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The role of Class II KNOX family in controlling compound leaf patterning in *Medicago truncatula*

Xiao Wang[1], Juanjuan Zhang[1], Maofeng Chai[2], Lu Han[1], Xiaohua Cao[1], Jing Zhang[1], Yiming Kong[3], Chunxiang Fu[4], Zeng-Yu Wang[2], Kirankumar S. Mysore[5], Jiangqi Wen[5] and Chuanen Zhou[1]*

1. The Key Laboratory of Plant Development and Environmental Adaptation Biology, Ministry of Education, School of Life Science, Shandong University, Qingdao 266237, China
2. Grassland Agri-Husbandry Research Center, College of Grassland Science, Qingdao Agricultural University, Qingdao 266109, China
3. College of Life Sciences, Shandong Normal University, Jinan 250014, China
4. Key Laboratory of Biofuels, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China
5. Institute of Agricultural Biosciences, Oklahoma State University, Ardmore 73401, Oklahoma, USA

*Correspondence: Chuanen Zhou (czhou@sdu.edu.cn)

Xiao Wang

Chuanen Zhou

ABSTRACT

Compound leaf development requires the coordination of genetic factors, hormones, and other signals. In this study, we explored the functions of Class II *KNOTTED*-like homeobox (*KNOXII*) genes in the model leguminous plant *Medicago truncatula*. Phenotypic and genetic analyses suggest that *MtKNOX4*, *5* are able to repress leaflet formation, while *MtKNOX3*, *9*, *10* are not involved in this developmental process. Further investigations have shown that MtKNOX4 represses the CK signal transduction, which is downstream of MtKNOXI-mediated CK biosynthesis. Additionally, two boundary genes, *FUSED COMPOUND LEAF1* (orthologue of *Arabidopsis* Class M *KNOX*) and *NO APICAL MERISTEM* (orthologue of *Arabidopsis CUP-SHAPED COTYLEDON*), are necessary for MtKNOX4-mediated compound leaf formation. These findings suggest, that among the members of MtKNOXII, MtKNOX4 plays a crucial role in integrating the CK pathway and boundary regulators, providing new insights into the roles of MtKNOXII in regulating the elaboration of compound leaves in *M. truncatula*.

Keywords: *KNOTTED*-like homeobox, KNOXM, NAM, cytokinin, compound leaf

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INTRODUCTION

Leaves have evolved into different forms, including variations in blade, petiole and stipule (Conklin et al., 2018). Leaves can be simple, with one leaf blade attached to the petiole, or compound with several distinct leaflets (Champagne and Sinha, 2004; Barkoulas et al., 2007). Both simple leaves and compound leaves arise from the periphery of the shoot apical meristem (SAM), followed by the establishment of leaf polarity, formation of the leaf blades, lobes or leaflets, and other attachments (Andriankaja et al., 2012; Bar and Ori, 2014; Ichihashi and Tsukaya, 2015; Sluis and Hake, 2015).

In some compound-leafed species, such as *Solanum lycopersicum* and *Cardamine hirsute*, the Class I *KNOTTED*-like homeobox (*KNOXI*) genes play key roles in leaf morphogenesis (Hareven et al., 1996; Bharathan et al., 2002; Hay and Tsiantis, 2006; Shani et al., 2009; Hay and Tsiantis, 2010; Gupta and Tsiantis, 2018). In these species, the expression of *KNOXI* genes can be detected in developing leaves, and suppression of *KNOXI* leads to a reduction of the leaflet number (Hay and Tsiantis, 2006; Shani et al., 2009). Moreover, increasing activity of *KNOXI* genes results in extra leaflets (Hareven et al., 1996; Parnis et al., 1997; Janssen et al., 1998; Hay and Tsiantis, 2006). These observations suggest that the KNOXI family is not only

necessary but also sufficient for the formation of leaflets in these species.

In contrast with *KNOXI*, Class II *KNOTTED*-like homeobox (*KNOXII*) genes display diverse expression patterns and functions. For example, members of *KNOXII* are involved in root development, ABA response, secondary cell wall biosynthesis, seed coat mucilage biosynthesis, as well as leaf serration development in *Arabidopsis* (Serikawa et al., 1997; Truernit et al., 2006; Zhong et al., 2007; Li et al., 2011; Li et al., 2012; Bhargava et al., 2013; Kim et al., 2013; Liu et al., 2014; Furumizu et al., 2015; He et al., 2018; Wang et al., 2020; Challa et al., 2021; Zhang et al., 2022). In rice, *KNOXII* family member *OsKNAT7* negatively regulates the grain size and the thickness of the cell wall, and *HOS59* functions as a negative regulator in grain size and plant architecture regulation (Wang et al., 2019b; Sheng et al., 2022). Additionally, *KNOXII* genes repress cell wall thickness during wood development in cotton and some Cactaceae plants, and control the physical dormancy of seeds in mung beans (Gong et al., 2014; Reyes-Rivera et al., 2017; Laosatit et al., 2022). In *Solanum lycopersicum*, *KNOXII* genes function redundantly in dry and fleshy fruits maturation (Keren-Keiserman et al., 2022).

In *Medicago truncatula*, five *MtKNOXI* genes have been identified. *MtKNOX1* and *MtKNOX6* are *STM-like* genes, *MtKNOX2* is a *BP-like* gene, *MtKNOX7* and *MtKNOX8* are *KNAT2/6-like* genes (Di Giacomo et al., 2008; Zhou et al., 2014). In addition, *MtKNOX3*, *MtKNOX4*, *MtKNOX5*, *MtKNOX9* and *MtKNOX10* belong to the *MtKNOXII* family (Zhou et al., 2014). *MtKNOX4* controls the seed physical dormancy, and mutation of *MtKNOX4* allows the seeds to absorb water easily (Chai et al., 2016). Other *MtKNOXII* genes mainly function in nodule development. *MtKNOX3* modulates symbiotic nodule development through the cytokinin (CK) biosynthesis pathway by activating the expression of *LONELYGUY1/2* (*MtLOG1/2*), and *ISOPENTENYL TRANSFERASE 3 (IPT3)* (Azarakhsh et al., 2015; Azarakhsh et al., 2020). Silencing the expression of *MtKNOX3*, *5*, *9*, and *10* promotes the formation of fused nodule organs, and decreases the expression of both the ethylene response gene *MtEFD* and the CK response gene *MtRR4* (Di Giacomo et al., 2016).

M. truncatula, as a widely studied compound-leafed species in legumes, provides a sensitive system to explore the mechanism of leaf complexity (Wang et al., 2008). However, *KNOXI* genes are not involved in compound leaf development in *M. truncatula* (Champagne et al. 2007). Accordingly, simultaneous disruption of *STM/BP-like MtKNOXI* genes, *MtKNOX1*, *2*, and *6*, shows normal leaf morphology (Zhou et al., 2014). *M. truncatula* belongs to the inverted repeat lacking clade (IRLC) of Fabaceae, in which the *LEAFY* orthologue, *SINGLE LEAFLET1* (*SGL1*), replaces *MtKNOXI* genes to determine the compound leaf patterning. The loss-of-function *sgl1* mutant exhibits a simple leaf, indicating that it is necessary for the formation of leaflets (Wang et al., 2008). However, overexpression of *SGL1* did not increase leaf complexity, suggesting that *SGL1* activity is not sufficient to prolong the window of morphogenetic plasticity during leaf development (Zhou et al., 2014). These observations imply that *LEAFY* orthologues in IRLC species and *KNOXI* orthologues in other compound-leafed species have different regulatory mechanisms in the elaboration of compound leaf patterning.

