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Expression of an expansin carbohydrate-binding module affects xylem and phloem formation

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Expansins are believed to be involved in disrupting the non-covalent adhesion of cellulose to matrix polysaccharides, thereby promoting wall creep. We have targeted a putative potato expansin (EXPA) carbohydrate-binding module (CBM) to the cell walls of tobacco plants. Histological examinations and electron microscopy indicated that 30% of the xylem cells of the transgenic stems with high expression of the expansin CBM are wider (radial surface area) than those of the controls. Similarly, 37% of the xylem cells of the stems of the high expressers have thinner cell walls than those of the controls. There were no such phenotypes in the low and none expressers, as well as in the control plants. The transgenic tobacco plants expressing the potato expansin CBM did not exhibit marked change in plant morphology. Analysis of cellulose content in the stem cell walls was similar between the high expresser of the transgene and the control plants. Nonetheless, our results taken together demonstrate that expansin CBM alone can bring about changes in the plant cell walls.

Key words: Expansin, carbohydrate binding modules, cell wall modification, homology modelling, cell expansion, transgenic.

INTRODUCTION

Expansins are plant proteins discovered over a decade ago, which have the capacity to induce extensibility and stress relaxation of plant cell walls *in vitro* and are proposed to mediate cell expansion (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove, 1995). The proteins are thought to facilitate wall extension by catalyzing the disruption of hydrogen bonds between cellulose microfibrils and matrix polysaccharides, thereby causing turgor-driven slippage between the microfibrils and other components of the wall (McQueen-Mason and Cosgrove, 1995). Expansins are expressed in growing tissues including hypocotyls, shoot meristems and internodes (Cho and Kende, 1997; Fleming et al., 1997; Reinhardt et al., 1998). They are also expressed in non-growing tissues such as ripening fruits and abscission zones or tissues where cell separation will take place (Brummell et al., 1999; Cosgrove et al., 1998). These findings suggest that the proteins have tissue-specific roles in addition to cell expansion (Chen and Bradford, 2000). Studies with transgenic plants indicate that endogenous expansins are involved in regulating growth and developmental processes in which rearrangements in the cell wall are thought to be important (Cho and Cosgrove, 2000; Choi et al., 2003; Pien et al., 2001; Zenoni et al., 2004).

Based on their phylogenetic relationship, expansins and expansin-like proteins can be classified into four families(website:http://www.bio.psu.edu/expansins/arabid opsis.htm and (Kende et al., 2004). The four protein families currently recognized are EXPA, EXPB, EXLA and EXLB. Members of the EXPA and EXPB families

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Abbreviations: Cryo-SEM, cryo-scanning electron microscopy; CBM, carbohydrate-binding module; CaMV, cauliflower mosaic virus; PCR, polymerase chain reaction; MS, Murashige-Skoog; NAA, α-naphtalene acetic acid; BAP, 6-benzylaminopurine; rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase.

have been shown to possess an *in vitro* weakening effect on cellulose paper. The amino acid sequences of the two expansin-like families EXLA and EXLB are divergent from EXPA and EXPB and no biological or biochemical function has been established for any member of these families. All members of the expansin superfamily consist of two domains: an N-terminal domain I that is distantly related to the catalytic domain of glycoside hydrolase family-45 (GH-45) and a C-terminal domain II that is distantly related to group-2 grass pollen allergens (Cosgrove, 2000). Plants also have single-domain proteins known as immunoreactant plant natriuretic peptides (irPNPs), which have marked sequence similarity with domain I of the expansins (Ceccardi et al., 1998; Gehring and Irving, 2003; Ludidi et al., 2002). The irPNP-like molecules can enhance osmoticum-dependent water transport across plasma membranes (Maryani et al., 2001; Pharmawati et al., 2001) and hence increases cell turgor (Ludidi et al., 2002). This has led to the idea that the irPNP-like molecules function cooperatively with expansins in bringing about cellular expansion. Similarly, rice possesses a group of truncated proteins that have high similarity to the carboxy-terminal domain II of expansins but lack the domain I part of these proteins (Li et al., 2003). However unlike the irPNP-like molecules, the function of these domain II-like proteins is unknown.

In theory, each module of a particular modular protein such as expansins is capable of folding independently into a functioning protein. Therefore one would expect to gain more insight into the function of the C-terminal domain II of the expansins by over- expressing it in a suitable host. It has been proposed that expansin's domain II resembles cellulose-binding domains of some cellulases; with respect to the spacing a series of noncontiguous conserved tryptophan residues (Gilkes et al., 1991). This has led to the growing speculation that this region may be responsible for expansin binding to wall polymers (Barre and Rouge, 2002; Cosgrove, 2000a; Shcherban et al., 1995). Expansin protein is thought to bind the interface between cellulose microfibrils and matrix polysaccharides (McQueen-Mason and Cosgrove, 1995). Evidence for the binding and interaction of expansin with cell wall polysaccharide is seen in its weakening effect on pure cellulose paper without evidence of hydrolytic activity (McQueen-Mason and Cosgrove, 1994). Besides, a cucumber expansin (EXPA) caused rapid weakening of artificial composites made of bacterial cellulose and xyloglucan (Whitney et al., 2000). The authors found that weakening was stronger in the cellulose-xyloglucan composite material than in cellulose-only material. However, the speculated binding properties of the expansin's domain II have not been demonstrated experimentally till date, because of the difficulty in producing active recombinant expansin, let alone the domain II. This explains why the protein has not been formally classified as a carbohydrate-binding module (website:http://www.bio.psu.edu/expansins/arabidopsis.

htm).

