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Full Length Research Paper

Expression of the C-terminal family 22 carbohydrate-binding module of xylanase 10B of *Clostridium themocellum* in tobacco plant

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Carbohydrate-binding modules have been shown to alter plant cell wall structural architecture. Hence, they have the potential application of being used to engineer the plant to produce tailor-made natural fibers in the cell wall. The *Clostridium thermocellum* xylanase, Xyn10B, contains two CBMs that belong to family 22 (CBM22). The C-terminal CBM22-2 of the glycoside hydrolase (GH) 10 had been characterized to interact with xylan, a major hemicellulosic component in the secondary cell wall of plants. In this work, the expression of the CBM22-2 in transgenic tobacco plants was evaluated. Histological examinations of the transgenic stems did not reveal marked cell wall phenotype. In addition, there were no observable changes in the height or the appearance of the transgenic plants expressing the CBM22-2 module. The results indicate that the family 22 carbohydrate binding module is not a potential candidate for use in *in planta* modification of the cell wall.

Key words: Carbohydrate binding module, xylan binding protein, cell wall modification, cellulose-hemicellulose network, *Nicotiana tabacum.*

INTRODUCTION

Plant cell walls are dynamic, highly complex extracyto-plasmic matrix consisting of various polysaccharides that interact with each other, through extensive and closely-knit networks (O'Neill and York, 2003). As a result of this intricate structure of the cell wall, access of cell wall degrading enzymes to their various target polysaccha-rides is restricted (Hall et al., 1995). In order to overcome this challenge, plant cell wall hydrolytic enzymes have evolved a complex modular architecture comprising a catalytic module appended to one or more non-catalytic carbohydrate-binding modules (CBMs) (Boraston et al., 2004). The CBMs do not only bind the enzyme to the polysaccharides, thereby increasing the local concen-tration of the enzyme and leading to more effective degradation (Bolam et al., 1998; Gill et al., 1999) but some are also involved in substrate disruption and therefore, making the substrate more available for the catalytic module (Din et al., 1994; Southall et al., 1999).

The work of Din et al. (1991) indicated that CBMs have potential in modification of natural fiber. They showed that modification of the polysaccharide structure could be achieved with isolated CBMs. It has been shown that CBMs can modulate cell wall structure (Obembe et al., 2007a) as well as growth of transgenic plants (Kilburn et al., 2000; Obembe et al., 2007b; Quentin, 2003; Safra-Dassa et al., 2006; Shoseyov, 2001). The expression of the CBM3 gene from *Clostridium cellulovorans* in potato plants (Safra-Dassa et al., 2006) and poplar tree plants (Shoseyov, 2001) was reported to enhance the growth rate of the transgenic plants. Similarly, Kilburn et al. (2000) reported that transgenic plants expressing a mannan-recognising CBM27 exhibited enhanced plant growth. However, a reduction in growth rate as well as delayed plant development was reported for transgenic tobacco plants expressing the promiscuous CBM29-1-2 from a non-catalytic protein1, NCP1, of the *Piromyces equi* cellulase/hemicellulase complex (Obembe et al., 2007b).

The family 22 CBMs are often found with a family 10 glycoside hydrolase catalytic module (GH10) (Devillard et

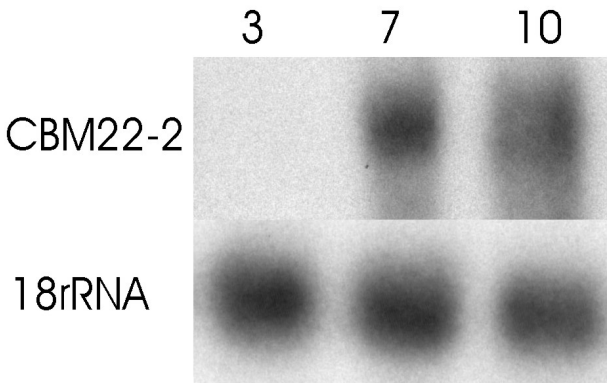

Figure 1. Transcript expression analysis of the CBM22-2 gene in transgenic tobacco leaves. A differential transcript expression pattern is shown in the upper panel with the representative of each class in the transgenic line CBM22-2. Lines 10, 7 and 3 represent high, low and non-expressers, respectively. Lower panel shows RNA blots for the ribosomal RNA internal control with comparable intensities.

