RESEARCH PAPER

The F-box protein MIO1/SLB1 regulates organ size and leaf movement in Medicago truncatula

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Abstract

The size of leaf and seed organs, determined by the interplay of cell proliferation and expansion, is closely related to the final yield and quality of forage and crops. Yet the cellular and molecular mechanisms underlying organ size modulation remain poorly understood, especially in legumes. Here, MINI ORGAN1 (MIO1), which encodes an F-box protein SMALL LEAF AND BUSHY1 (SLB1) recently reported to control lateral branching in Medicago truncatula, was identified as a key regulator of organ size. We show that loss-of-function of MIO1/SLB1 severely reduced organ size. Conversely, plants overexpressing MIO1/SLB1 had enlarged organs. Cellular analysis revealed that MIO1/SLB1 could form part of SKP1/Cullin/F-box (SCF) E3 ubiquitin ligase complex, to target BIG SEEDS1 (BS1), a repressor of primary cell division, for degradation. Interestingly, we found that MIO1/SLB1 also played a key role in pulvinus development and leaf movement by modulating cell proliferation of the pulvinus as leaves developed. Our study not only demonstrates a conserved role of MIO1/SLB1 in the control of organ size in legumes, but also sheds light on the novel function of MIO1/SLB1 in leaf movement.

Keywords: BIG SEEDS1 (BS1); F-box protein; MINI ORGAN1 (MIO1)/SMALL LEAF AND BUSHY1 (SLB1); SCF E3 ligase, proteasome-mediated degradation; organ size; pulvinus
Introduction

Organ size in plants, which is determined by their genetic make-up and environmental conditions, is one of the most important parameters for their adaptation and survival (Czesnick and Lenhard, 2015). In agriculture, the size of plant organs directly influences the final yield and quality of crops (Graham and Vance, 2003). In particular, legumes provide the most significant sources of plant-based protein for consumption by humans and animals. So, improving the production of legume crops and forage by increasing organ size is one promising way to meet the protein requirements of the rise in global population. However, the cellular and molecular mechanisms underlying the determination of organ size in plants remain largely unclear, especially in legume species.

In plants, organ size is controlled by a complex interplay of cell proliferation and expansion (Gonzalez et al., 2012; Hepworth and Lenhard, 2014; Czesnick and Lenhard, 2015). The leaf has served as an ideal system to understand the plasticity of organ size in a developmental context. For Arabidopsis, leaf growth is under spatiotemporal control at five overlapping phases: initiation phase, when the founder leaf growth is under spatiotemporal control at five overlapping phases: initiation phase, when the founder leaf primodium; Andriankaja et al., 2012); general cell division phase, when cell proliferation occurs throughout the entire primordium during primary morphogenesis (Gonzalez et al., 2012; Du et al., 2018); transition phase, when cell division ceases in a basipetal manner (from the tip to the base of leaf primordium; Andriankaja et al., 2012); cell expansion phase, when the cell size becomes enlarged, which generally coincides with endoreduplication, and remodeling of the cell wall and vacuole (Cosgrove, 2005; Cookson et al., 2006); and finally, meristemoid division phase, when the dispersed meristematic cells (DMC) undergo asymmetric division to form stomatal guard and stomatal-lineage ground cells (Donnelly et al., 1999; White, 2006; Pillitteri and Torii, 2012). The final leaf size would thus be coordinated by the number of cells of the primordium, the rate and duration of general cell division, as well as cell expansion and meristemoid division (Gonzalez et al., 2012). The disturbance of genetic pathways that control one or more of these cellular mechanisms could lead to altered cell proliferation and growth, which in some cases can trigger a compensatory mechanism. For example, in leaves of flowering plants, the total abundance of cells that results from abnormal mitotic cell division would induce corresponding changes in post-mitotic cell expansion, to ensure final leaf size is not significantly changed compared with that expected from variation in the quantity of leaf cells (Horiguchi and Tsukaya, 2011).

Recent studies have enhanced our understanding of the molecular mechanisms underlying organ size determination (Gonzalez et al., 2012; Hepworth and Lenhard, 2014; Czesnick and Lenhard, 2015). Notably, the ubiquitin-proteasome pathways have been revealed to play a significant role in the regulation of cell-cycle progression during plant organ morphogenesis (Vierstra, 2003). The F-box protein AtFBX92 represses leaf growth by influencing the cell division rate during the early stages of leaf development in Arabidopsis (Baute et al., 2017). Another F-box protein, FBOX-LIKE17 (FBL17), accelerates cell proliferation and endoreduplication by forming an Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex that targets KIP-RELATED PROTEIN2 (KRP2) for degradation (Noir et al., 2015). The peptidase DA1 is activated by the RING E3 ligases DA2 and ENHANCER OF DA1/BIG BROTHER (EOD1/BB) via mono-ubiquitination, thereby negatively regulating final organ size by destabilizing various positive growth factors (Disch et al., 2006; Li et al., 2008; Xia et al., 2013; Du et al., 2018; Dong et al., 2017; Vanhaeren et al., 2020). AtrKPT2a, being a subunit of the 26S proteasome, represses organ size by reducing both cell endoreduplication and expansion (Sonoda et al., 2009). It was also shown that STERILE APETALA-PEAPOD-KIX-TOPLESS (SAP-PPD-KIX-TPL), KIX-PEAPOD-MYELOCYTOMATOSIS-GRF-INTERACTING FACTOR 1 (KIX-PPD-MYC-GIF1) and STERILE APETALA-PEAPOD-NOVEL INTERACTOR OF JAZ-TOPLESS (SAP-PPD-NINJA-TPL) modules govern lateral organ size, seed size and leaf shape in Arabidopsis (White, 2006; Gonzalez et al., 2015; Wang et al., 2016; Baekelandt et al., 2018; Li et al., 2018; Liu et al., 2020). The F-box protein SAP integrates with other subunits to form the SCFASP E3 ubiquitin ligase; this marks the PPD2-KIX8/9 repressor complex for degradation to determine the final organ size, or for degradation of the PPD2-NINJA repressor complex to ensure that a normal leaf blade curvature develops in Arabidopsis (Byzova et al., 1999; Wang et al., 2016; Baekelandt et al., 2018; Li et al., 2018). Either knock down or knock out of PPD2 and its orthologs led to a reduction in the size of lateral organs (White 2006; Gonzalez et al., 2015; Ge et al., 2016; Wang et al., 2016; Naito et al., 2017; Kanazashi et al., 2018; Li et al., 2018; Yang et al., 2018).

