***Title***

Expression of a fungal *laccase* fused with a bacterial cellulose-binding module improves the enzymatic saccharification efficiency of lignocellulose biomass in transgenic *Arabidopsis thaliana*

***Authors***

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***Abstract***

Delignification is effective for improving the saccharification efficiency of lignocellulosic biomass materials. We previously identified that the expression of a fungal laccase (Lac) fused with a bacterial cellulose-binding module domain (CBD) improved the enzymatic saccharification efficiency of rice plants. In this work, to evaluate the ability of the Lac-CBD fused chimeric enzyme to improve saccharification efficiency in a dicot plant, we introduced the chimeric gene into a dicot model plant, *Arabidopsis thaliana*. Transgenic plants expressing the *Lac-CBD* chimeric gene showed normal morphology and growth, and showed a significant increase of enzymatic saccharification efficiency compared to control plants. The transgenic plants with the largest improvement of enzymatic saccharification efficiency also showed an increase of crystalline cellulose in their cell wall fractions. These results indicated that expression of the Lac-CBD chimeric protein in dicotyledonous plants improved the enzymatic saccharification of plant biomass by increasing the crystallinity of cellulose in the cell wall.

***Keywords***

Laccase; cellulose-binding module; saccharification; cell wall composition; lignocellulosic biomass.

## *Introduction*

Plant lignocellulosic biomass resources have great potential as materials alternative to fossil resources for biofuels and biorefinery products. However, these materials must undergo enzymatic saccharification before they can be used in bio-applications, and this process can be quite costly, since lignocelluloses are complex conjugates of crystalline cellulose, hemicellulose, and lignin. Our high dependency on petroleum-based production has led to global problems such as global warming and environmental pollution, and thus it is important to reduce this dependency by developing reproducible methods of bioresource-based production. To replace at least a portion of the products from petroleum, the production of fuels and chemicals from plant biomass by a bioconversion and chemo-bioprocess called biorefinement has been reported (Brethauer and Studer, 2015). Sugars and starch have long been used as feedstocks for the production of fuels and chemicals via microbial fermentation, but this practice is increasing the prices of foods and forage crops due to their competing use as food and feedstock, and thus the development of methods to use lignocellulosic materials as a feedstock for biorefinery operation is being promoted globally.

To use lignocellulosic materials as a feedstock for biorefinery production, cellulose and hemicelluloses must be isolated and digested into fermentable sugars. However, cellulose molecules bind each other via hydrogen bonds to form a rigid crystalline structure, and cellulose is covered by and linked tightly with lignin, and these structural characteristics make cellulose hard to digest. The crystallinity and lignin content of lignocellulosic biomass are determinants of its saccharification efficiency, because they limit the ability of cellulase to access the beta-1,4-glycoside bonds in cellulose molecules (Palonen *et al.*, 2004). For this reason, a variety of physico-chemical pretreatment methods are used to decrystallize and delignify the lignocellulosic biomass to improve its saccharification efficiency, such as steam explosion, sulfuric acid treatment and alkaline cooking (Li *et al.*, 2010; Zhu *et al.*, 2008; Vinzant *et al.*, 1997; Laureano-Perez *et al.*, 2005; Jeoh *et al.*, 2007; Min *et al.*, 2011; Papa

68 *et al.*, 2012). The pretreatment of lignocellulosic biomass is expected to be a low energy-input and
69 cost-effective process, provided that plants with low lignin content or low cellulose crystallinity can
70 be developed without any negative influences on their growth (such as decreases in the biomass by
71 dwarfing), tolerance to pathogens or physiological strength.

72 It has been reported that the downregulation of genes involved in lignin biosynthesis leads to a
73 reduction in lignin content (Chen and Dixon, 2007). However, this downregulation also causes male
74 sterility, a decrease in tolerance to pathogens, and inhibitions to growth such as dwarfing (Bonawitz
75 and Chapple, 2010; Sattler and Funnell-Harris, 2013; Malinovsky *et al.*, 2014), so it is important to
76 develop an alternative method to avoid these negative effects on plant physiology. As for decrystallinity,
77 there has been no report on the molecular breeding of plants with low crystalline cellulose, but in our
78 previous study we considered that the expression of a bacterial cellulose-binding module (CBM),
79 which was fused with a fungal laccase, in rice plants might reduce the crystallinity of cellulose
80 (Furukawa *et al.*, 2013). CBMs are proteins which bind cellulose molecules specifically via the
81 hydrogen bonds and the hydrophobic interaction between the glucopyranoses in cellulose molecules
82 and the aromatic amino acids in CBM polypeptides (Tormo *et al.*, 1996; Shoseyov *et al.*, 1992). It has
83 been reported that a recombinant protein produced using E. coli binds cellulose fibers (Goldstein *et al.*,
84 1993; Ciolacu *et al.*, 2010), and CBMs have been shown to promote the decrystallization of crystalline
85 cellulose (Din *et al.*, 1991; Abramsona *et al.*, 2010).

