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1 ***Title***

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

4
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25

26 ***Abstract***

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

39

40

41 ***Keywords***

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

43

44 ***Introduction***

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

58 To use lignocellulosic materials as a feedstock for biorefinery production, cellulose and
59 hemicelluloses must be isolated and digested into fermentable sugars. However, cellulose molecules
60 bind each other via hydrogen bonds to form a rigid crystalline structure, and cellulose is covered by
61 and linked tightly with lignin, and these structural characteristics make cellulose hard to digest. The
62 crystallinity and lignin content of lignocellulosic biomass are determinants of its saccharification
63 efficiency, because they limit the ability of cellulase to access the beta-1,4-glycoside bonds in cellulose
64 molecules (Palonen *et al.*, 2004). For this reason, a variety of physico-chemical pretreatment methods
65 are used to decrystallize and delignify the lignocellulosic biomass to improve its saccharification
66 efficiency, such as steam explosion, sulfuric acid treatment and alkaline cooking (Li *et al.*, 2010; Zhu
67 *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.

92

93

94 ***Materials and Methods***

95 **Construction of laccase fused with a bacterial cellulose-binding module**

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

101

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

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

110

111 **Quantitative reverse transcriptional polymerase chain reaction (qRT-PCR)**

112 A rosette or cauline leaf was sampled and RNA was extracted from the sample using an RNeasy
113 Plant Mini Kit (QIAGEN). RT reactions were performed using ReverTra Ace RT master mix
114 (TOYOBO). For qRT-PCR, the reaction volume of 10 µL contained 5 µL of Thunberbird™ SYBR®
115 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 90°C for 10 min, followed by 40
118 cycles consisting of denaturation at 95°C for 0.5 min, and annealing and extension at 60°C 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* primer
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 105°C 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 105°C. The dried samples were ground and
139 sieved with a 200 mesh (ϕ 77 μ m) stainless filter. The sieved powder prepared from each transgenic

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

146

147 **Cell wall composition analysis**

148 The cell wall material was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1 h at 121°C to yield
149 a non-crystalline polysaccharide fraction, including matrix polysaccharides such as hemicelluloses and
150 pectin. The TFA insoluble residues were hydrolyzed with 72% (w/w) H₂SO₄ for 2 h at room
151 temperature, after then the acid concentration was diluted to 3% and further incubated for 2 h at 100°C
152 to yield the crystalline cellulose fraction (Selvendran and O'Neill, 1987; Edashige and Ishii, 1997; Sato
153 *et al.*, 2001). The residual material was dried and weighed as the lignin (Klason lignin) fraction. The
154 amount of total sugar in each fraction was measured by the phenol-H₂SO₄ method (DuBois *et al.*,
155 1956) using a glucose standard. The composition of neutral sugar (glucose, xylose, galactose,
156 arabinose, mannose, rhamnose and fucose) in the non-crystalline polysaccharide fraction was analyzed
157 by the alditol acetate method (Blakeney *et al.*, 1983).

158

159

160 ***Results and discussion***

161 **Molecular breeding of a transgenic *Arabidopsis* plant expressing a laccase fused with a bacterial** 162 **cellulose-binding module**

163 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 T₁ 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

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

206

207 ***Lac-CBD* expression increases the crystalline cellulose component of the cell wall in *Arabidopsis***

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

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

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

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

239

240 **Conclusions**

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

257

258

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262 University Grant for Exploratory Research by Young Scientists (TS).

