Genetic control of compound leaf development in the mungbean (Vigna radiata L.)

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Abstract

Many studies suggest that there are distinct regulatory processes controlling compound leaf development in different clades of legumes. Loss of function of the LEAFY (LFY) orthologs results in a reduction of leaf complexity to different degrees in inverted repeat-lacking clade (IRLC) and non-IRLC species. To further understand the role of LFY orthologs and the molecular mechanism in compound leaf development in non-IRLC plants, we studied leaf development in *unifoliate leaf (un)* mutant, a classical mutant of mungbean (Vigna radiata L.), which showed a complete conversion of compound leaves into simple leaves. Our analysis revealed that UN encoded the mungbean LFY ortholog (VrLFY) and played a significant role in leaf development. In situ RNA hybridization results showed that STM-like KNOXI genes were expressed in compound leaf primordia in mungbean. Furthermore, increased leaflet number in heptafoliate leaflets1 (hel1) mutants was demonstrated to depend on the function of VrLFY and KNOXI genes in mungbean. Our results suggested that HEL1 is a key factor coordinating distinct processes in the control of compound leaf development in mungbean and its related non-IRLC legumes.

Introduction

Plant leaves are the primary photosynthetic organs that are initiated on the flanks of the shoot apical meristem (SAM). The class I KNOTTED1-like homeobox (KNOXI) genes are involved in the maintenance of the meristem activity of SAM, while the initiation of leaves requires downregulation of KNOXI genes at the incipient site1–3. In simple-leafed species such as *Arabidopsis thaliana*, downregulation of KNOXI genes in leaf primordia is permanent, whereas in most compound-leafed eudicot species, including the tomato (*Solanum lycopersicum*) and *Cardamine hirsuta*, KNOXI genes are reactivated in leaf primordia after initiation of leaf development4–6. In *C. hirsuta*, the leaflet number is reduced in mutants of the KNOXI gene SHOOTMERISTEMLESS (ChSTM) or BREVIPEDICELLUS (ChBP)5–8. In *S. lycopersicum*, ectopic expression of the KNOXI genes Tomato KNOTTED1 (Tkn1) or Tkn2 (orthologs of STM and BP in tomato, respectively) in transgenic lines, or upregulated expression of Tkn1 or Tkn2 in related mutants, results in ramification for compound leaves suggesting that regulatory processes mediated by KNOXI genes, especially STM/BP-like KNOXI genes, play pivotal roles in compound leaf development5,9,10.

However, in the inverted repeat-lacking clade (IRLC) of legumes, which includes *Pisum sativum* and *Medicago truncatula*, the expression of STM/BP-like KNOXI genes is excluded from leaf primordia11–13. Genetic analysis shows that single mutants, double mutants and triple mutants of 3 STM/BP-like KNOXI genes, namely, MtKNOX1, MtKNOX2, and MtKNOX6, in *M. truncatula* do not show obvious defects in compound leaves13. Thus,
STM/BP-like KNOXI genes may not be involved in compound leaf development in IRLC legume plants\(^{11-13}\). Instead, another type of transcription factor, UNIFOLIATA (UNI) in \textit{P. sativum} and SINGLE LEAFLET1 (SGL1) in \textit{M. truncatula}, orthologs of LEAFY (LFY) from \textit{Arabidopsis}, functions in controlling compound leaf development\(^{14-16}\). The \textit{uni} mutants in pea and \textit{sgl1} mutants in \textit{M. truncatula} exhibit single leaflet phenotypes, and inflorescence and floral defects\(^{15,16}\). Hence, the LFY orthologs appear to play a significant role in compound leaf development in IRLC legumes. Furthermore, it has been shown that the UNI cofactor UNUSUAL FLORAL ORGANS (UFO) in pea, and PALM1, an upstream transcription factor of SGL1 in \textit{M. truncatula}, are involved in the control of leaf complexity\(^{17,18}\). Recent studies show that the adaxial–abaxial regulators PHAN-TASTICA (PHAN), ARGONAUTE7 (AGO7), and AUXIN RESPONSIVE FACTOR 3 (ARF3) regulate the expression level of PALM1 and therefore control compound leaf development in \textit{M. truncatula}\(^{19-21}\).

