

Spatial and temporal regulation of the forisome gene *for1* in the phloem during plant development

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Received: 23 May 2007 / Accepted: 23 July 2007 / Published online: 11 August 2007
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Abstract Forisomes are protein aggregates found uniquely in the sieve elements of Fabaceae plants. Upon wounding they undergo a reversible, calcium-dependent conformational switch which enables them to act as cellular stopcocks. Forisomes begin to form in young sieve elements at an early stage of metaphloem differentiation. Genes encoding forisome components could therefore be useful as markers of early sieve element development. Here we present a comprehensive analysis of the developmental expression profile of *for1*, which encodes such a forisome component. The *for1* gene is highly conserved among Fabaceae species and appears to be unique to this phylogenetic lineage since no orthologous genes have been

found in other plants, including *Arabidopsis* and rice. Even so, transgenic tobacco plants expressing reporter genes under the control of the *for1* promoter display reporter activity exclusively in immature sieve elements. This suggests that the regulation of sieve element development is highly conserved even in plants where mature forisomes have not been detected. The promoter system could therefore provide a powerful tool for the detailed analysis of differentiation in metaphloem sieve elements in an unexpectedly broad range of plant species.

Keywords Immature sieve elements · Forisomes · Metaphloem development · Promoter analysis

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9217-0) contains supplementary material, which is available to authorized users.

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Abbreviations

<i>CC</i>	Companion cell
<i>EST</i>	Expressed sequence tag
<i>GFP</i>	Green fluorescent protein
<i>GUS</i>	β -Glucuronidase
<i>RACE</i>	Rapid amplification of cDNA ends
<i>SE</i>	Sieve element

Introduction

Phloem development has been investigated by expressing reporter proteins such as GFP (green fluorescent protein) and GUS (β -glucuronidase) under the control of phloem-specific promoters in transgenic plants (e.g., Haritatos et al. 2000; Stadler et al. 2005). One remarkable example is the well-characterized promoter of the *Arabidopsis thaliana* sucrose transport protein gene, *AtSUC2*, which is activated when the veins become functionally mature (Imlau et al. 1999; Wright et al. 2003). In contrast, little is known about cellular processes prior to the maturation of sieve element/

companion cell (SE/CC) complexes, and this mainly reflects the lack of markers specific for individual developmental stages. Recently, the *Arabidopsis thaliana* *Altered Phloem Development* (*APL*) gene was identified as a marker of early phloem cell determination. The *APL* protein is a MYB-family transcription factor which is required for division of the SE/CC-precursor, and subsequently for SE and CC differentiation (Bonke et al. 2003; Bonke 2004). In addition, a specific marker for immature tracheary elements was recently identified which will be useful for the analysis of early xylem development (Pyo et al. 2004).

Forisomes, formerly known as non-dispersive crystalline P-proteins (Behnke 1991), are macromolecular assemblies found uniquely in the Fabaceae. They alter their conformation in response to changes in Ca^{2+} levels (Knoblauch et al. 2001, 2003). Forisomes first appear in cells that are already recognizable as young sieve elements of the metaphloem (Wergin and Newcomb 1970; Palevitz and Newcomb 1971). At these specific developmental stages, the sieve elements are clearly immature as indicated by the presence of a nucleus, large numbers of ribosomes, abundant vesiculate endoplasmic reticulum and numerous dicotysomes. There is also an accumulation of finely granular and fibrillar material, which subsequently undergoes a series of morphological transformations to form forisomes. The forisomes assume their mature morphological characteristics by the time tonoplast degradation and nuclear degeneration take place. Thereafter, they remain unmodified for the lifetime of the sieve element (Palevitz and Newcomb 1971). The identification of genes encoding forisome proteins would facilitate future molecular studies of this unique protein complex and would also provide specific markers for immature sieve elements of the metaphloem in Fabaceae. To the best of our knowledge, no such markers have yet been reported.

Here, we present the molecular characterization of the forisome component ForI, from three different Fabaceae plants. We show that the *forI* gene is unique to the family Fabaceae and is expressed only in immature sieve elements. We study the expression profile of this gene using *forI* promoter-reporter gene constructs in transgenic plants and by direct analysis of *forI* mRNA at different developmental stages in *Vicia faba*.

Materials and methods

Purification of forisomes and determination of peptide sequences

Forisomes from 6–8-week-old *V. faba* cv. Witkiem major plants were purified as described previously (Knoblauch et al. 2003). Forisome-specific peptide sequences were

generated by nanoLC-ESI-MS/MS and MALDI-TOF-MS (Proteomfactory, Berlin).

Identification and cloning of *mtfor1*, *vffor1* and *cgfor1*; expression of GST-ForI fusion constructs and antibody production

Forisome peptide sequences were compared to sequences in two public databases: MENS (European Medicago EST Navigation System: <http://medicago.toulouse.inra.fr/Mt/EST/>) and NCBI (National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov>). To clone the corresponding genes, mRNA was isolated from *Medicago truncatula* cv. Jemalong A17, *Vicia faba* cv. Witkiem and *Canavalia gladiata* plants according to established protocols (Leiser et al. 1992). Based on the 478 bp EST sequence from the MENS database, the full-length cDNA for *mtfor1* was obtained using the BD GenomeWalker™ Universal Kit (BD Bioscience, Clontech) following the manufacturer's instructions and using forward primer 5'-CAC CTC CAT CAC ACG TCC ACA-3' and reverse primer 5'-TGT GGA CGT GTG ATG GAG GTG-3'. A similar strategy involving the same primers in combination with 5' and 3' RACE (BD SMART™ RACE cDNA Amplification Kit; BD Bioscience, Clontech) was used to identify the cDNAs for *vffor1* and *cgfor1*. All full-length clones were subsequently subcloned in the pGEM-T vector (Promega, Heidelberg) and verified by sequencing using established protocols (Sanger et al. 1977).

