

Ectopic expression of a wood-abundant expansin *PttEXPA1* promotes cell expansion in primary and secondary tissues in aspen

Madoka Gray-Mitsumune^{1,†}, Kristina Blomquist², Simon McQueen-Mason³, Tuula T. Teeri², Björn Sundberg^{1,*} and Ewa J. Mellerowicz^{1,*}

¹Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SE-90183 Umeå, Sweden

²Royal Institute of Technology, School of Biotechnology, AlbaNova University Centre, SE-10691 Stockholm, Sweden

³Department of Biology, University of York, PO Box 373, York YO10 5YW, UK

Received 21 May 2007;
revised 28 August 2007;
accepted 31 August 2007.

*Correspondence (fax +46 90 7868165;
e-mail ewa.mellerowicz@genfys.slu.se;
bjorn.sundberg@genfys.slu.se)

†Present address: Department of Biology,
Concordia University, 7141 Sherbrooke St
W, Montreal, QC, Canada, H4B 1R6

Keywords: cell expansion, expansin,
fibre length, poplar, *Populus*,
transgenic trees, wood formation,
xylogenesis.

Summary

Expansins are primary agents inducing cell wall extension, and are therefore obvious targets in biotechnological applications aimed at the modification of cell size in plants. In trees, increased fibre length is a goal of both breeding and genetic engineering programmes. We used an α -expansin *PttEXPA1* that is highly abundant in the wood-forming tissues of hybrid aspen (*Populus tremula* L. \times *P. tremuloides* Michx.) to evaluate its role in fibre elongation and wood cell development. *PttEXPA1* belongs to Subfamily A of α -expansins that have conserved motifs at the N- and C-termini of the mature protein. When *PttEXPA1* was over-expressed in aspen, an extract of the cell wall-bound proteins of the transgenic plants exhibited an increased expansin activity on cellulose–xyloglucan composites *in vitro*, indicating that *PttEXPA1* is an active expansin. The transgenic lines exhibited increased stem internode elongation and leaf expansion, and larger cell sizes in the leaf epidermis, indicating that *PttEXPA1* protein is capable of increasing the growth of these organs by enhancing cell wall expansion *in planta*. Wood cell development was also modified in the transgenic lines, but the effects were different for vessel elements and fibres, the two main cell types of aspen wood. *PttEXPA1* stimulated fibre, but not vessel element, diameter growth, and marginally increased vessel element length, but did not affect fibre length. The observed differences in responsiveness to expansin of these cell types are discussed in the light of differences in their growth strategies and cell wall composition.

Introduction

Wood tissue performs two essential functions in land plants: (i) support and elevation of the photosynthetic leaves and reproductive organs; and (ii) conduction of water and nutrients. In most extant angiosperms, conduction is performed by vessel elements and tracheids, whereas the libriform fibres provide support. These cell types terminally differentiate by autolysis, leaving only thick multilayered cell walls. The ability to perform their respective functions is largely determined by the dimensions of these cells. Thus, support is enhanced by the small diameter and large length of libriform fibres, whereas conductive properties are a function of vessel element

diameter (Tyree and Zimmermann, 2002). The ultimate size and shape of wood cells are determined early during their differentiation from the vascular cambium (reviewed by Larson, 1994; Mellerowicz *et al.*, 2001; Mellerowicz, 2006). Cell length is determined by the length of fusiform initials and, in the case of fibres, also by the degree of intrusive growth outside the meristem in the radial expansion zone. The diametric growth of fibres is dependent on the diffuse symplastic growth of the radial cell walls, because the cells are organized in radial files, in which the direction of growth is radial. Vessel elements, by contrast, frequently expand more than their surrounding cells, leading to the separation of cell contacts and lateral intrusion. This growth takes place mostly

in the radial expansion zone. Thus, wood cell enlargement involves a combination of symplastic and intrusive growth and is probably controlled by several different mechanisms.

Expansins are considered to be primary agents of acid growth affecting cell wall rheology (Cosgrove, 2000; Choi *et al.*, 2006). They induce cell wall stress relaxation in a pH-dependent manner by disrupting hydrogen bonds between cellulose microfibrils and cross-linking glycans, such as xyloglucan or xylan, causing a local stress release (McQueen-Mason *et al.*, 1992; McQueen-Mason and Cosgrove, 1994, 1995; Yennawar *et al.*, 2006). Expansins are also crucial for cell wall modification, for example during fruit ripening or organ abscission or pollen germination on a stigma (Rose and Bennett, 1999; Cho and Cosgrove, 2000; Li *et al.*, 2003; Belfield *et al.*, 2005). The action of expansins may affect cell wall composition and structure, probably by regulating the access of enzymes that degrade specific cell wall components (Brummell *et al.*, 1999) and/or by affecting cell wall deposition (Zenoni *et al.*, 2004).

Expansins form large multigene families in all land plant species studied so far, including angiosperms, gymnosperms, ferns and mosses, and are divided into α - and β -expansins (EXPA and EXPB, respectively) (Sampedro and Cosgrove, 2005; Choi *et al.*, 2006; Sampedro *et al.*, 2006). Genes sharing distant sequence similarity with expansins have been classified as expansin-like A (EXPLA) and B (EXPLB), but their biological function is unknown. From phylogenetic analyses, it can be deduced that at least 17 different expansin genes existed in a common ancestor of dicots and monocots, which indicates extensive specialization within the family, in particular amongst the α -expansins (Sampedro and Cosgrove, 2005). The tissue-specific expression patterns commonly observed for different α -expansins suggest functional diversification of the genes (Wu *et al.*, 2001; Lee and Kende, 2002), but it is not clear whether there are also functional differences between proteins belonging to different clades.

The activity of expansins extracted from plant cell walls can be assayed *in vitro* by measuring creep in a cellulose-xyloglucan composite submerged in a buffered expansin extract (Whitney *et al.*, 2000). Using this assay, we have observed high expansin activity in the cambial meristem and adjacent radial expansion zone in hybrid aspen, *Populus tremula* L. \times *P. tremuloides* Michx. (Gray-Mitsumune *et al.*, 2004), indicating a role for expansins in wall restructuring in primary-walled developing wood cells. The recent analyses of expansin genes in the *Populus* genome revealed at least 36 genes (Geisler-Lee *et al.*, 2006; Sampedro *et al.*, 2006). Transcripts of α - and β -expansin genes were found in actively growing wood-forming tissues, but were absent during

cambial dormancy, consistent with their proposed role in wood cell expansion (Geisler-Lee *et al.*, 2006). Some expansin genes were found to be expressed in secondary-walled xylem, which suggests that these genes function in cell wall remodelling and/or secondary wall formation.