86 In our work, we are focusing on the development of saccharification-efficient plants which can be
87 used as a feedstock for biorefinery operation. We previously reported that the expression of a fungal
88 laccase fused with a bacterial cellulose-binding module improved the saccharification efficiency of
89 rice plants (Furukawa *et al.*, 2013). In this study, we apply the same chimeric gene constructs to a
90 dicot model plant, *Arabidopsis thaliana*, and evaluate its potential effect on the saccharification
91 efficiency of dicot plants.

## *Materials and Methods*

### Construction of laccase fused with a bacterial cellulose-binding module

The chimeric genes of laccase and a bacterial cellulose-binding module were constructed in a previous study (Furukawa *et al.*, 2013). For expression in Arabidopsis, the chimeric genes were introduced into the *Nde*I and *Sac*I sites of pRI201-AN (Takara-bio, Shiga, Japan) (Fig. 1). Binary vectors into which either laccase or a cellulose-binding module had been inserted were prepared as a control (Fig. 1).

### Transformation of *Arabidopsis thaliana* and plant cultivation condition

*Arabidopsis thaliana* col-0 was used as the plant material. The binary plasmids were transformed into *Rhizobium radiobacter* (former called as *Agrobacterium tumefaciens*) EHA101, and the plant transformations were performed by the floral-dip method (Clough and Bent, 1998). T0 seeds were sown on MS agar (0.8% w/v) medium (Murashige and Skoog, 1962) with 50 μg/mL kanamycin, followed by screening for kanamycin-resistant T1 plants. The resistant plants were then transferred to pots and grown in a growth chamber (LH-350SP; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 22° C under a 16 h light/18 h dark cycle.

### Quantitative reverse transcriptional polymerase chain reaction (qRT-PCR)

A rosette or cauline leaf was sampled and RNA was extracted from the sample using an RNeasy Plant Mini Kit (QIAGEN). RT reactions were performed using ReverTra Ace RT master mix (TOYOBO). For qRT-PCR, the reaction volume of 10 μL contained 5 μL of Thunberbird™ SYBR® qPCR Mix (TOYOBO), and 0.2 μmol/L each of the primer pair, 1x ROX, and an aliquot of the template.

116 The real-time PCR reactions were performed with an ABI PRISM 7900 (Applied Biosystems)
117 according to the following step-cycle program: pre-incubation at 90ºC for 10 min, followed by 40
118 cycles consisting of denaturation at 95ºC for 0.5 min, and annealing and extension at 60ºC for 1 min
119 each. We used two different types of transgene-specific primer pairs, i.e., the *LAC* primer pair 5'-
120 CCGCGGACCTCTCTGTTATC -3' & 5'- TGAACGTGTAGTTGGGGTCG -3' and the *CBD* prime
121 pair 5'- TGACCATGCTGGTGCATTAT -3' & 5'- ATGTTGGGCTTGCTGTTTCT -3', plus a primer
122 pair for the internal control gene,, i.e., the *ACT2* primer pair 5'- TGATGCACTTGTGTGTGACAA -
123 3' & 5'- ACAATGGGACTAAAACGCAAA -3'. The transgene expressions were calculated as the
124 ratio of the quantity of transgene to that of *ACT2*.
125
126 **Preparation of cell wall fraction**
127 The aerial part of each transgenic line was dried in an oven at 105ºC for 20 h and cut into segments
128 of less than 1-cm length. Cell walls were prepared by a modified version of the method described by
129 Ishii *et al.* (2001). First, the aerial segments were suspended in 80% ethanol and shaken vigorously for
130 5 min at room temperature. After centrifugation, the pellet was re-suspended in 80% ethanol and
131 shaken vigorously for 5 min. Then the collected pellet was suspended in 95% ethanol, shaken for 5
132 min, and recovered. The resultant pellet was suspended in 100% ethanol and shaken vigorously for 5
133 min. After centrifugation of the suspension, the alcohol-treated pellet was extracted with chloroform-
134 methanol (1:1, v/v) to remove lipids and then treated with dehydrated acetone and dried. The remaining
135 alcohol insoluble residues were used as cell wall fractions in this study.
136
137 **Enzymatic saccharification analysis**
138 The prepared cell wall fractions were dried for 2 h at 105ºC. The dried samples were ground and
139 sieved with a 200 mesh ( $\phi$ 77 μm) stainless filter. The sieved powder prepared from each transgenic