For the construction of chimeric GST-ForI expression vectors the entire *mtfor1* and *vffor1* cDNAs were amplified by PCR using forward primer 5'-AGA CCC GGG ATG TCA TTG TCC AAT GGA ACT AAA C-3' and reverse primer 5'-AGA CCC GGG TCA TAT CTT GCC ATT CTG TGG AGC-3' for *mtfor1*, and forward primer 5'-CAT GCC ATG GGA ATG TCC TTT TCT AAC TCA-3' and reverse primer 5'-TAA AGC GGC CGC AAC ACC AAA GTT ATT TGG-3' for *vffor1*. The PCR products were subcloned in pGEX-3X, and their integrity confirmed by sequencing.

Overexpression of GST-MTForI and GST-VFForI in *E. coli* BL21 was performed according to established protocols (Smith and Johnson 1988). Overexpressed proteins were then used to generate polyclonal antisera in rabbits. This work was carried out by EUROGENTEC (Seraing, Belgium). The resulting antisera were designated anti-MTForI and anti-VFForI.

Construction of *Pmtfor1*-GUS, *Pvffor1*-GUS and *Pmtfor1*-GFP plasmids

To clone the *mtfor1* and *vffor1* promoter sequences, Genome-Walking PCR (Clontech Laboratories, Palo Alto, CA)

was performed on *M. truncatula* cv. Jemalong A17 and *V. faba* cv. Witkiem major genomic DNA using the primers 5'-GTT TAG TTC CAT TGG ACA ATG ACA T-3' (for *Pmtfor1*) and 5'-CTA ACT CAC CTG CTG CTG CTA CTG GCA-3' (for *Pvffor1*) according to the manufacturer's instructions. In the resulting PCR, we obtained genomic DNA fragments of approximately 1 kbp for *Pmtfor1* and 2.7 kbp for *Pvffor1*, which were cloned as promoter elements into a *EcoRI*–*NcoI*-digested pUC103-GUS (kindly provided by Dr. Klaus Fritze, Cologne) to obtain *Pmtfor1*-GUS and *Pvffor1*-GUS, respectively. *Pmtfor1*-GFP-Emerald was obtained by insertion of the 1 kbp *Pmtfor1 EcoRI*–*NcoI* fragment described above into the same restriction sites of pGJ2628 (kindly provided by G. Jach, Cologne; Cubitt et al. 1999). Subsequently, all constructs were inserted into the *EcoRI*/*HindIII* site of binary vector pBIN19 (Bevan 1984) for plant transformation.

For the construction of *Pmtfor1*-GFP_{ER}, the GFP_{ER} coding sequence was isolated via *XhoI*-*HindIII* digestion of pGJ1029 (kindly provided by G. Jach, Cologne) and inserted into *XhoI*–*HindIII*-linearized pBluescriptIIKS+ to obtain pBSGFP_{ER}. *Pmtfor1* was amplified by PCR using the forward primer 5'-AGA GGT ACC GTC CAT TCG TAC ATG CAG AAA-3' and reverse primer 5'-AGA CTC GAG TGA TAA ATT CAA CTT TAG GC-3' digested with *KpnI* and *XhoI* and subsequently inserted into the corresponding restrictions sites of pBSGFP_{ER}. The resulting construct was designated *Pmtfor1*-GFP_{ER}. Finally, the construct was inserted into the *HindIII*/*KpnI* site of binary vector pBIN19 (Bevan 1984) for plant transformation.

Immunodetection of For1 protein in purified forisomes

For in situ detection of MTFor1 and VFFor1, forisomes were purified from *Vicia faba* plants using a modified version of the method described by Knoblauch et al. (2003). After the first filtration process, forisomes were collected directly by centrifugation in a microtiter plate. Bound forisomes were washed twice with 1x PBS, 0.1% Tween-20 and incubated for 1 h in a 1:500 dilution of anti-MTFor1 or anti-VFFor1 in the presence of 5% milk powder. The preparations were then washed three times in 1x PBS containing 0.1% Tween-20 and incubated in a 1:5000 dilution of the secondary antibody, ALEXA FLUOR[®] 488 goat-anti-rabbit IgG (Molecular Probes). After three washes, fluorescence was monitored directly with a confocal laser scanning microscope (Leica TCS 4D, Wetzlar, Germany).

Plant transformation and analysis

Stable transformation of *Nicotiana tabacum* cv. SR1 plants was carried out as described previously (Horsch et al.

