

LNK1 and LNK2 Corepressors Interact with the MYB3 Transcription Factor in Phenylpropanoid Biosynthesis¹

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Subgroup 4 of R2R3-MYB transcription factors consists of four members, MYB3, MYB4, MYB7, and MYB32, which possess the conserved EAR repression motif (pdLHLD/LLxiG/S) in their C termini. Here, we show that MYB3 is a newly identified repressor in *Arabidopsis thaliana* phenylpropanoid biosynthesis. However, the repression mechanism of MYB3 is completely different from MYB4, MYB7, and MYB32. Yeast two-hybrid screening using MYB3 as a bait isolates NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED1 (LNK1) and LNK2, members of a small family of four LNK proteins. The repression activity of MYB3 to cinnamate 4-hydroxylase (*C4H*) gene expression is directly regulated by corepressors LNK1 and LNK2, which could facilitate binding of MYB3 with *C4H* promoter. The two conserved Asp residues in both region 1 and 2 domain of LNKs are essential to mediate protein-protein interaction. Importantly, the Extra N-terminal Tail domain plays a negative role in LNK-MYB3 transcription complex-dependent repression of the *C4H* gene. We conclude that LNK1 and LNK2 act as transcriptional corepressors necessary for expression of the phenylpropanoids biosynthesis gene *C4H* through recruitment to its promoter via interaction with MYB3.

The phenylpropanoid metabolic pathway is a well-described plant secondary metabolite pathway. Different classes of compounds are produced by the phenylpropanoid pathway and have different functions, such as lignin, which plays a structural role in the secondary cell walls formation (Vanholme et al., 2010), while flavonoids mediate plants against UV radiation and act as a visual signal for attracting pollinators (Mol et al., 1998; Bradshaw and Schemske, 2003). The biosynthesis of these phenylpropanoid compounds are initiated with deamination of Phe by Phe ammonia-lyase (PAL), followed by reactions catalyzed by a P450 enzyme, cinnamate 4-hydroxylase (*C4H*), to form β -coumaric acid (Winkel-Shirley, 2002; Du et al., 2010). The subsequent reactions catalyzed by a series of enzymes, such as 4-coumarate coenzyme A ligase (4CL), *O*-methyltransferase (OMT),

ferulate-5-hydroxylase (F5H), and cinnamyl alcohol dehydrogenase (CAD; Winkel-Shirley, 2002; Du et al., 2010). During the last few years, many efforts have been made to discover the transcription regulators that regulate the phenylpropanoid compounds biosynthesis in the model plant *Arabidopsis thaliana*, and an increasing amount of evidence reveals that R2R3-MYB type transcription factors (TFs) mainly positively increase anthocyanin biosynthesis (Zhou et al., 2015a). It has been reported that PAP1/MYB75, PAP2/MYB90, MYB113, and MYB114 positively regulate anthocyanin metabolism (Zimmermann et al., 2004; Teng et al., 2005; Stracke et al., 2007; Gonzalez et al., 2008; Qi et al., 2011). However, MYB4, MYB7, MYB32, and MYBL2 repress the phenylpropanoids biosynthesis (Jin et al., 2000; Preston et al., 2004; Dubos et al., 2008, 2010; Fornalé et al., 2014; Zhou et al., 2015b).

The R2R3-MYB TFs are classified into subgroups based on the presence of their C-terminal conserved domain and several studies demonstrate that same subgroup TFs share similar functions (Dubos et al., 2010; Zhou et al., 2015b). R2R3-MYB subgroup 4 TFs consist of four members, MYB3, MYB4, MYB7, and MYB32, which possess the conserved LLsrGIDP_xT/SHR_xI/L motif and the EAR repression motif (pdLHLD/LLxiG/S) in their C-termini (Jin et al., 2000). In addition, our previous report showed that MYB4, MYB7, and MYB32, but not MYB3, has a putative zinc-finger domain (CX₁₋₂CX₇₋₁₂CX₂C) and a conserved GY/FDFLGL motif in their C termini (Zhou et al., 2015b). Further experiments showed that the

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GY/FDFLGL motif contributes to the interaction between MYB TFs and the Sensitive to ABA and Drought 2 (SAD2) (Zhou et al., 2015b). SAD2, an importin β -like protein, mediates the transport of MYB4, MYB7, and MYB32 into the nucleus and then increases the repression activity on their target gene (such as *C4H*) expression (Zhou et al., 2015b). Until now, three of four R2R3-MYB subgroup 4 TFs, MYB4, MYB7, and MYB32, are involved in repression of the phenylpropanoid pathway (Jin et al., 2000; Preston et al., 2004; Dubos et al., 2010; Fornalé et al., 2014; Zhou et al., 2015b). MYB4 is responsive to UV-B irradiation and acts as a repressor in the phenylpropanoid pathway through negatively regulating the expression of the *C4H* gene (Jin et al., 2000; Zhao et al., 2007). MYB32 has been described as a pollen-specific repressor of lignin biosynthesis (Preston et al., 2004). MYB7 is partly involved in regulation of UV sunscreens through

repressing flavonol biosynthesis (Fornalé et al., 2014). Additionally, it has been reported that the R3-MYB-related factor MYBL2 also negatively regulates the expression of anthocyanin genes in *Arabidopsis* seedlings (Dubos et al., 2008). However, nothing is known about the function of MYB3, the closest MYB4, MYB7, and MYB32 homolog.

Therefore, we investigated the role of MYB3 in the regulation of the phenylpropanoid biosynthesis. Recently, NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED1 (LNK1) and LNK2 were demonstrated to play important roles in *Arabidopsis* period determination, circadian oscillator, and anthocyanin biosynthesis (Rugnone et al., 2013; Xie et al., 2014; Pérez-García et al., 2015). Here, we report that MYB3, like MYB4, MYB7, and MYB32, is involved in the repression of phenylpropanoid biosynthesis. Through the use of yeast two-hybrid (Y2H) screening, we identified the