Several key regulators have been identified during the formation of the compound leaf. *CUP-shaped COTYLEDON* (*CUC*)/*NO APICAL MERISTEM* (*NAM*) genes are required for the boundary formation between the leaflet primordia during the compound leaf development (Blein et al., 2008). The silencing or mutation of *CUC*/*NAM* genes results in fused leaflets or reduced leaflet number (Blein et al., 2008; Berger et al., 2009; Rast-Somssich et al., 2015; Jiao et al., 2019). In *M. truncatula*, *MtNAM* is also specifically expressed at the boundary regions of leaflets, and their mutation leads to the fusion of leaflets (Cheng et al., 2012). In addition, gain of function of *PETROSELINUM* (*PTS*), which encodes a Class M KNOX protein, increases the compound leaf complexity in tomato (Kimura et al., 2008). The orthologous gene of *PTS* in *M. truncatula* is *FUSED COMPOUND LEAF1* (*FCL1*), which is expressed at boundaries between the SAM and the leaf primordia. The leaves in *fcl1* are fused or clustered, suggesting a trend toward decreased leaf complexity (Peng et al., 2011).

During the developmental process of leaves, activation or repression of indeterminacy in developing leaves results in changes in compound leaf patterning. In this study, we comprehensively analyzed the function of the KNOXII (MtKNOXII) family in compound leaf development in *M. truncatula*. The roles of the *MtKNOXII* family showed considerable diversity, in which *MtKNOX4*, *5* function as repressors in controlling leaf complexity and *MtKNOX3*, *9*, and *10* are not involved in compound leaf formation. Genetic analysis showed that *MtKNOX4* plays a major role in the regulation of compound leaf formation, which is independent of *STM/BP-like MtKNOXI* activity. Moreover, MtKNOX4 acts antagonistically to FCL1, but functions in an *MtNAM*-dependent manner. Our work discovered that the members of MtKNOXII are not only necessary, but also sufficient, to repress leaf complexity, and sheds light on the conserved and diverged regulatory mechanisms of compound leaf patterning in IRLC species.

RESULTS

Genetic characterization of the *MtKNOXII* family in compound leaf development

To explore the roles of the *MtKNOXII* genes in *M. truncatula*, we analyzed the phylogenetic relationship of KNOXII proteins in *Arabidopsis thaliana*, *Solanum lycopersicum*, *Glycine max*, and *Medicago truncatula*. The KNOXII proteins were divided into a KNAT3/4/5 clade and a KNAT7 clade. MtKNOX3, 5, 9, and 10 were classified into the KNAT3/4/5 clade, whereas MtKNOX4 was grouped into the KNAT7 clade and separated from the other four members of MtKNOXII (Figure 1A). Previous studies have shown that the KNOX family is characterized by a

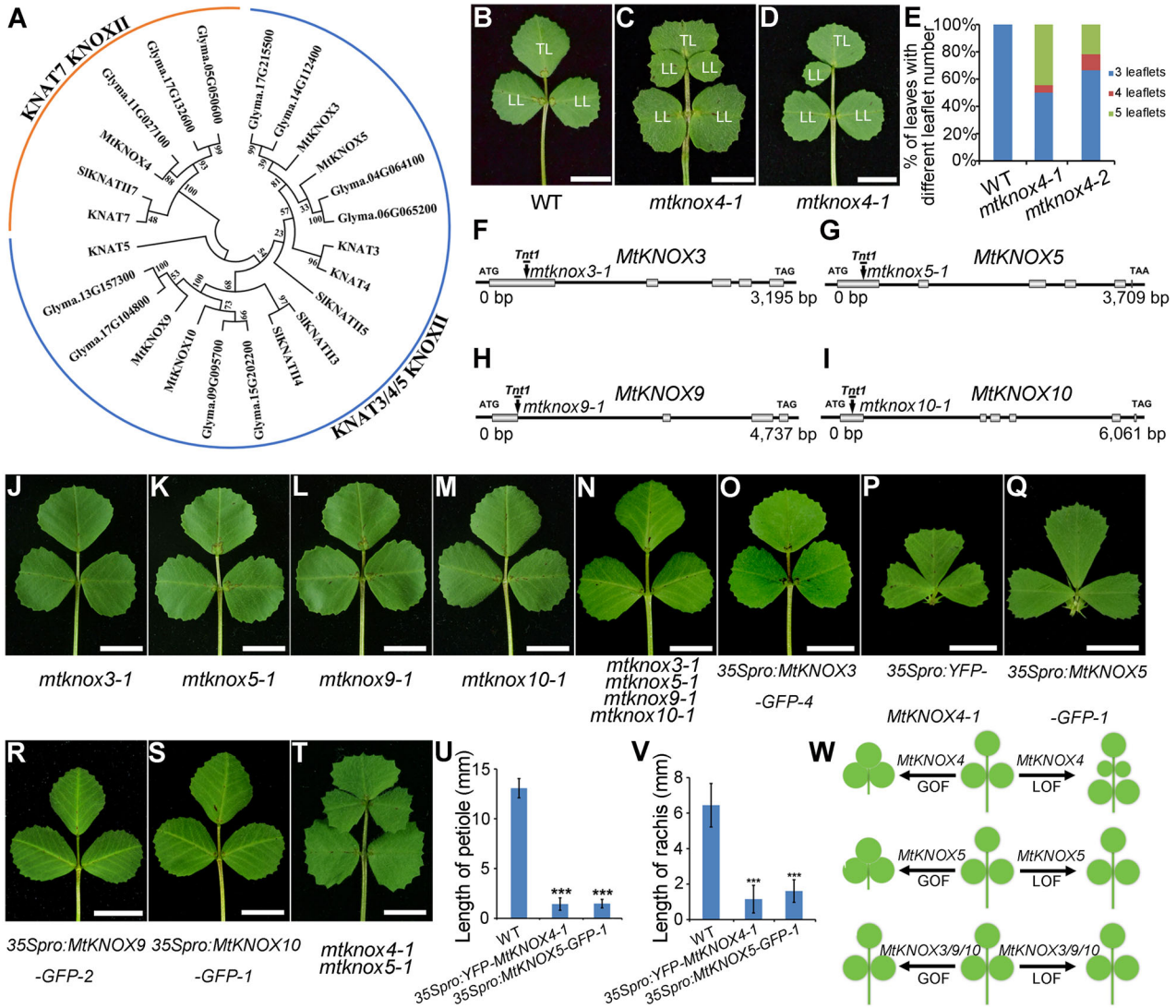

Figure 1. Genetic characterization of the *KNOXII* family in *M. truncatula*

(A) Phylogenetic tree of KNOXII proteins from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Glycine max*, and *Medicago truncatula*. The two distinct protein clades are named based on their homology with *Arabidopsis* genes. **(B–D)** Show 30-d-old leaves of the wild-type **(B)** and *mtknox4-1* **(C, D)**. TL, terminal leaflet; LL, lateral leaflet. Bars = 1 cm. **(E)** Proportion of leaves with different leaflet numbers in the wild-type and *mtknox4* mutants. **(F–I)** Schematic representation of the gene structures of *MtKNOX3*, *5*, *9*, and *10* showing the *Tnt1* insertion sites in *mtknox3-1*, *mtknox5-1*, *mtknox9-1* and *mtknox10-1*. The positions of the ATG start and TAA/TAG stop codons are shown. Vertical arrows mark the location of *Tnt1* retrotransposons in mutants. Introns are represented by lines, exons are represented by boxes. **(J–N)** Show 30-d-old leaves of the *mtknox3-1* **(J)**, *mtknox5-1* **(K)**, *mtknox9-1* **(L)**, *mtknox10-1* **(M)** and *mtknox3-1 mtknox5-1 mtknox9-1 mtknox10-1* **(N)** mutants. Bars = 1 cm. **(O–T)** Show 30-d-old leaves of the *35Spro:MtKNOX3-GFP-4* **(O)**, *35Spro:MtKNOX4-GFP-1* **(P)**, *35Spro:MtKNOX5-GFP-1* **(Q)**, *35Spro:MtKNOX9-GFP-2* **(R)**, *35Spro:MtKNOX10-GFP-1* **(S)** and *mtknox4-1 mtknox5-1* plants **(T)**. Bars = 1 cm. **(U, V)** Lengths of petiole **(U)** and rachis **(V)** at the third node of 45-d-old wild-type, *35Spro:MtKNOX4-GFP-1* and *35Spro:MtKNOX5-GFP-1* plants ($n = 16$). $^{***}P < 0.001$. **(W)** Schematic illustration of the functions of the *MtKNOXII* genes in leaf complexity regulation. Gain of *MtKNOX4* function (GOF) causes simplified leaves, while loss of *MtKNOX4* function (LOF) causes complex leaves. Gain of function of *MtKNOX5* leads to simplified leaves, and loss of function of *MtKNOX5* does not influence leaf complexity. *MtKNOX3*, *9*, *10* do not affect leaf complexity.

