

Original Article

Overexpression of carbohydrate binding modules (CBMs) of *Cellulomonas fimi* glucanase B (CenB) in tobacco modifies cellulose in the cell wall and xylem cell enlargement

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Abstract

Carbohydrate-binding modules (CBMs) have been shown to alter the structural architecture of plant cell walls. They have the potential of altering the characteristics of fibers in the cell wall. *Cellulomonas fimi* glucanase B belongs to family 2 CBM and it has been characterized as interacting with cellulose. However, its potential for altering plant cell walls and for modulating plant growth has not been explored. In this study, the expression of CBMs in tobacco plants was evaluated. There were no observable changes in the growth of the transgenic plants compared with the wild type. Histological examinations of transgenic plant stems revealed that xylem cells were bigger than the wild type. Moreover, the cellulose fiber lengths of transgenic plants were longer than the wild type. The results indicated that the family 2 CBM has the potential for modification of cellulose in the cell wall and inducing xylem cell expansion.

Keywords: plant cell walls, cell expansion, cellulose fiber, cellulose synthesis, carbohydrate binding modules

1. Introduction

Carbohydrate-binding modules (CBMs) appear to have the potential of modulating plant cell walls and enhancing plant growth. CBMs are commonly found within cell wall degrading enzymes as discrete domains separate from catalytic modules. CBMs are non-catalytic only domain in a protein molecule with the capacity to bind to soluble and crystalline carbohydrates (Shoseyov, Shani, & Levy, 2006). Currently, these modules are classified into 74 families in CAZy databases (Boraston, Bolam, Gilbert, & Davies, 2004). A CBM within the cellulase complex of *Clostridium cellulovorans* (CBPA) was the first one to be confirmed to be able to enhance plant growth. Purified CBPA protein was shown to

have a positive effect on the elongation of pollen tubes of peach and on the roots of Arabidopsis seedlings. In addition, CBPA protein was found to increase the rate of cellulose synthase in *Acetobacter xylinum* (Shpigel, Roiz, Goren, & Shoseyov, 1998). The expression of CBPA in transformed potato plants led to accelerated plant growth (Safrá-Dassa *et al.*, 2006). It was postulated that the binding of CBMs to polysaccharides in the plant cell wall might interfere with cellulose deposition and modify the interaction of cellulose with hemicellulose, thereby altering cell expansion and modifying plant growth (Darley, Forrester, & McQueen-Mason, 2001).

The CBMs used for expression in plants are currently divided into two groups. In the promiscuous group, CBMs bind to many types of polysaccharides in the plant cell wall. In contrast, in the non-promiscuous group, CBMs bind to specific polysaccharides, importantly cellulose (Charnock *et al.*, 2002). For example, promiscuous CBM29-1-2 from *Piromyces equi* binds hemicellulose polysaccharides including

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xyloglucans, xylans, and mannans and some insoluble cellulose (Freelove *et al.*, 2001), while non-promiscuous CBM *Cellulomona fimi*, coded as CBM2b-1-2, binds specifically to xylan and non-crystalline cellulose (Bolam *et al.*, 2001). In studies to date, it was reported that plants expressing both promiscuous and non-promiscuous CBMs were able to modulate the cell-wall structure and the development of transgenic tobacco plants (Bolam *et al.*, 2001). CBM-labelling studies indicated that promiscuous CBM binds indiscriminately to every tissue of the wild-type tobacco stem whereas binding of non-promiscuous CBMs was restricted to vascular tissue (Obembe *et al.*, 2007a). However, the binding of non-promiscuous CBMs was confined to specific sites in the cell wall or cell type, and this may have fewer effects on the wall as a whole compared to the use of promiscuous CBMs (Obembe *et al.*, 2007a).

The general phenotype of a plant expressing CBMs is characterized by accelerated growth, enhanced cell enlargement, and increased or decreased cellulose polymerization. These responses occur in many types of plants, for example tobacco (Shani *et al.*, 1999), poplar (Levy, Shani, & Shoseyov, 2002), and potato (Safra-Dassa *et al.*, 2006). Expression of CBMs derived from *C. cellulovorans* bacteria had growth promoting effects in potato and increased xylem cell size in tobacco (Obembe, Jacobsen, Visser, & Vincken, 2007b). The growth promoting effects could be obtained when the *cbm* gene was expressed at a low level (Safra-Dassa *et al.*, 2006), whereas high expression of this gene caused delayed growth and collapsed tissues (Obembe *et al.*, 2007a) and even cell death (Safra-Dassa *et al.*, 2006).