A. thaliana was used for the following enzymatic saccharification experiment. Fifteen mg of the samples were suspended gently with 1.0 mL of 50 mM citrate buffer (pH 4.8) containing 14 μL of Celluclast 1.5L (60 FPU; Sigma Aldrich, St. Louis, MO) and 6 μL of Novozyme 188 (64 pNGU; Sigma Aldrich), and incubated at 50ºC with gentle rotation. After 6, 24 and 48 h of incubation, each reaction mixture was centrifuged and the supernatant was collected. The glucose concentration in each supernatant was measured with a Biosensor BF-5 (Oji Scientific Instruments, Hyogo, Japan).

**Cell wall composition analysis**

The cell wall material was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1 h at 121ºC to yield a non-crystalline polysaccharide fraction, including matrix polysaccharides such as hemicelluloses and pectin. The TFA insoluble residues were hydrolyzed with 72% (w/w) $H_2SO_4$ for 2 h at room temperature, after then the acid concentration was diluted to 3% and further incubated for 2 h at 100°C to yield the crystalline cellulose fraction (Selvendran and O'Neill, 1987; Edashige and Ishii, 1997; Sato *et al.*, 2001). The residual material was dried and weighed as the lignin (Klason lignin) fraction. The amount of total sugar in each fraction was measured by the phenol-$H_2SO_4$ method (DuBois *et al.*, 1956) using a glucose standard. The composition of neutral sugar (glucose, xylose, galactose, arabinose, mannose, rhamnose and fucose) in the non-crystalline polysaccharide fraction was analyzed by the alditol acetate method (Blakeney *et al.*, 1983).

## *Results and discussion*

### Molecular breeding of a transgenic *Arabidopsis* plant expressing a laccase fused with a bacterial cellulose-binding module

In our previous study, we reported that expression of the laccase-CBD fusion protein in rice plants

164 improved the efficiency of their enzymatic saccharification. In the present study, to evaluate the
165 feasibility of inserting a chimeric gene into dicot plants, we applied the same chimeric gene constructs
166 to a dicot model plant, *Arabidopsis thaliana*. Then, we conducted samples from transgenic
167 *Arabidopsis* plants expressing *laccase*-alone (designated TF009), *CBD*-alone (designated TF010), and
168 a *laccase-CBD* (*Lac-CBD*) chimeric gene (designated TF012 and TF013), respectively (Fig. 1). The
169 two *Lac-CBD* fusion genes exhibited a difference in the hinge sequence between laccase and *CBD*, but
170 showed no differences in the enzymatic saccharification or cell wall compositions (Furukawa *et al.*,
171 2013). In $T_1$ generation, we obtained 19 independent lines for TF009; 6 independent lines for TF010;
172 and 11 and 17 independent lines for TF012 and TF013. As we discussed above, the negative impact
173 of laccase expression on the growth of plants may have decreased the transformation efficiency of this
174 construct. Four plants from each line were selected from each line and used for the following
175 experiments. The expression levels of these transgenes are shown in Figure 2. In our previous study,
176 we obtained no transgenic plants expressing *CBD*-alone, and discussed that higher expression of *CBD*
177 would be lethal (Furukawa *et al.*, 2013). In addition, lignin deficiency is commonly known to result
178 in the dwarfing and growth inhibition of plants (Bonawitz and Chapple, 2010). In this study, we
179 obtained transgenic plants expressing *CBD*-alone and *Lac*-alone, respectively, but the expression levels
180 of *CBD* and *Lac* were lower than that of *Lac-CBD* in the transgenic plants expressing *Lac-CBD*.
181 These results supported the idea that the expression of *Lac-CBD* was a lesser burden on plants than the
182 expressions of *CBD*-alone and *Lac*-alone.
183
184 ***Lac-CBD* expression improved enzymatic saccharification of the cell wall fraction from**
185 ***Arabidopsis***
186 Because it was possible that serious phenotypes occurred in transgenic plants with high expression
187 of *laccase*, we conducted enzymatic saccharification assay and cell wall component analyses in T1

