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The carbohydrate-binding module (CBM)-like sequence is crucial for rice CWA1/BC1 function in proper assembly of secondary cell wall materials

Kanna Sato,[1,*] Sachiko Ito,[1] Takeo Fujii,[1] Ryu Suzuki,[1] Sachi Takenouchi,[1] Satoshi Nakaba,[2] Ryo Funada,[2] Yuzou Sano,[3] Shinya Kajita,[1] Hidemi Kitano[4] and Yoshihiro Katayama[5]

[1]Graduate School of Bio-Applications and Systems Engineering; Tokyo University of Agriculture and Technology; Koganei; [2]Graduate School of Agriculture; Tokyo University of Agriculture and Technology; Fuchu; [3]Graduate School of Agriculture; Hokkaido University; Sapporo; [4]Bioscience and Biotechnology Center; Nagoya University; Nagoya; [5]College of Bioresource Sciences; Nihon University; Fujisawa, Kanagawa Japan

We recently reported that the *cwa1* mutation disturbed the deposition and assembly of secondary cell wall materials in the cortical fiber of rice internodes. Genetic analysis revealed that *cwa1* is allelic to *bc1*, which encodes glycosylphosphatidylinositol (GPI)-anchored COBRA-like protein with the highest homology to Arabidopsis COBRA-like 4 (COBL4) and maize Brittle Stalk 2 (Bk2). Our results suggested that CWA1/BC1 plays a role in assembling secondary cell wall materials at appropriate sites, enabling synthesis of highly ordered secondary cell wall structure with solid and flexible internodes in rice. The N-terminal amino acid sequence of CWA1/BC1, as well as its orthologs (COBL4, Bk2) and other BC1-like proteins in rice, shows weak similarity to a family II carbohydrate-binding module (CBM2) of several bacterial cellulases. To investigate the importance of the CBM-like sequence of CWA1/BC1 in the assembly of secondary cell wall materials, Trp residues in the CBM-like sequence, which is important for carbohydrate binding, were substituted for Val residues and introduced into the *cwa1* mutant. CWA1/BC1 with the mutated sequence did not complement the abnormal secondary cell walls seen in the *cwa1* mutant, indicating that the CBM-like sequence is essential for the proper function of CWA1/BC1, including assembly of secondary cell wall materials.

Key words: carbohydrate-binding module, COBRA-LIKE, CWA1/BC1, glycosylphosphatidylinositol-anchored protein, secondary cell wall formation

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*Correspondence to: Kanna Sato; Email: ikanna@cc.tuat.ac.jp

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The main function of carbohydrate-binding modules (CBMs) of microbes and plants is to attach the enzyme to a variety of cell surface glycans and thereby increase the local concentration of substrate, leading to more efficient catalysis.[1-4] Almost all CBMs studied to date contain surface-exposed aromatic rings, which have been shown to be the main sites of interaction with polysaccharides. These residues form face-to-face hydrophobic stacking interactions in which a Trp residue or ring of a Tyr residue interacts with the non-polar face of a sugar ring.[5-9] CBMs have been classified into families based on amino acid sequence similarity. Currently, there are 59 defined families of CBMs and these CBMs display substantial variation in ligand specificity (http://www.cazy.org/Carbohydrate-Binding-Modules.html). Among these CBM families, the large family of CBM2 has been further classified into two subgroups, CBM2a and 2b, which have shown to bind cellulose and xylan, respectively.[10-12] CBM2a characteristically possess three exposed Trp residues,[13] whereas CBM2b have two Trp residues,[14] which are conserved among the CBM2 members (**Fig. 1A**).