DNA-binding homeodomain (HD) domain and a MEINOX interacting domain (Hay and Tsiantis, 2010; Liu et al., 2014). According to this, the amino acid sequence alignment of the MtKNOXII proteins showed two types of conserved domains, including one HD domain and one MEINOX domain (KNOXI domain and KNOXII domain) (Figure S1).

To determine the function of the MtKNOXII family in compound leaf development, the loss-of-function mutants were characterized. We first observed the leaf phenotype of *mtknox4* mutants that had been reported previously to be involved in the physical dormancy of seeds (Chai et al., 2016). Compared with the trifoliate leaf form in the wild-type, *mtknox4* mutants exhibited an extra one or two leaflets on rachises (Figure 1B–D). Two independent mutant lines of *MtKNOX4* were selected to count the leaflet numbers, and about 50% of leaves in *mtknox4-1* and 33.5% in *mtknox4-2*

produced extra leaflets (Figure 1E). To further explore the functions of other members of MtKNOXII, loss-of-function mutants of *MtKNOX3*, *5*, *9*, and *10* were isolated by a reverse genetic screening in a *Tnt1* retrotransposon insertion mutant collection of *M. truncatula* (Cheng et al., 2014). Sequence analysis showed that a single *Tnt1* was inserted in the first exon of four mutants (Figure 1F–I). Reverse transcription-polymerase chain reaction (RT-PCR) data showed that transcripts were completely interrupted by the *Tnt1* insertion in these mutants (Figure S2), indicating that they were knockout mutants. Phenotypic observation showed that *mtknox3*, *5*, *9*, and *10* mutants did not exhibit obvious defects in leaf morphology and compound leaf pattern (Figure 1J–M). To assess the functional redundancy between them, quadruple mutants were generated. Simultaneous disruption of *MtKNOX3*, *5*, *9*, and *10* led to the slightly downward-curled leaves, but the leaflet number was unchanged (Figure 1N). These data demonstrated that MtKNOX4 plays a dominant role in suppressing the indeterminacy of developing leaves to elaborate the leaflet number, while MtKNOX3, 5, 9, and 10 have limited involvement in the process.

To test whether increasing activities of MtKNOXII are able to affect leaf complexity, they were overexpressed in the wild-type under the control of the *CaMV35S* promoter. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses showed that their transcriptional levels were significantly increased in transgenic plants (Figure S3). The leaves of *MtKNOX3*, *9*, and *10*-overexpressing lines were similar to those of the wild-type (Figure 1O, R, S). However, the transgenic plants overexpressing *MtKNOX4* and *MtKNOX5* showed clustered leaflets, shortened petioles and rachises (Figure 1P, Q, U, V), suggesting a trend toward decreased leaf complexity. These data indicated that *MtKNOX4* and *5* probably have a similar role in the regulation of the window of morphogenetic plasticity. To further study the possible redundancy between them, *mtknox4-1 mtknox5-1* double mutants were generated. The leaves of double mutants were similar to those in *mtknox4* (Figure 1T), further indicating that *MtKNOX4* plays a major role in regulating the compound leaf pattern. Taken together, the elaboration of compound leaf development requires the necessary and sufficient activity of MtKNOX4, while MtKNOX5 is only sufficient for reducing leaf complexity. It is possible that MtKNOX3, 9, and 10 are not involved in leaf development patterning (Figure 1W).

Phenotype of *mtknox4* is independent of *STM/BP-like MtKNOXI* activities

To explore the mechanism of MtKNOXII underlying compound leaf patterning, we focused on *MtKNOX4* as it plays a major role in this process. First, the expression pattern of *MtKNOX4* was analyzed. An *MtKNOX4* promoter-β-glucuronidase (GUS) reporter was generated and introduced into wild-type plants. The transgenic plants of *MtKNOX4pro:GUS* exhibited the GUS signals in young leaves as well as in mature leaves (Figure 2A, B). To gain a better spatial expression pattern of *MtKNOX4*, RNA *in situ* hybridization was performed. Strong *MtKNOX4* transcripts were detected in the developing leaflets at stage 5, but not at the early stages (Figure 2C). At stage 7, *MtKNOX4* transcripts mainly accumulated in the leaf lamina (Figure 2D). The sense probe was hybridized as the control and did not show any signal (Figure 2E). These observations suggest that *MtKNOX4* functions during the relatively late stages of leaf development in *M. truncatula*.

In compound leaves, leaflets are generated along the leaf margins (Du et al., 2020). As the loss-of-function mutant of *mtknox4* exhibited extra leaflets, this probably hints toward its role in leaf marginal patterning. Auxin is essential for the initiation of leaflet primordia and *SMOOTH LEAF MARGIN1* (*SLM1*), encoding an auxin efflux carrier, is responsible for driving the accumulation of auxin in leaflet primordia in *M. truncatula* (Zhou et al., 2011). To investigate the possible relationship between MtKNOX4 and auxin, RNA *in situ* hybridization of *SLM1* was performed in the shoot apices of wild-type, *mtknox4-1* and *35Spro:YFP-MtKNOX4-1*. *SLM1* mRNA was detected in the leaf vein precursors and vascular tissues of petioles in all plants, and relatively weak *SLM1* expression was detected in *35Spro:YFP-MtKNOX4-1* compared with that in wild-type (Figure S4). Moreover, strong hybridization signals were detected at the leaf marginal region where ectopic leaf primordia were initiated in the *mtknox4-1* mutant, indicating that the auxin accumulation mediated by *SLM1* was associated with the initiation of ectopic leaflets in *mtknox4-1* (Figure S4B).

Our previous report showed that the leaflet number was significantly increased in overexpressing *STM/BP-like MtKNOXI* (*MtKNOX1*, *2*, and *6*) transgenic plants in *M. truncatula*. The *35S:MtKNOX2* transgenic plants with higher expression levels exhibited the reiteration of higher order leaflets (Zhou et al., 2014), and the transgenic plants with lower *MtKNOX2* expression normally produced two ectopic leaflets, which is similar to those of *mtknox4* mutant. To study the relationship between *MtKNOX4* and *STM/BP-like MtKNOXI* genes, the expression levels of *MtKNOX1*, *2*, and *6* in wild-type and *mtknox4* were checked. qRT-PCR data showed that the transcriptional levels of *MtKNOX1*, *2*, and *6* remained unchanged in the *mtknox4-1* mutant compared with those in the wild-type (Figure 2F). To further determine whether *STM/BP-like MtKNOXI* was responsible for the formation of additional leaflets in *mtknox4*, the *mtknox4-1 mtknox2-1* double mutant was generated. The results showed that knocking out *MtKNOX2* in *mtknox4* could not rescue the defects in *mtknox4* (Figure 2G, H). Furthermore, knocking out all *MtKNOX1*, *2*, and *6* genes in the *mtknox4* background did not recover the leaf patterning of *mtknox4* (Figure 2I, J), suggesting that the leaf phenotype of *mtknox4* is independent of the *STM/BP-like MtKNOXI* activities.

