Different expression patterns of duplicated *PHANTASTICA*-like genes in *Lotus japonicus* suggest their divergent functions during compound leaf development

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ABSTRACT

Recent studies on leaf development demonstrate that the mechanism on the adaxial-abaxial polarity pattern formation could be well conserved among the far-related species, in which *PHANTASTICA* (*PAHN*)-like genes play important roles. In this study, we explored the conservation and diversity on functions of *PHAN*-like genes during the compound leaf development in *Lotus japonicus*, a papilionoid legume. Two *PHAN*-like genes in *L. japonicus*, *LjPHANa* and *LjPHANb*, were found to originate from a gene duplication event and displayed different expression patterns during compound leaf development. Two mutants, *reduced leaflets1 (rel1)* and *reduced leaflets3 (rel3)*, which exhibited decreased adaxial identity of leaflets and reduced leaflet initiation, were identified and investigated. The expression patterns of both *LjPHANs* in *rel* mutants were altered and correlated with abnormalities of compound leaves. Our data suggest that *LjPHANa* and *LjPHANb* play important but divergent roles in regulating adaxial-abaxial polarity of compound leaves in *L. japonicus*.

Keywords: adaxial identity, *LjPHANa*, *LjPHANb*, gene duplication, *rel* mutants, *Lotus japonicus*.

INTRODUCTION

The leaf organs of higher plants can be classified as simple or compound leaves. Compound leaves are found in distantly related groups, and differ from simple leaves in that each petiole bears multiple leaflets lacking auxiliary buds [1, 2]. Most leaves possess three-dimensional polarities that can be dissected into the adaxial-abaxial, proximodistal and mediolateral polarities, among which the adaxial-abaxial polarity is established early in leaf primordia and determines the leaf expansion [3]. Many regulators are involved in adaxial-abaxial patterning of *Arabidopsis* leaves, including the adaxial identity genes *PHANTASTICA*, *PINHEAD*, *ARGONAUTE1*, *PHABULOSA* and *PHAVOLUTA* [4-7]; the abaxial identity genes *YABBYs*

and *KANADIs* [8, 9].

PHANTASTICA was the first gene found to be required for establishment of adaxial-abaxial polarity; when *PHAN* was mutated in *Antirrhinum*, the adaxial identity of leaves was lost and needle-like leaves were formed [5, 10]. Orthologs of *PHAN* have been reported in many species, such as *ROUGH SHEATH2 (RS2)* in maize [11, 12] and *Asymmetrical Leaves1 (AS1)* in *Arabidopsis* [13, 14]. *PHAN* genes are also showed to be involved in proximodistal elaboration [15] and palisade development [16]. Another important function of *PHAN* genes is responsible for suppressing expression of class 1 *KNOTTED1-like homeobox I (KNOXI)* genes in leaf primordia, which are often expressed in meristems. Loss of functional *PHAN* genes causes ectopic expression of *KNOXI* genes and ectopic meristematic activity in the leaf primordia, which result in lobed leaves or ectopic growth on leaf [11-13, 16, 17]. The complementary expression patterns between *PHAN* and *KNOXI* were also found in leaves of *Selaginella kraussina*,

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a lycophyte with microphyllous leaves [18], which suggested that *PHAN-KNOXI* interaction is a common mechanism in determining leaf development.

In compound leaf development, *PHAN* genes were also found to reserve the function in building the adaxial domain in both compound leaf primordia and leaflet primordia; proper expression of those genes is required for correct patterning of leaflet arrangement that is used for proper differentiation between pinnate and palmate compound leaves in eudicots [19]. On the other hand, over-expression of *KNOXI* genes caused lobed leaf phenotype in *Arabidopsis* as well as enhanced complexity of compound leaves in tomato, suggesting that *KNOXI* genes might play an important role in forming a compound leaf [20-23]. Further studies revealed that re-establishment of *KNOXI* gene expression in the compound leaf primordia was responsible for the maintenance of meristematic activity and the initiation of leaflets, which was conserved in the compound leaf development of most vascular plants, with the exception of legumes [24, 25]. In tomato, the expression domain of the *PHAN* genes overlaps with that of the *KNOXI* genes, and their proper expression balance appears to be required for accurate regulation of compound leaf development [26, 27].

In legumes, most studies on compound leaf development have been carried out in pea. It has been shown that *UNIFOLIATA* (*UNI*), an ortholog of *LEAFY*, rather than *KNOXI* genes play an important role in maintaining the meristematic activity in compound leaf primordia [24, 28]. *STP*, an ortholog of *UNUSUAL FLORAL ORGANS* involved in floral development, also regulates the complexity of compound leaves in pea [29]. *CRISPA* (*CRI*), the pea ortholog of *PHAN*, is believed to be required for adaxial-abaxial polarity of leaflets and suppress expression of *KNOXI* genes in compound leaf primordia in pea [30]. The relation between *CRI* and *KNOXI* is similar to that in *Arabidopsis*, indicating that mechanism controlling pea compound leaves is different from that in tomato. However, compound leaves in pea are highly specialized because their lateral organs show distinct identities: stipules, leaflets and tendrils [30], and this raises the question about the function of *PHAN*-like genes and their interaction with *KNOXI* genes in other papilionoid legumes with relatively simple form of compound leaf.