Various leaf movements have been observed in higher plants in response to endogenous and environmental signals. These intriguing behaviors are proposed to provide advantages for plant adaptation, survival, and evolution (Darwin, 1880; Satter et al., 1990; Koller, 2011; Mancuso and Shabala, 2015; Minorsky, 2019). Reversible leaf movement relies on the pulvinus, a specialized motor organ in higher plants (Satter et al., 1990; Coté, 1995; Ueda and Nakamura, 2007). Yet, surprisingly, the molecular mechanisms underlying pulvinus development are still poorly understood. It is known that ELONGATED PETIOLULE1 (ELP1), a plant-specific lateral organ boundaries domain (LBD) transcription factor, determines the pulvinus identity in legumes. Disruption of ELP1 expression or that of its orthologs in related legume species...
completely abolished both pulvinus development and leaf movement (Marx, 1987; Kawaguchi, 2003; Chen et al., 2012; Zhou et al., 2012). The gene Glycine max Increased Leaf Petiole Angle1 (GmILPA1) encodes an APC8-like protein that is a component of the APC/C complex and able to interact with GmAPC13a to promote motor cell division and growth in soybean (Glycine max). The mutation of GmILPA1 results in defective pulvinus formation and diminishes leaf movement (Gao et al., 2017).

Here we report on the identification and characterization of the Medicago truncatula mutant mini organ1 (mio1), which has a decreased organ size and a defective pulvinus. MIO1 encodes an F-box protein, SLB1, which has been shown to control lateral branching in M. truncatula (Yin et al., 2020). MIO1/SLB1 is the ortholog of SAP and highly expressed in both leaf and floral primordia. The transformation of MIO1/SLB1 into the mio1 mutant completely rescued the latter's traits of smaller organ size and defective pulvinus. Protein–protein interaction assays revealed that MIO1/SLB1 could directly interact with Medicago truncatula Arabidopsis SKP (MtASK), and with BIG SEEDS1 (BS1) via its C terminal WD40 repeat domain. The protein degradation assay indicated that BS1 could be degraded by MIO1/SLB1 in an MG132-sensitive manner. Loss-of-function of MIO1/SLB1 induced down-regulation of expression of the cell-cycle marker genes. Our work reveals that MIO1/SLB1 plays a key role not only in the determination of lateral organ size, which would be valuable for improving biomass production and yield of legume crops and forages, but also in the regulation of normal pulvinus development that is necessary for leaf movement in M. truncatula.

Materials and methods

Plant materials and growth conditions

Arabidopsis (Col-0) and M. truncatula (R108) plants were grown in a chamber and greenhouse, respectively, under the following conditions: a 16 h/8 h day/night photoperiod, 150 μE m⁻² s⁻¹ light intensity, temperatures of 22 °C/18 °C at day/night, and under 70% humidity. The shoot apices of three-week-old and 10-week-old WT plants were paraffinized with toluidine blue. Their images were captured with a microscope (Olympus BX63M).

Phenotypic analysis

The length, width, and area of leaflets were measured using ImageJ. For the time-course analysis of leaf development, a juvenile leaf was imaged daily over a 12-day period with a digital camera (Nikon); data were collected with ImageJ. To analyse the leaf epidermal cell area, stomatal index, and for the follow-up investigation of cell area, images of epidermal cells were captured under a microscope (BX63, Olympus, Japan). From these, the cell area and stomatal index were determined using ImageJ.

Whole-genome resequencing and molecular cloning of MIO1/SLB1

The heterozygous plants of mio1-1, mio1-2, and mio1-3 were backcrossed with WT (R108) for two generations. The ensuing homozygous mutant plants of mio1-1, mio1-2, and mio1-3 were then used for whole-genome resequencing (next-generation sequencing). Specifically, their sequence data was analysed with the ITIS (Identification of Transposon Insertion Sites) tool, to identify all Tnt1 insertion sites as described previously (Jiang et al., 2015). Both PCR and RT–PCR were used for generating cosegregation analysis and verification of Tnt1 insertions responsible for the mutant phenotype. The primers used are listed in Supplementary Table S1.

Phylogenetic analysis and sequence alignment

The protein homologs of MIO1/SLB1 were identified from phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#). All the candidates were verified to contain the conserved F-box and WD40 repeat motif. Next, phylogenetic trees were constructed by using the neighbor-joining algorithm implemented in MEGA5, with 1000 bootstrap replications performed. The sequence alignment of MIO1/SLB1 and its homologs was carried out with DNAMAN software.

Scanning electron microscope (SEM) analysis

The pulvini from the mio1-1, proMIO1::MIO1/mio1-1 transgenic plants, and WT were collected. The collected tissues were subjected to vacuum infiltration in FAA fixative solution for 30 min and then kept at 27 °C overnight. The fixed tissues were dehydrated in a graded ethanol series (45%, 55%, 65%, 75%, 85%, 90%, 95%, each concentration for about 1 h), and ending with 100% ethanol overnight. The dehydrated tissues were dried until critical-point in liquid CO2 with a critical point drier (Samdri-PVT-3D, Tousimis, USA), mounted on aluminum stubs, dissected with the stereomicroscope (SZX16, Olympus, Japan), sputter-coated with gold, and examined under SEM (SIGMA 300, Zeiss, Germany).

Paraffin sectioning

The fixed and dehydrated pulvinus samples were embedded in paraplast (EG1150 H, Leica, Germany). These plant tissues were sectioned at an embedder (RM2235, Leica, Germany), to an 8 μm thickness and stained with toluidine blue. Their images were captured with a microscope (Olympus BX63M).

RNA in-situ hybridization

Shoot apices of three-week-old and 10-week-old WT plants were paraffin embedded and sectioned. These sections were hybridized with a digoxigenin-labeled anti-sense probe, using the sense probe to serve as the control. The hybridization signal was visualized under a microscope (Olympus BX63M).