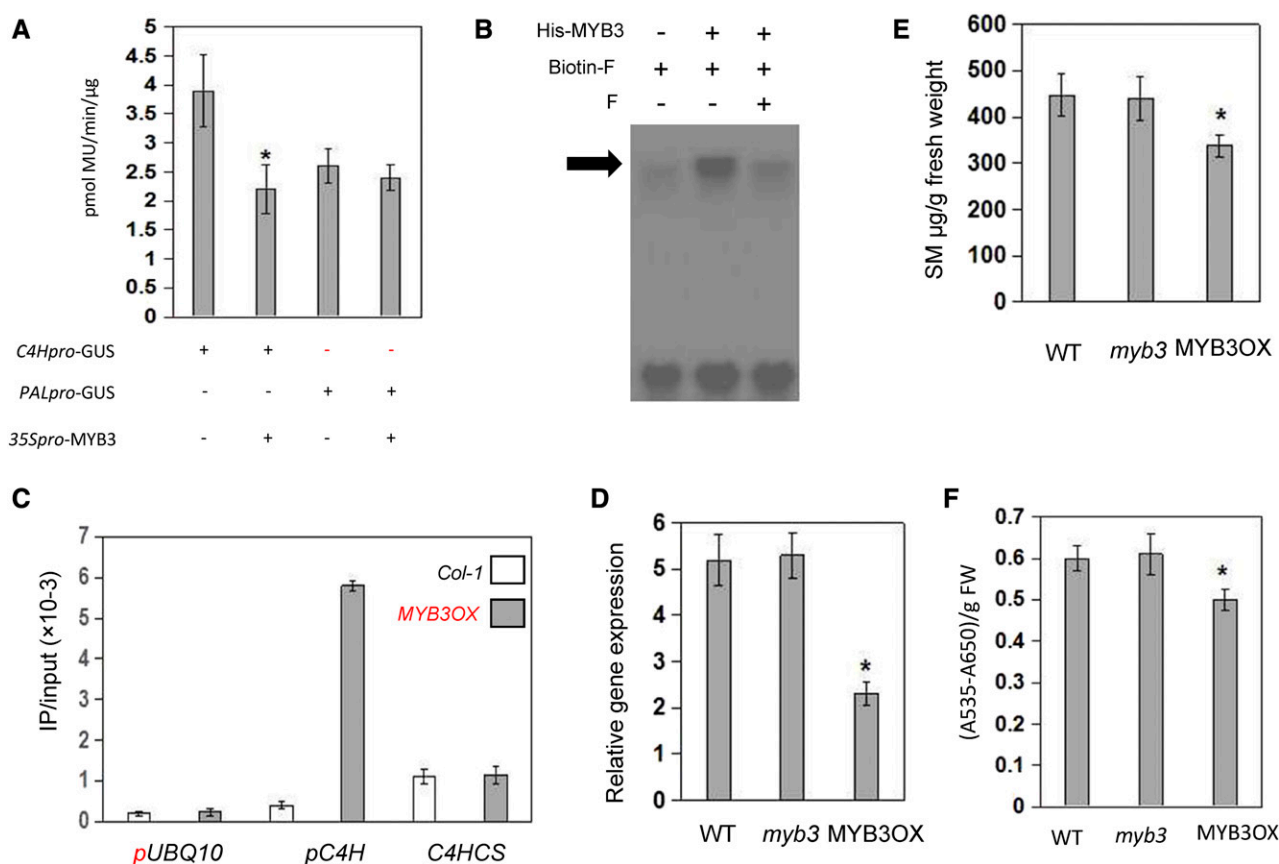


Figure 1. MYB3 represses phenylpropanoid biosynthesis. **A**, Transactivation assays in *Arabidopsis* protoplasts that were cotransfected with plasmids carrying *C4Hpro-GUS* and overexpression vectors containing 35S:MYB3, as indicated. Values are means \pm SD of three biological repeats. Asterisks indicate statistically significant differences compared with *C4Hpro-GUS* ($P < 0.05$, Student's *t* test). **B**, EMSA of a probe including the MYB3 binding site AATAGTT with His-MYB3 purified from *Escherichia coli* BL21 (DE3). The arrow indicates the protein probe complex. **C**, Direct binding of MYB3 with the promoter of *C4H*. ChIP assays were conducted by real-time PCR after normalizing with the input DNA. The fragment of *C4H* coding sequence and the reference gene *UBQ10* promoter were used as a negative control. **D** to **F**, Measurement of *C4H* gene expression levels (**D**), SM (**E**), and anthocyanin (**F**) contents in indicated genotypes by LC/MS and quantitative RT-PCR, respectively. Values are means \pm SD of three biological repeats of three independent transgenic lines. Asterisks indicate statistically significant differences compared with wild-type and *myb3* plants ($P < 0.05$, Student's *t* test). FW, Fresh weight.

MYB3-interacting factors, LNK1 and LNK2, known to function in the control of circadian rhythm-associated gene expression as a transcriptional coactivator and anthocyanin biosynthesis-associated gene expression as a transcriptional repressor, respectively (Xie et al., 2014; Pérez-García et al., 2015). We demonstrated that the repression activity of MYB3 is directly regulated by corepressors LNK1 and LNK2, which could facilitate binding of MYB3 with its target gene *C4H* promoter. We also found that the two conserved Asp residues in both region 1 and 2 domains of LNKs are essential to mediate LNK-MYB3 interactions. Importantly, we provide evidence that the Extra N-terminal Tail (ENT) domain of LNKs plays a negative role in LNK-MYB3 transcription complex-dependent repression of *C4H* gene. Together, our results suggest that LNKs act as corepressors for the regulation of MYB3-dependent gene expression.

RESULTS

MYB3 Represses Phenylpropanoids Biosynthesis

MYB4, MYB7, and MYB32 have been described as transcriptional repressors of the phenylpropanoid pathway (Jin et al., 2000; Preston et al., 2004; Dubos

et al., 2010; Fornalé et al., 2014; Zhou et al., 2015b). Thus, we hypothesized that MYB3 may also repress the phenylpropanoids biosynthesis. As a first step to test this hypothesis, the effect of MYB3 on *C4H* gene expression was tested by protoplast transactivation assays. Cotransformation of Arabidopsis protoplasts with a *C4Hpro*-GUS reporter construct (Zhou et al., 2015b) and an effector CaMV35S-MYB3 resulted in significant repression of around 2-fold, while MYB3 did not affect the GUS activity of *PALpro*-GUS reporter, as expected (Fig. 1A). To test whether the directly interaction between MYB3 protein and *C4H* promoter, electrophoresis mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP) were performed. As shown in Figure 1, B and C, MYB3 was able to bind to the *C4H* promoter fragment. As a negative control, MYB3 did not bind to the reference gene *UBQ10* promoter and *C4H* CDS fragments (Fig. 1C). These results indicate that MYB3 directly represses the *C4H* gene expression. Furthermore, to functionally test the role of MYB3 on phenylpropanoids metabolism in planta, we analyzed the *MYB3-HA* overexpressing and *myb3* mutant Arabidopsis plants (Supplemental Fig. S1). A *myb3* T-DNA insertion line did not express the *MYB3* transcript and was also morphologically

