# The *REDUCED LEAFLET* Genes Encode Key Components of the trans-Acting Small Interfering RNA Pathway and Regulate Compound Leaf and Flower Development in *Lotus japonicus*[1][W][OA]

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The endogenous trans-acting small interfering RNA (ta-siRNA) pathway plays a conserved role in adaxial-abaxial patterning of lateral organs in simple-leafed plant species. However, its function in compound-leafed species is largely unknown. Using the compound-leafed species *Lotus japonicus*, we identified and characterized two independent mutants, *reduced leaflet1* (*rel1*) and *rel3*, whose most conspicuous defects in compound leaves are abaxialized leaflets and reduction in leaflet number. Concurrent mutations in *REL* genes also compromise flower development and result in radial symmetric floral organs. Positional cloning revealed that *REL1* and *REL3* encode the homologs of Arabidopsis (*Arabidopsis thaliana*) *SUPPRESSOR OF GENE SILENCING3* and *ARGONAUTE7/ZIPPY*, respectively, which are key components of the ta-siRNA pathway. These observations, together with the expression and functional data, demonstrated that the ta-siRNA pathway plays conserved yet distinct roles in the control of compound leaf and flower development in *L. japonicus*. Moreover, the phenotypic alterations of lateral organs in ta-siRNA-deficient mutants and the regulation of downstream targets by the ta-siRNA pathway in *L. japonicus* were similar to those in the monocots but different from Arabidopsis, indicating many parallels between *L. japonicus* and the monocots in the control of lateral organ development by the ta-siRNA pathway.

Plant endogenous small RNAs can be categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs) according to their mechanism of biogenesis (Vaucheret, 2006). trans-Acting siRNAs (ta-siRNAs) are one type of siRNA, and their biogenesis requires several key components, such as SUPPRESSOR OF GENE SILENCING3 (SGS3), RNA-DEPENDENT RNA POLYMERASE6 (RDR6), DICER-LIKE4 (DCL4), ARGONAUTE7 (AGO7)/ZIPPY (ZIP), and dsRNA-BINDING4 (Peragine et al., 2004; Vazquez et al., 2004; Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005; Adenot et al., 2006; Nakazawa et al., 2007). Recent studies revealed that the ta-siRNA pathway is integrated into different processes of plant development, such as vegetative phase transition in Arabidopsis (*Arabidopsis thaliana*; Hunter et al., 2003; Peragine et al., 2004; Xie et al., 2005; Nakazawa et al., 2007) and shoot apical meristem (SAM) initiation in rice (*Oryza sativa*; Satoh et al., 1999; Itoh et al., 2000; Nagasaki et al., 2007). Parallel studies of this pathway in simple-leafed species also showed that the ta-siRNA pathway plays critical roles in patterning of leaves and floral organs.

In flowering plants, leaves and flowers are produced on the periphery of the apical meristem. These lateral organs are structurally asymmetric with regard to the apical meristem. The adaxial side is adjacent to the meristem, while the abaxial side is away from the meristem. The ta-siRNA pathway was found to play a conserved role in specifying the adaxial identity of lateral organs in both monocots and dicots, but defects in the ta-siRNA pathway caused more severe phenotypes in monocots than in dicot Arabidopsis. In Arabidopsis, no clear leaf polarity defects were detected in the ta-siRNA-defective mutants. However,

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blocking the ta-siRNA pathway in *asymmetric1* (*as1*) or *as2* background, which are regulators of leaf adaxial identity (Lin et al., 2003; Xu et al., 2003), results in enhanced adaxial-abaxial leaf defects (Li et al., 2005; Xu et al., 2006; Garcia et al., 2006). In addition, the *as2rdr6* double mutants also display aberrant flowers with sepals failing to enwrap the inner whorl organs and some sepals and petals becoming needle-like structures (Li et al., 2005). In maize (*Zea mays*), mutations in *LEAFBLADELESS1* (*LBL1*), which encodes the Arabidopsis *SGS3* ortholog, give rise to abnormal leaves with partial or complete loss of adaxial cell identity (Timmermans et al., 1998; Nogueira et al., 2007). In severe *lbl1* mutants, leaf-like lateral organs of inflorescences and flowers develop as symmetric, thread-like organs, and the immature ear is exposed and arrested in development (Timmermans et al., 1998). In rice, the *osdcl4-1* mutants display an abaxialized epidermis in coleoptiles and in the first leaf, and knockdown of *OsDCL4* can lead to the awn-like lemma with a radial abaxialized identity and the stamens and carpel not enwrapped by the lemma and pelea (Liu et al., 2007). Transgenic rice plants with ectopic expression of *SHOOTLESS4* (*SHL4*), the homolog of Arabidopsis *AGO7*, exhibit partially adaxialized leaves (Nagasaki et al., 2007; Shi et al., 2007).

In addition to the ta-siRNA pathway, other components have also been shown to be involved in the adaxial-abaxial patterning of lateral organs. The *Antirrhinum majus PHANTASTICA* (*PHAN*) gene (Waites et al., 1998; Byrne et al., 2000; Xu et al., 2003; Qi et al., 2004), which is the ortholog of Arabidopsis *AS1*, and *CLASS III HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIP III*) gene family members (McConnell et al., 2001; Emery et al., 2003) contribute to adaxial pattern formation of lateral organs, whereas members of *YABBY* (*YAB*; Sawa et al., 1999; Siegfried et al., 1999) and *KANADI* (Eshed et al., 2001; Kerstetter et al., 2001) gene families, *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4* (Pekker et al., 2005), and the miRNAs miR165/166 (Emery et al., 2003; Eshed et al., 2004; Mallory et al., 2004) are required for specifying abaxial identity. How the activities of these adaxial and abaxial determinants are coordinated has been extensively studied. It was found that *ARF3* and *ARF4* are regulated by the *TAS3* ta-siRNA, and this regulation is conserved in both monocots and dicots (Allen et al., 2005; Williams et al., 2005). Recent studies in Arabidopsis suggest that ta-siRNAs act in a non-cell-autonomous manner to spatially restrict ARF activity (Chitwood et al., 2009; Schwab et al., 2009).