hemizygous generation. Remarkable phenotypic abnormality was observed in all of the T1 transgenic plants expressing *Lac*-alone, *CBD*-alone, and *Lac-CBD* chimeric genes. Cell wall fractions were extracted and degreased/dehydrated cell wall fractions were treated with amylase. The efficiency of enzymatic saccharification was evaluated by treatment with an enzyme cocktail consisting of Celluclast 1.5L (Sigma-Aldrich) and Novozyme 188 (Sigma-Aldrich) for 48 h. During the enzymatic treatments, the eluted glucose concentrations at 6, 24, and 48 h after treatment and the total eluted glucose contents at 48 h after treatment were quantitated (Fig. 3). We found that some of the transgenic plants expressing the *Lac-CBD* chimeric gene showed a significant increase in glucose elution by enzymatic saccharification. In particular, the amounts of eluted glucose from TF012 L-4, TF012 L-7, TF013 L-2, and TF013 L-7 were 2-7%, 5-8%, 24-26%, and 29-43% higher than those from the vector control plants, respectively (Fig. 3). In contrast, no significant difference in enzymatic saccharification efficiency was observed in transgenic plants expressing *Lac*-alone or *CBD*-alone (Fig. 3). On the other hand, poor correlation was shown between expression levels of *Lac-CBD* and enzymatic saccharification levels (Fig.2 and Fig3). We suggest that the poor correlation was possibly caused by differences in the organs and age of the plants for each analysis, i.e. fresh rosette leaves and mature stems were used for expression analysis and saccharification analysis, respectively. These results indicated that expression of the *Lac-CBD* chimeric gene improved the enzymatic saccharification of lignocellulosic biomass in transgenic *Arabidopsis thaliana*.

**_Lac-CBD_ expression increases the crystalline cellulose component of the cell wall in _Arabidopsis_**

To evaluate the mechanism underlying the improvement of enzymatic saccharification efficiency of *Lac-CBD*-expressing transgenic plants, we conducted an analysis of the cell wall components of TF012 L-7 and TF013 L-7 plants. The contents of the cell wall components in transgenic plants are shown in Figure 4. The crystalline cellulose contents of plants expressing *Lac-CBD* were 9.9-12.0% higher

than that of the control plant, and this difference was statistically significant (Fig. 4). Because crystalline cellulose is a substrate suitable for the attachment of saccharification enzymes, it was suggested that the increase in cellulose in the transformed plants contributed to the improvement of enzymatic saccharification. Increases in crystalline cellulose in the cell wall have been reported in hybrid aspens with repressed expression of genes related to lignin synthesis, e.g., *4-coumarate-CoA ligase* (*4CL*) and *Catechol-O-methyltransferase* (*COMT*), and increases of crystalline cellulose have been suggested to be a response to a decrease in physical strength due to a decrease in lignin (Hu *et al.*, 1999) (Jouanin *et al.*, 2000). In transgenic plants expressing *Lac-CBD*, an 8.6-9.4% reduction of lignin content in the cell wall was observed, although this change was not statistically significant (Fig. 4). We previously showed that crystalline cellulose contents were decreased in transgenic rice plants expressing *Lac-CBD* (Furukawa *et al.*, 2013). In that study, we observed an increase of silicon components in the cell wall of transgenic rice plants expressing *Lac-CBD*, which were suggested to reinforce the physical strength in response to the decrease in cell-wall strength caused by a reduction of lignin (Furukawa *et al.*, 2013). It is interesting that *Lac-CBD* expression improved the efficiency of enzymatic saccharification of both *Arabidopsis* and rice plants, despite the opposite changes in crystalline cellulose contents.

There was no significant difference in non-crystalline polysaccharide content in the cell wall of transgenic plants expressing *Lac-CBD* compared to the control (Fig. 4). Next, we conducted analyses of the monosaccharide component of the non-crystalline polysaccharide fluxes and evaluated the structural changes in the matrix polysaccharides in transgenic plants expressing *Lac-CBD* (Fig. 5). Comparison of the content of neutral sugars in non-crystalline polysaccharide between transgenic plants expressing *Lac-CBD* and the control plants revealed a significant difference in glucose and xylose contents (Fig. 5). An increase of xylose content and reduction of glucose contents in non-crystalline polysaccharide in the *Lac-CBD*-expressing plants indicated that the ratio of the change in

branched chain sugar to the change in hemicellulose was increased. Thus, the lignin degradation adjacent to cellulose by *Lac-CBD* expression affected not only the primary cellulose augmentation but also the hemicellulose structure in the lignocellulose matrix of dicot plants.