1986). Histochemical localization of GUS activity in transgenic plants was performed using the substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide without an oxidative catalyst according to established protocols (Jefferson 1987). GFP-Emerald and GFP_{ER} expression (Ex. 487 nm, Em. 509 nm) in plant tissue was analyzed with a confocal laser scanning microscope (Leica DMIRE2, Wetzlar, Germany). In order to obtain clear images of immature sieve elements, the abaxial epidermis of the midrib was removed. CLSM detection (Ex. 355 nm, Em. 665 nm) of the nucleus was performed after vacuum infiltration of 5 μ g/ml Bisbenzimidide H 33342 (Fluka, Munich, Germany) in sodium phosphate buffer (pH 7) supplemented with 0.1% Tween-20 (Fang and Spector 2007).

For the analysis of *vffl* gene expression in *Vicia faba* by RT-PCR, total RNA was isolated using the Total RNA Purification from Plants Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. First strand cDNA synthesis was carried out by reverse transcription of 1.5 μ g of total RNA in the presence of an oligo(dT) primer and 1 μ l of SuperScript[™] II reverse transcriptase (Invitrogen, Karlsruhe, Germany). After digesting the RNA with 2 units of RNase H (Roche, Penzberg, Germany) for 20 min at 37°C a 769 bp *vffl*-specific PCR fragment was amplified using forward primer 5'-GCT GAT AGT CCT CAG ATC TAT G-3' and reverse primer 5'-CTT GAA TCT CAC AAT CCA CAG C-3'. The same strategy was applied to the *PPO* gene (accession number: Z11702) using forward primer 5'-GAA CCC CAG GGA GCT GGA TC-3' and reverse primer 5'-GTC ACT CAA CAC AGA CGG TAC AT-3'. RT-PCR products and total RNA samples were fractionated by non-denaturing agarose gel electrophoresis and visualized by ethidium bromide staining. The integrity of all RT-PCR fragments was verified by sequence analysis.

Results and discussion

Identification and molecular characterization of genes encoding forisomes

Although most published Fabacean gene sequences are from the model legume *Medicago truncatula*, we needed to use forisome proteins from *V. faba* (Fig. 1A) in our experiments due to the absence of efficient protocols for isolating the small forisomes from *M. truncatula* (Fig. 1B). Several *V. faba* peptide sequences were generated (data not shown) and used to search public databases. Computer analysis revealed that two peptides, with the amino acid sequences QGWVILSK (peptide I) and EGFDIATK (peptide II), matched parts of a *M. truncatula* 478 bp EST (expressed sequence tag) from the MENS database

(Medicago EST Navigation System), encoding a partial sequence of 157 amino acids. Peptide I showed 87.5% identity to the corresponding section of the EST, reflecting the substitution of threonine for serine at position 7, whereas peptide II was 100% identical to its corresponding section (see supplementary data S1). Based on this sequence information, we cloned either the corresponding full length genomic sequence by genome walking or the cDNA sequence by 5' and 3' RACE (rapid amplification of cDNA ends) using *M. truncatula* genomic and cDNA.

Comparison of the genomic and cDNA sequences revealed the presence of a 1944 bp open reading frame, interrupted by six introns, encoding a ~75 kDa protein. The resulting cDNA clone was designated *mtfor1* (protein MTFor1; accession number EU016204). In parallel, 5' and 3' RACE produced homologous cDNA clones for two other Fabaceae plants, *V. faba* (*vffor1*, 2055 bp; VFFor1, 79 kDa; accession number EU016203) and *Canavalia gladiata* (*cgfor1*, 2007 bp; CGFor1, 77 kDa; accession number EU016202). These clones were of similar length and shared protein sequence identity of ~60% (see supplementary data S1). The *for1* gene appears to be unique to the Fabaceae since none of the above sequences showed greater than 25% identity to sequences from other plants, such as *Arabidopsis thaliana* and rice, for which complete genome sequences and extensive EST resources are available. In addition, we used standard PCR as well as 5' and 3' RACE with both specific and degenerate primers in an attempt to isolate orthologous sequences from non-Fabacean plants such as tobacco. However, no amplification products were detected indicating that the *for1* gene is restricted to the Fabaceae (data not shown).

We next attempted to demonstrate the presence of VFFor1 (Fig. 1C) in purified *Vicia faba* forisomes directly. The anti-VFFor1 antiserum specifically detects an antigen on the forisome body, indicating that For1 is most likely to be a structural component of the forisome. However, we cannot completely rule out the possibility that For1 is a secreted protein that accumulates in the sieve element and spontaneously associates with assembled forisomes, although the fact that the protein is found on the surface of forisomes broken during extraction suggests otherwise (Fig. 1D). Due to its reciprocal cross-reactivity with GST-VFFor1 expressed in bacteria we also tested the capacity of the anti-MTFor1 antiserum to detect the homologous VFFor1 protein in *Vicia faba* forisomes (Fig. 1D). As shown in Figs. 1C and D, both antisera are able to recognize their antigens and the staining appears to be restricted to the surface of intact forisomes. However, the internal staining of the broken forisome in Fig. 1D (arrow) indicates that the For1 protein is distributed throughout the forisome body and the lack of internal staining in intact forisomes suggests the antigen is not accessible, perhaps

due to its dense packaging. Control experiments with pre-immune sera (Figs. 1E and F), secondary antibodies and anti-GST antiserum (data not shown) showed no staining.

