Received: 16 August 2023 | Revised: 19 September 2023 | Accepted: 4 October 2023

DOI: 10.1111/ppl.14046

ORIGINAL RESEARCH

Physiologia Plantarum

The MIO1-MtKIX8 module regulates the organ size in *Medicago truncatula*

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Funding information
Strategic Priority Research Program of Chinese Academy of Sciences, Grant/Award Numbers: XDA26030301, XDB27030106; Cross-Team Project-Key Laboratory Cooperative Research Project, Grant/Award Number: xbzg-zdsys-202016; High-end Scientific and Technological Talents in Yunnan Province, Grant/Award Numbers: 2015HA031, 2015HA032; National Natural Science Foundation of China, Grant/Award Numbers: 32170839, 32200290; Youth Innovation Promotion Association CAS, Grant/Award Number: 2021395; Yunnan Revitalization Talent Support Program, Grant/Award Numbers: E3YN103K01, XDYC-QNRC-2022-0179

Edited by T. Greb

Abstract

Plant organ size is an important agronomic trait tightly related to crop yield. However, the molecular mechanisms underlying organ size regulation remain largely unexplored in legumes. We previously characterized a key regulator F-box protein MINI ORGAN1 (MIO1)/SMALL LEAF AND BUSHY1 (SLB1), which controls plant organ size in the model legume *Medicago truncatula*. In order to further dissect the molecular mechanism, MIO1 was used as the bait to screen its interacting proteins from a yeast library. Subsequently, a KIX protein, designated MtKIX8, was identified from the candidate list. The interaction between MIO1 and MtKIX8 was confirmed further by Y2H, BiFC, split-luciferase complementation and pull-down assays. Phylogenetic analyses indicated that MtKIX8 is highly homologous to *Arabidopsis* KIX8, which negatively regulates organ size. Moreover, loss-of-function of *MtKIX8* led to enlarged leaves and seeds, while ectopic expression of *MtKIX8* in *Arabidopsis* resulted in decreased cotyledon area and seed weight. Quantitative reverse-transcription PCR and in situ hybridization showed that *MtKIX8* is expressed in most developing organs. We also found that MtKIX8 serves as a crucial molecular adaptor, facilitating interactions with BIG SEEDS1 (BS1) and MtTOPLESS (MtTPL) proteins in *M. truncatula*. Overall, our results suggest that the MIO1-MtKIX8 module plays a significant and conserved role in the regulation of plant organ size. This module could be a good target for molecular breeding in legume crops and forages.

Yawen Mao and Shaoli Zhou contributed equally to this study.

1 | INTRODUCTION

Plant organ size is coordinately fine-tuned by a combination of internal genetic and external environmental cues (Hepworth & Lenhard, 2014; Vierstra, 2003). In cereal and legume crops, the size of the plant organs, particularly the seed, is closely related to the final yield of the crops. However, the molecular mechanisms underlying organ size control in legumes are still poorly understood. Legumes contribute significantly to the human diet by providing a majority of plant protein resources and a significant portion of oils (Reddy et al., 2005). Increasing the plant organ size is an efficient approach for breeders to potentially improve crop yields to meet the demand of the growing global population.

From a cellular point of view, the size of plant organs is determined by the interplay of cell division and expansion. The development of plant organs is a sequential process, starting with the recruitment of founder cells and leading to the formation of the primordium, followed by cell proliferation and subsequent cell expansion (Czesnick & Lenhard, 2015; Gonzalez et al., 2012; Hepworth & Lenhard, 2014; Powell & Lenhard, 2012). The final organ size is also depends on the number of cells and the rate and duration of cell proliferation and expansion (Gonzalez et al., 2012). Several genes have been reported to regulate these processes in *Arabidopsis*. For example, *STRUWWELPETER* (*SWP*) plays a significant role in determining the leaf primordium size (Autran et al., 2002). The rate of cell division is controlled by cell cycle genes such as *ANAPHASE PROMOTING COMPLEX10* (*APC10*) and *CELL DIVISION CYCLE PROTEIN 27 HOMOLOG A* (*CDC27a*) (Eloy et al., 2011; Rojas et al., 2009). AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE-AINTEGUMENTA-AINTEGUMENTA-LIKE-CYCLIN D3 (ARGOS-ANT-AIL-CYCD3) module, *DA1*, *ENHANCER OF DA1-1* (*EOD1*), *KLUH* (*KLU*) and *DELLA* were reported to mainly regulate the duration of cell division (Dewitte et al., 2003; Fleet & Sun, 2005; Hu et al., 2003; Krizek, 2009; Li et al., 2008; Nole-Wilson et al., 2005; Stransfeld et al., 2010). Furthermore, *EXPANSIN10* (*EXP10*), *ErbB-3 EPIDERMAL GROWTH FACTOR RECEPTOR BINDING PROTEIN* (*EBP1*), *ARGOS-LIKE* (*ARL*), *TARGET OF RAPAMYCIN* (*TOR*), *ZINC FINGER HOMEODOMAIN5* (*ZHD5*) and *AUXIN RESPONSE FACTOR2* (*ARF2*) have been shown to control the cell size (Cho & Cosgrove, 2000; Deprost et al., 2007; Hong et al., 2011; Horváth et al., 2006; Hu et al., 2006; Schruff et al., 2006).

In recent years, a series of studies have revealed that the STERILE APETALA-PEAPOD-KIX-TOPLESS (SAP-PPD-KIX-TPL) module plays a critical role in regulating organ size and shape in the model plant *Arabidopsis*. The F-box protein SAP, which forms an SCF E3 ubiquitin ligase complex to target substrates for degradation, promotes organ size by positively regulating meristemoid cell proliferation (Byzova et al., 1999; Wang et al., 2016). The TIFY proteins PPD1 and PPD2 are the first reported regulators that affect meristemoid cell proliferation (Bai et al., 2011; Cuéllar Pérez et al., 2014; Gonzalez et al., 2015; White, 2006). Knock-out or knock-down of the *PPD* genes produces larger, dome-shaped leaves (Gonzalez et al., 2015; White, 2006). INDUCIBLE DOMAIN INTERACTING8 (KIX8) and KIX9 act as an adaptor between PPD and co-repressor TOPLESS (TPL) to form the PPD-KIX-TPL repressor complex. SAP can target KIX and PPD for degradation to disrupt the stability of PPD-KIX-TPL repressor complex, thereby positively regulate the meristemoid cell proliferation and organ size in *Arabidopsis* (Gonzalez et al., 2015; Kagale et al., 2010; Li et al., 2018; Pillitteri & Dong, 2013; Swinnen et al., 2022; Thakur et al., 2013; White, 2006). Moreover, the phenotypes of *kix8* single mutant, *kix8 kix9* double mutant, and *SAP*-overexpression line are both similar to the phenotype of the *ppd* mutant (Gonzalez et al., 2015; Wang et al., 2016), which further confirms that the SAP-KIX-PPD complex is indeed related to the regulation of plant organ size. Similarly, transcription factors MYC3/4 could recruit the TPL-KIX-PPD complex to repress the *GRF-INTERACTING FACTOR 1* (*GIF1*) to specifically regulate the seed size (Liu et al., 2020). PPD-KIX-TPL also interacts with the NOVEL INTERACTOR OF JAZ (NINJA) to repress the down-stream cell cycle gene *CYCD3* to regulate the leaf flatness (Baekelandt et al., 2018).

In other plant species, the orthologous genes *SAP*, *KIX* and *PPD* play important roles in regulating plant organ size. *LITTLELEAF* (*LL*) is an ortholog of *SAP* and was reported to positively regulate organ size in cucumber (*Cucumis sativus*). Loss-of-function and ectopic expression of *LL* result in decreased and increased organ size, respectively (Yang et al., 2018). In the model legume *Medicago truncatula*, *MIO1/SLB1*, the ortholog of *SAP*, was uncovered to positively regulate the lateral organ size by controlling the primary cell division during plant development (Yin et al., 2020; Zhou et al., 2021). Similar to KIX8/9, SlKIX8/9 acts as an adaptor between SlPPD and SlTOPLESS to negatively regulate organ size in tomatoes (*Solanum lycopersicum*). Loss-of-function of SlKIX8 and SlKIX9 leads to enlarged leaves and fruits (Swinnen et al., 2022). *GmKIX8-1* in soybean (*Glycine max*) negatively regulates the size of aerial plant organs, such as seeds and leaves, by repressing cell proliferation during development (Nguyen et al., 2021). *BIGGER ORGANS* (*BIO*) gene, the homologs of *KIX8/9* in pea (*Pisum sativum*), negatively regulates the size of lateral organs like leaves, flowers and pods (Li et al., 2019). The *PPD* orthologous genes *BIG SEED1* (*BS1*) in *M. truncatula* and soybean, *VmPPD* in *Vigna mungo* and *ELEPHANT EAR-LIKE LEAF1* (*ELE1*) in pea all generate larger leaves and seeds by negatively regulating primary cell proliferation (Ge et al., 2016; Li et al., 2019; Naito et al., 2017). From the above clues, it appears that the SAP-KIX-PPD module is conserved in organ size regulation in dicot plants.