## Conclusions

We showed that the expression of a fungal laccase fused with a bacterial cellulose-binding module domain improved the enzymatic saccharification efficiency of a dicot model plant, *Arabidopsis thaliana* (Fig. 3). Cell wall component analysis revealed that the Lac-CBD-expressing plants showed no alteration in lignin contents, but did exhibit an alteration in primary cellulose contents and hemicellulose structure (Figs. 4 and 5). These results suggest the *Lac-CBD* chimeric gene is one of the potential solutions to improve the plant biomass utilization. Heretofore the transformed abnormal phenotype was not observed in transgenic *Arabidopsis* or rice expressing the *Lac-CBD* chimeric gene (Furukawa *et al.*, 2013). Thus, the transformation employed herein might be applicable to woody plants to improve access to their lignocellulosic biomass as an important technology toward a fuller utilization of biomass resources. One of our collaborators has developed a transformation system using eucalyptus plants, and plans to conduct the transformation of the *Lac-CBD* chimeric gene in the near future. If the chimeric gene yields an improvement of the enzymatic saccharification of the lignocellulose biomass of eucalyptus, this technology would be one of the major solutions for the utilization of woody biomass resources. Nonetheless, many evaluations, including studies on the growth characteristics, physical strength, and tolerance to diseases and insects, will be required before any practical applications in the future.

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***Figure legends***

**Fig. 1 Schematic models of the T-DNAs used in this study.**

TF012 and TF013 are mostly equivalent but exhibit difference in the intermediate hinge region, designated PTa and PTb. Pentagons, squares, circles, and triangles indicate the promoter, transcribed sequences, terminators, and T-DNA border sequences, respectively. p35S, promoter of Cauliflower mosaic virus 35S; *AtADH* 5'-UTR, sequence of the 5'- untranslated region of the *Alcohol Dehydrogenase* gene derived from *A. thaliana* as a translational enhancer; *Lac*, *Lacase III* derived from *Trametes vericolor*; PT, proline- or threonine-rich sequence of *Clostridium cellulovorans*; *tHSP*, terminator of *Heat-shock protein 18.2* derived from *A. thaliana*; *pNOS* and *tNOS*, promoter and terminator of nopaline synthase derived from *Rhizobactor tumefaciens*; *NPTII*, *neomycin transferase II* derived from *Escherichia coli*; R and L, right and left border sequences of Ti-plasmids.

**Fig. 2 Expression levels of transgenes.**

Expressions were assayed by quantitative RT-PCR targeted to the Lac and CBD segments. The target gene expressions were normalized by expression of the endogenous Actin 2 gene. Dark and light gray bars indicated the relative expression levels of *Lac* and *CBD*, respectively. Error bars indicate the standard deviation (n=4).

**Fig. 3 Enzymatic saccharification efficiency.**

Cellulose fractions derived from dried inflorescence stems were provided for the enzymatic saccharification assay. Eluted glucose concentrations were measured at 6, 24, and 48 h after the reaction and are indicated with white, gray, and black bars. The letters atop the bars indicate significant differences among lines by the Tukey-HSD test ( $\alpha$ =0.05).

**Fig. 4 Cell wall components of *Lac-CBD*-expressing plants.**

Cell wall components of *Lac-CBD*-expressing plants with alternative saccharification efficiency were analyzed. The concentrations of each component for TF012 (L-7), TF013 (L-7), and a vector control plant (L-6) are indicated with light gray, dark gray, and white bars, respectively. Error bars indicate the standard deviation (n=3). The letters atop the bars indicate significant differences among lines by the Tukey-HSD test ( $\alpha$ =0.05).

**Fig. 5 Sugar contents in the non-crystalline polysaccharide fraction of *Lac-CBD*-expressing plants.**

Sugar contents in the non-crystalline polysaccharide fraction of *Lac-CBD*-expressing plants with altered saccharification efficiency were analyzed. The concentrations of each component for TF012 (L-7), TF013 (L-7), and a vector control plant (L-6) are indicated with light gray, dark gray, and white bars, respectively. Error bars indicate the standard deviation (n=3). The scale of the vertical axis is shown at left for all components except xylose; the scale for xylose is shown at right. The letters atop the bars indicate significant differences among lines by the Tukey-HSD test ( $\alpha$ =0.05).