In this study, we have targeted the expression of a putative potato expansin CBM to tobacco cell walls. The expression of the gene encoding this CBM was driven by 35S cauliflower mosaic virus (CaMV) promoter. In this paper, we describe the cellular events that are associated with the heterologous over-expression of a potato expansin CBM in transgenic tobacco plants. Our observations indicate that the expansin CBM alone can alter cell wall structure.

MATERIALS AND METHODS

Plant transformation vector

The EST clone (EST accession number BG097738) of the putative expansin CBM was obtained from an EST library of potato leaves and petioles. Standard polymerase chain reaction (PCR) was performed on the EST fragment encoding the expansin CBM, using primers that included BamHI and EcoRI restriction sites (5'-cgc<u>ggatcc</u>ggtggaataaggtttacaattaacgg-3' and 5'-cttcttttc<u>gaattca</u>ctatttaaactctg -3'; the BamHI and EcoRI sites are underlined, respectively). The three bases highlighted in bold type represent the stop codon. The amplified 319 base pairs fragment of the potato expansin CBM was digested with BamHI and EcoRI (Invitrogen, The Netherlands) and cloned into a similarly digested binary vector pGreen7k (Hellens et al., 2000). Upstream of the expansin fragment in the binary vector, two more gene fragments were cloned. The first sequence codes for a tobacco transit peptide for transporting a cellular glycoprotein NTP303 across the plasma membrane into the cell wall (Wittink et al., 2000), while the second sequence encodes a hexa-histidine tag. The sequence of the transit peptide was obtained as a product of annealing two oligonucleotide primers,TP1(5' <u>agctt</u>atgggaagtggtaaagtaacatttgtggctttgctactttgcctctccgtaggggtgatagctt-3') and TP2 (5'-<u>ctaga</u>agctatcacccctacggagaggcaaagtagcaaagccacaaatgttactttaccacttcccata-3'). The underlined nucleotides produced overhangs of HindIII and XbaI, respectively. This fragment was then cloned into the HindIII and XbaI cloning sites of the vector. Similarly, the sequence of hexa-histidine tag was obtained and ligated into XbaI and BamHI sites as annealing product of His1 (5'-<u>ctagaa</u>gaggatcgcatcaccatcaccatcacg-3') and His2 (5'-<u>gatcc</u>gtgatggtgatggtgatgcgatcctctt-3'), with the underlined producing overhangs of XbaI and BamHI, respectively. The purpose of using the hexa-histidine tag was to facilitate affinity purification of the potato expansin's CBM. The control construct did not contain any of the CBMs, the transit peptide and the hexa-histidine epitope tag. All constructs were sequenced to verify that the sequences encoding the transpeptide, hexa-histidine tag, and expansin CBM were in-frame.

Sequence alignment and homology modelling

The program DNA-Star was used to compare the amino acid sequences of a pollen allergen Phl P2 of timothy grass (*P43214*) and domain II sequences of *Arabidopsis* expansins EXPA1 (*AAG60095*), EXPB1 (*AY084478*), EXLA1 (*AY058142*), EXLB1 (TAIR accession *2130444*), and the potato expansin CBM. Multiple amino acid sequence alignment was based on Clustal W, with gap penalties of 10 and a PAM250 matrix.

The amino acid sequences of the representatives of the four expansin families above were obtained from the expansins web site (http://www.bio.psu.edu/expansins/arabidopsis.htm). This site was

linked to the ExPASy (Expert Protein Analysis System) proteomic server (http://us.expasy.org/), designed for detecting protein modular domains. The alignment was manually edited for structurally homologous (according to Risler's matrix) residues occurring in the CBM.

An alignment, based on Clustal W, of the amino acid sequence of the potato expansin CBM and the Phl P2, together with SWISS-MODEL Version 36.0003 program (available from http://swissmodel.expasy.org//SWISS-MODEL.html) (Guex and Peitsch 1997; Peitsch, 1995; Schwede et al., 2003) were used for homology modelling, in which the atomic coordinates of Phl P2 (*1WHO*) served as a template.