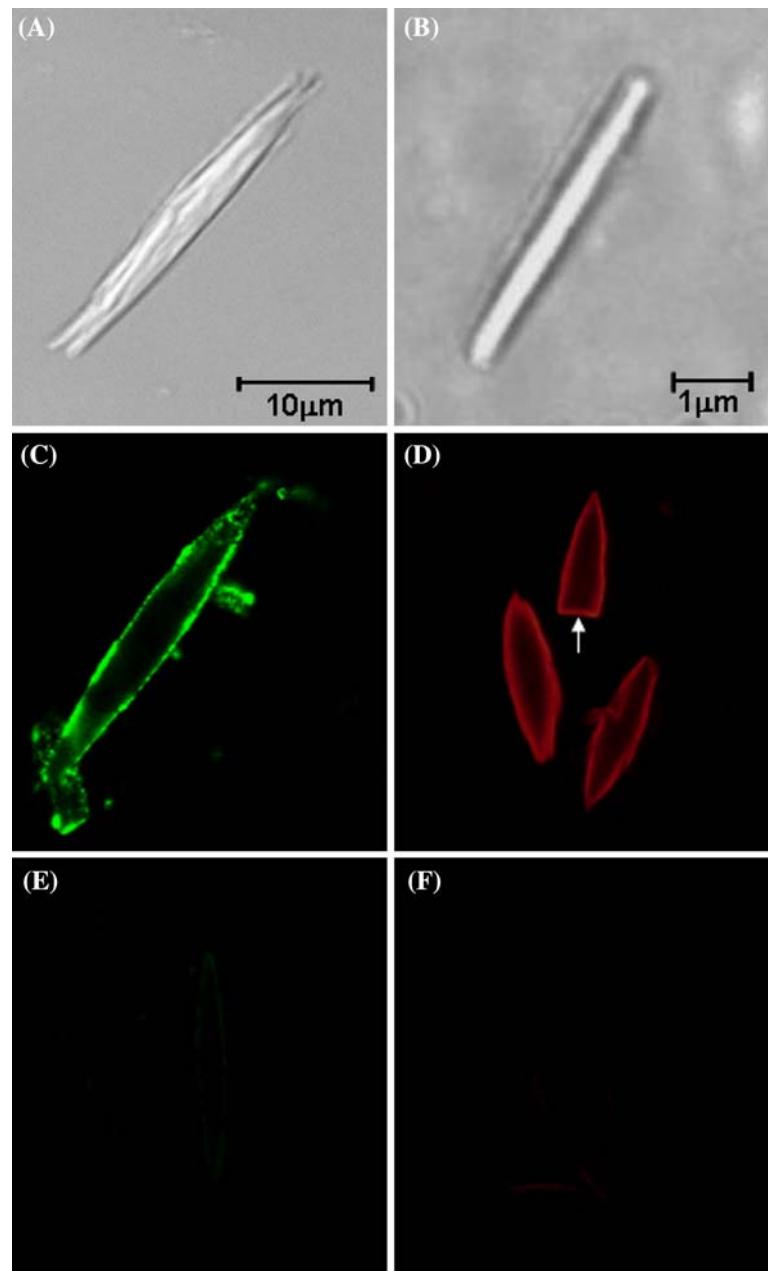
Forisomes are complex macromolecular structures and are likely to comprise several or many different proteins. We note that six additional full-length or partial *Medicago truncatula* ESTs were identified in the MENS database which showed significant identity (~50%) to *mtfor1* and subsequently *vffor1* at the amino acid level. Further experiments will be required to determine whether the encoded gene products are also forisome components.

Analysis of for1 promoter activity in transgenic tobacco plants

We next set out to confirm that the For1 protein is expressed in young sieve elements at an early stage of metaphloem differentiation, as previously reported for the formation of the entire protein body (Zee 1969; Wergin and Newcomb 1970; Palevitz and Newcomb 1971). Our approach was to study *mtfor1* and *vffor1* promoter activity in transgenic tobacco plants. The promoter sequences were isolated from *V. faba* and *M. truncatula* genomic DNA by genome walking (see Materials and Methods, see supplementary data S2) and were designated *Pvffor1* and *Pmtfor1*, respectively. Comparison with known phloem-specific promoter sequences such as those from coconut foliar decay virus (CFDV) (Hehn and Rohde 1998), *Agrobacterium rhizogenes rolC* (Schmülling et al. 1989), pumpkin *PP2* (Guo et al. 2004), pea *GS3A* (Brears et al. 1991) and rice tungro bacilliform virus (Yin et al. 1997) did not show any significant similarities.

In order to study the promoter activities in detail, *Pvffor1* and *Pmtfor1* were subcloned as regulatory sequences upstream of the *Escherichia coli uidA* (GUS) reporter gene to obtain *Pvffor1*-GUS and *Pmtfor1*-GUS. For each construct, ten individual transgenic tobacco plants were regenerated and subsequently analyzed for GUS activity by histochemical staining. A representative set of stained leaves from a *Pvffor1*-GUS and *Pmtfor1*-GUS T₀-transgenic plant is shown in Fig. 2. In immature leaves, GUS activity was strong, but staining was restricted to the vascular system indicating spatial regulation of the promoter (Fig. 2A for *Pmtfor1*-GUS, Fig. 2B for *Pvffor1*-GUS). In contrast, GUS activity was detected in only a few cells in the vein network of older leaves, showing that the promoter was also temporally regulated, and was largely inactive at this stage (Figs. 2C and D). Thin sections of stained petioles showed that GUS activity was restricted to the phloem (Figs. 2E and F), but clear distinction between companion cells and sieve elements was not possible due to diffusion of the GUS reaction products.