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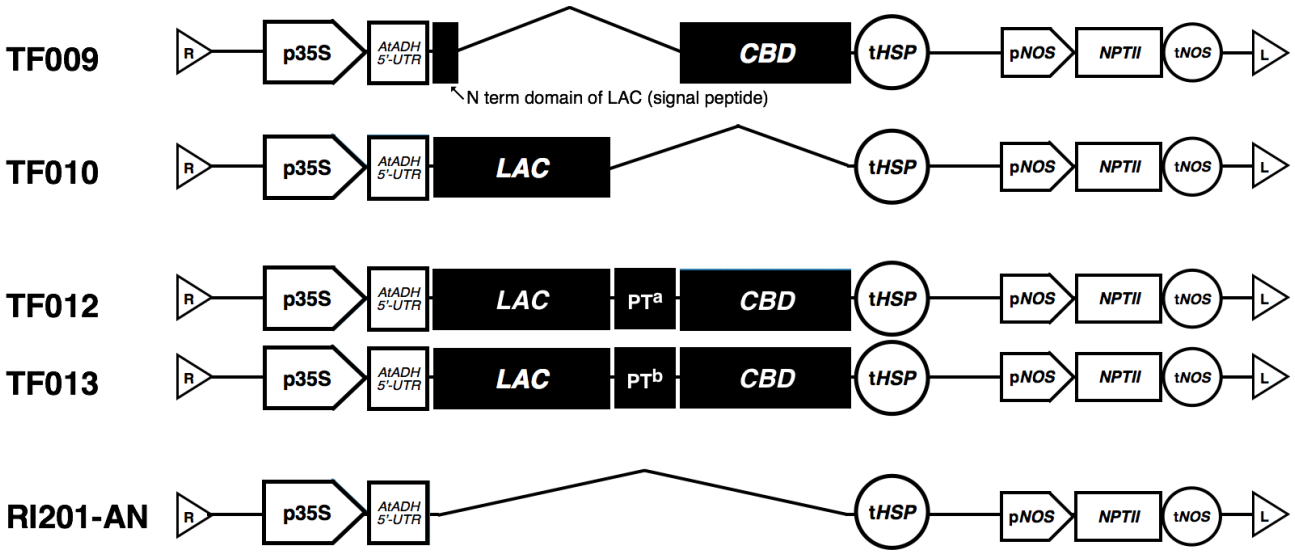
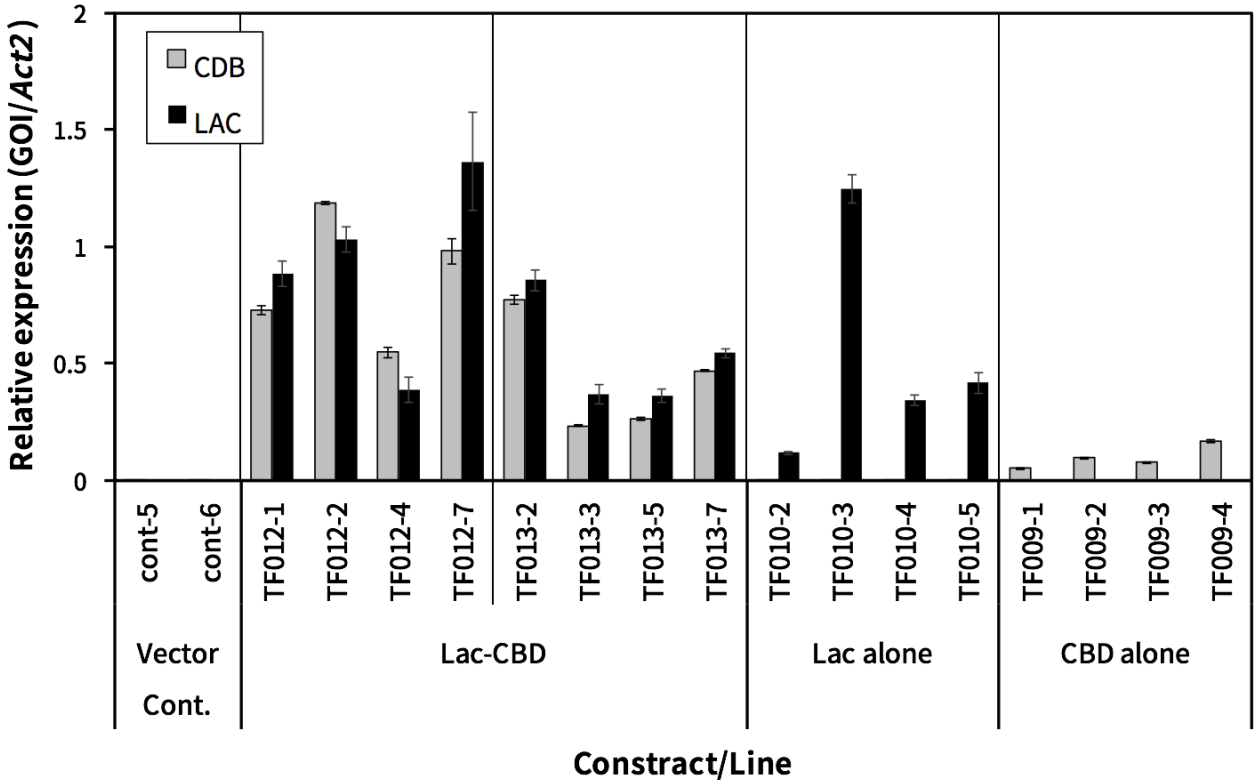