Recently, we found that MIO1, the ortholog of SAP in *M. truncatula*, positively regulates organ size by promoting primary cell proliferation (Zhou et al., 2021). To further explore how the MIO1 protein regulates organ size, we used MIO1 as the bait to screen its interacting proteins. Ultimately, 23 potential interactors were identified. Among them, Medtr4g114900 was predicted to encode a typical KIX domain-containing protein. Phylogenetic analysis showed that Medtr4g114900 is the homolog of *Arabidopsis* KIX8. Protein sequence analysis indicates that MtKIX8 harbor the conserved KIX domain, R domain and EAR domain, and then we named it MtKIX8. In addition, the protein interaction assays showed that MtKIX8 directly interacts with MIO1 in vitro. Expression pattern analysis indicates high *MtKIX8* expression levels in flower, pod, seed and shoot. To verify the gene function of *MtKIX8* in

M. truncatula, a reverse genetic screening was performed to identify the mutant that harbors the retrotransposon *Tnt1* in the *MtKIX8* gene locus. The *mtkix8* mutant produces enlarged leaves and seeds. When *MtKIX8* was ectopically expressed in *Arabidopsis*, it rescued the increased organ size phenotype in the *kix8* mutant. These results confirm that MtKIX8 acts as a negative regulator of organ size. Y2H results showed that MtKIX8 also directly interacts with BS1 and MtTOPLESS (MtTPL), suggesting that MtKIX8 represses organ size by forming the conserved repressor complex in *M. truncatula*. This study demonstrates that MtKIX8 plays an important and conserved role in regulating organ size, and it would be a promising locus for molecular design breeding in legumes.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Medicago truncatula ecotype R108 and *Arabidopsis* ecotype Col-0 were used as the wild type in this study. The *mio1-1* mutant and *35S::MIO1* transgenic lines were described previously (Zhou et al., 2021). The *mtkix8* (NF19103) mutant of *M. truncatula* was isolated from the tobacco *Tnt1* retrotransposon tagged mutant collection at Oklahoma State University. The *kix8* (SALK_206915C) mutant was ordered from AraShare (Fujian Agriculture and Forestry University). *M. truncatula* seeds were rubbed with sandpaper and placed in water dishes at 4°C for 7 days and then sown in the greenhouse. *Arabidopsis* seeds were imbibed and kept at 4°C for 2 days before being sown in the greenhouse. Plants were grown under the following conditions: 16/8 h day/night photoperiod, 22/18°C day/night temperature, 150 μmol m^{-2} s^{-1} light intensity and 50%–60% relative humidity.

2.2 | Phylogenetic analysis and sequence alignment

Sequence data were downloaded from Phytozome (https://phytozome-next.jgi.doe.gov/) and pea genome database (https://urgi.versailles.inra.fr/blast/). Phylogenetic trees were generated using the neighbor-joining method, implemented in MEGA 7.0 with 1000 bootstrap replications. Multiple alignments of the sequences were performed using the DNAMAN software.

2.3 | Yeast two-hybrid screening

The yeast library screening was performed following the manufacturer's instructions (Matchmaker® Gold Yeast Two-Hybrid System User Manual, Clontech). The tissues for cDNA library construction were shoot tips during the reproductive stage of *M. truncatula*. The *MIO1* sequence was amplified and cloned into the *Bam*HI-*Sma*I site of the pGBKT7 bait vector. The bait plasmid was transformed into yeast strain Y2H Gold and expressed as a fusion to the yeast GAL4 BD-MIO1. Before the screening, the bait protein was assessed for autoactivity and toxicity. Then, the Y2H Gold strains containing the bait protein and the Y187 library proteins were mixed for mating. The mated cells were spread on the 150 mm SD/-Leu/-Trp/-His/-Ade medium at 30°C for 4–6 days. Each clone was selected individually in 10 μL sterile water and then loaded for colony PCR after repeating freezing and thawing at −37°C three times. The amplified fragments greater than 200 bp were selected for sequencing. To confirm the positive interaction, the complete coding sequence of *MtKIX8* was cloned into pGADT7 as a prey plasmid. The bait plasmid BD-MIO1 and prey plasmid AD-MtKIX8 were co-transformed into Y2H Gold strain and cultured on SD/-Leu/-Trp medium at 30°C for 3–4 days. Cultured colonies were collected when the OD_{600} reached 1.3, then the cultures were diluted to make 1×, 0.1×, 0.01× and 0.001× culture solutions. The diluted cultures were spread onto the SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade medium at 30°C for 3–4 days. Other yeast vectors were also constructed according to the above method. Notably, due to the low expression of the TPL gene, the reverse primer at the end of the TPL gene was used as a specific reverse transcription primer to obtain cDNA. The empty pGBKT7 and pGADT7 vectors were used as controls.

2.4 | Bimolecular fluorescence complementation assay

The complete coding regions of *MIO1* and *MtKIX8* were fused into the C-terminal fragment of the pFGC–cYFP vector and the N-terminal fragment of the pFGC–nYFP vector, respectively. The plasmids were transformed into *Agrobacterium* strain EHA105 and co-infiltrated into the leaves of *N. benthamiana* plants. The plants were incubated at 22°C in the dark for 48 h, and then the YFP signals were observed with a confocal laser scanning microscope (Zeiss LSM900).

2.5 | Split-luciferase complementation imaging assay

The coding sequence of MtKIX8 was cloned into the *Bam*HI-*Bgl*II site of pCAMBIA1300-nLUC, and the coding sequences of MIO1 and WD40 were cloned into the *Bgl*II-*Xba*I site of pCAMBIA1300-cLUC. All plasmids were transformed into the *Agrobacterium tumefaciens* EHA105 strain and then infiltrated into *N. benthamiana* leaves. The *N. benthamiana* plants were cultured in the dark for 72 h. The 0.5 mM fluorescein was applied to the abaxial side of leaves and kept in the dark for 5 minutes, and the signal was observed under the CCD camera (Tanon 5200).

2.6 | In vitro pull-down assay

The coding sequences of *MtKIX8* and *WD40* were amplified and cloned into the *Bam*HI-*Hin*dIII site of the pET-28a vector and the

*Bam*HI-*Eco*RI site of the pGEX-4T-1 vector, respectively. The bacterial lysates containing the His-MtKIX8 and GST-WD40 fusion proteins were mixed and incubated with glutathione sepharose beads (GE Healthcare) at 4°C for 2 h. The isolated proteins were separated with 10% SDS-polyacrylamide gel and detected by immunoblot analysis with anti-GST and anti-His antibodies, respectively. The empty GST protein was used as a negative control.

2.7 | RNA extraction and quantitative reverse-transcription PCR

Total RNA was isolated from plant tissues with a TransZol Kit (Tiangen Biotech). Agarose gel electrophoresis and Nanodrop Analyzer (Thermo Scientific) were used to check the quality and concentration of RNA. Subsequently, 1.5 μg of total RNA was used for reverse transcription with HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme). Quantitative reverse-transcription PCR was conducted using TransStart Tip Green qPCR SuperMix (TransGen) on the LightCycler 480II device (Roche). *AtUBQ10* and *AtACTIN* genes were used as internal controls in *Arabidopsis*, and *MtACTIN* and *MtGAPDH* genes were used as internal controls in *M. truncatula*. Primer sequences are listed in Table S1, Supporting Information.

2.8 | RNA *in situ* hybridization

Shoot apices of 8-week-old plants were collected for RNA in situ hybridization as previously described (Coen et al., 1990). The hybridization signal was visualized under a microscope (FV1000, Olympus).

2.9 | Subcellular localization

To generate the plasmid *35S::MtKIX8-GFP*, the full-length coding sequence of *MtKIX8* was amplified and cloned into the pYS22 vector between the *Xho*I and *Kpn*I restriction sites. The *35S::MtKIX8-GFP* construct was transformed into the *Agrobacterium tumefaciens* EHA105 strain and transiently expressed in *N. benthamiana* leaves. GFP signals were examined using a confocal laser scanning microscope.

