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Letter

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

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is: Ying Fu (yingfu@cau.edu.cn).

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 (Torney et al., 2007). 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* L.), 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 1, A 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 1, A–C and Supplemental Figure

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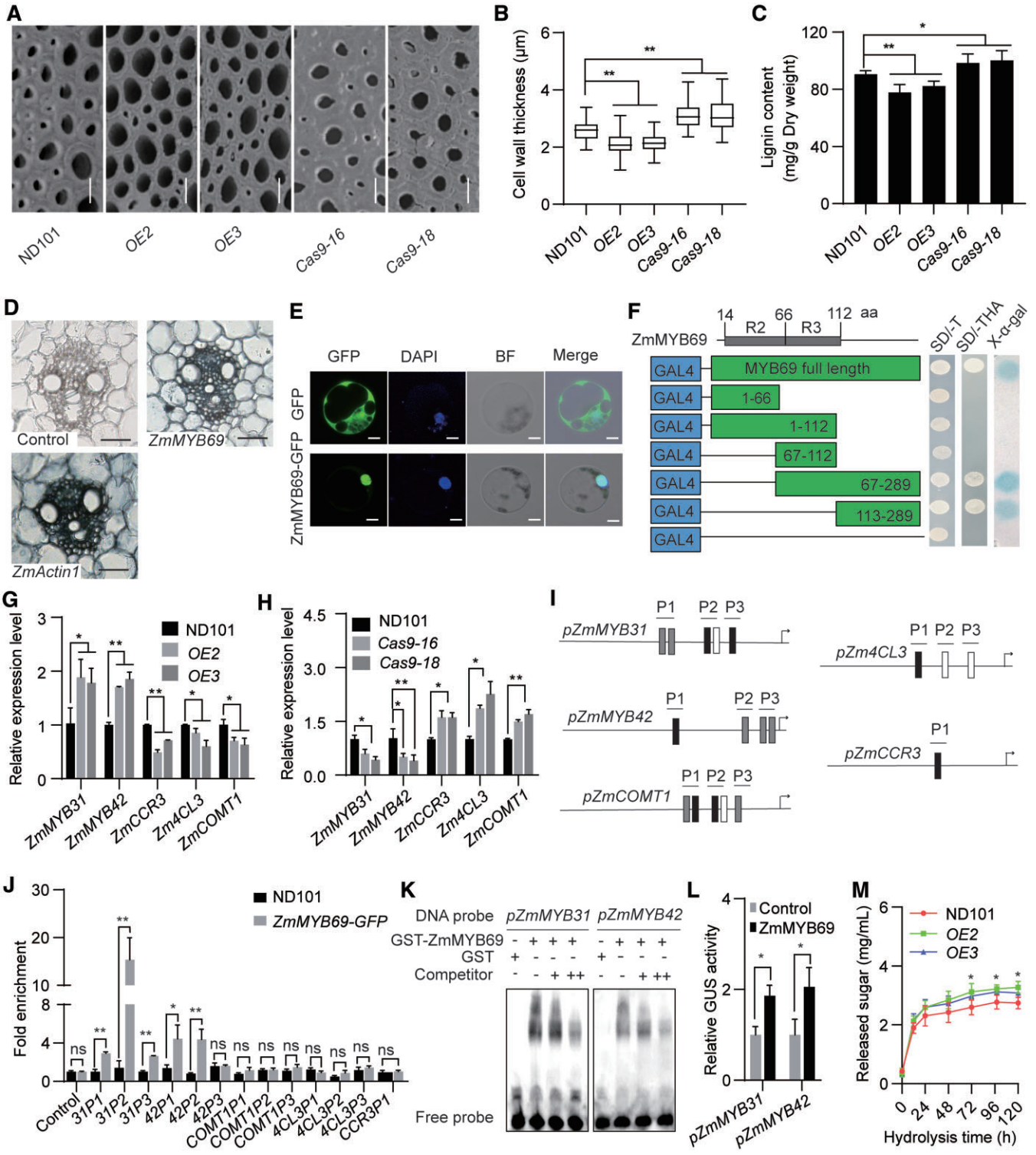

Figure 1 ZmMYB69 regulates lignin biosynthesis in maize. A, Scanning electron microscopy images of cross-sections of the fourth internodes from ND101, *ZmMYB69-OEs*, and *Cas9* mutants, showing the cell wall of vascular cells. Bar = 10 μm. B, Quantitative analysis of the cell wall thickness shown in (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*,

(Continued)

S2). 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 pGBKT7 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-α-Gal medium (Figure 1F). Additionally, X-α-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 1J). 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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Conflict of interest statement. None declared.

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 Ubi::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 ± SD; not significant (ns), $P > 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t* test.

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