In contrast to simple leaves with their single lamina, compound leaves are composed of one petiole and several leaflets. It is found that genes required for the adaxial-abaxial patterning of lateral organs in simple-leafed species also play critical roles in compound-leafed species, but these genes play multiple roles in compound leaf development. In tomato (*Solanum lycopersicum*), down-regulation of *PHAN* ortholog disturbs the leaf polarity as well as leaflet formation (Kim et al., 2003). Extensive studies of the *PHAN* expression in diverse compound-leafed species suggest that the function of *PHAN* in maintaining leaf adaxial identity is associated with leaflet formation in compound leaves and reduced adaxial identity of leaf primordia by down-regulation of *PHAN* could change pinnate compound leaves into palmate leaves (Kim et al., 2003). In pea (*Pisum sativum*), the role of *PHAN* in compound leaf development has also been elucidated by characterization of the *phan* mutant *crispa* (*cri*; Tattersall et al., 2005). However, unlike antisense *PHAN* transgenic tomato leaves, the *cri* mutant has the individual leaflet abaxialized, rather than the whole leaf. The number of lateral organs on the *cri* mutant compound leaves, including leaflets, is not altered, and the leaves remain pinnate. Apart from leaf development, the *cri* mutation also affects flower development. Although the floral organ identity and organ number are not altered, the laminar floral organ display abaxialized identity (Tattersall et al., 2005).

The ta-siRNA pathway plays a critical role in simple-leafed species, but its role in compound-leafed species is not understood. Here, we address this question by analyzing loss-of-function *reduced leaflet* (*rel1*) and *rel3* mutants in the compound-leafed species *Lotus japonicus*. Phenotypic characterization shows compound leaves of *rel* mutants exhibit a conspicuous disturbance in leaflet polarity as well as reduction in leaflet number. Besides the abnormal compound leaves, flower development is also severely affected in *rel* mutants, showing radial symmetric petals. *REL1* and *REL3* were identified by map-based cloning and were shown to be homologs of Arabidopsis *SGS3* and *AGO7*, respectively. *REL1* and *REL3* act in the same genetic pathway and are both required for the biogenesis of *TAS3* ta-siRNA. Further investigation reveals that the homolog of the Arabidopsis *ARF3* is duplicated in the *L. japonicus* genome and that the duplicate *ARF3* homologs and the *ARF4* homolog are all negatively regulated by the ta-siRNA pathway. Furthermore, we found that the expression of *LjYAB1*, a homolog of Arabidopsis *YAB1*, was decreased in *rel* mutants, which may be associated with the reduced lamina.

Taken together, our data reveal that the ta-siRNA pathway is integrated into the regulatory networks in the control of lateral organ development in *L. japonicus* and further emphasize the importance of the ta-siRNA pathway in compound leaf development. Moreover, our results also indicate many parallels between *L. japonicus* and monocots for the ta-siRNA pathway in the regulation of lateral organs.

## RESULTS

### Phenotypic Characterization of Compound Leaves and Flowers in *rel1* and *rel3*

From large scale mutagenesis screens in *L. japonicus* (Feng et al., 2006), we isolated a series of mutants with

disrupted leaf polarity and reduction in leaflet number in compound leaves, designated as *rel*. Apart from the conspicuous leaf abnormality, the mature *rel* plants also display aberrant flowers.

Wild-type *L. japonicus* plants display classical compound leaves, with one rachis and five leaflets: one top and two lateral leaflets are clustered at the tip of petiole, and two basal ones are arranged at the base of rachis (Fig. 1A). In *rel1* mutants, loss of one or two basal leaflets occurs in nearly all mature compound leaves (Fig. 1, A and G), and the leaflets are narrower than those of the wild type (Fig. 1, A and H). In addition, needle-like leaflets are found occasionally (Fig. 1A). However, the length of the rachis is not altered, and the distinct adaxial domain is still present throughout the rachis region (Fig. 1, B and C). Anatomical analysis of the needle-like leaflet showed that the cell type around the central vasculature was homogeneous and that phloem tissue surrounded the xylem tissue as a ring (Fig. 1D). This is in contrast to the wild-type leaflet, where palisade mesophyll cells and xylem are on the adaxial side, and spongy meso-

