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Research paper

Pathway-specific genetic pretreatment strategy to improve bioenergy feedstock

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ABSTRACT

The plant secondary wall is composed of a complex mixture of cellulose, hemicellulose (e.g., xylan), and lignin. The transcription factor MYB46 (At5g12870) has been reported as a central regulator of the secondary wall formation. However, constitutive overexpression of *MYB46* results in a severe growth penalty, possibly due to ectopic lignification in the parenchymatous and photosynthetic cells. To test this hypothesis, we constitutively overexpressed *MYB46* in the genetic background of a T-DNA insertion mutant *4cl1-2* of 4-coumarate-CoA ligase 1 (4CL1), a key lignin biosynthesis gene. As expected, the transgenic *Arabidopsis* plants 35S::MYB46/*4cl1-2* grow normally, similarly to the *4cl1-2* mutant, with elevated expressions of three secondary wall cellulose synthase genes and xylan biosynthesis genes. Consistent with the gene expression changes, the immunohistological staining of stem tissues showed the increased accumulation of both cellulose and xylan contents in the 35S::MYB46/*4cl1-2* plants. Further biochemical analyses confirmed that 35S::MYB46/*4cl1-2* plants have higher level accumulations of crystalline cellulose and xylan in both leaf and stem tissues than wild-type control plants as well as the *4cl1-2* mutant, up to 1.18- and 1.13-fold respectively, with considerably reduced lignin content (up to 0.40-fold). Subsequent analysis of enzymatic glucose release from cell wall materials revealed that 35S::MYB46/*4cl1-2* plants have higher yields up to 1.27-fold increase in both leaf and stem tissues than that of control plants and *4cl1-2* mutant. Our results showed that overexpression of *MYB46* with simultaneous reduction of lignin biosynthesis significantly increases both fermentable sugar contents and cell wall digestibility without growth penalty associated with *MYB46* overexpression.

1. Introduction

In the past decade, cellulosic ethanol, produced from carbohydrates sequestered in plant tissues that are not in competition with our food supply, has become an alternative to fossil fuels for use in transportation. A prerequisite for sustainable production of cellulosic ethanol is to have high yielding biofuel crops with increased efficiency of conversion of the biomass to biofuel. Thus, the genetic modification of biofuel crops offers a wide range of options for improving the composition and yield potential of biomass [1].

Plant secondary cell walls (SCW) account for the majority of biomass feedstock for bioenergy. The secondary wall is formed in a highly coordinated manner by successive encrustation and deposition of cellulose, hemicelluloses and lignin as soon as the cell has ceased growth [2–4] and thus requires a coordinated transcriptional activation of the genes involved in the biosynthesis of the secondary wall components, such as cellulose, hemicellulose and lignin.

Recently, the transcription factor MYB46 (At5g12870) has been reported to be a central regulator of secondary wall formation in *Arabidopsis thaliana* [5–8]. Overexpression of *MYB46* or its functional paralog *MYB83* (At3g08500) has been shown to activate the genes involved in secondary wall biosynthesis and results in ectopic deposition

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of secondary walls. This deposition can occur in multiple tissue types including parenchymatous and other photosynthetic tissue types that normally do not form secondary walls [6]. In contrast, dominant suppression of MYB46 significantly reduces secondary wall thickening in the fibers and vessels of the transgenic plants [5]. Alternatively, double knockout mutants of *MYB46* and *MYB83* resulted in severe growth arrest subsequent to germination [9,10], further suggesting the critical role of MYB46 in secondary wall formation. Several MYB46 orthologs from other plant species (e.g., pine, eucalyptus, poplar and maize) have also been shown to function as a master switch for secondary wall formation [7,8,11–14].

In addition, genome-wide analysis of promoter sequences in *Arabidopsis* have revealed that many MYB46-induced secondary wall biosynthetic genes have one or more MYB46 binding *cis*-acting regulatory motifs (called M46RE [15]; SMRE [16]) in their promoter region. This supports the hypothesis that MYB46 'directly' regulates these genes, SMRE [15,16]. Indeed, we have also recently reported that MYB46 directly up-regulates the expression of the three secondary wall cellulose synthases, *CesA4*, *CesA7*, and *CesA8* [17]. Furthermore, MYB46 is an obligate component of the transcriptional regulatory complex for functional expression of the three *CesA*s [10]. In fact, ectopic overexpression of *MYB46* significantly increased cellulose content in the leaves of the plants [17]. In addition, MYB46 is a direct regulator of glucomannan synthase CSLA9 [18] and xylan biosynthesis genes (*FRA8*, *IRX8*, *IRX9*, and *IRX14*) as well as nine monolignol biosynthesis genes (*PAL*, *C4H,* 4CL, *HCT*, *C3H*, *CCoAOMT*, *F5H*, *CCR* and *CAD*) [19]. In summary, multiple research provides support that MYB46 is a direct regulator of the genes responsible for the biosynthesis of the major components of secondary walls.

To reduce overall biomass recalcitrance and improve saccharification yield without compromising biomass yield, various approaches to the modification of lignin structure, content or distribution has been made (for a recent review [20]). Among them, Yang et al. [21] reported an interesting strategy to enhance polysaccharide deposition in *Arabidopsis* stems without over-lignifying them by restoring lignin in xylem vessels in the *c4h* mutant with the overexpression of a fiber transcription factor *NST1* by the promoter of *IRX8* gene, a secondary cell wall glycosyltransferase [22] for an artificial positive feedback loop (APFL). The resulting transgenic *Arabidopsis* stems had higher sugar yields after enzymatic hydrolysis.