Fig. 1 Purified forisomes in the condensed state from *Vicia faba* (A) and *Medicago truncatula* (B). Detection of For1 in forisomes from *Vicia faba* using either the anti-VFFor1 (C) or anti-MTFor1 (D) antisera. Incubation of forisomes with pre-immune sera of anti-VFFor1 (E) and anti-MTFor1 (F). Single pictures in (D) and (F) represent compositions of several forisomes analyzed independently. The distribution of VFFor1 is shown in green and that of MTFor1 is shown in red. White arrow in (D) indicates the internal staining of the broken forisome



To address this issue, we produced and analyzed transgenic tobacco plants expressing GFP-Emerald under the control of the *mtfor1* promoter (Fig. 2G and H). In immature leaves, GFP-Emerald activity was restricted to a pipeline-like assembly of fairly narrow, elongated cells located within the phloem of the vascular bundle (Fig. 2H). These displayed the characteristic morphology of sieve elements, as confirmed by their typical end-to-end connections, which are the sieve-plate precursors (Fig. 2G). The immature nature of the sieve elements is clearly demonstrated by their ability to carry out active protein synthesis (indicated by GFP-Emerald expression) and the presence of a nucleus and one or more vacuoles within the

cytoplasm (Fig. 2I; Cronshaw 1981). However, we note that some weak GFP-Emerald fluorescence was detected in companion cells (Fig. 2J). GFP is known to be transported as soon as companion cell/sieve element complexes become functionally mature (Wright et al. 2003), as is its derivative GFP-Emerald (G. Jach, personal communication). In order to prevent the transport of GFP between immature sieve elements and companion cells, a KDEL-tagged form of GFP (GFP_{ER}, kindly provided by G. Jach) was placed under the control of the *mtfor1* promoter and expressed in transgenic tobacco plants. Detailed confocal laser scanning microscopy of *Pmtfor1*-GFP_{ER} transgenic plants confirmed that the *mtfor1* promoter is active exclusively in immature