In this study, we choose *L. japonicus* as the experiment system, whose compound leaf typically consists of five visible leaflets. We herein isolated two *PHAN*-like genes, *LjPHANa* and *LjPHANb*, and three *KNOXI* genes, *LjKN1*, *LjKN2* and *LjKN3*, in *L. japonicus*. Two mutants with abnormal compound leaves, *reduced leaflets1* (*rel1*) and *reduced leaflets3* (*rel3*), were identified, which exhibited decreased adaxial identity of leaflets and reduced leaf-

let initiation. The expression patterns of *LjPHANs* and *LjKNs* were examined in wild type as well as in *rel* mutants. Our results suggest that duplicated *PHAN*-like genes have divergent functions in regulating compound leaf development and possibly act together to repress the *KNOXI* gene expression in *L. japonicus* compound leaves.

MATERIALS AND METHODS

Plant material and growth conditions

The *rel1* and *rel3* mutants used for the analysis were M4 segregated offspring with mutant phenotypes. The wild-type *L. japonicus* (Gifu-B129 ecotype), *rel1* and *rel3* mutants were grown in a growth chamber at 22°C on a 16 h:8 h light:dark cycle.

Isolation of homologs and sequence analysis

RNA was prepared from vegetative shoots of *L. japonicus* (Gifu-B129 ecotype) and soybean (Jilin21 variety) using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from total RNA using the SL0066 primer (5'-gac tcg agt cga cat cgt t₁₆3') and AMV retrotranscriptase enzyme (Promega). *LjPHANa* and *LjPHANb* were amplified by 3'-RACE using degenerate primers SL0445 [5'-cac gg(a/g/c) aac aa(a/g) tgg a(a/g) aa(a/g) at(t/c) gc3'] and SL0446 [5'-aag tgg tgg ga(a/g) gt(a/t/g) tt(c/t) aa(a/g) gag3'], respectively, as the 5' primer and SL0065 (5'-gac tcg agt cga cat cg3') as the 3' primer. The full length ORF of *LjPHANa* was amplified from cDNA with specific primers SL0711 (5'-cta gag aat ggc atc tcg tgt c3') and SL0712 (5'-tgt aaa act gga ggt tag gca c3'), and the full length ORF of *LjPHANb* was amplified with SL0713 (5'-cac aga aat atg att agt tca aga t3') and SL0519 (5'-gaa tag ata gga aat ttg cac aa3'). *GmPHANa* was amplified from cDNA with specific primers SL0913 (5'-ctt gga aat gaa aga tag gca a3') and SL0914 (5'-taa ttt aac tga cct gtg tga gct a3'), and *GmPHANb* was amplified with SL0915 (5'-ctt gga aat gaa aga gag gca3') and SL0916 (5'-tag tca gaa gag cac caa tat cca t 3'). *LjKN1* was amplified from cDNA using SL0161 (5'-atc acc gtc tct tgg cag c 3') as the 5' primer, and SL0065 and SL0162 (5'-tgg-gta-agg-cca-ttt-gta-atg 3') as the 3' primers. *LjKN2* was amplified by SL0512 [5'-gc(t/c)-tac-atg-ga(c/t)-tg(c/t)-caa-aag3'] as the 5' primer, and SL0065 and SL0513 [5'-gaa cca (ga)tt (g/a) tt tat ttg (c/t)tt (t/c)tg 3'] as the 3' primers. *LjKN3* was amplified by SL0514 [5'-atc aa(a/g) gc(c/t) aaa atc g(c/t)(c/t) tci ca(c/t) cct3'] as the 5' primer, and SL0065 and SL0515 [5'-gca t(g/a)t t(c/t)t cig aig g(c/t)t tcc aat g3'] as the 3' primers. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced. Sequence analyses were carried out, using the GenBank database along with the Bioedit 6.0 [31], ClustalX 1.83 [32], and TreeView 1.6.6 [33] software packages.

Genomic southern blotting

Genomic DNAs of *L. japonicus* (Gifu-B129 ecotype), soybean (Jilin21 variety), *Medicago truncatula* (Jemalong ecotype) and pea (*Hortense Poir.* variety) were digested with *HindIII*, *EcoRI*, *EcoRV*, and double digested with combination of any two enzymes mentioned as above, or with *BamHI*. The digested DNA fragments were transferred onto Hybond N+ nylon membranes by alkaline transfer method. The probe was labelled by random primer labelling (Promega) and *GmPHANa* cDNA was used as template. The membranes were hybridized at 55°C and washed at 55°C with 0.1×SSC and 0.1% SDS after 1×SSC and 0.1% SDS, and then exposed to phosphor image plate or X-film.

Construction of transgenic Arabidopsis

LjPHANa (1.1Kb) and *LjPHANb* (1.2Kb) cDNA fragments were inserted into the pMON530 binary T-DNA vector downstream of the 35S promoter (generously gifted by the Lab of Dr. Hai HUANG). Plasmids 35S::*LjPHANa* or 35S::*LjPHANb* were introduced into Arabidopsis plants (*Landsberg erecta*) by *Agrobacterium*-mediated transformation. Sixteen 35S::*LjPHANa* transgenic lines and ten 35S::*LjPHANb* transgenic lines were obtained after *Km^r* selection on MS medium; they were transferred to soil and grown in a growth chamber at 22°C on a 16 h:8 h light:dark cycle. Transgenic plants were verified by PCR with specific primers SL0711 and SL0712 for *LjPHANa* and SL0713 and SL0519 for *LjPHANb*.