Tobacco transformation, regeneration and growth

The expansin CBM construct and the empty vector control construct were used for *Agrobacterium tumefaciens*-mediated transformation of *in vitro* leaf explants of *Nicotiana tabacum* cv. Samsun NN as follows. Cloned binary vector pGreen7k was co-transformed with the helper plasmid, pSoup (Hellens et al., 2000) into *A. tumefaciens* strain LBA4404 by electroporation. This was plated out on LB-agar plates containing kanamycin (100 μg/mL) and rifampicin (30 μg/mL), and incubated for three days at 28°C, to obtain single colonies. The integrity of the binary plasmids was tested by restriction analysis of plasmids isolated from *A. tumefaciens* cultures used for plant transformation. 20 mL LB medium without selection was inoculated with a single colony and incubated overnight at 28°C. 100 μL of the grown culture was added to a petridish containing 10 mL Murashige-Skoog (MS30). Leaf explants, without major veins and edges, from young seedlings were transferred upside down in to the MS30 medium. *Agrobacterium* infection was done in the dark for 2 days at 24 - 25°C. Two controls were used for the procedure, untransformed wild type control and empty pGreen7k control without an insert. Leaf explants were washed in three changes of liquid washing medium of MS30 with 250 mg/L carbenicillin. The washed explants were transferred upside-up to MS30-phytagel plates containing 0.1 mg/L α-naphtalene acetic acid (NAA), 1 mg/L 6-benzylaminopurine (BAP), 200 mg/L kanamycin and 250 mg/L carbenicillin, and incubated overnight in the dark at 28°C. Plants were transferred to a growth chamber (25°C), where they were gradually adapted to light and incubated for callus induction. After two weeks in culture, calli generated were transferred to shoot-inducing medium, MS20-phytagel, containing 0.2 mg/L NAA, 2 mg/L BAP, 200 mg/L kanamycin and 250 mg/L carbenicillin. Well-formed shoots were harvested and transferred to root-inducing medium, MS15-phytagel plate containing 100 mg/L kanamycin, 250 mg/L carbenicillin and 200 mg/L vancomycin. Twelve transformed plantlets were transferred to the greenhouse to generate mature plants.

Semi-quantitative RT-PCR analysis of transgenic plants

Total RNA was isolated from 3 - 5 g of transformed *in vitro* shoots as described elsewhere (Kuipers et al., 1995). For first strand cDNA synthesis, 1 μg of total RNA was treated with 0.5 μL DNAse I RNase free (10 U/μL; Invitrogen) and incubated with 5 μL of 10 x Taqman RT buffer, 11 μL of 25 mM $MgCl_2$, 10 μL of 10 mM dNTP mix, 2.5 μL of 50 μM random hexamer primers, 1μL RNAse inhibitor (20 U/μL) and H_2O until a final volume of 50 μL for 30 min at 37°C and 5 min at 75°C. The mixture was then incubated for 10 min at 25°C and 30 min at 48°C with 1 μL of MultiScribe reverse transcriptase (50 U/μL; Applied Biosystems). The reaction was then terminated by heating the sample for 5 min at 95°C. 5 μL of cDNA was then used as a template in standard PCR using construct-specific primers. The forward primer, SPFOR 5'-agcttatgggaagtggta-3', was specific for the signal peptide while the reverse primer, HISREV 5'- tccgtgatggtgatggt- 3', was specific for the hexa-histidine tag. Rubisco-specific primers, RUBFOR 5'-cagaaatcatcaggaaaggaaaca - 3' and RUBREV 5'-tctcttcacgttttcttgctcttg - 3' were used to amplify rubisco cDNA, as internal control.

Light microscopy

Three individual plants from the high expressing and two plants from the low/none expressing class were used for microscopic examination.

The empty pGreen7k transformants were used as the control. Stem samples were taken from the second internode from the top of the plant. 1 mm-thick stem sections were fixed in 3% glutardialdehyde (Merck) and 3% paraformaldehyde (Merck) in 0.1 M phosphate buffer containing 0.1% Triton x100 for 2 h. The samples were then washed and dehydrated in an ethanol series. After dehydration, they were embedded in Technovit 7100 resin (USA) (Kuroiwa et al., 1990). 4 μm-thick sections were stained with 0.1% toluidine blue (Aldrich) and examined under a bright field microscope.

Cryo-scanning electron microscopy

Transgenic line 16 and one individual plant from the empty vector control were used for cryo-scanning electron microscopy (cryo-SEM) examination. Stem sampling was the same as for the light microscopy. 6 mm-thick stem samples were mounted in a brass cylindrical sample holder with TBS (Tissue Freezing Medium EMS, Washington, PA, USA). The frozen samples were placed in a sample holder in a cryo-ultra microtome (Reichert Ultracut E/FC4D) and cut at a specimen temperature of 100°C. These samples were first planed with a glass knife, after which the surface was planed with a diamond knife (Histo no trough, 8 mm 45°C, Drukker International, The Netherlands). After planing, the samples were placed in a dedicated cryo-preparation chamber (CT 1500 HF, Oxford instruments, UK). All the samples in the cryo-preparation chamber were freeze dried for 3 min at -90°C and 8 x 10^{-4} Pa, and subsequently sputtered with a layer of 10 nm Pt. The samples were cryo-transferred into the field emission scanning microscope (JEOL 6300F, Japan) on the sample stage at -190°C. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 s (full frame) at the size of 2528 x 2030, 8 bit. The images were optimized and resized for publication by Adobe Photoshop CS.

Quantification of cell size and cell wall diameter

To quantify the micrographic images, files were opened with Image J software developed at the National Institute of Health, USA (http://rsb.info.nih.gov/ij/). The surface area of three hundred xylem cells was measured as shown by the inset in Figure 6A. One hundred cells per field of view were measured in three replicates. Similarly, cell wall thickness of one hundred and fifty cells was quantified as shown by the inset in Figure 6B. Fifty cells per field of view were measured in three replicates. Standard deviation of three replicates was determined.