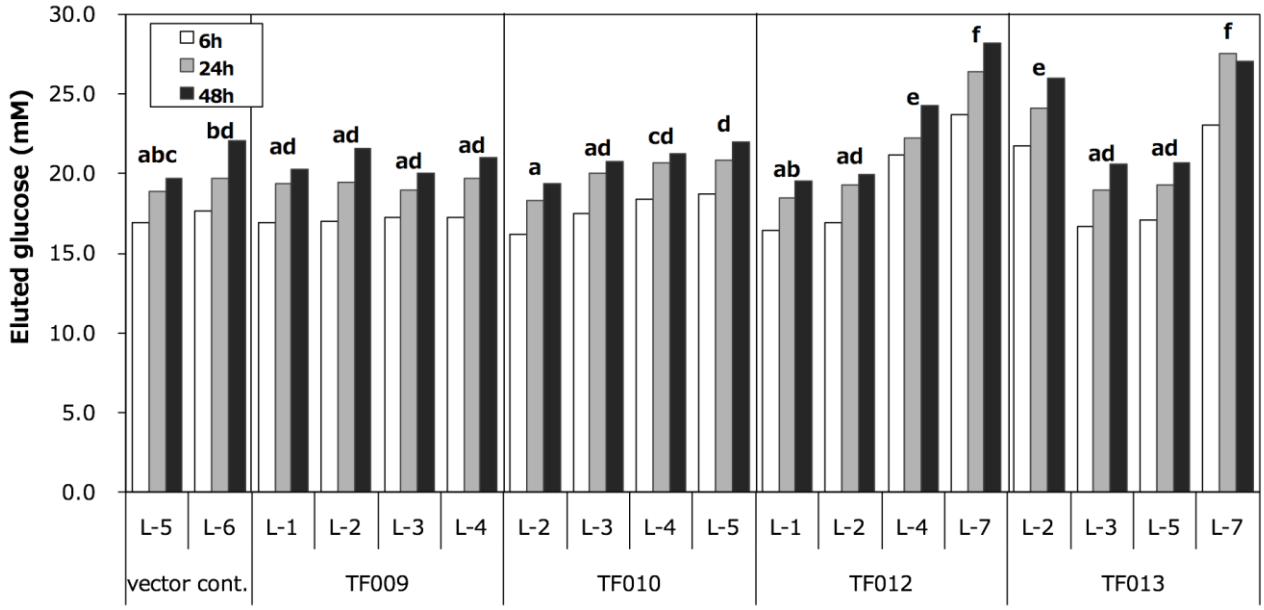

384

385 Figure 1



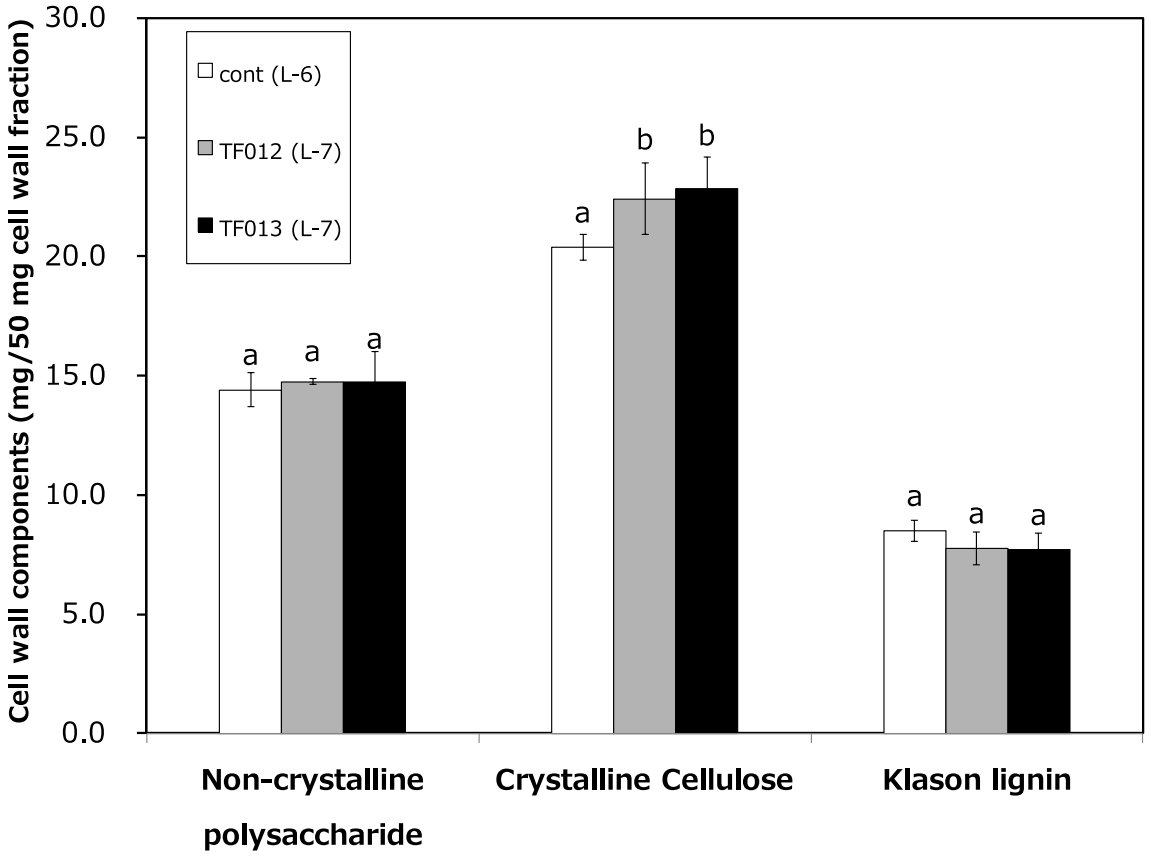

386