Interestingly, expansins highly expressed in wood-forming and vascular tissues share conserved motifs of unknown function at the C- and N-termini of the mature protein (Im *et al.*, 2000; Gray-Mitsumune *et al.*, 2004; Geisler-Lee *et al.*, 2006). These expansins form a distinct group within the α -expansin family, known as subfamily A (Link and Cosgrove, 1998), that forms a branch of clade IV with representatives from several plant species (Sampedro and Cosgrove, 2005; Sampedro *et al.*, 2006). In *Populus*, there are five members of this branch, including EXPA1, EXPA5, EXPA7, EXPA15 and EXPA16 (renamed after Sampedro *et al.*, 2006). To address the function of this branch in cell wall remodelling and wood cell development, we over-expressed *PttEXPA1*, formerly published as *PttEXP1* (Gray-Mitsumune *et al.*, 2004), in hybrid aspen, and studied the effects of this expression during primary and secondary growth. We found that proteins extracted from the developing wood cells of the trees over-expressing *PttEXPA1* showed increased expansin activity when compared with corresponding protein extracts from wild-type (WT) trees. This supports the proposed expansin activity of clade IV expansins. The over-expression of *PttEXPA1* promoted the growth of developing wood cells and also increased the length of stem internodes, leaf epidermal cell size and leaf size, supporting a universal function of *PttEXPA1* in cell growth.

Results

Generation of lines over-expressing *PttEXPA1*

To functionally characterize *PttEXPA1 in planta*, transgenic hybrid aspen lines were generated that expressed the *PttEXPA1* coding sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The majority of the lines examined showed a two- to threefold increase in *PttEXPA1* mRNA in the leaves compared with the WT level, as estimated by relative quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1a). On the basis of this analysis, two independent lines (6 and 14) showing high *PttEXPA1* expression and one line (3) showing a WT or slightly lower level of *PttEXPA1* expression were selected for more detailed analysis. The selected lines and WT were clonally multiplied *in vitro*, and ten plants of each genotype were grown in soil until they reached about 1.5 m in height

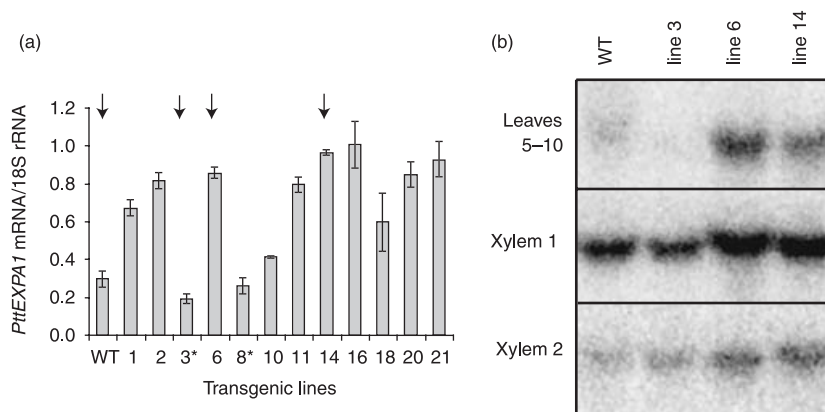


Figure 1 Expression of *PttEXPA1* in transgenic aspen. (a) Relative quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of leaves of *in vitro*-grown plants. *PttEXPA1* expression is presented as a ratio of the PCR products obtained with gene-specific primers, which were derived from *PttEXPA1* mRNA and 18S rRNA. The values are the means of three RT-PCRs \pm standard errors. Transgenic lines denoted with asterisks are those with no transgene expression. Transgenic lines indicated by arrows were selected for detailed analysis. (b) Northern blot analysis of different tissues of 4-month-old plants. RNA was extracted from young leaves (leaves 5–10), primary-walled developing xylem (xylem 1) and secondary-walled developing xylem (xylem 2). WT, wild-type.

and had well-established secondary growth. Northern blot analysis of *PttEXPA1* expression in young expanding leaves, developing primary-walled xylem, including the cambial meristem and expanding xylem cells (denoted 'xylem 1'), and developing secondary-walled xylem, containing xylem cells undergoing secondary thickening (denoted 'xylem 2'), showed that, in all tissues, transgenic lines 6 and 14 exhibited increased transcript levels, whereas line 3 showed slightly reduced expression, compared with WT tissues (Figure 1b). WT aspen exhibited a much higher level of *PttEXPA1* mRNA in the primary-walled developing xylem compared with other tissues, confirming our previous observations (Gray-Mitsumune *et al.*, 2004).

PttEXPA1 shows expansin activity *in vitro*

To determine whether *PttEXPA1* shows measurable expansin activity, ionically bound cell wall proteins were extracted from lines over-expressing *PttEXPA1*, from WT plants and from transgenic line 3 that was slightly suppressed for *PttEXPA1*. The proteins extracted from young leaves and primary-walled xylem were applied to the cellulose–xyloglucan composite, and the creep behaviour of the composite was measured in an extensometer, as described by Whitney *et al.* (2000). In the case of proteins extracted from primary-walled xylem, the expansin activity corresponded well to the level of *PttEXPA1* transcripts of transgenic lines (cf. Figure 2 and Figure 1), suggesting that *PttEXPA1* protein is a *bona fide* expansin. In the case of proteins extracted from leaves, the expansin activity was generally at least one order of magnitude less than in the primary-walled xylem and close to the detection limit in all

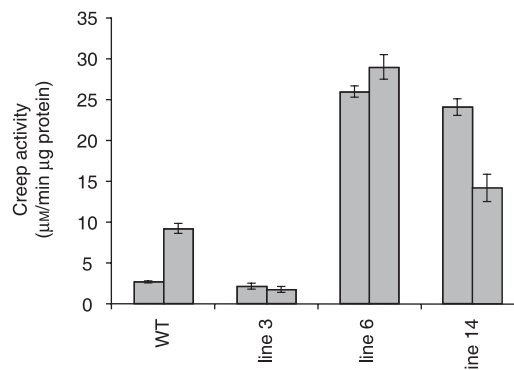


Figure 2 Expansin activity in *PttEXPA1* over-expressing plants. Cell wall proteins were extracted from the primary-walled developing xylem isolated from the 35th to 40th internodes counting from the apical bud (xylem 1). Two extracts from each line were obtained and the expansin activity was assayed using a cellulose–xyloglucan composite. Values are the means of four assays \pm standard error. WT, wild-type.

the lines. The activity was reduced in line 3 compared with WT (data not shown).

Effects of altered *PttEXPA1* expression on primary growth

To examine the impact of expansin over-expression, the phenotypes of lines ectopically expressing *PttEXPA1* during primary growth were studied. In addition to the lines selected for detailed analysis, a group of ten transgenic plants, each representing an independent transgenic line carrying the same construct as the selected lines, was phenotyped and presented as 'assorted'.

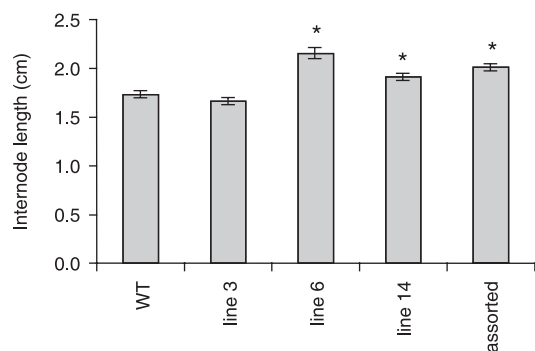


Figure 3 *PttEXPA1* over-expressing plants have longer internodes.