The function of the LFY orthologs during compound leaf development has also been investigated in non-IRLC legumes, including soybean and \textit{L. japonicus} in which KNOXI proteins are expressed in leaves, and are likely associated with compound leaf development\(^{12,22}\). In \textit{L. japonicus}, a mutant of the LFY ortholog \textit{Proliferating Floral Meristem} (PFM) exhibits one or two reduced basal leaflets\(^{12}\). In soybean LFY-RNAi-silenced lines, only the leaflet number of the compound leaves produced at the second node is reduced\(^{12}\). This would indicate that there is a minor role in compound leaf development for LFY orthologs in non-IRLC legume species\(^{12,22,23}\).

In this study, we described the compound leaf developmental processes in a non-IRLC legume species, mungbean (\textit{Vigna radiata} L.), a fast-growing (60–90 days) warm-season grain legume, and characterized the \textit{unifoliate leaf} (\textit{uni}) mutants that showed a complete conversion of compound leaves into simple leaves. Four alleles of \textit{uni} carried mutations in the LFY ortholog, indicating that the LFY ortholog in mungbean played a significant, rather than a minor role in compound leaf development. Phylogenetic analysis of the KNOX gene family in legumes was conducted, and the expression of four \textit{STM/BP-like KNOXI} genes was characterized in mungbean using in situ RNA hybridization. Furthermore, genetic interaction and gene expression analysis showed that increased leaflet number in \textit{heptafoliate leaflets}1 (\textit{hel1}) mutants involved regulatory processes mediated by \textit{VrLFY} and \textit{STM/BP-like KNOXI} genes in mungbean. This study showed that the LFY ortholog might play a more significant role in the control of compound leaf development earlier than the time estimated by Champagne et al.\(^{12}\).

**Materials and methods**

**Plant material and growth conditions**

All the mutants were isolated from the M\(_2\) population of a mutagenized mungbean cultivar, Sulu, generated in Nanjing, China. The gamma irradiator was calibrated to irradiate the seed lots with 400 Gy of gamma rays. The \textit{M\(_1\)} seeds were sown in the field, and the \textit{M\(_2\)} seeds were individually harvested from the population. Approximately 36 seeds of each \textit{M\(_2\)} line were planted in individual rows in the field, with a distance of 0.3 m between rows. The mutant plants were then individually harvested and sown for further observation in the greenhouse at 26–30 °C with a 16-h light/8-h dark photoperiod at 200 μmol m\(^{-2}\) s\(^{-1}\). The allelic nature of genes was confirmed by crosses among \textit{uni1-1, uni1-2, uni1-3}, and \textit{uni1-4}, using heterozygote parents because the mutants were sterile (the mutant plants were found in \textit{F\(_1\)} plants of the crosses). The \textit{L. japonicus} ecotype Gifu B-129 was grown at 20–22 °C with a 16-h light/8-h dark photoperiod at 150 μmol m\(^{-2}\) s\(^{-1}\) in the greenhouse.

**Scanning electron microscopy**

Mungbean shoot apices at different developmental stages were fixed in FAA solution containing 3.7% (v/v) formaldehyde, 50% (v/v) ethanol, and 5% (v/v) acetic acid. Before vacuum freeze drying, fixed samples were dehydrated in an ethanol/tert-butanol series. The materials were transferred to a vacuum freeze dryer overnight. The preparation of shoot apices for scanning electron microscopy (SEM) analysis was as described by Chen et al.\(^{24}\). Samples were examined in a JEOL JSM-6360LV (JEOL) SEM at 10–15 kV of acceleration voltage.

**Molecular cloning of the full-length LFY gene (\textit{VrLFY}) from mungbean**

To clone the full-length mungbean LFY gene, we first searched the genome sequence database of mungbean (http://plantgenomics.snu.ac.kr) using sequences for \textit{UNI} and SGL1. Finally, sequence alignment with \textit{UNI/SGL1/LFY/FLO} coding sequences allowed the open reading frame of \textit{VrLFY} to be defined. Polymerase chain reaction (PCR) was carried out using the primers in Supplementary Table 3. PCR products were cloned into the pGEM-T easy vector (Promega), and inserts were characterized by nucleotide sequencing.