Plasmid construction and transformation

The 4.7 kb promoter sequence of MIO1/SLB1 was generated from genome sequence and cloned into the pCAMBIA3301 vector to obtain the proMIO1::GUS construct. The coding sequence of MIO1/SLB1 was inserted into the proMIO1::GUS vector in which the GUS gene was replaced with MIO1/SLB1 coding sequence, to obtain the proMIO1::MIO1 construct. The coding sequence of MIO1/SLB1 was cloned into the pCAMBIA3301 vector to generate the 35S::MIO1 construct. For the construction of 35S::GFP-MIO1, the coding sequence of MIO1/SLB1 was cloned into pCAMBIA3301. To generate 35S::MIO1-GFP, the MIO1/SLB1 coding sequence was inserted into pYSS22. To derive the plasmids used for the yeast two-hybrid (Y2H) assay, the full-length MIO1/SLB1 coding sequence and the truncated sequence that corresponds to the F-box or WD40 repeat domain of MIO1/SLB1 were inserted into the bait vector pGBK7 (Clontech, USA). Similarly, the coding sequences of BS1, MtASK,
and MKIX were inserted into the prey vector pGADT7. For the bimolecular fluorescence complementation (BiFC) assay, the MIO1/SLB1 coding sequence was inserted into the pFGC-YN173 vector while MtASK and BS1 were inserted into the pFGC-YC155 vector. To obtain a plasmid that expresses the GST-MIO1 WD40 fusion, the truncated coding sequence of Medtr5g097060 corresponding to the WD40 repeat domain was cloned into pGEX4T-1 that harbored the GST coding sequence. Similarly, the coding sequence of BS1 was inserted into pET28a that contained the His tag, to generate the His-BS1 fusion protein. All the primers used for the above are listed in the Supplementary Table S1.

Sub-cellular localization

The 35S::GFP-MIO1 and 35S::MIO1-GFP constructs were transformed into the Agrobacterium tumefaciens EHA105 strain, and then infiltrated into tobacco (Nicotiana benthamiana) leaves. The MIO1-GFP and GFP-MIO1 signals were examined under a confocal laser scanning microscope (Olympus, FV1000).

RNA isolation, RT–PCR, and quantitative RT–PCR

Total RNA was isolated from plant tissues with a plant total RNA isolation kit (Generay, China). The quantity and quality of extracted RNA were assessed with Nanodrop 2000 (Thermo Scientific, USA). Next, the cDNA synthesis was performed with PrimeScript™ RT reagent kit (Generay, China). The quantity and quality of extracted RNA isolation, RT–PCR, and quantitative RT–PCR

Results

Isolation and characterization of the M. truncatula mio1 mutant

To identify the key regulators that control the organ size of legumes, we screened the Tnt1 retrotransposon insertion mutant population of the model legume M. truncatula (R108) for any organ size mutants. Three independent mutant lines—mini organ 1 (mio1)-1, mio1-2, and mio1-3—exhibiting similarly decreased organ size, were isolated. A genetic analysis confirmed that they were allelic mutants. Then the mio1-1 mutant was backcrossed with the WT plants. In the BC1F2 population, the plants displaying the WT-like and mutant phenotype showed a 3:1 (149:51) segregation ratio; this indicated that the mutant phenotype was caused by the mutation of a single recessive gene. The mio1-1 was backcrossed with WT for three generations, and then the BC3F2 plants were used for phenotypic analysis. Compared with the WT, the mio1 mutant was evidently reduced in its plant and organ size in both its vegetative (Fig. 1A, B) and reproductive phases (Fig. 1C, D). The sizes of the representative lateral organs, namely the leaves and flowers, were markedly smaller in the mio1 mutant than the WT (Fig. 1E-G). The terminal leaflet length (TLL), terminal leaflet width (TLW), terminal leaflet area (TLA), and terminal leaflet perimeter (TLP) of the mio1 mutant exhibited significant (P<0.01) reductions compared with those of the WT plants (Fig. 1H). These results indicated that a functional MIO1 gene is essential for proper organ size regulation in M. truncatula.

Molecular cloning and characterization of MIO1/SLB1

The MIO1 gene was cloned with the ITIS tool by identifying the Tnt1 retrotransposon insertion that caused the mutant phenotype (Jiang et al., 2015). The mio1-1, mio1-2, and mio1-3 mutants were backcrossed with WT, after which the mutant plants of BC2F2 were used for whole genome resequencing (He et al., 2020). The Tnt1 insertion sites were then isolated, by analysing the resequencing data with the ITIS tool, as
previously described (Jiang et al., 2015). These isolation results showed that all three mutant alleles were homozygous for the Tnt1 insertions at the same ORF (open reading frame) of the Medtr5g097060 locus at different sites (Fig. 2A; Supplementary Table S2). These insertions co-segregated with the mutant phenotype, according to the PCR-based genotyping results of homozygous recessive mutants of the BC2F2 population, in all the three alleles. In contrast with WT, the transcript of Medtr5g097060 could not be detected in the mio1 mutant by RT–PCR analysis (Supplementary Fig. S1). Collectively, these data suggested that the Medtr5g097060 locus defines the gene corresponding to MIO1.

To further confirm that the Medtr5g097060 locus corresponds to the MIO1 gene, we reverse-screened the Tnt1 insertion population for additional alleles, and also carried out genetic complementation. Three new mutant lines harboring the Tnt1 insertions at different sites of the Medtr5g097060 locus were isolated; they displayed similar phenotypes to the mio1-1 mutant and were named mio1-4, mio1-5, and mio1-6 (Fig. 2A; Supplementary Table S2). Subsequently, the coding sequence (CDS) of MIO1 driven by its native promoter was introduced into the mio1-1 mutant via A. tumefaciens-mediated transformation. The transgenic plants resulted in full complementation of the mutant phenotype in two independent complemented lines (Fig. 2B–D). Importantly, in these lines, the reduced leaf size trait was completely rescued (Fig. 2E–G), and the decreased floral organ size was also recovered (Fig. 2H–J), to match those of the WT phenotype. The expression of MIO1 was evidently restored in these complemented plants (Fig. 2K). The Medtr5g097060 locus has been reported to also encode the SMALL LEAF AND BUSHY1 (SLB1; Yin et al., 2020); we confirmed that the MIO1 gene was allelic to SLB1 by comparing the gene sequence and mutant phenotypes. Taken together, these results confirmed that the Medtr5g097060

Fig. 1. Phenotype comparisons between the wild type (WT) and mio1 mutant. (A, B) Four-week-old seedlings of the WT (A) and mio1-1 mutant (B). Scale bar=2 cm. (C, D) Branch of a 12-week-old plant of the WT (C) and mio1-1 mutant (D). Scale bar=2 cm. (E, F) The fifth compound leaf of five-week-old seedlings of the WT (E) and mio1-1 mutant (F). Insets provide a close-up view of the stipules. Scale bar=2 mm. (H) Comparison of the terminal leaflet length (TLL), terminal leaflet width (TLW), terminal leaflet area (TLA), and terminal leaflet perimeter (TLP) of the WT and mio1-1 mutant plants. Values indicate the mean±SD (n=3 biological replicates, with 20 plants per replicate); asterisks indicate significant differences from the WT (**P<0.01; Student’s t-test).
locus corresponds to MIO1 that is allelic to the SLB1 gene and required for organ size determination in *M. truncatula*.