MtKNOX4 regulates compound leaf development by repressing CK signal transduction

Previous studies have shown that *KNOXI* genes positively regulate CK biosynthesis during leaf development in *Arabidopsis*, rice, and tomato (Jasinski et al., 2005; Sakamoto et al., 2006; Shani et al., 2010), and that a reduction in the CK

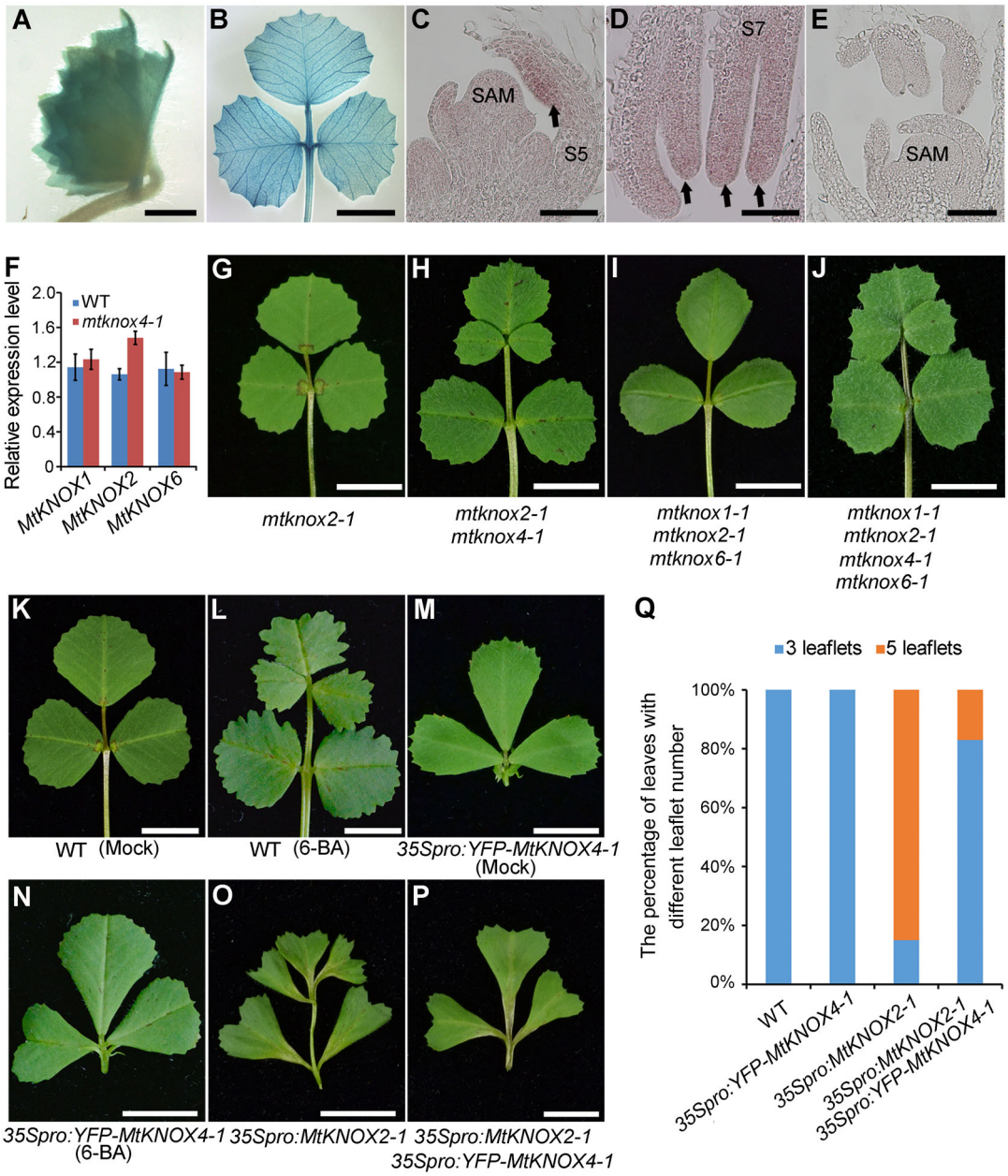

Figure 2. Genetic interaction between *MtKNOX4* and *STM/BP-like MtKNOXI*, and regulatory relationship between *MtKNOX4* and CK signal transduction pathway

(A, B) GUS histochemical staining of the 30-d-old transgenic plants harboring the *MtKNOX4pro:GUS* construction. The promoter of *MtKNOX4*-driven GUS is expressed in the unexpanded leaf **(A)** and fully expanded leaf **(B)**. Bars = 2 mm. **(C, D)** RNA *in situ* hybridization analyses of *MtKNOX4* mRNA in the wild-type. Longitudinal sections of shoot apical meristem and leaf primordia are shown in **(C)**. Transverse sections of developed leaflets are shown in **(D)**. SAM, shoot apical meristem; S, stage. Arrows indicate the signals for *MtKNOX4*. Bars = 50 μm. **(E)** The sense probe was hybridized and used as a control. Bar = 50 μm. **(F)** Expression levels of *MtKNOX1*, *2*, and *6* genes in the wild-type and *mtknox4-1* determined by qRT-PCR. Transcript levels were measured using vegetative buds of 30-d-old plants. *MtUBIQUITIN* was used as the internal control. Bars represent the mean ± SD of three biological replicates. **(G–J)** Show 30-d-old leaves of the *mtknox2-1* **(G)**, *mtknox2-1 mtknox4-1* **(H)**, *mtknox1-1 mtknox2-1 mtknox6-1* **(I)** and *mtknox1-1 mtknox2-1 mtknox4-1 mtknox6-1* **(J)** mutants. Bars = 1 cm. **(K–N)** Show 45-d-old leaves of the wild-type **(K, L)** and *35Spro:YFP-MtKNOX4-1* **(M, N)** treated with 0.1 mM 6-benzyladenine and the same concentration of Tween 20 as the controls. Bars = 1 cm. **(O)** and **(P)** 30-d-old leaves of the *35Spro:MtKNOX2-1* **(O)** and *35Spro:YFP-MtKNOX4-1 35Spro:MtKNOX2-1* plants **(P)**. Bars = 1 cm. **(Q)** The proportion of leaves with different leaflet numbers was compared among wild-type, *35Spro:YFP-MtKNOX4-1*, *35Spro:MtKNOX2-1* and *35Spro:YFP-MtKNOX4-1 35Spro:MtKNOX2-1* plants.

level could suppress the phenotype of *KNOXI* overexpression plants in tomato (Shani et al., 2010). Moreover, exogenous CK treatment was sufficient for increasing leaf serration or leaf complexity (Greenboim-Wainberg et al., 2005; Sun et al., 2022). To test whether the ectopic leaflets in *mtknox4* mutants were related to the CK pathway, exogenous spraying of 6-benzyladenine (6-BA) solution was manipulated in *35Spro:YFP-MtKNOX4* and wild-type plants, and the same

concentration of Tween 20 was used as a control. Extra leaflets and lobed leaves were formed in the wild-type, but not in *35Spro:YFP-MtKNOX4* plants (Figure 2K–N). These results indicated that *MtKNOX4* represses the signal transduction of CK in leaf development. In addition, transcript profiling analyses were performed by RNA sequencing, and the differentially expressed genes (DEGs) in *mtknox4-1* mutant and *35Spro:YFP-MtKNOX4-1* are shown in Table S1 and Table S2, respectively. The data showed that many genes involved in the CK signal transduction displayed the expression changes in an opposite manner in *mtknox4-1* and *35Spro:YFP-MtKNOX4-1* (Figure S5), indicating that the function of *MtKNOX4* was associated with CK signaling. In tomato, heightening the CK signals resulted in increased leaf complexity (Shwartz et al., 2016). Similarly, six response regulators (RRs) involved in cytokinin signaling were upregulated in the *mtknox4-1* mutants and downregulated in the *35Spro:YFP-MtKNOX4-1* (Figure S5), further suggesting that *MtKNOX4* repressed the CK signal transduction pathways. As overexpressing *MtKNOX4* and *MtKNOX2* in the wild-type displayed opposite effects on leaf complexity, the relationship between them was investigated by crossing the *35Spro:YFP-MtKNOX4-1* and *35Spro:MtKNOX2-1* plants. The percentage of five leaflets in *35Spro:MtKNOX2-1* plants was 85%, and decreased to 17% when the *35Spro:YFP-MtKNOX4-1* was introduced (Figure 2O–Q). The recovery of ectopic leaflets in overexpressing MtKNOXI plants by the activities of MtKNOX4 further demonstrated that MtKNOX4 functioned downstream of MtKNOXI and negatively regulated CK signal transduction.

