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A fasciclin-domain containing gene, *ZeFLA11*, is expressed exclusively in xylem elements that have reticulate wall thickenings in the stem vascular system of *Zinnia elegans* cv Envy

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Abstract The vascular cylinder of the mature stem of *Zinnia elegans* cv Envy contains two anatomically distinct sets of vascular bundles, stem bundles and leaf-trace bundles. We isolated a full-length cDNA of *ZeFLA11*, a fasciclin-domain-containing gene, from a zinnia cDNA library derived from in vitro cultures of mesophyll cells induced to form tracheary elements. Using RNA in situ hybridization, we show that *ZeFLA11* is expressed in the differentiating xylem vessels with reticulate type wall thickenings and adjacent parenchyma cells of zinnia stem bundles, but not in the leaf-trace bundles that deposit spiral thickenings. Our results suggest a function for this cell-surface GPI-anchored glycoprotein in secondary wall deposition during differentiation of metaxylem tissue with reticulate vessels.

Keywords Arabinogalactan protein · Fasciclin domain · In situ hybridization · Secondary wall · Vascular bundles · *Zinnia elegans*

Abbreviations AGP: Arabinogalactan protein · FLA: Fasciclin-like arabinogalactan protein · GPI: Glycosylphosphatidylinositol · TE: Tracheary element

Introduction

The newly formed vascular cylinder in the subapical zone of the stem of *Zinnia elegans* consists of anatomically identical juvenile bundles. However, as the plant

matures, two types of vascular bundles, the leaf-trace bundles and the stem bundles, acquire distinct morphological features (Dahiya et al. 2005). Previously, we showed that a RING-domain-containing gene was expressed in xylem of leaf-trace bundles and in phloem of stem bundles, correlated with transport activity in transfer cells and in the companion cells of sieve elements (Dahiya et al. 2005). In this paper, we describe a second molecular marker that is expressed exclusively in xylem of stem bundles.

The tracheary elements (TEs) of xylem have secondarily-thickened lignified walls with a variety of pits and are nonliving at maturity. The secondary wall thickenings of TEs are highly characteristic and appear in an orderly ontogenetic series of forms. During xylem development, protoxylem formed in younger parts of the primary plant body contains annular or spiral vessels embedded in parenchyma. As tissues elongate, the shoot protoxylem undergoes severe stretching and is eventually destroyed. Metaxylem is initiated in the still-growing primary plant body, but matures largely after stem elongation is completed. Metaxylem may have spiral, scalariform, reticulate, or pitted elements. In plants lacking secondary growth, the metaxylem remains functional in the mature plant (Esau 1962).

Here, we report that the expression of a zinnia gene, *ZeFLA11*, is confined only to reticulate xylem vessels and adjacent parenchyma cells with secondary wall thickenings. The *ZeFLA11* gene fragment was isolated during a cDNA-AFLP analysis (Miloni et al. 2002) of the time-course of differentiation to TEs in an in vitro system. In the zinnia mesophyll cell system, between 60 and 80% of freshly-isolated leaf mesophyll cells trans-differentiate into TEs (Fukuda and Komamine 1980; Miloni et al. 2002). In vitro TEs exhibit all of the different patterns of wall thickening (Pesquet et al. 2003). Roberts and Haigler (1994) observed that the proportion of reticulate-patterned metaxylem-like TEs in the culture could be increased by prolonging a phase of cell expansion before differentiation. We obtained a full-length cDNA for *ZeFLA11* using a cDNA library derived from the in vitro

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system. The *ZeFLA11* gene is a member of a subclass of glycosylphosphatidylinositol (GPI)-anchored arabinogalactan proteins (AGPs), the fasciclin-like AGPs (FLAs; Johnson et al. 2003). Proteins containing fasciclin domains have been shown to function as adhesion molecules in the animal extracellular matrix (Huber and Sumper 1994; Kawamoto et al. 1998; Kim et al. 2000). In *Arabidopsis*, the salt overly sensitive five mutant, mutated in an AGP with two fasciclin domains, has thinner cell walls and abnormal cell swelling at the root tip (Shi et al. 2003). Based on the expression pattern, we propose a role for *ZeFLA11* in deposition of wall thickenings in reticulate vessels of metaxylem.

Materials and methods

Plant material

Seeds of *Zinnia elegans* cv Envy were obtained from Stokes Seeds, Chiltern, UK. Plants were grown in short day conditions with 60% humidity at 26°C. Plants with 2–3 pairs of fully expanded leaves were used for in situ hybridization and anatomical studies. Node 1 is the first node with fully expanded leaves, when counted from the stem apical meristem. The young zone is defined as the stem above node 1, and the bundles in this zone are referred to as juvenile bundles. The mature zone is between node 1 and the hypocotyl, containing stem and leaf-trace bundles together with recently branched small bundles (Dahiya et al. 2005). Mesophyll cells were isolated from zinnia leaves and induced to differentiate to tracheary elements in vitro as described previously (Domingo et al. 1998).

Amplification of cDNA fragments by 3'- and 5'-RACE PCR

Total RNA and poly(A⁺) RNA was prepared as described by Milioni et al. (2002). For 3' and 5'-RACE (rapid amplification of cDNA ends), adapter-ligated double-stranded cDNA was synthesized using a Clontec MarathonTM cDNA amplification kit (Clontec, Palo Alto, USA) following the manufacturer's instructions. RACE reactions were performed as per manufacturer's instructions. Amplified cDNAs were subcloned using TOPO TA cloning kit for sequencing (Invitrogen, Groningen, The Netherlands) and sequenced using ABI PRISM dye terminator cycle sequencing-ready reaction kit with fluorescent sequencing AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut).

Sequence analysis

Database searches were performed using GCG version 10 software using the Basic Local Alignment Search Tool (BLAST) network services (Altschul et al. 1997). Phy-

logeny and ClustalW alignment (Higgins et al. 1994) were generated using software at the European Bioinformatics Institute web site (<http://www.ebi.ac.uk>). Fasciclin, H1, and H2 domain and adhesion sites were determined based on similarity with FLA11 (Johnson et al. 2003) and repetitive AP sites were identified as described in Schultz et al. (2002). N-terminal signal peptide and cleavage sites were predicted using SignalP 3.0 server (Bendtsen et al. 2004), <http://www.cbs.dtu.dk/services/SignalP>. The transmembrane domain was identified using TMPRED (Hofmann and Stoffel 1993), <http://www.ch.embnet.org/cgi-bin/TMPRED>, and PHDhtm (Rost et al. 1996), <http://www.cubic.bioc.columbia.edu>. The C-terminal GPI-anchor and cleavage site were predicted using big-PI predictor version 1.5 (Eisenhaber et al. 2003); http://www.mendel.imp.univie.ac.at/gpi/plant_server.html. N-glycosylation sites were identified using PROSITE version 99.07 (Bairoch et al. 1997), <http://www.expasy.ch/prosite>.