CBM endoglucanase B from *C. fimi* was classified into family 2 CBM. CBMs in this family have been shown to bind to crystalline cellulose (Gilkes, Warren, Miller, & Kilburn, 1988), insoluble chitin (Nakamura *et al.*, 2008), and xylan (Black, Hazlewood, Millward-Sadler, Laurie, & Gilbert, 1995). These modules can bind to cellulose and, in the case of cellulase CenA from *C. fimi*, have activity against crystalline forms of the polysaccharide (Gilkes, Warren, Miller, & Kilburn, 1988). In the case of CBM2b-1-2 from *C. fimi*, xylanase 11A can bind in a way that restricts growth of the vascular tissue and altered cell expansion can be observed only in xylem cells.

In this study, CBM cellulase CenB from *C. fimi* was examined for its effects on tobacco. Since the binding of CBMs to cellulose is a key factor for modulating plant cell wall development, CBMs have the potential to induce accelerated plant growth. In this study, the cauliflower mosaic virus (CaMV) 35S was used as a promoter to control the expression of the CBM. The CBM was fused at the N-terminal with the Arabidopsis expansion 4 (*AtEXP4*) signal peptide. Transgenic tobacco, using the *CaMV35:CBM* construct were generated. The transgenic plants that were produced were grown in greenhouse conditions and subsequently analyzed for growth by measuring plant height and weight. Finally, the transgenic plant was investigated by xylem cell sizes, cellulose fiber length, and cellulose content.

2. Materials and Methods

2.1 Gene cloning and plasmid constructions

In order to achieve CBM expression, a 306 bp coding sequence was cloned from the *C. fimi* CenB gene

(NCBI number M64644.1; at the base position 3110-3416 of the coding sequence) before insertion into pCAMBIA1305.1, where it was fused with the coding sequence of the *AtEXP4* signal peptide, under the control of a CaMV35S promoter. CBM was amplified for gDNA of *C. fimi* CenB gene by PCR using Cen1 (5'-GGTACCTGCACGGTCGTGTACTCG-3') and Cen2 (5'-CACGTGTCAGCCGCAGACCTCACCG-3') primers, cloned into a pGEM-T vector, and confirmed by sequencing. A verified clone was amplified by PCR using Cen1 and Cen2 primers and the product was purified using column purification. For CBM with a C-terminus HA tag (hemagglutinin tag), the amplification was performed using Cen1 and Cen2-HA (5'-CACGTGTCAGCGTAATCTGGAACATCGTATGGGTAGCCGCAGACCTCACCGTTC-3'; HA tag coding sequence is underlined). The amplified CBM fragment and pCAMBIA1305.1 carrying the CaMV35S promoter *AtEXP4* signal peptide were then digested using *NcoI* and *PmlI*, purified using gel electrophoresis and a gel purification column and ligated together using T4 ligase (New England Biolabs) at 4 °C overnight before transforming into *Escherichia coli*. Positive clones were selected by PCR screening. Plasmids were verified by sequencing before being used for transforming *Agrobacterium tumefaciens* strain EHA105.

2.2 Gene expression in transgenic plants

Real-time PCR was carried out to investigate the transcription levels of the *cbm* gene in the transgenic tobacco plants. Total RNA was extracted from leaf tissue from the transgenic plants (300 mg fresh weight) using TriPure Isolation Reagent (Roche) according to the manufacturer's protocol. RNA was treated with DNaseI to eliminate any DNA before column purification. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). The same amount of 5 µg RNA per sample was used to prepare cDNA using a ProtoScript II First Strand cDNA synthesis kit (New England Biolabs) following the manufacturer's protocol with 20-mer-encored oligo-dT and 18-mer-encored oligo-dT in a total 20 µL reaction.