Interestingly, constitutive overexpression of *MYB46* results in severe growth defects including ectopic and irregular secondary cell wall thickening in epidermal, cortical and pith tissues [6,17]. We hypothesized that this growth retardation is the result of MYB46-induced ectopic lignification in the parenchymatous and photosynthetic cells. To test this hypothesis, we constitutively overexpressed *MYB46* using the 35S promoter in the background of a T-DNA insertion mutant *4cl1-2* of *4-COUMARATE-COA LIGASE 1* (*4CL1*, *At1g51680*), a key lignin biosynthesis gene. In this report, we describe experimental evidence in support of this hypothesis. Furthermore, these results provide a proof-of concept for pathway-specific biomass engineering, which offers an attractive 'genetic pretreatment' strategy using an alternative genetic approach to improve bioenergy feedstock by increasing both fermentable sugar contents as well as cell wall digestibility.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia Col-0 and Col were used in the wild-type and transgenic experiments. The 35S::MYB46/*4cl1-2* plants were generated by crossing the 35S::MYB46/Col-0 (#8 line from Ref. [17]) with the *4cl1-2* mutant, which is a T-DNA insertion mutant of *4CL1* gene (*At5g13160*, WiscDsLox473B01, Supplemental Fig. S1). The 35S::MYB46/Col plants were also prepared by introducing the 35S::MYB46 construct [17] into Col ecotype since the *4cl1-2* mutant has Col background. Homozygous lines were generated and confirmed by PCR using the primers listed in Supplemental Table S1. Plants were grown on soil (Sunshine Mix#4, Sun Gro Horticulture, MA, USA) in an environmental growth chamber (16 h light/8 h dark) at 23 °C. All experiments were performed in triplicate and repeated at least three times.

For the *4 cl* mutant experiments, seeds were sterilized in 6 kg/m^3 sodium hypochlorite plus 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), rinsed three times with sterile water, and plated on 1/2MS medium without sugar (Sigma-Aldrich, St. Louis, MO, USA) containing 8 kg/m^3 agar (Sigma-Aldrich, St. Louis, MO, USA). After treatment at 4 °C for 3–5 days, the plates were placed vertically (~80° angle) under constant light (100 μE). Seven days subsequent to germination, the seedlings were transplanted to soil consisting of Jiffy Mix #901 (Jiffy Co. Lorain, OH) plus Krum horticultural perlite (Silbrico, Hodgkins, IL, USA) in a two to one ratio. Plants from each line were transplanted into two 4-inch (10.2 cm) pots with three plants per pot, for a total of six plants per line per experiment. Potted plants were grown in the greenhouse at the University of Wisconsin-Madison Biotron (Madison, WI, USA) under a light cycle of sixteen hours on and eight hours off, at a fluency of at least 250–300 μE. Plants were watered four times per week, using an ebb and flow bench, with half-strength Hoagland's solution [23]. The greenhouse temperature was maintained at 18 °C ± 1° during the dark period and 21 °C ± 1° during the light period.

2.2. RNA extraction, RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instruction. For RT-PCR analysis, total RNAs were first treated with DNaseI before the first-strand cDNA synthesis by SuperScript II Reverse Transcriptase (Invitrogen, http://www.thermofisher.com). RT-PCR was carried out using 1 cm^3 of the reaction products as a template. Amplified DNA fragments were separated on 10 kg/m^3 agarose gel and stained with EtBr (ethidium bromide). The qRT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, http://www.takara.co.kr) and the ABI Prism 7900HT Sequence Detection System (ABI, http://www.lifetechnologies.com). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the *ACTIN8* reference gene. Three biological replicates were used in each experiments. The primers used for PCR are listed in Supplemental Table S1.

2.3. Labeling for CBM3a and LM10 and immunofluorescence microscopy

Arabidopsis thaliana plants, ecotype Columbia (Col-0 and Col) wild type, *4cl1-2*, 35S::MYB46/Col and 35S::AtMYB46/*4cl1-2* plants were grown on soil (Sunshine Mix#4, Sun Gro Horticulture, MA, USA) in a growth chamber (16 h light/8 h dark) at 23 °C for 5 weeks. Lower parts of the stems (ca. 3 cm from rosette level) were fixed in FAA solution (50 mass % ethanol, 5 mass % glacial acetic acid and 3.7 mass % formaldehyde) for 12 h at 4 °C. After fixation, the fixed stems were embedded in paraffin and sectioned into 20 μm thin sections. The stem sections were labeled with anti-xylan (LM10, http://www.plantprobes.net) or crystalline cellulose-specific carbohydrate-binding module CBM3a as described by Ref. [24]. In brief, the sections were incubated in PBS (phosphate-buffered saline) containing 30 kg/m^3 milk protein (MP/PBS) with LM10 diluted 1:100 in the blocking buffer for overnight or 50 kg/m^3 milk protein (MP/PBS) with 10 μg/cm^3 CBM3a for 1.5 h at 4 °C. The incubated samples in CBM3a were washed in PBS at least three times and incubated with a 100 –fold dilution of mouse anti-his monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) in MP/PBS for 1.5 h. After all samples washed with PBS, antibody linked to fluorescein isothiocyanate (anti-rat-IgG (LM10), anti-mouse FITC (CBM3a); Sigma-Aldrich, St. Louis, MO, USA) was applied for 1.5 h as a 50 –fold dilution in MP/PBS in darkness. The samples were washed with PBS, mounted in a ProLong® Gold anti-fade solution (Invitrogen,