Ten consecutive internodes located from approximately 50–90 cm above ground level on each of ten plants per line were measured. In addition to the four transgenic lines (lines 3, 6, 14 and 16), the internodes of ten other transgenic lines (lines 1, 5, 18, 20, 21, 23, 27, 28, 29 and 30), each represented by one plant, were also measured, and shown as 'assorted' in the figure. The values are the means of 100 internodes \pm standard error. Data points denoted by asterisks are significantly different from the wild-type (WT) (Dunnett's test, $P = 0.05$).

The plant height in the over-expressing lines 6 and 14 and in the 'assorted' group was not significantly different from that in WT or line 3 (data not shown), but the average internode length was significantly increased (up to 124% of the WT level) (Figure 3). Although the leaf number was not counted directly, this suggests that over-expressing plants produced fewer leaves than WT plants. When the number of leaves was estimated by comparing the height divided by the average internode length, over-expressing lines were predicted to have produced 10%–24% fewer leaves compared with WT (data not shown). In spite of this, the phyllotactic pattern of the leaves did not show any obvious change (data not shown).

The growth of the leaves was examined by measuring every fifth successive leaf from the apex (Figure 4a, b). In WT and line 3, the leaves numbered 5–10 were in their rapid growth phase, and leaf 15 was almost fully expanded. However, in the over-expressing and 'assorted' lines, leaf 15 was still growing rapidly and some growth of leaf 20 was evident in line 14 and the 'assorted' lines (Figure 4a). The width increment between leaf 15 and 20 was significantly ($P = 0.05$) higher in over-expressing lines than in WT. Thus, in lines over-expressing *PttEXPA1*, the leaves grew for a longer period, resulting in a larger final leaf size (Figure 4c). In addition, the growth in width appeared to be more stimulated than the growth in length, resulting in a small but noticeable change in the leaf shape (Figure 4e).

To establish whether a larger leaf size in *PttEXPA1* over-expressing lines was a result of increased cell expansion, the epidermal cell size was measured on the adaxial leaf side in lines 6 and 14 and compared with that of WT. The

over-expressing lines had larger cells (Figure 4d). A rough estimate was also made of the cell number per leaf by comparing cell areas with leaf areas. Using this method, it was determined that leaves of WT and over-expressing lines contained a similar number of cells (data not shown). Thus, the changes in leaf size in *PttEXPA1* over-expressing lines was explained by enhanced cell expansion alone.

Effects of altered *PttEXPA1* expression on secondary growth

To deduce the role of *PttEXPA1* in secondary tissues, stems of transgenic lines were sectioned and the radial width of the secondary xylem (wood) and bark was measured. Over-expression of *PttEXPA1* induced a small but consistent increase in the amount of secondary xylem at the expense of bark (Figure 5a, b). Increased wood diameter could be a result of either an increased rate of xylem cell formation by the cambium and/or an increased radial expansion of the xylem cells. To determine whether cell radial expansion was increased, wood cells were separated by maceration and their width was measured under the microscope. All over-expressing lines had wider fibres (9%–16% wider than the WT level), but their vessel diameters did not differ from the WT level (Figure 6a, b).

Expansins have been implicated in tip growth on the basis of their presence at the tip of the cells during tip growth and the accumulation of their transcripts at the tip (Baluška *et al.*, 2000; Gray-Mitsumune *et al.*, 2004). Furthermore, cosegregation of root hair elongation and the expression of *AtEXPA7* and *AtEXPA18* in *Arabidopsis*, and *HvEXPB1* in barley, also support the role of expansins in tip growth (Cho and Cosgrove, 2002; Kwasniewski and Szarejko, 2006). The availability of transgenic aspen lines with increased expansin activity in secondary tissues, where intrusive tip growth is prevalent (Mellerowicz, 2006), was used to test the hypothesis of the involvement of expansins in the intrusive tip growth of developing wood cells. Vessel element lengths (with tails) were significantly affected by *PttEXPA1* over-expression, as demonstrated by the significant effect ($P = 0.0018$) of genotype in the analysis of variance (ANOVA) test, although the effects were small, ranging from a 2% to 7% increase compared with the WT level (Figure 6d). Thus, when the individual lines were compared with WT, only the 'assorted' lines showed a significant effect. Nevertheless, a positive correlation between the vessel element length and expansin activity of the lines (data from Figure 2), with a coefficient of determination $R^2 = 0.99$, suggests a role for expansin in vessel length determination. The effects of the genotype on