Quantitative realtime RT-PCR was performed in a 25 µL reaction mixture comprised of SsoFast™ EvaGreen® Supermix (Bio-Rad, USA) with 1 µL of cDNA and 0.5 µM for each of *cbm2* gene specific primer: 5'-TCACGGGGTCCGGTGAAGAT-3' and 5'-GTGACCGTCGTCCCGTCT-3' forward and reverse, respectively or actin specific primer: 5'-CTGGCATTGCAGATCGTATGA-3' and 5'-GCGCCACCACC TTGATCTT-3' forward and reverse, respectively (Faize *et al.*, 2010). The reaction used the CFX96 Touch Real-Time PCR Detection System (Bio-RAD, USA) with the following conditions: 3-min denaturation step at 98 °C; 40 cycles of 5 s denaturation at 98 °C; 30 s annealing at 60 °C; and 30 s synthesis at 72 °C. Analysis of the relative gene expression data using real-time quantities of PCR were carried out as described by Livak and Schmittgen (2001).

2.3 Protein extraction and western blot analysis

Total protein was extracted from 10 mg fresh leaves from 60-day-old transgenic tobacco plants and from the wild type by grinding in liquid nitrogen and incubating in 500 µL of 6X SDS buffer at 95 °C for 10 min. After incubation the sample was centrifuged at 12,000 rpm for 5 min, the

supernatant containing the soluble protein was collected, and the protein sample was then separated with 12% (w/v) SDS-PAGE polyacrylamide gels (Mini-Protean II apparatus, Bio-Rad, USA). The separated proteins were electroblotted onto a nitrocellulose membrane in 192 mM glycine, 25 mM Tris base and 20% (v/v) ethanol, pH 8.3. Blocking against non-specific binding was performed for 1 h with 3% (w/v) skim milk in Tris-buffered saline (TBS) (10 mmol/L Tris, 150 mmol/L NaCl, pH 7.5). The membrane was incubated with a 1:1000 dilution of the primary antibody HA-tag rabbit and secondary anti-rabbit IgG Ap-linked antibody (Cell Signaling Technology) in TBS solution containing 3% (w/v) skim milk for 1 h. The membrane was washed three times at 10 min each in TBS buffer. Washed blots from the nitrocellulose membrane were developed using 50 mL alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM MgCl₂) with 33 μ L 5-bromo-4-chloro-3-indolyl-phosphate and 66 μ L nitro blue tetrazolium. Finally, the membrane was incubated at room temperature.

2.4 Monitoring the growth of the transformed plants in the greenhouse

To monitor the growth of the transformed tobacco plants, three replications per transgenic line and their controls were grown in aseptic conditions for 40 days and then in a greenhouse for 90 days. The stem length, fresh weight, and dry weight were measured at 60 days and 90 days after the planting in the greenhouse to evaluate the growth. The data were analyzed using a one-way analysis of variance (ANOVA) ($\alpha=0.05$) and means were compared using the Tukey method.

2.5 Light microscopy

Three individual plants per transgenic tobacco line and three wild-type plants as the control were used for microscopic examination. Three transgenic lines were used per high expresser, low expresser, and very low expresser. Stem samples were taken from the third internode from the top of the plant of 60-day-old plants. Stem sections 1 mm thick were fixed in 50% formaldehyde-alcohol-acetic acid. The samples were then washed. After washing they were stained with Safranin O and examined under a bright field microscope. Xylem vessels were measured using the Axio Vision SP1 program (Carl Zeiss Microscopy). Measurements were taken of 360 xylem cells.

The dry stems of transgenic line were prepared using a modified maceration method (Franklin, 1945). First, a 1 cm long section of dry stem was added to the solvent containing equal volumes of glacial acetic acid (99.7%) and hydrogen peroxide (30%). The mixture was then incubated at 100 °C for 6 h. After maceration, the stem sample was washed several times using deionized water until the pH was neutral. Finally, the stem was added into the deionized water (4 mL) and mixed by a vortex mixer. The cellulose fibers were stained with chorazol black. The cellulose fiber length was measured by ZEISS Primo Star Microscopy and AxioVision Rel. 4.8 (ZEISS Microscopy, Germany). In each plant line, more than 250 fibers from three different plants were measured and the data were analyzed.

2.6 Cellulose content of plant raw material

Three replicates per line of dry stem from nine transgenic plants and the wild type control were ground to a fine powder by a blender. For each isolation, 1 g of the stem material was used to determine the cellulose content using the forage fiber analysis method (Van Soest, Robertson, & Lewis, 1991) and the data were analyzed.