**In situ hybridization**

For in situ probes, gene-specific regions of \textit{VrLFY}, \textit{VrKNOXI}, and \textit{LjKNOXI} genes were generated by PCR with primer sets (see Supplemental Table 4) and cloned into a pGEM-T vector (Promega, A1360). In situ probes were synthesized from these clones by in vitro transcription using the Digoxigenin RNA Labeling Kit (Roche) from either the T7 or SP6 promoter flanking the
insert, generating either sense or anti-sense probes. Mungbean shoot apices were fixed overnight in 4 \% (wt/vol) paraformaldehyde, pH 7.0, and then embedded in Paraplast (Sigma). RNase-free slices of the shoot apices were hybridized to digoxigenin-labeled probes and used for subsequent immunological detection as previously described\textsuperscript{25}.

**Expression analysis by quantitative reverse transcription PCR**

Shoot apices from 2-week-old mutant and wild-type plants were collected in RNase-free tubes and stored in a −80°C freezer. Samples were taken in triplicate as biological replicates. Total RNA was extracted using the Plant RNA Kit (Omega) following the manufacturer’s instructions. Samples were then treated with RNase-free DNase I (Promega) for 30 min.

For quantitative reverse transcription PCR (qRT-PCR), first-strand cDNA was synthesized from total RNA using the First Strand cDNA Synthesis Kit (Fermentas). Real-time RT-PCR analysis was performed as three technical replicates in 384-well plates using SYBR\textsuperscript{\textregistered} Premix ExTaq\textsuperscript{TM} (Takara), on an ABI StepOnePlus machine, according to the manufacturer’s protocol (Applied Biosystems). The relative expression level of genes was determined by the $2^{-\Delta\Delta CT}$ method. Amplification of VrTUB (Vra-dio05g13910), a constitutively expressed gene, was used as an internal control to normalize all data\textsuperscript{26}. Shoot apices from a single genotype were represented by nine samples; independent total RNA isolations were generated from the three biological replicates, and three technical qRT-PCR replicates were performed on each of the total RNA preparations. The primers used for qRT-PCR are given in Supplementary Table 5.

**Transcript profiling by deep-sequencing**

For Illumina sequencing, mRNA was purified from shoot apices of 2-week-old seedlings of un1-1 mutant and wild-type plants and then fragmented into small pieces. Random hexamer primers and reverse transcriptase (Invitrogen) were used to carry out Random hexamer primers and reverse transcriptase wild-type plants and then fragmented into small pieces.

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**Phylogenetic analysis**

The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0) by the neighbor-joining method (JFF Matrix model) with 1000 bootstrap replications.

**Data availability**

Sequence data from this article can be found in the GenBank data libraries under the following accession numbers: XP_014491863.1 (VrLFY); XP_017441945.1 (VrLFY); XP_007137848.1 (VrLFY); XP_003526918.2 (VrLFY1); XP_014630701.1 (VrLFY2); AAX13294.1 (PFM); AAC49782.1 (UNI); XP_003602745.1 (SGL1); XP_002284664.1 (VFL); AF197934.1 (FALSIFLORA); AAM27941.1 (LEAFY); AAA62574.1 (FLORICAULA); AQQ16908.1 (ChLFY); XP_015635355.1 (RFL); and O04407.1 (NEEDLY).

**Results**

**Compound leaf development in mungbean**

Similar to *M. truncatula*, *L. japonicus*, and other compound leaf-producing species, leaf development in mungbean was heteroblastic. The first pair of juvenile leaves with simple leaf morphologies emerged in opposite phyllotaxy on the first node of a developing mungbean plant and was succeeded by adult trifoliate leaves in alternate phyllotaxy (Fig. 1a–c). The wild-type trifoliate leaves of mungbean consisted of a pair of stipules, a petiole, 2 lateral leaflets, a rachis, and a single terminal leaflet (Fig. 1c). To facilitate the characterization of leaf mutants in mungbean, we investigated leaf developmental processes by scanning electron microscopy (SEM). The morphological changes during compound leaf development in mungbean can be divided into seven distinct stages. At Stage 0 (S0), cells along the periphery of SAM were recruited as founder cells and became an incipient leaf primordium (Fig. 1d). At S1, a common leaf primordium formed as a strip of cells grew out along the periphery of SAM (Fig. 1d). At the subsequent S2, a pair of stipule primordia emerged at the proximal end of the common leaf primordium (Fig. 1e), enlarged, and then separated away from the common leaf primordium so that boundaries were established between the two stipules and the common leaf primordium at S3 (Fig. 1f). At S4, a pair of lateral leaflet primordia emerged between the stipule and the common leaf primordium; and then the common leaf primordium differentiated into a terminal leaflet primordium (Fig. 1g).
Subsequently, at S5, the lateral leaflet primordia were separated away from a terminal leaflet primordium so that a boundary was established, and trichomes initiated from the abaxial surface of the terminal leaflet primordium (Fig. 1h). Following S5, the lateral leaflets and terminal leaflet primordium became folded (Fig. 1i), and the region between the stipule and lateral leaflet primordia expanded to become a petiole as a result of cell division and cell expansion at the S6 stage.