2.10 | Plasmid constructions and plant transformation

The full-length coding sequence of *MtKIX8* was amplified by PCR from WT cDNA of the reproductive shoot apex and was inserted into the *pCAMBIA3301* vector between the *Nco*I and *Eco*72I restriction sites to generate the *35S::MtKIX8* construct. The 654 bp native promoter of *AtKIX8* was amplified from WT Col-0, and the full-length *MtKIX8* coding sequence was amplified from the plasmid *35S::MtKIX8*. The above two fragments have a 23 bp overlapping sequence. Overlapping PCR was carried out using these two fragments as templates to obtain the recombinational fragment. The fragment was cloned into the *Hin*dIII and *Pml*I sites of the *pCAMBIA3301* vector to generate the plasmid *pAtKIX8::MtKIX8*. All PCR reactions were carried out using the Phanta Max Super-Fidelity DNA Polymerase (P505, Vazyme) and the homologous recombination process was achieved using the ClonExpress II One Step Cloning Kit (C112, Vazyme). The constructed vectors were transformed into *A. tumefaciens* EHA105 strain and introduced into Col-0 and *kix8* mutant via the floral dipping method. 20 mg L^{-1} Basta was used to screen positive transgenic plants. Next, PCR was used to verify the positive results. Primer sequences are listed in Table S1.

2.11 | Morphological analysis

Arabidopsis seeds were harvested from the fifth to fifteen siliques on the stem. Cotyledons were taken from 8-day-old seedlings. The picture of cotyledons and seeds were captured with the stereomicroscope (SZX16, Olympus) and measured by ImageJ software.

3 | RESULTS

3.1 | Identification and characterization of MIO1 interactors

We previously reported that the F-box protein MIO1 plays a prominent role in the regulation of lateral organ size by forming the SCF E3 ubiquitin ligase complex in *M. truncatula* (Zhou et al., 2021). Mutation of *MIO1* resulted in a significant reduction in plant organ size at both vegetative (Figure S1A,C) and reproductive development stages (Figure S1B,D). In contrast, ectopic expression of *MIO1* gave rise to enlarged plant and lateral organ sizes, such as leaves, flowers, pods and seeds (Figure S1A–I).

To further get insight into how MIO1 regulates organ size in *M. truncatula*, a Y2H screening was performed. We firstly examined whether MIO1 could be autoactivated in this system and found that MIO1 protein displayed a weak autoactivation on SD/-Leu/-Trp/-His media but none on SD/-Leu/-Trp/-His/-Ade media. Next, we checked the toxicity of MIO1 protein on yeast strain Y2H Gold and found that MIO1 did not impact yeast cell growth (Figure S2A,B). This observation suggests that MIO1 is appropriate to screen the yeast library on SD/-Leu/-Trp/-His/-Ade media. During the screening processes, the number of yeast mating cells (similar to shamrock) gradually increased with time (Figure S3A,B). Positive colonies were selected for the PCR test, and the PCR products greater than 200 bp were selected for sequencing (Figure S4A,B). Finally, 23 candidates of MIO1 interacting proteins were identified from the sequencing results (Table 1). Among them, the gene *Medtr4g114900* encodes a typical KIX domain protein. Previously the KIX domain-containing proteins KIX8 and KIX9 had been reported to negatively regulate organ size in *Arabidopsis* (Gonzalez et al., 2015). Based on these clues, it suggests that Medtr4g114900 encoding a conserved KIX protein could been involved in organ size regulation by interacting with MIO1.

TABLE 1 Genes identified from the yeast library screening and their functions annotations

A17 ID	Frequency	Conserved domain	Homologous gene function	Reference
Medtr7g009330	9	Porin/voltage-dependent anion-selective channel protein	Metabolite transport, Programmed cell death	Robert et al. (2012)
Medtr5g097280	5	Chlorophyll A-B binding	Photosynthesis	
Medtr4g114900	2	KIX domain	Plant organ size	Li et al. (2018)
Medtr1g051895	2	EF-hand motif		
Medtr8g015580	2	PWWP domain		
Medtr4g098490	1	2Fe-2S iron–sulfur cluster binding domain		Pfeifer et al. (1993)
Medtr4g088615	1	Ribosomal protein S5, N-terminal domain	Structural constituent of ribosome	Ramakrishnan et al. (1992)
Medtr1g010290	1	Enhancer of polycomb-like	fungus-host interactions	Seidl et al. (2006)
Medtr7g100450	1	Heavy metal-associated domain	Optimal use of Cu for photosynthesis	Abdel-Ghany et al. (2005)
Medtr4g073400	1	C2 domain	Plasma membrane integrity and plant fitness	Yamazaki et al. (2008)
Medtr4g029390	1	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain	Intracellular lipid transfer	Molina et al. (1993)
Medtr4g021880	1	Ankyrin repeat-containing domain	Lateral root initiation, leaf development	Nodzon et al. (2004)
Medtr1g094680	1	Anion-transporting ATPase-like domain	Photosynthesis	Formighieri et al. (2013)
Medtr7g082940	1	Translation initiation factor IF2/IF5, W2 domain	GTPase-activator protein	Paulin et al. (2001)
Medtr2g005870	1	NAD(P)-binding Rossmann-fold domains	Nod factor transduction, nodulation and mycorrhization	Ané et al. (2002)
Medtr7g032240	1	CCT domain	Regulation of flowering by photoperiod	Ben-Naim et al. (2006)
Medtr1g019240	1	Helix–loop–helix domain	Mediates the germination response to temperature	Penfield et al. (2005)
Medtr8g012410	1	Mediator-associated protein 2	Coactivator complex regulates cold-responsive gene expression	Hemsley et al. (2014)
Medtr8g098485	1	BTB domain	BTB–Cullin 3–Roc1 ubiquitin ligases	Furukawa et al. (2003)
Medtr2g082640	1	Myc-type, basic helix–loop–helix domain	Plant development, senescence, iron metabolism and reactive oxygen species (ROS) homeostasis	Selote et al. (2015)
Medtr8g075260	1	The tetratrico peptide repeat region (TPR)		
Medtr7g103620	1	Enolase, C-terminal TIM barrel domain	Catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate	Van der Straeten et al. (1991)
Medtr1g093240	1	AUX/IAA domain	Repressor of auxin pathway, lateral root formation	Fukaki et al. (2002)

3.2 | MtKIX8 is the homolog of AtKIX8 and AtKIX9

To uncover the phylogenetic relationship of Medtr4g114900 with other reported typical KIX domain proteins, we selected KIXs from *Arabidopsis*, soybean, pea and tomato to build a phylogenetic tree (Gonzalez et al., 2015; Li et al., 2019; Nguyen et al., 2021; Swinnen et al., 2022; Thakur et al., 2013). The result showed that Medtr4g114900 is the homolog of the previously reported KIX proteins. Thus, we named Medtr4g114900 as *MtKIX8* (Figure 1A). Compared to *Arabidopsis*, tomato and soybean, we found only one KIX protein in this subgroup in *M. truncatula*. Protein sequence analysis showed that the N-terminus of MtKIX8 harbors a conserved KIX domain, which is a protein interaction domain (Kumar et al., 2018;