3. Results and Discussion

3.1 Expression of *cbm* gene in transgenic tobacco plants

To investigate the function of CBM (CenB), a construct was made for the *cbm* gene (306 base pairs), which was part of the endoglucanase CenB from *C. fimi*. It was linked with the *AtEXP4* signal peptide which was inserted into a pCambia1305.1 expression vector containing the *CaMV35S* promoter (Figure 1). The resultant sequence was used to transform leaves of tobacco. After 2 months on Murashige and Skoog medium supplemented with hygromycin B, nine independent transgenic tobacco lines were regenerated after *Agrobacterium*-mediated transformation. The presence of *cbm* gene in these plants was confirmed by PCR (data not shown).

Quantitative mRNA analysis was used to detect expression of the introduced *cbm* gene in the transgenic tobacco plants. Three classes of transcript expression (high, low, and very low) were determined (Figure 2). Five of the CBM transgenic plants were classified as high expressers (CBM1, CBM2, CBM4, CBM8, and CBM9), three as low (CBM3, CBM5, and CBM7) and one as very low (CBM6 which was only 0.0001-fold of its actin). The quantitative mRNA analysis disclosed that the highest *cbm* transcript level was in the plant line CBM8. These results showed that the levels of CBMII mRNA had no lethal effect on the transgenic plant. In

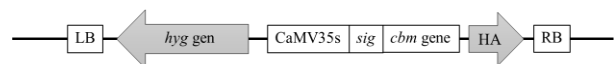


Figure 1. Structure of the chimeric binary plant expression cassette (pCambia1305.1) used for tobacco transformation.

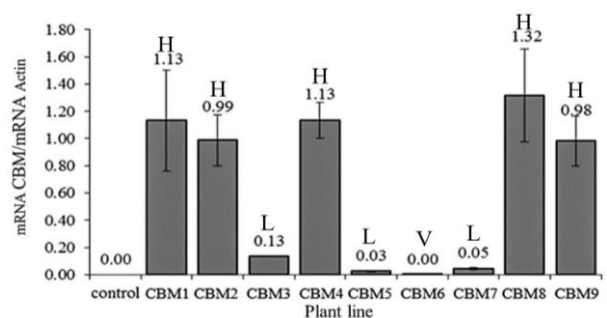


Figure 2. RT-PCR analysis of CBM2-expression in the transformed and control tobacco plants. Values of *cbm* transcript level are presented as *cbm*:*actin* ratios. Three classes of transformation (high = H, low = L and very low = V) are apparent from the data presented. Error bars indicate \pm SE.

contrast, Safra-Dassa *et al.* (2006) found that the levels of CBMIII mRNA affected the survival rates of the transformed plants in that study as high levels of expression were lethal.

From the quantitative mRNA and western blot analysis, it was shown that these constructs could be expressed in the transgenic tobacco plants. Further, it was possible to purify the CBM protein from some of the transgenic tobacco plants. It is important to note that the expression level of *cbm* transcripts was also reflected in the translation level of CBM protein. The expression level of *cbm* transcripts was high in high expressers because the western analysis was sensitive enough to detect the CBM protein. However, in low expressers, the western analysis was not sensitive enough to detect the CBM protein (Figure 3). Previously, attempts at purifying the protein of CBMIII with hexa-histidine tag were not successful because the hexa-histidine tag was removed from the fusion protein in the plant (Safra-Dassa *et al.*, 2006). However, when the HA-tag was used, it could successfully detect the CBM protein.

