEZ of *F. pratensis*. If such expansins are responsible for the control of tissue elongation in the basal part of the LEZ, they are expressed at significantly lower levels than expansins expressed in adjacent tissue within the LEZ. Thus, *FpExp2* is expressed at relatively high levels in the distal part of the LEZ and, based on its expression pattern, the encoded protein is likely to be involved in the differentiation of the vascular tissue. This complexity in expression patterns and levels of different expansin transcripts poses a major challenge in determining the physiological function of the various α-expansins proposed to be involved in tissue elongation.

A further complication arises from the consideration that although transcript expression studies can provide important information on the mRNA abundance and the specific expression pattern of a gene, its physiological relevance can only be determined by measuring the amount and the activation state of the encoded protein. This will be particularly difficult for α-expansin proteins located in the basal part of the LEZ. For example, in order that the highly abundant *FpExp2* protein does not obscure signal from basal expressed α-expansins in any immunocytochemical assay, non-cross reacting isozyme specific antibodies will be required (Rose *et al.* 2000).
Figure 22. Southern blot analysis of α- and β-expansins. Genomic DNA (7.5 μg) was digested with either PstI (P), EcoRI (E) or HindIII (H), separated by gel electrophoresis and hybridised with a 32P radiolabelled full length α- (a) and β-expansin (b) cDNA. Lane C shows undigested DNA as control. DNA size standards (kb) are given on the left.

(a) (b)

In addition, to determine accurately the activity of such specific α-expansins, appropriate isozyme specific activity assays have to be developed or methods for separating closely related expansin proteins during biochemical purification. There are thus significant technological problems to be overcome before precise physiological functions can be assigned to individual expansins.

The expression of the major leaf expressed α-expansin, FpExp2, was strictly restricted to cells of the xylem parenchyma in the distal non-growing part of the LEZ. This is another example for the growing body of evidence that the activity of α-expansin is not just delimited to the control cell wall relaxation during cell expansion. Other studies also provide evidence in which α-expansin gene expression does not correlate with a proposed simple role in the regulation of cell wall extensibility during growth (e.g. Caderas et al. 2000). Although some of these observations can be interpreted as indicating that, as tissue matures, the cell walls may loose their sensitivity to α-expansin action.
(Cosgrove and Li 1993). There is evidence that particular α-expansins are involved in altering the cell wall architecture during differentiation. These processes include weakening of the endosperm during tomato seed germination (Chen and Bradford 2000), pedicel abscission of Arabidopsis (Cho and Cosgrove 2000) and tissue softening during ripening of tomato (Brummell et al. 1999b) and peach fruits (Hayama et al. 2000). The significant level of FpExp2 protein and activity in tissues of the LEZ of F. pratensis that have ceased growing but which are undergoing differentiation provides further evidence that some members of the α-expansin gene family have functions other than the control of elongation growth.

However, it is remarkable, that the participation of α-expansins in differentiation processes does not preclude a role as primary wall loosening agents (Cosgrove 1999). Differentiation involves, similar to cell expansion, substantial modification of the cell wall structure and is thus a process that has to be highly controlled and coordinated. As wall loosening agents, α-expansins could initiate or modulate differentiation processes via rendering the cell wall architecture more susceptible to other wall-modifying enzymes. In such a scenario, the effected change would depend strongly on the enzymatic milieu present in the cell wall at that time and place. α-Expansins should therefore perhaps best be viewed as keys to unlock the structure of the cell wall, but the actual result of α-expansin action would depend on the battery of cell wall modifying enzymes available in specific cell types at specific stages of development (Cosgrove 1997; Rose et al. 1997).

Development of α-expansin research has shown a tremendous increase during the last years. Although complicated by the potential redundancy within the α-expansin gene family, the production of transgenic plants with altered expression of specific α-expansin genes has finally proven successful (Brummell et al. 1999a; Rochange and McQueen-Mason 2000; Cho and Cosgrove 2000). These transgenic systems offer a great potential to further investigate the in vivo role of α-expansins. It is likely that the future will provide
us with new important information about the physiological roles and the biochemical basis of \( \alpha \)-expansin activity.