**Isolation and characterization of un mutants in mungbean**

Four similar leaf mutants (Supplementary Table 1), which mimicked the phenotype of the classical mutant un, were isolated from a mutant population of mungbean generated by gamma irradiation. Genetic analyses demonstrated that they were allelic (Materials and Methods section). In these mutants, all adult leaves consisted of a short petiole bearing a single terminal leaflet (Fig. 2a, b). Compared with the terminal leaflet of the wild type, the...
single terminal leaflet of the mungbean un mutant was larger (Fig. 2b). We found that the heteroblastic progression was also delayed in un mutants such that two simple leaves were produced at the first and second nodes in opposite phyllotaxis. Flowers that developed in un mutants were abnormal and infertile. un mutants produced sepal-like proliferating structures that lacked petals and stamens, and the number of whorls of organs within the flowers was increased (Fig. 2c). Because of their infertility, un mutants were maintained as heterozygotes. Progeny from self-pollination of heterozygous lines segregated in a 3:1 ratio (33 wild-type plants and 9 mutant plants, $\chi^2 = 0.28 < \chi^2_{0.05} = 5.99$), suggesting that the mutant phenotype was controlled by a single-recessive gene.

To characterize leaf development defects in mungbean un mutants, SEM analysis of leaf development was conducted. This analysis indicated that in un mutants, leaf development was initially normal until S4, at which point, the pair of lateral leaflet primordia failed to initiate between the stipule and the common leaf primordium (Fig. 2d, e). All four alleles of the mungbean un mutants exhibited this identical defect. The defect in the initiation of the lateral leaflet primordia was persistent throughout subsequent developmental stages, resulting in the formation of simple adult leaves in the un mutants of mungbean (Fig. 2f, g).

**Molecular cloning of VrLFY in mungbean**

The floral homeotic defects and single-leaf phenotype of the un mutants resembled that of the uni mutant in pea and the sgl1 mutant in M. truncatula. The full-length DNA sequence of the LFY ortholog (VrLFY) in mungbean was obtained from the mungbean genomic database; the genomic sequence of VrLFY was 2155 bp in length. Alignment of the genomic sequence of VrLFY with its cDNA sequence showed the existence of two introns (Supplementary Figure 1a). PCR amplification of mungbean genomic LFY from the un mutants and from the wild-type plants indicated that three un alleles (un1-1, un1-2, and un1-3) carried deletions (Fig. 3a). Nucleotide sequencing showed that another allele, the un1-4 mutant, had only a single base-pair substitution from the wild type gene (GAA to GGA, Supplementary Figure 1a). This resulted in an amino acid change (E112G, where the acidic amino acid Glu was replaced by a neutral amino acid Gly) in the N terminal domain of the protein (Supplementary Figure 1b).

Segregation analysis of an F2 population of the un1-1 allele indicated that 50 out of a total of 208 individuals were homozygous for the deletion and exhibited both simple leaf and floral homeotic defects, suggesting that the deletion in the corresponding VrLFY gene cosegregated with the mutant phenotype. Thus, the locus of
un in mungbean was allelic to VrLFY, which encoded a putative plant-specific transcription factor closely related to UNI in pea and SGL1 in *M. truncatula* (Fig. 3b). In situ RNA hybridization data revealed that the VrLFY gene was expressed in the SAM and in the emerging leaf primordia (Fig. 3c) and showed relatively high expression in the distal portion of leaf primordia (Fig. 3d).