3.2 Monitoring the growth of the transformed plant in a greenhouse

The growth of transgenic lines was monitored in detail by measuring stem height, fresh weight, and dry weight of leaves, stems, and the whole plants at age 60 and 90 days after planting. No significant alteration ($P > 0.05$) in growth was observed (data not shown). There was also no visible morphological or developmental change in any of the transgenic plant lines that were assessed and compared with the controls. The average plant height of the transgenic plants at 60 days old was 20.79 ± 0.34 cm which was comparable with the 17.72 ± 1.60 cm average height for the controls. No changes in plant development with regard to stem elongation and flower formation were observed (Figure 4). These results indicated that the expression of CBM from *C. fimi* CenB had no effect on plant growth and development in any of the transgenic plants. The CBM of endoglucanase B from *C. fimi* contains a unique sequence which was demonstrated to be in the family 2 CBM. This cellulose binding domain (CBD) protein is responsible for cellulose binding (Shani *et al.*, 1999). Similar phenotypes were also previously described when expressing *CBM2b-1-2* of xylanase 11A from *C. fimi* in *N. tabacum* (Obembe *et al.*, 2007a; Obembe, Jacobsen, Visser, & Vincken, 2007b). These same results might be because of the CBM of endoglucanase B in this investigation and the *CBM2b-1-2* of xylanase 11A are both in the same family of CBM. Both belong to the promiscuous family 2 CBM. In contrast, the report of Safra-Dassa *et al.* (2006) showed that enhanced growth occurred in transgenic potato plants expressing a non-promiscuous bacterial CBM3 in family 3. The fact that the experiments were performed on different plant species may also have contributed to the different observations. Nonetheless, these data indicate that different effects on plant growth and plant morphology are likely to depend on the type of CBM.

3.3 Light microscopy; Expression of *cbm* enhanced xylem vessel cell sizes and cellulose fiber length in tobacco

Light microscopy was used to examine the xylem length to determine whether the transgenic stems had larger

xylem vessel cells than the control plants. Almost 56.7% of the xylem vessel cells of the wild-type control were grouped into the small size class ($500\text{--}1500 \mu\text{m}^2$), whereas less than 35% of the xylem vessel cells of the CBM1 (CBM high expresser) plant were grouped in this small class. In contrast, the opposite was observed in the large size class ($>1500 \mu\text{m}^2$), i.e. less than 50% of the xylem vessel cells from the control stems were grouped into the large size class compared to more than 60% of those from the CBM1 plant (Figure 5). It can be inferred from these results that the xylem vessel cell sizes of the transgenic tobacco plant stem are larger than those of the xylem vessel cell sizes of wild-type control stems (Figure 6).

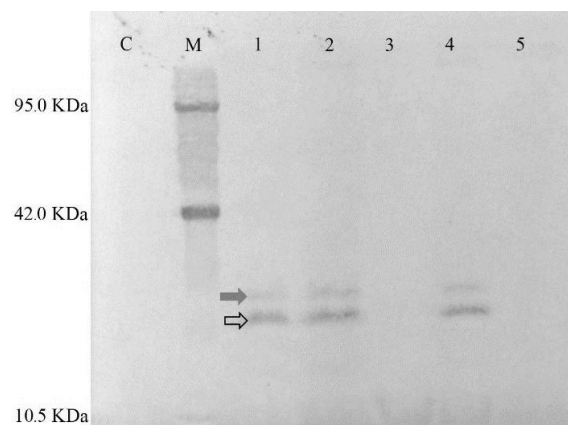


Figure 3. Analysis of leaf protein extracts from the transgenic plants using anti-HA antibody showed bands corresponding to CBM protein: the gray arrow = 13.74 kD with signal peptide; the white arrow = 11.44 kD without signal peptide; C = wild type control; M = Marker; 1-5 = transgenic tobacco lines CBM 1-5. The protein was only detectable in those lines that showed high expression.

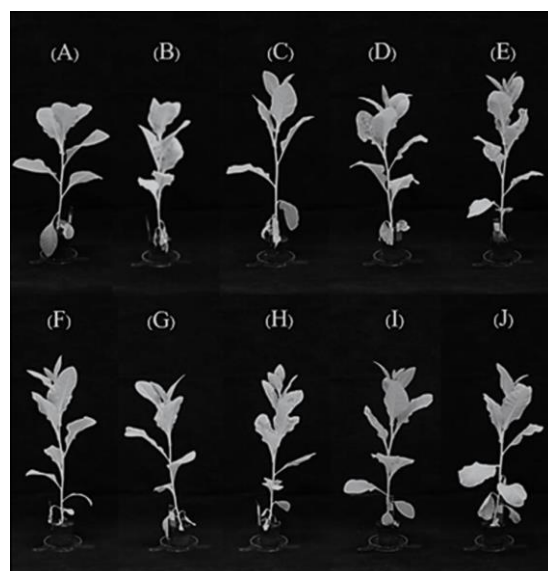


Figure 4. Control and transgenic tobacco plant 60 days after plantings: (A) wild type control; (B) CBM1; (C) CBM2; (D) CBM3; (E) CBM4; (F) CBM5; (G) CBM6; (H) CBM7; (I) CBM8; and (J) CBM9. No differences in morphology or development were observed.