1.1.2 The unusual composition of the grass cell wall

The primary cell wall composition of members of the Poaceae plant family ('type II' cell wall, (Carpita and Gibeaut 1993)) differs strongly from that of most other plant species ('type I' cell wall) including monocots (Carpita 1996). Although both wall types respond to the same cues and perform the same physical functions during growth, they are remarkably different in the makeup of their major matrix and protein constituents. A similar network of cellulose microfibrils coated and interlocked by hemicelluloses embedded in a matrix of pectins and structural proteins provides the gross architectural structure. However, while in the typical flowering plant cell wall ('type I' cell wall) the microfibrils connecting hemicelluloses consists mainly of xyloglucan, grass cell walls contain only limited amounts of xyloglucans, but significant levels of glucuronoarabinoxylans, which tether the cellulose microfibrils together. Differences are also found in the composition of the pectic and protein constituents (Carpita 1996). These structural differences between type I and type II cell walls, and the observation that \( \alpha \)-expansins had a relatively low effect in restoring the extension capacity of heat inactivated 'type II' grass cell walls (Li et al. 1993), raised new questions. Cosgrove (2000a) suggested that in the course of evolution the function of \( \alpha \)-expansins had been replaced in grasses by the closely related \( \beta \)-expansin gene family. \( \beta \)-Expansins were initially reported to be exclusive to grasses, consistent with their proposed role as effectors of loosening in this special molecular environment.

1.1.3 Are \( \beta \)-expansins in grasses substitutes for \( \alpha \)-expansins?

We were not able to identify specific \( \alpha \)-expansins whose expression pattern correlated with the pattern of tissue elongation in the LEZ of \( F. \) pratensis. Due to the unusual polymer composition of the grass cell wall and the
proposed role of \( \beta \)-expansins in modulating this cell wall, we investigated whether \( \beta \)-expansin gene expression correlated with the growth pattern in the LEZ of \textit{F. pratensis}.

Similar to \( \alpha \)-expansins, \( \beta \)-expansins are encoded by a multigene family in \textit{F. pratensis} (Figure 22). The characterisation of three members of this gene family might thus represent only a minor fraction of the leaf expressed \( \beta \)-expansins. However, neither an exhaustive PCR screening of cDNA libraries prepared from the basal part of the LEZ, nor the use of cross-hybridising heterologous \( \beta \)-expansin probes (such as an EST from rice) in Northern blot analysis led to the detection of \( \beta \)-expansins expressed in the basal part of the LEZ. This indicates that, similar to our findings for \( \alpha \)-expansins, any \( \beta \)-expansin involved in the control of tissue elongation during leaf growth of \textit{F. pratensis} must be relatively lowly abundant. Or, alternatively, it must display such a high degree of sequence variation that the conservative probes used failed to detected it even under low stringency hybridisation conditions.

The differential expression of the three \textit{FpExpB} genes observed in the LEZ of \textit{F. pratensis} provides the first insight into the gene and tissue-specific regulation of \( \beta \)-expansins expressed in vegetative tissues of grasses. In particular, they show that these proteins do not simply represent substitutes of \( \alpha \)-expansins in grasses. This is also supported by the now completed \textit{Arabidopsis} genome-sequencing project. In addition to the so far described single \( \beta \)-expansin, a BLAST search now reveals several other \( \beta \)-expansins in \textit{Arabidopsis}. Whether the evolutionary emergence of \( \beta \)-expansins is likely to be associated with the unusual composition of the matrix polymers of grass cell walls remains, however, unclear. Until we have a more detailed picture of the substrate specificity and biochemical mechanism of \( \alpha \)- and \( \beta \)-expansin action, our understanding of the significance of the divergence of these two gene families will remain limited.

The data reported here that transcripts for \textit{FpExpB2} accumulate in the tiller primordia is reminiscent of the expression pattern of \textit{LeExp18}, an \( \alpha \)-expansin.
expressed in the apical meristem of tomato and associated with leaf initiation (Reinhardt et al. 1998). Thus, the expression of at least some members of both α- and β-expansins is associated with organogenesis. Whether this similarity in expression pattern relates to a similarity in function remains to be investigated. Our knowledge and understanding of the β-expansins is still very rudimentary. Access to the sequenced rice genome will provide a complete inventory of the β-expansin gene family in this grass species and thus the determination of the tissue and gene-specific expression pattern of the β-expansins. Rice can also be used as a model system to explore the in vivo functions of β-expansins by reverse genetic experiments (antisense and tagging approaches) as well as overexpression, although the potential for functional redundancy is high. Similar to α-expansins, there is still a need for greater understanding of expansin biochemistry. For example, it is still unclear whether purified protein extracts of vegetatively expressed β-expansins posses the capacity to induce the extension of heat inactivated grass cell walls.

1.2 **FpXET1 and FpXET2 participate in different processes during leaf growth of *F. pratensis***

Expansins were primary candidates as agents involved in the control of tissue elongation and leaf growth in *F. pratensis*. However, our investigations revealed no such correlation between expression and tissue elongation for several members of the two gene families. We therefore extended our research to other candidate genes that have been proposed to be involved in the control of tissue elongation, in particular xyloglucan endotransglycosylases.