263

264

265 ***Figure legends***

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

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

276

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

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

282

283 **Fig. 3 Enzymatic saccharification efficiency.**

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

288

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

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

295

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

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

304

305

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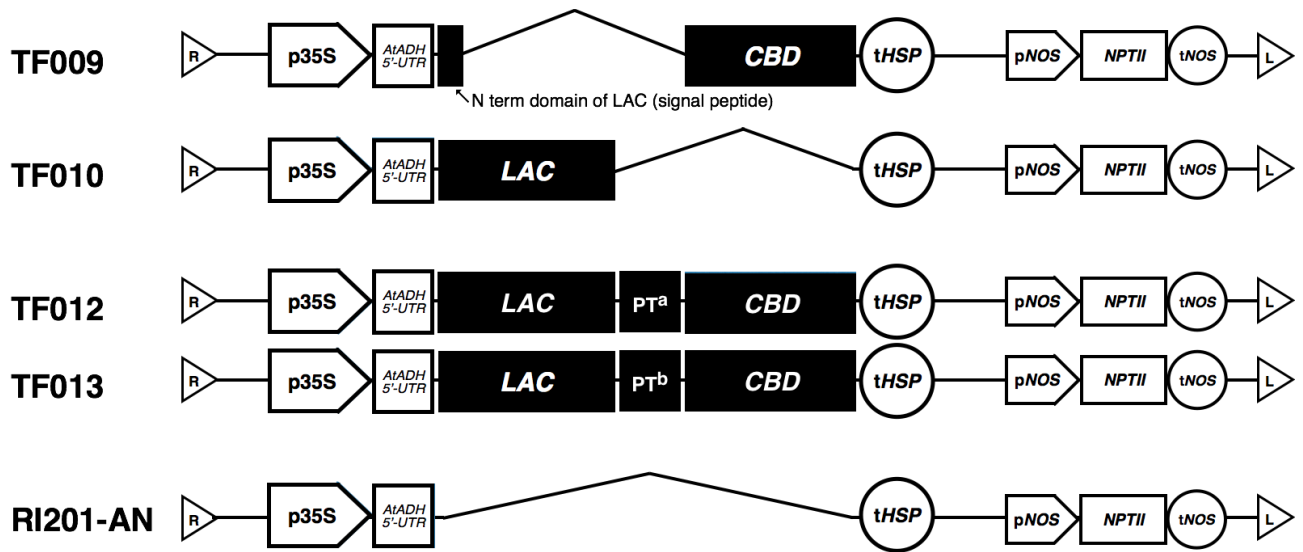
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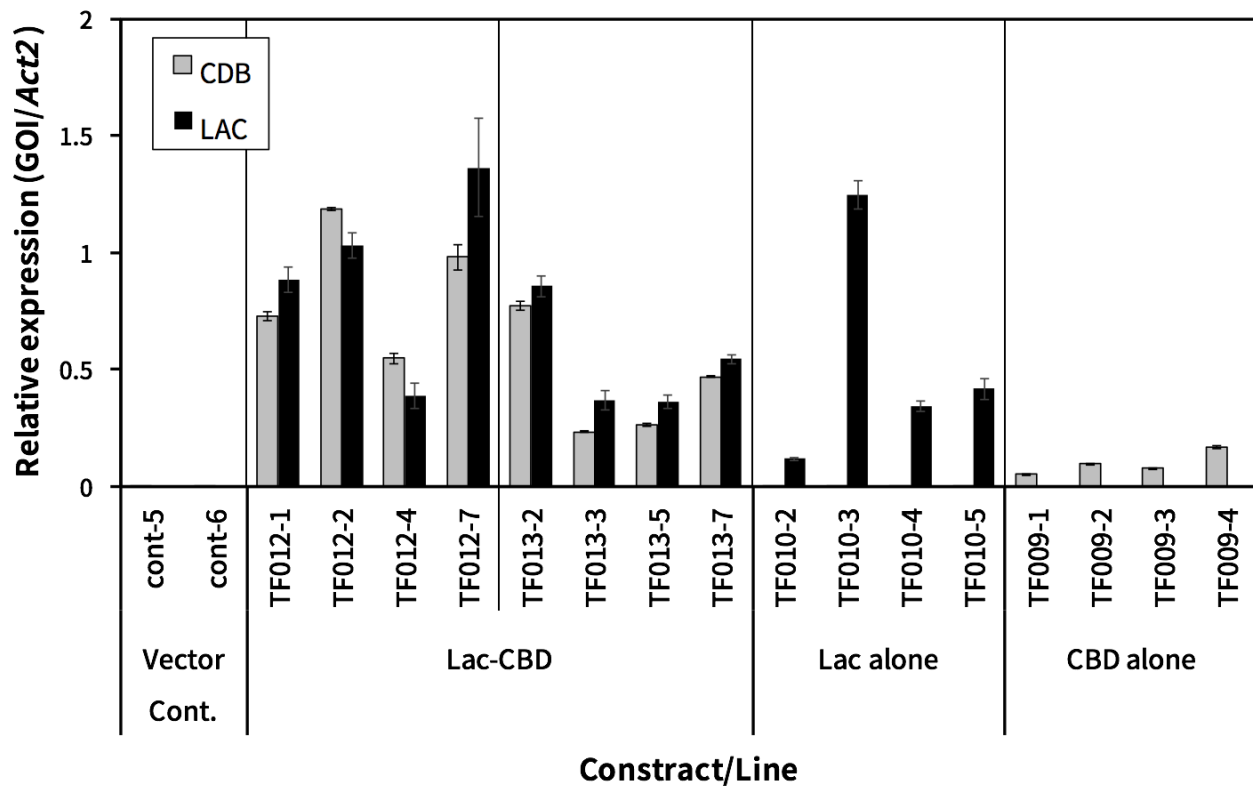
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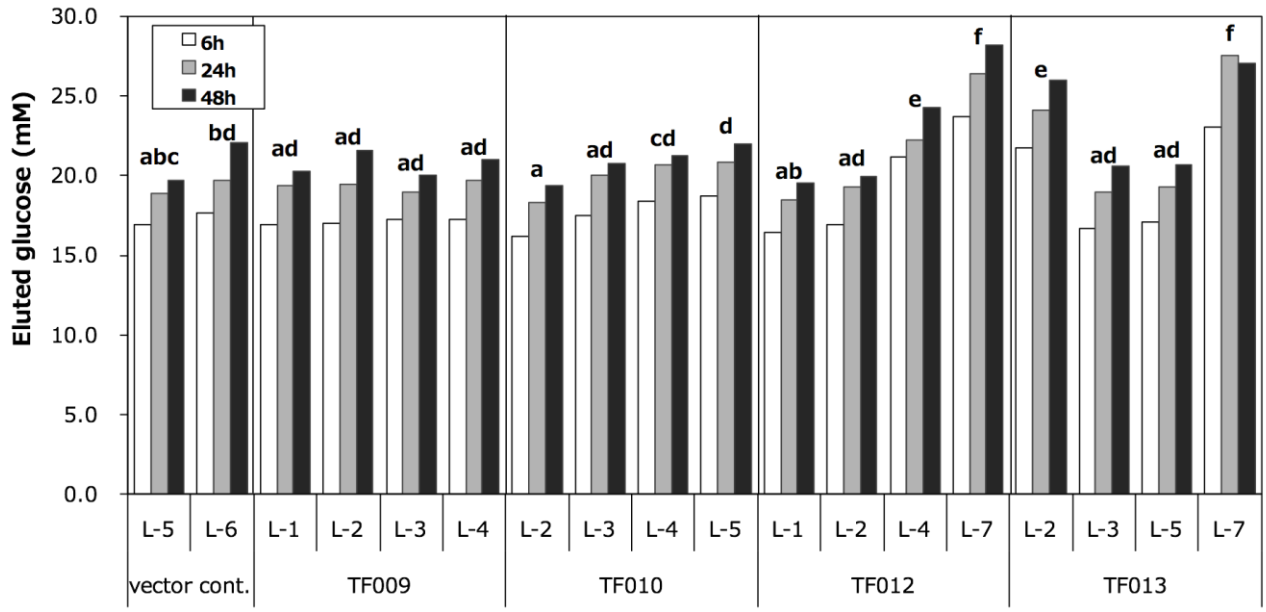
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385 Figure 1