Phylogenetic analysis revealed MIO1/SLB1 as the ortholog of STERILE APETALA (SAP) and LITTLE LEAF (LL; Fig. 2L; Supplementary Fig. S2), the key organ size regulators in Arabidopsis and cucumber (*Cucumis sativus*), respectively (Wang et al., 2016; Yang et al., 2018). Multiple amino acid sequence alignments of MIO1/SLB1 with its orthologs indicated
that they shared the highly conserved N-terminal F-box and C-terminal WD40 repeat domains (Fig. 2M; Supplementary Fig. S3). All of these features suggested that MIO1/SLB1 encodes an F-box protein. F-box proteins are known to form the SCF E3 ubiquitin ligase with ASK and Cullin through its N-terminal F-box domain, and recognize specific substrates for ubiquitination through its C-terminal domain, just like the WD40 repeat domain (Vierstra, 2003; Wang et al., 2016).

Ectopic expression of MIO1/SLB1 rescues the mutant phenotype of Arabidopsis sod3-3

The Arabidopsis MIO1/SLB1 ortholog, STERILE APETALA (SAP)/SUPPRESSOR OF DA1 (SOD3), is known to positively regulate organ size during plant growth and development (Wang et al., 2016). Loss-of-function of SAP gave rise to decreased organ size in Arabidopsis (Wang et al., 2016). To investigate whether MIO1/SLB1 performs a similar function as SAP for organ size control, the CDS of MIO1/SLB1 driven by the cauliflower mosaic virus 35S (CaMV35S) promoter was introduced into the Arabidopsis sod3-3 mutant (35S::MIO1/SLB1) and the corresponding WT (35S::MIO1/Col-0). The smaller organ size of sod3-3 was rescued in 35S::MIO1/Col-0 transgenic plants (Fig. 3A-F). Furthermore, the 35S::MIO1/Col-0 transgenic lines exhibited increased organ size compared with Col-0 (Fig. 3G, H). Interestingly, most of the transgenic plants had twisted and dome-shaped leaves (Fig. 3E, H). This phenotype was similar to that reported for the Arabidopsis ppd mutant (White, 2006). The expression of MIO1/SLB1 was detected in both 35S::MIO1/Col-0 and 35S::MIO1/Col-0 transgenic plants (Fig. 3I). These results suggested that MIO1/SLB1 and SAP play a conserved role in organ size regulation.

Expression pattern of MIO1/SLB1 and sub-cellular localization of MIO1/SLB1

Quantitative RT-PCR revealed that MIO1/SLB1 was highly expressed in leaves, floral organs, and immature seeds (Fig. 4A; Supplementary Fig. S4). Further analysis with RNA in-situ hybridization showed that the MIO1/SLB1 transcripts mainly accumulated in the early leaf primordia (P0-P2; Fig. 4B, C), pulvinus primordia (Fig. 4D), floral meristem, axillary bud (Fig. 4E), and petal meristem, developing carpel, and ovule (Fig. 4F, G), with no signals detected in the control which hybridized with a sense probe (Fig. 4H). To further confirm this, but using a different approach, we introduced the GUS reporter gene driven by the MIO1/SLB1 promoter into WT (proMIO1::GUS). GUS signals were detected in both single and trifoliate leaves, and the signals were stronger in vascular tissue and pulvinus (Fig. 4I-L). Taken together, the above expression pattern suggested a crucial role for MIO1/SLB1 during lateral organ morphogenesis in M. truncatula.

To determine its sub-cellular localization, we transiently expressed MIO1/SLB1 fused with a green fluorescent protein (MIO1-GFP or GFP-MIO1) under the control of a 35S promoter in tobacco leaves. Both the MIO1-GFP and GFP-MIO1 fusion proteins were localized to the nuclei of tobacco leaf epidermal cells (Fig. 4M, N). Free GFP driven by the 35S promoter was used as the negative control (Fig. 4O).

MIO1/SLB1 positively regulates primary cell division

Loss-of-function mutation of MIO1/SLB1 resulted in reduced lateral organ size compared with the WT (Fig. 5B, C; Supplementary Fig. S5A, B).

To gain insight into the mechanism by which MIO1/SLB1 functions to regulate organ size, MIO1-overexpressing plants were generated by introducing the 35S::MIO1 construct into WT. Ten independent transgenic lines exhibited enlarged organs, especially for leaf (Fig. 5D; Supplementary Fig. S5C), floral organ (Fig. 5E), pod (Fig. 5F), and seed (Fig. 5G); these phenotypes were very similar to the reported M. truncatula bs1 mutant (Ge et al., 2016). MIO1/SLB1 expression increased by more than twelve-fold when compared with the WT in the MIO1-overexpressing transgenic lines (Fig. 5A; Supplementary Fig. S6).

Final organ size in plants is determined by a complex coordination of cell division and expansion (Gonzalez et al., 2012). Microscopic examination of mature leaf epidermal cells revealed that cell size was opposite to MIO1/SLB1 activity: cellular size increased in the mio1 mutant while it decreased in the MIO1-overexpressing plants in comparison with the WT (Fig. 5H-J; Supplementary Fig. S5D-F). Using the measurements of epidermal cell area (Fig. 5K) and leaflet area (Fig. 5L), we calculated the total number of epidermal cells per given leaflet (Fig. 5M). On average, this value for MIO1-overexpressing plants increased by more than two-fold compared with WT on both adaxial and abaxial sides (Fig. 5M). However, the number of cells in the mio1 mutant was only about half that of the WT (Fig. 5M). These results suggested that MIO1/SLB1 augments leaf size mainly by increasing the abundance of cells (and not their individual area).