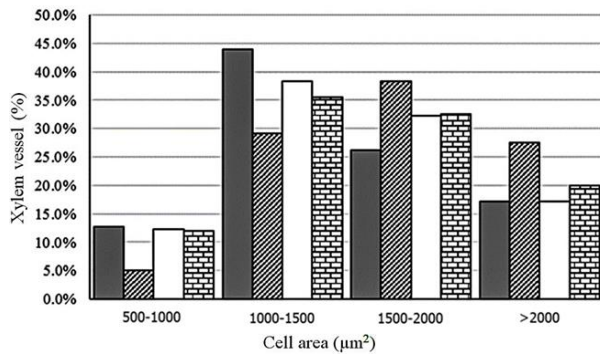


Figure 5. Size of xylem vessel cells in stems of the transgenic and wild-type tobacco plants. Grey bars represent the wild-type control. Diagonal bars represent CBM1 (high expresser). Open bars represent CBM3 (low expresser). Horizontal brick bars represent CBM 5 (very low expresser).

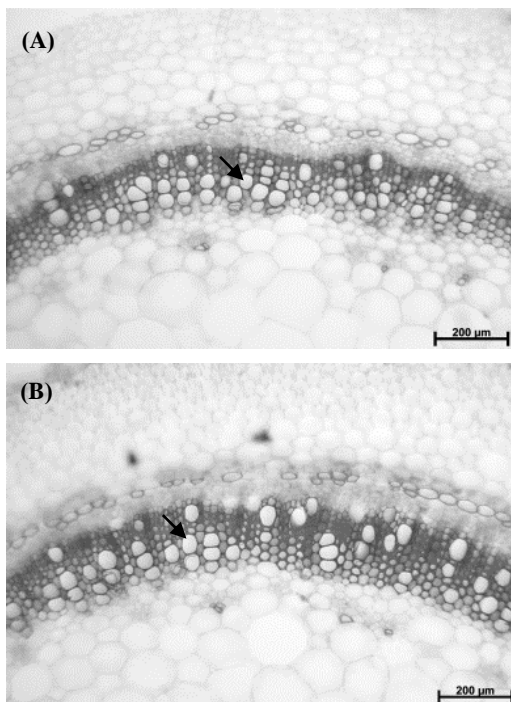


Figure 6. Cross section of representative stems from transgenic and control plants. Section of wild type control (A) and transgenic line 1, high expresser of tobacco CBM (B) were stained with Safranin O. A and B show the vascular tissue, xylem cells of the stem sections of the control and the CBM high expresser, respectively. Average xylem cross-sectional area is larger in the transformed plant stems. The black arrow = xylem.

Examination of cellulose fiber length showed that the cellulose fibers in the transgenic lines were significantly longer than those from the wild type controls (Figure 7). The highest cellulose fiber length was in plant lines CBM1 (Figure 8) and CBM2. It is clear that CBM increased the cellulose fiber length in the transgenic tobacco plants.

3.4 Analysis of cellulose content of stem plant raw material

Some previous reports indicated that CBD may play a role in the structure of the cell wall by affecting cellulose synthesis (Zenoni *et al.*, 2004). The cellulose content of the stem plant cell walls of the CBM-expressing plants and in the control plants was determined. The cellulose content of the transgenic plants was generally higher than the control plants (Figure 9). However, these differences were not statistically significant.

The results from this study showed abnormal enlargement of the xylem vessel cells in the high CBM expressing transgenic tobacco plants. Significantly, the increase

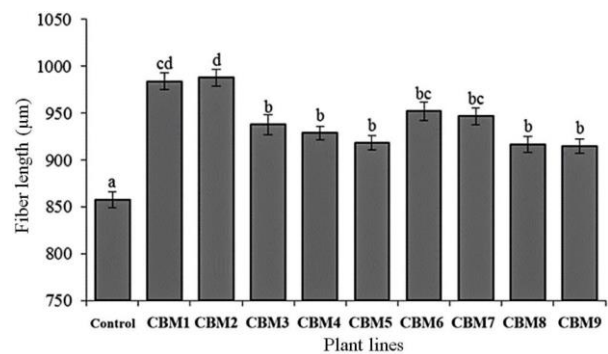


Figure 7. Cellulose fiber length of wild type control and transgenic plant lines: Error bars indicate ±SE. Identical letters mean that differences between values are not significant at P=0.05 using Tukey.