MtKNOX4 is antagonistic to FCL1 at the protein level in compound leaf development

A previous study showed that the loss-of-function mutant of *fcl1* displayed abnormal leaves with clustered leaflets (Peng et al., 2011), which was similar to the leaves in *35Spro:YFP-MtKNOX4* transgenic plants (Figure 3A, B). To investigate whether *FCL1* was involved in *MtKNOX4*-mediated compound leaf development, the expression level of *FCL1* was first detected. qRT-PCR data showed no obvious change between wild-type and *mtknox4* (Figure 3C), suggesting that *MtKNOX4* did not regulate *FCL1* activity at the transcriptional level. To better understand the potential regulatory mechanism between *MtKNOX4* and *FCL1*, their interaction at the protein level was investigated. A yeast-two-hybrid (Y2H) assay showed that MtKNOX4 could interact with FCL1 (Figure 3D), which was confirmed by the bimolecular fluorescence complementation (BiFC) assay (Figure 3E). Moreover, MtKNOX4 protein contains the MEINOX domain (KNOXI domain and KNOXII domain) and homeodomain (Figure 3F). Y2H assay showed that the KNOXI portion of the MEINOX domain was sufficient for MtKNOX4 interaction with FCL1 (Figure 3G). To confirm their interaction *in vivo*, we performed a co-immunoprecipitation (Co-IP) assay by transiently expressing MYC-tagged MtKNOX4 (MYC-MtKNOX4) and GFP-tagged FCL1 (FCL1-GFP) in *Nicotiana benthamiana* leaves. The MYC-MtKNOX4 fusion protein co-immunoprecipitated with FCL1-GFP (Figure 3H), further supporting the interaction between MtKNOX4 and FCL1.

To investigate the biological significance of the MtKNOX4-FCL1 complex regulatory mechanism, the subcellular localization of these proteins was observed. *35Spro:YFP-FCL1* plasmids were transformed into *M. truncatula* protoplasts and YFP signals were observed in the cytoplasm and nucleus (Figure 3I). Then, *35Spro:YFP-FCL1* was co-expressed with *35Spro:MtKNOX4* in protoplasts, and the intensity of YFP fluorescence decreased significantly, indicating that MtKNOX4 represses the activities of FCL1 at the protein level (Figure 3I, K). Moreover, *35Spro:YFP-MtKNOX4* was co-expressed with *35Spro:FCL*, and the fluorescence intensity of YFP was also decreased, compared with that of *35Spro:YFP-MtKNOX4* (Figure 3J, L). These results demonstrated that MtKNOX4 and FCL1 mutually inhibit their activities at the protein level. To further confirm such antagonism at the genetic level, the *mtknox4-1 fcl1-1* double mutant was generated. *mtknox4-1 fcl1-1* displayed normal trifoliate leaves (Figure 3M, N), suggesting that *MtKNOX4* and *FCL1* antagonistically regulated the compound leaf patterning.

MtKNOX4 transcriptionally regulates *MtNAM* to modulate compound leaf development

MtNAM, the homologue of *CUC2* in *M. truncatula*, plays important roles in lateral organ separation and compound leaf development (Cheng et al., 2012). The leaves of *mtnam* displayed two types, including the fused leaflets and clustered leaflets without rachis. The clustered leaflets resembled the leaf patterning observed in *35Spro:YFP-MtKNOX4-1* plants (Figure 4A–C). qRT-PCR data showed that the expression level of *MtNAM* was markedly reduced in the *35Spro:YFP-MtKNOX4-1* (Figure 4D), indicating that *MtKNOX4* negatively regulated *MtNAM* at the transcription level. Then, *mtknox4-1 mtnam-2* double mutants were generated, and the leaves of *mtknox4-1 mtnam-2* were similar to those of *mtnam-2* (Figure 4E, F). This finding suggests that *mtnam* is genetically epistatic to *mtknox4*, implying that the formation of ectopic leaflets in *mtknox4* depends on the presence of *MtNAM*.

DISCUSSION

KNOX gene duplication in a common ancestor of land plants produced two classes of *KNOX* genes, *KNOXI* and *KNOXII*, which attend distinct biological processes. *KNOXI* genes are involved in shoot meristem maintenance and cell proliferation, while *KNOXII* genes regulate the haploid-to-diploid developmental transition and organ differentiation (Sakakibara et al., 2008; Sakakibara et al., 2013; Tsuda and Hake, 2015). *KNOXI* and *KNOXII* show an opposite function during the leaf's primary morphogenesis (Tsuda and Hake, 2015). On the one hand, KNOXI maintains an undifferentiated state in developing tissues in flowering plants. Modulation of KNOXI activity can change the shape of the leaf margin in simple leaved *Arabidopsis* and