Scanning electron microscopy and histology

The samples used for SEM were treated as previously described [34] and examined at an acceleration voltage of 15 kV on Hitachi S-2460. The tissues for histology were fixed and sectioned as described previously [35]. Sections of leaflet blades were stained by Toluidine blue.

In situ hybridization

Material for *in situ* hybridization was fixed, sectioned, and hybridized to digoxigenin-labeled RNA probes as described previously [36]. Templates for the *LjPHANa*- and *LjPHANb*-specific probes were obtained from amplification with specific primers [for *LjPHANa*, SL516 (5'tcg cgg aga agc tag tga aa3') and SL0517 (5'atc cca atg gaa atg gaa cat a3'); for *LjPHANb*, SL518 (5'taa gga gaa gca gag aga3') and SL0519 (5'gaa tag ata gga aat ttg cac aa3')]. The partial cDNAs of *LjKN1* and *LjKN3* were used directly as templates.

RESULTS

Isolation of PHAN-like genes in *L. japonicus* genome

PHAN-like genes belong to a subgroup of *MYB* gene family and share a conserved C-terminal domain in addition to the N-terminal *MYB* domain [37]. We designed degenerate primers aimed at the conserved domains and used them to amplify cDNA from the shoots of Gifu-B129. Two *PHAN*-like genes, *LjPHANa* and *LjPHANb*, were identified in the *L. japonicus* genome. Comparison between the two cDNA sequences and genomic sequences of *L. japonicus* [38] revealed that *LjPHANa* and *LjPHANb* were located in a tandem *cis*-cluster on chromosome 3, which indicated that the two *PHAN*-like genes likely derived from gene duplication. The ORFs of *LjPHANa* and *LjPHANb* were separated by a 4kb region, which did not encode another ORF and presumably contained the promoter of *LjPHANb* and intergenic sequences (Fig. 1A).

Analysis of the putative protein sequences showed that the N-terminal *MYB* domain and C-terminal domain are highly conserved in both *LjPHANs* (Fig. 1B). *LjPHANa* shared 75.8% and 70.8% identity, and 82.9% and 77.4% similarity to PsPHAN (*PHAN* ortholog in pea) and MtPHAN (*PHAN* homolog in *Medicago truncatula*) respectively, while *LjPHANb* shared 67.3% and 66.0% identity, and 74.2% and 71.9% similarity to PsPHAN and MtPHAN respectively.

In contrast, *LjPHANa* and *LjPHANb* only shared 68.8% identity and 76% similarity with each other.

We next investigated whether the genomes of other legume species may contain two or more copies of the *PHAN*-like genes. Publicly available database searching yielded two soybean EST sequences (BF598488 and BE330204) showing high similarities with the C-termini of *LjPHANa* and *LjPHANb* respectively. We isolated the two *PHAN*-like genes from soybean cDNA and named them *GmPHANa* and *GmPHANb*. The results of Genomic Southern hybridization revealed that there is likely to be more than one *PHAN*-like gene in all four-legume species, including *L. japonicus*, soybean, medicago and pea (Fig. 1D). Therefore, we assume that the duplication of *PHAN*-like genes should be generally occurred in papilionoid legumes.

A phylogenetic tree of *PHAN*-like proteins was built using the Neighbour Joining method on predicted protein sequences from the variable region between the *MYB* domain and the CTD domain (C-terminal conserved domain). In the resulting tree with SvARP1 (*PHAN* proteins from *Selaginella viticulosa*) as the outgroup, the legume *PHAN*-like proteins clustered together and were divided into two large clades. The first clade included PsPHAN, MtPHAN, *GmPHANa*, *LjPHANa* and AhPHAN (*PHAN*-like protein from *Acacia hindisii*) while *LjPHANb* and *GmPHANb* belonged to the second clade. The first clade was further divided into two small sub-clades, one shared by PsPHAN, MtPHAN and *GmPHANa* while AhPHAN and *LjPHANa* rooted in another one (Fig. 1C).

Genbank Accession Numbers: SvARP1 (*Selaginella viticulosa*), AY667453; SkARP1 (*Selaginella kraussiana*), AY667452; RS2 (maize), AF143447; PaPHAN (*Pachira aquatica*), AY180135; FaPHAN (*Fraxinus americana*), AY180136; SaPHAN (*Schefflera actinophylla*), AY180133; VcPHAN (*Vitex cannabifolia*), AY180132; PHAN (*Antirrhinum*), AJ005586; AhPHAN (*Acacia hindisii*), AY180134; MtPHAN (*Medicago truncatula*), AF308453; PsPHAN (pea), AF299140; NtPHAN (*Nicotiana tabacum*), AY559043; LePHAN (tomato), AF148934; AS1 (*Arabidopsis*), AF175996; EcPHAN (*Eschscholzia californica*), AY228766; AfPHAN (*Aquilegia formosa*), AY180131; *LjPHANa* (*L. japonicus*), AY790244; *LjPHANb* (*L. japonicus*), AY790245; *GmPHANa* (soybean), AY790252; *GmPHANb* (soybean), AY790253.