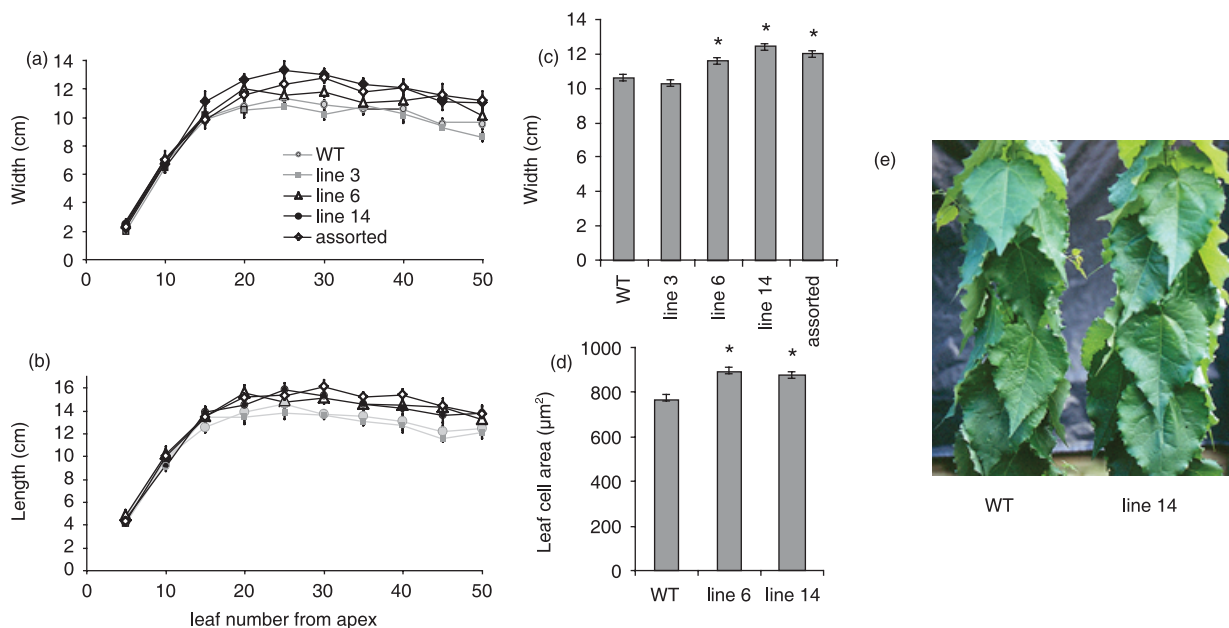


Figure 4 *PttEXPA1* over-expressing plants have larger leaves. (a, b) The sizes of the leaves at different developmental stages were compared in 3-month-old plants. The widths (a) and lengths (b) of leaves were measured every fifth leaf from the apex. The values are the means of ten plants \pm standard error. The group 'assorted' represents a mixture of ten transgenic lines, as explained in Figure 3. (c) Comparison of fully expanded leaves. The width measurements of the 20th to 45th leaves were collected to compare fully expanded leaves. The values are the means of 60 leaves \pm standard error. Data points denoted by asterisks were significantly different from the wild-type (WT) (Dunnett's test, $P = 0.05$). (d) Epidermal cell areas of fully expanded leaves. The values are the mean of 260 cells from the upper epidermis \pm standard error. Data points denoted by asterisks are significantly different from WT (Dunnett's test, $P = 0.05$). (e) Three-month-old plants showing fully expanded leaves.

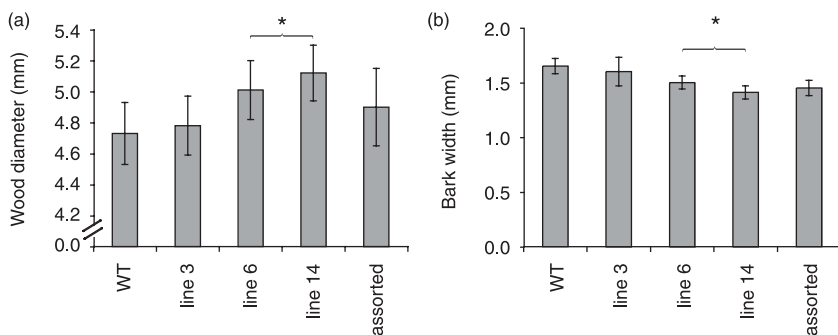


Figure 5 Secondary growth of *PttEXPA1* over-expressing plants. Effects on the radial width of wood (a) and bark (b). Internode 41 of the stem of each plant was cross-sectioned and the radial distances were measured under a dissecting microscope. Values are the means of ten plants \pm standard error. Transgenic lines 6 and 14 were significantly different from the wild-type (WT) when the values from the two lines were pooled (Dunnett's test, $P = 0.05$).

fibre length were less evident (Figure 6c). By comparing fibre length with vessel element length, the amount of intrusive growth of the fibres during xylem differentiation can be estimated. Using this method, we did not observe any consistent effect of expansin activity on fibre intrusive growth in *PttEXPA1* over-expressing lines.

Discussion

Subfamily A member *PttEXPA1* has expansin activity *in vitro* and *in vivo*

PttEXPA1 belongs to Subfamily A in clade IV of α -expansins that are expressed in vascular tissue (Im *et al.*, 2000;

Gray-Mitsumune *et al.*, 2004; Geisler-Lee *et al.*, 2006; Sampedro *et al.*, 2006). Aspen lines over-expressing *PttEXPA1* showed a markedly increased expansin activity in creep assays using cellulose–xyloglucan composites. This is similar to a cucumber α -expansin of clade II, CsEXP1 (Whitney *et al.*, 2000). Moreover, the over-expression of *PttEXPA1* in primary tissues induced typical expansin effects *in vivo*; both organs, leaf and internode, grew larger when the *PttEXPA1* transcript level was increased in transgenic lines. A larger leaf size corresponded to a similarly increased epidermal cell size, indicating that the primary effect of *PttEXPA1* was on cell expansion rather than on cell division. These observations indicate that, similar to other expansins, the *PttEXPA1* protein has a general wall-loosening activity *in vivo* (Cosgrove *et al.*,

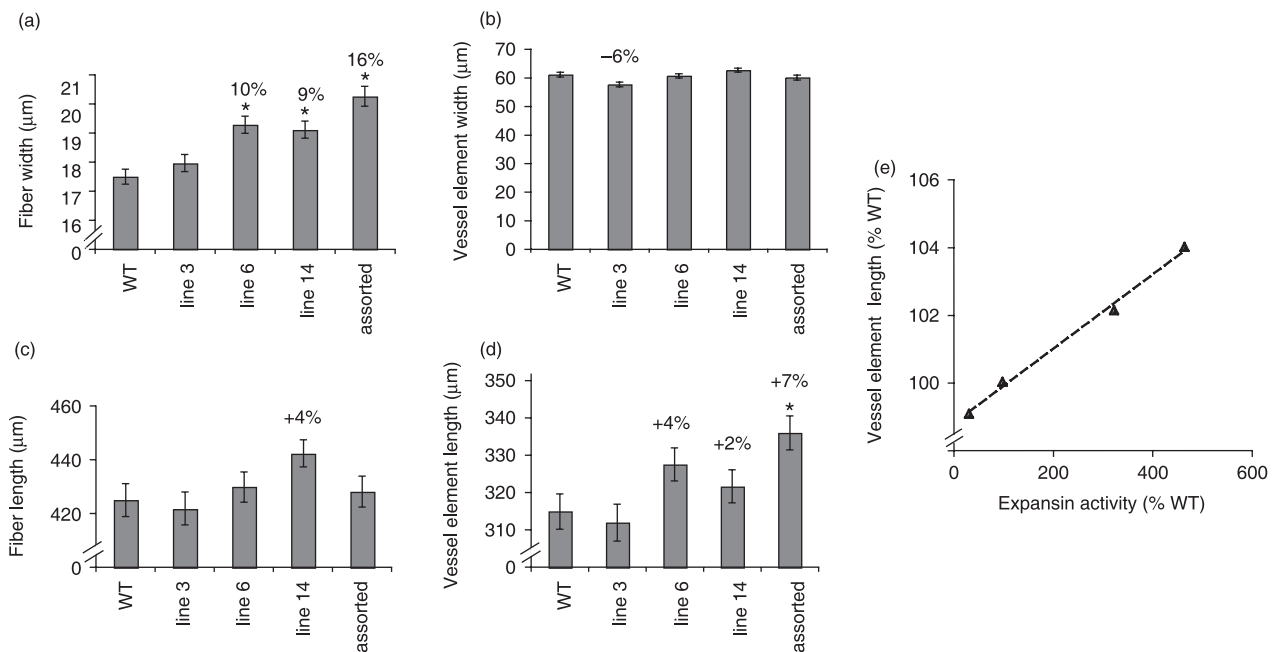


Figure 6 *PttEXPA1* over-expressing lines exhibit changes in xylem cell size. Effects on the width of fibres (a) and vessel elements (b). Effects on the length of fibres (c) and vessel elements (d). Cells were separated by maceration, and images of the cells were captured and measured using AxioVision software. The values are the means of 250 cells \pm standard error. Fibre width and vessel element length (with tails; a similar result was obtained without tails) were significantly (ANOVA, $P = 0.05$) affected by genotype. Data points denoted by asterisks were significantly different from the wild-type (WT) (Dunnett's test, $P = 0.05$). (e) Relationship between vessel element length and expansin activity recorded in proteins extracted from primary-walled developing xylem of lines 3, 6, 14 and WT, as expressed as a percentage of the WT level.