The follow-up investigation of abaxial epidermal cell area (Fig. 5N), leaflet area (Fig. 5O), and the calculated total number of cells per leaflet (Fig. 5P) indicated that differences in leaf epidermal cell size among the MIO1-overexpressing, mio1 mutant, and WT plants had its origin around the P6 leaf development stage, and this became magnified in later stages. Differences in cell abundance began to emerge at P5, and reach a plateau at P6 (Fig. 5N, P). All of these findings implicated MIO1/SLB1 in influencing primary cell division during leaf growth and development. Although the cell size and number differed, the leaf epidermal cell pattern (Fig. 5H-J; Supplementary Figs S5D-F; S7) and stomatal index (Fig. 5Q) were similar among the MIO1-overexpressing, mio1 mutant, and WT plants, further pointing to the promotion of primary cell proliferation by MIO1/SLB1 activity. The time point at which leaflet length, width, and area peaked in size was likely
delayed for MIO1-overexpressing plants and occurred sooner for the mio1 mutant, when compared with the WT, but the duration of leaflet expansion showed no clear differences (Supplementary Fig. S8). This indicated that meristemoid cell division is not altered when MIO1/SLB1 is overexpressed or knocked out. Taken together, these results suggested that
MIO1/SLB1 positively regulates organ size mainly by promoting the process of primary cell proliferation during plant development.

**F-box protein MIO1/SLB1 regulates the stability of BS1 in vitro**

Previous reports demonstrated that SAP interacts with ASK1/2 and CUL1 (Cullin1), to form a conserved SCF E3 ubiquitin ligase, which targets the repressor PPD for degradation to thereby enlarge the size of organs (Wang et al., 2016). The loss-of-function of BS1, the ortholog of PPD in *M. truncatula*, also led to enlarged lateral organs similar to the *ppd* mutant (Ge et al., 2016). These clues suggest that the role of MIO1/SLB1 in regulating organ size may involve the conserved ubiquitin-proteasome at its core.

To uncover the mechanism underlying the control of organ size by MIO1/SLB1, we performed protein-protein interaction assays to analyse the interaction between MIO1/SLB1 and other potential components of the ubiquitin-proteasome pathway. MtASK, the ortholog of ASK1/2 in *M. truncatula*, was identified by phylogenetic analysis and its interaction with MIO1/SLB1 was tested using Y2H and BiFC assays. According to these results, MIO1/SLB1 physically interacted with MtASK in both yeast and tobacco leaf cells (Fig. 6A, B). Similarly, the interaction between MIO1/SLB1 and BS1 was also verified by the Y2H and BiFC assays (Fig. 6C, D). Further analysis of the Y2H results revealed that MIO1/SLB1 is capable of interacting with BS1 through its C-terminal WD40 repeat domain (Fig. 6C), which was confirmed by the results from the *in vitro* pull-down experiment (Fig. 6E). Hence, MIO1/SLB1 could interact not only with MtASK, which is predicted to
Fig. 5. MIO1/SLB1 positively regulates primary cell division during leaf development. (A) MIO1/SLB1 transcript abundance in the wild type (WT) and MIO1-overexpressing line, with MtActin used as an internal control. Values indicate the mean±SD (n=3); asterisks indicate significant differences with respect to the WT (**P<0.01; Student’s t-test). (B-D) The fifth compound leaf of five-week-old seedlings of WT (B), mio1 mutant (C), and MIO1-overexpressing line 35S::MIO1 (D). Insets are close-up views of stipules. Scale bars=1 cm for leaves, 0.5 cm for stipules. (E) Flower (open petal) of the WT (left), mio1 mutant (middle), and MIO1-overexpressing (right) plants. Scale bar=1 cm. (F, G) The pod (F) and seed (G) of the WT (upper) and MIO1-overexpressing (bottom) plants. Scale bars=1 cm (E), 0.5 cm (F). (H–J) The epidermal cell outlines for the abaxial epidermis of a mature leaflet of the WT

- **A**: MIO1/SLB1 expression
- **B**: WT
- **C**: mio1
- **D**: 35S::MIO1
- **E**: WT
- **F**: WT
- **G**: WT
- **H**: WT
- **I**: mio1
- **J**: 35S::MIO1
- **K**: Cell area (μm²)
- **L**: Leaflet area (mm²)
- **M**: Cell number
- **N**: Cell area (μm²)
- **O**: Leaflet area (mm²)
- **P**: Cell number
- **Q**: Stomatal Index
form an SCF E3 ubiquitin ligase, but also with BS1 in a way possibly related to protein degradation.

Given the similar phenotypes of MIO1-overexpressing and bs1 mutant plants (Ge et al., 2016), we suspected that MIO1/SLB1 might regulate the stability of BS1 protein to control organ size in Medicago truncatula. To test this, total proteins were extracted from WT, mio1 mutant, and MIO1-overexpressing plants, and mixed with equal amounts of E. coli-expressed His-BS1 fusions, followed by incubation at 4°C with gentle shaking. Samples were then removed at different time points for a gel quantification analysis. Evidently, the amount of His-BS1 decreased after 30 min of incubation compared with what it was at the start (time 0; time point=0 min) in both the WT and MIO1-overexpressing plants (Fig. 6F). However, the His-BS1 protein degraded more slowly when incubated with the proteasome inhibitor MG132 (Fig. 6F), indicating that the stability of BS1 is affected by the proteasome.

Additionally, the degradation of His-BS1 fusion protein appeared to be much slower in samples incubated with total proteins from the mio1 mutant but faster in samples incubated with proteins from the MIO1-overexpressing lines, when compared with the WT (Fig. 6F), indicating that the stability of BS1 protein is negatively regulated by MIO1/SLB1 in vitro. Taken together, these results suggested that MIO1/SLB1 might form an SCF E3 ubiquitin ligase complex with MtASK so as to modulate BS1 stability and control the lateral organ size in Medicago truncatula. MtKIX8 was identified during the Y2H library screening for potential interacting proteins of MIO1/SLB1, and was verified to physically interact with MIO1/SLB1 (Fig. 6G). Since MIO1/SLB1 remarkably influences cell proliferation and the number of cells, the expression of several representative cell-cycle genes were analysed in the mio1 mutant and WT plants. The expression of MtCYCD3;2, MtCDKB1;1, MtE2Fβ, and MtKRP3 were analysed by qRT–PCR in SAM (shoot apical meristem) and P1 (plastochron 1)-P8 of six-week-old mio1 mutant and WT plants (Supplementary Fig. S9). The expression of the predicted cell division activators MtCYCD3;2 (Fig. 6H; Supplementary Fig. S10A), MtCDKB1;1 (Fig. 6I; Supplementary Fig. S10B), and MtE2Fβ (Fig. 6J; Supplementary Fig. S10C) were all decreased in the mio1 mutant when compared with the WT, especially in the juvenile leaves. By contrast, the expression of the predicted cell division repressor MtKRP3 (Fig. 6K; Supplementary Fig. S10D) was increased in mio1 mutant at the P4 and P5 stages, where cell division activity is supposed to be high. We then carried out an in-situ hybridization assay to analyse the expression of MtH4 (Medicago HISTONE4) (Fig. 6L–O) and MtCYCD3;3 (Fig. 6P–S) at the early leaf development stages of WT and mio1 mutant plants. These results showed that both MtH4 and MtCYCD3;3 were expressed in the SAM and P1 to P4 of both the WT and mio1 mutant, but the mio1 mutant had much weaker signals. These findings suggested that MIO1/SLB1 could influence the expression of the core cell-cycle genes; hence, it was suspected to regulate cell proliferation and lateral organ size.