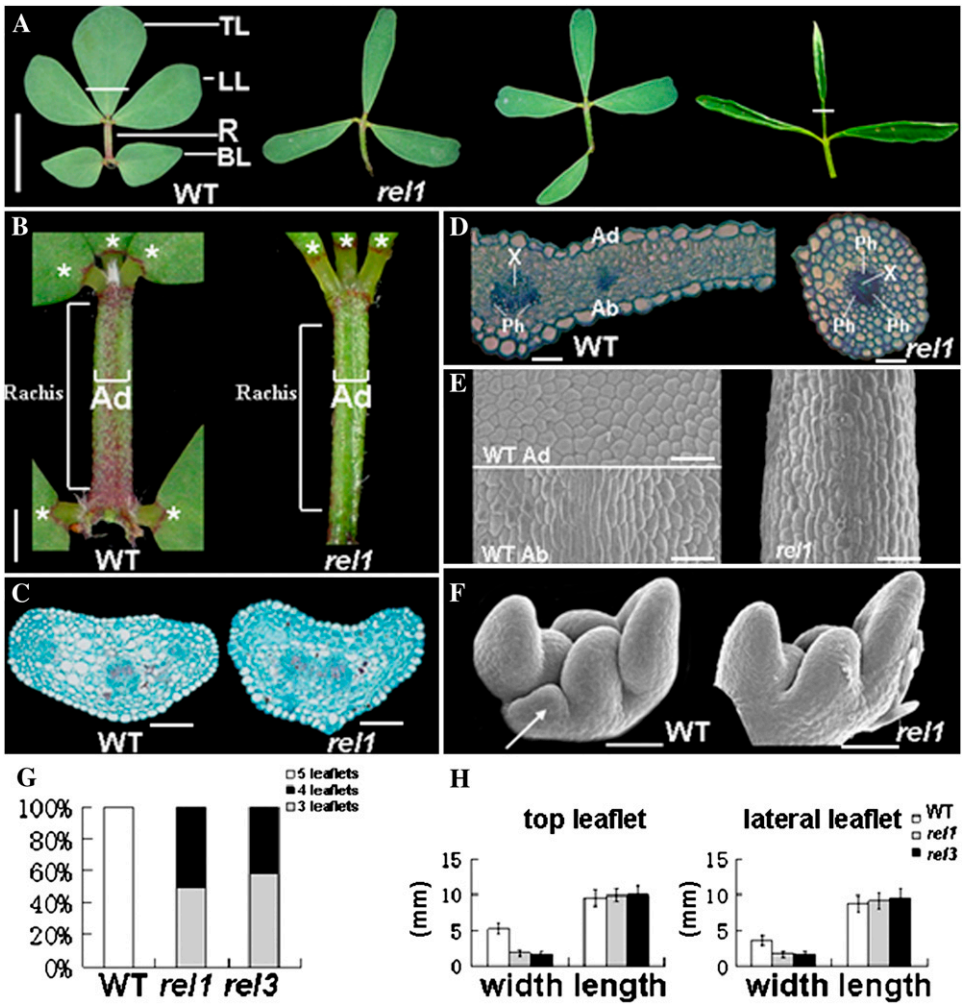


**Figure 1.** Different effects of *rel* mutations on compound leaf. A, In the wild type (WT), the mature compound leaf consists of one top (TL), two lateral (LL), and two basal leaflets (BL). In *rel* mutants, the leaflets are narrow, and one or two BL leaflets are absent. Needle-shaped leaflets can be found. B, Adaxial domain on the rachis of wild-type and *rel1* compound leaves. The adaxial domain could be observed throughout the rachis of both wild-type and *rel1* compound leaves. Asterisks, leaflets; Ad, adaxial domain. C, Transverse sections of rachis in the wild type and *rel1*. D, Transverse sections of TL leaflets in the wild type and *rel1* (the sectioning regions in the TL leaflets are indicated with white lines in A). E, Wild-type adaxial and abaxial epidermal patterns of leaves and epidermal patterns of needle-shaped leaflets in *rel1* mutants. Ad, Adaxial; Ab, abaxial. F, SEM analysis of initiation of leaflet primordia. The initiation of BL primordia commences in the wild type but is blocked in *rel1* (arrows). Asterisks, meristem; TL, top leaflet primordium; LL, lateral leaflet primordium; BL, basal leaflet primordium. Bars = 1 cm in A, 1 mm in B, and 100 $\mu$m in C to F. G, Quantitative analysis of leaflet numbers on wild type ($n = 12$) and *rel* mutants ($n = 12$) between nodes 6 and 10. Bars show SD. H, Quantitative analysis of the top and lateral leaflet width and length on the wild type ($n = 12$) and *rel* mutants ($n = 12$) between nodes 6 and 10. Bars show SD.

phyll cells and phloem are on the abaxial side (Fig. 1D). Scanning electron microscopy (SEM) analysis showed that the needle-like leaflets were covered with long and narrow epidermal cells, which are similar to the epidermal cells observed on the abaxial side of the wild-type leaflet (Fig. 1E). These data clearly indicate that in *rel1* mutants, specification of the leaflet adaxial-abaxial polarity is disturbed and leaflets are abaxialized. SEM was also used to examine vegetative shoots and revealed the absence of the basal leaflets commenced at the early stage when leaflet primordia were initiated, and the basal leaflet primordia were normally not found in *rel1* mutants (Fig. 1F).

Another prominent phenotype of *rel1* mutants was their infertile flowers (Fig. 2A). In comparison with floral organs in wild-type *L. japonicus* plants, most *rel1* flowers were dramatically reduced in size. We examined more than 300 *rel1* flowers, and only two *re11* flowers were as large as wild-type flowers, but these two flowers opened prematurely (Fig. 2A). The phenotypic abnormalities of *rel1* flowers were further analyzed by SEM. During early stages of flower development, the initiation of floral organ primordia in the floral meristem was normal and no defects in floral organ number and organ identity were detected (Fig. 2B). In the later stage when floral organs were further differentiated, the carpel and stamens were enclosed by the petals in the wild type (Fig. 2C). However, all petals in *rel1* mutants were open, and the inner two reproductive organs were exposed (Fig. 2C). In addition, the carpel were frequently split (Fig. 2D), and some petal primordia formed a nearly symmetric and trumpet-shaped structure containing small undifferentiated epidermal cells (Fig. 2E), indicating that the adaxial-abaxial patterning of floral organ was also disturbed in *rel1* mutant. The phenotypic analysis of *rel3* mutant leaves and flowers showed that the developmental defects in *rel3* were very similar to those in *rel1* (data not shown).

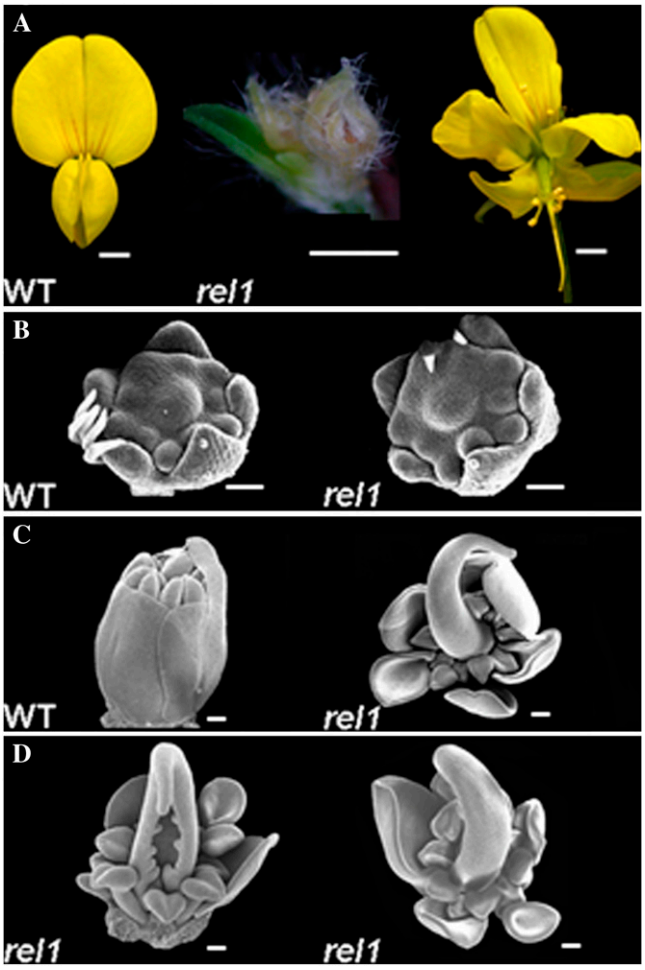


**Figure 2.** Flowers of the wild type (WT) and the *rel1* mutant. A, In the *rel1* mutant, only a few infertile flowers reached the full size as does the wild type, and most are degenerate. B, No detectable difference between the wild type and the *rel1* mutant is found during the primordium initiation of floral organs. C to E, SEM analysis of floral organ differentiation in the wild type and the *rel1* mutant. At the stage when the petals enclose stamens and carpel in the wild type (C), the inner two whorls of reproductive organs were exposed (C–E). In the *rel1* mutant, the carpel is frequently split (D), and symmetric petals are occasionally observed (E). Bars = 1 mm in A and 100 $\mu$m in C to E.