Expression patterns of PHAN-like genes in *L. japonicus*

The existence of two *PHAN*-like genes in *L. japonicus* is in contrast to most other species characterized thus far, which generally contain one copy of *PHAN*-like gene [18, 19]. To explore whether the two *PHAN*-like genes have different biological functions during compound leaf development, we assessed the expression patterns of

Fig. 1 Duplicated *PHAN*-like genes in *L. japonicus*. **(A)** Tandem arrangement of the two *PHAN*-like genes on LjT11G09. **(B)** Multiple alignment of *PHAN*-like proteins or fragments; residues identical in six of the proteins are shown in black. **(C)** A phylogenetic tree of *PHAN*-like proteins based on Neighbour Joining method with SvARP1 as outgroup, the analyzed sequence covering the variable region between the MYB domain and CTD domain. **(D)** Genomic Southern hybridization with *GmPHANa* cDNA as the probe. E1: *EcoRI*; H3: *HindIII*; E5: *EcoRV*; H3/E5: *HindIII* and *EcoRV*; E1/E5: *EcoRI* and *EcoRV*; B1/E5: *BamHI* and *EcoRV*; H3/E1: *HindIII* and *EcoRI*.

Fig. 2 Expression patterns of *LjPHAN* genes in *L. japonicus*. (A–C) Expression pattern of *LjPHANa* in compound leaf primordia and leaflet primordia (A, B) and developing floral bud (C). (D–E) Expression pattern of *LjPHANb* in compound leaf primordia and leaflet primordia. Signal of both *LjPHANs* was detected in p0, and stronger expression was observed at the adaxial domains of p1 (A, D). In p4 and p5, *LjPHANb* was detected at the bases of leaflet primordia (as indicated by arrows in E). (F) No *LjPHANb* signal was detected in developing floral bud. p0–p5: compound leaf primordia at different developmental stages. Scale bar = 50 μ m.

LjPHANa and *LjPHANb* by RNA *in situ* hybridization.

LjPHANa and *LjPHANb* were dynamically expressed during compound leaf development in *L. japonicus*. The expression of both genes first appeared in the compound leaf founder cells (p0) located in the peripheral zone of the SAM (Fig. 2A, D). Then their transcripts were detected in p1 compound leaf primordia, with stronger level in the adaxial domain (Fig. 2A, D). Therefore, *LjPHANa* and *LjPHANb* displayed similar expression patterns during the earlier phase of compound leaf development.

Later when leaflets initiated, expression pattern difference between the two *LjPHANs* was observed. In newly initiated leaflet primordia, *LjPHANa* expression was detected with stronger level at the middle region of leaflet primordia in p2 (Fig. 2A, B), and then dispersed in the older leaflet primordia of p3 and p4 (Fig. 2A, B). On the other hand, *LjPHANb* expression was continually confined to the adaxial domain of the incipient leaflet primordia in p2 and maintained at the adaxial domain in p3. Then it gradually dispersed in leaf primordia of p4 and p5 (Fig. 2D, E). However, expression of *LjPHANb* was also found at the base of leaflets from P4, and this strong expression still be seen at the leaflet base in much older leaflets (Fig. 2E). During the reproductive developmental stage, *LjPHANa* was expressed in the bracts and developing flower organs (Fig. 2C) whereas no *LjPHANb* transcript was detected in the floral organs (Fig. 2F). Taken together, expression pattern of *LjPHANa* is similar to *PHAN* in *An-*

Fig. 3 Phenotypes of transgenic Arabidopsis lines overexpressing *LjPHANa* or *LjPHANb*. (A) Transgenic Arabidopsis transformed with empty vector; (B) 35S::*LjPHANa* transgenic Arabidopsis and PCR confirmation of *LjPHANa* insertion; (C) 35S::*LjPHANb* transgenic Arabidopsis and PCR confirmation of *LjPHANb* insertion.

tirrhinum and *AS1* in *Arabidopsis* [5, 13], whose expression is detected in both compound leaves and floral organs whereas *LjPHANb* is only expressed in compound leaf primordia and leaflet primordia, indicating that the *LjPHANb* gene plays a more specific role restricted to compound leaf development.

Fig. 4 Analysis of *KNOXI* genes in *L. japonicus*. **(A)** Multiple alignment of *KNOXI* proteins or fragments; residues identical in five of the proteins are shown in black. **(B)** A phylogenetic tree of *KNOXI* proteins based on Neighbour Joining method.

Overexpression of *LjPHANa* and *LjPHANb* in Arabidopsis

To investigate biochemical functions of the *LjPHANa* and *LjPHANb* proteins, we constructed plasmids containing *LjPHANa* or *LjPHANb* ORF driven by the 35S promoter respectively, and transformed wild type Arabidopsis (Landsberg *erecta* ecotype). 16 independent transgenic lines for *LjPHANa* and 10 independent transgenic lines for *LjPHANb* were obtained. Similar leaf phenotypes in various extents were observed in T1 seedlings of transgenic plants transformed with either 35S::*LjPHANa* or 35S::*LjPHANb*, but not in T1 seedlings of transgenic plants transformed with empty vectors. The phenotypes observed in the transgenic lines were segregated well with the transgenes in the manner of either single or multi transgene insertions (data not shown). These transgenic plants typically displayed elongated leaves with narrower, longer blades and longer petioles (Fig. 3B, C), as compared with the leaves of wild type Landsberg *erecta*. The transgenic phenotypes mimicked to the ones of a previous study, in which the overexpression of *RS2* and *ASI* in Arabidopsis caused narrower leaves with longer petioles [37]. These results suggested that the function of either *LjPHANa* or

LjPHANb should be the same as other PHAN orthologs at protein level.