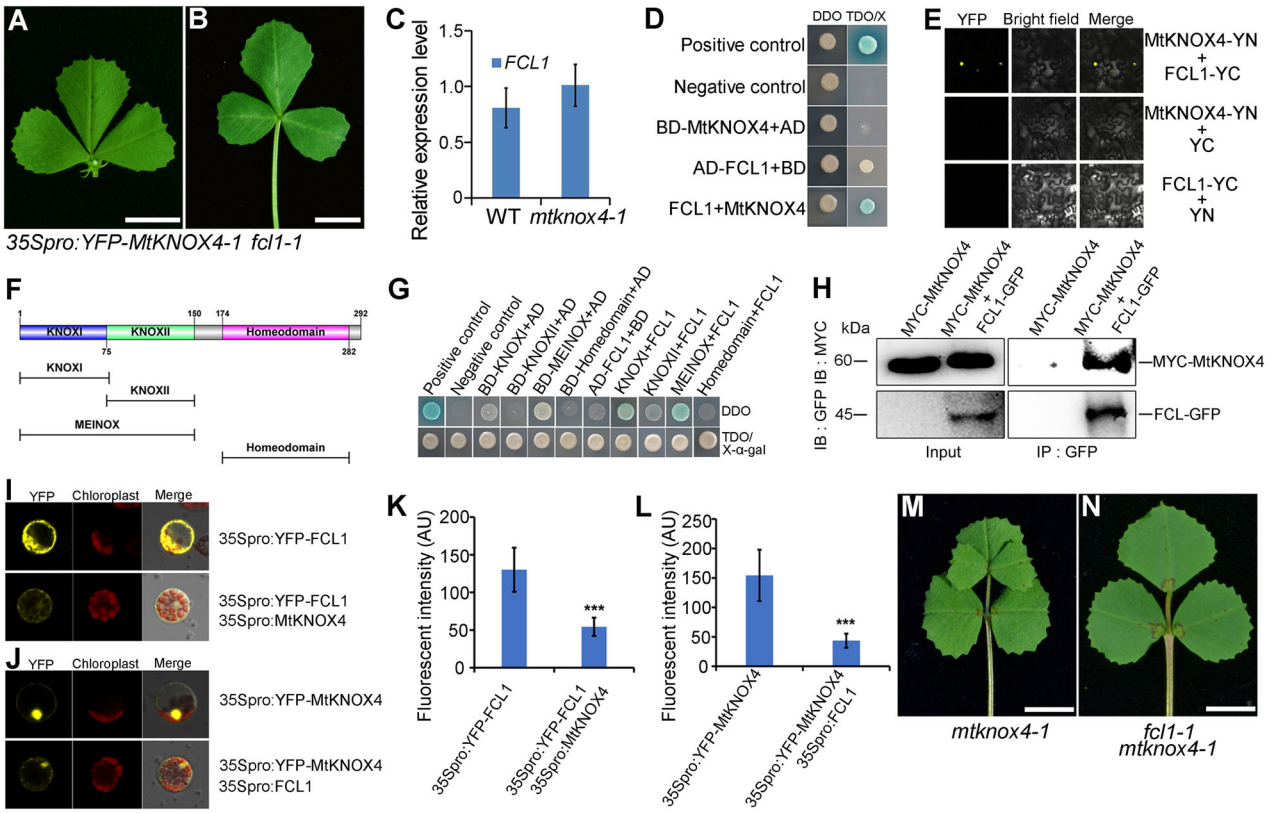

Figure 3. MtKNOX4 acts antagonistically to FCL1 at the protein level

(A, B) Show 30-d-old leaves of the *35Spro:YFP-MtKNOX4-1* **(A)** and *fcl1-1* **(B)**. Bars = 1 cm. **(C)** Expression levels of *FCL1* in the wild-type and *mtknox4-1*. Transcript levels were measured using the leaf buds of 30-d-old plants by qRT-PCR. *MtUBIQUITIN* was used as the internal control. Bars represent the mean ± SD of three biological replicates. **(D)** Interaction between MtKNOX4 and FCL1 tested using a yeast-two-hybrid assay. DDO, SD−Leu−Trp. TDO, SD −Leu−Trp−His. X, X-α-Gal. **(E)** BiFC assay indicated the interaction between MtKNOX4 and FCL1 in tobacco. YC, C-terminal of YFP protein. YN, N-terminal of YFP protein. **(F)** Schematic diagram of MtKNOX4 with four domains, including the KNOXI domain, the KNOXII domain, the MEINOX domain and the homeodomain. **(G)** Interaction between MtKNOX4 fragments and FCL1 tested using the yeast-two-hybrid assay. **(H)** Interaction between MtKNOX4 and FCL1 in *N. benthamiana* using a Co-IP assay. Immunoblots of the total protein extracts (Input) and the IP products were detected using anti-MYC antibody or anti-GFP antibody. **(I, J)** The observation of fluorescence intensity in protoplasts transformed with different construct combinations. **(K, L)** The fluorescence intensity of YFP was measured using ImageJ software ($n = 15$). ***$P < 0.001$. **(M, N)** Show 30-d-old leaves of the *mtknox4-1* **(M)** and *mtknox4-1 fcl1-1* double mutant **(N)**. Bars = 1 cm. ***$P < 0.001$.

the leaf complexity in dissected leaved *Cardamine* (Hareven et al., 1996; Janssen et al., 1998; Das Gupta and Tsiantis, 2018). On the other hand, simultaneous disruption of *KNOXII* genes, *KNAT3* and *KNAT4* or *KNAT3* and *KNAT5*, results in serrated leaf margins (Furumizu et al., 2015), and a reduction in the expression of *KNAT3*, *KNAT4* and *KNAT5* produced deeply lobed leaves in *Arabidopsis*, suggesting that the loss of *KNOXII* functions leads to more complex leaves (Furumizu et al., 2015; Challa et al., 2021). Similarly, suppression of the expression of the homologous genes of *KNAT3*, *4*, *5* increased the leaf complexity in *C. hirsuta* (Furumizu et al., 2015). In this study, we found that functional diversity exists among the members of MtKNOXII. Loss-of-function *mtknox4* mutant displayed an increase in leaf complexity, while simultaneous disruption of *MtKNOX3*, *5*, *9*, and *10* did not affect leaflet number. These data suggested that only *MtKNOX4* is necessary for the regulation of the elaboration of trifoliate leaves. According to the leaf defects of *mtknox4* and *knat3;4;5-amiR* lines in *Arabidopsis* and *Cardamine*, we propose that MtKNOX4 has a closer relationship with KNAT3, 4, and 5 in the regulation of leaf complexity during evolution. This evolutionary relationship also implies the functional specificity of MtKNOX4 among the MtKNOXII family in *M. truncatula*. In addition, overexpression of *MtKNOX4* or *MtKNOX5*, instead of *MtKNOX3*, *9*, and *10*, produced fused leaflets, which indicated a decrease in leaf complexity. The different contributions to leaf simplification of overexpressing *MtKNOXII* genes are likely to be due to the distinct downstream regulatory network between MtKNOX4, 5 and MtKNOX3, 9, 10 (Figure 5).

Previous studies have shown that KNOXI proteins increase leaf complexity by promoting CK biosynthesis by inducing the expression of *ISOPENTENYL TRANSFERASE* (*IPT*) genes (Jasinski et al., 2005; Sakamoto et al., 2006; Shani et al., 2010). Consistent with these findings, overexpression of *MtKNOXI* genes in the wild-type was able to promote leaf complexity in *M. truncatula*. Surprisingly, the function of MtKNOXI was repressed by MtKNOXII, as evidenced by the compound leaf patterning of simultaneous

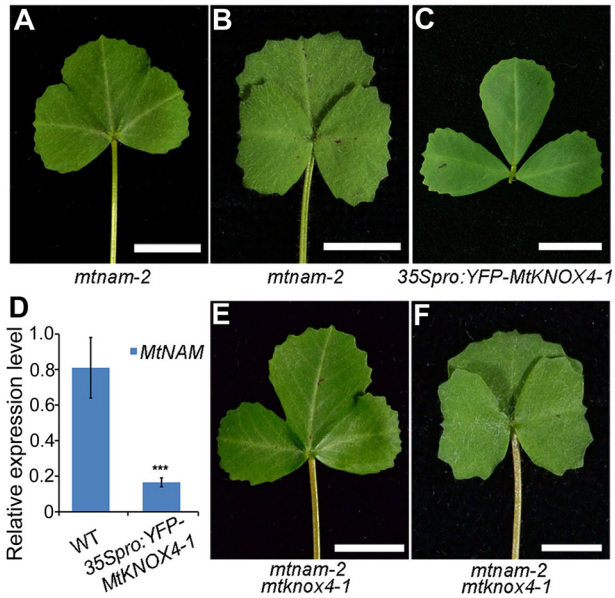

Figure 4. *MtKNOX4* regulates *MtNAM* to modulate the compound leaf development

(A–C) Show 30-d-old leaves of *mtnam-2* with fused leaflets **(A)** and clustered leaflets without rachis **(B)**, and *35Spro:YFP-MtKNOX4-1* **(C)**. Bars = 1 cm. **(D)** Expression levels of *MtNAM* in the wild-type and *35Spro:YFP-MtKNOX4-1* determined by qRT-PCR. Transcript levels were measured using the leaf buds of 30-d-old plants. *MtUBIQUITIN* was used as the internal control. Bars represent the mean ± SD of three biological replicates. **(E, F)** Show 30-d-old leaves of the *mtnam-2 mtknox4-1* double mutant with fused leaflets **(E)** and clustered leaflets **(F)**. Bars = 1 cm. ***$P < 0.001$.