MIO1/SLB1 influences pulvinus development and leaf movement

Leaf movement is driven by a motor organ— the pulvinus — that is commonly observed in legume species (Darwin, 1880; Satter et al., 1990; Koller, 2011; Mancuso and Shabala, 2015). Besides its decreased organ size, the mio1 mutant also showed defective leaf movement when compared with the WT (Fig. 7A, B).

Generally, all the leaflets of WT engaged in reversible movement, that is, remaining in a horizontal (open) position during the day and vertical (closed) at night (Supplementary Fig. S11). In contrast to the WT, leaves of the mio1 mutant featured a suite of defects in terms of leaf closure (Supplementary Fig. S11C, D). Generally, leaf movement was absent in the first and second trifoliate leaves (L1, L2), after which the degree of leaflet rotation gradually increased in the third to sixth trifoliate leaves (L3–L6), followed by it progressively decreasing in the seventh to tenth trifoliate leaves until it was completely lost (L7–L10; Supplementary Fig. S11A, C, D). These defects in leaf movement were rescued when the coding sequence of MIO1/SLB1, which was driven by its native promoter, was expressed in the mio1 mutant (Fig. 7C; Supplementary Fig. S12).

To investigate the causes behind the defective leaf movement in the mio1 mutant, we analysed the structure of its pulvinus. The pulvini of mio1 mutants were shortened or

\[ \text{Fig. S10D} \]
even completely absent from the base of its leaflets, and this followed a developmental stage-dependent manner that matched the defects in leaf movement described above (Supplementary Fig. S13). The pulvinus was completely absent in the L1 and the L2 leaves, but progressively recovered from the L3 to L6, yet it gradually disappeared again from the L7 to L10 until it was lacking entirely (Supplementary Fig. S13). From the SEM observations, we...

Fig. 6. F-box protein MIO1/SLB1 physically interacts with, and regulates BS1 stability. (A) Yeast two-hybrid (Y2H) assay showing the interaction between MIO1/SLB1 and MtASK. -2, SD/-Leu/-Trp; -4, SD/-Ade/-His/-Leu/-Trp. (B) Bimolecular fluorescence complementation (BiFC) assay showing the interaction between MIO1/SLB1 and MtASK in the nuclei of tobacco (Nicotiana benthamiana) leaf epidermal cells. MIO1-nYFP and MtASK-cYFP were coexpressed in the leaves of tobacco; DAPI signals indicate the nuclei. (C) Y2H assay showing the interaction between MIO1/SLB1 and BS1. MIO1/SLB1 interacts with BS1 through its WD40 repeat domain. AD+BD was used as the control. -2, SD/-Leu/-Trp; -4, SD/-Ade/-His/-Leu/-Trp. (D) BiFC assay showing the interaction between MIO1/SLB1 and BS1 in the nuclei of tobacco leaf epidermal cells. MIO1-nYFP and BS1-cYFP are coexpressed in leaves of tobacco, DAPI signals indicate the nuclei. (E) N-terminal WD40 repeat domain of MIO1/SLB1 (MIO1 WD40) interacts with BS1 in vitro. His-BS1 was pulled down (PD) by GST-MIO1 WD40 immobilized on glutathione sepharose, and analysed by immunoblotting (IB) using an anti-His antibody. GST was used as a negative control. (F) MIO1/SLB1 regulates BS1 stability in vitro. The His-BS1 fusion protein was detected with the His antibody. MG132 was used to inhibit the proteasome activity. The total proteins extracted from plants were used as a loading control. (G) Yeast two-hybrid (Y2H) assay showing the interaction between MIO1/SLB1 and MtKIX. -2, SD/-Leu/-Trp; -4, SD/-Ade/-His/-Leu/-Trp. (H-K) Expression of MtCYCD3;3 (H), MtCDKB1;1 (I), MtE2Fb (J), and MtKRP3 (K) in the WT and mio1 mutant, for which MtActin served as the internal control. SAM, shoot apical meristem; P, plastochron. Values indicate the mean±SD (n=3); asterisks indicate significant differences from the WT (*P<0.05, **P<0.01; Student’s t-test). (L-U) RNA in situ hybridization of MtH4 (Medicago truncatula HISTONE4) and MtCYCD3;3. Longitudinal sections of shoot apices (SAM+P1-P3) and P4 of the wild type (WT) and mio1 mutant were hybridized with anti-sense probe of MtH4 (L to O) and MtCYCD3;3 (P to S). The shoot apices and P4 were collected from three-week-old WT and mio1 seedlings. P+number, plastochron; asterisks denote the shoot apical meristem. The MtH4 and MtCYCD3;3 sense probes were used as the control (T and U). Scale bar=50 μm.
found that the knitted wool-like structure of the pulvinus was partially or completely absent from the boundary region between the leaflet and petiole in the mio1 mutant in comparison with the WT (Fig. 7D-G; Supplementary Fig. S14). This abnormal pulvinus structure was restored to a WT-like phenotype in the complemented transgenic lines (Fig. 7H, I).

Moreover, the anatomical analysis uncovered a substantially decreased number of motor cells in the typically defective pulvinus of mio1 mutant when compared with those of the WT (Fig. 7J, K), but this was also fully rescued in the complemented plants (Fig. 7L). These results indicated that the defective pulvinus of mio1 mutant likely arose from reduced motor cell division and final cell counts. Auxin was able to specifically accumulate in the pulvinus of WT (Zhou et al., 2012; Supplementary Fig. S15A, B), but this signal disappeared in the mio1 mutant (Supplementary Fig. S15C, D). In conclusion, we have shown that MIO1/SLB1 is also necessary for robust pulvinus development and leaf movement in M. truncatula.