Expression patterns of *KNOXI* genes in *L. japonicus*

Previous studies showed *PHAN-KNOXI* interaction in pea is similar to that in Arabidopsis [30]. To investigate whether it is the same case in *L. japonicus*, we isolated *KNOXI* genes and examined their expression patterns by RNA *in situ* hybridization.

Three *KNOXI* gene fragments were isolated from vegetative apex cDNA and named *LjKNO1*, *LjKNO2* and *LjKNO3*, all belonging to the *KNOXI* subgroup of *KNOX* gene family. Sequence analysis and phylogenetic tree indicated that *LjKNO1* shared high similarity with *PSK1* in pea and *STM* in Arabidopsis; *LjKNO2* resembled to *PSKN2* in pea and *KNAT1* in Arabidopsis; and *LjKNO3* was similar to *KNAT2* or *KNAT6* in Arabidopsis (Fig. 4A, B).

In wild-type plants, *LjKNO1* and *LjKNO3* were simultaneously expressed in the shoot apical meristems (SAM) and the axillary meristems (Fig. 5A, D) during the vegetative and reproductive stages, provascular tissue, inflorescences (Fig. 5A, D), floral meristems (Fig. 5B, E) and indeterminate tissues of developing floral buds (Fig. 5C,

Fig. 5 Expression patterns of *LjKN* genes in *L. japonicus*. (A–C) Expression patterns of *LjKN1* in SAM and inflorescence meristem (A), floral meristems (B), developing floral buds (C). (D–F) Expression patterns of *LjKN3* in SAM and inflorescence meristem (D), floral meristems (E), developing floral bud (F). S: SAM; I: inflorescence meristem; FM: floral meristem; F: floral bud. Scale bar = 50 μ m.

F). However, some differences were found: the signal for *LjKN1* was always stronger and in much restricted area near the central zone of SAM, but the one for *LjKN3* was generally weaker and more dispersed. No *LjKN1* and *LjKN3* transcripts were detected in the compound leaf primordia or leaflet primordia at any stage, consistent with previous reports about no expression of *KNOX1* genes in the compound leaf primordia in pea [24, 30]. On the other hand, we failed to detect the expression of *LjKN2* in any tissues at both vegetative and reproductive stages by RNA *in situ* hybridization.

Genbank Accession Numbers: MKN2 (*Physcomitrella patens*), AF285147; MKN4 (*Physcomitrella patens*), AF284817; STM (Arabidopsis), ATU32344; KNAT1 (Arabidopsis), AF482994; KNAT2 (Arabidopsis), ATU14175; KNAT6 (Arabidopsis), NM160620; PsKN1 (pea), AF080104; MtKN1 (*Medicago truncatula*), AF308454; PsKN2 (pea), AF080105; SBH1 (soybean), L13663; KN1 (maize), AY312169; RS1 (maize), L44133; LeT6 (tomato), AF000141; TKN1 (tomato), U33247; OSH1 (rice), D16507; OSH15 (rice), AB016071; NHT15 (tobacco), AB004785.1; NTH22 (tobacco), AB025715; NTH9 (tobacco), AB025713; LjKN1 (*L. japonicus*), AY790246; LjKN2 (*L. japonicus*), AY790247; LjKN3 (*L. japonicus*), AY790248.

The *reduced leaflets* mutants with decreased adaxial identity of compound leaves

We tried to identify mutants with decreased adaxial identity of compound leaves in *L. japonicus* from ethyl

methanesulfonate (EMS) mutant collection. Two mutants with abnormal compound leaves were identified, one being isolated from the *SL256* mutated line [39] and the other from the *D0013* mutated line (unpublished data). In both cases, the F1 backcross progeny had the wild-type phenotype, and the F2 progeny yielded phenotypes at a Mendelian segregation ratio of 3:1 (132 wild type: 46 mutant for *SL256*, $P < 0.5$; 117 wild type: 42 mutant for *D0013*, $P < 0.5$), indicating that the phenotype of each mutant was caused by a single recessive mutation. The two mutants exhibited nearly identical phenotypes and the most distinct defects were abnormal compound leaves with narrow leaflets and reduced leaflet numbers. According to their phenotypes, we named the two mutants *reduced leaflets 1* (*rel1*) and *reduced leaflets 3* (*rel3*). Genetic analysis showed that the corresponding loci *Rel1* and *Rel3* were not allelic and none of them were linked to the *LjPHANs* (unpublished data).

In wild-type *L. japonicus*, nodes from one to five produce compound leaves with three or four leaflets. Usually from the sixth node, plants produce mature compound leaves containing five visible leaflets: one top leaflet, two lateral leaflets and two basal leaflets (Fig. 6A). In contrast, most of the compound leaves in both *rel1* and *rel3* were composed of three narrow leaflets, regardless of the node (Fig. 6B, C). In both mutants, the leaflets were narrower and thicker than wild type; the proximal parts of the leaflets being rod-like, and the leaflet edges slightly curled up. The most severely affected leaflets were not completely expanded, displaying the needle shape (Fig. 6B, C).