Accession numbers for the *Arabidopsis* FLA genes are: *FLA6*, At2g20520; *FLA7*, At2g04780; *FLA9*, At1g03870; *FLA11*, At5g03170; *FLA12* At5g60490; *FLA13*, At5g44130. The accession number for the poplar *FLA11* gene (*PopFLA11*) is AY607763, and for the zinnia *FLA11* gene (*ZeFLA11*) is AJ972920.

RT-PCR

Total RNA was extracted from zinnia cells as described by Milioni et al. (2002). Endogenous DNA was removed by adding 0.2 µl DNaseI/µl (Pharmacia, Uppsala, Sweden) at 37°C for 10 min. RT-PCR was then performed as described by Wisniewski et al. (1999). One hundred mM dNTPs were added to the reaction. For studying the differential expression of *ZeFLA11*, primer 5'-tcctacatacctaacaacctc-3' and reverse primer 5'-cgcttccatcgtcttttgc-3', derived from the 3' end of *ZeFLA11* cDNA, were used. Amplification was performed at 94°C 30 s, 60°C 30 s, and 72°C 30 s. Aliquots (4 µl) were taken after every third cycle from the seventeenth to thirty-second cycle, loaded on a 1.2% (wt/vol) agarose gel (Sigma, Poole, UK) and separated by electrophoresis at 80 V in TBE buffer for 1 h. The gel was stained with ethidium bromide (Sigma, Poole, UK) and imaged using Molecular Analyst Software (BioRad, USA).

The 26S rRNA fragment, isolated by cDNA-AFLP, was used as a control for equal loading of total RNA. Primers for the 26S rRNA (5'-aaaggattctaccagtcgcttgatggga-3' and 5'-acgctctaagtcagaatccgggctaga-3') were mixed with the above reaction.

Tissue fixation and embedding for in situ hybridization

Zinnia plants, with 2–3 pairs of open leaves, were used for fixation and embedding as described by Dahiya et al.

(2005). Tissue fixation was performed as described by Wisniewski et al. (1999).

Ribo-probe synthesis and in situ hybridization

Gene-specific, digoxigenin-labeled riboprobes were generated from a 578-bp fragment derived from the 3' end of the *ZeFLA11* cDNA sequence. T3 and T7 sites of the TOPO sequencing vector were used to make sense and antisense riboprobes. The plasmid was linearized using the *SpeI* and *NotI* sites, for antisense and sense probes, respectively. Linearized plasmid was purified by phenol–chloroform extraction and quantified using a BioRad spectrometer. Ribo-probe synthesis and in situ hybridization was performed as described by Wisniewski et al. (1999). Photographs were taken on a Nikon Microphot SA microscope using Nomarski (DIC) optics.

Field-emission scanning electron microscopy

The zinnia stems were mounted vertically in a rivet on the aluminum SEM specimen holder using O.C.T. compound (BDH Laboratory Supplies, Poole, England). The sample holder was then immediately plunged into liquid nitrogen slush at approximately -210°C to cryopreserve the material. The sample was transferred onto the cryostage of a CT1500HF cryo-transfer system (Gatan, Oxford, England) attached to a Philips XL30 FEG scanning electron microscope (FEI UK Ltd, Cambridge, England). After the sample and stage had equilibrated to -100°C , the stems were fractured then immediately cooled to below -110°C before sputter-coating with platinum for 2 min at 10 mA. After sputter-coating, the sample was moved onto the cryo-stage in the main chamber of the microscope, held at approximately -140°C . The sample was viewed at 3 kV and digital TIFF files were stored.

Vascular strand arrangement

Serial hand sections of the zinnia plant were used to determine the vascular strand arrangement.

Results

ZeFLA11 contains one fasciclin domain and two AGP-like domains

Using complementary DNA-amplified fragment length polymorphisms, Milioni et al. (2002) isolated over 600 transcript-derived fragments that showed differential expression during TE formation in vitro. One of these was an 87 bp-long transcript-derived fragment with