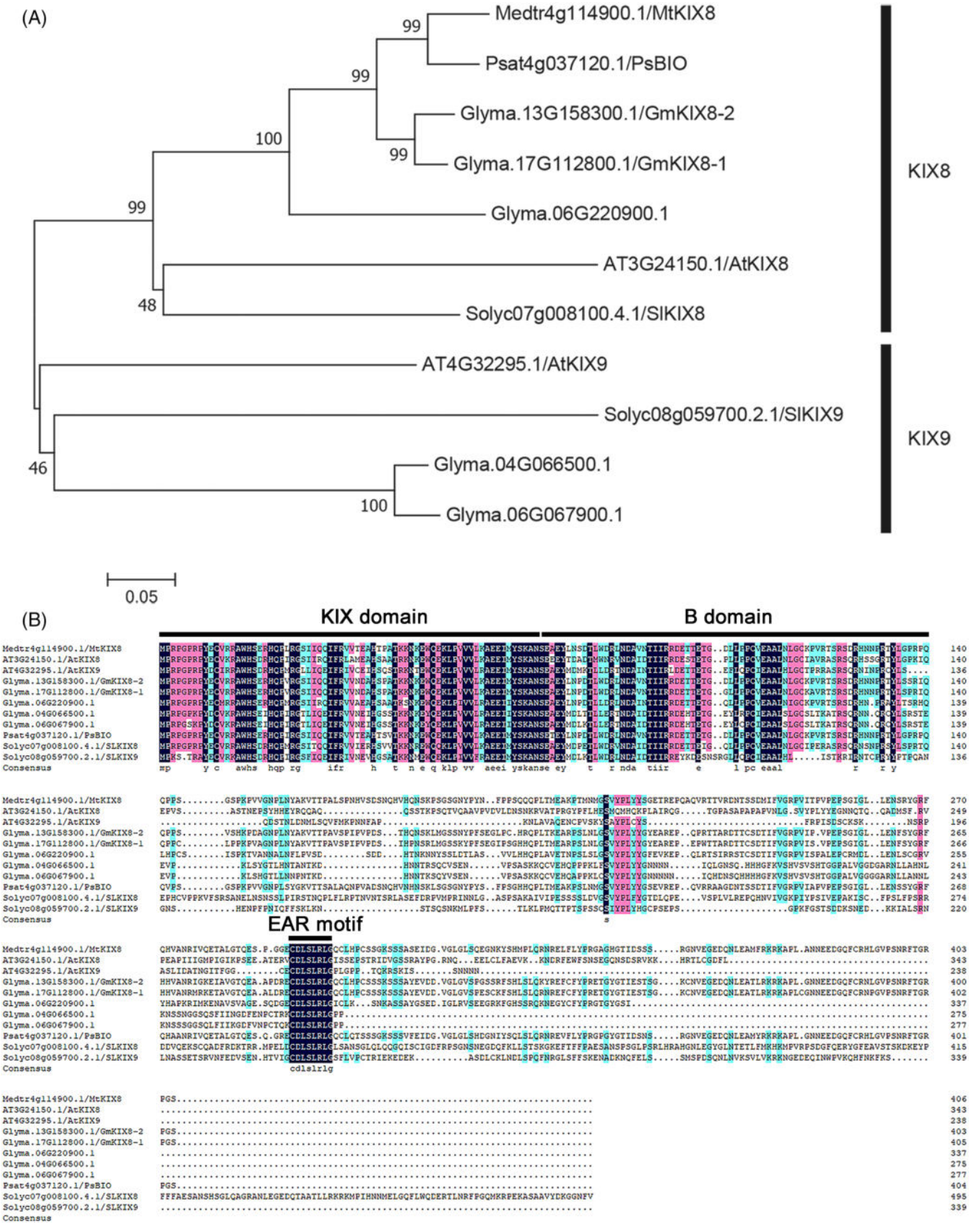

FIGURE 1 Phylogenetic analysis and sequence alignment of KIX8 and KIX9 proteins. (A) Phylogenetic tree and (B) amino acid sequence alignments of MtKIX8 and its orthologues from *Arabidopsis thaliana*, *Glycine max*, *Pisum sativum* and *Solanum lycopersicum*

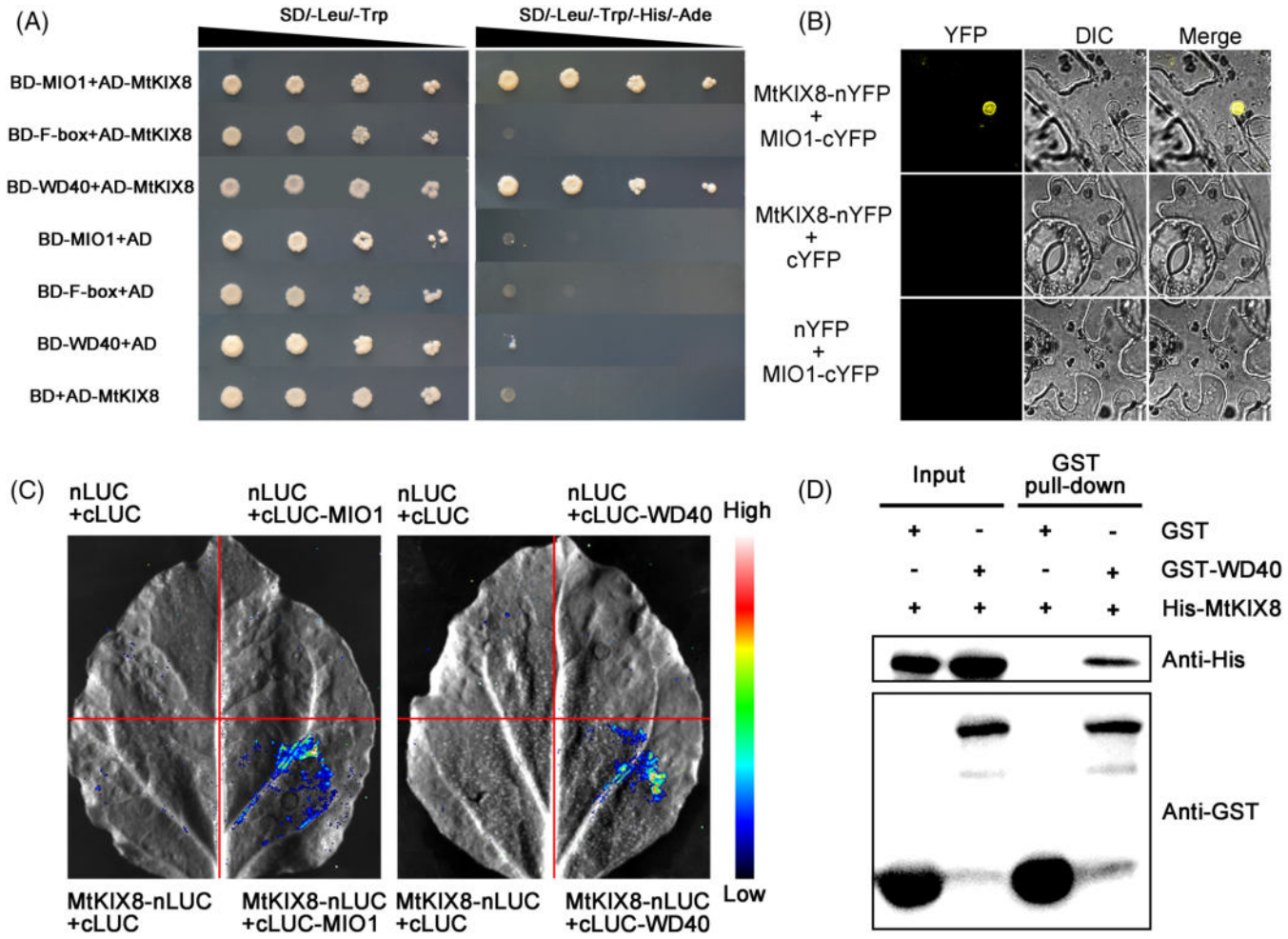

FIGURE 2 The interaction between MIO1 and MtKIX8. (A) Yeast two-hybrid assay showing the interaction between MIO1 and MtKIX8. BD represents GAL4 binding domain; AD represents GAL4 activation domain. pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. Black triangle represents the range of yeast concentrations from dilution of 10^0 (OD600 = 1.3) to 10^{-3}. (B) BiFC assay showing the interaction between MIO1 and MtKIX8 in epidermal cells of *N. benthamiana*. (C) Split-luciferase complementation imaging assay showing the interaction between MIO1 and MtKIX8. (D) C-terminal WD40 repeat domain of MIO1 interacts with MtKIX8 in vitro. The empty GST protein is used as a negative control

Thakur et al., 2013), and the C-terminus also has a conserved EAR domain (Figure 1B). In *Arabidopsis*, the N-terminal KIX domain of KIX8/9 interacts with PPD, and the C-terminal EAR motif recruits the co-repressor TPL to form the PPD-KIX-TPL transcriptional repressor complex to regulate organ size (Gonzalez et al., 2015; Swinnen et al., 2022). These results suggest that MtKIX8 might play a conserved role in organ size regulation in *M. truncatula*.

3.3 | MIO1 physically interacts with MtKIX8 in vitro

To further confirm the results from Y2H screening, we used the individual Y2H method to verify the interaction between MIO1 and MtKIX8. The results showed that MtKIX8 interacts with the full length of MIO1 in yeast cells. Further analysis of the Y2H results revealed that MIO1 is capable of interacting with MtKIX8 through its C-terminal WD40 repeat domain (Figure 2A). Furthermore, the interaction between MIO1 and MtKIX8 was also verified by the BiFC and LUC complementation imaging assays (Figure 2B,C). Subsequently, the pull-down assay was performed. We expressed the C-terminal WD40 protein of MIO1 fused with GST tag and MtKIX8 protein fused with His tag in vitro and found that His-MtKIX8 bound to GST-WD40 but not the GST control, suggesting that MtKIX8 can directly interact with the WD40 domain of MIO1 (Figure 2D). Taken together, these results demonstrate that MIO1 can form a protein complex with MtKIX8.