1997; Cho and Cosgrove, 2000; Choi *et al.*, 2003; Lee *et al.*, 2003). Another member of Subfamily A, *PhEXPA1*, has also been shown to be involved in cell expansion, as its down-regulation in petunia resulted in a reduced cell size in petals (Zenoni *et al.*, 2004). Thus, the presence of conserved motifs at the beginning and end of the protein sequence in Subfamily A expansins does not seem to interfere with their function as cell wall-loosening agents.

Over-expression of expansin affects different meristems to different degrees

The induction of leaf primordia formation by expansins has been elegantly shown by local expansin delivery in the apical meristem (Fleming *et al.*, 1997; Pien *et al.*, 2001). Localized expression of native *LeEXPA18* has also been shown to mark the leaf primordium location in tomato (Reinhardt *et al.*, 1998). Thus, a localized induction of expansin causes local bulge formation and growth. However, when expansins are expressed ectopically, it is the responsiveness of cells to expansin action that determines their reaction. Cells would not respond to increased expansin levels if: (i) expansins were non-limiting, although cell walls were capable of expansion; or (ii) cell walls had matured, rendering them unresponsive to

expansin action. In rice, the over-expression of *OsEXPA4* stimulated the rate of leaf primordia formation (Choi *et al.*, 2003). This is in stark contrast with the action of *PttEXPA1* in aspen, where all the over-expressing lines formed fewer leaves when their internodes grew longer. Expression from the CaMV 35S promoter in aspen is both stable over time and uniform for different organs, when expressed per protein level as opposed to histochemical staining (Hawkins *et al.*, 2003). Silencing of other expansins in the apex that are encoded by genes sharing stretches of 22 or more nucleotides that are identical to *PttEXPA1* is not very likely, as we did not observe the silencing of *PttEXPA1* in primary-walled xylem where it is most highly expressed, although we cannot discount this possibility entirely. Thus, the reasons for a reduced rate of leaf formation in over-expressing lines are not clear. However, it can be concluded that, in aspen, the subapical and intercalary meristems responsible for internode elongation are sensitive to the over-expression of expansin, and thus the availability of expansin activity may effectively limit internode elongation.

Kinetic analysis of leaf growth in transgenic plants sheds some light on the importance of expansins in leaf growth. Although *PttEXPA1* was ectopically expressed, the effect on leaf size was detectable only at the late stage when WT plants had completed leaf growth. We speculate that endogenous

expansin is highly abundant early in leaf development, as indicated by the abundance of transcripts for *PttEXPA3* in young leaves (Geisler-Lee *et al.*, 2006), but becomes limiting later in leaf development, causing growth cessation. In addition, the sensitivity of aspen leaves to ectopic expansin was greater for the growth in width than in length, resulting in wider blades in the *PttEXPA1* over-expressing lines. This suggests that coordinate expression of expansin in different leaf meristems may contribute to the generation of specific leaf shapes in aspen, as also suggested in tobacco (Pien *et al.*, 2001).

Role of expansin in wood cell growth

Several expansin genes are highly expressed in the developing wood of deciduous and conifer tree species, with Subfamily A α -expansins dominating in these tissues (Gray-Mitsumune *et al.*, 2004; Geisler-Lee *et al.*, 2006; Sampedro *et al.*, 2006). However, the action of any of these proteins in xylogenesis is largely speculative. We found that over-expression of a member of Subfamily A, *PttEXPA1*, in developing wood resulted in increased fibre diameter but, surprisingly, had no effect on vessel diameter. The differential effect is unlikely to be a result of differential expression of the CaMV 35S promoter. According to studies using the β -glucuronidase (GUS) reporter gene, the CaMV 35S promoter drives gene expression in all cell types in developing xylem (Chen *et al.*, 2000; Hawkins *et al.*, 2003). Instead, this result suggests that *PttEXPA1* has a typical wall-loosening function in fibre symplastic growth, i.e. interacting with cross-linking glycans, such as xyloglucan, causing their detachment from cellulose microfibrils (McQueen-Mason and Cosgrove, 1994, 1995; Whitney *et al.*, 2000; Yennawar *et al.*, 2006). By contrast with vessel elements, fibres continue to deposit xyloglucan into their primary walls late during their differentiation (Bourquin *et al.*, 2002) when the native expansin activity is already reduced (Gray-Mitsumune *et al.*, 2004). Thus, it is possible that the xyloglucan cross-links formed at this stage may be susceptible to the over-expression of expansin. The observation that vessel diameters were not affected by *PttEXPA1* over-expression indicates that the radial expansion of vessel elements is not limited by expansin activity. Vessel elements expand very rapidly in response to changes in K⁺-controlled turgor pressure (Langer *et al.*, 2002). The extent of radial growth is very substantial in vessels compared with fibres, and also involves lateral intrusive growth between adjacent cells. The nature of this process is therefore very different from the symplastic and gradual cell expansion observed for other cell types. Moreover, vessel walls mature ahead of fibres by oxidative cross-linking with phenolic

compounds and lignification (reviewed by Mellerowicz *et al.*, 2001). This would render them non-responsive to expansin at an earlier stage than fibres.

Although correlative evidence from expression studies has implicated expansins in tip growth in several species and tissues (Baluška *et al.*, 2000; Cho and Cosgrove, 2002; Gray-Mitsumune *et al.*, 2004; Kwasniewski and Szarejko, 2006), such a role has yet to be demonstrated. Transgenic lines with increased expansin activity in secondary tissues, where fibres elongate solely by intrusive tip growth (Mellerowicz, 2006, submitted for publication), did not exhibit any increase in fibre length. There are several possibilities explaining this result. As the over-expression affected fibre expansion in the radial dimension, we can exclude the possibility that the transgene was inactive in the target cells. However, it is possible that tip growth requires the targeting of expansin transcript to the site of expansin action, i.e. to the cell tip, as observed in developing xylem cells (Im *et al.*, 2000; Gray-Mitsumune *et al.*, 2004). If this is the case, so far unknown elements governing targeting would be required in the transgene constructs. Our previous study has indicated that the *PttEXPA1* transcript is enriched in fibre tips (Gray-Mitsumune *et al.*, 2004). However, it is possible that another expansin gene may be much more specifically involved in tip growth. It is also possible that the intrusive growth of fibres may be primarily limited by the middle lamella through which the growing fibre tips penetrate. Indeed, we have previously observed that increasing pectin methylesterification of homogalacturonan promotes fibre elongation, whereas decreasing methylesterification has the opposite effect (A. Siedlecka, S. Wiklund, A.-M. Péronne, F. Micheli, J. Lesniewska, I. Sethson, U. Edlund, L. Richard, B. Sundberg, E. J. Mellerowicz, submitted for publication). Thus, the strategy of combining the increase in expansin expression with the modification of pectin methylesterification in the middle lamella may be a promising route for fibre length engineering in the future.