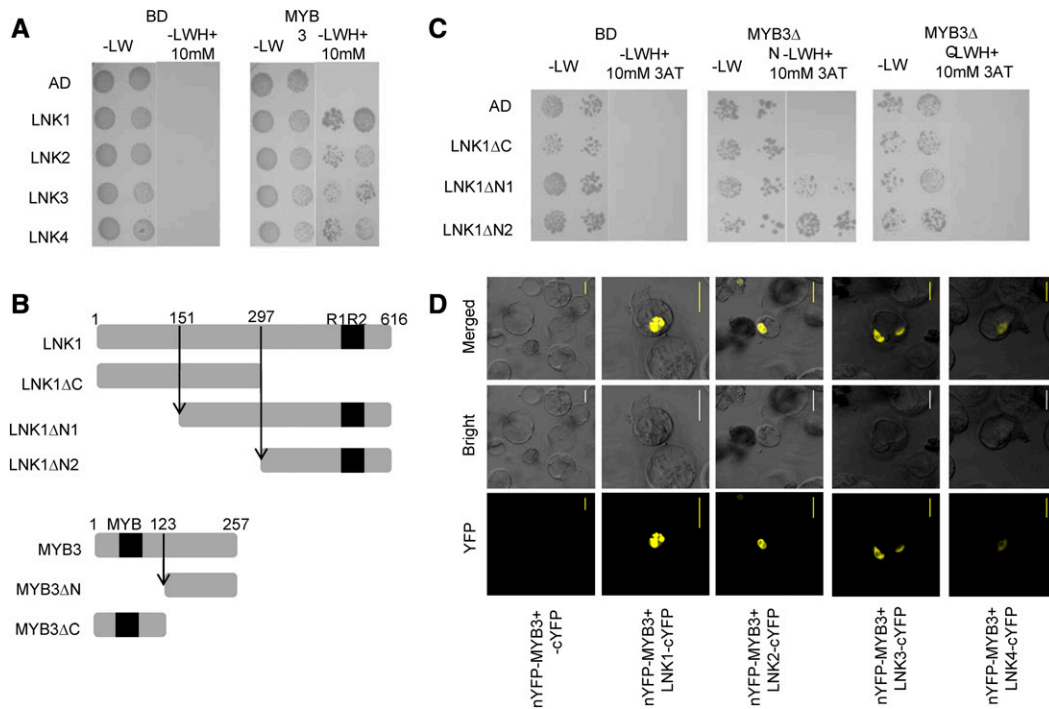


Figure 2. MYB3 interacts with a small family of four LNK proteins in yeast and in planta. A to C, LNK1, LNK2, LNK3, LNK4, and LNK1 deletion derivatives (LNK1ΔN1 and LNK1ΔN2) interact with MYB3 and MYB3ΔN in yeast. B, Schematic representation of the deletion derivatives of both LNK1 and MYB3. Yeast cells expressing LNK1, LNK2, LNK3, LNK4, LNK1ΔN1, LNK1ΔN2, and LNK1ΔC proteins fused to the GAL4 AD and MYB3, MYB3ΔN, and MYB3ΔC fused to the GAL4 BD were spotted on SD/-LW medium to select for the plasmids and on SD/-LWH medium containing 10 mM 3-amino-1,2,4-triazole (3AT) to select for transcriptional activation of the *His-3* gene. Growth was monitored after 5 d. Yeast cells transformed with empty plasmids pAS2.1 and pACT2, expressing GAL4 BD and AD, respectively, were used as controls in A and C. D, BiFC assays in planta. YFP fluorescence images alone or merged with bright-field images of Arabidopsis cell suspension protoplasts cotransfected with constructs encoding the indicated fusion proteins with YFP at the C terminus or the N terminus. Bar = 20 μm.

indistinguishable from wild-type plants (Supplemental Fig. S1). qRT-PCR analysis performed with 2-week-old seedlings showed that the *C4H* gene was highly repressed in *MYB3-HA*-overexpressing plants compared with *myb3* and wild-type plants (Fig. 1D). Additionally, overexpression of MYB3 also affected the key enzyme genes, such as *CHS*, *4CL1*, and *4CL3*, which were unaffected in the *myb3* mutant plants (Supplemental Fig. S2). The transcript levels of *PAL2*, *OMT*, *F5H*, and *CAD1* were not regulated by MYB3 factor (Supplemental Fig. S2). Therefore, we want to functionally test the role of MYB3 on the sinapoyl malate (SM) and anthocyanin accumulation (Jin et al., 2000; Zhou et al., 2015b). As shown in Figure 1, E and F, the level of SM and anthocyanin were significantly reduced in plants overexpressing *MYB3-HA* compared with *myb3* and wild-type plants. It has been reported that the accumulation of SM in plants are more tolerant of UV-B (Jin et al., 2000). Our results showed that overexpression of *MYB3* enhanced the sensitivity to UV-B (Supplemental Fig. S3).

Identification of LNKs That Interact with MYB3

To identify proteins that interact with MYB3, Y2H screenings were performed using full-length MYB3 fused to the GAL4 DNA-binding domain (BD) in the vector pAS2.1 as a bait. Screening of 5.4×10^5 yeast transformants obtained with Arabidopsis cDNA library generated from ecotype Col-0 seedlings in the vectors pACT2 resulting in nine colonies that were able to grow on medium lacking His. Recovered prey plasmids were retransformed and five plasmids conferred growth on selective medium. From these candidate MYB3 interactors, only one cDNA sequence was in frame with the GAL4 activation domain (AD) and encoded the C-terminal 294 amino acids of the protein named LNK1 (At5g64170). LNK1 is one of a small family of four LNK proteins, which has been shown to interact with Myb-like REVEILLE TFs (RVE4 and RVE8) and regulate some circadian rhythm and anthocyanin biosynthesis-associated genes expression as a transcriptional coregulator (Rugnone et al., 2013; Xie et al., 2014; Pérez-García et al., 2015). To test whether all four LNKs interact with MYB3, Y2H assays were performed. These results showed that all four LNKs interacted with MYB3 (Fig. 2A). To investigate the functional domains mediating the interaction between MYB3 and LNKs, we tested the interaction of three LNK1 deletion derivatives (LNK1 Δ N1, LNK1 Δ N2, and LNK1 Δ C) with two MYB3 deletion derivatives (MYB3 Δ N and MYB3 Δ C; Fig. 2B). As shown in Figure 2C, the interaction observed between LNKs and MYB3 requires the C-terminal domain of both LNKs and MYB3. However, LNKs did not interact with another subgroup 4 MYB TF MYB4, MYB7, and MYB32 (Supplemental Fig. S4).

To confirm the interaction of MYB3 with LNKs in planta, a bimolecular fluorescence complementation (BiFC) assay was performed. The constructs were transiently coexpressed in all possible combinations of YN and YC fusion proteins in Arabidopsis suspension cell

protoplasts. As shown in Figure 2D, strong YFP signals were observed in the nucleus of Arabidopsis protoplasts upon coexpression of LNK-cYFP with nYFP-MYB3. No YFP fluorescence was detected upon coexpression of single plasmids and any combination of empty YFP vectors. These results demonstrated that MYB3 can interact with LNKs in the nucleus of plant cells.

Two Conserved Asp Residues of LNKs Are Required for Protein Interactions

The two plant-specific conserved regions (R1 and R2) of C termini of LNK family members have previously been shown to interact with the Myb-like TF RVE4 (Xie et al., 2014). Mutation of two conserved residues in either R1 or R2 region (Arg-555Asp-556 to GlyGly or Asp-614Arg-615 to GlyGly) of LNK1 significantly diminished the interaction between LNK1 and RVE4 (Xie et al., 2014). Several studies demonstrated the conserved Asp residue contributes to the protein-protein interaction, such as the Asp residue in the JAZ interaction domain of MYC2 and the SID motif of MYB4 (Goossens

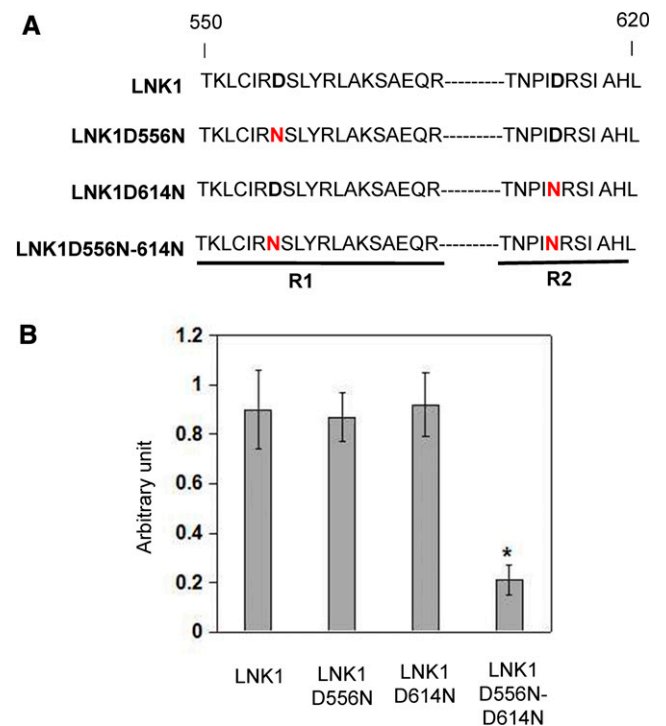


Figure 3. Two conserved Asp residues of LNKs are required for protein interactions. A, Schematic representation of mutation of two conserved residues in either R1 or R2 region of LNK1. B, LNK1D556N, LNK1D614N, and LNK1D556N-D614N interact with MYB3 in quantitative Y2H assays. A liquid culture β -galactosidase assay was performed on the transformed yeasts. The activity of β -galactosidase was measured in arbitrary units. Values are means \pm sd of three biological repeats. Asterisks indicate statistically significant differences compared with MYB3, MYB3D556N, and MYB3D614N ($P < 0.05$, Student's *t* test).

et al., 2015; Zhou et al., 2015b). To obtain insight into the role of the Asp residue in LNK1 interaction, we tested the interaction of Asp-556 to Asn (D556N), Asp-614 to Asn (D614N), or Asp-556-Asp-614 to Asn-Asn (D556N-D614N) mutated LNK1 with MYB3 protein (Fig. 3A). Immunoblot analysis revealed that LNK1 and LNK1 derivatives were present at similar levels in yeast (Supplemental Fig. S5), indicating that LNK1 protein stability was not affected by this mutation. Quantitative Y2H assays showed that the interaction of LNK1D556N or LNK1D614N with MYB3 was not significantly diminished (Fig. 3B). However, LNK1D556N-D614N greatly reduces the ability of LNK1 to interact with MYB3 (Fig. 3B). Taken together, these results suggest that the two conserved Asp residues in R1 and R2 of LNKs' C termini are important for interaction of MYB3 with LNKs.