Controlled cellular expansion has been proposed to occur by XET mediated cleavage and rejoicing of xyloglucan polymers which tether the cellulose microfibrils together. However, it has not yet been possible to demonstrate that XET can catalyse tissue elongation, either in vitro or in vivo (McQueen-Mason and Cosgrove 1994). Although the role of XET in plant growth remains thus debatable, the differential expression pattern of the three
FpXET observed in this study is consistent with a role for the encoded XET-related proteins in cell wall modification processes in the LEZ of F. pratensis. However, the data also indicate that FpXET1 and FpXET2 are likely to participate in different physiological processes.

FpXET1 expression displays a close correlation with the measured SER and thus could be directly involved in the control of tissue elongation. However, the delay between the measured maximal and minimal peak of the LER and the subsequent altered expression pattern of FpXET1 weigh against a simple causal relationship between FpXET1 expression and tissue elongation. In addition, the expression of FpXET1 was not limited to specific cell types such as e.g. the epidermal cells, generally believed to act as growth limiting tissue (Kutschera 1992). It is noticeable that such a specific expression pattern has been reported for a specific growth correlating α-expansin which is expressed in the epidermal cells of elongating rice internodes (Cho and Kende 1998). Thus, although it is possible that FpXET1 plays an important role during cell expansion, that function might be more associated with cell wall biogenesis for the maintenance of growth rather than cell wall loosening. In this scenario, FpXET1 would be involved in the continuous integration of new cell wall material into the existing cell wall structure necessary for the maintenance of cell wall thickness and, thus, biophysical integrity (McCann and Roberts 1994; Thompson et al. 1997).

The contrasting spatial expression pattern of FpXET2 compared to FpXET1 provides evidence that the encoded FpXET2 protein is not associated with the control of tissue elongation. The accumulation of the FpXET2 transcript in regions of the LEZ where the biosynthesis of secondary cell wall occurs (MacAdam et al. 1992) suggests that the encoded protein is more likely to be involved in processes associated with secondary cell wall differentiation. FpXET2 could be involved in the cessation of elongation growth observed in this part of the LEZ via an increased xyloglucan mediated cross-linking of the secondary cell wall, possibly working in association with wall-associated
peroxidases. These enzymes have been proposed to be responsible for the decline of tissue elongation in *F. arundinacea* (MacAdam et al. 1992) and *L. perenne* (Bacon et al. 1997).

Although the abundance and pattern of the particular *FpXET* mRNAs in the LEZ of *F. pratensis* are consistent with a role for the encoded proteins in cell wall modification processes associated with leaf growth, it is absolutely necessary in future studies to determine the activity and the spatial abundance of the encoded proteins. While the interpretation of data obtained by measuring the *in vitro* XET activity extracted from segments along the growing grass leaf has been shown to be complicated by the spatial superposition of different isozymes (Palmer and Davies 1996; Smith et al. 1996b; Schünmann et al. 1997), *in vivo* XET activity measurements could help to circumvent this problem. Recently, Vissenberg et al. (2000) published a novel method that allows the co-localisation of XET activity and donor substrate *in vivo* in roots of *Arabidopsis*. Analogous to *in situ* mRNA hybridisations, this method allows the delimitation of XET activity to specific cell types with a high spatial resolution. An adaptation of this new assay to the LEZ of grasses might therefore help to solve the problem of analysing spatially overlapping XET activities and provide a better insight into the physiological functions of XET during tissue elongation and leaf growth of *F. pratensis*.

2 CELL EXPANSION VS CELL DIVISION: LEAF GROWTH AS INFLUENCED BY NITROGEN SUPPLY

Nitrogen is a major determinant of dry matter yield in forage grasses influencing strongly tillering and leaf growth. The effect on leaf growth is closely associated with the dynamics within the LEZ. Significant effects on the cell division as well as on the cell expansion process have been reported from several studies focusing on the influence of nitrogen supply on the cellular
dynamics in the LEZ (Volenc and Nelson 1983; MacAdam et al. 1989; Fricke et al. 1997). To study the expression of expansin and XET genes potentially associated with the control of leaf growth, we used this specific nitrogen dependent growth response as a tool to alter the spatial distribution of growth within the LEZ. Consequently, we observed significant differences in the cellular growth dynamics depending on the genotype and the supplied nitrogen level (Table 1). On the one hand, the inherently lower LER (-23%) of the SL genotype was the result of a shorter LEZ (-13%) with only a limited effect on the maximal SER_{max} (-4%) compared to the LL genotype. Thus, a similar relative decreased cell expansion (-14% reduced final cell length) and cell division (-10% reduced cell flux) contributed to the significantly shorter LEZ. On the other hand, the significant nitrogen-dependent reduction of the LER in the LL genotype (-60%) was brought about by a significant reduction in the SER_{max} (-42%), resulting in a considerably shorter LEZ (-45%). However, it is important to note that nitrogen deprivation had a much greater impact on cell division (-60% decrease in cell flux) than on cell expansion, as indicated by only a 3% decrease of the final epidermal cell length. These results are a striking example demonstrating the importance of cell division within the basal meristem for the control of leaf growth in grasses. In order to obtain a more complete picture of the molecular regulation of leaf growth in grasses, particularly in response to different environmental conditions, attention must therefore also be paid to cell division within the basal meristem of the growing leaf.