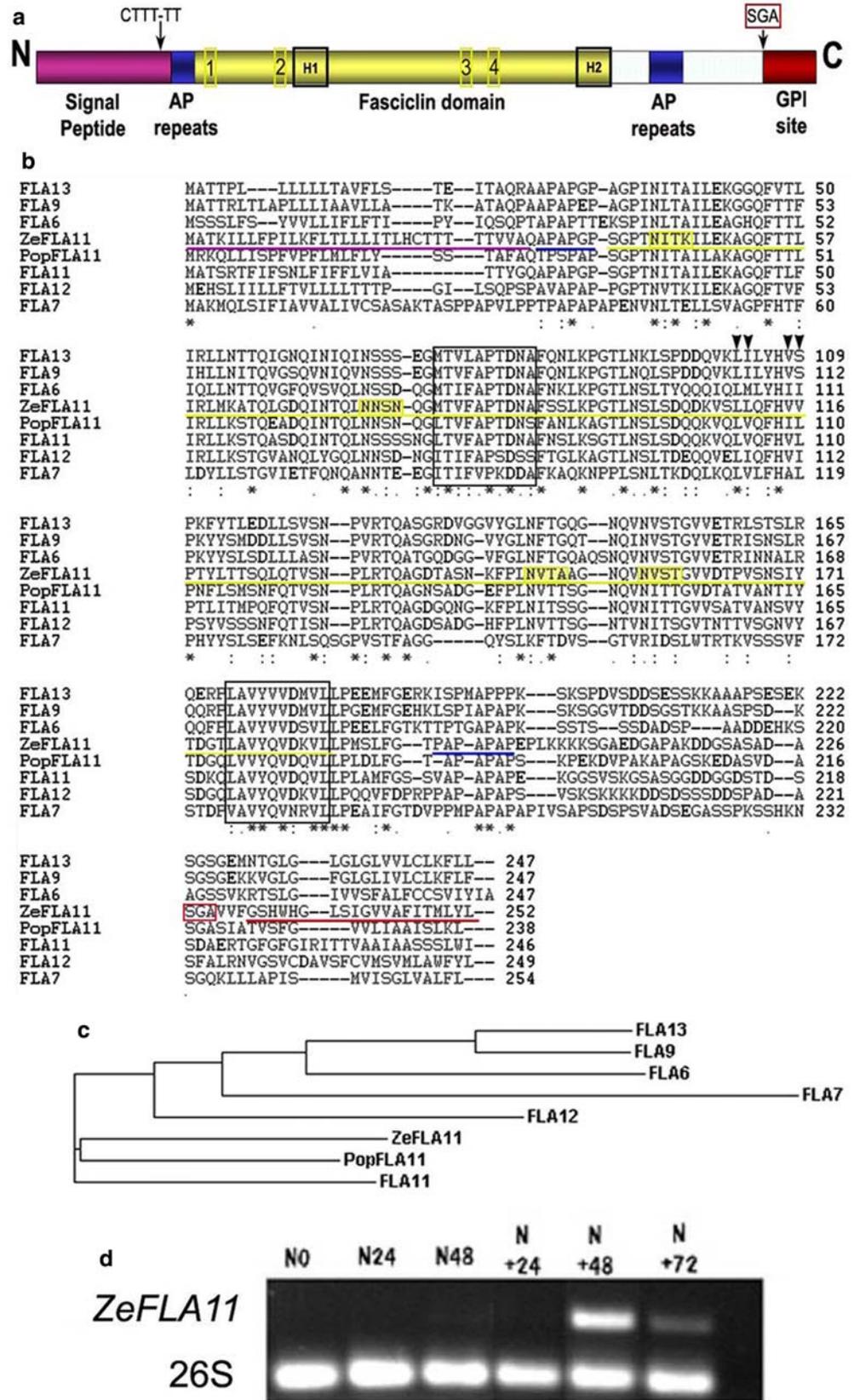
sequence similarity to the *Arabidopsis* gene *FLA11* (Fasciclin-Like Arabinogalactan-protein; *At5g03170*). The corresponding full-length zinnia cDNA was obtained by RACE-PCR, using an adapter-ligated cDNA library as the base template, which was prepared from zinnia cells differentiating in vitro. We obtained a 976 bp-long sequence (Accession Number AJ972920) with an open reading frame of 756 bp. The translated peptide showed 61.4% pairwise sequence identity and 67.6% sequence similarity at the amino acid level to *AtFLA11*. We, therefore, named the zinnia gene *ZeFLA11* (*Zinnia elegans* homologue of FLA11). The *ZeFLA11* peptide also shares 63.1% pairwise sequence identity and 67.4% sequence similarity with the translated sequence of a poplar gene *PopFLA11* (Lafarguette et al. 2004). Johnson et al. (2003) classified *Arabidopsis* FLA proteins into four groups (A–D), based on phylogenetic analysis and sequence characteristics. FLA11 belongs to group 'A', together with five other FLAs (FLA6, FLA7, FLA9, FLA12, and FLA13). The alignment of *ZeFLA11* sequence with group 'A' FLAs of *Arabidopsis* and the poplar homologue, and their phylogenetic relationship, is shown in Fig. 1b and c, respectively.

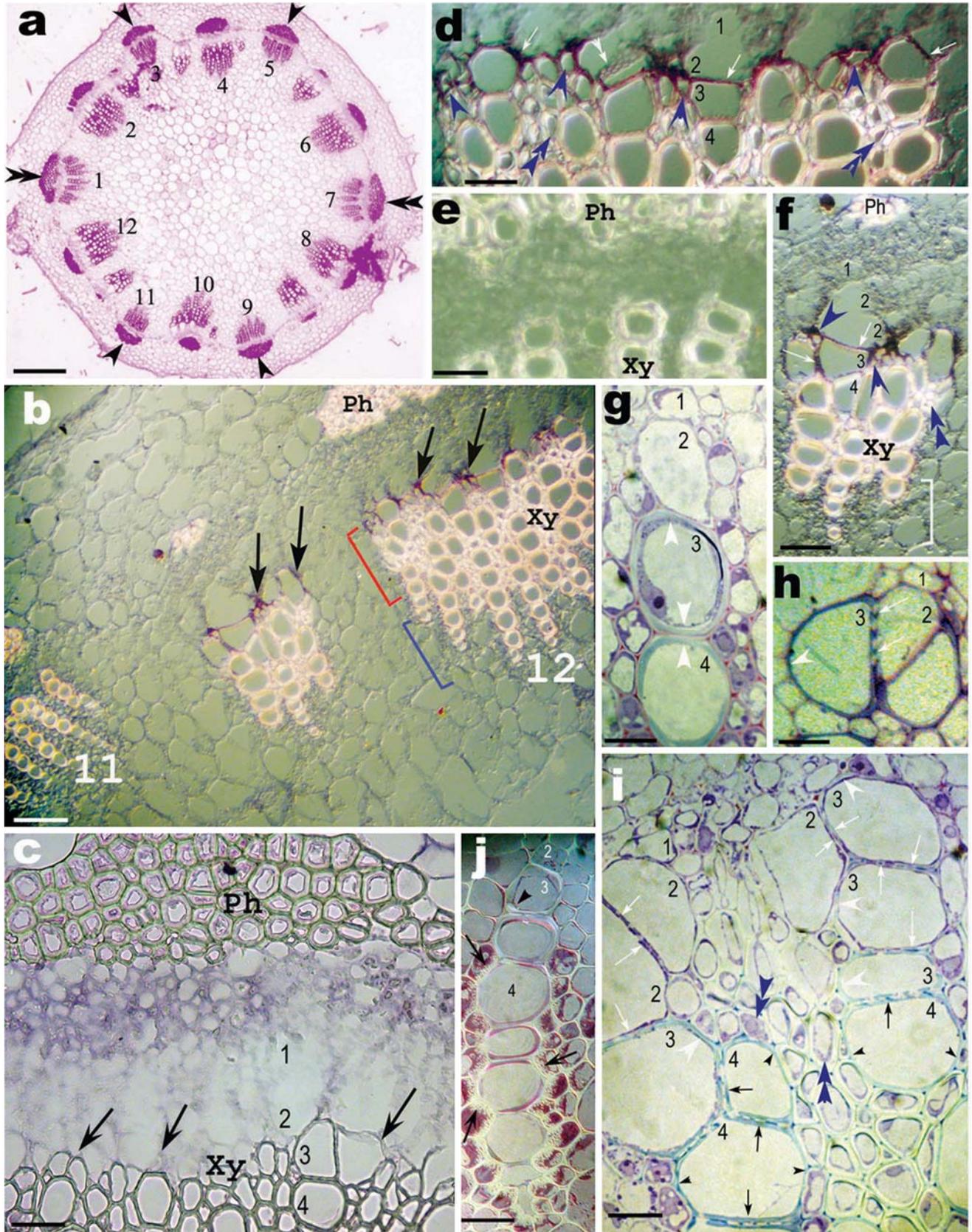
Figure 1a shows a schematic representation of the domain structure and key features of the *ZeFLA11* peptide. A fasciclin domain, which contains highly conserved H1 and H2 motifs (Kawamoto et al. 1998), accounts for most of the open reading frame. Putative adhesion sites (Kim et al. 2000) are marked by arrowheads in Fig. 1b. Fasciclin domains of FLAs are also predicted to have 2–8 *N*-glycosylation sites (Johnson et al. 2003). We identified four potential *N*-glycosylation sites, NITK, NNSN, NVTA, and NVST, in the fasciclin domain of the *ZeFLA11* peptide (Fig. 1a, b). The fasciclin domain is flanked by short stretches of AP repeats, the core structure of the arabinogalactan glycomodules that are characteristic of AGPs (Schultz et al. 2002). Sequence analysis of the *ZeFLA11* protein predicted a cleavable signal peptide and a C-terminal site for the GPI anchor (Fig. 1a, b).