MYB3 Repressor Activity Is Enhanced by LNK1 and LNK2

In order to elucidate the functional significance of the interaction between LNKs and MYB3, transactivation assays were performed. Effector constructs for MYB3, LNK1, LNK2, LNK3, LNK4, or LNK1 derivatives were expressed under the control of the CaMV 35S promoter. Arabidopsis cell suspension protoplasts were transiently cotransformed with a *C4Hpro*-GUS reporter (Zhou et al., 2015b) and effectors. As shown in Figure 4A and Supplemental Figure S6, we noticed that all four LNKs and LNK1 derivatives failed to affect the *C4Hpro*-GUS activity. Therefore, we asked whether LNKs were coregulators necessary for the repression of *C4H* transcription by MYB3. The MYB3 protein repressed the expression of *C4Hpro*-GUS about 2-fold,

whereas coexpression of LNK1, LNK2, LNK1D556N, or LNK1D614N with MYB3 greatly reduced the expression of *C4Hpro*-GUS about 4-fold (Fig. 4A), indicating that LNK1 and LNK2, but not LNK3 and LNK4, act as corepressors to enhance MYB3 repression activity (Supplemental Fig. S6).

The data presented above indicate that LNK1 and LNK2 serve as transcriptional corepressors for *C4H* transcription, which suggests that LNK1 and LNK2 should be recruited to the *C4H* promoter. We then investigated whether LNK1 affected the interaction between MYB3 and *C4H* promoter using competitive EMSA. As shown in Figure 4B, the interactions between MYB3 and *C4H* promoter was inhibited by the addition of His-LNK3, but not His-LNK1 and His-ENT-LNK3. Taken together, these results reveal that LNK1 probably facilitate binding of MYB3 with *C4H* promoter.

The N-Terminal Domain of LNK1 Is Required for MYB3/LNK1 Complex-Dependent Repression of *C4H*

As noted above, both LNK3 and LNK4 did not enhance the MYB3 repression activity, suggesting that both LNKs lack the transcription regulation domain. The amino acid sequence alignment of four LNK proteins shows that LNK1 and LNK2 possess the ENT domain that is around 300 amino acids long compared to LNK3 and LNK4 (Supplemental Fig. S7). Therefore, we hypothesized that the ENT domain is essential for LNK transcription activity. To test this hypothesis, we generated a chimeric protein ENT-LNK3 that is the ENT domain of LNK1 fused to the N termini of LNK3, and ENT-LNK3 derivatives (Fig. 5A). As shown in Figure 5B,

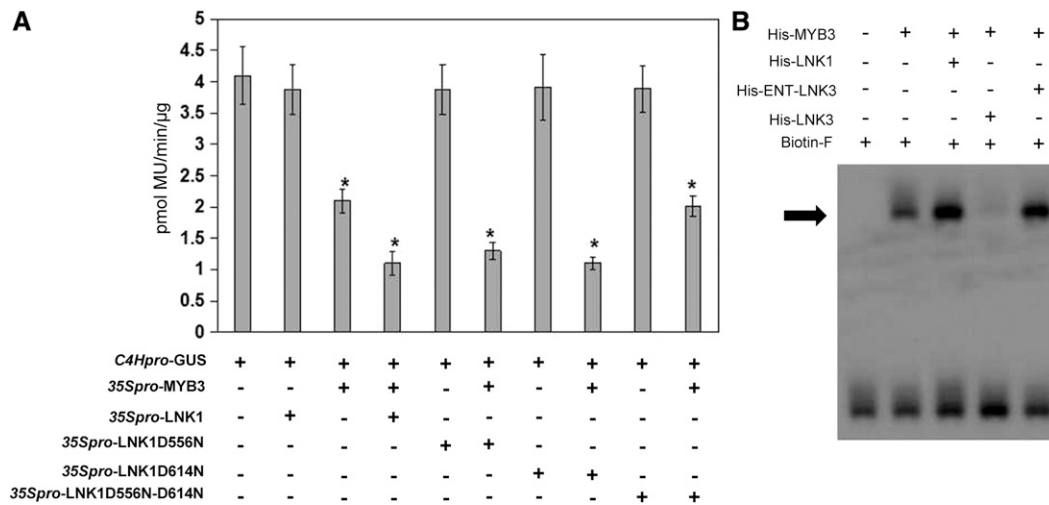


Figure 4. MYB3 repressor activity is enhanced by LNK1 and LNK2. A, Transactivation assays in Arabidopsis protoplasts that were cotransfected with plasmids carrying *C4Hpro*-GUS and overexpression vectors containing 35S:MYB3, 35S:LNK1, 35S:LNK1D556N, 35S:LNK1D614N, or 35S:LNK1D556N-D614N, as indicated. Values are means \pm sd of three biological repeats. Asterisks indicate statistically significant differences compared with *C4Hpro*-GUS, 35S:MYB3 or 35S:LNK1 ($P < 0.05$, Student's *t* test). B, EMSA of a probe including the MYB3 binding site AATAGTT with His-MYB3, His-LNK1, His-ENT-LNK3, and His-LNK3 purified from *E. coli* BL21 (DE3). The arrow indicates the protein probe complex.