By contrast with a lack of induction of fibre elongation by increased levels of *PttEXPA1*, a weak positive effect of this expansin on vessel element length was observed. The length of vessel elements is determined by a different mechanism from that of fibres and, by and large, reflects the length of the fusiform initial from which they originate (Mellerowicz, 2006). The complex regulation of the fusiform initial length depends on the length of the procambium cell from which it originates, the rate of intrusive growth between divisions, the frequency of anticlinal divisions, the orientation of the cell plate and the rate of fusiform initial selection (reviewed in Larson, 1994; Mellerowicz *et al.*, 2001; Mellerowicz, 2006). We presume that *PttEXPA1* could have affected the size of procambial cells from which the fusiform initials developed,

as the internodes of *PttEXPA1* over-expressing lines were substantially longer.

Conclusion

Using a transgenic approach in aspen, we have shown that *PttEXPA1*, an abundant α -expansin of wood-forming tissues belonging to conserved Subfamily A, enhances the creep of cellulose–xyloglucan composites *in vitro* and promotes cell wall expansion *in planta*. The effects on cell growth were seen during both primary and secondary growth in lines over-expressing *PttEXPA1*. In wood-forming tissues, expansin over-expression affected mostly the symplastic radial growth of fibres without affecting vessel radial expansion or fibre elongation. This supports previous observations indicating that there are many kinds of growth in developing wood, each subject to different types of regulation, and determining the final cell size. Therefore, technologies for the genetic engineering of fibre length should consider using an integrated approach that combines the modification of wall extensibility and cellular adhesion.

Experimental procedures

Plant transformation and growth conditions

Hybrid aspen, *P. tremula* L. \times *P. tremuloides* Michx., clone T89, was transformed by *Agrobacterium*-mediated gene transfer using the binary vector pPCV702kana (Walden *et al.*, 1990), with the coding sequence of *PttEXPA1* (GENBANK accession number AY435099) fused behind the CaMV 35S promoter as described previously (Nilsson *et al.*, 1992). Twelve independent kanamycin-resistant lines were multiplied *in vitro* by transferring shoot segments on to fresh half-strength Murashige and Skoog (MS) medium containing 1% agar every 4 weeks.

Expression of the transgene was confirmed by RT-PCR analysis as described below. Rooted plantlets were transferred to a mixture of peat and perlite (5 : 1) when they were approximately 10 cm in length. They were grown in a glasshouse under natural light conditions, supplemented with metal halogen lamps, with an 18-h light/6-h dark photoperiod at a temperature of 22 °C/15 °C (day/night). They were watered daily and fertilized once a week with a SuperbraS nutrient solution (Supra Hydro AB, Landskrona, Sweden).

Tissue isolation

Young leaves, unless otherwise indicated, were collected from internodes 5–10, counting from the apical bud. These leaves were rapidly expanding. Developing secondary xylem tissues were isolated from internodes 35–50 from the apical bud. These internodes had a well-developed secondary growth with a round wood core underneath the bark. The bark was peeled, and the exposed surfaces were scraped to isolate developing primary-walled xylem from the bark

side, denoted xylem 1, and developing secondary-walled xylem from the wood core side, denoted xylem 2 (Gray-Mitsumune *et al.*, 2004). The tissues were frozen in liquid nitrogen immediately after isolation, and were stored at –80 °C until use.

RNA extraction

Plant tissues were ground into a fine powder in liquid nitrogen. Two hundred milligrams of powdered tissues were suspended in 0.9 mL of the RNA extraction buffer described by Chang *et al.* (1993) [2% cetyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP), 100 mM Tris-HCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl, 0.5 g/L spermidine, 2% β -mercaptoethanol, pH 8.0] and incubated at room temperature for 5 min. The solution was mixed with an equal volume of chloroform–isoamylalcohol (24 : 1) and vortexed for complete suspension. The aqueous phase was separated from the organic phase by centrifugation at 17 000 *g* for 5 min. Nucleic acids in the aqueous phase were precipitated by adding 1.2 vol of isopropanol, followed by incubation at –20 °C for 30 min, and were collected by centrifugation at 17 000 *g* for 20 min at 4 °C. The precipitate was dissolved in RNeasy[®] RNA extraction buffer (Qiagen, Valencia, CA, USA), and RNA was purified using RNeasy[®] columns according to the manufacturer's recommendation.

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from the leaves of plants grown *in vitro*. The RT reactions contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM deoxynucleoside triphosphate (dNTP) mix, 0.01 μ g/ μ L random oligohexamers, 0.5 U/ μ L RNAGuard[™] (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 U/ μ L M-MLV reverse transcriptase (GibcoBRL, Gaithersburg, MD, USA) and 0.1 μ g/ μ L RNA. In a PCR tube, the RNA sample was mixed with an appropriate amount of random hexamers and RNase-free water to make a final volume of 12 μ L. The tube was incubated at 95 °C for 3 min for denaturation, and then cooled at 4 °C for 3 min in a thermal cycler with a heated lid (PTC100, MJ Research, Watertown, MA, USA). The other components of the RT reaction were added to the denatured RNA–primer mixture to make a final volume of 20 μ L. The tubes were incubated at 37 °C for 60 min, followed by 90 °C for 5 min. The cDNA was used immediately for PCR or stored at –20 °C.

To confirm the expression of the transgene, PCRs were performed using a forward primer specific for the *PttEXPA1* sequence (5'-AGGGTTCAAAGACTGGTTGGATGAG-3') and a reverse primer specific for the nopaline synthase (NOS) terminal sequence 5'-CTTTATTGCCAAATGTTTGAACGA-3'). The PCR mixture contained 1 μ L of cDNA sample, 0.4 μ M forward and reverse primers, 200 μ M dNTP, 1 \times Taq buffer (Roche Diagnostics, Basel, Switzerland) and 0.025 U/ μ L Taq DNA polymerase (Roche Diagnostics). PCR was performed according to the following thermal cycling regime: one cycle of 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; one cycle of 72 °C for 10 min. To examine the overall expression level of *PttEXPA1*, relative quantitative RT-PCRs were performed using forward and reverse primers specific for *PttEXPA1* (5'-AGGGT-TCAAAGACTGGTTGGATGAG-3' and 5'-TAGACAGCGGGCTAAC-CATCTTTG-3') and QuantumRNA[™] 18S Internal Standards (Ambion, Austin, TX, USA). The specificity of the primers for *PttEXPA1*

cDNA was verified by BLAST searches in the *Populus* genome (<http://genome.jgi-psf.org/>). PCR conditions were optimized according to the manufacturer's recommendations. PCR products were separated by 2% agarose gel electrophoresis, visualized by staining with ethidium bromide, and the intensities of staining were quantified using GelDoc software (Bio-Rad, Hercules, CA, USA).