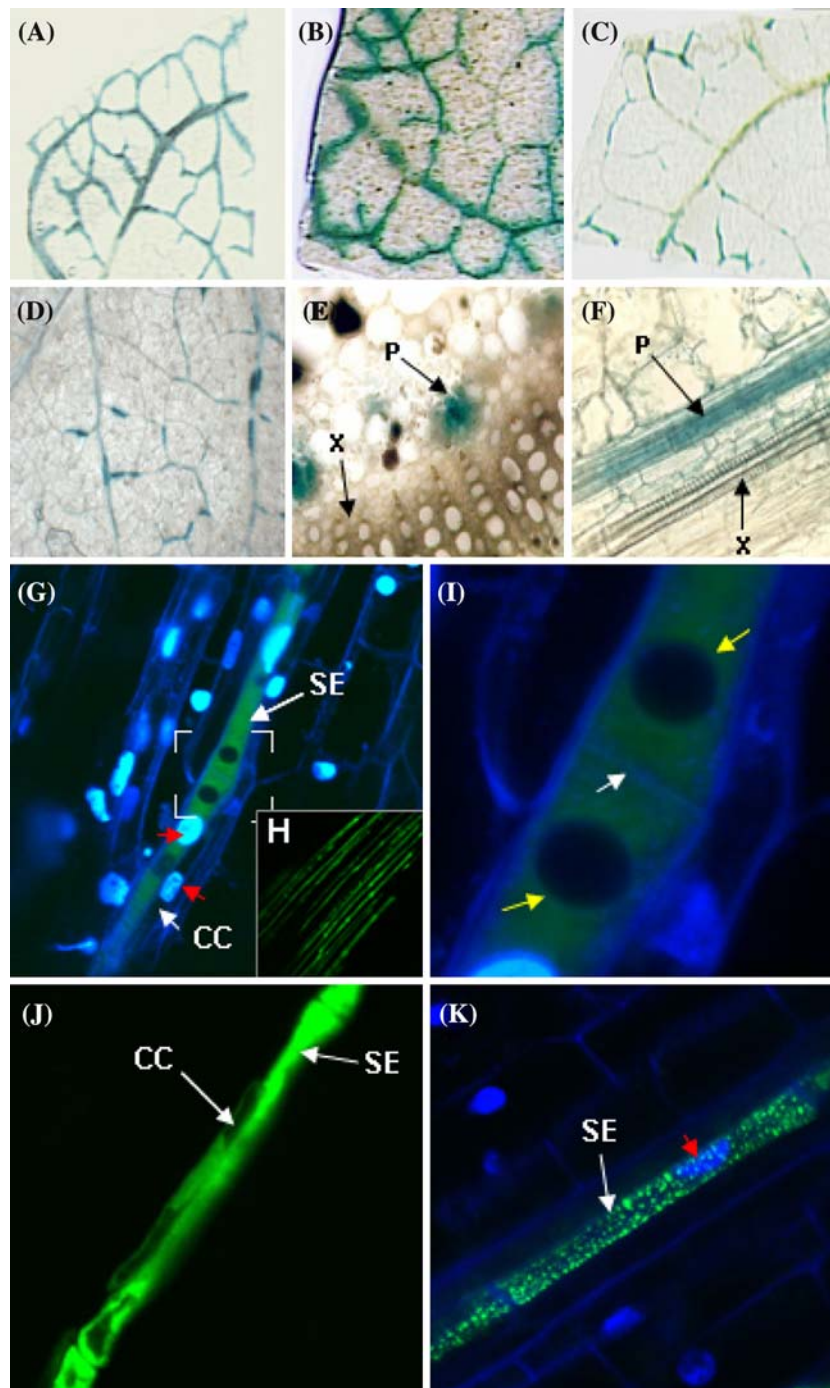


Fig. 2 Histochemical analysis of individual leaves from *Pmtfor1*-GUS, *Pvffor1*-GUS, *Pmtfor1*-GFP-Emerald and *Pmtfor1*-GFP_{ER} T₀-transgenic *Nicotiana tabacum* plants. Stained section of young (A) and old (C) leaves of transgenic *Pmtfor1*-GUS plants or young (B) and old (D) leaves of *Pvffor1*-GUS transgenic plants. (E) and (F) show cross and longitudinal sections, respectively, of the petiole vein of young *Pmtfor1*-GUS transgenic leaves, indicating the phloem-restricted activity of the corresponding promoter (P = Phloem; X = Xylem). (G) GFP expression in an immature sieve element. The nuclei are indicated by red arrows. The area enlarged in panel I is edged in white (H) *Pmtfor1*-driven GFP-Emerald expression in several “pipeline-like” assemblies of fairly narrow, elongated cells

located within the phloem of the vascular bundle of young *Pmtfor1*-GFP-Emerald transgenic tobacco plants. (I) Magnification of G. Yellow arrows indicate the tonoplast. White arrows indicate sieve plates. (J) GFP expression obtained in an immature sieve element where fluorescence was also visible in the companion cell. (K) ER-tagged GFP expression obtained exclusively in immature sieve elements. The stained nucleus is indicated by a red arrow. Pictures in G-K were taken from the phloem of the major vein at the base of immature leaves (~ 5 cm in size) of greenhouse cultivated plants. For simultaneous representation of stained nuclei and GFP expression an overlay of two individual pictures is presented in G and K

sieve elements because GFP fluorescence could no longer be detected within companion cells (Fig. 2K).

We also observed a significant reduction in GFP-Emerald activity in leaves described by Avery (1933) as fully mature. Only a few immature sieve elements could be found within the vascular bundle. The distribution of cells expressing GFP-Emerald was very similar to the pattern of GUS staining observed in leaves from T₀ *Pvfor1*-GUS transgenic plants (Figs. 2C and D). Furthermore, and as expected, *for1* promoter activity could be detected in developmental stages characterized by the secondary growth of vascular tissues (see supplementary data S3).

Developmental regulation of *for1* promoter activity

Since the *for1* promoter is active exclusively in immature sieve elements, we next carried out a series of experiments to study the promoter activity in detail during vein maturation. Therefore, five *Pvffor1*-GUS and *Pmtfor1*-GUS T₁-transgenic tobacco plants derived from independent transformants were grown from seeds in sterile culture, and GUS activity was analyzed at different stages of development. The individual clones from all transgenic lines showed identical GUS expression patterns, as represented by the *Pmtfor1*-GUS plant shown in Fig. 3. Approximately 7 days after germination (Fig. 3A) cotyledons showed strong staining only visible in the phloem. At this stage there was no detectable staining in the roots demonstrating that the *for1* promoter is not active in the protophloem, in which forisomes also could not be identified (Esau and Gill 1971; Lawton 1978). Later, there was a significant reduction in GUS activity, with only a few stained phloem cells remaining at ~9 days post-germination (Fig. 3B) and none at all at ~11 days post-germination (Fig. 3C). This reflected the pattern of GUS activity observed with true leaves from the T₀ plants. The first staining in the roots became evident at this stage, exclusively in the maturation region where cells begin to differentiate (arrow in Fig. 3C).

Next, we analyzed the promoter activity in true leaves. In developing leaves, GUS expression first became visible in class I veins (Fig. 3D) followed by class II-III veins (Fig. 3E). In the next development phase, GUS expression over the entire vein network was clearly visible, although it was strongest at the leaf tip including class III-V veins (Fig. 3F). Shortly after veins at the leaf tip reached functional maturity (indicated by loss of GUS activity), staining appeared in the vein network at the middle part (Fig. 3G) and subsequently at the base of leaf (Fig. 3H). This revealed basipetal progression in functional vein development. When the entire vein network reached functional maturity, no significant GUS activity was observed (Fig. 3I). At all developmental stages described above, the

maturation region of primary and secondary roots showed strong GUS activity (Fig. 3J and K).