Expression pattern of *ZeFLA11* during TE formation in the zinnia model system

Analysis of complementary DNA-amplified fragment length polymorphisms indicated that the transcript-derived fragment of *ZeFLA11* was expressed 48 h after addition of auxin and cytokinin to the culture medium in the zinnia system. RT-PCR analysis of the expression pattern of *ZeFLA11* in the zinnia system confirms this expression pattern. As shown in Fig. 1d, transcript only appears at 48 h, at the time when most differentiating cells are depositing their secondary walls, before declining at 72 h. Most of the cells that trans-differentiate to TEs in the zinnia system have autolyzed their cell contents by 72 h.

Fig. 1 a Schematic diagram showing features of the translated sequence of *ZeFLA11*. A fasciclin domain (yellow) accounts for most of the open reading frame and contains two highly conserved domains, H1 and H2, (black rectangles), and four potential *N*-glycosylation sites, (1) NITK, (2) NNSN, (3) NVTA, and (4) NVST (yellow rectangles). Two small AGP domains (AP repeats) (blue) flank the fasciclin domain. A cleavable signal peptide is predicted to be present at the N- (magenta) terminus, marked by an arrow. At the C-terminus, a GPI anchor site at SGA (arrow), is predicted to anchor the protein to the plasma membrane. **b** Alignment of the *ZeFLA11* peptide sequence with putative *Arabidopsis* and poplar homologues. The *ZeFLA11* sequence is marked with underlines and rectangles for characteristic domains, following the same color scheme as in **a**. Black rectangles mark the highly conserved H1 and H2 domains of all of the aligned proteins. Arrowheads show potential adhesion sites. "Asterisk" indicates identical residues in all alignments, "colon" indicates conserved substitution, "dot" indicates semi-conserved substitution. **c** Phylogenetic tree of *ZeFLA11* with putative *Arabidopsis* and poplar homologues. **d** Reverse transcription PCR showing the expression pattern of the *ZeFLA11* gene at different stages of TE differentiation in vitro: N0, immediately after isolation of mesophyll cells; N24 and N48, 24 and 48 h after isolation; +24, +48, +72 h after induction by addition of auxin and cytokinin at 48 h (N48). The 26S ribosomal subunit is amplified as a positive loading control





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Fig. 2 a Transverse section from the mature zone of the zinnia stem, showing the arrangement of leaf-trace and stem bundles. Numbered bundles represent the 12 vascular bundles in the zinnia stem (Dahiya et al. 2005). Recently, branched bundles are *not numbered* and can be recognized by comparatively smaller areas of phloem. Bundles 1 and 7 (*double arrowheads*) are leaf midrib bundles supplying opposite leaves at the same node. *Arrowheads* indicate each pair of siderib bundles (3 and 11, 5 and 9, respectively) that flank the midribs. The colors in this picture have been enhanced using Adobe Photoshop software to show morphological details. *Bar* = 330 μm . **b** In situ hybridization of *ZeFLA11* antisense riboprobe showing stem bundle 12 and a young bundle in the interfascicular region (*unnumbered*) with abundant *ZeFLA11* transcript in two to three cell layers, at the peripheral end of the xylem (*arrows*). Leaf-trace bundle 11 does not contain the transcript. *Blue bracket* marks protoxylem, *red bracket* marks metaxylem. *Bar* = 66 μm . **c–j** Cells numbered 1–4 show different developmental stages of xylem vessels, described in **g**. **c** In situ hybridization of *ZeFLA11* sense riboprobe with an equivalent serial section as shown in **b**, showing nonspecific background labeling in stem bundle 12. *Arrows* mark differentiating xylem cells, as in **B**, that are not labeled with the sense riboprobe. *Bar* = 33 μm . **d–f** In situ hybridization of transverse sections of stem with *ZeFLA11* antisense riboprobe. **d** Higher magnification of stem bundle 12. Vessels at stage 2 and 3 of differentiation contain abundant transcript (*white arrows*). Neighboring parenchyma cells also express *ZeFLA11* (*blue arrowheads*). *Double arrowheads* mark older parenchyma cells without transcript. *Double white arrowheads* indicate the patterned secondary wall of vessels. *Bar* = 33 μm . **e** Higher magnification of leaf-trace bundle 3 showing absence of *ZeFLA11* transcript. *Bar* = 33 μm . **f** Higher magnification of a