Discussion

MIO1/SLB1 positively regulates primary cell proliferation to control organ size

The final organ size of multicellular plants is determined by a complex coordination of cell division and expansion (Gonzalez et al., 2012). Our results demonstrate that MIO1/SLB1 acts as a positive regulator of organ size by promoting the number of cells in M. truncatula. The time-course analysis of epidermal cell counts in developing leaflets indicated that MIO1/SLB1 promoted cell proliferation during the early development stages (Fig. 5P); the stomatal index (a credible indicator of meristemoid cell division), epidermal cell pattern, and duration of leaf area expansion were not influenced by either loss-of-function or overexpression of MIO1/SLB1 when compared with the WT (Fig. 5H–J, Q; Supplementary Figs S5; S7; S8). These results suggest that MIO1/SLB1 functions as a facilitator of primary cell division, rather than meristemoid cell proliferation, to modulate organ size during development.

MIO1/SLB1 and its orthologs SAP (in Arabidopsis) and LL (in cucumber) perform a conserved function - to positively regulate organ size during plant development, but interestingly, their effects on cell proliferation and cell size seem to follow different pathways. While MIO1/SLB1 positively controls primary cell division, SAP mainly acts to promote meristemoid cell proliferation during development (Wang et al., 2016). This functional variation might be caused by the differences in function of their downstream regulators between the Fabaceae and Brassicaceae. Previous studies have confirmed that PPD (the substrate of SAP) and its ortholog BS1 in M. truncatula act as negative regulators of meristemoid proliferation and primary cell division, respectively (White, 2006; Ge et al., 2016).

The loss-of-function of MIO1/SLB1 in M. truncatula and LL in C. sativus led to an increased and decreased cell size, respectively, when compared with the WT (Yang et al., 2018). A probable explanation for this functional divergence in cell size is the different mutational patterns and compensatory effects of mio1 and ll mutants. The mio1 mutant arose from the Tnt1 retrotransposon insertion, which completely eliminated the MIO1/SLB1 transcript, so its cells may expand in an attempt to compensate for a drastic loss in cell division (Horiguchi and Tsukaya, 2011). Since the ll mutant arose from a single nucleotide substitution (Yang et al., 2018), LL might retain some partial functioning; it might remain below the threshold to confer full function, yet still above the threshold needed to resist compensation. Irrespective of compensation and species differences, both cell proliferation and cell expansion clearly contribute to determining organ size, and they account for the conserved function of MIO1/SLB1, SAP, and LL in their native plant species.

MIO1/SLB1 controls final organ size by regulating BS1 stability and expression of cell-cycle genes

Based on previous reports (Ge et al., 2016; Wang et al., 2016), we proposed that the F-box protein MIO1/SLB1 could form part of an SCF E3 ubiquitin ligase complex, to regulate organ size via targeting its potential substrate BS1 for ubiquitination and degradation in M. truncatula. Protein-protein interaction assays indicated that MIO1/SLB1 could directly interact with MtASK and BS1 (Fig. 6A–E). Our analysis also showed that the protein stability of BS1 could be modulated by MIO1/SLB1 with sensitivity to the inhibitor MG132 (Fig. 6F). These observations are consistent with BS1 being recruited by MIO1/SLB1 into the ubiquitin-proteasome pathway to control its stability.

Nevertheless, the His-BS1 fusion could still be degraded by proteins extracted from the mio1 mutant, albeit at a slower rate (Fig. 6F), which suggests that additional factors may co-exist with MIO1/SLB1 to regulate the abundance of BS1. It is thus conceivable that in M. truncatula, MIO1/SLB1 positively regulates organ size, in part, by forming the SCF E3 ubiquitin ligase to target the organ size repressor BS1 for degradation. Identifying the additional factors that co-regulate BS1 and elucidating their molecular mechanisms will enlighten our understanding of organ size regulation in legumes.

Cyclin-dependent kinases (CDKs) are the primary regulators of eukaryotic cell cycle progression, whose catalytic activity depends on the binding and activation of cyclins (CYCs; Joubès et al., 2000). For example, the plant-specific CDKs are necessary for proper cell cycle progression (Andersen et al., 2008; Nowack et al., 2012), and CYCD3s are vital regulators that control cell division and expansion by regulating the duration of mitotic phase and the mitosis-to-endocycle transition (Dewitte et al., 2007).

Kip-related proteins (KRPs), another kind of essential cell cycle regulator, which usually exhibit cyclin-dependent
kinase binding specificity, are the negative regulators of CDKs (Verkest et al., 2005; De Almeida Engler et al., 2009). Other work has revealed that the conserved transcription factors E2Fs play crucial roles in several pathways related to plant cell division and differentiation; for instance, E2Fb is an activator of cell cycle progression in Arabidopsis (Magyar et al., 2005; Sozzani et al., 2006). The expression of three genes encoding predicted positive regulators of the cell cycle in *M. truncatula*, namely *MtCYCD3;2, MtCDKB1;1*, and *MtE2Fb*, were down-regulated in juvenile leaves of *mio1* mutants (Fig. 6H-J; Supplementary Fig. S10A-C). In contrast, the expression of *MtKRP3*, the predicted cell cycle repressor, was up-regulated in *mio1* mutant (Fig. 6K; Supplementary Fig. S10D). The in-situ hybridization assay further confirmed the disrupted expression profiles of cell cycle genes in the *mio1* mutant (Fig. 6L–U). These results
suggest that MIO1/SLB1 could regulate the expression of cell cycle genes to manipulate cell proliferation and expansion in *M. truncatula*. The variation in expression profiles of cell cycle genes in the *mio1* mutant is similar to those resulting from the breakdown of LL, the ortholog of *MIO1/SLB1* in cucumber (Yang et al., 2018). BS1 and its ortholog PPD also influence cell cycle gene expression in *M. truncatula* and *A. thaliana*, respectively (Gonzalez et al., 2015; Ge et al., 2016). So, collectively, these clues suggest that the MIO1/SLB1-related pathway that is relevant to organ size control through regulation of cell cycle gene expression during plant development, might be conserved.