3.4 | MtKIX8 is a negative regulator of leaf and seed size

To check the role of MtKIX8 in regulating organ size in *M. truncatula*, we isolated a *mtkix8* mutant from the *Tnt1* retrotransposon insertion

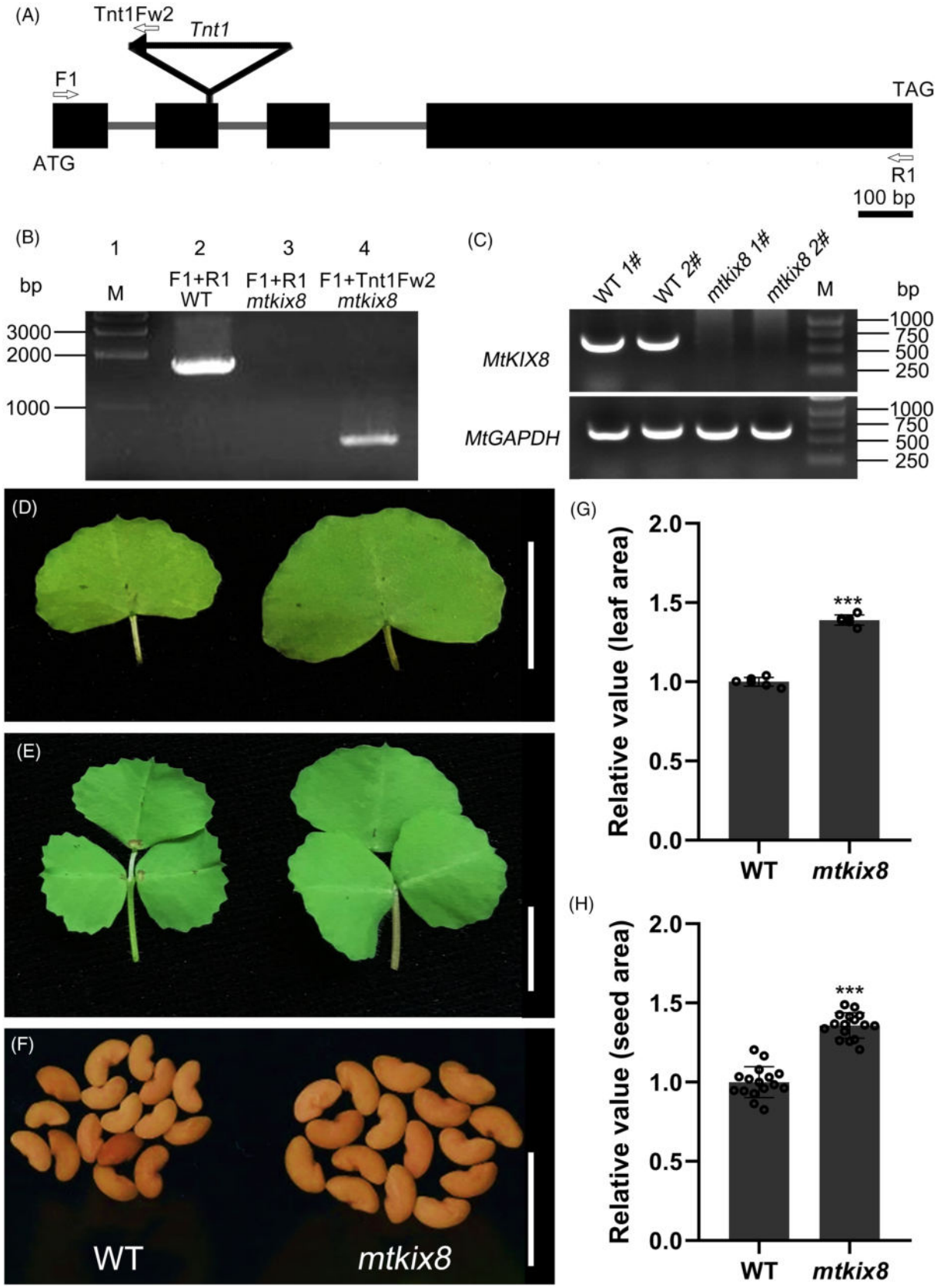

FIGURE 3 Legend on next page.

mutant population by the reverse genetic approach in the model legume *M. truncatula*. While examining the mutation site in *mtkix8*, we found that there was a reverse *Tnt1* insertion in the *MtKIX8* gene (Figure 3A,B). The loss-of-function of *MtKIX8* was confirmed by the absence of detectable *MtKIX8* transcripts in the *mtkix8* mutant (Figure 3C). At the seedling stage, the first true leaf of *mtkix8* was significantly larger than that of wild type (Figure 3D) and the trifoliate leaflets at the same node were also significantly larger (Figure 3E,G). We also observed that the size of mutant seeds was significantly larger than wild-type seeds (Figure 3F,H). These genetic and biochemical data provide direct and solid evidence to support the role of MtKIX8 as an important regulator of organ size in *M. truncatula*.

3.5 | Expression pattern of *MtKIX8* and subcellular localization of MtKIX8

To further gain insight into the gene function of *MtKIX8*, we performed quantitative reverse-transcription PCR to analyze the expression pattern of this gene. The results revealed that *MtKIX8* was expressed in all vegetative and reproductive organs of *M. truncatula* and with a higher expression level in flowers, pods, seeds and shoots (Figure 4A). This expression pattern of *MtKIX8* is reminiscent of the expression pattern of *MIO1*, which was highly expressed in leaves, floral organs and immature seeds of *M. truncatula* (Zhou et al., 2021). RNA in situ hybridization further showed that *MtKIX8* transcript signals were detectable at whole reproductive shoot apical meristem (R-shoot) (Figure 4B,C). Interestingly, although *MtKIX8* is diffusely expressed in the early floral primordia, a very high expression level of *MtKIX8* was detected in the tapetum during the development of anthers (Figure 4D,E). The tapetum provides necessary nutrition for pollen development and maturation, and the defect of the tapetum will lead to male sterility of plants (Zhang et al., 2006, 2007; Zhu et al., 2008). This result suggests that *MtKIX8* might affect the development of anthers. Meanwhile, we used the *MtKIX8* sense probe as a control and found that the endogenous signal could not be detected, confirming the accuracy of the *MtKIX8* expression pattern (Figure 4F). We transiently expressed MtKIX8-GFP in *N. benthamiana* epidermal cells and demonstrated that MtKIX8 is a nuclear localization protein, which is consistent with the localization of MIO1 protein (Figure 4G). The expression pattern of *MtKIX8* suggests an expression preference in developing organs, and subcellular localization of the MtKIX8 protein coincides with the gene function of *MtKIX8* in the regulation of lateral organ size in *M. truncatula*.

3.6 | Ectopic expression of *MtKIX8* results in decreased organ size in *Arabidopsis*

In order to rapidly explore the gain-of-function of MtKIX8, we ectopically expressed *MtKIX8* in *Arabidopsis*. The coding sequence of *MtKIX8* was fused with a 35S promoter and was transformed into Col-0. The transgenic plants had decreased cotyledon size and seed weight (Figure 5A–D). By the way, the transformation of the coding sequence of *MtKIX8* driven by the *KIX8* promoter into the *kix8* mutant restored the mutant phenotypes in three independent transgenic lines (Figure 5A–D). The PCR results confirmed that all three overexpressing lines harbor the target gene, and the expression level of *MtKIX8* was significantly higher in transgenic plants than that of Col-0 (Figure S5A,B). Moreover, another three transgenic lines driven by the *KIX8* promoter have been validated as positive transgenic plants in a homozygous mutant background. The expression level of *MtKIX8* has also increased accordingly (Figure S5C,D). These results indicate that MtKIX8 plays a conserved function in negatively regulating the organ size.

In *Arabidopsis*, KIX8 form a transcriptional repressor complex with PPD2 to regulate the downstream cell cycle D3-type cyclin gene, thus controlling lateral organ size (Gonzalez et al., 2015; Li et al., 2018). Whether *MtKIX8* also affects organ development in the transgenic *Arabidopsis* plants through regulating cell cycle genes, we examined the transcriptional level of the typical cell cycle marker genes *CYCD3;2* and *CYCD3;3* in *Arabidopsis* transgenic plants. Consistent with this hypothesis, the transcriptional level of the two cell cycle genes coincided with organ size changes, for example, the transgenic line with larger organ size also had higher expression level of *CYCD3;2* and *CYCD3;3* than that of WT (Figure 5E,F). These findings indicate that *MtKIX8* negatively regulates organ size by influencing cell cycle gene expression and eventually influences cell proliferation and cell number during development.