young bundle, formed in the interfascicular region. *White arrows* indicate xylem vessels, at stages 2 and 3, with abundant transcript. *Arrowheads* and *double arrowheads*, as in **d**. *White bracket* marks TEs with spiral wall thickenings at the internal end of the xylem. The remainder of the TEs have reticulate thickenings. *Bar* = 33 μm . **g–j** Anatomy of the TEs. **g** Light micrograph showing four stages of xylem cell development in spiral-patterned vessels in juvenile bundles: (1) small cell, with thin primary wall, recently differentiated from the cambium, (2) enlarged cell, with thin primary wall, (3) enlarged cell, with recently developed thick secondary wall (*arrowhead*) and cytoplasmic contents, and (4) completely differentiated, dead xylem cells with thick secondary walls (*arrowhead*) and without cytoplasm. *Bar* = 14 μm . **h** Light micrograph of developing reticulate vessels in the mature zone of the stem. The vessel at stage 2 is a younger cell with thin primary walls, except for the shared wall with the neighboring vessel at stage 3 (*white arrows*), which is being secondarily thickened with characteristic wall pits. The vessel at stage 3 has deposited secondary wall on all faces and has flat secondary wall on sides adjoining parenchyma cells (*arrowhead*). *Bar* = 22 μm . **i** Light micrograph showing reticulate-patterned vessels in similar developmental stages as described in **g**. *White arrows* mark shared secondary wall with wall pits between neighboring vessels at stage 2 and 3 of differentiation. *Black arrows* mark shared pitted secondary wall between two fully differentiated vessels. *Black* and *white arrowheads* mark secondary walls without pits. *Double arrowheads* indicate secondarily-thickened parenchyma cells with cytoplasm. *Bar* = 14.4 μm . **j** Light micrograph of developing spiral xylem in the leaf-trace bundle, as in **g**. A thin layer of cytoplasm is visible in the cell at stage 3 (*arrowhead*). Fully differentiated cells at stage 4 are surrounded by transfer cells (*arrows*). *Bar* = 22 μm . *Ph*, phloem, *Xy*, xylem

ZeFLA11 is selectively expressed in the differentiating xylem cells of the stem bundle

The mature zone of the zinnia stem (between node1 and hypocotyl) is ideal for anatomical and molecular analyses of xylem as both functional leaf-trace and stem bundles, which contain protoxylem and metaxylem elements, respectively, are represented in transverse sections (Dahiya et al. 2005).

Figure 2a shows a transverse section of zinnia stem from the internode of the mature zone. The numbered bundles represent the 12 vascular bundles in the zinnia stem (Dahiya et al. 2005). Recently, branched small bundles are not numbered and can be recognized by comparatively smaller areas of phloem. Bundles 1 and 7 (*double arrowheads*) are leaf midrib bundles supplying opposite leaves at the same node. *Arrows* indicate each pair of siderib bundles (3 and 11, 5 and 9, respectively) that flank the midribs. The remainder of the numbered bundles are stem bundles.

In situ hybridization of the *ZeFLA11* antisense riboprobe with transverse sections of mature stem shows that the transcript is expressed only in the xylem of stem bundles. For example, bundle 12 with labeled xylem cells is a stem bundle and leaf-trace bundle 11 does not accumulate *ZeFLA11* transcript (Fig. 2b). Higher magnification of stem bundle 12 (Fig. 2d) shows that the *ZeFLA11* transcript is localized to the peripheral two cell layers of xylem vessels and parenchyma cells. Cells marked by numbers 1–4 are TEs at different stages of differentiation: (1) differentiation from cambium; (2) cell enlargement; (3) deposition of secondary wall thicken-

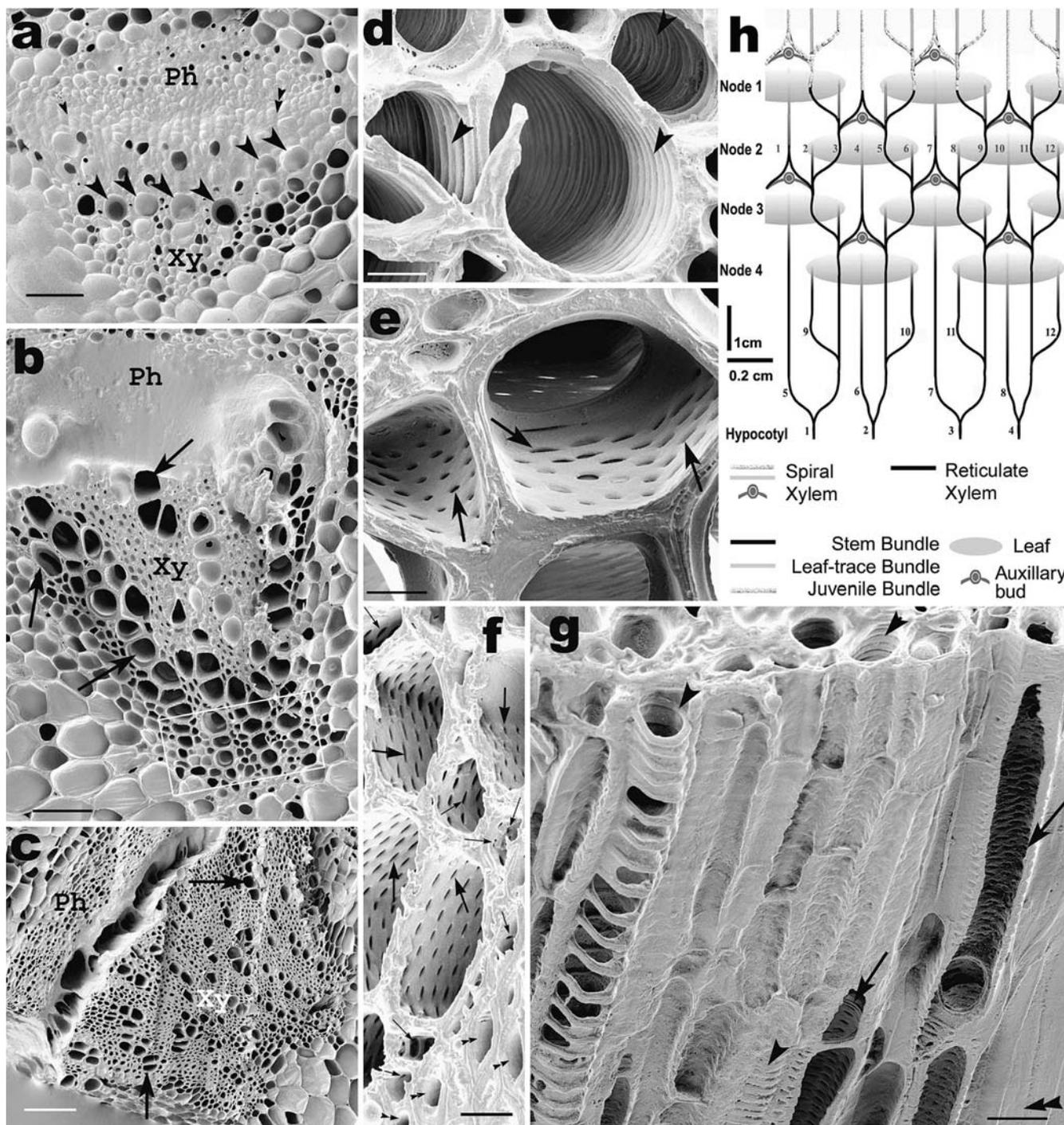
ing, and (4) cell death as a terminal differentiation event. Of these, cells at stages 2 and 3, and their neighboring small parenchyma cells, contain abundant transcript. Cells at stage 1 and cells surrounding vascular bundles (Fig. 2b, d, f) show background staining, which is also present in the control experiment with sense riboprobe (Fig. 2c). The red bracket in Fig. 2b indicates newly formed metaxylem at the peripheral end of the xylem, whereas the blue bracket indicates protoxylem at the internal end of the stem bundle. Leaf-trace bundles contain only protoxylem and do not show expression of *ZeFLA11* gene, as shown for leaf-trace bundles 3 (Fig. 2e) and 11 (Fig. 2b).