Isolation of cell wall material and analysis of the cellulose content

Stem samples from transgenic line 16, pGreen7k vector control and a wild type control plant were separately ground to a fine powder in liquid nitrogen. For each isolation, 1 g of this stem material was extracted in a 50 mM Tris[HCl] pH 7.2 solution containing 1% SDS, acetone and air-dried. 10 mg of the different cell wall materials was
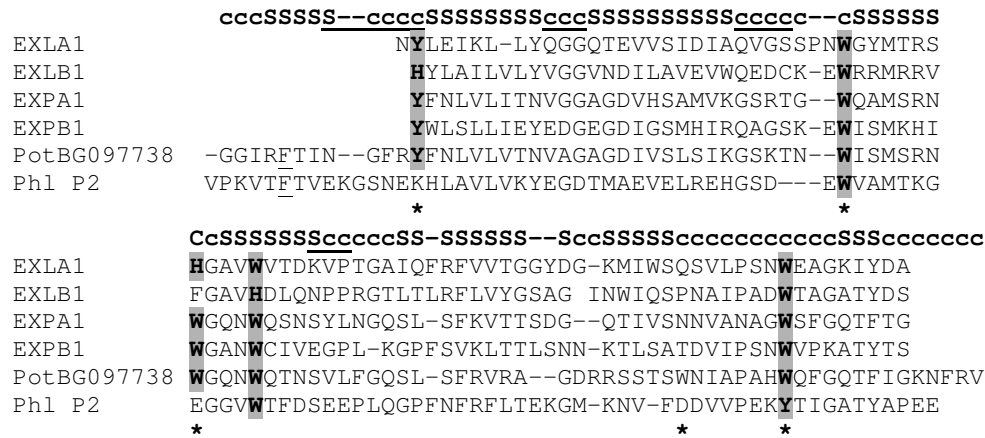
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        cccSSSSS--ccccSSSSSSSSScccSSSSSSSSSSSccccc--cSSSSSS
EXLA1                    NYLEIKL-LYQGGQTEVVSIDIAQVGSSPNWGYMTRS
EXLB1                    HYLAILVLYVGGVNDILAVEVWQEDCK-EWRRMRRV
EXPA1                    YFNLVLITNVGGAGDVHSAMVKGSRTG--WQAMSRN
EXPB1                    YWLSLLIEYEDGEGDIGSMHIRQAGSK-EWISMKHI
PotBG097738  -GGIRFTIN--GFRYFNLVLVTNVAGAGDIVSLSIKGSKTN--WISMSRN
Phl P2       VPKVTFTVEKGSNEKHLAVLVKYEGDTMAEVELREHGSD---EWVAMTKG
                    *                                        *

        CcSSSSSSSCcccccSS-SSSSSS--SccSSSSSccccccccccccSSScccccccc
EXLA1        HGAVWVTDKVPTGAIQFRFVVTGGYDG-KMIWSQSVLPSNWEAGKIYDA
EXLB1        FGAVHDLQNPPRGTLTLRFLVYGSAG INWIQSPNAIPADWTAGATYDS
EXPA1        WGQNWQSNSYLNGQSL-SFKVTTSDG--QTIVSNNVANAGWSFGQTFTG
EXPB1        WGANWCIVEGPL-KGPFSVKLTTLSNN-KTLSATDVIPSNWVPKATYTS
PotBG097738  WGQNWQTNSVLFGQSL-SFRVRA--GDRRSSTSWNIAPAHWQFGQTFIGKNFRV
Phl P2       EGGVWTFDSEEPLQGPFNFRFLTEKGM-KNV-FDDVVPEKYTIGATYAPEE
             *                                  *     *
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Figure 1. Amino acid sequence alignment of At-EXPA1, At-EXPB1, At-EXLA1, At-EXLB1 (all four from *Arabidopsis thaliana*), Phl p2 (pollen allergen from *Phleum pratense*) and the putative potato expansin CBM (EST accession number BG097738). Conserved aromatic residues are shaded and shown in bold letters. Secondary structure elements of Phl P2 and of the putative potato cellulose-binding domain are indicated with letter 'S' and 'c' above the sequences. Asterisk denotes exposed aromatic residues and histidine on the modelled structure. Underlining of secondary structure elements indicates that this region does not have good match with the 3D structure of the template used for modeling.

hydrolyzed in 1 ml 2 M TFA. The TFA-insoluble cellulose was spun down at 13000 rpm for 15 min, and pellet was dissolved in 67% for 3 h at room temperature (RT) with continuous shaking. The cell wall material (CWM) was spun down by centrifugation at 13000 rpm for 15 min.