387 Figure 2



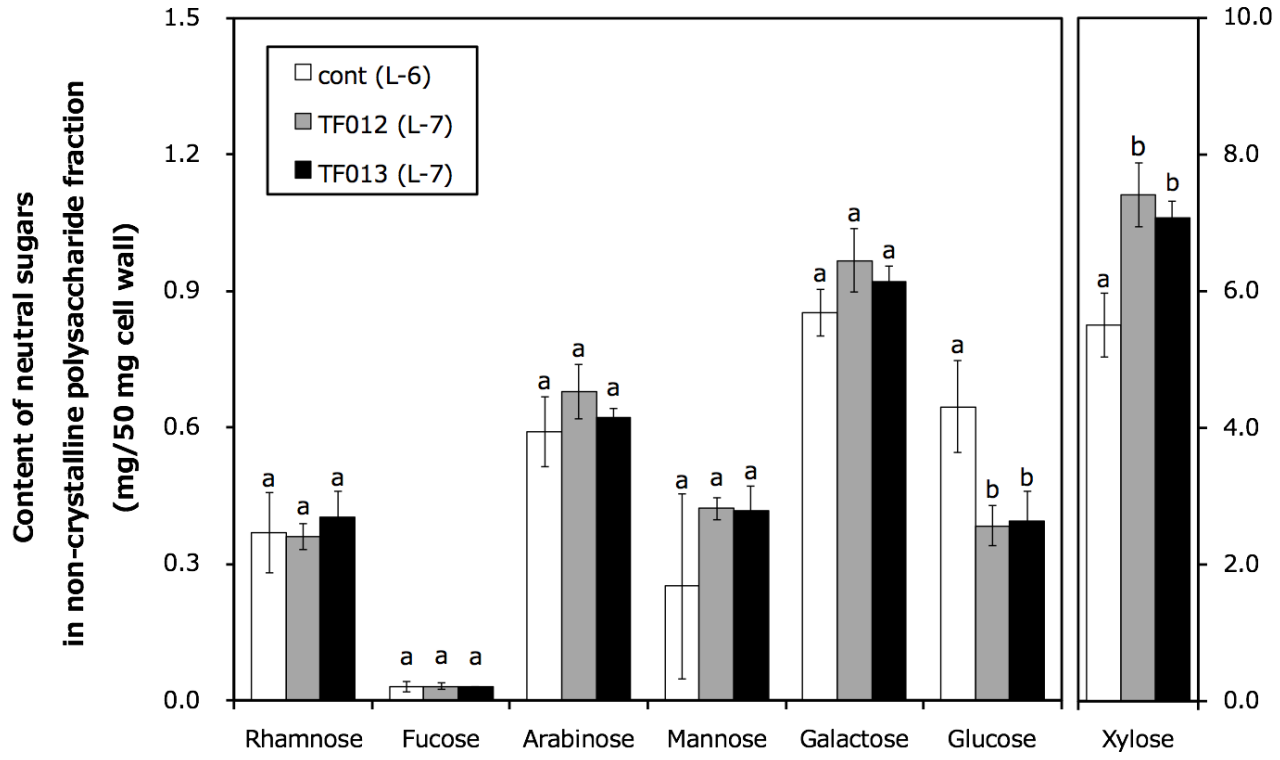

388

389 Figure 3




390

391 Figure 4




392

393 Figure 5