Our recent study showed that the defect of the rice *CWA1/BC1* (*CELL WALL ARCHITECTURE 1*/*BRITTLE CULM 1*) gene induced abnormal secondary cell wall formation with amorphous and bulky structures at the cytoplasm side and *CWA1/BC1* encodes one of COBRA-like glycosylphosphatidylinositol (GPI)-anchored proteins, which are specifically found in plants, suggesting that CWA1/BC1 regulates

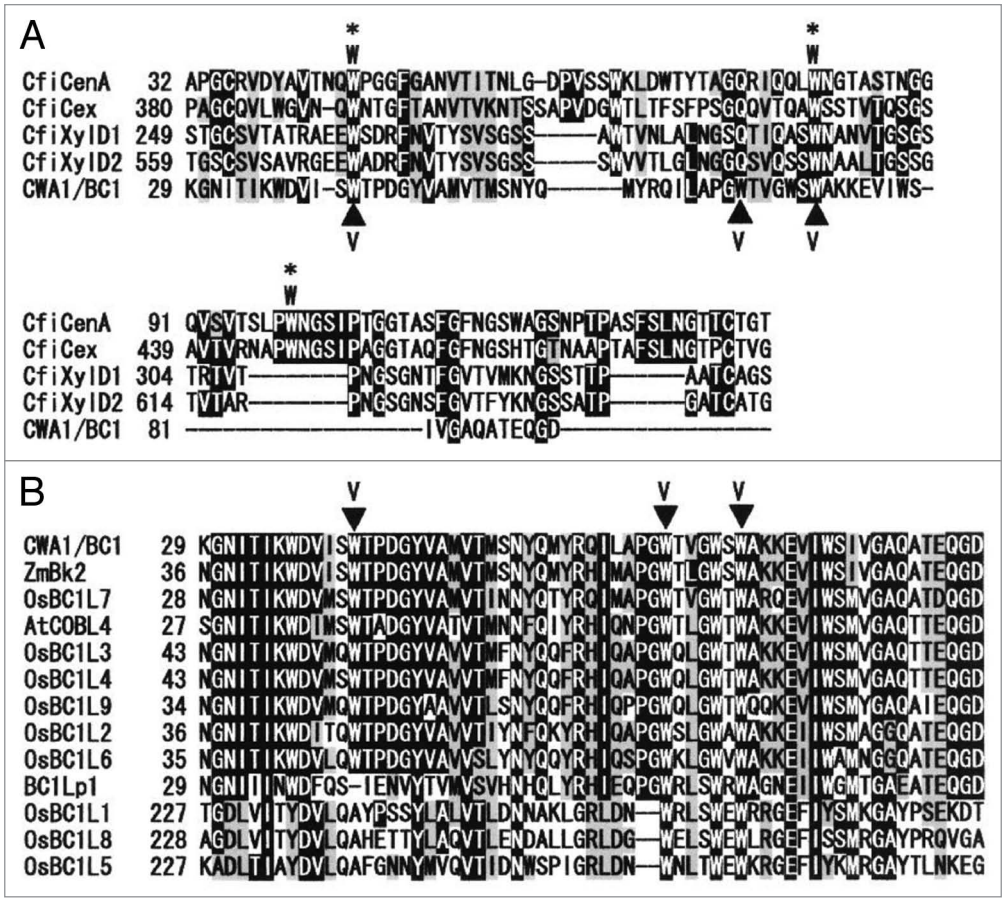

Figure 1. Sequence alignment of the CBM-like sequence of CWA1/BC1, the BC1L proteins and bacterial CBM2 members. (A) Sequence alignment between bacterial CBM2a, 2b and CWA1/BC1. The three surface-exposed Trp residues of CBM2a members are indicated by asterisks and W. The CBM sequences of CBM2a are: CfiCenA, *Cellulomonas fimi* endo-1,4-glucanase; CfiCex, *C. fimi* exo-beta-1,4-glucanase. Those of CBM2b are: CfiXylD1, *C. fimi* endo-1,4-beta-xylanase D; CfiXylD2, *C. fimi* endo-1,4-beta-xylanase. CWA1/BC1 shows weak similarity to CBM2, and some Trp residues are conserved with bacterial CBM2 members. (B) Sequence alignment of CWA1/BC1, the BC1L proteins and CWA1/BC1 orthologs, *Zea maiz* Brittle Stalk 2 (ZmBk2) and *Arabidopsis thaliana* COBRA-LIKE 4 (AtCOBL4). The CBM-like sequence of CWA1/BC1, especially the Trp residues, is highly conserved among the analyzed sequences. Substituted Trp (W) residues to Val (V) in CWA1/BC1 are indicated by closed triangles. Numbers at the left are the positions of the amino acids in each protein, with gaps (dashes) included to maximize alignments. Identical and similar amino acids are shaded and gray, respectively.