Subsequently, the residue was washed with water, ethanol and Sulphuric acid. The suspension was heated and diluted appropriate ly to determine the cellulose content colorimetrically using anthrone as a colouring agent according to Updegraff (1969). The acid hydrolysis was performed in quadruplicate

RESULTS

Characteristics of the putative potato expansin CBM

The size of a complete expansin protein is about 25 - 28 kDa. The potato CBM that was targeted to tobacco cell wall was about 15 kDa(133 amino acids) in size,including the transit peptide and the hexa -histidine epitope tag.
A blast search with the amino acid sequence of the EST clone of the potato expansin CBM revealed that it has high homology with the *Arabidopsis* expansin 18 (e-value, 2e–50), which belongs to the EXPA family. We were faced with the question of which part of the sequence should be used for engineering a construct expressing a functional domain II of the potato expansin.

Meanwhile, the putative CBM of an EXPB family Lol P2 from rye grass was found to have about 50% sequence identity with a pollen allergen, Phl P2 from timothy grass (Cosgrove, 2000b), whose structure was solved (De Marino et al., 1999). An amino acid alignment with the potato expansin EST clone and Phl P2 was made, in order to deduce the start of the potato expansin CBM more precisely. The 3-D structure of Phl P2 starts with

Val27 (Val1 in the alignment presented in Figure 1), which is five amino acids before the conserved Phe6 (underlined). We deduced an amino acid sequence (96 amino acids) for a potentially functional putative CBM of the potato expansin, starting with Gly1, four positions before the conserved Phe5 (underlined). We particularly singled out Gly1 as the starting point because it is directly preceded by an exposed two-basic residue RR sequence (data not shown), which is a conserved cleavage signal between domain I and domain II of expansins (Barre and Rouge, 2002). An amino acid sequence alignment of the potato expansin CBM, with four Arabidopsis expansin CBMs (representing each of the four classes of the expansins family), and a pollen allergen Phl P2 from Timothy grass, was made (Figure 1). The amino acid sequences of the four representatives above were obtained from a web site (http://www.bio.psu.edu/expansins/arabidopsis.htm), and correspond to the domain II or CBM as determined by the ExPASy proteomic server (http://us.expasy.org/). These four sequences are shorter than the sequence we deduced for potato CBM, which explains the gaps left in the alignment at the beginning of these sequences. We have incorporated the extra GGIRFTINGFR sequence at the N-terminal side to reduce the risk of an incorrectly folding domain, when expressing the construct in tobacco. The alignment information revealed that the potato CBM shared varying homologies with the CBMs of the four Arabidopsis expansin families; 64% identity with the CBM of EXPA1, 24% with EXPB1, 28% with EXLA1 and 26% with EXLB1. Furthermore, the alignment result revealed that aromatic amino acids (tryptophan and tyrosine) are mostly conserved in the different sequences
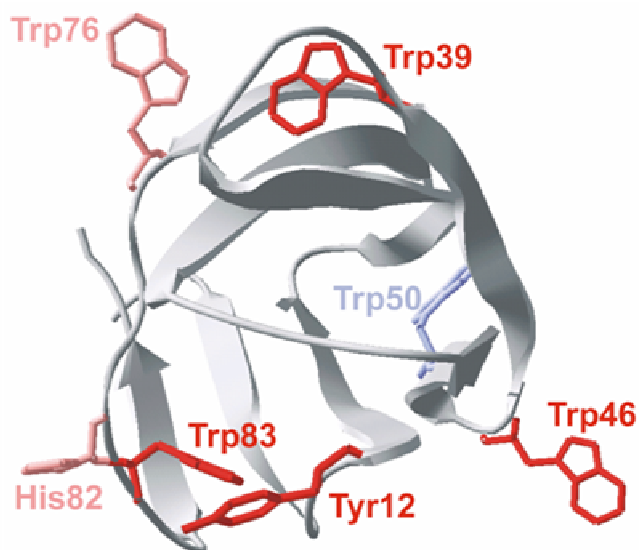
Figure 2. Putative 3-D structure of potato expansin CBM, obtained by homology modelling, using the 3-D structure of pollen allergen Phl P2 as template. Conserved aromatic residues, which are exposed and might be involved in polysaccharide binding, are: Trp39, Trp46, Trp76, Trp83, Tyr12 and His82. Trp50 is mostly buried, hence it is indicated with light colour. Side chains of none conserved residues His82 and Trp76 are also indicated in light colour. Conserved residues are presented in dark colour.

Figure 3. Semi quantitative RT-PCR analysis of the selected transformants and the wild type leaf RNA. The upper panel shows the product amplified using the hexa-histidine tag-specific forward primer and pGreen7k construct-specific reverse primer. The lower panel shows the product amplified using specific primers for ribulose 1,5-biphosphate carboxylase/oxygenase (rubisco), as internal control. Plant lines 5, 7, and 17 are classified as low/none expresser, because of low or none visible intensities. The rest are classified as high expressers. EVC = empty vector negative control, PL = CBM-containing plasmid, as positive control.

particularly among the four expansins families. Our particular interest in the aromatic residues was premised on their involvement in carbohydrate binding (Toone, 1994) and reviewed in (Boraston et al., 2004).