We further compare the compound leaf development in both wild type and mutant plants by scanning electron microscopy (SEM). In the wild type plants, the leaflet primordia basipetally initiated from the compound leaf primordia, leading to formation of the 5 leaflet primordia (Fig. 6D). In *rel1* and *rel3* mutants, fewer leaflet primordia were initiated. Once the top leaflet primordia initiated, the compound leaf primordia normally stopped initiating new leaflet primordia in the mutants (Fig. 6E, F).

SEM analysis was also conducted to analyze the epidermis of compound leaves in *rel* mutants. The adaxial surface of a fully expanded wild-type leaflet consisted of the multiangular cells (Fig. 6G), whereas the abaxial surface possessed multiangular cells and elongated narrow cells located in the middle part of the leaflets (Fig. 6J). The boundary between the adaxial and abaxial surfaces also consisted of several rows of elongated cells. By comparison, the adaxial and abaxial epidermis of leaflets in *rel1* and *rel3* were similar to each other, but drastically different from wild type. The abaxial surface of the mutant leaflets, especially on the proximal parts, was only composed of elongated narrow cells (Fig. 6K, L) that were

Fig. 6 Analysis of abnormal compound leaves in the *rel* mutants. (A–C) Comparison of compound leaves among wild type (*L. japonicus*, Gifu ecotype) (A), *rel1* (B), and *rel3* (C); TL: top leaflet; LL: lateral leaflet; BL: bottom leaflet. (D–F) Comparison of leaflet initiation by SEM among wild type (D), *rel1* (E) and *rel3* (F); Numbers indicate the sequential initiation of leaflet primordia (The fourth leaflet primordia of wild type is shielded by the next compound leaf primordia). (G–I) Comparison of adaxial epidermis by SEM among wild type (G), *rel1* (H) and *rel3* (I). (J–L) Comparison of abaxial epidermis by SEM among wild type (J), *rel1* (K) and *rel3* (L); only the proximal parts being showed, in which arrow indicates the elongated cells. (M–O) Histological transverse sections of leaflet blade among wild type (*L. japonicus*, Gifu ecotype) (M), *rel1* (N), and *rel3* (O). Scale bar = 50 μ m.

also proliferating to the adaxial surface (Fig. 6H, I); resulting in narrowed and approximately round leaflet bases. The appearance of elongated narrow cells on the proximal part of the adaxial surface (Fig. 6H, I) in mutant leaflets suggests that adaxial identity was reduced in *rel1* and *rel3* compound leaves.

Sections of leaflets further revealed reduced adaxial-abaxial polarity in mutant compound leaves. On wild type leaf sections, elongated palisade mesophyll cells are lo-

cated in the adaxial region and round spongy mesophyll cells in the abaxial region (Fig. 6M). Whereas in both mutants, palisade mesophyll in the adaxial domain became almost round and difficult to distinguish from spongy mesophyll in the abaxial domain, as well as mesophyll cells were arranged sparsely and disorderly as compared with wild type (Fig. 6N, O). These results confirmed that the adaxial identity was decreased in the compound leaves in *rel1* and *rel3* mutants.

Altered expression patterns of *LjPHAN* genes in *rel* mutants

The *Rel1* and *Rel3* loci were not linked to *LjPHANa* and *LjPHANb*. However, the most prominent phenotype of the *rel1* and *rel3* mutants was decreased adaxial-abaxial polarity and reduced complexity of compound leaves, suggesting the possible involvement of abnormal *PHAN*-like gene function. Thus, we further characterized the relationship between the *rel* mutant phenotypes and the *PHAN*-like genes by examining the expression patterns of *LjPHANa* and *LjPHANb* in the mutants using RNA *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR).

The results of RT-PCR revealed that the expression levels of *LjPHANa* and *LjPHANb* were slightly increased in both vegetative shoot tips and matured leaves of *rel1* and *rel3* mutants as compared with wild type, suggesting that the overall expression level of both *LjPHANs* was not dramatically changed in the mutants (data not shown).

However, RNA *in situ* hybridization showed that expression patterns of *LjPHANa* and *LjPHANb* were altered in *rel1* and *rel3* mutants in comparison with wild type. In both mutants, *LjPHANa* was diffusely expressed throughout p1 compound leaf primordia (Fig. 7B, C). In p2, the *LjPHANa* signal continued to be dispersed in leaflet primordia without the normally observable stronger expression at the middle region of leaflets (Fig. 7B, C). Similarly, *LjPHANb* expression was also diffused throughout P1 compound leaf primordia (Fig. 7E, F). After leaflet initiation, *LjPHANb* expression was still dispersed in the newly initiated leaflet primordia of p2 and p3 (Fig. 7E, F). After leaflets expanded, the expression domains for both *LjPHANa* and *LjPHANb* were progressively confined to the adaxial side at the distal part of the leaflets (Fig. 7B, C, E). The expression of *LjPHANb* at the bases of the leaflet primordia was much weaker or lost in both *rel* mutants (Fig. 7H, I).