overexpressing *MtKNOX4* and *MtKNOX2*. The pentafoliate leaves in *35S:MtKNOX2* plants were significantly changed to the trifoliate form in *35S:MtKNOX2 35S:MtKNOX4* plants. As the mutant phenotype of *mtknox4* is not dependent on the *MtKNOX1*, *2*, and *6*, the CK signaling-related genes in the *mtknox4-1* mutant and *35Spro:YFP-MtKNOX4-1* transformants are oppositely regulated, and *35S:MtKNOX4* plants showed less sensitivity to exogenous treatment of CK in compound leaf development, we propose that MtKNOX4 represses CK signal transduction, which is downstream of MtKNOXI-mediated CK biosynthesis (Figure 5). The interactions between KNOX and co-factors are critical for their diverse functions, and such modes are frequently proposed. In *Arabidopsis*, KNOX proteins BP and STM interact with PNF to form heterodimers that are required for inflorescence architecture (Kanrar et al., 2006). Additionally, KNAT7 can interact with MYB75, OVATE FAMILY PROTEIN4 (OFP4), OFP1, BLH6, and MYB6 to form functional complexes that regulate the development of secondary cell walls in *Arabidopsis* (Bhargava et al., 2010; Li et al., 2011; Liu et al., 2014; Wang et al., 2019a). KNATM is a unique member of the KNOX family, it lacks a homeodomain and interacts with other proteins to modulate their activities. For example, KNATM interacts with BP to function synergistically in plant development (Magnani and Hake, 2008). In *M. truncatula*, *FCL1* encodes a KNOXM protein, and is expressed at the boundary regions as well as in the developing leaf primordia (Peng et al., 2011). We found that MtKNOX4 interacted with FCL1, and reduced their activities mutually. For example, the combined overexpression of *MtKNOX4* and *FCL1* antagonized the fluorescent intensity elicited by each gene alone. The genetic evidence of *fcl1 mtknox4* demonstrated that MtKNOX4 and FCL1 play an opposite role in compound leaf development through, at least partially parallel, pathways. A previous study showed that the KNATM orthologue PTS interacted with SAW-like protein BIP in tomato, and regulated the proportion of BIP–KNOX heterodimers, which are involved in compound leaf formation (Kimura et al., 2008). Such protein-protein interactions governing KNOX activity raises a hypothesis that FCL1 interacts with MtKNOX4 to release the unknown BELL proteins to further modulate the complexity of the compound leaf. Characterization of the mutant with pentafoliate leaves in *M. truncatula* may help to provide insight into the roles of the MtKNOX4-FCL1 dimers in compound leaf patterning.

Compound leaf patterning is dependent on genetic networks involving multiple transcription factors. Previous studies have shown that NAM/CUC plays a conserved role in compound leaf development among species (Blein et al., 2008). In addition, *CUC2* is the shared downstream target of both *KNOXI* and *KNOXII*, as evidenced by the antagonistic relationship between KNOXI and KNOXII in *Arabidopsis* (Furumizu et al., 2015). In this study, we demonstrated that the pentafoliate leaves of *mtknox4* were dependent on *MtNAM*, implying that *MtNAM* may also be a target of both *MtKNOXI* and *MtKNOXII* in *M. truncatula*. Furthermore, *GOBLET*, the *NAM/CUC2* orthologue in tomato, affects the compound leaf elaboration and modulates leaflet morphogenesis together with auxin response inhibitor *ENTIRE* through a redundant pathway (Berger et al., 2009; Ben-Gera et al., 2012). Therefore, auxin-related regulators are probably involved in the MtKNOX4-mediated elaboration of leaves.

Taken together, we propose a possible regulation mechanism in which MtKNOX4 plays a crucial role in integrating the CK pathway and boundary regulators, providing new insight into the roles of MtKNOXII in regulating the elaboration of compound leaves in *M. truncatula* (Figure 5).

MATERIALS AND METHODS

Plant materials and growth conditions

Medicago truncatula R108 plants were used in this study. *mtknox4-1* and *mtknox4-2* (Chai et al., 2016), *mtknox3-1* (NF8825), *mtknox5-1* (NF4659), *mtknox9-1* (NF5569), *mtknox10-1* (NF18109), *fcl1-1* (Peng et al., 2011), *mtnam-2* (Cheng et al., 2012), *mtknox2-1* and *mtknox1-1 mtknox2-1 mtknox6-1* mutants (Zhou et al., 2014) were identified from the *Tnt1* retrotransposon-tagged mutant populations of *M. truncatula* (Tadege et al., 2008; Cheng et al., 2014). Plants were grown in a greenhouse at 22°C–24°C, 16 h light and 8 h dark, with a relative humidity of 70%–80%.

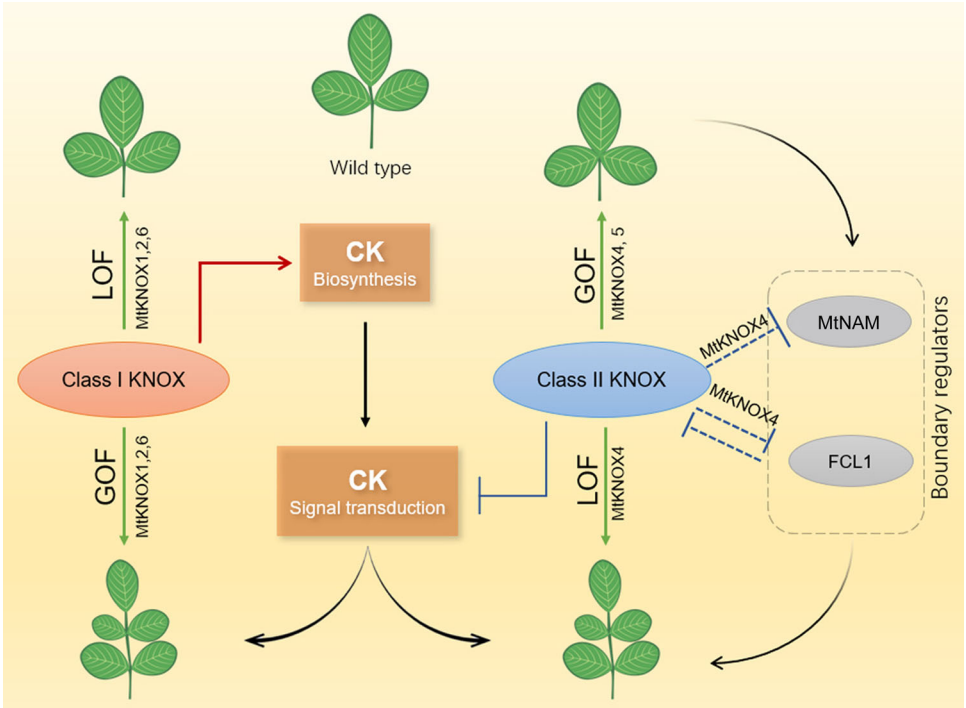

Figure 5. A proposed model illustrating the functional role of MtKNOXII in compound leaf development
During compound leaf development of *M. truncatula*, loss of MtKNOX1, 2, and 6 function (LOF) causes normal trifoliate leaves, while gain of MtKNOX1, 2, and 6 function (GOF) leads to complex leaves by promoting CK biosynthesis. Compared with MtKNOXI, MtKNOXII shows an opposite effect on leaf complexity. Loss of MtKNOX4 function results in the development of extra leaflets, while gain of MtKNOX4 or MtKNOX5 function causes simplified leaves by repressing CK signal transduction, which is downstream of MtKNOXI. In addition, boundary genes are involved in this developmental process, in which MtKNOX4 represses the expression of *MtNAM* at the transcriptional level, and acts antagonistically to FCL1 at the protein level to regulate compound leaf development.

Plasmid construction and plant transformation

For the *35Spro:YFP-MtKNOX4*, *35Spro:FCL1-GFP*, *35Spro:MtKNOX3-GFP*, *35Spro:MtKNOX5-GFP*, *35Spro:MtKNOX9-GFP* and *35Spro:MtKNOX10-GFP* constructs, the coding sequences (CDS) of *MtKNOX4*, *FCL1*, *MtKNOX3*, *MtKNOX5*, *MtKNOX9* and *MtKNOX10* were obtained by PCR amplification and then inserted into the pENTR/D-TOPO cloning vector (Invitrogen). The positive clones were transferred into the pEarleyGate 103 or pEarleyGate 104 vectors, using the Gateway LR recombination reactions (Invitrogen) (Earley et al., 2006). All final binary vectors were introduced into the *Agrobacterium tumefaciens* EHA105 strain, and leaves of ecotype R108 were used for stable transformation (Earley et al., 2006). Primer sequences are listed in Table S3.