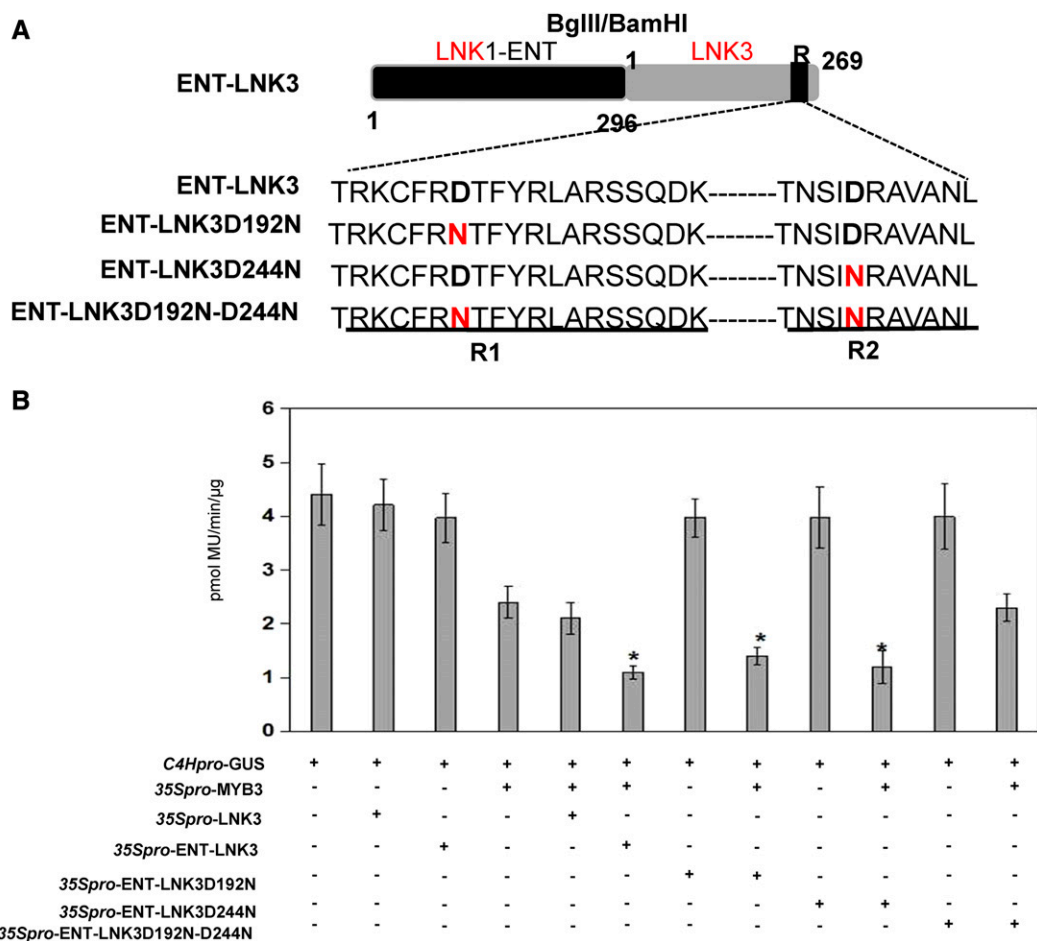


Figure 5. ENT domain of LNK1 promotes the activity of MYB3/LNK1 complex-dependent repression of *C4H*. A, Schematic representation of mutation of two conserved residues in either the R1 or R2 region of LNK3 and chimeric protein of ENT-LNK3. B, Transactivation assays in Arabidopsis protoplasts that were cotransfected with plasmids carrying C4Hpro-GUS and overexpression vectors containing 35S:MYB3, 35S:LNK3, 35S:ENT-LNK3, 35S:ENT-LNK3D192N, 35S:ENT-LNK3D244N, or 35S:ENT-LNK3D192N-D244N, as indicated. Values are means \pm SD of three biological repeats. Asterisks indicate statistically significant differences compared with C4Hpro-GUS, 35S:MYB3, 35S:LNK3, or 35S:ENT-LNK3 ($P < 0.05$, Student's *t* test).

cotransformation of ENT-LNK3, ENT-LNK3D192N, or ENT-LNK3D244N and MYB3 significantly enhanced the ability of MYB3 to repress the expression of *C4Hpro-GUS* in Arabidopsis cell suspension protoplast. As shown in Figure 4B, LNK3 inhibited MYB3 binding to *C4H* promoter, but chimeric protein ENT-LNK3 facilitated the MYB3 binding activity. These results indicate that ENT-LNK3, like LNK1, can be as a corepressor to enhance MYB3 repression activity. Taken together, we conclude the ENT domain of LNKs is required for MYB3/LNK complex-dependent repression of *C4H*.

LNK1/2-MYB3 Complex Represses Phenylpropanoids Biosynthesis

As noted above, overexpression of *MYB3* represses phenylpropanoids biosynthesis. Our results from transactivation assays indicated that LNK1 or LNK2

acts as corepressor to enhance MYB3 repression activity. Therefore, we wanted to functionally test the role of these proteins in plants. First, we generated double-mutant plants homozygous for T-DNA insertion alleles of *lnk1* and *lnk2* to circumvent possible redundant roles of these two proteins. Then, the triple mutant *lnk1/lnk2/myb3* plants were generated from a cross between *lnk1/lnk2* and *myb3* plants. qRT-PCR analysis confirmed that mutant seedlings failed to accumulate *LNK1*, *LNK2*, and *MYB3* transcripts (Supplemental Fig. S8). The double mutant *lnk1/lnk2* was morphologically indistinguishable from wild-type plants, which is agreement with the previous reports (Xie et al., 2014; Pérez-García et al., 2015). Additionally, the triple mutant *lnk1/lnk2/myb3* plants were also morphologically indistinguishable from wild-type plants. To further investigate whether the two conserved Asp residues in R1 and R2 of LNK C termini and ENT motif affect the activity of LNKs, Arabidopsis *lnk1/lnk2* and

wild-type plants constitutively overexpressing *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, *LNK1D556N/D614N-HA*, and *ENT-LNK3-HA* were generated. qRT-PCR analysis of 2-week-old seedlings showed that the *C4H* gene expression level was significantly higher in *lnk1/lnk2* plants and *LNK1D556N/D614N-HA/lnk1/lnk2* plants compared to wild-type plants *LNK1-HA/lnk1/lnk2* plants, respectively (Fig. 6A). In Arabidopsis wild-type background, the *C4H* expression level was reduced in *MYB3-HA*, *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, and *ENT-LNK3-HA*-overexpressing plants compared with plants overexpressing *LNK1D556N/D614N-HA* and wild-type plants. Interestingly, the *C4H* gene expression level was the highest in *lnk1/lnk2/myb3* plants compared with wild-type and *lnk1/lnk2* plants (Fig. 6A). It has been reported that the REV8 directly regulated anthocyanin biosynthesis-related gene *UGT79B1* (*At5g54060*, anthocyanin 3-*O*-glucoside 2'-*O*-

xylosyltransferase) is significantly up-regulated in *lnk1/lnk2* plants (Pérez-García et al., 2015). Here, we found that the *UGT79B1* gene was not changed in *MYB3-HA*-overexpressing plants compared with wild-type plants, indicating that MYB3 does not regulate the *UGT79B1* gene expression (Supplemental Fig. S9A). Additionally, the *UGT79B1* expression level was down-regulated in *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, and *ENT-LNK3-HA*-overexpressing plants, while significantly up-regulated in *lnk1/lnk2* and *LNK1D556N/D614N-HA/lnk1/lnk2* plants, compared with plants overexpressing *LNK1D556N/D614N-HA* and wild-type plants, respectively (Supplemental Fig. S9A). These results are in agreement with data showing that LNK1 and LNK2 act as transcriptional repressors of RVE8-activated *UGT79B1* gene expression (Pérez-García et al., 2015). Taken together, our observation suggests that LNK1 and LNK2 act as corepressors of MYB3 to