**Characterization and expression analysis of STM/BP-like KNOXI genes in mungbean**

To characterize the KNOXI genes in mungbean, we conducted a BLAST search of its genome (http://plantgenomics.snu.ac.kr), verifying candidate genes with the public transcriptome dataset30. Sixteen KNOX proteins were identified from mungbean and these were divided into three classes (class I, class II, and class M) based on phylogenetic analysis (Supplementary Figure 2)30. Nine proteins were classified as STM-like KNOXI proteins (Vradi07g26830, Vradi10g07810 and Vradi06g03570), BP-like KNOXI protein (Vradi06g14320) and KNAT2/6-like KNOXI proteins (Vradi08g11380, Vradi03g07470, Vradi05g04350, Vradi11g09640, and Vradi0322S00070). Five KNOXII proteins were classified as KNAT3/4/5-like proteins (Vradi05g1039, Vradi05g03240, and Vradi07g21010) and KNAT7-like proteins (Vradi11g02470 and Vradi07g13210). In addition, there were two members of class M KNOX proteins found in our mungbean sequence search (Vradi01g05360 and Vradi11g11780).

It has been reported that STM/BP-like KNOXI genes in tomato and *C. hirsuta* are expressed in the compound leaf and are involved in the control of lateral leaflet development4–6. However, STM/BP-like KNOXI genes are not associated with compound leaf development in *M. truncatula* and pea11–13. Previously, accumulation of KNOXI proteins in compound leaf primordia of non-IRLC legumes was detected by polyclonal KNOXI-specific antibodies12. To compare the expression patterns of STM/BP-like KNOXI genes in mungbean with those of IRLC-legume and model plant species, in situ RNA hybridization of four STM/BP-like KNOXI genes (*Vradi07g26830*, Vradi10g07810, Vradi06g03570, and Vradi06g14320) was carried out on sections of apices from 2-week-old mungbean seedlings (Fig. 4). The results showed that there were different expression patterns among the four STM/BP-like genes in mungbean (Fig. 4a–d). The expression of the two STM-like genes *Vradi10g07810* and *Vradi06g03570* was strongly detected in the shoot apical
meristem, and transcripts were also observed in the leaf primordia (Fig. 4b, c). However, expression of the STM-like gene *Vradi07g26830* and the BP-like gene *Vradi06g14320* was detected in the SAM but not in the leaf primordia (Fig. 4a, d).

Therefore, our data showed that the expression patterns of 2 STM-like KNOXI genes from mungbean differed from pea and *M. truncatula*, as in those species, no STM-like genes have been shown to be expressed in any stage of the compound leaf primordia 

Moreover, BP-like genes were not expressed in the compound leaf primordia of mungbean, *L. japonicus*, pea and *M. truncatula*, which is a different situation from the expression of these genes in *C. hirsuta* and tomato.

**Transcript profiling of VrLFY downstream targets**

To address the molecular function of *VrLFY* during leaf development, the transcriptome of shoot apices of 2-week-old seedlings from *un1-1* mutant and wild-type plants was studied using RNA-Seq. A total of 538 differentially expressed genes (300 downregulated and 238 upregulated) were identified between the mutant and wild-type plants (Supplementary Table 2). The results revealed a significant representation of genes associated with circadian rhythm and plant hormone signal transduction (Supplementary Table 2). In the *un1* mutants, the genes encoding proteins with high similarity to GIGANTEA 3 (GI3), GIGANTEA-like, Phytochrome A (PHYA), TIME OF CAB EXPRESSION 1 (TOC1), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), EARLY FLOWERING 3 (ELF3), and Adagio protein 3 (ADO3) were significantly downregulated, while those with high similarity to LATE ELONGATED HYPOCOTYL (LHY) were significantly upregulated. It has been reported that these are key factors in the regulation of the circadian clock and regulate important developmental transitions such as flowering, which was consistent with the involvement of LFY and its orthologs in controlling flowering time and phase transition.

Previous studies have reported that plant hormones, including auxin and gibberellins (GA), play critical roles in leaflet initiation and compound leaf development. In our expression studies, auxin, GA, ethylene and cytokinin-related genes were significantly differentially expressed in mutants compared to wild-type plants (Supplementary Table 2). In addition, a number of receptor-like protein kinases were differentially expressed, which might imply that several signaling cascades involved in cell proliferation and differentiation play a significant role in the control of compound leaf development in mungbean.