al., 2003; Feng et al., 2000; Fontes et al., 1995; Sunna et al., 2000). Xylanase Xyn10B from *C. thermocellum* is a modular enzyme that contains two of such family 22 carbohydrate binding modules, one at the N-terminal (CBM22-1) and the other at the C-terminal (CBM22-2) of the GH10 (Fontes et al., 1995). The C-terminal CBM22-2 was shown to interact with purified xylan through its Trp 53, Tyr 103, and Glu 138 (Xie et al., 2001). It is of interest to note that members of the family CBM22 also exist in *Arabidopsis* and *Populus* (Henrissat et al., 2001; Suzuki et al., 2002; Kallas, 2006).

It had been previously suggested that specific CBMs, such as CBM22-2 could be used especially to prevent the binding of xylan to cellulose during biosynthesis, with a view to producing cellulose fibers with enhanced properties (Obembe, 2006). In this work, the effect of expressing the CBM22-2 in transgenic tobacco plants was investigated.

MATERIALS AND METHODS

Preparation of transformation construct

The CBM22-2 construct was prepared by amplifying the gene fragment from pET21a, recombinant plasmid vector used for cloning and expression in Escherichia coli (Xie et al., 2001). The polymerase chain reaction (PCR) was performed using primers that included BamHI and SmaI recognition sites (5'-cgggatccgttcatgctactaactgttgtt-3' and 5'-tcccccgggccttttaattaattgcgtcatcgt-3'; the BamHI and SmaI sites, respectively, are underlined). The three bases highlighted in bold type are the stop codon. The amplified fragment of the tandem CBM22-2 was digested with BamHI and SmaI (Invitrogen, The Netherlands) and cloned into a similarly digested binary vector pGreen7k (Hellens et al., 2000) as described (Obembe et al., 2007a). Upstream of the expansion fragment in the binary vector, two more gene fragments. 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 tag was placed so as to facilitate affinity purification of the expressed protein. Cloning of these upstream components was done as described by Obembe et al. (2007a). 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 transit peptide, hexa-histidine tag, and expansion CBM were in frame.

Tobacco transformation, regeneration and growth

The CBM22-2 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 as described by Obembe at al. (2007a). Thirteen transformed plantlets were transferred to the green house to generate mature plants.

RNA gel blot analysis of transgenic plants

Total RNA was isolated from 3 to 5 g transformed *in-vitro* shoots as described elsewhere (Kuipers et al., 1995). Separation of RNA on agarose gel, blotting onto a nylon membrane, hybridizing with labeled probes of CBM22-2 and the exposure of radioactively labeled to imaging films were carried out as described (Obembe et al., 2007a).

Light microscopy

Three individual plants per transgenic tobacco line and three wild-type plants, as control, were used for microscopic examination. Three transgenic lines were used (two high expressers and one low expresser). Stem sampling, fixing, embedding, sectioning and examination under a bright field microscope were carried out as described (Obembe et al., 2007a).

RESULTS AND DISCUSSION

It has been shown previously that CBMs has the capabilities of being used to engineer the plant to produce tailor-made natural fibers in the cell wall (Obembe et al., 2007b). To express the *C. thermocellum*-derived xylan-binding module in tobacco plants, I cloned the CBM22-2 gene into a binary plasmid pGreen 7k, the expression of which was driven by the CaMV 35S promoter. The plasmid pGreen 7 k without the CBM gene was used as a vector control. Thirteen antibiotic-resistant tobacco transformants carrying the CBM22-2 transgene were regenerated and transferred to the green house. Total RNA from leaves of the transgenic plants was isolated to analyze the expression level of the *C. thermocellum* CBM22-2 in each transgenic line by Northern blot analysis using tobacco 18S ribosomal RNA gene as a control. Varying hybridization intensities with the transgenic plants revealed differential expression of the CBM22-2 gene in the individual plants. Based on the level of expression, transgenic plants have been categorized into three classes as high, low and none expressers; the respective representatives for the classes are shown in Figure 1. Five plants each were classified as high and