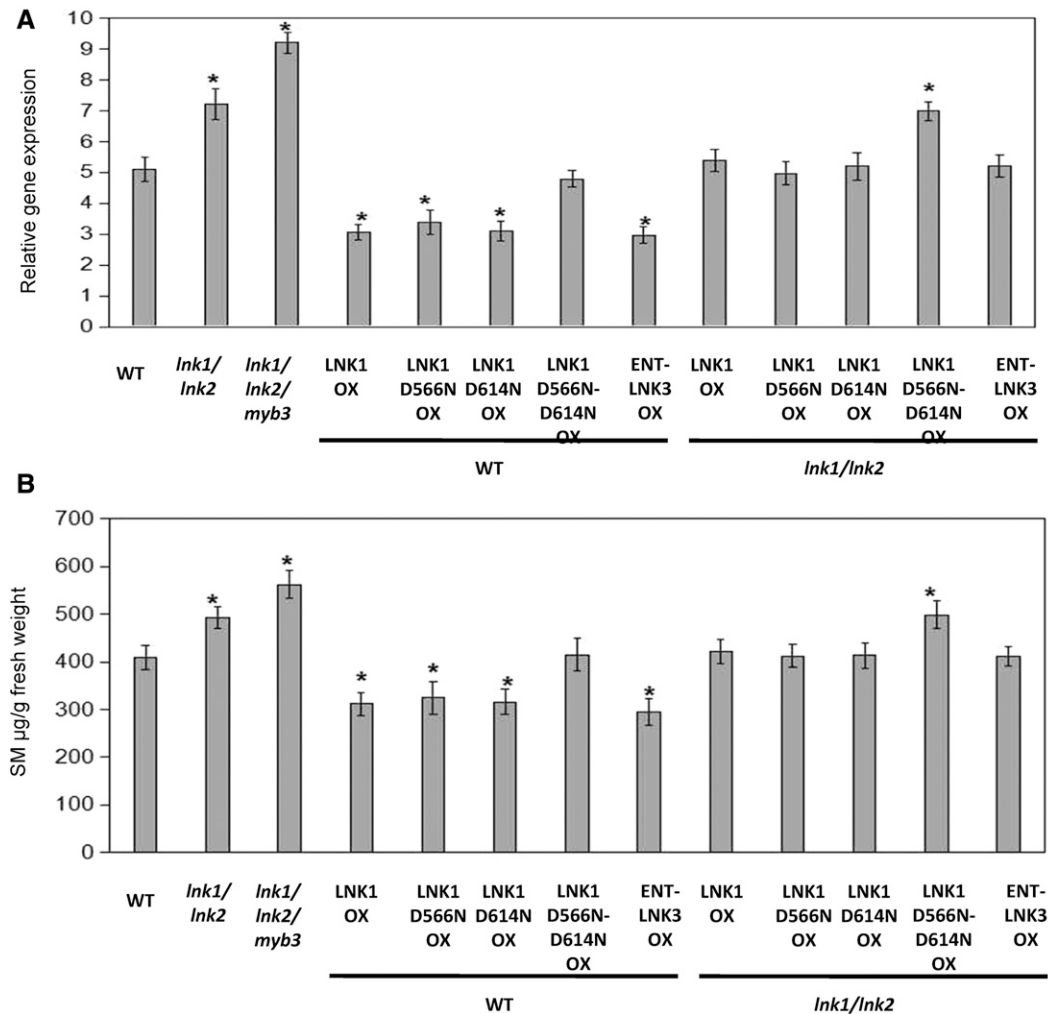


Figure 6. LNK1 and LNK2 repress phenylpropanoids biosynthesis. A and B, Measurement of *C4H* gene expression (A) and SM (B) levels in indicated genotypes by LC/MS and qRT-PCR, respectively. Values are means \pm SD of three biological repeats. Asterisks indicate statistically significant differences compared with wild-type and *lnk1/lnk2* plants ($P < 0.05$, Student's *t* test).

modulate *C4H* gene expression in plants, and this regulation depends on the two conserved Asp residues in R1 and R2 of LNKs' C-termini and the ENT domain of N termini.

To determine whether modulation of LNK1 and LNK1 derivatives activity affects the MYB3-regulated phenylpropanoid metabolism, the content of SM and anthocyanin were analyzed for all genotypes. Liquid chromatography/mass spectrometry (LC/MS) assays showed that SM accumulated at a significantly higher level in *lnk1/lnk2* plants and *LNK1D556N/D614N-HA/lnk1/lnk2* plants than wild-type and *myb3* plants. The level of SM was significantly reduced in plants overexpressing *MYB3-HA*, *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, and *ENT-LNK3-HA* compared with plants overexpressing *LNK1D556N/D614N-HA* and wild-type plants (Fig. 6B). As shown in Supplemental Figure S9B, decreased anthocyanin accumulation was observed in *MYB3-HA*, *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, and *ENT-LNK3-HA*-overexpressing plants, whereas the anthocyanin content was significantly higher in *lnk1/lnk2* plants and *LNK1D556N/D614N-HA/lnk1/lnk2* plants. The contents of both SM and anthocyanin were the highest in *lnk1/lnk2/myb3* plants compared to all other plants (Fig. 6; Supplemental Fig. S9), as expected. We did not observe the change of the anthocyanin concentration in the *LNK1D556N/D614N-HA*-overexpression plants. These results are consistent with the transcriptional changes observed in these plants and with the negative role for MYB3 and the corepressors of LNK1 and LNK2 in the control of the phenylpropanoid pathway.

DISCUSSION

R2R3-MYB TFs possess an N-terminal DNA-binding domain and a C-terminal regulation domain. Based on the highly varied C-terminal domains, R2R3-MYB TFs have been divided into subgroups. The function is broadly conserved for the R2R3-MYB TFs of the same subgroup in different angiosperms. Subgroup 4 R2R3-MYB TFs, MYB3, MYB4, MYB7, and MYB32 are considered as transcriptional repressors due to the presence of the EAR repression motif (pdLHLD/LLxiG/S) in their C termini (Dubos et al., 2010). MYB4 is responsive to UV-B irradiation and controls sinapate ester biosynthesis through negatively regulating the expression of the *C4H* gene (Jin et al., 2000), while MYB32 regulates pollen wall composition due to be a pollen-specific repressor of lignin biosynthesis (Preston et al., 2004). Additionally, MYB7 represses flavonol biosynthesis and partly involved in regulation of UV sunscreens (Fornalé et al., 2014). Here, we show that MYB3, like MYB4, negatively regulates the expression of the *C4H* gene and represses the sinapate ester biosynthesis. However, the regulatory activity of four members of subgroup 4 R2R3-MYB TFs is different. MYB4, MYB7, and MYB32 possess a conserved GY/FDFLGL motif in their C termini, which can be

recognized by an importin β -like protein SAD2, and then transports the SAD2-MYBs complex into the nucleus (Zhou et al., 2015b). Here, we demonstrated that the repression activity of MYB3 is directly regulated by LNKs, and this interaction depends on the varied C-terminal domain. However, the R3-MYB factor MYBL2 repressed flavonoid biosynthesis through directly inhibiting the activity of MYB-BHLH-WDR complexes (Dubos et al., 2008). Therefore, the same subgroup R2R3-MYB TFs share similar functions but have different regulation mechanisms.

The regulation of gene expression needs interlocked transcriptional feedback loops with both activating and repressive components. In the Arabidopsis circadian clock, many interlocked transcriptional components have been identified. For example, transcription of the morning phased *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) gene is repressed by the sequential binding of PSEUDORESPONSE REGULATOR (PRR) family protein to the *CCA1* and *LHY* promoters, and this repression requires a corepressor, encoded by members of the *TOPESS/TOPESS-RELATED* gene family (Nakamichi et al., 2010; Wang et al., 2013). LNK1 and LNK2 have