In contrast, expression patterns of *LjKN1* and *LjKN3* displayed no significant changes in the *rel1* and *rel3* mutants. Like for wild type, neither of these genes was detectable by *in situ* hybridization, in any compound leaf primordia and leaflet primordia of the *rel1* and *rel3* mutants (Fig. 7K, L, N, O). Also, transcripts of *LjKN1* and *LjKN3* could be detected by RT-PCR from the vegetative shoot tips in wild type and in the *rel* mutants as well, without significant difference between them (data not shown).

DISCUSSION

PHAN-like gene duplication in *L. japonicus*

Gene duplication has occurred widely in the genomes of disparate life forms, contributing greatly to evolution and diversity and elaboration of basic developmental form.

Except for genome duplication, gene duplication often occurs in three ways: unequal crossing over, retroposition and chromosomal rearrangement [40].

We isolated two *PHAN*-like genes in *L. japonicus* genome, *LjPHANa* and *LjPHANb*, and found them in a tandem arrangement on chromosome 3, which strongly indicated that the two genes likely originated from gene duplication caused by unequal crossing over. As duplication of *PHAN* gene had not been reported in species other than *L. japonicus*, we investigated whether it might have also occurred in other legumes. Database searching allowed us to identify a similar pair of *PHAN*-like genes in soybean and genomic Southern blotting showed that there was possibility of more than one copy of *PHAN*-like gene in *M. truncatula* and *Pisum sativum* genomes. Phylogenetic analysis of *PHAN* proteins revealed that duplication of *PHAN*-like genes possibly was an ancient duplication and most likely occurred before the species divergence of legumes. Consistently, it has been suggested unaffected tendrils and stipules never being radialized in pea *cri* mutants, are likely to result from redundancy of *PHAN*-like genes [30]. Genome sequencing data in *M. truncatula* and other papilionoid legumes will be required to adequately address this possibility.

Sequence analysis indicated that *LjPHANa* shared higher similarity with *PHAN* ortholog in pea than with *LjPHANb*. In the phylogenetic tree based on putative sequences of *PHAN*-like proteins, *LjPHANa* and *LjPHANb* rooted in different clades. Expression patterns of both *LjPHANs* also differ from each other. However, the transgenic phenotypes caused by overexpression of either *LjPHANa* or *LjPHANb* in *Arabidopsis*, resembled to each other and to the phenotypes of *35S::ASI* [37], which suggested that both *LjPHAN* proteins could exert similarly conserved biochemical functions as *AS1* in *Arabidopsis*. These results suggested that the duplicated *PHAN*-like genes were functional and the biological function difference between them, if there is any, was likely to be the outcome of their specific expression patterns.

Divergent functions of *LjPHAN* genes in *L. japonicus* compound leaf development

As the previous research showed, like for simple leaf formation, correct establishment of adaxial-abaxial polarity is important for compound leaf development. *PHAN*-like genes were found to reserve functions in setting up adaxial identity in compound leaf primordia and leaflet primordia [19, 27]. It has been shown that adaxial-abaxial polarity of the compound leaves was reduced in pea mutant *cri* and *CRI* was a pea ortholog of *PHANTASTICA* [30].

During *L. japonicus* compound leaf development,

Fig. 7 Expression patterns of *LjPHANs* and *LjKNs* in wild type and *rel* mutants. (A-C) Expression patterns of *LjPHANa* in wild type (A), *rel1* (B) and *rel3* (C). (D-F) Expression patterns of *LjPHANb* in wild type (D), *rel1* (E) and *rel3* (F). (G-I) Expression patterns of *LjPHANb* in older leaflets of wild type (G), *rel1* (H) and *rel3* (I); star indicates expression of *LjPHANb* at leaflet bases in wild type (G), and arrows indicate no expression of *LjPHANb* at leaflet bases in *rel* mutants (H, I). (J-L) Expression patterns of *LjKN1* in wild type (J), *rel1* (K) and *rel3* (L). (M-O) Expression patterns of *LjKN3* in wild type (M), *rel1* (N) and *rel3* (O). Scale bar = 50 μ m.

LjPHANa and *LjPHANb* displayed different expression patterns. Like all *PHAN*-like genes described so far, both *LjPHAN* transcripts were firstly detected in leaf founder cells (p0), which was believed to be responsible for leaf

determination [5, 13, 16, 18]. In p1 compound leaf primordia, they were expressed with stronger level at the adaxial domain, suggesting their functions in establishment of adaxial identity in compound leaf primordia. After leaf-

let primordia initiated, *LjPHANa* was detected with stronger level at the middle region of leaflet primordia whereas *LjPHANb* expression continued at the adaxial domain of leaflet primordia where it perhaps helped specify adaxial identity of leaflets. In expanded leaflets, *LjPHANb* displayed a unique expression pattern at bases of leaflets, possibly involved in stabilizing the boundary between leaflets and petiolules. Consistently, expression of *LjPHANa* was detected in both compound leaf and flower, but the one of *LjPHANb* was exclusive from flower, strongly indicating that *LjPHANb* may not play an important role in the floral development.

Therefore, based on their expression pattern difference, *LjPHANb* and *LjPHANa* should be defined as paralogs with divergent functions and play different roles in *L. japonicus* compound leaf development. Recent studies showed that evolutionary switch of some basic morphological forms could only involve the regulatory regions of some master genes. In the case of evolutionary switch from teosinte to maize, the regulatory region of *Tb1*, a gene encoding for a putative transcription factor, was changed but not in the coding region of the gene [41, 42]. It is possible that the similar mechanism occurred in *L. japonicus*, the divergent biological functions of duplicated *LjPHAN* genes depending on their distinct expression patterns rather than their protein differences.