The transcripts of the KNOX family genes showed no obvious differential expression (data not shown), with the exception that one of the class M KNOX genes, *Vradi11g11780*, was decreased by approximately eightfold in vegetative shoot apices of the *un1*-1 mutant compared with the wild-type plant (Supplementary Table 2), and this was confirmed by qRT-PCR (data not shown). It has been reported that increasing the expression of class M KNOX genes in *Arabidopsis* and tomato results in serrated leaves and a larger number of leaflets, respectively.

**Genetic interactions affecting compound leaf development in mungbean mutants**

Other mutants that showed an increase in the number of leaflets were identified, including heptafoliate
leaflets1 (hel1) and small-pentafoliate leaflets1 (smp1), with 2 and 3 alleles, respectively (Fig. 5a–c; Supplementary Table 1). The juvenile leaves of the hel1 mutant showed an extreme dissection and leaflet-like structures, sometimes associated with stipules, which developed in the proximal part of the blade (Supplementary Figure 3). The juvenile leaves of the smp1 mutant were normal, but the adult leaves exhibited five leaflets of small size (Fig. 5c). We made crosses between hel1 and smp1 to examine genetic interactions. In the resulting F2 population, there were four classes of leaflet size and number: trifoliate, heptafoliate, small-heptafoliate, and small-pentafoliate. The numbers of plants in the different leaflet classes approximated a 9:3:3:1 ratio of the respective phenotypes (data not shown), suggesting that there were 2 unlinked genes controlling the multiple leaflet trait in mungbean.

Further genetic analysis showed that the small-heptafoliate plants were hel1 smp1 double mutants (Fig. 5d), indicating that there was no additive phenotype of the double mutation in terms of leaflet number and that HEL1 interacted genetically with SMP1 in the control of leaflet number in mungbean compound leaf development.

To genetically test the involvement of un in the proliferation of lateral leaflet primordia in the hel1 mutant, we generated hel1-1 un1-1 double mutants. Juvenile plants of the double mutant were similar to that of a hel1 single mutant. However, all adult leaves in the hel1-1 un1-1 double mutants consisted of a short petiole bearing two lateral leaflets and one terminal leaflet (Fig. 5f), indicating the requirement for the VrLFY gene in the proliferation of lateral leaflet primordia in the hel1 mutant. In addition to the alterations in leaflet number, the hel1 and un mutants also exhibited alterations in the proximal-distal axis of compound leaves. Compared with wild-type leaves, the petiole length of mature leaves in the hel1 mutant was increased by approximately 10%, while the mature leaves of the un mutant exhibited short petioles (Fig. 5b, e). Interestingly, the petiole length of mature leaves of hel1-1 un1-1 double mutants was short (Fig. 5f), resembling those of the un single mutant, suggesting that un is genetically epistatic to hel1 in leaf petiole development.

HEL1 regulates the expression of VrLFY and STM/BP-like KNOXI genes

The genetic analysis described above indicated that HEL1 controls trifoliate development via 2 distinct
pathways, either dependent or independent of LFY. We first examined the expression of VrLFY and its putative upstream transcription factor PALM1 (VrPALM1) and cofactor UFO (VrUFO) in the wild type and hel1 mutants. qRT-PCR data revealed that in vegetative shoot apices of the hel1 mutant compared with wild-type plants, VrLFY and VrUFO transcript levels were increased by threefold and fourfold, respectively (Fig. 6a). However, VrPALM1 transcript levels showed no obvious change in the hel1 mutant compared with wild-type plants. Because some KNOXI genes expressed in the compound leaf were likely associated with compound leaf development in mungbean, we further examined the expression of KNOXI genes in the hel1 mutant and wild-type plants. The results showed that the expression of the 4 STM/BP-like KNOXI genes was significantly upregulated (Fig. 6b), while other KNOXI genes were not upregulated (data not shown). Interestingly, a KNOXM gene, Vradi11g11780 (Supplementary Figure 2), was also upregulated by fourfold in the hel1 mutant (Fig. 6a). Taken together, these data suggest that HEL1 regulates the expression of LFY and KNOXI genes to determine the leaflet number in mungbean.