We have modelled a putative 3D-structure for the potato expansin CBM by homology modelling using the structure of Phl P2 as a template. This was with a view to revealing the location of its conserved aromatic residues. The modelled 3-D structure of the potato expansin CBM consisted of β strands and coils (Figure 2). Accompanied information on the modelled CBM revealed that the putative structure had 85% identity to the template Phl P2. The identical regions include all regions containing the eight β strands (indicated in Figure 1 with letter 'S'). Only four coil-constituting regions of the model are structurally divergent to Phl P2 as indicated in Figure 1 by underlining of the secondary structure elements. All aromatic amino acid residues (tyrosine, trptophan) of the sequence except one are exposed. The exposed histidine residue was also visualized because of its ability to mediate pH-dependent binding of proteins to carbohydrates (Linder et al., 1999). One side of the modelled CBM revealed a patch of three exposed residues, Tyr12, His82, and Trp83. It should be noted from the alignment result that the His82 is not conserved, which raises the question as to what extent it is involved in binding. At the opposite side of the protein is a straight line of exposed aromatic residues Trp39, Trp46, and Trp76. It should be noted as well that, even though Trp76 is not conserved
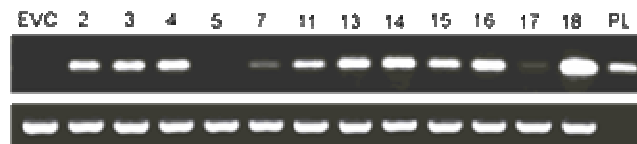
across the four families, it is quite conserved within the EXPA family (data not shown). The well-conserved Trp50 is mostly buried, and therefore its involvement in binding is not very likely. It may have a role in maintaining protein structure through hydrophobic interactions.

Characterization of tobacco transformants expressing the putative potato expansin CBM

Twelve antibiotic-resistant tobacco transformants carrying the expansin CBM transgene were regenerated and transferred to the green house. RNA was isolated from leaves of the transgenic plants to analyze the expression level of the expansin CBM in each transgenic line by semi-quantitative RT-PCR analysis, using the constitutively expressed rubisco gene as an internal control. Varying amplification intensities of the different transgenic plants revealed differential expression of the potato expansin CBM gene in the individual tobacco transformants, thus making it possible to categorize them into two classes, as high and low/none expressers (Figure 3). Transgenic lines 5, 7 and 17 represent the low/none expressing transformants, while the remaining nine lines represent the high expressers. Preliminary attempts at purifying CBM proteins using affinity purification with the hexa-histidine tag were not successful (data not shown). Similarly, western detection of a hexa-histidine epitope-tagged fungal elicitor protein ECP2 that was infiltrated into the apoplast of leaves did not succeed in the tomato plants but succeeded in *Arabidopsis* (van Esse et al., 2006)

The results indicated that the histidine tag was cleaved off in the tomato plants, but not in *Arabidopsis*. These observations have now led to the idea that the hexa-histidine epitope tags have cleavage sites that are being recognized by proteases, which are specific for the cell walls of the solanaceous species. There was no obvious phenotype in the transgenic plants when compared to the pGreen7k vector control. The average plant height of the transgenic plants expressing the potato expansin CBM
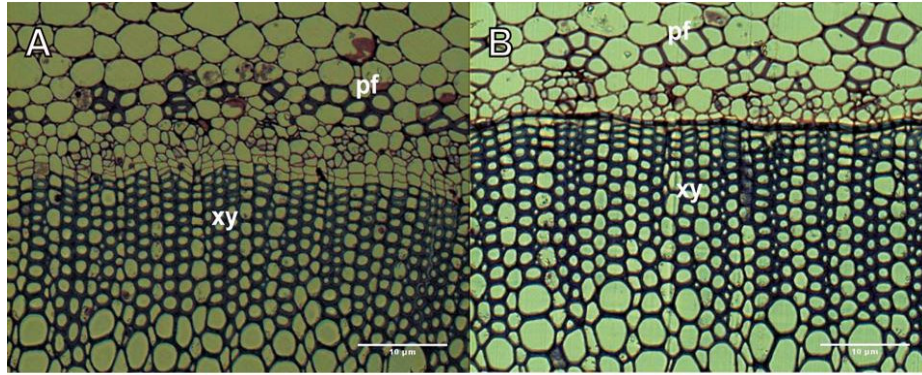
Figure 4. Cross sections of representative transgenic and control stems. Sections of empty Pgreen7k control (A) and transgenic line 16, high expresser of potato expansin CBM (B) were stained with toluidine blue. A and B show the vascular tissue, phloem fibre and xylem cells of the stem sections of the control and the expansin CBM high expresser, respectively. Pf = phloem fibre, xy = xylem.
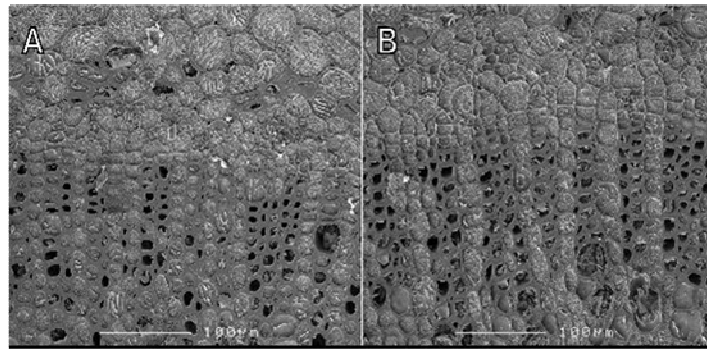

Figure 5. Scanning electron micrographs of stem vascular tissue of the empty Pgreen7k control (A) and the transgenic tobacco line 16 (B), at x300 magnification. Micrographs are representatives of several fields of view.

gene at maturity was 70 cm, which was comparable to 75 cm average height for the pGreen7k vector control plants. There was no particular trend in plant development with respect to stem elongation and flower development.