The morphology of compound leaf in *L. japonicus* is simpler than the one of pea, since it consists of five leaflets with the same identity (Fig. 6A). However, functions of *PHAN*-like genes are well conserved; both *LjPHANs* being expressed in compound leaf primordia indicated that they likely reserve the functions in establishment of adaxial identity. On the other hand, the expression pattern difference between *LjPHANa* and *LjPHANb* suggested that in *L. japonicus*, adaxial identity of compound leaves could be specified at two levels, adaxial identity of compound leaf primordia built by both *LjPHAN* genes while adaxial identity of leaflet primordia only by *LjPHANb*.

The functions of *Rel1* and *Rel3* in compound leaf development of *L. japonicus*

It is believed that compound leaf specification consists of two aspects, leaflet initiation and leaflet pattern formation. Correct leaflet initiation determines the leaflet number and placement, which is regulated by marginal meristem maintenance and the adaxial-abaxial polarity of the compound leaf primordia [1, 19, 25]. Although *KNOXI* and *PHAN*-like genes have been shown to be involved in the process [19, 25], there are likely other factors regulating compound leaf development. In this study, we present novel candidates *Rel1* and *Rel3*, which induce disturbances in compound leaf development when mutated.

The phenotypes of the *rel1* and *rel3* mutants are highly similar to each other, suggesting that *Rel1* and *Rel3* genes might function in the same genetic pathway. The *rel1* and *rel3* mutants displayed abaxialization of adaxial epidermis and palisade mesophyll of leaflets (Fig. 6G-O), indicating decreased adaxial identity in compound leaves. Although *Rel1* and *Rel3* loci were not linked to the *LjPHANs*, the abnormalities of compound leaves in both mutants were associated with altered expression patterns of *LjPHANa* and *LjPHANb*, the newly isolated *PHAN*-like genes in *L. japonicus*. In *rel* mutants, both *LjPHAN* transcripts were dispersed throughout compound leaf primordia and leaflet primordia, which lost their distinct expression patterns (Fig. 7B, C, E, F, H, I). As a result, adaxial epidermis and palisade mesophyll of compound leaves were differentiated improperly. Only in the expanded leaflet primordia, both *LjPHAN* expressions had gradually been confined to the adaxial domain in the distal parts, which was also consistent with mutant phenotypes that the most leaflets still have narrow blades. The correlation between expression pattern and compound morphology in both wild type and mutants further strongly support the notion that *LjPHANa* and *LjPHANb* genes played important roles in adaxial identity specification in the development of *L. japonicus* compound leaves.

These data also suggest that *Rel* loci are epistatic to *LjPHANs* in regulating their spatial expression pattern and controlling the adaxial-abaxial polarity during compound leaf development in *L. japonicus*, although the clear relationship between them needs to be further investigated. Apart from the defects in compound leaf development, the *rel* mutants also exhibited abnormalities in cotyledons, floral development and stem vascular pattern formation (data not shown). The pleiotropic developmental defects in both mutants suggest that *Rel* loci are possibly involved in a fundamental regulation and responsible for controlling many aspects of *L. japonicus* ontogeny.

The relationship between *LjKN* genes and *LjPHAN* genes in *L. japonicus*

Previous studies in pea have questioned the role of *KNOXI* genes in compound leaf development in papilionoid legumes [24]. However, recent study showed that the *PHAN-KNOXI* relation in pea is like that in Arabidopsis rather than in tomato, *PsPHAN* suppressing *PsKN2* expression in wild-type compound leaf primordia [30].

In *L. japonicus*, expression of *LjKN1* and *LjKN3* was restricted to the SAM and exempt from the compound leaf primordia and leaflet primordia. The mutually exclusive expression patterns between *LjKNs* and *LjPHANs* in wild type might suggest that the duplicated *PHAN*-like genes could be involved in repressing the expression of *KNOXI*

genes in compound leaf primordia, being consistent with *PHAN-KNOX1* relation in pea [30]. Considering conservation of re-establishment of *KNOX1* expression in compound leaf primordia among species from fern to angiosperm [25], we could speculate *KNOX1* genes were expressed in the ancient compound leaf primordia, which have been maintained through evolution in most species but repressed by redundant expression of *PHAN*-like genes after gene duplication in *L. japonicus* and possibly in other papilionoid legumes. Therefore, it is likely that the duplication of *PHAN*-like genes contributes to the special developmental mechanism in legumes compound leaves. Loss of function mutants of *LjPHANa* and *LjPHANb* remain to be identified to explore these possibilities.

On the other hand, other factors, such as *Rel1* and *Rel3*, are expected to play important roles in the mechanism. Based on the results of *in situ* hybridization and RT-PCR, we found that *LjPHAN* genes were mainly affected on spatial expression domain whereas expression of *LjKN* genes was not altered, when *Rel1* or *Rel3* was mutated. This might reflect the specific function of *Rel* genes as the regulators for *LjPHANs*. With cloning of both *Rel1* and *Rel3* in the near future, it will provide a clear insight into the interaction among *Rel*s, *LjPHANs* and *LjKNs*, and lead to a better understanding for the molecular mechanism controlling compound leaf development in legumes.

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