Discussion

The role of the LFY orthologs in compound leaf development in legumes

In most compound-leaved species, activation of KNOXI gene expression in the leaf primordia is correlated with the development of compound leaves. However, in IRLC legume species with compound leaves such as pea and M. truncatula, the STM/BP-like KNOXI genes are excluded from the leaf primordia, and their expression is not correlated with compound leaf development. In these plants, the LFY orthologs appear to function in place of the KNOXI genes in controlling compound leaf development. The pea uni mutants and M. truncatula sgl1 mutants exhibit compound leaf defects, with all adult leaves changed to simple leaves, indicating that the LFY orthologs play a significant role in compound leaf development in IRLC legumes. The available information on the role of LFY orthologs in compound leaf development in non-IRLC legumes comes from phenotypic analysis of L. japonicus pfm mutants and soybean LFY transgenic lines. In the pfm mutants, compound leaves lack 1 or 2 basal leaflets. In transgenic soybean lines in which the endogenous LFY genes are downregulated, only the leaflet number of the compound leaves produced at the second node is reduced. In tomato, the mutant of the LFY ortholog, fa, has a reduced number of small leaflets present on the compound leaf. Recently, it has been reported that the lfy mutant in C. hirsuta shows a lower number of leaflets than the wild type. Therefore, it seems that the single leaflet phenotype caused by mutations of the LFY orthologs is only exhibited in IRLC legumes. It is thought that the LFY orthologs acquired a more significant role no earlier than the divergence of the Hologalegina clade from the other legumes.
However, our results showed that the *un* mutants in mungbean, with a complete conversion of compound leaves into simple leaves, were loss-of-function mutations of *VrLFY*. This finding indicated that *VrLFY* could play a significant rather than a minor role in compound leaf development in mungbean, a member of the non-IRLC legumes (Fig. 2a–c). Interestingly, mutants exhibiting simple leaves and malformed flowers have also been reported in other non-IRLC legumes, including adzuki bean and cowpea. While unproven, one could speculate that some of these mutant phenotypes in adzuki bean and cowpea, with similarities to the *un* mutants in mungbean, could also be caused by mutations of *LFY* orthologs. If so, *LFY* orthologs could play a significant role in compound leaf development in other non-IRLC legumes, not just in mungbean. In addition, the question of when in evolution *LFY* orthologs acquired a significant role in compound leaf development might need to be re-addressed. Further investigation of the function of *LFY* orthologs in leaf development of different clades of non-IRLC legumes would be helpful in answering this question.

**Expression pattern of STM/BP-like KNOXI genes in legumes**

Phylogenetic analysis showed that there were duplications of *KNOXI* genes in legumes (Supplementary Figure 2). There was 1 *STM* gene and 1 *BP* gene in *Arabidopsis*, tomato and *C. hirsuta*. However, in IRLC legumes such as *M. truncatula* and pea, there were 2 *STM*-like genes and 1 *BP*-like *KNOXI* gene. In non-IRLC legumes such as mungbean and *L. japonicus*, there were 3 *STM*-like genes and 1 *BP*-like *KNOXI* gene. *In situ* RNA hybridization showed that the 2 *STM*-like *KNOXI* genes in mungbean were expressed at the compound leaf primordia (Fig. 4), which was different from that of pea and *M. truncatula* in which none of the *STM*-like genes were expressed in any stage of the compound leaf primordia. *BP*-like genes were not expressed in the compound leaf primordia of mungbean (Fig. 4) and *L. japonicus* or in pea and *M. truncatula*. This result was in contrast to findings of *BP* orthologs from *C. hirsuta* and tomato, which were expressed in the compound leaf primordia. It has been shown that differences in expression patterns between *BP* from *Arabidopsis* and *ChBP* from *C. hirsuta* are attributable to their cis-regulatory regions. When *KNOXI* genes are overexpressed in *M. truncatula* and *Alfalfa*, there is an increase in leaflet number. The loss of the role for *KNOXI* genes in compound leaf development of IRLC legumes also likely occurred due to a loss of expression. However, this loss of expression of the *STM*-like and *BP*-like genes in compound leaf primordia in IRLC legumes could have occurred at different times in evolution because *BP*-like genes were also not expressed in compound leaf primordia in non-IRLC legumes, such as mungbean and *L. japonicus*.