Figure 2b and f show recently branched small bundle, positioned between bundles 11 and 12. Like stem bundles, xylem cells at stages 2 and 3 of these young bundles also contain *ZeFLA11* transcript (Fig. 2f).

Anatomy of TEs in the stem and the leaf-trace bundles

The expression of the *ZeFLA11* gene is confined to two cell layers of the differentiating xylem cells of the stem bundles, and small bundles that have recently branched from existing bundles, and transcript is absent from the leaf-trace bundle. To explain this expression pattern, we have imaged xylem elements by light and scanning electron microscopes.

Figure 2g shows protoxylem vessels represented in the leaf-trace and the juvenile bundles at the four different stages of differentiation. A similar sequence of events occurs during formation of the metaxylem vessels



in the stem and in the hypocotyl bundles (Fig. 2h, i). Figure 2j shows differentiation of transfer cells adjacent to fully differentiated vessels at stage 4, in the leaf-trace bundle.

The patterns of secondary wall thickenings deposited in protoxylem and in metaxylem vessels are different. In protoxylem vessels, a uniform layer of secondary wall is deposited all around the inner surfaces of the cell wall at stage 3 of differentiation (arrowheads, Fig. 2g). In metaxylem vessels, however, secondary wall is deposited

in two phases (Fig. 2h, i): the vessel cell, at stage 2, first develops secondary thickenings with pits, on the wall (arrows, Fig. 2h, i) shared with the neighboring vessel, which is at stage 3. Vessel cells at stage 3 show flat secondary thickenings on the sides adjoining the parenchyma cells (arrowheads, Fig. 2h, i). Completely differentiated vessel cells, at stage 4 (Fig. 2i) show a differentially patterned secondary wall. The shared wall between the two neighboring vessels (black arrows, Fig. 2i) shows secondary wall thickening with pits,



Fig. 3 a–g Scanning electron micrographs of xylem cells of zinnia leaf-trace and stem bundles. **a** Leaf-trace bundle with radially arranged rows of xylem vessels (*arrowheads*), a characteristic shared with juvenile bundles. *Double arrowheads* mark small cambial cells. *Bar* = 50 μm . **b** Stem bundle showing irregularly arranged clusters of metaxylem vessels (*arrows*), surrounded by small parenchyma cells with secondarily thickened walls. The internal end of the bundle contains protoxylem (*white rectangle*). *Bar* = 67 μm . **c** Hypocotyl bundle showing the dense network of clusters of xylem vessels (*arrows*) interspersed between small parenchyma cells. Most of the protoxylem at the inner end of the xylem tissue has been degraded. *Bar* = 200 μm . **d** Higher magnification of xylem vessels, showing spiral wall thickening (*arrowheads*). *Bar* = 7.7 μm . **e** Higher magnification of metaxylem vessels, showing reticulate wall thickening, with characteristic pits (*arrows*). *Bar* = 7.7 μm . **f** Higher magnification showing stem bundle vessels and parenchyma cells. *Small arrows* mark specific parenchyma cells sandwiched between vessels (*large arrows*), which develop reticulate type thickenings with pits, resembling neighboring vessels. *Double*

arrowheads indicate parenchyma cells, without immediate neighboring vessels, that have flat secondarily thickened walls. Most of the stem bundle parenchyma cells do not develop wall pits. *Bar* = 15 μm . **g** Longitudinal fracture of a stem vascular bundle showing the transition from spiral vessels (*arrowheads*) to reticulate vessels (*arrows*). *Double arrowhead* marks young xylem cells. *Bar* = 18.5 μm . **h** Zinnia stem vascular network showing the arrangement and interconnections of the leaf and stem bundles and the distribution of spiral- and reticulate-patterned TEs. Juvenile bundles (*dotted lines*), formed in the young zone (above node 1), differentiate to form leaf-trace bundle (*gray lines*) and stem bundle (*black lines*) segments of the vascular strands in the mature zone (between node 1 and the hypocotyl). During this transition, the xylem acquires different characteristics: spiral vessels of the juvenile bundle continue in the leaf-trace bundles, but are displaced by reticulate vessels in the stem bundles. In long internodes, the leaf traces also acquire stem bundle morphology and newly formed reticulate vessels displace spiral vessels of leaf-trace bundles, indicated by the transition of *gray lines* into *black* (also shown in **b** and **g**). *Ph*, phloem, *Xy*, xylem

whereas, the wall adjoining the parenchyma cells (black arrowheads, Fig. 2i) develops flat secondary thickening, devoid of any wall pits. Therefore, precise cell-cell interactions are involved in reticulate wall patterning.