### Cloning of *REL1* and *REL3*

A map-based cloning strategy was utilized to clone the *REL1* and *REL3* loci. The *REL1* locus was positioned on chromosome 1 between markers BM1800a and BM1800b, which contains nine putative genes by annotation analysis (Fig. 3A). Sequence comparison of the wild type and *rel1* mutants in this region revealed a nucleotide substitution in *rel1*, leading to a premature stop codon in the coding region of the homolog of Arabidopsis *SGS3* (Fig. 3B). The *REL3* locus was mapped to chromosome 2 and then was narrowed down to a region between markers BM1861 and TM1805, where eleven 11 putative genes were identified (Fig. 3C). Further analysis revealed that *rel3* carried a retrotransposon insertion in the coding region of the gene encoding an ARGONAUTE protein with PAZ and PIWI domains (Fig. 3D). The coding sequence of *REL1* and *REL3* was obtained by reverse transcription (RT)-PCR. Phylogenetic analysis further indicated that REL1 is a likely homolog of Arabidopsis SGS3 and maize LBL1, while REL3 was closely related with Arabidopsis ZIP/AGO7 and rice SHL4 (Supplemental Fig. S1, A and B).

A 10-kb genomic fragment containing the *L. japonicus SGS3* homolog in the *REL1* region was transformed into the *rel1* mutant by stable *Agrobacterium tumefaciens*-mediated gene transfer. In resulting transformants, the mutant compound leaf and flower phenotypes were fully recovered (Supplemental Fig. S2). To test the possible genetic interaction between *rel1* and *rel3*, genetic analysis was conducted, and the *rel1rel3* double mutants were obtained. No detectable difference was found between the double mutants and the single

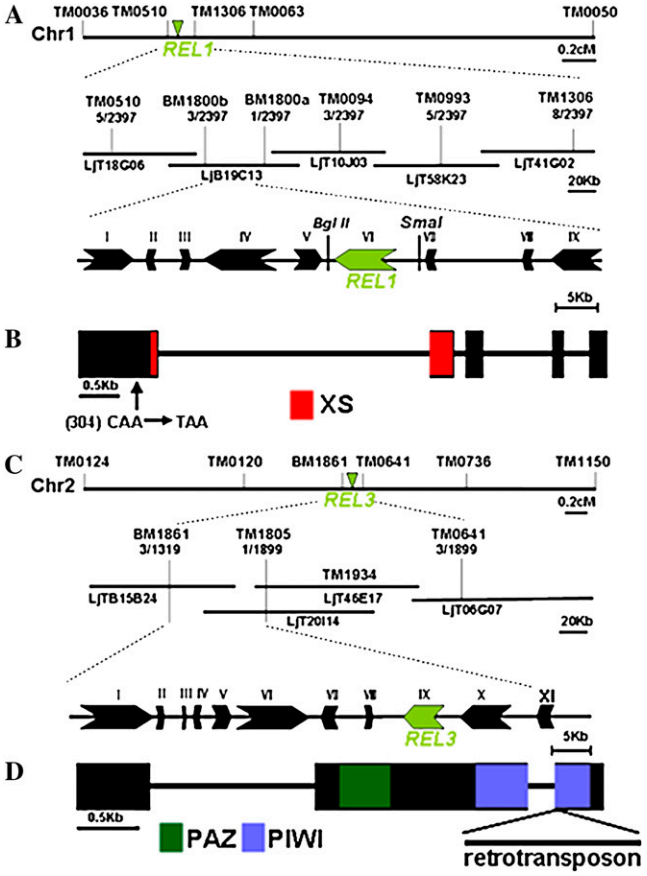


**Figure 3.** Cloning of *REL1* and *REL3* genes. A, Fine structure mapping to localize *REL1* on chromosome 1 between markers BM1800a and BM1800b; the *Bgl*II-*Sma*I genomic fragment containing the *REL1* gene was used in the complementation experiment. B, The *REL1* gene structure and position of nucleotide changes in *rel1* mutants. The red box represents a region encoding the XS domain. C, Fine structure mapping to localize *REL3* on chromosome 2 between markers BM1861 and TM1805. D, The *REL3* gene structure and position of insertion in *rel3* mutants. The green and blue boxes indicate the region encoding the PAZ domain and PIWI domain, respectively.

mutant (data not shown), indicating that *REL1* and *REL3* act in the same genetic pathway. Accordingly, we conclude that *REL* genes should encode different components in the same ta-siRNA pathway, and their mutations are responsible for the *rel* phenotypes.

### Molecular Characterization of *REL1* and *REL3*

We analyzed the expression of *REL1* and *REL3* by RT-PCR, and both transcripts were detected in all tissues tested, including aerial organs and roots (Fig. 4A). The broad mRNA expression profiles of *REL* genes were consistent with their roles in regulating multiple developmental processes. Transcript abundance of *REL1* and *REL3* was dramatically reduced in *rel1* and *rel3* mutants, respectively (Fig. 4B), indicating that the mutations in *rel1* and *rel3* plants may affect both protein function and mRNA stability.

RNA in situ hybridization was conducted to further examine spatial expression patterns of *REL1* and *REL3* in the wild type. At the vegetative stage, transcripts of *REL1* were detected throughout the SAM, young leaf primordial, and developing leaflets (Fig. 4, C and D). However, the domain of *REL3* expression was largely limited to central region of the SAM (Fig. 4G). In the newly emerging leaf primordium, *REL3* accumulated preferentially on the adaxial side, and the expression was retained on the adaxial side of emerging leaflet primordium (Fig. 4, G and H). At the reproductive stage, higher levels of expression of both *REL1* and *REL3* were found in the floral meristem and developing floral organs (Fig. 4, E, F, I, and J), but in the carpel, transcripts of *REL1* and *REL3* localized mainly on the inner side (Fig. 4, F and J).