assembly of secondary cell wall materials in rice sclerenchyma. Furthermore, several reports have shown that the N-terminus of rice CWA1/BC1 and other COBRA-like GPI-anchored proteins in Arabidopsis (12 members) and maize Brittle Stalk 2 (Bk2) share weak similarity to a CBM2 in several bacterial cellulases.[15,16] However, the importance of CBM-like sequence in COBRA family members has not been clarified. To investigate the nature of CWA1/BC1, we compared the CBM-like sequence in rice CWA1/BC1 with bacterial CBM2, 10 members of the BC1-like (BC1L) protein in rice and CWA1/BC1 orthologs, Arabidopsis COBL4 and maize Bk2. Furthermore, we constructed three-point mutated CWA1/BC1, in which three conserved Trp residues in CBM-like sequence were substituted to Val residues (CWA1/BC1$^{W\rightarrow V}$), and introduced it into the *cwa1* mutant to evaluate the necessity of the CBM-like sequence for proper function of CWA1/BC1. We discuss a putative explanation, based on our results, of the properties and possible functions of CWA1/BC1.

The Trp Residues in CBM-Like Sequence in CWA1/BC1 is Conserved between Bacterial CBM2, CWA1/BC1 Orthologs and BC1-Like Proteins

A database search for the amino acid sequence of CWA1/BC1 has shown weak similarity of the amino acids from 30 to 91 with those of bacterial CBM2 (www.sbg.bio.ic.ac.uk/phyre/index.cgi).[17] To investigate the relationship between this CBM-like sequence within CWA1/BC1 and bacterial CBM2, sequence alignment was analyzed to determine their homology. CBM2a and 2b have been shown to bind cellulose and xylan, respectively.[10-12] The three exposed Trp residues of CBM2a[13] and the two on CBM2b[14] were conserved among the CBM2 members (**Fig. 1A**). The CBM-like sequence of CWA1/BC1 shows weak similarity with those of CBM2 members and a few Trp residues were conserved among the CWA1/BC1 and CBM2 members (**Fig. 1A**). To confirm whether the CBM-like sequence of CWA1/BC1 is conserved among CWA1/BC1 orthologs (Arabidopsis COBL4 and maize Bk2) and BC1L proteins in rice, N-terminal amino acid sequences of these