Northern hybridization

Twenty micrograms of RNA were separated by electrophoresis using a formaldehyde–agarose gel and blotted onto a Hybond-N nylon membrane (AP Biotechnology, Uppsala, Sweden). Equal concentrations of RNA were verified by visualizing ribosomal bands with ethidium bromide. Gene-specific probes of *PttEXPA1* were synthesized using PCR fragments amplified from the 3' end of non-coding regions. The fragments were labelled with α -³²P-dATP using a Strip-EZ™ DNA labelling kit (Ambion). Hybridizations were performed in ULTRAHYB™ (Ambion) buffer at 42 °C overnight, followed by washing in 2 × standard saline citrate (SSC) twice, 0.5 × SSC twice and 0.1 × SSC twice. The temperature of the final wash was increased to 65 °C. Hybridization signals on the membrane were analysed using a GS-525 Molecular Imager® Storage Phosphor Imaging System (Bio-Rad).

Expansin activity

Cell wall proteins were extracted from young leaves and developing primary-walled xylem tissues of internodes 35–40 from the apex. Proteins were extracted in duplicate samples from each line and assayed for expansin activity using a protocol described by McQueen-Mason *et al.* (1992) with modifications (Gray-Mitsumune *et al.*, 2004). Briefly, frozen tissue was homogenized in a Wareing blender with 4 mL/g fresh weight of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 1% PVP (MW = 40 000), 1% Triton X100, 3 mM sodium metabisulphite, 2 mM EDTA and 2 mM DTT, adjusted to pH 6.8. Wall fragments were collected on a 50- μ m nylon mesh, washed three times in the same volume of homogenizing buffer without Triton X100, re-suspended in 2 mL/g original fresh weight of 1 M NaCl, 25 mM HEPES, 3 mM sodium metabisulphite, 2 mM EDTA, 2 mM DTT, pH 6.8, and left to extract for 1 h at room temperature. The salt extract was filtered through the nylon mesh, and proteins were precipitated by the gradual addition of 0.39 g/mL of solid ammonium sulphate. After 10 min on ice, the protein precipitates were recovered by centrifugation at 10 000 *g* for 10 min at 4 °C, and the pellets were stored at –20 °C until assay for expansin activity. For expansin assays, precipitates were re-suspended in 1 mL of the assay buffer (50 mM sodium acetate, pH 4.5), desalted through a 5-mL column of Sephadex G25 (Amersham Bioscience, Uppsala, Sweden) in the same solution, and brought to a final volume of 1 mL/g original fresh weight. Expansin assays were carried out in an extensometer using a cellulose–xyloglucan composite (Whitney *et al.*, 2000). Two-millimetre-wide strips of composite were placed between the clamps of the extensometer, bathed in the assay buffer and extended by the application of a 5 g weight to the lower clamp. After 15 min of extension, the bathing solution was replaced with 100 μ L of the solution of desalted cell wall proteins. The extension rates were measured for 10 min prior to and 10 min after protein addition. The expansin activity was calculated as the rate of extension after protein addition minus the rate prior to protein addition.

Plant growth analysis

To obtain the length of fully elongated internodes, ten consecutive internodes located from approximately 50–90 cm above the ground level on each of the ten plants per line were measured.

The sizes of the leaves at different developmental stages were compared in 3-month-old plants. The widths and lengths of leaves were measured every fifth leaf from the apex. To compare the fully expanded leaves, the width measurements of leaves 20–45 were collected.

The epidermal cell areas of fully expanded leaves were measured. To obtain imprints of the leaf surfaces, nail polish was applied to the leaf upper surface and the dried nail polish was carefully removed using a clear adhesive tape. The adhesive tape with the leaf imprint was then placed on a glass slide for observation under a microscope. Images of leaf imprints were captured using an AxioVision camera (München-Hallbergmoos, Germany), and the areas of epidermal cells were measured using AxioVision software.

Effects on the radial width of the wood and bark were analysed by sectioning internode 41 from the top of the stem of each plant and measuring the radial width under a dissecting microscope.

To obtain isolated wood cells, wood of internode 40 was macerated in glacial acetic acid–30% H₂O₂ (1 : 2) at 65 °C for 5 days (Berlyn and Miksche, 1976), and cells were observed using the Nomarski optics of a Zeiss (Göttingen, Germany) AxioPlan2 microscope. Images of the cells were captured by an AxioVision camera and measured using AxioVision software.

Statistical analysis

One-way ANOVA was performed using SigmaStat 2.03 (Jandel Corporation, San Rafael, CA, USA). Differences between sample groups and the control group were further tested using the simultaneous 95% confidence limit (Dunnett's test).

Acknowledgements

We thank Dr Alicja Banasiak for her advice on phyllotaxy and Kjell Olofsson for technical assistance. This work was supported by grants from Formas, the Swedish Research Council, the Wallenberg Foundation, European project Eden QLK5-CT-2001-00443, the Wood Ultrastructure Research Centre and the UPSC Excellence Centre.

References

- Baluška, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Šamaj, J., Chua, N.H., Barlow, P.W. and Volkmann, D. (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol.* **227**, 618–632.
- Belfield, E.J., Ruperti, B., Roberts, J.A. and McQueen-Mason, S. (2005) Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in *Sambucus nigra*. *J. Exp. Bot.* **56**, 817–823.
- Berlyn, G.P. and Miksche, J.P. (1976) *Botanical Microtechnique and Cytochemistry*. Ames, IA: Iowa State University Press.