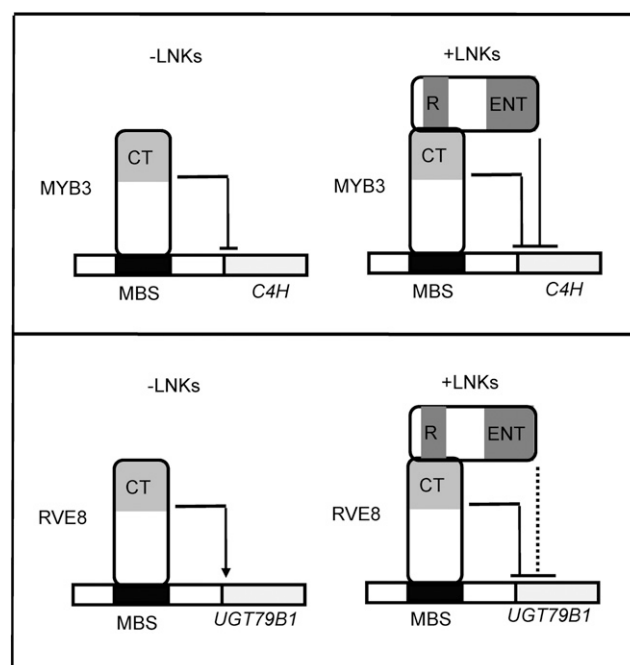


Figure 7. Simplified transcriptional networks of LNKs as corepressors in Arabidopsis phenylpropanoids biosynthesis. LNKs serve as transcriptional corepressors recruited to the promoters of target genes *C4H* and *UGT79B1* (Pérez-García et al., 2015), via protein-protein interaction with the DNA binding MYB TFs MYB3 and RVE8. Both regions 1 and 2 (R1 and R2) of C termini of LNKs contribute to the interaction with the C termini of MYB3. The ENT domain of LNKs is required for its repression activity. Arrows indicate activation, T-shaped lines indicate inhibition, and broken lines indicate that this regulation was not reported to date. CT, C termini; MBS, MYB binding site; UGT79B1, anthocyanin 3-O-glucoside.

been demonstrated to be as transcriptional coactivators via protein-protein interactions to enhance the transcription activity of RVE8 and RVE4 in the control of evening-expressed clock genes (Xie et al., 2014). However, it has been reported recently that LNK1 and LNK2 act as repressors of the expression of anthocyanin structural genes (such as *UGT79B1*), a repressive role that counteracts RVE8-activating function (Pérez-García et al., 2015). These studies thus unravel a dual sign for the regulatory activity of RVE8-LNK interaction. Here, we identified MYB3 as a new binding partner of LNK1 and LNK2. Our results showed that LNK1 and LNK2 serve as transcriptional corepressors interacting with MYB3 and facilitating binding of MYB3 with *C4H* promoter. Additionally, we found that LNK1 alone does not directly activate the *C4H* promoter (Fig. 4A), which contradicts the reported data showing LNK1 is recruited to elements of *PRR5* promoter and affects their activity (Xie et al., 2014). Therefore, LNKs probably have the different regulatory functions in different biological processes.

LNKs are plant-specific family proteins and consist of two conserved regions (R1 and R2). Both R1 and R2 domains contribute to the interaction between LNKs and multiple clock TFs (Xie et al., 2014). Mutation of two residues in either R1 or R2 domain (Arg-555Asp-556 to GlyGly or Asp-614Arg-615 to GlyGly) of LNK1 reduces the ability of LNK1 to interact with RVE4 (Xie et al., 2014). It has been widely accepted that the conserved Asp residue plays an important role in mediating protein-protein interaction. For example, the conserved Asp residue in the JAZ interaction domain of MYC2 and MYC3 plays a key role in binding to most JAZ proteins (Fernández-Calvo et al., 2011; Frerigmann et al., 2014; Goossens et al., 2015). Our recent publication showed that the conserved Asp residue mutation of SID motif of MYB4, MYB7, and MYB32 greatly reduces the interaction with SAD2 and is not transported into the nucleus (Zhou et al., 2015b). Here, we mutated the conserved Asp residue in either the R1 or R2 domain of LNK1, which does not affect the interaction between LNK1 and MYB3. However, two conserved Asp residues mutation from both R1 and R2 domains of LNK1 completely abolishes the interaction with MYB3. Thus, we postulate that the conserved Asp residue mutation of LNK1 greatly reduces the protein-protein interaction, probably by perturbing the LNK1 protein structure and thereby indirectly compromising the MYB3 binding domain.

Many plant nucleotide-binding Leu-rich repeat proteins possess an N-terminal domain that plays distinct roles in regulating downstream signaling pathways (Collier and Moffett, 2009). For example, the extended N-terminal domain of tomato (*Solanum lycopersicum*) Prf interacts with Pto kinase that directly interacts with AvrPtoB, which mediates downstream resistance response (Gutierrez et al., 2010). Tomato Mi-1.1 and 1.2 also contain an extra N-terminal domain play dual regulatory roles in signaling host cell death (Lukasik-Shreepaathy et al., 2012). Here, we also found that LNK1 and LNK2

possess an ENT domain around 300 amino acids long that is essential for LNK transcription activity. Therefore, we postulate that LNK1 and LNK2 serve as coregulators connecting the components of the transcription complex to regulate the downstream signaling pathway. It would be interesting to learn in the future whether the multi-layered regulatory mechanism exists for LNKs protein. Further investigation of the ENT domain and the identification of LNKs' ENT domain interaction partners could provide the potential for discovering new links into a range of plant signaling pathways.

In summary, the subgroup 4 R2R3-MYB TF MYB3 is a novel identified repressor in the phenylpropanoid biosynthesis. The repression activity of MYB3 to *C4H* gene expression is directly regulated by corepressors LNK1 and LNK2, which also act as corepressors of the expression of anthocyanin structural gene *UGT79B1* activated by MYB TF RVE8. The two conserved Asp residues in both R1 and R2 domains of LNKs are essential to mediate protein-protein interaction. Importantly, the ENT domain became a negative regulator in LNK-MYB3 transcription complex-dependent repression of *C4H* gene expression. Therefore, our data can be combined into a coherent model (Fig. 7) and may ultimately be useful for the design of artificial TFs for plant metabolic engineering.

MATERIALS AND METHODS

Yeast Two-Hybrid Assays

Full-length MYB3 cloned in pAS2.1 was used as bait for yeast two-hybrid screening. Using the Stratagene cDNA synthesis kit, amplified cDNA libraries representing 2×10^6 primary transformants were prepared from an equal mixture of RNA from stems, leaves, roots, and flowers of mature ecotype Col-0 *Arabidopsis* (*Arabidopsis thaliana*) plants. The cDNA in this library had been fused to the GAL4 activation domain (GAL4 AD) into pACT2 plasmid library (James et al., 1996). Cotransformation of bait and cDNA library was performed into yeast strain PJ64-4A according to a modified yeast transformation protocol (James et al., 1996). Transformants were plated on minimal synthetic defined (SD)-Glc medium containing 10 mM 3-amino-1,2,4-triazole and lacking Trp, Leu, and His (-LWH). The full-length genes *LNK1*, *LNK2*, *LNK3*, and *LNK4*, the deletion derivatives *LNK1ΔN1*, *LNK1ΔN2*, *LNK1ΔC*, *MYB3ΔN*, and *MYB3ΔC*, and the mutated LNK1 genes *LNK1D556N*, *LNK1D614N*, and *LNK1D556N-D614N* were cloned into pACT2 or pAS2.1. The point mutations were constructed using the GeneTailor site-directed mutagenesis system (Life Technologies). All constructed plasmids were sequenced. All primer sequences are presented in Supplemental Table S1. Interaction assays were performed by cotransformation of bait and prey plasmids into yeast strain PJ69-4A according to the protocol as previously described (Zhou et al., 2015b). The β -galactosidase assay was performed as described in the Yeast Protocols Handbook (Clontech).

Arabidopsis Protoplast Transactivation Assays

Full-length *MYB3-HA*, *LNK1-HA*, *LNK2-HA*, *LNK3-HA*, and *LNK4-HA*, or mutated *LNK1D556N-HA*, *LNK1D614N-HA*, *LNK1D556N/D614N-HA*, and chimeric *ENT-LNK3-HA* (HA tag, influenza virus hemagglutinin tag) were PCR amplified cloned into effector plasmid pRT101 under the CaMV 35S promoter (Töpfer et al., 1987). The promoter fragments of *C4H*-fused GUS cloned in reporter plasmid GusXX was described previously (Zhou et al., 2015b). All primer sequences are presented in Supplemental Table S1. Arabidopsis protoplast suspensions were cotransformed with a reporter plasmid combined with effector plasmids by polyethylene glycol-mediated transfection as previously described (Schirawski et al., 2000). GUS activity assays were performed as described (van der Fits and Memelink, 1997).