Previous studies showed that *TAS3* ta-siRNA and miR390, which direct the cleavage of *TAS3* ta-siRNA precursor, are conserved between monocots and dicots (Allen et al., 2005; Liu et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). Using the Arabidopsis *TAS3* ta-siRNA sequence as a query, we BLAST-searched the current available *L. japonicus* genome database (www.kazusa.or.jp/lotus/) and identified sequence BP047016, which matched the criteria to be a precursor of *TAS3* ta-siRNA and should be a putative *TAS3* gene in *L. japonicus* (Fig. 5A). The predicted *L. japonicus TAS3* ta-siRNA carried only one nucleotide mismatch with the Arabidopsis *TAS3* ta-siRNA (Fig. 5B). The precursor of miR390 with the ability to form stem-loop structures was also found (Fig. 5C). The accumulation of *TAS3* ta-siRNA in the wild type and *rel* mutants was compared using small RNA blots. The accumulation of *TAS3* ta-siRNA was evident in the wild type but was abolished in *rel* mutants (Fig. 5D). By comparison, the expression level of a miRNA, namely miR164, did not show any difference between the wild type and *rel* mutants (Fig. 5D). These results confirmed that *REL1* and *REL3* were the key components required for the biogenesis of *L. japonicus TAS3* ta-siRNA.

### Isolation and Expression Analysis of Direct and Indirect Targets Regulated by *REL1* and *REL3*

*ARF3* and *ARF4* are direct targets of *TAS3* ta-siRNA, and the mechanism of this regulation is well conserved in both monocots and dicots (Allen et al., 2005; Williams et al., 2005). In *L. japonicus*, several partial sequences homologous to *ARF3* were identified from the current *L. japonicus* genome database. Based on this sequence information, we cloned two copies of *ARF3* homolog using RACE PCR. Further identification and analysis of the corresponding TAC clones revealed that the *ARF3* homologs in *L. japonicus* genome were duplicated, and the two *ARF3* homologs shared 69.1% similarity and 64.0% identity, respectively. One putative *ARF4* homolog in *L. japonicus* was also identified. Phylogenetic analysis confirmed their close relationship with *ARF3* and *ARF4* in *Arabidopsis*, respectively (Supplemental Fig. S1C). Therefore, these *ARF* genes

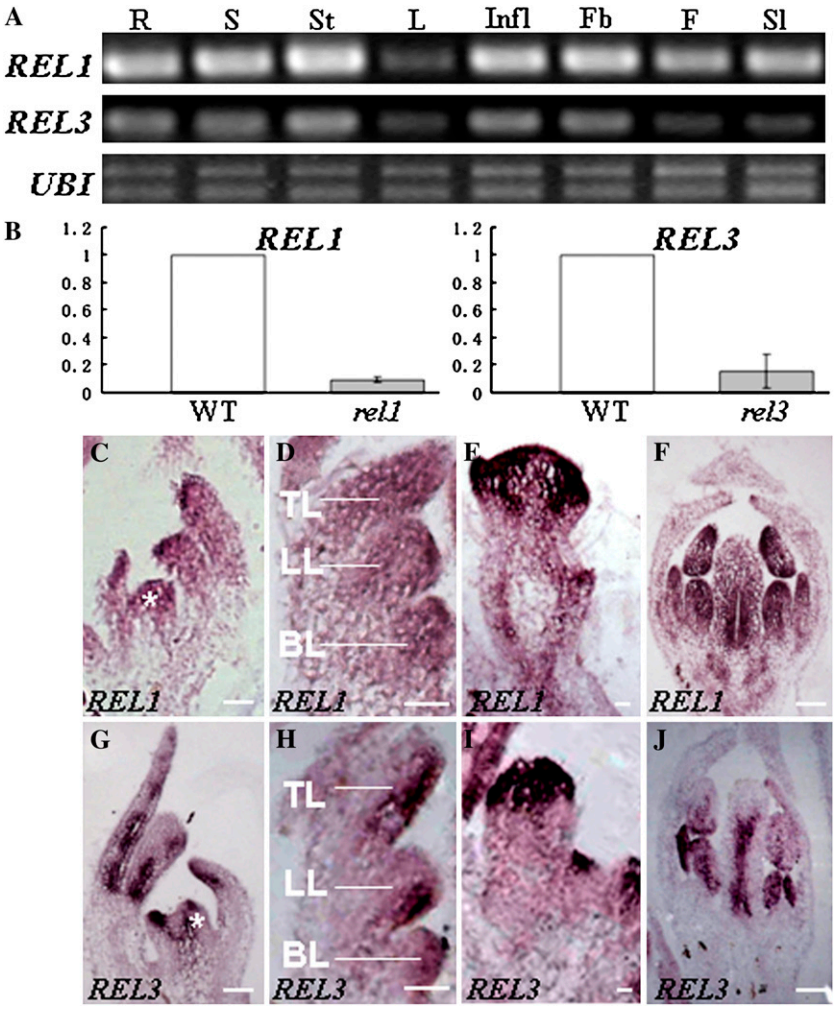


**Figure 4.** Expression analysis of *REL1* and *REL3*. A, RT-PCR analysis of *REL1* and *REL3* expression in all plant tissues examined. R, Root; S, seedling; St, stem; L, leaf; Infl, inflorescence; Fb, floral bud; F, flower; Sl, silique. B, Real-time PCR analysis of the transcript levels of *REL1* and *REL3* in the wild type (WT) and mutants. Quantification was normalized to that of *UBI* and then to the value of wild-type Gifu plants, whose value was arbitrarily fixed at 1. Bars show SE. C to F, An antisense probe of *REL1* is used in the RNA in situ hybridization on longitudinal sections of vegetative and reproductive shoot apices of the wild type. G to J, An antisense probe of *REL3* is used. Asterisks, meristem; TL, top leaflet primordium; LL, lateral leaflet primordium; BL, basal leaflet primordium. Bars = 50 $\mu$m.

were named *LjARF3a*, *LjARF3b*, and *LjARF4*, respectively. Both *LjARF3a* and *LjARF3b* contain two adjacent sequences in the coding region that are complementary to *TAS3* ta-siRNA, whereas *LjARF4* carries only one complementary sequence (Fig. 6A). We then compared the expression level of these three *ARF* genes in the wild type and *rel* mutants. We found that the transcript levels of these genes were all up-regulated in seedlings, leaves, and inflorescence of *rel* mutants (Fig. 6B), though the up-regulation of *LjARF3a* and *LjARF3b* was more dramatic than that seen for *LjARF4* (Fig. 6B).