**VrLFY could interact with KNOXI in mungbean to regulate compound leaf development**

In simple-leafed species, *LFY* orthologs play a key role in phase transition and floral development. Many downstream targets and DNA binding motifs of *LFY* have been identified at the genomic level in the model plant *Arabidopsis*. Despite its key role in compound leaf development in species such as pea and *M. truncatula*, how the *LFY* orthologs regulate downstream genes to affect lateral organ development, especially that of compound leaf development, remains elusive. In this study, transcriptomic analysis uncovered a total of 538 differentially expressed genes between mutants and the wild type. Several types of key factors, such as *CCA1* and *ELF3*, involved in flowering time and phase transition were among the significantly differentially expressed genes (Supplementary Table 2), which was consistent with the conserved function of *LFY* orthologs in plants. *LFY* has been reported to regulate the expression of some *KNOXI* genes such as *BP* and *KNAT2* in *Arabidopsis* during pedicel and flower development. However, the transcription levels of the *KNOXI* genes showed no obvious change in the *un* mutant compared with the wild-type plant (data not shown), suggesting that *VrLFY* does not regulate the expression of *KNOXI* genes at the transcriptional level in mungbean. Nevertheless, 1 class M *KNOX* gene, *Vradi11g11780*, with high similarity to *PETROSELIUM* (PTS) in tomato and *FUSED COMPOUND LEAF* (*FCL2*) in *M. truncatula*, was downregulated 3.5-fold in the *un*-1 mutant. It has been shown that the class M *KNOX* proteins in *Arabidopsis* and tomato could form heterodimers with *BEL1*-like homeodomain (BEL) proteins and interfere with the regulatory networks of *KNOXI-BELL* complexes during leaf development. Transgenic *Arabidopsis* lines overexpressing *KNATM-B* exhibit serrated leaves, and a mutant with upregulated expression of the *PTS* gene in tomato exhibits a proliferation of compound leaves. In *M. truncatula*, the class M *KNOX* gene *FCL1* has been shown to control boundary establishment and petiole length of compound leaves and is required for the development of extra leaflet primordia in a *palm1* mutant. Therefore, our results indicated that *VrLFY* might modulate *KNOXI* regulatory networks by regulating the expression of a class M *KNOX* gene (*Vradi11g11780*) to control compound leaf development. Mutant databases for model legume plants such as *M. truncatula* and *L. japonicus* and legume crops such as soybean are available. It would be worth identifying mutants of *Vradi11g11780/FCL2* orthologs and dissecting their roles in compound leaf development in
different legumes. Furthermore, it will also be necessary to identify mutations of the STM/BP-like KNOXI genes in mungbean and other non-IRLC legumes to investigate gene and protein interactions between KNOXI genes and the LFY orthologs during compound leaf development in non-IRLC plants.

**HEL1 could orchestrate VrLFY and KNOXI to control compound leaf development in mungbean**

Our results suggested that there were 2 distinct regulatory processes mediated by the LFY ortholog and KNOXI proteins during compound leaf development in mungbean. It also raised the question of how the two processes were coordinated during compound leaf development in mungbean. The HEL1 gene was a key locus of mungbean in the control of leaflet number, whose mutation resulted in dissected juvenile leaves and heptafoliate adult leaves (Fig. 5b and Supplementary Figure 3). Double mutant analysis showed that hel1 genetically interacted with un and smp1 to control the leaflet number of the compound leaves, indicating that lateral leaflet formation in the hel1 mutant was dependent not only on LFY but also on other regulators in the control of compound leaf development. Consistently, gene expression analysis showed that the STM/BP-like KNOXI genes and a KNOXM gene, VrLFY, as well as its putative cofactor VrUFO, were significantly upregulated in the hel1 mutant compared to the wild type (Fig. 6). Therefore, these results suggested that HEL1 could coordinate the regulatory processes mediated by VrLFY and KNOXI to control compound leaf development in mungbean.

Interestingly, heptafoliate-leaf-like mutants similar to the hel1 mutant in mungbean were also identified and characterized in other legumes, including soybean and cowpea. In soybean, the seven-leaflet character is a single recessive trait conditioned by the \( i_{2} \) locus. Preliminary mapping results in mungbean revealed that the HEL1 gene was located to a region of chromosome 11, which showed synteny with the \( i_{2} \) locus in soybean (data not shown). Future work to clone the HEL1 gene and its ortholog in soybean and related legumes could provide new insights into the molecular mechanisms orchestrating the two regulatory processes mediated by the LFY ortholog and KNOXI genes during compound leaf development in mungbean and other legumes.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supplementary Information**

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