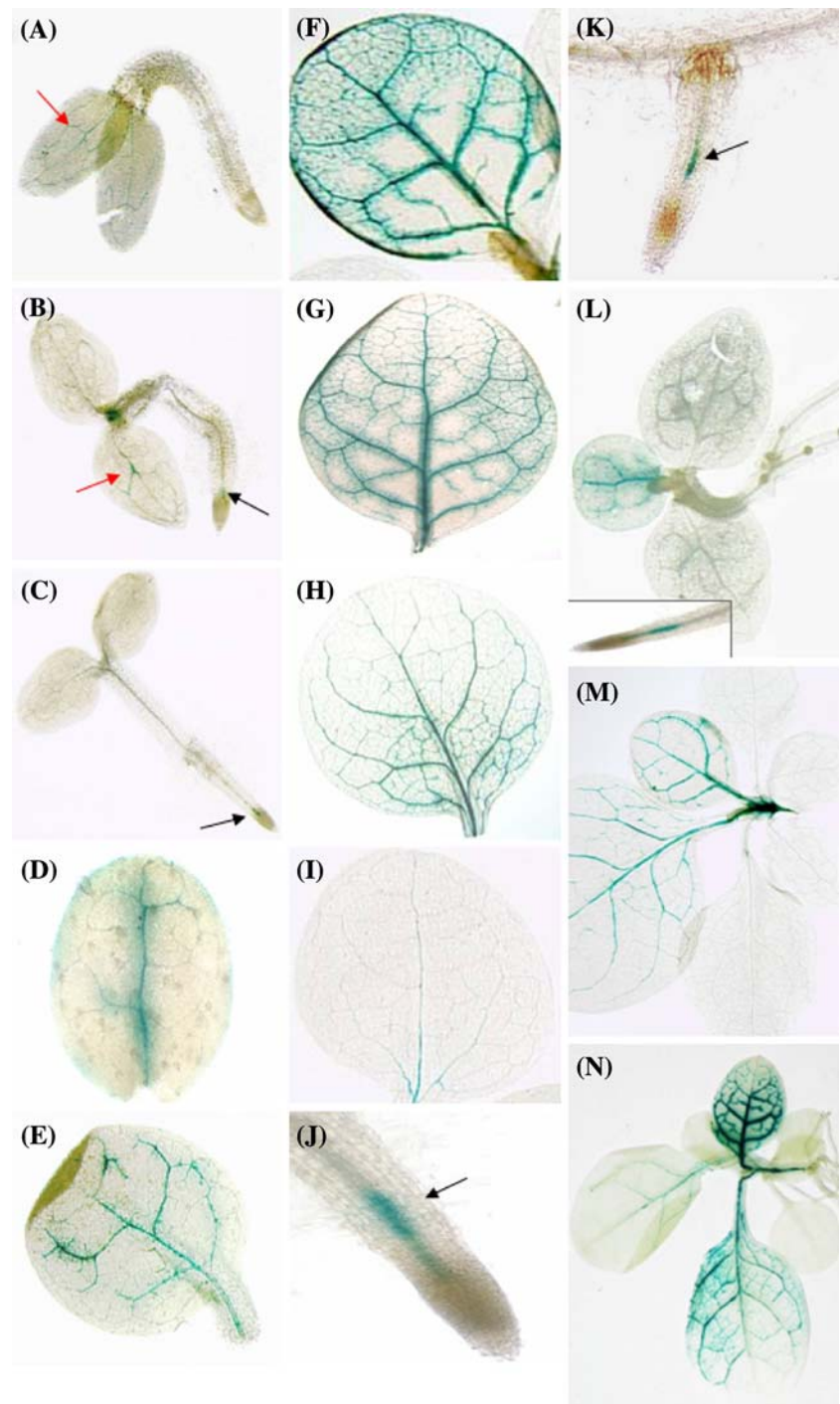
The progression of GUS staining during development was discussed above in the context of individual leaves taken at different developmental stages. To gain further insight into the developmental regulation of the *for1* gene, we next stained complete transgenic seedlings (Fig. 3L–N). The strongest GUS activity was observed in young leaves, with much weaker staining evident in older leaves, thus reflecting the same temporal regulation observed in individual leaves. This pattern is almost diametric to the activities of the *Arabidopsis thaliana* *AtSUC2* promoter (Imlau et al. 1999) and the *Cucumis melo* galactinol synthase promoter (Haritatos et al. 2000), both of which first become active when the companion cell/sieve element complex reaches functional maturity, reflecting the same pattern as the source-sink transitions of photoassimilate transport (Turgeon 1989). We also note that similar experiments involving a reporter gene driven by the phloem-specific promoter of the *Arabidopsis thaliana* *APL* gene revealed reporter activity not only in immature sieve elements, but also in companion cells and throughout the entire vascular strands in seedlings and true leaves. This indicates that the *APL* promoter has a less restricted pattern of activity compared to the *for1* promoter (Bonke et al. 2003; Bonke 2004).

In summary, the lack in GUS expression in the proto-phloem strongly indicates that the *for1* promoter is exclusively active in immature sieve elements of the metaphloem during functional phloem development.