Among the genes involved in the adaxial-abaxial patterning of lateral organs in simple-leafed species, *YAB* genes are the common downstream targets of other polarity genes (Lin et al., 2003; Eshed et al., 2004; Li et al., 2005; Garcia et al., 2006; Xu et al., 2006) and had been reported to be regulated indirectly by ta-siRNA pathway in both Arabidopsis and maize (Juarez et al., 2004; Li et al., 2005; Garcia et al., 2006). The expression of homologs of *YAB* genes was also analyzed in wild-type and *rel* mutant plants in *L. japonicus*. Based on the sequence information from the current *L. japonicus* genome database, a full-length cDNA of *LjYAB1*, which shares the highest similarity with Arabidopsis *YAB1/FILAMENTOUS* (Supplemental Fig. S1D), was first isolated to allow expression analysis. In the vegetative stage, *LjYAB1* accumulated predominantly in the abaxial region of leaf primordia and leaflet primordia (Fig. 7A). In reproductive stage, transcripts of *LjYAB1* were detected in floral organ primordia, but in the sepal and petal primordia, they were mainly confined to the abaxial region (Fig. 7B). In *rel* mutants, the *LjYAB1* expression pattern was not altered in the primordia of lateral organs (Supplemental Fig. S3); however, its expression level was dramatically reduced (Fig. 7C).

## DISCUSSION

### Many Parallels between the Dicot *L. japonicus* and the Monocots in the Regulation of Lateral Organs by the ta-siRNA Pathway

In this study, we cloned two *REL* genes in *L. japonicus* (Fig. 3, A–D) and showed that in these

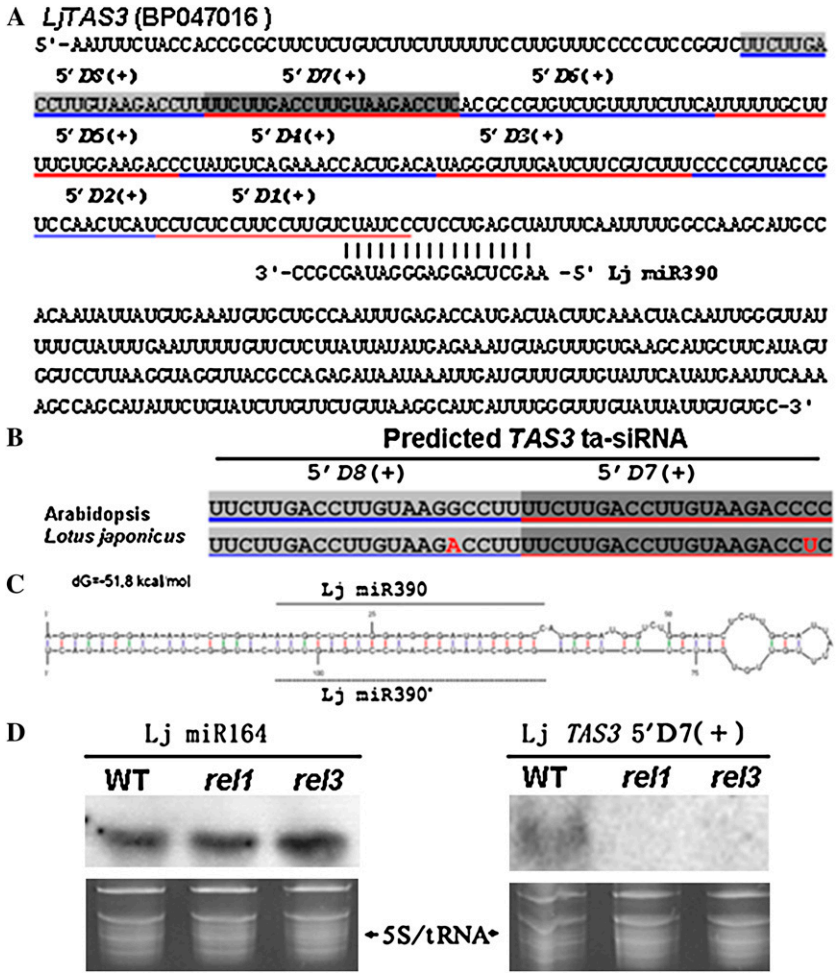


**Figure 5.** Characterization of the *TAS3* ta-siRNA precursor and *TAS3* ta-siRNA in *L. japonicus* (Lj). A, Predicted ta-siRNAs in *LjTAS3* (BP047016; underlined in red or blue). B, Alignment of the sequences of *AtTAS3* ta-siRNA and *LjTAS3* ta-siRNA (one base mismatch is indicated in red). C, Predicted stem-loop structure of *LjmiR390*. The positions of mature LjmiR390 and complementary LjmiR390* are shown. D, Northern-blot analysis of the accumulation of *LjTAS3* ta-siRNA in the wild type (WT), *rel1*, and *rel3*. *L. japonicus* miR164 is used as a control.

mutants, the production of *TAS3* ta-siRNAs was affected (Fig. 5D). We conclude that *REL1* and *REL3* are the functional orthologs of *SGS3* and *ZIP* in Arabidopsis, respectively. Both *REL1* and *REL3* are key components of the ta-siRNA pathway in *L. japonicus*. It has been shown that the ta-siRNA pathway is involved in different developmental processes in both monocots and dicots. In Arabidopsis, it is found that *SGS3* and *ZIP* play an important role in the control of vegetative phase change but have subtle effect on lateral organ morphology (Hunter et al., 2003; Peragine et al., 2004). However, when the ta-siRNA pathway is blocked in monocots, dramatic and pleiotropic effects on plant development, including disruption of SAM formation and patterning of lateral organs, were observed (Timmermans et al., 1998; Satoh et al., 1999; Itoh et al., 2000; Liu et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). In *L. japonicus*, the *rel* mutants also displayed severe developmental defects in lateral organs, suggesting similarity between *L. japonicus* and the monocots for the ta-siRNA pathway in the control of lateral organ development.