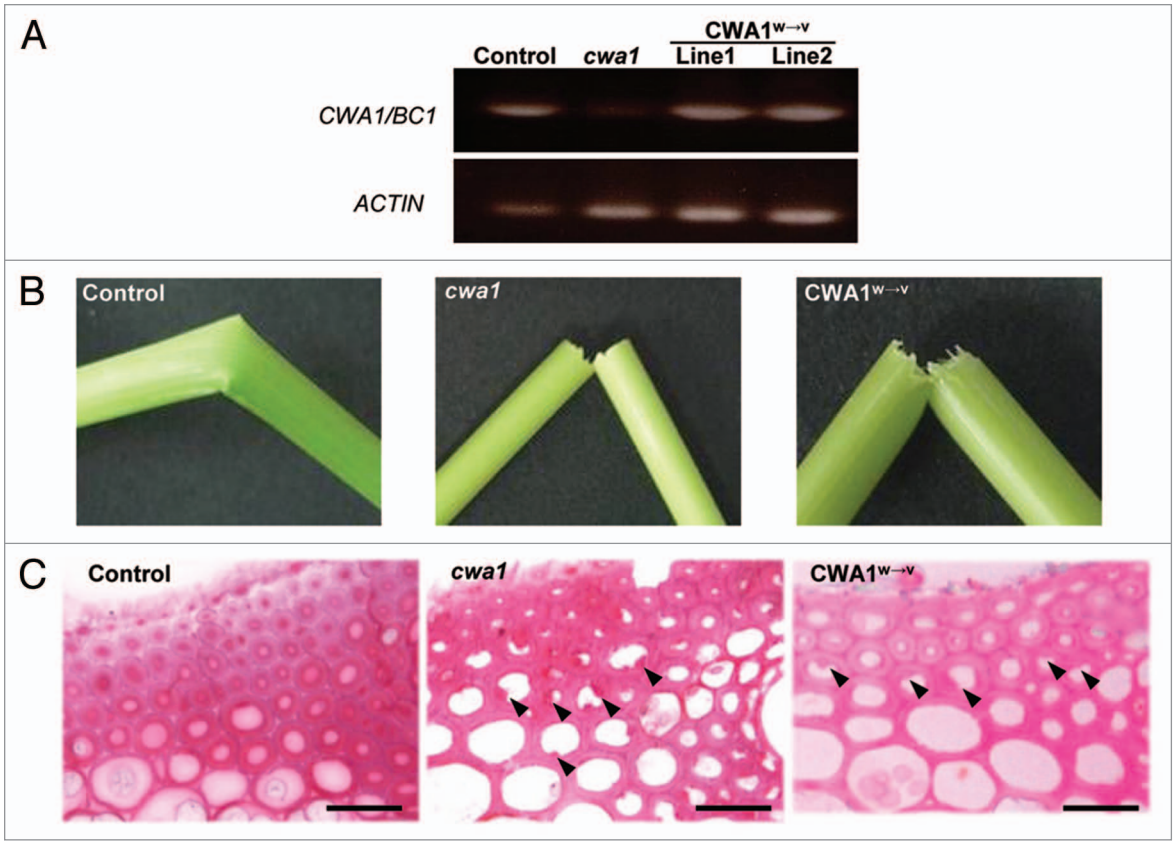

Figure 2. Evaluation of transgenic and *cwa1* mutant plants. (A) Expression of the *CWA1/BC1* gene in the transgenic *cwa1* plant expressing the wild-type *CWA1/BC1* gene (control), *cwa1* mutant and transgenic *cwa1* plants expressing mutated *CWA1/BC1*$^{W\rightarrow V}$ (CWA1$^{W\rightarrow V}$, lines 1 and 2). Expression of the *CWA1/BC1* gene is rarely detected in the *cwa1* mutant, whereas obvious expression is detected in the transgenic *cwa1* plants introduced with each construct. (B) Mature internodes of transgenic *cwa1* plants expressing wild-type *CWA1/BC1* (Control), the *cwa1* mutant (*cwa1*) and transgenic *cwa1* plant expressing mutated *CWA1/BC1*$^{W\rightarrow V}$ (CWA1$^{W\rightarrow V}$) after hand flexing. The brittle culm phenotype of transgenic *cwa1* plant was rescued by expression of wild-type *CWA1/BC1* (Control), but not by expression of *CWA1/BC1*$^{W\rightarrow V}$ (CWA1$^{W\rightarrow V}$). (C) Transverse section at the cortical fiber of mature internodes from transgenic *cwa1* plant expressing wild-type *CWA1/BC1* (Control), the *cwa1* mutant (*cwa1*) and transgenic *cwa1* plant expressing mutated *CWA1/BC1*$^{W\rightarrow V}$ (CWA1$^{W\rightarrow V}$). The transgenic *cwa1* plant expressing *CWA1/BC1*$^{W\rightarrow V}$ has abnormal and amorphous cell wall structures (arrowheads) as seen in the *cwa1* mutant. Scale bars = 20 μm.

proteins were compared. The CBM-like sequence of each protein shared high homology; most of the Trp residues were especially conserved (**Fig. 1B**). Therefore, it is possible that the CBM-like sequence in CWA1/BC1 plays a role in carbohydrate binding, which may be essential for the CWA1/BC1 function in assembling secondary cell wall materials in rice sclerenchyma.