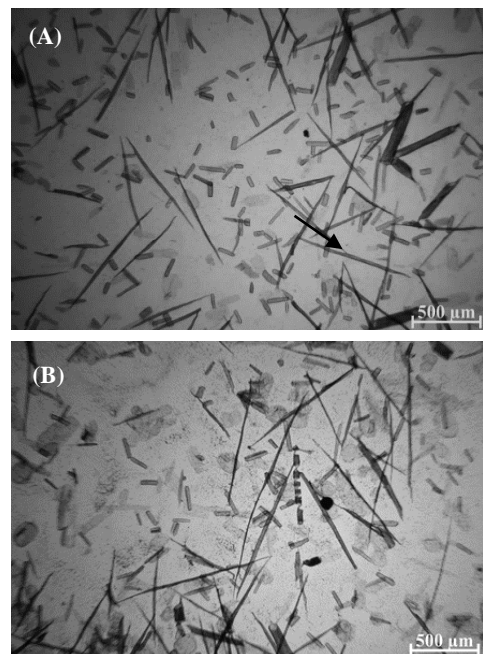


Figure 8. Cellulose fiber length: (A) wild type control; (B) transgenic plant lines CBM 1. The black arrow = cellulose fiber.

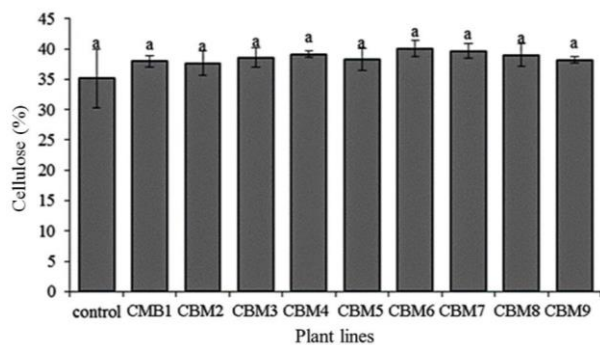


Figure 9. Composition of cellulose in stem of wild type control and transgenic plants. Differences in cellulose content between the control and transformed plants were not statistically significant. Error bars indicate \pm SE. Identical letters mean that the differences between values are not significant at $P=0.05$ using Tukey.

in cell size in tobacco did not result in an increase in plant growth. In a previous study, the expression of *CBM29-1-2* showed increases in cell size, but those with expression of *CBM2b-2-2* showed only slight increases (Obembe *et al.*, 2007a). The xylem cell enlargement of the CBM expressing plants in this study might be attributable to the influence of CBM on the networks of plant cell wall polysaccharides. CBMs affect cellulose synthesis by interaction with cellulose at an early stage of crystallization. CBM enables cellulose polymerization to proceed freely, and it removes feedback inhibition caused by the crystallization process (Shpigel, Roiz, Goren, & Shoseyov, 1998; Levy, Shani, & Shoseyov, 2002; Obembe *et al.*, 2007a). Moreover, binding of CBM to cellulose may interfere with the cellulose-xyloglucan interaction during cell growth. It is assumed that such interference will enhance cell wall loosening, resulting in smaller mechanical restraint of developing cell wall turgor-pressure-imposed expansion of the cell wall (Obembe *et al.*, 2007a). The effect of CBM on cellulose synthesis was observed by the increased cellulose fiber length and percent cellulose content in CBM expression in all of the transgenic plants. However, the increase in percent cellulose content in transgenic plants did not significantly impact growth.

4. Conclusions

The results of our investigation suggest that expression of the CBM protein of endoglucanase B from *C. fimi* under the control of *CaMV35S* promoter in tobacco might be suitable to induce cellulose fiber modification but that it is not a potential candidate for use in *in planta* modification of plant growth and development.

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