*Medicago truncatula* KINASE-INDUCIBLE DOMAIN INTERACTING 8 (MtKIX8), the homolog of KIX8 in Arabidopsis, was identified during the Y2H library screening for potential interacting proteins of MIO1/SLB1, and then verified to physically interact with MIO1/SLB1 via the Y2H assay (Fig. 6G). In Arabidopsis, SAP interacts with PPD and KIXs to target the PPD-KIX complex for degradation (Wang et al., 2016; Li et al., 2018). Accordingly, it is likely that the putative MIO1/SLB1-BS1-MtKIX module is playing a vital role in plant organ size determination analogous to the SAP-PPD-KIX module. Yet interestingly, it is possible that the MIO1/SLB1-BS1-MtKIX module has disappeared from the Poaceae, based on our inspection of available genome sequences (Supplementary Fig. S2). Further investigation of this complex may advance our understanding of differences in molecular mechanisms of lateral organ expansion between eudicot and monocot plant species.

The organ size of legume crops and forage is one of the most important agronomic traits because it is closely related to final yield and quality. Those previously reported genes in *M. truncatula*, such as SGL1 (SINGLE LEAFLET1), PALM1, LLS1 (LATERAL LEAFLET SUPPRESSION1), and PINNA1 (PENTAFOLIATA1), all provide important clues as to how the total leaf area and biomass might be promoted through manipulating the number and size of leaflets (Wang et al., 2008; Chen et al., 2010; He et al., 2020; Zhao et al., 2020). From the present study, the predicted MIO1/SLB1-BS1 module would be a useful candidate for the genetic manipulation of crop yields, via increased seed size or total biomass.

**MIO1/SLB1 influences pulvinus development in *M. truncatula***

The reversible leaf movement of legumes is driven by the pulvinus, a specialized motor organ (Satter et al., 1990; Mancuso and Shabala 2015). An abnormal pulvinus initiation or development would directly influence leaf movement (Chen et al., 2012; Zhou et al., 2012; Gao et al., 2017). Loss-of-function of MIO1/SLB1 led to impaired leaf movement which resulted from the extremely shortened pulvinus in *M. truncatula* (Fig. 7B, F; Supplementary Fig. S13). However, this defect was fully rescued when the coding sequence (CDS) of *MIO1/SLB1* was transformed into the *mio1* mutant (Fig. 7C, H). These results provide evidence that MIO1/SLB1 function is necessary to ensure the pulvinus develops normally in *M. truncatula*. Although the molecular mechanism underpinning how MIO1/SLB1 regulates pulvinus development is still unclear, there are several possibilities based on the available clues. On the one hand, MIO1/SLB1 might directly or indirectly regulate certain pulvinus identity genes, such as ELP1 and GmILPA1 (Chen et al., 2012; Zhou et al., 2012; Gao et al., 2017). ELP1 is a plant-specific lateral organ boundaries domain (LBD) transcription factor that determines the pulvinus identity in legumes through its conserved function (Chen et al., 2012). GmILPA1 was found to ensure the normal progression of the motor cell cycle during pulvinus development in soybean (Gao et al., 2017). It would be worth analysing the genetic interaction between MIO1/SLB1 and ELP1, or the ortholog of GmILPA1, during pulvinus morphogenesis. On the other hand, our anatomical examinations showed that the shortened pulvini of *mio1* mutant mainly originate from a vastly decreased number of motor cells (Fig. 7K). It seems likely that MIO1/SLB1 regulates pulvinus development through modulating the cell division of the motor organ, similar to the regulation of organ size, by taking part in the genetic pathway of cell cycle control. Loss-of-function of MIO1/SLB1 led to defective leaf movement in a developmental stage-dependent manner (Supplementary Fig. S11); however, this differs from the completely lost pulvinus as found in the *elp1* mutant (Chen et al., 2012). This suggests that additional extant but unknown regulators ought to function synergistically with MIO1/SLB1 to regulate pulvinus development in *M. truncatula*. Another attractive scenario is that auxin specifically accumulated in the WT pulvinus but disappeared in the *mio1* mutant (Supplementary Fig. S15). Auxin is known to have significant roles in the activation of cell cycle processes (Perrot-Rechenmann, 2010). It would be very interesting to test how MIO1/SLB1 may regulate pulvinus development through auxin-related cell proliferation processes.

**Supplementary data**

The following supplementary data are available at JXB online.

Fig. S1. The expression of *MIO1/SLB1* in WT and *mio1* mutant.

Fig. S2. MIO1/SLB1 is the ortholog of LL and SAP.

Fig. S3. The distribution of F-box and WD40 repeat domain in MIO1/SLB1.

Fig. S4. The expression pattern of *MIO1/SLB1* in different organs of WT.

Fig. S5. Size changes of whole plants and epidermal cells in different backgrounds.

Fig. S6. The expression of *MIO1/SLB1* in 35S::MIO1 transgenic plants.
Fig. S7. Time-course analysis of the adaxial leaf epidermal cell pattern of WT and mio1 mutant.

Fig. S8. Quantitative analysis of leaflet growth during different stages.

Fig. S9. Different stages of leaves were used to monitor the expression of cell-cycle genes.

Fig. S10. The expression of cell-cycle genes in WT and mio1 mutant.

Fig. S11. Loss-of-function of MIO1/SLB1 results in the defect of leaf movement at different stages.

Fig. S12. Complementation of leaf movement phenotype in mio1 mutant.

Fig. S13. Loss-of-function of MIO1/SLB1 results in a series of defective pulvini.

Fig. S14. Morphological changes of epidermal cells of pulvini in mio1 mutant.

Fig. S15. The auxin reporter DR5rev::Green Fluorescent Protein (GFP) shows the distribution of auxin in the pulvini of WT and mio1 mutant.

Table S1. Sequence information of primers used in this study.

Table S2. Detailed information of different mio1 mutant alleles.

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Author contributions

ZSL, YTQ and CJH conceived and designed the experiments; ZSL, YTQ, MYW, LY, WRR, and FYGW performed the experiments; ZSL, YTQ, ZBL, HLL, LYH, BQZ, WDE, WQ, YYF, and CJH analysed the data and wrote the paper. All authors reviewed and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests in relation to this study.

Data availability

No new sequence data were published in the present paper. All sequence data included in our manuscript can be obtained from the publicly available genome of M. truncatula (Mt4.0v1) (http://bioinfo3.noble.org/doblast/) under the following accession numbers: MIO1 (Medr5g097060), BS1 (Medr1g102900), MtASK (Medr5g022710), MtKIX8 (Medr4g114900).

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