The Trp Residue in the CBM-like Sequence is Essential for Proper Function of CWA1/BC1

To evaluate the importance of conserved Trp residues in the CBM-like sequence of CWA1/BC1, we constructed mutated CWA1/BC1 with three substituted Trp residues to Val residues (designated as CWA1/BC1$^{W\rightarrow V}$) as shown in **Figure 1**. For constructing CWA1/BC1$^{W\rightarrow V}$, we used QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and cloned the sequence into the binary vector, pBI121Hm. The mutations were confirmed by sequencing. Wild-type *CWA1/BC1* or mutated *CWA1/BC1*$^{W\rightarrow V}$ genes were expressed in the *cwa1* mutant plants under the control of the 1973-bp *CWA1/BC1* promoter. Transgenic *cwa1* plants are produced by *Agrobacterium tumefaciens*-mediated transformation.[18] To confirm the expression of these introduced genes in the *cwa1* plants, we performed RT-PCR of the *CWA1/BC1* gene. The expression of the *CWA1/BC1* gene in the *cwa1* mutant was hardly detected (**Fig. 2A**), whereas transgenic *cwa1* plants introducing *CWA1/BC1* (Control) or *CWA1/BC1*$^{W\rightarrow V}$ (CWA1$^{W\rightarrow V}$) expressed each gene in their young internodes (**Fig. 2A**), confirming that the transgenic *cwa1* plants express each introduced gene.

The *cwa1* plants showed a brittle culm phenotype (**Fig. 2B**) and abnormal and amorphous secondary cell wall structures in the cortical fiber of rice internodes (**Fig. 2C** and arrowheads). In contrast, the brittle phenotype (**Fig. 2B** and control) and abnormal secondary cell wall structures in the cortical fiber (**Fig. 2C** and control) of transgenic *cwa1* plants were rescued by introduction of wild-type *CWA1/BC1*. On the other hand, the *cwa1* plants introduced with *CWA1/BC1*$^{W\rightarrow V}$ still exhibited the brittle phenotype and abnormal secondary cell walls with uneven thickness and a rough surface on the cytoplasmic side of the cortical fiber as seen in the *cwa1*

mutant (**Fig. 2B and C**, CWA1$^{W\rightarrow V}$). These results strongly suggest that the Trp residues in the CBM-like sequence of CWA1/BC1 is essential for its function, especially for proper assembly of secondary cell wall materials at appropriate sites. Therefore, at least one of the three mutated Trp residues should have the capacity to binding polysaccharides in the cell walls, such as cellulose or hemicelluloses in microbes.[8,14]

How Does CWA1/BC1 Regulate Secondary Cell Wall Assembly via CBM?

GPI-anchored proteins including the COBRA family are predicted to be localized in the outer surface of the plasma membrane or cell wall.[19,20] Arabidopsis COBRA protein has also been shown to be abundantly localized in the cell wall.[21] Therefore, it is possible that the proper assembly of secondary cell wall materials by the CWA1/BC1 requires binding between the CBM-like sequence within CWA1/BC1 and cell wall polysaccharides such as cellulose and/or hemicelluloses. Furthermore, it was proposed that the nascent cellulose microfibrils are oriented by binding to a scaffold of cell wall polysaccharides or plasma membrane proteins.[22] Our previous study revealed that *CWA1/BC1* is expressed before the start of secondary cell wall formation. Consequently, CWA1/BC1 may play an important role at the initial stage of secondary cell wall formation and act as a scaffolding protein for regulating the orientation of cellulose microfibrils and/or reserving space for secondary cell wall thickening between the plasma membrane and the cell wall or between cell wall polysaccharides, by binding cell wall polysaccharides via the CBM-like sequence. In order to elucidate the role of CWA1/BC1 in the assembly of secondary cell wall materials, the target polysaccharide of the CBM-like sequence and cellular localization of the CWA1/BC1 protein need to be investigated.

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