Analysis of *for1* expression in Fabaceae

We next set out to determine whether the activity of the *for1* promoter in a homologous (Fabacean) background was the same as that established in the heterologous (Solanaceous) background of our transgenic tobacco plants. To do this, we compared the GUS activity observed in *Pvffor1*-GUS and *Pmtfor1*-GUS transgenic plants with the steady state mRNA levels of the corresponding *vffor1* gene in *V. faba* with both species grown under greenhouse conditions. Total RNA was extracted from the first (young) to the fourth (old) leaves of *V. faba* plants and used to amplify *vffor1* cDNA by RT-PCR. In parallel, GUS expression was evaluated in leaves of the same developmental stage from the *Pvffor1*-GUS and *Pmtfor1*-GUS transgenic tobacco lines. Figure 4 confirms that the highest level of *vffor1* mRNA (column B) was found in young leaves (L1), with a steady decline as the leaves became older (L2–L4). GUS activity in the *Pvffor1*-GUS transgenic tobacco plants (column A) and the *Pmtfor1*-GUS tobacco plants (data not shown) declined in parallel. In addition,

Fig. 3 GUS staining of *Pfor1*-GUS T₁-transgenic *Nicotiana tabacum* plants at different developmental stages. (A–C) show *Pmtfor1*-GUS activity in seedlings 7 (A), 9 (B), 11 (C) days after germination. Red arrows show GUS activity in the phloem of leaves. Black arrows indicate GUS activity in the maturation region of primary roots. (D–I) show the first six leaves taken from individual *Pmtfor1*-GUS transgenic seedlings. A basipetal (tip-to-base) progression in vein maturation is clearly visible. (J–K) show *Pmtfor1*-driven GUS activity in the maturation region of primary and secondary roots, as indicated by black arrows. Staining of complete *Pmtfor1*-GUS (L–M) and *Pvffl*-GUS (N) T₁-transgenic seedlings shows the same GUS activity pattern observed for individual leaves (D–I). At all developmental stages, GUS staining was observed in the maturation region of primary (magnification in L) and secondary roots



GUS activity and *vffl* expression could be detected in root tips in both individual experiments (Fig. 4, RT). In order to confirm that the observed decline in GUS activity and *vffor1* expression did not reflect a general decline in mRNA levels in ageing leaves, the polyphenoloxidase (*PPO*), was analyzed as a positive control. This was chosen as an alternative to typical housekeeping genes such as actin or glyceraldehyde 3-phosphate dehydrogenase, which are not

currently available for *V. faba* due to the paucity of sequence data.

The similar *PPO* mRNA levels throughout development showed that transcription and mRNA turnover remained constant (column C), although the RT-PCR product could not be detected in root tips. In summary, the expression of *vffor1* in *V. faba*, a Fabaceae plant, reflects entirely the pattern observed in transgenic tobacco plants expressing a

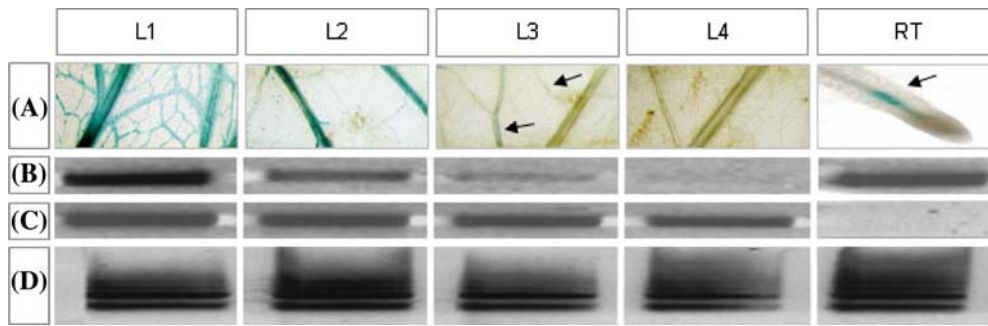


Fig. 4 Analysis of *vffl* gene expression in *Vicia faba*. (A) P*vffl*-driven GUS activity in the first (L1), second (L2), third (L3), and fourth (L4) leaves as well as in root tips (RT) of a single T₁-transgenic plant. Weak GUS activity in L3 veins is indicated by arrows. (B) RT-

PCR amplification of the *vffl* gene from total RNA of *V. faba* leaves and root tips following the same strategy as described in (A). RT-PCR amplification of the *PPO* gene (C) and total RNA (D) from *Vicia faba*, used as controls

reporter enzyme under the control of either the *vffor1* or *mtfor1* promoter. This indicates that these promoters function under a conserved regulatory system, presumably with common elements between species.

Although forisomes are thought to exist only within the Fabaceae, the results of our heterologous promoter system analysis might suggest that more distantly related protein structures exist in other families, and that their genes utilize similar promoter sequences for expression during metaphloem differentiation. In support of this theory, the formation of tubular P-protein bodies in immature sieve elements has been reported in tobacco (Cronshaw 1975; Cronshaw et al. 1973) and other plants (Cronshaw and Esau 1968; Sabnis and Hart 1973), which might implicate an evolutionarily conserved expression strategy. Plugging by clogging proteins is seen as a universal tool for temporary sieve-plate occlusion (van Bel 2006). Further experiments will need to be performed in order to gain more insight into these P-protein bodies and the genes encoding them, as well as the regulation of genes encoding phloem-specific proteins.

Acknowledgements We thank Dr. Richard M Twyman for helpful discussion and critical reading of the manuscript. The technical assistance of Christiane Fischer and Eva Aguado is gratefully acknowledged. This work was supported by the BMBF grant 0312014A and B and the Fraunhofer MAVO program.

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