Although extremely complex in nature, our understanding of the mechanisms underlying the control of the plant cell cycle has progressed lately through the identification and characterisation of enzymes involved in the control of cell division, such as CDKs and cyclins (reviewed in Huntley and Murray 1999). Consequently, during the last years several studies have unequivocally demonstrated that the regulation of this fundamental process is indeed, a very important checkpoint for the control of leaf growth.
Table 1. LER, maximal SER, size of the LEZ, final cell length and cell flux of the 2nd intact leaf that developed on the main tiller of two contrasting F. pratensis grown under two nitrogen levels. Cell flux at the distal end of the LEZ, used as indication of the epidermal cell division rate was calculated by dividing the LER by final epidermal cell length (Schäufele and Schnyder 2000). SE indicates the standard error of the LER.

<table>
<thead>
<tr>
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<th>LLNN</th>
<th>LLN</th>
<th>SLNN</th>
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<tbody>
<tr>
<td></td>
<td>absolute</td>
<td>%</td>
<td>absolute</td>
</tr>
<tr>
<td>LER [mm h(^{-1})] (SE)</td>
<td>1.99 (0.063)</td>
<td>100</td>
<td>0.79 (0.063)</td>
</tr>
<tr>
<td>(SER_{\text{max}}) [mm mm(^{-1}) h(^{-1})]</td>
<td>0.076</td>
<td>100</td>
<td>0.044</td>
</tr>
<tr>
<td>Size of the LEZ [mm]</td>
<td>40</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>Final cell length [(\mu m)]</td>
<td>622</td>
<td>100</td>
<td>601</td>
</tr>
<tr>
<td>Cell flux [cells h(^{-1})]</td>
<td>3.20</td>
<td>100</td>
<td>1.31</td>
</tr>
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</table>

For example, it has been shown that the overexpression of a specific D-type cyclin that increases cell division leads to an overall increase in the growth rate of Arabidopsis plants (Cockcroft et al. 2000). In addition, it has been well demonstrated that, especially under stress conditions such as drought, the activity of cyclin-dependent kinases are an important factor in the control of leaf growth (Schuppler et al. 1998; Granier et al. 2000).

Future studies focusing on the molecular regulation of leaf growth in forage grasses should therefore also include a quantitative analysis of cell division, as proposed by Tardieu and Granier (2000). Combined with measurements of key enzymes involved in the regulation of the plant cell cycle, such as specific cyclins and cyclin-dependent kinases (e.g. Granier et al. 2000) or inhibitors of these enzymes (e.g. Wang et al. 1998), such approaches will allow to assess the role of cell division for the control of leaf growth in forage grasses.
3 CONCLUSION

In conclusion, our results demonstrate that the control of tissue elongation during leaf growth of *F. pratensis* is a highly coordinated process, influenced by various factors. Although α- and β-expansins were primary gene candidates responsible for the control of cell expansion and leaf growth, we were able to demonstrate that the predominantly leaf expressed α- and β-expansins are associated with differentiation events rather than the control of tissue elongation. Our results thus provide evidence that the physiological function of α- and β-expansins is not purely limited to the control of tissue elongation. In contrast to our findings for expansins, we were able to identify a specific XET-related gene (*FpXET1*) with an expression pattern that tightly correlated with the spatial distribution of tissue elongation within the LEZ. This specific expression pattern was maintained in two genotypes with contrasting leaf growth characteristics and under two levels of nitrogen supply. The results suggest that this gene plays an important role in cell wall modification processes during tissue elongation. However, as revealed by an analysis of the cellular growth dynamics in the LEZ of *F. pratensis*, nitrogen deprivation had a stronger impact on cell division than on cell expansion. In order to obtain a more realistic picture of the molecular regulation of leaf growth in *F. pratensis*, future studies should therefore also focus on key enzymes involved in the control of the cell cycle.
VII REFERENCES


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