3.7 | MtKIX8 act as an adaptor between the BS1 and MtTPL

In *Arabidopsis*, the adaptors KIX8/9 interact with PPD to form a PPD-KIX complex, which is involved in the regulation of lateral organ size

FIGURE 3 *MtKIX8* negatively regulates organ size in *M. truncatula*. (A) The gene structure of *MtKIX8* and *Tnt1* insertion site of *mtkix8* mutant. The black boxes represent the exons and the gray line represents the intron. The triangle structure indicates the *Tnt1* retrotransposon insertion sites and the arrow indicates the insertion direction. (B) Genotyping of retrotransposon *Tnt1* insertion of *mtkix8* mutant by PCR. Lane 1 represents Marker, lane 2 represents the PCR results of WT plants with F_1 primer and R_1 primer, lane 3 represents the PCR results of *mtkix8* mutant with F_1 primer and R_1 primer, lane 4 represents the PCR results of *mtkix8* mutant with F_1 primer and Tnt1Fw2 primer, the position of the primer is shown in A. F_1 and R_1 primer span *Tnt1* sequence, owing to the length of *Tnt1* sequence is 5334 bp, the lines without a *Tnt1* insertion can amplify the band. (C) The expression level of *MtKIX8* in the WT and *mtkix8* mutant plants. *MtGAPDH* was used as an internal control. (D) The first true leaf of WT and *mtkix8* mutant. (E) The third compound leaves of 4-week-old plants of WT and *mtkix8* mutant. (F) The seed of the WT and *mtkix8* mutant. Scale bar, 1 cm (D–F). (G, H) The leaf area and seed area of the WT and *mtkix8* mutant. (G, $n = 6$, H, $n = 15$), data are mean ± SD, Student's *t* test, *p*-values: $p < 0.001$

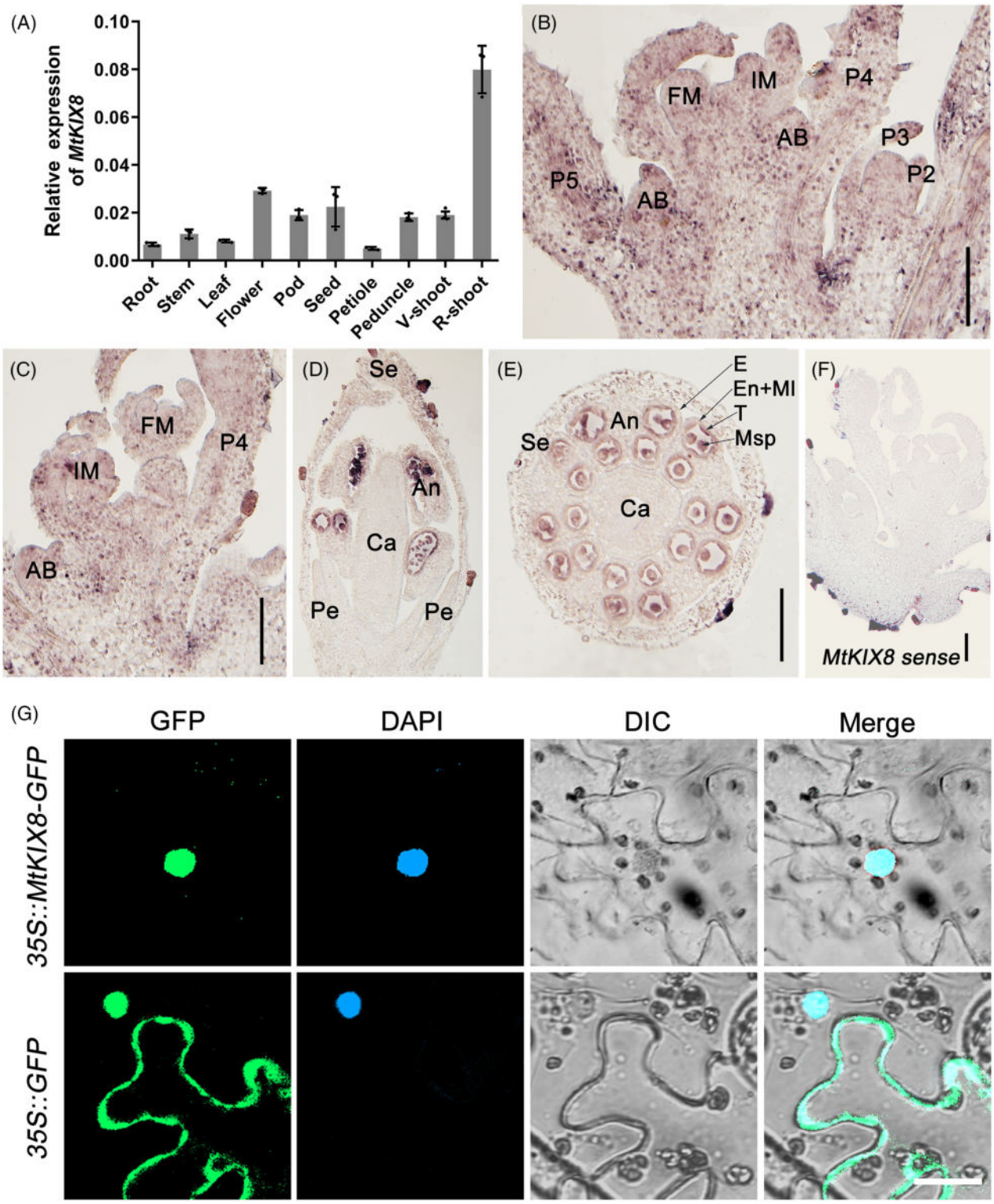

FIGURE 4 Expression patterns of *MtKIX8* and subcellular localization of MtKIX8 protein. (A) Relative expression of *MtKIX8* in different tissues. *MtACTIN* was used as an internal control. Root, stem, leaf, flower, pod, seed, petiole and peduncle were sampled from 8-week-old plants. V-shoot and R-shoot are vegetative shoot apical meristem (SAM) and reproductive shoot apical meristem (SAM), respectively. V-shoot and R-shoot were sampled from 4-week-old plants and 8-week-old plants, respectively. Data are mean ± SD. (B–E) In situ hybridization of *MtKIX8*. The longitudinal sections of the reproductive shoot apical meristem (R-shoot) (B, C), and longitudinal sections (D), and cross sections (E) of flower were sampled from 8-week-old plants. (F) The *MtKIX8* sense probe was used as the control. P + number, plastochron; FM, floral meristem; IM, inflorescence meristem; AB, axillary buds; Se, sepal; Pe, petal; An, Anther; Ca, Carpel; E, epidermis; En, endothecium; MI, middle layer; T, tapetum; Msp, microspores. Scale bar, 100 μm (B–F). (G) Subcellular localization of MtKIX8-GFP in *N. benthamiana* epidermal cells. Scale bar, 20 μm. Free GFP driven by the CaMV35S promoter was used as the control