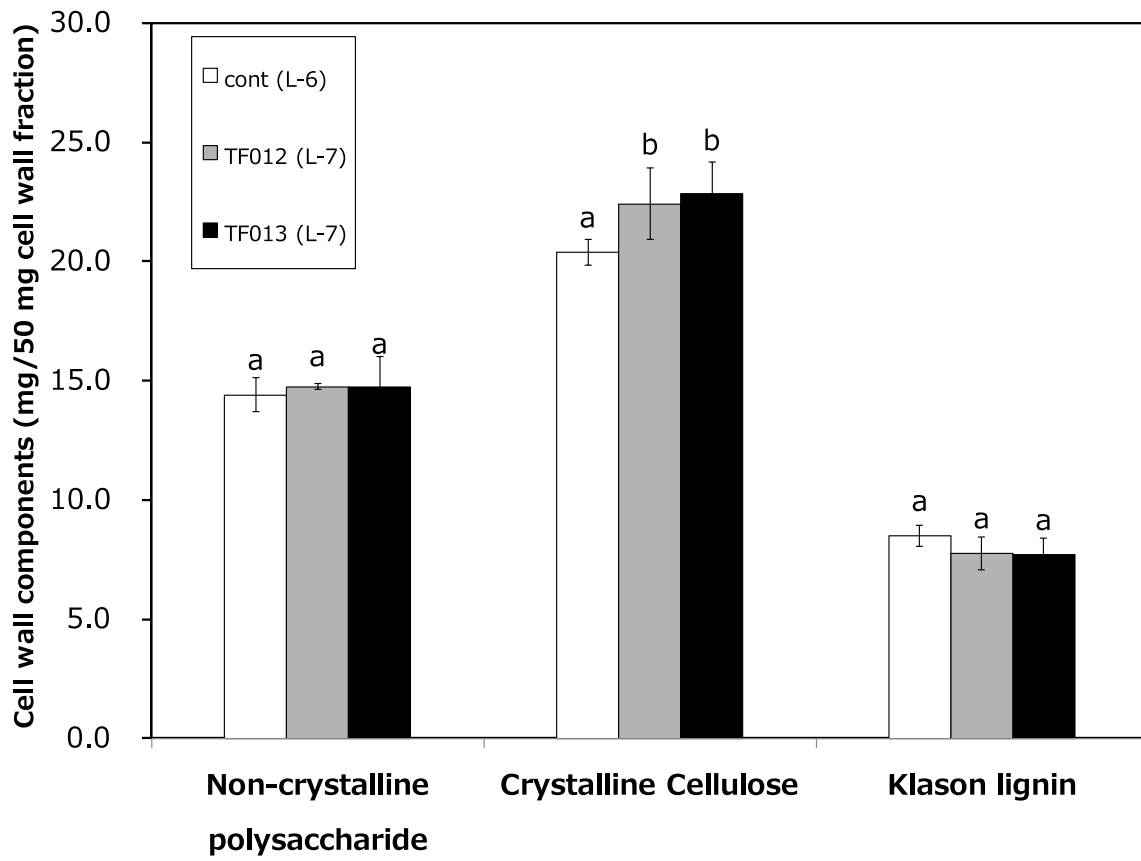
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387 Figure 2