Histochemical GUS assay and RNA *in situ* hybridization analysis

For the GUS assay, the transgenic plants were generated in a previous study (Chai et al., 2016). Leaves were collected for GUS staining, and the GUS activity was detected as previously reported (Chai et al., 2016). For RNA *in situ* hybridization, the probe for *MtKNOX4* was generated according to a previous study (Chai et al., 2016) and the probe for *SLM1* was generated in a previous study (Zhou et al., 2011). RNA *in situ* hybridization was performed on the vegetative buds of the 30-d-old wild-type as previously described (Zhou et al., 2014). Primer sequences are listed in Table S3.

RNA extraction, RT-PCR, qRT-PCR and transcriptomic analysis

Total RNA from leaves or vegetative buds was extracted from 30-d-old plants. Total RNA was isolated using TRIzol-RT Reagent (Molecular Research Center, INC) following the manufacturer's protocol. RNA extraction, cDNA synthesis, RT-PCR and qRT-PCR were performed as described previously (Wang et al., 2021). The primers are listed in Table S3. For transcriptomic analysis, the shoot buds were harvested from 30-d-old wild-type, *mtknox4-1* mutant and *35Spro:YFP-MtKNOX4-1* plants. The transcriptomic assay was performed as previously described (Wang et al., 2023), and the heatmap was created by GraphPad Prism 8.0.

CK treatment

The 45-d-old wild-type and *35Spro:YFP-MtKNOX4-1* plants growing in soil were sprayed with a solution containing 0.1 mM 6-benzyladenine (6-BA) with 0.01% Tween 20. The same concentration of Tween 20 was used as a control. 6-BA and Tween 20 were sprayed every 2 days, 6–8 times.

Phylogenetic analysis and protein sequence alignment

To construct the phylogenetic tree, the KNOXII protein sequences of *Glycine max* and *Medicago truncatula* were obtained from Phytozome (https://phytozome-next.jgi.doe.gov/), KNOXII protein sequences of *Solanum lycopersicum* were obtained from the Sol Genomics Network (https://solgenomics.net/), and KNAT3, 4, 5, and 7 were obtained from The Arabidopsis Information Resource (https://www.arabidopsis.org/). A phylogenetic tree was constructed using the neighbor-joining method and MEGA 6.06 software. MtKNOXII protein sequences were aligned using ClustalX 2.1 software, visualized and edited with GENEDOC.

Y2H assay and BiFC assay

The Y2H assays were performed according to the manufacturer's instructions for the Matchmaker GAL4-based two-hybrid system 3 (Clontech). *FCL1* was cloned into the pGADT7 vector. The CDS of *MtKNOX4* was cloned into the pGBKT7 vector. To verify the interaction domain, the CDS of the KNOXI domain (1–231 bp), KNOXII domain (225–450 bp), MEINOX domain (1–450 bp) and the homeodomain (540–846 bp) in *MtKNOX4* were cloned into pGBKT7. The bait and prey constructs were transformed into the yeast strain AH109 (Clontech). Protein-protein interactions were tested by stringent (SD/−Leu/−Trp/−His) selection (Clontech) supplied with 3-amino-1, 2, 4-triazole (Sigma) and X-α-Gal (Clontech) according to the manufacturer's protocol (Clontech).

For the BiFC assay, the full-length CDS of *MtKNOX4* was subcloned into the Gateway vector pEARLEY201-YN, while *FCL1* was cloned into the Gateway vector pEARLEY202-YC using the LR reaction. The *MtKNOX4-YN* and *FCL1-YC* constructs were transformed into tobacco cells. After incubation for 48–60 h, the leaves were dissected for yellow fluorescent protein (YFP) signal observation under a confocal laser scanning microscope (Leica).

Co-IP assay

The *MYC-MtKNOX4* and *FCL1-GFP* constructs were made using Gateway cloning and pCAMBIA1390 and pEarleyGate 103 vectors for the production of the fusion protein. The GV3101 strain harboring the recombinant plasmids was introduced into the *N. benthamiana* leaves via infiltration. The infiltrated leaves were collected after 48 h and lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM DTT) containing protease inhibitor cocktail. After centrifuging at 14,000 r/min for 10 min at 4°C, the supernatant was incubated with GFP Trap magnetic beads (ChromoTek) for 2 h. Then the beads were washed five times with wash buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). Proteins were eluted with SDS sample buffer, and boiled for 10 min at 95°C, followed by western blot using anti-MYC antibody (ABclonal, catalog number AE010) and anti-GFP antibody (TransGen Biotech, catalog number HT801-02).

Subcellular localization and quantification of protein fluorescence signals

For the *35Spro:YFP-MtKNOX4* and *35Spro:YFP-FCL1* constructs, the CDS were transferred into the pEarleyGate 104 vector. For the *35Spro:MtKNOX4* and *35Spro:FCL1* constructs, the CDS were transferred into pEarleyGate 100, using the Gateway LR recombination reactions (Invitrogen). The plasmids were transformed into *Medicago* protoplasts as described previously (Yoo et al., 2007) with some modification. YFP signals were observed using an LSM700 laser scanning confocal microscope, and the fluorescence intensity was determined using ImageJ software.

Accession numbers

Sequence data from this article can be found in the National Center for Biotechnology Information GenBank following the accession numbers: MtKNOX4, Medtr5g011070; MtKNOX3, Medtr1g012960; MtKNOX5, Medtr3g106400; MtKNOX9, Medtr4g116545; MtKNOX10, Medtr2g461240; MtKNOX1, Medtr2g024390; MtKNOX2, Medtr1g017080; MtKNOX6, Medtr5g085860; MtKNOX7, Medtr5g033720; MtKNOX8, Medtr1g084060; FCL1, HQ695002; MtNAM, JF929904; SlKNATII3, Solyc07g007120; SlKNATII4, Solyc12g010410; SlKNATII5, Solyc08g041820; SlKNATII7, Solyc08g080120.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

X.W. and C.Z. designed the experiments; X.W., J.J.Z., M.C., L.H., X.C., and J.Z. conducted the experiments; K.S.M. and J.W. contributed to the generation of *Tnt1*-tagged mutants; X.W., J.J.Z., Y.K., C.F., Z.W. and C.Z. analyzed the data and edited the manuscript; X.W. and C.Z. wrote the paper. All authors read and approved this manuscript.

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SUPPORTING INFORMATION

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Figure S1. Multiple alignments of KNOXII proteins in *M. truncatula*

Figure S2. RT-PCR analyses of the *MtKNOX3* (A), *5* (B), *9* (C) and *10* (D) transcripts in the leaves of wild-type and mutants

Figure S3. The expression levels of *MtKNOX3* (A), *MtKNOX4* (B), *MtKNOX5* (C), *MtKNOX9* (D) and *MtKNOX10* (E) in the leaves of 30-d-old wild-type and their transgenic plants determined by qRT-PCR

Figure S4. The expression patterns of *SLM1* in leaf primordia of the wild-type (A), *mtknox4-1* (B) and *35Spro:MtKNOX4-1* (C), as determined by RNA *in situ* hybridization

Figure S5. A heatmap showing differentially expressed CK signal transduction-related genes in shoot buds of *mtknox4-1* and *35Spro:MtKNOX4-1*

Table S1. Differentially expressed genes (DEGs) in the shoot buds of *knox4-1* compared to wild-type

Table S2. DEGs in the shoot buds of *35Spro:YFP-MtKNOX4-1* compared to wild-type

Table S3. Primers used in this research

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