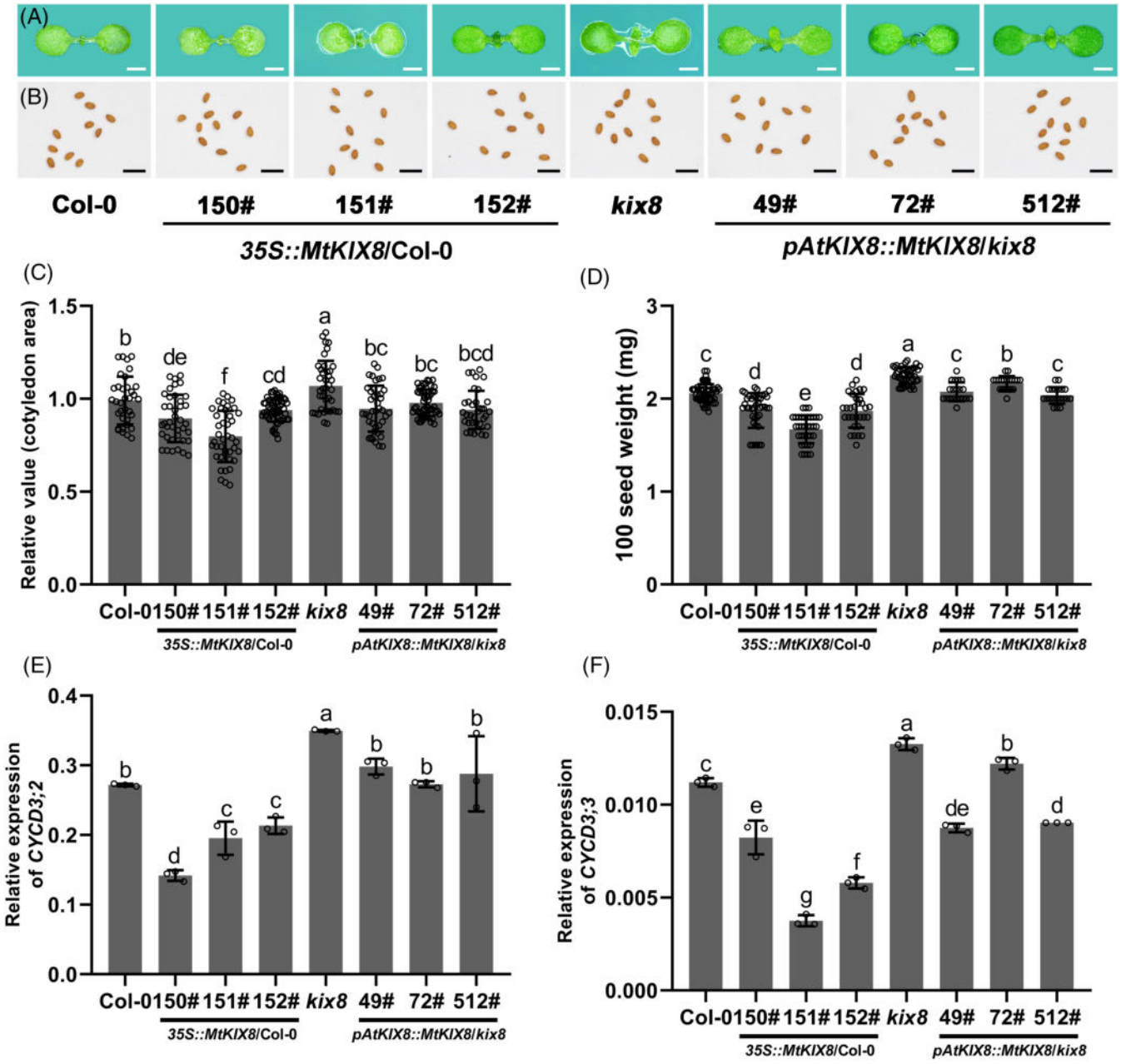

FIGURE 5 Ectopic expression of *MtKIX8* in *Arabidopsis*. (A) 8-day-old plants of Col-0, three transgenic lines of *35S::MtKIX8/Col-0* (150#, 151#, 152#), *kix8* mutant, and three transgenic lines of *pAtKIX8::MtKIX8/kix8* (49#, 72#, 512#). Scale bar, 1 mm. (B) The seeds of Col-0, three transgenic lines of *35S::MtKIX8/Col-0*, *kix8* mutant and three transgenic lines of *pAtKIX8::MtKIX8/kix8*. Scale bar, 1 mm. (C, D) The relative cotyledon area (C, $n \geq 35$) and 100 seed weight (D, $n \geq 20$) of Col-0, three transgenic lines of *35S::MtKIX8/Col-0*, *kix8* mutant and three transgenic lines of *pAtKIX8::MtKIX8/kix8*. (E, F) The relative expression level of *CYCD3;2* and *CYCD3;3* in the inflorescence tissue from Col-0, three transgenic lines of *35S::MtKIX8/Col-0*, *kix8* mutant, and three transgenic lines of *pAtKIX8::MtKIX8/kix8*. *AtACTIN* was used as an internal control. Data are mean ± SD, one-way ANOVA and LSD post hoc test, *p*-values: $p < 0.05$

by modulating cell-cycle gene expression (Baekelandt et al., 2018; Gonzalez et al., 2015). Beside in *Arabidopsis*, the PPD-KIX complex was also reported to regulate organ size in tomato and pea (Li et al., 2019; Swinnen et al., 2022). The above studies suggest that a similar repressor complex involved in organ size regulation may exist in *M. truncatula*. We first performed a Y2H assay and found that MtKIX8 interacts with BS1 (Figure 6), suggesting the conservation function of the MtKIX8-BS1 complex on the organ size regulation in *M. truncatula*. Furthermore, the TOPLESS (TPL) family of co-repressors interact with specific proteins to mediate transcriptional repression of specific target genes (Plant et al., 2021). It was previously reported in *Arabidopsis* and tomatoes that PPD-KIX recruits TPL to form a PPD-KIX-TPL transcriptional repressor complex (Gonzalez et al., 2015; Swinnen et al., 2022). Therefore, we redefined the TPL

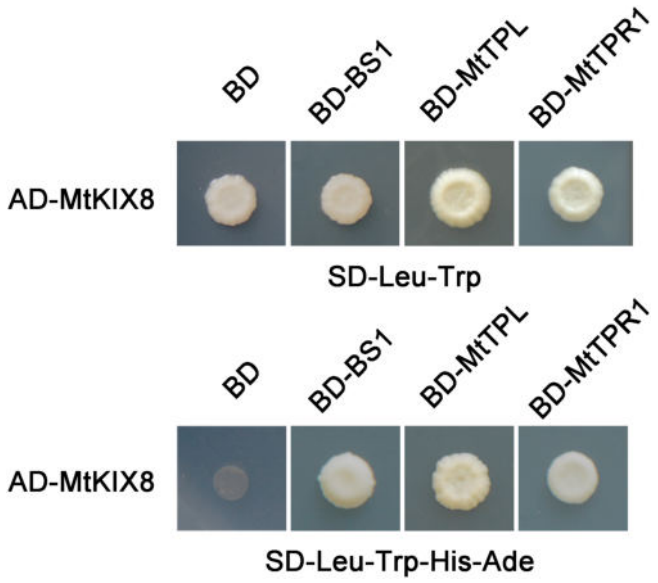

FIGURE 6 Yeast two-hybrid assay of interaction between BS1, MtKIX8 and MtTPL proteins. Yeast cells were grown on dropout medium SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade for 4 days after plating. pGBKT7 (BD) was used as negative controls

family proteins in *M. truncatula* based on previous research (Zhang et al., 2014). There are seven TPL proteins, all with the same conserved domain (Figure S6A,B). We selected two TPL proteins from *M. truncatula* with the closest homology to *Arabidopsis* TPL1, MtTPL and MtTPR1 to explore the interaction function of MtKIX8. These two TPL proteins directly interact with MtKIX8 but not BS1 (Figures 6 and S7). Thus, MtKIX8 acts as a molecular adaptor to interact with BS1 and MtTPL in *M. truncatula*.

4 | DISCUSSION

The molecular mechanism of plant organ size is controllable and stable, which provides the genetic basis for the improvement of crops and forages. Recently, we reported that MIO1, the ortholog of SAP in the model legume *M. truncatula*, plays a vital role in the regulation of organ size (Zhou et al., 2021). To further explore how MIO1 regulates organ size, we performed a yeast library screening and isolated a potential interactor, MtKIX8 (Table 1). The protein sequence alignment reveals that MtKIX8 and its orthologs contain a conserved N-terminal KIX domain and a C-terminal EAR domain (Figure 1B). The KIX domain usually serves as a docking site for transcription factors, which could improve the stability of protein–protein interaction and is much important for the conformation of transcriptional apparatus and transcriptional regulation of genes (Thakur et al., 2013). The EAR domain usually recruits transcriptional co-inhibitors to form transcriptional inhibition complexes. The loss function of *MtKIX8* gives rise to enlarged seeds and leaves (Figure 3), fitting with the assumption that MtKIX8 plays a role within the repressor complex to negatively regulate the organ size. Additionally, ectopic expression of *MtKIX8* in the *kix8* mutant partially rescues the mutant phenotypes, and overexpression of *MtKIX8* in Col-0 leads to decreased seed and cotyledon size (Figure 5). These results indicate that *MtKIX8* plays a conserved function both in *Arabidopsis* and *M. truncatula*. Based on the previous report about the function of *MIO1/SLB1* in the organ size regulation (Yin et al., 2020; Zhou et al., 2021), the MIO1/SLB1-MtKIX8 module is a key component to fine-tune the organ size in *M. truncatula*.