- Bourquin, V., Nishikubo, N., Abe, H., Brumer, H., Denman, S., Eklund, M., Christiernin, M., Teeri, T.T., Sundberg, B. and Mellerowicz, E.J. (2002) Xyloglucan endotransglycosylases have a function during the formation of secondary cell walls of vascular tissues. *Plant Cell*, **14**, 3073–3088.
- Brummell, D.A., Harpster, M.H., Civello, P.M., Palys, J.M., Bennett, A.B. and Dunsmuir, P. (1999) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. *Plant Cell*, **11**, 2203–2216.
- Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Reporter*, **11**, 113–116.
- Chen, C., Meyermans, H., Burggraave, B., De Rycke, R.M., Inoue, K., De Vleeschauwer, V., Steenackers, M., Van Montagu, M., Engler, G.J. and Boerjan, W.A. (2000) Cell-specific and conditional expression of caffeoyl-coenzyme A-3-O-methyltransferase in poplar. *Plant Physiol.* **123**, 853–867.
- Cho, H.T. and Cosgrove, D.J. (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **97**, 9783–9788.
- Cho, H.T. and Cosgrove, D.J. (2002) Regulation of root hair initiation and expansin gene expression in *Arabidopsis*. *Plant Cell*, **14**, 3237–3253.
- Choi, D., Cho, H.T. and Lee, Y. (2006) Expansins: expanding importance in plant growth and development. *Physiol. Plant.* **126**, 511–518.
- Choi, D.S., Lee, Y., Cho, H.T. and Kende, H. (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. *Plant Cell*, **5**, 1386–1398.
- Cosgrove, D.J. (2000) Loosening of plant cell walls by expansins. *Nature*, **407**, 321–326.
- Cosgrove, D.J., Bedinger, P. and Durachko, D.M. (1997) Group I allergens of grass pollen as cell wall-loosening agents. *Proc. Natl. Acad. Sci. USA*, **94**, 6559–6564.
- Fleming, A.J., McQueen-Mason, S., Mandel, T. and Kuhlemeier, C. (1997) Induction of leaf primordia by the cell wall protein expansion. *Science*, **276**, 1415–1418.
- Geisler-Lee, J., Geisler, M., Coutinho, P.M., Segerman, B., Nishikubo, N., Takahashi, J., Aspeborg, H., Djerbi, S., Master, E., Andersson-Gunnerås, S., Sundberg, B., Karpinski, S., Teeri, T.T., Kleczkowski, L.A., Henrissat, B. and Mellerowicz, E.J. (2006) Poplar carbohydrate-active enzymes. Gene identification and expression analyses. *Plant Physiol.* **140**, 946–962.
- Gray-Mitsumune, M., Mellerowicz, E.J., Abe, H., McQueen-Mason, S., Winzell, A., Sterky, F., Blomqvist, K., Schrader, J., Teeri, T.T. and Sundberg, B. (2004) Expansins abundant in secondary xylem belong to Subgroup A of the α -expansin gene family. *Plant Physiol.* **135**, 1552–1564.
- Hawkins, S., Leplé, J.C., Cornu, D., Jouanin, L. and Pilate, G. (2003) Stability of transgene expression in poplar: a model forest tree species. *Ann. For. Sci.* **60**, 427–438.
- Im, K.H., Cosgrove, D.J. and Jones, A.M. (2000) Subcellular localization of expansin mRNA in xylem cells. *Plant Physiol.* **123**, 463–470.
- Kwasniewski, M. and Szarejko, I. (2006) Molecular cloning and characterization of beta-expansin gene related to root hair formation in barley. *Plant Physiol.* **141**, 1149–1158.
- Langer, K., Ache, P., Geiger, D., Stinzing, A., Arend, M., Wind, C., Regan, S., Fromm, J. and Hedrich, R. (2002) Poplar potassium transporters capable of controlling K⁺ homeostasis and K⁺-dependent xylogenesis. *Plant J.* **32**, 997–1009.
- Larson, P.R. (1994) *The Vascular Cambium*. Berlin: Springer Verlag.
- Lee, D.K., Ahn, J.H., Song, S.K., Choi, Y.D. and Lee, J.S. (2003) Expression of an expansin gene is correlated with root elongation in soybean. *Plant Physiol.* **131**, 985–997.
- Lee, Y. and Kende, H. (2002) Expression of alpha-expansin and expansin-like genes in deepwater rice. *Plant Physiol.* **130**, 1396–1405.
- Li, L.C., Bedinger, P.A., Volk, C., Jones, A.D. and Cosgrove, D.J. (2003) Purification and characterization of four β -expansins (Zea m 1 isoforms) from maize pollen. *Plant Physiol.* **132**, 2073–2085.
- Link, B.M. and Cosgrove, D.J. (1998) Acid-growth response and alpha-expansins in suspension cultures of bright yellow 2 tobacco. *Plant Physiol.* **118**, 907–916.
- McQueen-Mason, S.J. and Cosgrove, D.J. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc. Natl. Acad. Sci. USA*, **91**, 6574–6578.
- McQueen-Mason, S.J. and Cosgrove, D.J. (1995) Expansin mode of action on cell-walls – analysis of wall hydrolysis, stress-relaxation, and binding. *Plant Physiol.* **107**, 87–100.
- McQueen-Mason, S.J., Durachko, D.M. and Cosgrove, D.J. (1992) Two endogenous proteins that induce cell wall extension in plants. *Plant Cell*, **4**, 1425–1433.
- Mellerowicz, E.J. (2006) Xylem cell expansion – lessons from poplar. In: *The Science and Lore of the Plant Cell Wall*. (Hayashi, T., ed.), pp. 267–275. Boca Raton, FL: Universal Publishers BrownWalker Press.
- Mellerowicz, E.J., Baucher, M., Sundberg, B. and Boerjan, W. (2001) Unravelling cell wall formation in the woody dicot stem. *Plant Mol. Biol.* **47**, 239–274.
- Nilsson, O., Aldén, T., Sitbon, F., Little, C.H.A., Chalupa, V., Sandberg, G. and Olsson, O. (1992) Spatial pattern of cauliflower mosaic virus 35S promoter luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.* **1**, 209–220.
- Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C. and Fleming, A. (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proc. Natl. Acad. Sci. USA*, **98**, 11 812–11 817.
- Reinhardt, D., Wittwer, F., Mandel, T. and Kuhlemeier, C. (1998) Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. *Plant Cell*, **10**, 1427–1437.
- Rose, J.K.C. and Bennett, A.B. (1999) Cooperative disassembly of the cellulose–xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. *Trends Plant Sci.* **4**, 176–183.
- Sampedro, J. and Cosgrove, D.J. (2005) The expansin superfamily. *Genome Biol.* **6**, Art. No. 242.
- Sampedro, J., Carey, R.E. and Cosgrove, D.J. (2006) Genome histories clarify evolution of the expansin superfamily: new insights from the poplar genome and pine ESTs. *J. Plant Res.* **119**, 11–21.
- Tyree, M.T. and Zimmermann, M.H. (2002) *Xylem Structure and the Ascent of Sap*, 2nd edn. Berlin: Springer Verlag.
- Walden, R., Koncz, C. and Schell, J. (1990) The use of gene vectors in plant molecular biology. *Methods Mol. Cell Biol.* **1**, 175–194.
- Whitney, S.E.C., Gidley, M.J. and McQueen-Mason, S.J. (2000) Probing expansin action using cellulose/hemicellulose composite. *Plant J.* **22**, 327–334.
- Wu, Y.J., Meeley, R.B. and Cosgrove, D.J. (2001) Analysis and

- expression of the α -expansin and β -expansin gene families in maize. *Plant Physiol.* **126**, 222–232.
- Yennawar, N.H., Li, L.C., Dudzinski, D.M., Tabuchi, A. and Cosgrove, D.J. (2006) Crystal structure and activities of EXPB1 (*Zea m 1*), a β -expansin and group-1 pollen allergen from maize. *Proc. Natl. Acad. Sci. USA*, **103**, 14 664–14 671.
- Zenoni, S., Reale, L., Torielli, G.B., Lanfaloni, L., Porceddu, A., Ferrarini, A., Moretti, C., Zamboni, A., Speghini, S., Ferranti, F. and Pezzotti, M. (2004) Downregulation of the *Petunia hybrida* α -expansin gene *PhEXP1* reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. *Plant Cell*, **16**, 295–308.