*ARF3* and *ARF4* are the key effectors in the ta-siRNA pathway. In this study, two copies of *ARF3* homolog were identified in the genome of *L. japonicus*, and both were regulated by the ta-siRNA pathway (Fig. 6, A and B). In rice and maize, *ARF3* homologs are also duplicated (Liu et al., 2007; Nogueira et al., 2007), while the Arabidopsis genome contains only one copy of *ARF3*. It is possible that the severe developmental defects in the ta-siRNA-deficient mutants are partially due to the increased dosage effects of the duplicated *ARF3* homologs in maize, rice, and *L. japonicus*. In Arabidopsis ta-siRNA-deficient mutants, the up-regulation of endogenous single-copy *ARF3* accounts for the altered vegetative phase transition but causes only subtle aberrant phenotype in lateral organs (Fahlgren et al., 2006; Hunter et al., 2006). However, severe developmental defects in leaf and floral organs, which are similar to those observed in maize, rice, and *L. japonicus* ta-siRNA-deficient mutants, could be found when extra copies of *ARF3* were introduced into the *rdr6* mutant (Fahlgren et al., 2006). Therefore, duplication of *ARF3* homologs during evolution may be one of the molecular bases underlying the functional diversity of the ta-siRNA pathway in different species.

In addition to directly regulating the *ARF* genes, the ta-siRNA pathway also regulates members of the *YAB*

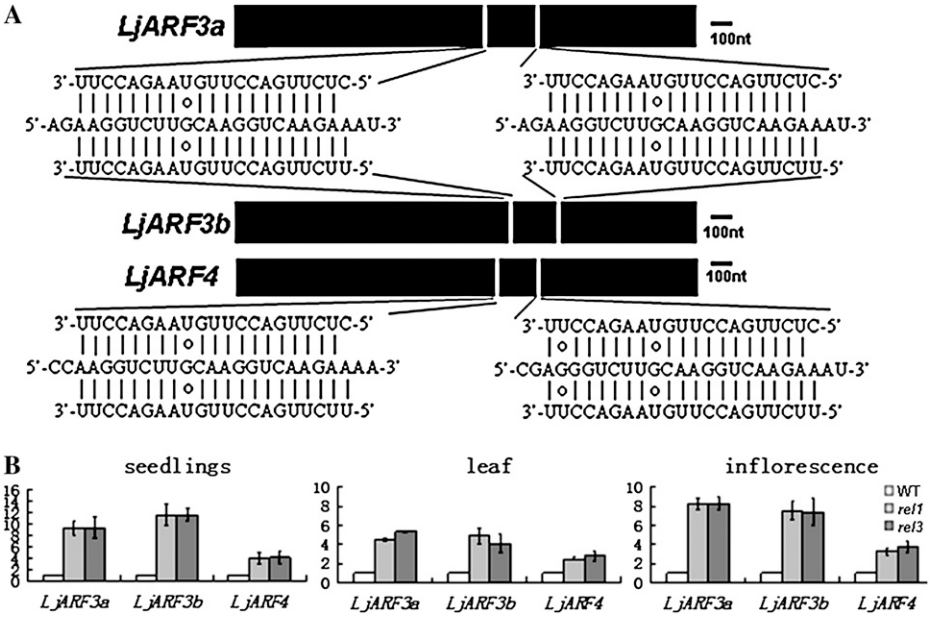


**Figure 6.** Isolation and expression analysis of *LjARF3a*, *LjARF3b*, and *LjARF4*. A, Diagrammatic representation of coding sequence of *LjARF3a*, *LjARF3b*, and *LjARF4* with *TAS3* ta-siRNA complementary sites. B, Real-time PCR analysis of transcript levels for *LjARF3a*, *LjARF3b*, and *LjARF4* in the wild type (WT) and *rel* mutants. Quantification was normalized to that of *UBI* and then to the value of wild-type Gifu plants, whose value was arbitrarily fixed at 1. Bars indicate SE.

gene family indirectly, but the regulatory mode appears to be different in different species. Arabidopsis *YAB* genes are expressed on the abaxial side of the developing leaf and contribute to the specification of abaxial identity and laminar expansion (Siegfried et al., 1999; Eshed et al., 2004). Although *YAB* expression is not altered in single ta-siRNA-defective mutants, nor in the *as1* or *as2* single mutants, the expression of *YAB* gene is up-regulated in mutants in which *AS1* or *AS2* and key components of the ta-siRNA pathway are simultaneously inactivated, suggesting that the ta-siRNA pathway negatively regulates *YAB* genes (Li et al., 2005; Garcia et al., 2006). In maize, the function of *YAB*-like genes may be associated with lateral blade outgrowth rather than specifying adaxial cell fate. Expression of *YAB*-like genes is limited to the adaxial side of leaf primordia and is repressed in *lbl1* mutants, indicating that *YAB* genes are positively regulated by the ta-siRNA pathway (Juarez et al., 2004). In *L. japonicus*, we found that *LjYAB1* is predominantly expressed in the abaxial side of both vegetative and reproductive organ primordia (Fig. 7, A and B). In *rel* mutants, the expression level of the *LjYAB1* gene is dramatically reduced in both narrow leaves and tiny flowers (Fig. 7C). The reduction in *LjYAB1* expression in *rel* mutants may be associated with the reduced lamina.

### Multiple Roles for the ta-siRNA Pathway in Controlling Compound Leaf Development in *L. japonicus*

The ta-siRNA pathway plays a conserved role in specifying leaf adaxial identity in Arabidopsis, maize, and rice (Garcia et al., 2006; Xu et al., 2006; Liu et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). However, all these species possess simple leaves, and the contribution of the ta-siRNA pathway in controlling compound leaf development is largely unknown. *L. japonicus*, like most legume species, has compound leaves consisting of one petiole and several leaflets (Fig. 1A). In *rel* mutants, narrow and needle-like leaflets with loss of adaxial identity are observed (Fig. 1, A, D, and E). Analysis of expression patterns

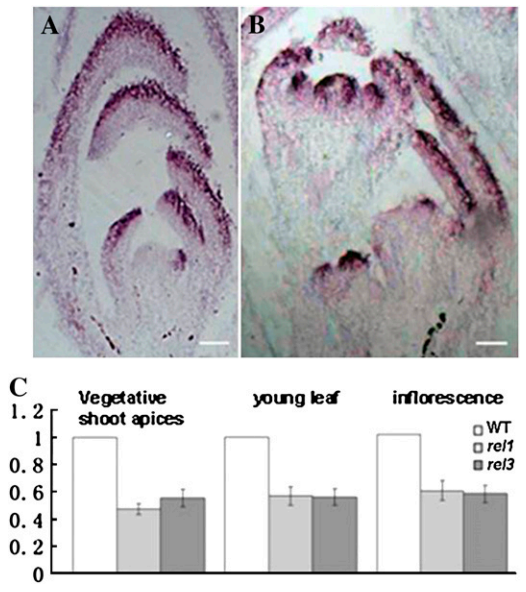


**Figure 7.** Expression analysis of *LjYAB1*. A and B, RNA in situ hybridization on longitudinal sections of vegetative and reproductive shoot apices in the wild type (WT) using an antisense *LjYAB1* probe. Bars = 50 $\mu$m. C, Real-time PCR analysis of the transcript levels of *LjYAB1* in the wild type and *rel* mutants. Quantification was normalized to that of *UBI* and then to the value of wild-type Gifu plants, whose value was arbitrarily fixed at 1. Bars indicate SE.

of *REL* genes demonstrates that *REL1* is expressed more broadly than *REL3*, but both genes accumulate in the adaxial domain of leaf primordia, where the transcripts of *REL3* are predominantly accumulated (Fig. 4, C, D, G, and H). These data indicate that *REL* genes are required for specifying leaf adaxial identity; thus, the ta-siRNA pathway has a conserved function in the establishment of leaf adaxial-abaxial polarity in both simple and compound leaf development.

