The transcription factor ZmMYB69 represses lignin biosynthesis by activating ZmMYB31/42 expression in maize

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Dear Editor,

Lignin is a phenylpropanoid-derived polymer that is directly deposited on the secondary walls of specialized cells, such as vessel elements and fibers, in plants. Lignification has vital roles in mechanical support, long-distance water transport, and plant defense (Barros et al., 2015). However, lignin is undesirable for biotechnological applications because its covalent interaction with cell wall polysaccharides limits the enzymatic digestion of agricultural biomass feedstocks (Foster et al., 2010). A hierarchical transcriptional network comprising various transcription factors (TFs), including NACs (NAM, ATAF, and CUC) and MYBs (myeloblastosis-related proteins), controls lignin biosynthesis in Arabidopsis (Arabidopsis thaliana). In this network, different MYBs act as activators (e.g. MYB58 and MYB63) or repressors (e.g. MYB4 and MYB32) of lignin biosynthesis genes (LBGs) and these MYBs are also downstream targets of MYB46 and MYB83 (Xie et al., 2018). In maize (Zea mays), ZmMYB31 and ZmMYB42 were repressors of LBGs (Sonbol et al., 2009; Fornale et al., 2010). However, the upstream regulation mechanism is still unrevealed. Here, we report that ZmMYB69 is a regulatory factor at the upper level of ZmMYB31 and ZmMYB42 in the hierarchical network that controls lignin biosynthesis in maize. We provide evidence that ZmMYB69 is a transcriptional activator and directly targets ZmMYB31 and ZmMYB42 promoters. Thereafter, it can repress lignin biosynthesis by activating ZmMYB31 and ZmMYB42 expression.

In this study, we overexpressed UBI-driven ZmMYB69 in maize ND101 inbred plants. Two lines (OE2 and OE3) were obtained, which displayed reduced plant height (Supplemental Figure S1) and decreased vascular bundle cell wall thickness (Figure 1A and B). By measuring the lignin content in the cell wall (Foster et al., 2010), we detected significantly reduced lignification in the overexpression lines compared with that in ND101 (Figure 1C). In contrast, in ZmMYB69 loss-of-function lines generated by the CRISPR/Cas9 technique (Cas9-16 and Cas9-18), we observed thicker cell walls and higher lignin content, but no visible growth defect was detected (Figure 1A–C and Supplemental Figure S1).
Figure 1  ZmMYB69 regulates lignin biosynthesis in maize. A, Scanning electron microscopy images of cross-sections of the fourth internodes from ND101, ZmMYB69-OEs, and Cas9 mutants, showing the cell wall of vascular cells. Bar = 10 μm. B, Quantitative analysis of the cell wall thickness shown in (A). Data are presented as box plots that reflect 25%, 50%, 75%, and the maximum/minimum of total values (n = 100 cells). **P < 0.01 by Student's t test. C, Quantitative analysis of the lignin content in ND101, ZmMYB69-OEs, and Cas9 mutants (n = 5). D, Transcript of ZmMYB69 in stem vascular tissues was revealed by in situ PCR. The ZmMYB69 transcripts were visualized with alkaline phosphatase-conjugated anti-DIG antibody (dark blue). ZmActin1 was a positive control. Bars = 50 μm. E, Transient expression of ZmMYB69-GFP in maize protoplasts, showing the nuclear localization of ZmMYB69. Bars = 25 μm. BF, bright field; DAPI, 4',6-diamidine-2'-phenylindole dihydrochloride. F, Transactivation assay in yeast demonstrated a transcription activation feature of ZmMYB69. R2 and R3 are DNA-binding domains of ZmMYB69. G and H, Transcripts of ZmMYB31, ZmMYB42, and lignin structural genes in 1-month-old stem of ND101, ZmMYB69-OEs (G), and Cas9 mutants (H) analyzed by RT-qPCR. Internal reference: ZmActin1 (n = 3). I, Schematic diagrams of the promoters of ZmMYB31, ZmMYB42, ZmCOMT1,
These results suggest a negative role of ZmMYB69 in lignin biosynthesis.

To dissect the function of ZmMYB69, we first performed an in situ PCR analysis on stem cross sections. ZmMYB69 was mainly expressed in highly lignified tissues (e.g., vascular tissue) (Figure 1D). Next, we demonstrated the nuclear localization of ZmMYB69 by transiently expressing ZmMYB69 fused with green fluorescent protein (GFP) in maize protoplasts (Figure 1E). We then investigated the transcriptional activity of ZmMYB69 using a transactivation assay in yeast. Full-length and various truncated ZmMYB69 CDSs were fused with the GAL4 DNA-binding domain in pGKT7 and transformed into AH109 yeast cells. Yeast cells harboring either the full-length ZmMYB69 protein or the C-terminal fragments containing amino acids 113–289 grew normally on SD/Trp/His/Ade/X-a-Gal medium (Figure 1F). Additionally, X-a-gal was activated, implicating ZmMYB69 as a transcription activator with a transcriptional activation domain at the C-terminus. Considering that it repressed lignin biosynthesis, we suspected that ZmMYB69 may activate a repressor(s) in lignin biosynthetic pathways. To test this hypothesis, we analyzed the expression levels of ZmMYB31 and ZmMYB42 (known repressors of LBGs) in ZmMYB69 OEs and Cas9 mutants. ZmMYB31 and ZmMYB42 expression was upregulated in OE2 and OE3 but downregulated in Cas9-16 and Cas9-18 (Figure 1, G and H). Moreover, the expression of direct targets of ZmMYB31 and ZmMYB42 (ZmCOMT1, Zm4CL3, and ZmCCR3) was significantly decreased in OE2 and OE3 but increased in Cas9-16 and Cas9-18.