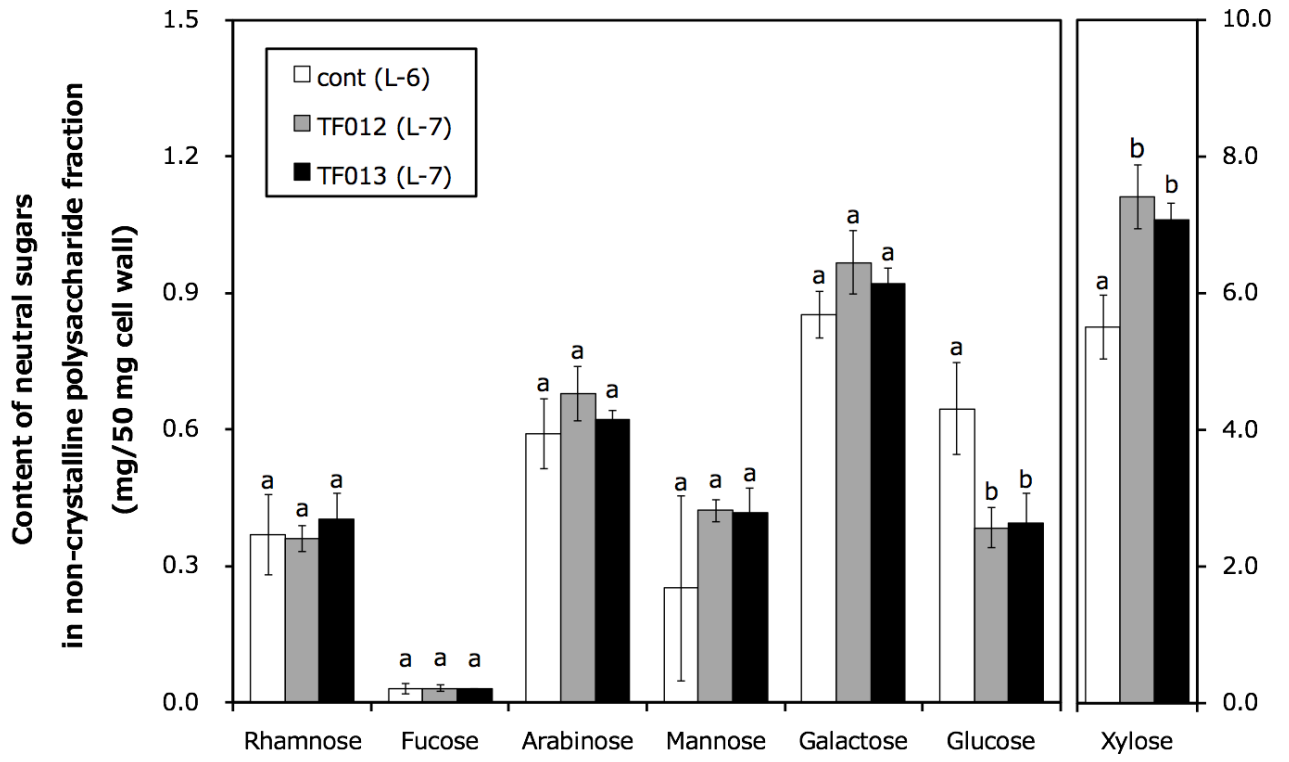
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389 Figure 3



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391 Figure 4



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393 Figure 5