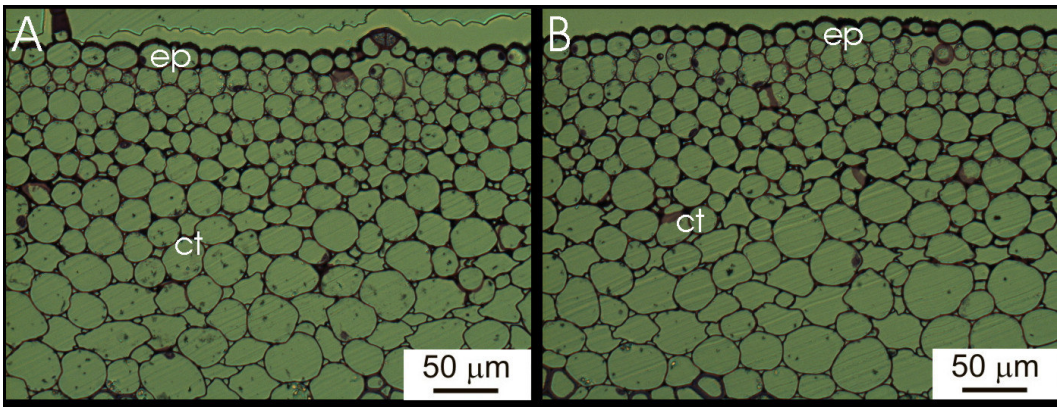

Figure 2. Cross sections of representative transgenic and control stems. Sections of the transgenic line 10, high expresser of CBM22-2 module (A) and empty pGreen7k control (B) were stained with toluidine blue. Figures 2 A and B show the cortex of the sections of the transgenic and the control plants, respectively. Figures reveal no marked difference in the cortex of the two stem sections. Ct = cortex, ep = epidermis. Scale bar = 50 µm.

low expressers, whereas three plants were classified as non-expressers. It should be noted that these non-expressers may include plants with very low RNA expression, which could not be detected. Attempts at purifying the protein of the CBM22-2 module using affinity purification with hexa-histidine tag were not successful for reasons previously discussed (obembe et al., 2007b).

The transgenic plants appeared normal in that there was no morphological or developmental change when compared to the pGreen 7k vector control. The average plant height of the transgenic plants expressing the CBM22-2 at maturity was 73 cm, which was comparable to 75 cm average height for the pGreen 7k vector control plants. There was also no particular trend in plant development with respect to stem elongation and flower formation.

To determine whether there were cellular events resulting from the expression of the CBM22-2 in the tobacco plant, light microscopic examinations of stem sections stained with toluidine blue was made. Three representatives of each expression class were examined. Figure 2 shows representative micrographs of the cortex of the transgenic and the control stems, which reveal no marked cellular phenotype in the transgenic stems. One argument for the unexpected result could be that there had been some sort of post-translation silencing. This is especially suggestive, since the transcript expression analysis was good. This argument apparently appears strong when one considers the evidence of xylan-binding attribute of the CBM22-2, on which the present investigation was premised. The evidence was based on *in vitro* binding and site directed mutagenesis studies (Charnock et al., 2000; Xie et al., 2001). However, recent immunohistochemical evidence provided by McCartney et al. (2006) gives new insight as to why the transgenic plants did not exhibit marked cellular phenotypes. In their approach, recombinant CBMs, which were fusion proteins of the CBMs and histidine tags, were used as molecular probes to localize their ligands *in planta*. The detection of the polysaccharide-CBM interactions within the plant cell wall was facilitated with the use of anti-histidine antibodies against the histidine tags. The investigation revealed that CBM22-2 has restricted secondary cell wall recognition; in that whereas it bound effectively to pea stem, it only bound weakly to flax and tobacco stems (McCartney et al., 2006). This likely reflects the context of xylan in different cell walls (McCartney et al., 2006), especially since CBM22-2 can readily recognize isolated xylan polymers and also because xylan was found to be present in all of the secondary cell walls of the plant materials used, as indicated by the binding of other xylan-binding CBMs.

Conclusion

Although it is well established that CBMs are capable of altering plant cell wall structure and could be potentially used as a tool for tailoring cellulose fiber with enhanced properties for specific industrial applications, it is strongly suggested that their appropriateness for such a task should be empirically determined. This study suggests that the CBM22-2 of xylanase 10B of the *C. thermocullum* might not be suitable for use in *in planta* modification of cellulose fiber.

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