Plant Materials and Growth Conditions

The T-DNA insertion lines *myb3* (CS478221), *lnk1-3* (SALK_024353), *lnk2-5* (CS807006), *lnk3-2* (SALK_085551C), and *lnk4-2* (CS120858) were obtained from the ABRC and had been described previously (Xie et al., 2014; Pérez-García et al., 2015). Pollen from homozygous *lnk1-3* plants were used to pollinate emasculated homozygous *lnk2-5* flowers to generate *lnk1/lnk2* double homozygous plants. The triple mutant *lnk1/lnk2/myb3* plants were generated from a cross between *lnk1/lnk2* and *myb3* plants. For construction of transgenic lines constitutively overexpressing *MYB3-HA*, *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, *LNK1D556N/D614N-HA*, and *ENT-LNK3-HA*, the CaMV 35S cassettes containing the open reading frames of these genes were digested from pRT101 using *SphI* and cloned into pCAMBIA1300 digested using *SphI*. The binary vectors pCAMBIA1300 and empty vector were introduced into *Agrobacterium tumefaciens* strain LBA4404. *A. tumefaciens* was then used to transform Arabidopsis homozygous *lnk1/lnk2* plants and ecotype Col-0 by floral dip (Clough and Bent, 1998). Transgenic Arabidopsis plants were identified using the methods that were described by our previous report (Zhou et al., 2015b).

BiFC

The N-terminal (YN) or C-terminal (YC) fragments of the yellow fluorescent protein (nYFP or cYFP) were fused either N-terminally or C-terminally with MYB3, LNK1, LNK2, LNK3, and LNK4 and cloned in pRTL2-YNEE (nYFP-) or pRTL2-HAYC (-cYFP). All primer sequences are presented in Supplemental Table S1. The constructs were transiently coexpressed in all possible combinations of nYFP and cYFP fusion proteins in Arabidopsis cell suspension by polyethylene glycol-mediated transfection as previously described (Schirawski et al., 2000). Images of triplicate transfected protoplasts were acquired with a Leica DM IRBE confocal laser scanning microscope as described previously (Zhou et al., 2015b). Microscopy images were analyzed using the ImageJ software (Abramoff et al., 2004).

qRT-PCR Analyses

Total RNA extraction and reverse transcription (Revert Aid first-strand cDNA synthesis kit; Fermentas) were described in the manufacturer's instructions and our previous publication (Zhou et al., 2010). qRT-PCR was performed as previously described (Zhou et al., 2010). The PCR primers for *C4H*, *CHS*, *4CL1*, *4CL3*, *PAL*, *F5H*, *OMT*, *CAD*, *UGT79B1*, and reference gene *UBQ10* were as previously described (Jin et al., 2000; Fornalé et al., 2014; Xie et al., 2014; Pérez-García et al., 2015).

ChIP Assay

Detached leaves from 4-week-old Col-0 and MYB3OX (35S::MYB3-HA) plants were incubated in darkness for 24 h. HA antibody (Roche) was used for immunoprecipitation. ChIP assays were quantified by real-time PCR after normalizing with the input DNA. qPCR was performed using primers flanking the MYB-binding sequence of *C4H* promoter. Primers used for qRT-PCR presented in Supplemental Table S1. The coding sequence region of *C4H* gene and the reference gene *UBQ10* promoter were used as a negative control. *ACTIN2* was used as a reference gene. ChIP assays were performed as previously described (Saleh et al., 2008).

Protein Purification and Western Blot

Overexpression of HA-tagged genes *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, *LNK1D556N/D614N-HA*, and *ENT-LNK3-HA* in Arabidopsis seedlings were ground in protein extraction buffer (25 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA, 7 mM β -mercaptoethanol, 1% Triton X-100, and 10% glycerol). Proteins were separated on 10% (w/v) SDS-polyacrylamide gels, transferred to Protran nitrocellulose by semidry blotting, and detected with anti-HA antibody (Roche). The detailed extraction method and western blot were described in our previous publication (Zhou et al., 2015b).

EMSA

To produce His-tagged proteins, MYB3, LNK1, LNK3, and ENT-LNK3 were amplified and cloned in pASK-IBA45plus. All primer sequences are presented in Supplemental Table S1. These constructs were transformed into *Escherichia coli* strain BL21 (DE3) pLysS selecting on 50 μ g/mL chloramphenicol and 200 μ g/mL carbenicillin, and protein expression was induced by 0.2 μ g/mL

anhydrotetracycline. The soluble His fusion proteins were extracted and immobilized onto Ni-NTA agarose beads (Qiagen). The 200-bp probe sequence including MYB3 binding site is presented in Supplemental Table S1. EMSAs were performed using biotin-labeled double-stranded probes and the Light Shift Chemiluminescent EMSA Kit according to the manufacturer's instructions (Thermo Fisher).

Measurement of Sinapoyl Malate and Anthocyanin

Arabidopsis seedlings were grown in solid acidified Murashige and Skoog medium at pH 5.4 by addition of HCl. Then, 2-week-old leaves were frozen and ground in liquid nitrogen. Total phenolics were extracted in dry ice-cold methanol. The anthocyanin content was measured as described by Swain and Hillis (1959) and presented as $(A_{535} - A_{650})/g$ fresh weight. The sinapoyl malate content was analyzed by LC/MS as described previously (Tamagnone et al., 1998). The experiment was repeated three biological times.

Statistical Analysis

Data were analyzed using Student's *t* test. A *P* value < 0.05 was considered to be significant.

Accession Numbers

Sequence data for the genes described in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: MYB3 (At1g22640), LNK1 (At5g64170), LNK2 (At3g54500), LNK3 (At3g12320), LNK4 (At5g06980), C4H (At2g30490), UGT79B1 (At5g54060), and UBQ10 (At4g05320).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Description and Identification of *myb3* Mutant and *MYB3*-Overexpressing Plants.

Supplemental Figure S2. Measurement of the Transcripts of Phenylpropanoids Biosynthesis Genes in Wild-Type, *myb3*, and *MYB3OX* Plants by Quantitative RT-PCR.

Supplemental Figure S3. Effect of Overexpression of *MYB3* on the Tolerance of Arabidopsis Seedlings to UV-B.

Supplemental Figure S4. MYB4, MYB7, and MYB32 Do Not Interact with a Small Family of Four LNK Proteins in Yeast.

Supplemental Figure S5. The LNK1D556N, LNK1D614N, and LNK1D556N-D614N Mutations Do Not Affect LNK1 Protein Stability in Transformed Yeasts.

Supplemental Figure S6. MYB3 Repressor Activity Is Not Enhanced by LNK3 and LNK4.

Supplemental Figure S7. Amino Acid Sequence Alignment of a Small Family of Four LNK Proteins.

Supplemental Figure S8. Identification of *lnk1/lnk2* Mutant, *lnk1/lnk2/myb3* Mutant, and *LNK1-HA*, *LNK1D556N-HA*, *LNK1D614N-HA*, *LNK1D556N/D614N-HA*, and *ENT-LNK3-HA*-Overexpressing Plants.

Supplemental Figure S9. LNK1, LNK2, and MYB3 Repress Anthocyanin Accumulation.

Supplemental Table S1. Primers Used in This Study.

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