The SAP-KIX-PPD signaling pathway plays a prominent role in the control of lateral organ size in *Arabidopsis* (Gonzalez et al., 2015; Wang et al., 2016; White, 2006). Moreover, this regulatory pathway also regulates organ size in tomatoes and peas (Li et al., 2019; Swinnen et al., 2022). There are few reports about the organ size regulation in legumes. Therefore, we try to dissect its function in the model legume *M. truncatula*. The biochemistry data indicate that MtKIX8 acts as a molecular adaptor to link MIO1 and BS1 to form the MIO1-MtKIX8-BS1 complex (Figures 2 and 6). The SAP-KIX-PPD complex regulates organ size by negatively regulating secondary cell proliferation in *Arabidopsis* (Gonzalez et al., 2015; Wang et al., 2016; White, 2006; Yang et al., 2018). However, the changes in organ size reported in soybean, *M. truncatula* and blackgram are achieved by repressing primary cell proliferation (Ge et al., 2016; Naito et al., 2017; Zhou et al., 2021). These findings suggest differences in the organ size control regulatory processes between Fabaceae and Brassicaceae. The above-studied plants belong to the rosids and asterids clade, which constitute most of the core eudicot species. It seems that this pathway has been recruited to determine the organ size before the explosion of eudicots or independently recruited in different plant lineages during the diversification of core eudicots. Furthermore, the SAP-KIX-PPD complex is absent in Poaceae (Gonzalez et al., 2015; Wang et al., 2016), which suggests that the molecular mechanism of organ size regulation in monocots could be different from dicots (Schneider et al., 2021). In addition, MtKIX8 functions as an adaptor to interact with BS1 and MtTPL to form a repressor complex (Figures 6 and 7), which is consistent with the functional hypothesis of MtKIX8 protein domain and similar to that of their orthologs in *Arabidopsis* and tomato (Baekelandt et al., 2018; Swinnen et al., 2022). Based on these clues, this conserved complex among legumes might shed light on biomass and yield improvement by molecular design breeding in important legume crops and forages.

The ubiquitin-proteasome pathways are often involved in the regulation of plant organ size by controlling the cell-cycle progression (Baute et al., 2017; Hao et al., 2021). In the *mio1* mutant, the downregulation of genes associated with cell division results in organ size changes (Zhou et al., 2021). In fact, increased expression of *MtKIX8* leads to the decreased expression of downstream D3-type genes (Figure 5E,F), adding an extra layer to understanding the regulatory pathway of MtKIX8 on plant organ size control. Based on our research results and previous studies, we proposed a working model in *M. truncatula*. MIO1/SLB1 and other proteins could form an $SCF^{MIO1/SLB1}$ E3 ubiquitin ligase complex to degrade the MtKIX8-BS1 repressor complex, which affects the expression level of downstream cell cycle genes to regulate the plant organ size (Figure 7).

Until now, there have been few studies on the roles of KIX proteins in plant development. The KIX domain protein NRB4 is involved

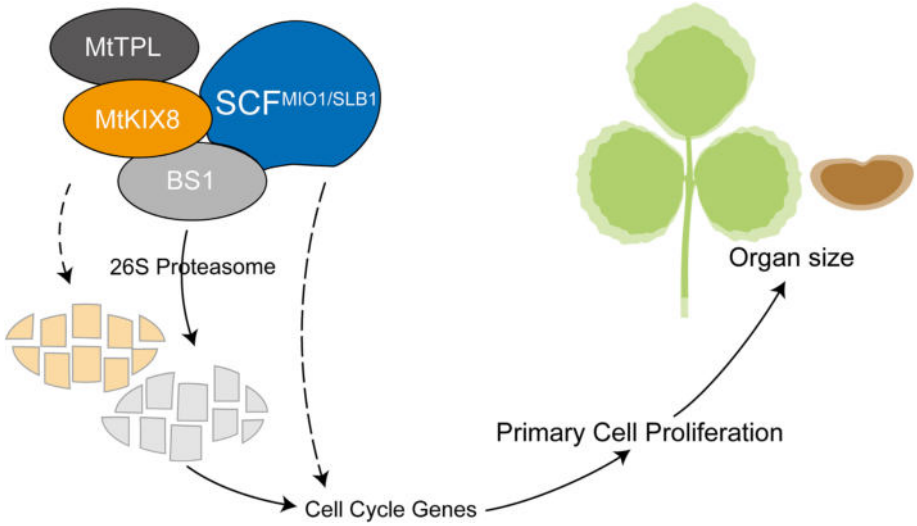

FIGURE 7 A working model for MtKIX8 in the organ size regulation in *M. truncatula*. The F-box protein MIO1/SLB1 could form part of an SCF E3 ubiquitin ligase complex to target the organ size repressor complex MtKIX8-BS1 for degradation, which regulates the expression level of cell cycle genes, resulting in significant changes in plant organ size. MtKIX8 also acts as an adaptor to recruit BS1 and MtTPL protein to form a transcriptional repressor complex to regulate the organ size

in plant response to salicylic acid, while another KIX domain protein, CBP/p300-like, can promote flowering time in plants by inhibiting FLC (Canet et al., 2012; Han et al., 2007; Radhakrishnan et al., 1997). In addition, KIX8/9 is involved in regulating organ size in plants (Gonzalez et al., 2015). The above studies suggest that the KIX domain-containing proteins could regulate different biological processes. Interestingly, when we examined the expression pattern of *MtKIX8* in *M. truncatula*, we found that *MtKIX8* is not only expressed in developing tissues and organs like SAM, leaf and floral primordia but also is highly expressed in the tapetum of flowers (Figure 4A–E). These results suggest that *MtKIX8* not only regulates cell proliferation and organ size but may also is recruited to regulate tapetum cell development and maturation. Previous studies showed that there are two distinct binding sites within the same KIX domain, which could interact with two different kinds of transcription regulators at the same time. This structural basis of the KIX protein further endows the KIX protein with multi-function (Campbell & Lumb, 2002; Goto et al., 2002). These clues would drive us to explore the function and molecular mechanism of the *KIX* genes in the future.

Furthermore, MIO1 also affects the development of pulvinus through the auxin pathway (Zhou et al., 2021). Pulvinus is an important motor organ essential for leaf movement in legumes (Chen et al., 2012; Zhou et al., 2012). The Y2H library screening also identifies the auxin-related gene (Medtr1g093240), which provide a clue to dissect the molecular mechanism of MIO1 on control of pulvinus development. In addition, we also identified a flower-related gene (Medtr7g032240) from the screening. We know that loss-of-function MIO1 also leads to the severe flower phenotype (Figure S1D), which provides evidence for future research on the mechanism of flower development. In conclusion, we identified several target genes from the Y2H library screening, which are helpful in comprehensively deciphering the regulatory mechanism of the MIO1 in *M. truncatula*.

The *kix8* single mutant in *Arabidopsis* produces enlarged cotyledons and seeds, and the *kix8 kix9* double mutant has a more significant phenotype than the single mutant (Liu et al., 2020). Based on our analysis, there is only one *KIX* gene in the KIX8 subgroup in *M. truncatula*. In contrast, there are two homologous genes in soybean and tomato, and *SlKIX8* and *SLKIX9* are functionally redundant in tomato (Swinnen et al., 2022). The single copy of MtKIX8 works as a negative regulator of organ size (Figure 3). Consequently, we did find smaller cotyledons and seeds by ectopic expression of *MtKIX8* in *Arabidopsis* (Figure 5A–D), which further illustrates the key role of MIO1-MtKIX8 module on the organ size regulation. In summary, this study provides a useful target gene for the future genetic improvement of legume forage and crops.

AUTHOR CONTRIBUTIONS

Jianghua Chen, Yawen Mao and Shaoli Zhou designed the research. Yawen Mao performed most experiments, analyzed data and wrote the first draft. Shaoli Zhou and Jianghua Chen assisted with some experiments and revised the manuscript. Jing Yang, Jiangqi Wen, Dongfa Wang, Xuan Zhou, Liangliang He, Baolin Zhao, Liling Yang, Million Tadege and Changning Liu provided advice and revised the manuscript. Jing Yang, Jiangqi Wen, Xinyuan Wu, Mingli Liu, Huan Wu and Yu Liu helped collecting some data. All authors read and approved the submitted version.

ACKNOWLEDGMENTS

This work was supported by the Strategic Priority Research Program of Chinese Academy of Sciences (Grants Nos: XDA26030301 and XDB27030106), the National Natural Science Foundation of China (32200290 and 32170839), the CAS-Western Light "Cross-Team Project-Key Laboratory Cooperative Research Project" (Grant xbzg-zdsys-202016), the "Yunnan Revitalization Talent Support Program" (Grant Nos: E3YN103K01, XDYC-QNRC--79 and XDYC-QNRC-2022-0179), the Youth Innovation Promotion Association CAS (Grant 2021395) and "High-end Scientific and Technological Talents in Yunnan Province" (2015HA031 and 2015HA032).

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available within the paper and in the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Mao, Y., Zhou, S., Yang, J., Wen, J., Wang, D., Zhou, X. et al. (2023) The MIO1-MtKIX8 module regulates the organ size in *Medicago truncatula*. *Physiologia Plantarum*, 175(5), e14046. Available from: https://doi.org/10.1111/ppl.14046