Transgenic stems exhibit enlarged xylem cells and thin cell walls

To examine cellular events resulting from the expression of the potato expansin CBM in the tobacco plant, stem sections from three representatives from the high expressers and two from the low/none expressers were stained with toluidine blue and examined by light microscope. Figure 4 reveals relatively larger xylem and phloem fibre cells in a representative high expresser of the potato CBM (Figure 4B) than those of the Pgreen7k control (Figure 4A). Furthermore, the micrographs indicated that the cell walls of the phloem fibres of the transgenic plants were thinner than those of the control. Other cell types of

the transgenic stems were the same as those of the control. No intermediate effect was observed with the two low/none expressers, as they were comparable with the control plants.

In order to quantify the cellular observations made with the light microscopy, cryo-SEM was done with the stem samples. The micrographs confirmed that the xylem cells of the transgenic stem of line 16 (Figure 5B) were larger than those of the control stems (Figure 5A). To validate our SEM observations, we subjected the images to quantitative measurements. Figure 6A shows that 40% of the xylem cells in the pGreen7k control stem were grouped in the small class ($0 - 30$ μm^2), whereas only 10% of the xylem cells of the transgenic stems grouped in this class. It also shows that 25% of the xylem cells in the transgenic stems grouped in the large class ($60 - 90$ μm^2), whereas only 5% of the cells of the pGreen7k control stems grouped in this class. There was no large difference between transgenic and the control plants, with respect to the percentage of xylem cells that were group-
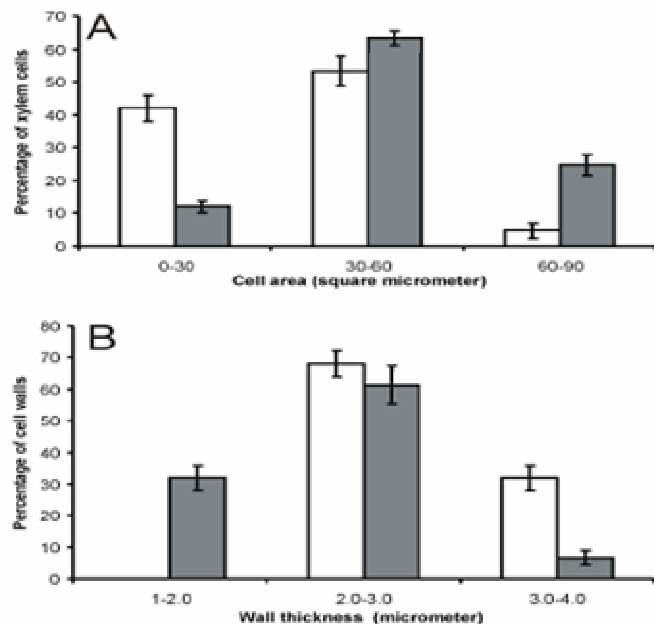
Figure 6. Cell size distribution (A) and cell wall thickness (B) of xylem cells in the stems of transgenic tobacco plants. The surface area of three hundred xylem cells was measured, as shown by the inset in Fig. 6A. One hundred cells per field of view were measured in three replicates. Similarly, cell wall thickness of one hundred and fifty cells was quantified, as shown by the inset in Fig. 6B. Fifty cells per field of view were measured in three replicates. Data were expressed as percentages of xylem cells and cell walls in the different size classes. Open bars represent pGreen7k empty control. Grey bars represent expansin CBM transgenic stems.

Figure 7. Cellulose content in transgenic and control tobacco plants. Cellulose content of stem wall material of transgenic line 16 was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material. All measurements were performed as quadruplicate and standard errors of the means are represented by the error bars

ed in the medium class (63 and 54%, respectively).

We thus can infer that the stem xylem cells of the potato expansin CBM-expressing plants were larger than those of the pGreen7k control plants. Similarly, for cell wall quantification, we measured the thickness of the cell walls of the xylem cells of the transgenic and the pGreen7k control plants as presented in Figure 6B. We observed that 32% of the cell walls of the stem xylem cells of the transgenic plants expressing the expansin CBM grouped in the thin class (1.0 – 2.0 μm), whereas 0% of the cell walls of the pGreen7k control stem xylem cells grouped in this class. Figure 6B also shows that only 5% of the cell walls of the stem xylem cells of the transgenic plant grouped in the thick classes (3.0 – 4.0 μm), whereas 32% of the cell walls of the pGreen7k control stem xylem cells grouped in same class. Similarly as for the cell size, there was no difference between the transgenic and the control, with respect to the distribution of cell wall thickness in the medium class (2.0 – 3.0 μm).