New xylem vessels are continuously being formed by the meristematic tissue layer, the cambium, sandwiched between the xylem and the phloem tissues of a vascular bundle (double arrowhead, Fig. 3a). The continuous addition of new cells changes the anatomy of the vascular bundle. Figure 3a–c show progressive differentiation of the xylem elements in maturing vascular bundles. The xylem of juvenile bundles in the young zone (above node 1) of the plant is composed of radially arranged protoxylem vessels (Dahiya et al. 2005). These bundles later differentiate into the leaf-trace bundles and the stem bundles in the mature zone (between node 1 and hypocotyl) of the plant (Dahiya et al. 2005; Fig. 3h). Leaf-trace bundles maintain the protoxylem vessels (Fig. 3a; for differences between juvenile and leaf-trace bundles, see Dahiya et al. 2005). As the juvenile and the leaf-trace bundles grow older, newly formed metaxylem (arrow, Fig. 3b) gradually displaces the protoxylem to the internal end of the bundle (rectangle, Fig. 3b). The bundles are now termed stem bundles (Fig. 3b; Dahiya et al. 2005). As the stem bundle matures, the cambium continues to form metaxylem, and a large network of vessels interspersed with small parenchyma cells comprise the xylem (Fig. 3c).

At higher magnification, the protoxylem vessels show spirally-thickened secondary walls (Fig. 3d) and there are no inter-connections with the neighboring vessels. Metaxylem vessels have reticulate secondary thickenings, with characteristic wall pits (Fig. 3e). Unlike spiral vessels, reticulate vessels are positioned in clusters (arrow, Fig. 3b, c) and are inter-connected by wall pits (arrow, Figs. 3e, 2h, i). The vessel clusters are embedded into a dense network of small parenchyma cells (Fig. 3b, c), which develop secondary wall thickening but maintain their cytoplasmic contents (double blue arrowheads, Fig. 2i). A few parenchyma cells that are sandwiched between vessels also develop wall pits (small arrows,

Fig. 3f) although most of the parenchyma cells have flat secondary walls devoid of pits (double arrowheads, Fig. 3f).

Figure 3g shows a longitudinal fracture of the transition point when the cambial layer starts to form reticulate xylem in the stem bundles. The internal end of the bundle displays 3–4 cell layers of the spiral vessels (arrowheads), and the proximal end shows 2–3 cell layers of the newly formed reticulate vessels (arrows). Young cells marked by double arrowheads are likely to be the newly differentiated xylem cells and the meristematic cambium cells. In addition to spiral and reticulate thickenings, a few cell layers of xylem vessels with annular thickenings were also observed in plants with one pair of open leaves (data not shown). One to two cell layers of scalariform thickening is often observed before formation of reticulate thickening (data not shown).

Figure 3h shows the vascular network of the zinnia plant and schematically demonstrates the transition from formation of spiral vessels (dotted and gray lines), to forming reticulate vessels (black lines). The transition of the juvenile bundles (dotted lines) into leaf-trace bundles (gray lines) and stem bundles (black lines) is shown above node 1. The transition of the long running leaf-trace bundles (gray lines) into stem bundles (black lines) is shown between node 1 and the hypocotyl. Long running stem bundles between node 4 and the hypocotyl acquire mature stem bundle morphology, as shown in Fig. 3c.

Discussion

We have isolated a full-length cDNA of a putative homologue of a fasciclin-like gene, *AtFLA11*, from zinnia. Two recent studies using a broad analysis of public microarray data have identified a small set of genes that are co-regulated with secondary cell wall cellulose synthase genes (Brown et al. 2005; Persson et al. 2005). Both of these studies highlight *AtFLA11* and implicate this gene in secondary wall formation.

PopFLA11, the closest putative homologue of *ZeFLA11*, is expressed in the secondary xylem of poplar (Lafarguette et al. 2004). In situ hybridization of the *ZeFLA11* riboprobe with transverse sections shows a differential expression pattern in the mature zone (between node 1 and hypocotyl) of the zinnia stem. The transcript is expressed in the stem bundles and newly branched small bundles, and is absent from the neighboring leaf-trace bundles. In stem and small bundles, *ZeFLA11* is expressed in the two peripheral cell layers of xylem. This expression pattern raises two questions: (1) what differentiates the peripheral 2–3 cell layers of stem bundles from the rest of the xylem and (2) how does the xylem of stem bundles differ from that of leaf-trace bundles.

Closer examination of the *ZeFLA11* gene expression pattern in stem bundles (Fig. 2d), and in small bundles (Fig. 2f), shows that both xylem vessels and the parenchyma cells contain abundant transcript. However, the transcript is limited to xylem cells at developmental stages two and three. These xylem cells are recently formed by the cambium and are enlarging and depositing secondary wall thickenings. This result is consistent with the RT-PCR expression pattern observed in the time-course of TE formation in the zinnia mesophyll cell system (Fig. 1d), where the transcript peaks at +48 h. At this time point, differentiating TEs deposit secondary wall thickenings (Milioni et al. 2002).

However, xylem cells of the leaf-trace bundle, at stage 2 and 3 of differentiation, do not express the transcript (Fig. 2e). Other riboprobes hybridize to xylem cells in all bundles of the mature zone of the stem (Ye and Varner 1994, 1995) demonstrating that the leaf-trace protoxylem is developmentally active. FESEM images of xylem show that leaf-trace bundles contain protoxylem vessels with spiral thickenings (Fig. 3d). In contrast, newly formed metaxylem vessels of the stem and small bundles contain reticulate thickenings (Fig. 3e), and protoxylem vessels are displaced to the internal end of the bundle. Unlike the radial arrangement of the protoxylem vessels (Fig. 3a), metaxylem vessels are arranged in clusters (Fig. 3b, c) and are inter-connected by wall pits (Fig. 2h, i). The abundant expression of the *ZeFLA11* transcript in the stem and the small bundles (Fig. 2b, d, f), and its corresponding absence from the leaf trace bundles (Fig. 2b, e), suggests that *ZeFLA11* is exclusively expressed in metaxylem with reticulate thickenings.