MYBs regulate lignin biosynthesis by directly targeting the conserved AC elements, including AC-I (ACCTACC), AC-II (ACCAACC), and AC-III (ACCTAAC), in the promoters of the majority of LBGs (Raes et al., 2003; Geng et al., 2020). We also identified multiple AC elements within the promoters of ZmMYB31 and ZmMYB42 (Figure 1I). We performed a chromatin immunoprecipitation assay and detected the recruitment of ZmMYB69 to the AC elements within ZmMYB31 and ZmMYB42 promoters but not the ZmCOMT1, Zm4CL3, and ZmCCR3 promoters (Figure 1I). The direct binding of ZmMYB69 to the promoters of ZmMYB31 and ZmMYB42 was also confirmed by an electrophoretic mobility shift assay (Figure 1K). Moreover, a transient transcription activation assay in Nicotiana benthamiana leaf cells showed that ZmMYB69 promoted the GUS reporter gene expression driven by the ZmMYB31 and ZmMYB42 promoters (Figure 1L). These results demonstrated that ZmMYB69 repressed lignin biosynthesis by regulating ZmMYB31 and ZmMYB42 expression.

We further tested whether the reduction of lignin content in OE2 and OE3 could improve the hydrolysis efficiency of maize stalk. Cell wall materials were isolated and treated with a commercial cellulase (C9748; Sigma–Aldrich, St. Louis, USA) as previously described. Biomass conversion was determined by calculating the amount of glucose and xylose released from cell wall materials (Foster et al., 2010; Yang et al., 2018). More sugars were released from OE2 and OE3 materials than from ND101, indicating an enhanced efficiency of biomass conversion caused by ZmMYB69 overexpression (Figure 1M; see also Supplemental Materials and Methods and Supplemental Table S1).

In summary, we demonstrate that ZmMYB69, a MYB TF in maize that negatively regulates lignin biosynthesis by directly activating the expression of transcription repressors ZmMYB31 and ZmMYB42, can subsequently repress the expression of LBGs. Moreover, the findings also implicate ZmMYB69 as a good candidate for the manipulation of lignin biosynthesis in biotechnological applications.

Supplemental data
Supplemental Figure S1. Overexpression of ZmMYB69 leads to reduced plant height.
Supplemental Figure S2. Plant height analysis of ZmMYB69-Cas9 mutants.
Supplemental Materials and Methods. Methodology used in this study.
Supplemental Table S1. Primers used in this study.

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Figure 1 (Continued)
Zm4CL3, and ZmCCR3. Black, gray, and white boxes represent the AC-I, AC-II, and AC-III elements, respectively. P1, P2, and P3 indicate the DNA fragments containing AC elements subjected to chromatin immunoprecipitation (ChIP)-qPCR analysis. J, Seven-day-old ND101 seedlings expressing Ubc:ZmMYB69-GFP and ND101 seedlings were used for ChIP analysis using anti-GFP antibody. The precipitated DNA was analyzed by qPCR assays. ZmActin1 was used as control (n = 3). K, Electrophoretic mobility shift assay revealed that ZmMYB69 binds to the promoters of ZmMYB31 and ZmMYB42. GST, glutathione-S-transferase. L, Transactivation analysis in N. benthamiana leaves showing that ZmMYB69 activated the expression of GUS reporter genes driven by the promoters of ZmMYB31 and ZmMYB42. The empty vector was used as control and set to 1 (n = 3). M, Release of sugars from cell wall materials of ZmMYB69-OEs plants and ND101 in the fourth maize internodes after treatment with a commercial cellulase (n = 5). Asterisks indicate significant differences in both OE lines compared with ND101. Data in (C), (G), (H), (J), (L), and (M) are shown as mean ± so; not significant (ns), P > 0.05; *P < 0.05, **P < 0.01 by Student’s t test.
References

Barros J, Serk H, Granlund I, Pesquet E (2015) The cell biology of lignification in higher plants. Ann Bot 115: 1053–1074
Fornale S, Shi X, Chai C, Encina A, Irar S, Capellades M, Fuguet E, Torres JL, Rovira P, Puigdomenech P, et al. (2010) ZmMYB31 directly represses maize lignin genes and redirects the phenylpropanoid metabolic flux. Plant J 64: 633–644
Foster CE, Martin TM, Pauly M (2010) Comprehensive compositional analysis of plant cell walls (Lignocellulosic biomass) part I: lignin. J Vis Exp 37: e1837–e1837
Geng P, Zhang S, Liu J, Zhao C, Wu J, Cao Y, Fu C, Han X, He H, Zhao Q (2020) MYB20, MYB42, MYB43, and MYB85 regulate phenylalanine and lignin biosynthesis during secondary cell wall formation. Plant Physiol 182: 1272–1283
Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol 133: 1051–1071
Sonbol FM, Fornale S, Capellades M, Encina A, Tourino S, Torres JL, Rovira P, Ruel K, Puigdomenech P, Rigau J, et al. (2009) The maize ZmMYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in Arabidopsis thaliana. Plant Mol Biol 70: 283–296
Torney F, Moeller L, Scarpa A, Wang K (2007) Genetic engineering approaches to improve bioethanol production from maize. Curr Opin Biotechnol 18: 193–199
Xie M, Zhang J, Tschaplinski TJ, Tuskan GA, Chen JG, Muchero W (2018) Regulation of lignin biosynthesis and its role in growth-defense tradeoffs. Front Plant Sci 9: 1427
Yang Y, Yang J, Liu J, Wang R, Liu L, Wang F, Yuan H (2018) The composition of accessory enzymes of Penicillium chrysogenum P33 revealed by secretome and synergistic effects with commercial cellulase on lignocellulose hydrolysis. Bioresour Technol 257: 54–61