We can also infer from this result that the thickness of the cell walls of the stem xylem cells of the transgenic tobacco plants expressing the potato expansin CBM are thinner than those of the pGreen7k control plants.
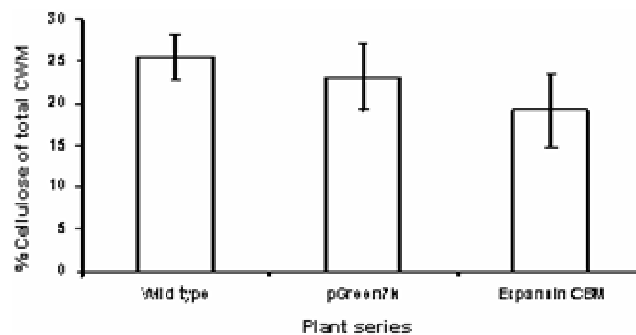
Analysis of cellulose content of transgenic stems

It has been suggested that expansins may play a role in the assembly of the cell wall by affecting cellulose synthesis or deposition (Zenoni et al., 2004). We have determined the cellulose content of the stem cell walls of the expansin CBM-expressing plant and the control plants. Colorimetric assays were performed on four independently hydrolyzed cell wall samples from transgenic tobacco line 16, the empty vector control, and a wild type control (Figure 7). It seems that the cellulose content of the transgenic plant is lower than that of the control plants, but given the large variation in the measurements (due to heterogeneity of the fibrous cell wall material of the stem), the differences are not significant.

DISCUSSION

We have over-expressed a putative CBM of a potato expansin in tobacco, to investigate whether expansin's domain II can bring about changes in the cell wall on its own. Results of the investigation are discussed with respect to its binding specificity and its ability to alter cell wall structure.

Homology modelling of the 3-D structure of the C-terminal domain II of the expansins has been possible with the resolution of the 3-D structure of Phl P2 (De Marino et al., 1999). The modelled three-dimensional structure of the putative potato expansin CBM showed that most of its conserved aromats were located on the surface. The patch of three "aromatic" residues on one side and the linear strip of another three aromatic residues on the opposite side of the protein are consistent with the 3-D structure of CBMIII (CBM of the cellulosome subunit S1 from *Clostidium thermocellum* (1NBC) as resolved by Tormo et al. (1996), which showed a groo-

ve and a planar cleft at opposite sides of the protein. They proposed that the planar cleft binds crystalline cellulose while the groove binds the amorphous single cellulose chain. Shoseyov et al. (2006) speculated further that the parallel arrangement of the binding grooves would enable the protein to slide between two cellulose chains and facilitate the disruption of the hydrogen bonding between adjacent chains in a wedge-like action. The structure of the C-terminal domain II of Lol PI (EXPB) had been modelled by others using the Phl P2 as template (Barre and Rouge, 2002; Cosgrove, 2000a). That model also revealed that most of the conserved aromatic residues were exposed around a linear strip and a groove (Barre and Rouge, 2002).

Even though our *in planta* characterization of the putative CBM is not conclusive, with respect to its binding specificity, it is interesting to note that the effect of over-expression of the protein was mainly observed in the cellulose- and xylan-rich vascular tissue. We observed an abnormal enlargement (radial expansion) of cells of the xylem and phloem fibre cells in the high expressing transgenic tobacco plants. These observations are consistent in the three independent high-expressing lines examined, suggesting that they are related to the expression of the transgene rather than to other factors. Apart from cell enlargement, the cell walls of the vascular tissue were observed to be thinner in the transgenics as compared to the control plants. In contrast to our observations, which were restricted to the vascular tissue, a heterologous expression of high levels of a recombinant cucumber expansin in transgenic tomato was reported to cause altered expansion of the cortical and epidermal cells of the stems (Rochange et al., 2001). These localised cellular events in both cases seem to support the view that the expansin proteins have tissue-specific roles in addition to cell expansion (Brummell et al., 1999; Chen and Bradford, 2000; Cosgrove et al., 1998). It should be noted that the EST clone that was used for this investtigation was obtained from an EST library of potato petioles and leaves. These matured organs are rich in secondary walled vascular tissue. The expansin CBM that was targeted to the tobacco cell walls may have preferentially interacted with the secondary wall of the vascular tissue. This interaction might disrupt cellulose-hemicellulose networks, culminating in wall thinning, which in turn might lead to wall weakening and hence abnormal cell enlargement (Kutschera, 1990). The foregoing might explain the more obvious phenotypes in the vascular tissue than in any other tissue of the stem of the transgenic tobacco plants.

In spite of the cellular phenotypes, transgenic tobacco plants exhibit normal morphology and development. However, in contrast to our observation of normal growth phenotype of the transgenic plants, high levels of over-expression or heterologous expression of expansin genes in transgenic plants was shown to lead to impaired growth phenotypes (Choi et al., 2003). A sense expres-

sion of AtEXP10 was also shown to enhance leaf growth in the transgenic *Arabidopsis* plants (Cho and Cosgrove, 2000). Similarly, local expansin expression within the meristem was shown to induce the process of leaf formation (Pien et al., 2001). It is worth noting, however, that all these reports had used the complete expansin protein, while only the CBM has been used in this work. This suggests that both domains of expansins are needed to bring about developmental changes in the plant.

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