One difference between spiral and reticulate thickenings is that deposition of the spiral thickenings occurs on all cell surfaces of a given cell, whereas, the reticulate thickenings of neighboring vessels develop in a more complex fashion. Spiral thickenings develop in cells at stage 3 of differentiation (arrowheads, Fig. 2g). Secondary thickening of reticulate vessels is, however, more elaborate and the vessels show two types of wall thickenings in one plane of section: (1) patterned secondary wall with pits, on the shared wall between neighboring vessels (arrows, Fig. 2h, i), and (2) flat secondary wall without pits, on the sides adjacent to the small parenchyma cells (arrowheads, Fig. 2h, i). A differentiating

reticulate vessel at stage 2 is largely a thin-walled cell except on the sides shared with the neighboring vessels at stage 3 (white arrows, Fig. 2h, i), where deposition of patterned secondary wall with pits is evident.

Reticulate vessels at stage 2 (Fig. 2d, f) have a higher abundance of *ZeFLA11* transcript, which is concentrated at the shared patterned wall with cells at stage 3 (arrow, Fig. 2d, f). Parenchyma cells surrounding the vessels at stages 2 and 3 also contain abundant transcript (blue arrowheads, Fig. 2d, f). Unlike xylem cells, the differentiated parenchyma cells maintain their cytoplasmic contents (double blue arrowheads, Fig. 2i). However, fully differentiated parenchyma cells do not express the *ZeFLA11* transcript (double blue arrowheads, Fig. 2d, f), suggesting that the gene is switched off following secondary wall deposition. These results suggest that the *ZeFLA11* gene product may be required specifically during the deposition of secondary wall thickenings, but only in metaxylem. Using semi-quantitative PCR, transcription of *popFLA11* has been shown to be upregulated in differentiating xylem and absent from the cambial xylem (Lafarguette et al. 2004).

The mechanism of secondary wall deposition is coordinated among metaxylem cells to generate reticulate-patterned thickenings on specific adjacent wall surfaces of neighboring vessels and parenchyma cells. These metaxylem cells are, therefore, tightly interlocked. The presence of a fasciclin domain with conserved protein–protein interaction sites in the *ZeFLA11* glycoprotein suggests that the protein may interact at the cell surface with other proteins involved in wall deposition. Sequence analysis of the putative *ZeFLA11* glycopeptide suggests that it may be attached to the outer face of the plasma membrane by a C-terminal GPI anchor. This linkage has been confirmed in *Arabidopsis* FLA proteins and is phospholipase C sensitive (Borner et al. 2003). Such GPI-anchored proteins may be preferentially located in lipid rafts. Since *ZeFLA11* has a fasciclin-like domain, that may facilitate protein–protein interactions, and two AG glycomodules (Borner et al. 2003) that may confer lectin-like properties and the ability to bind to wall polysaccharides (Nothnagel 1997), this cell surface glycoprotein could function as a bridge between wall and plasma membrane proteins involved in wall production and deposition. This proposed function, in helping to ensure fidelity in wall deposition, is supported by the phenotype of the *Atfla4* mutant, also called *sos5*, where root cell expansion is the result of thinner, less well organized cell walls being deposited (Shi et al. 2003). Given the co-regulation of *AtFLA11* with the secondary wall cellulose synthase genes (Brown et al. 2005; Persson et al. 2005), one such possible interaction target may be the cellulose synthase complex itself, possibly in the context of lipid rafts.

Other *FLA* gene family members may function in other cell types that are depositing secondary walls, for example, during the deposition of spiral thickenings in protoxylem or in forming the thick walls of phloem sieve tubes. It is not known whether there are compositional

differences between secondary walls of different cell types. However, even cell types that are developmentally related, the trichoblasts and atrichoblasts of root epidermis, express different complements of proteins and polysaccharides in their cell walls (Puhlmann et al. 1994; Bernhardt and Tierney 2000; Baumberger et al. 2001; Andeme-Onzighi et al. 2002) and, notably, a member of the larger arabinogalactan protein gene family is expressed only in atrichoblasts (van Hengel et al. 2004). A recent paper (Kubo et al. 2005) underscores the developmental distinction between the pathways of metaxylem and protoxylem differentiation. The switch from protoxylem to metaxylem differentiation may be at least partly under the control of two closely related NAC-domain containing transcription factors, raising the possibility that *ZeFLA11* may be a direct target gene of the zinnia equivalent of VND6.

In the zinnia stem, spiral thickenings of juvenile and leaf-trace bundles are gradually displaced by reticulate thickenings to form stem bundles (Fig. 3g, h). The increasing distance from the apical meristem of the elongating vascular strands apparently triggers the transition of the juvenile bundles into the stem bundle. Similarly, long running leaf-trace bundles acquire stem bundle morphology as the distance from the supply leaf increases. It is likely that the switch from spiral vessels to reticulate vessels is controlled by long distance hormone/auxin signaling. Recently branched small bundles, in the mature zone, also contain clusters of large metaxylem vessels with reticulate thickenings, a characteristic resembling the neighboring stem bundles (Fig. 2b, f). Similarly, the phloem is composed of companion cells and sieve elements, a characteristic of metaphloem (Dahiya et al. 2005). The xylem elements of the small bundle follow an ontogenic transition from initially formed spiral vessels to reticulate vessels, albeit a short one. Only one to two spiral vessels have formed at the internal end (Fig. 2f), indicating a swift transition to vessels with reticulate thickenings, which in turn suggests that the developmental switch from spiral to reticulate type vessel is permanent in the mature zone of the plant, under standard growth conditions. A change in the growth conditions, such as the absence of light, will promote formation of spiral vessels in the mature zone of the etiolating plants (Esau 1962).

In conclusion, the differential expression pattern of *ZeFLA11* suggests that the gene product is required specifically during deposition of secondary wall thickenings in metaxylem tissues containing reticulate vessels. The GPI anchor sequence suggests localization at the outer face of the plasma membrane whilst the presence of the fasciclin domain may indicate a function in interaction with outer cell surface proteins involved in wall assembly. The use of *ZeFLA11* as a molecular marker for the developmental switch from spiral to reticulate thickenings has revealed further anatomical heterogeneity within vascular bundles. Taken together with our previous description of a molecular marker expressed in xylem parenchyma of leaf-trace bundles but

phloem companion cells in stem bundles (Dahiya et al. 2005), it is clear that more complexity exists in vascular cell differentiation at the molecular level than is currently understood.

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