The conspicuous loss of basal leaflets in *rel* mutants suggests that the ta-siRNA pathway is also involved in the determination of leaflet formation during compound leaf development in *L. japonicus* and the basal leaflet formation seems to be more sensitive to the ta-siRNA pathway than the top leaflet and lateral leaflet formation. Unlike most compound-leafed species, in which Class 1 *KNOTTED1-like* (*KNOX1*) genes play a critical role in the determination of leaflet formation (Bharathan et al., 2002), legume species such as pea and alfalfa (*Medicago sativa*) utilize the *FLORICAULA* (*FLO*)/*LEAFY* (*LFY*) ortholog in place of the *KNOX1* genes to control leaflet formation (Champagne et al., 2007). Loss-of-function mutations in the *FLO/LFY* ortholog, such as *UNI* of pea and *SGL1* of *Medicago truncatula*, cause dramatic reduction in leaflet number (Hofer et al., 1997; Wang et al., 2008). Slightly reduced leaflet number is also observed in soybean (*Glycine max*) RNA interference transgenic lines with decreased expression of *FLO/LFY* ortholog (Champagne et al., 2007). Consistently, the *proliferating floral meristem* (*pfm*) mutant of *FLO/LFY* ortholog in *L. japonicus* gives rise to aberrant compound leaves lacking one or two basal leaflets (Dong et al., 2005). Thus, it raises a question whether the reduced leaflet formation in *rel* mutants is associated with altered action of the *PFM*. Our preliminary data indicated that no detectable alteration of both expression level and expression pattern of *PFM* was found in *rel* mutants (data not shown). Furthermore, subtle differences in the petiole can be found between *rel* mutants and the *pfm* mutant. The petiole length of *rel* mutants is not altered, and this is in contrast to the shortened petiole phenotype observed in *pfm* (Dong et al., 2005). This indicates that regulation of compound leaf development by the ta-siRNA pathway may be independent of the *FLO/LFY* pathway in *L. japonicus*.

Recent work revealed that apart from the typical *KNOXI* and *FLO/LFY* pathways, other factors were also found to be required for the leaflet formation in compound leaf development. In tomato, TCP transcription factors play a central role in the modulation of compound leaves and prevent leaflet formation via limiting growth (Ori et al., 2007). In *Cardamine hirsuta*, the promotion of leaflet formation was shown to be correlated with auxin activity (Barkoulas et al., 2008). It has been shown that the *NO APICAL MERISTEM/CUP-SHAPED COTYLEDON* boundary genes could have a non-cell-autonomous effect on leaflet formation within different eudicot compound-leafed species, including legume (Blein et al., 2008). Thus, future work to discover possible connections of these factors with the ta-siRNA pathway will provide new insight into the function of the ta-siRNA pathway in the control of compound leaf development.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Lotus japonicus* Gifu B-129 was used as the wild-type control and the parental line in the mutagenesis experiments. *L. japonicus* Miyakojima MG-20 was used as another parental line in the crossing to construct mapping populations. All plants were grown at 20°C to 22°C with a 16-h-light/8-h-dark photoperiod at 150 mE $m^{-2}$ $s^{-1}$. The *rel1* and *rel3* mutants were isolated from an ethyl methanesulfonate-mutagenized M2 population. Offspring segregating in the M4 generations with 3:1 ratios of the wild type and mutants were backcrossed thrice to Gifu B-129.

### Map-Based Cloning

Mapping of the *REL1* and *REL3* loci was performed by analysis of F2 populations generated by crossing the *Rel1rel1* and *Rel3rel3* heterozygotes with MG-20. F2 homozygous mutant plants were selected, and genotyping was conducted using simple sequence repeats markers from the *L. japonicus* genome Web site.

### Transgenic and Complementation Experiments

For genetic complementation, a *Sma*I-*Bgl*II fragment (10.0 kb) containing the entire *REL1* gene was excised from BAC clone LjB19C13 (BM1800) and ligated into the *Sma*I-*Spe*I site of binary vector pCAMBIA1301. A derived cleaved amplified polymorphic sequence marker was designed to isolate *rel1* homozygotes using primers 5′-CTTCGGGAAATCCCTGGCATGCA-3′ and 5′-CATCCATGCTCCAACGGAGGTCGA-3′ and the enzyme EcoT22I. The resultant construct was introduced into *rel1* plants. Transformation was carried out as described (Feng et al., 2006). Twelve independent transformants were obtained, of which eight displayed the complemented phenotype.

### Microscopy

SEM was conducted according to the methods described previously (Chen et al., 2000). The tissues for histology analysis were fixed and sectioned as described previously (Luo et al., 2005).

### Gene Cloning and RT-PCR

RACE PCR, semiquantitative RT-PCR, and real-time PCR were performed as described (Feng et al., 2006). Sequences of all primers are given in Supplemental Table S1.

### In Situ Hybridization

Tissues for in situ hybridization was fixed, sectioned, and hybridized to digoxigenin-labeled RNA probes as described previously (Coen et al., 1990). The probes were generated from the relevant cDNA fragment with gene-specific primers (sequences of all primers are given in Supplemental Table S1).

### Small RNA Filter Hybridization

Vegetative shoot apices were used to extract RNA. RNA extraction and filter hybridization were conducted as described previously (Liu et al., 2007). $^{32}$P-end labeled LNA probe complementary to *TAS3* ta-siRNA (5′-TTCTTGACCTTGTAAGACCTC-3′) and probe to miR164 (5′-TGGAGAAGCAGGGCACGTCA-3′) were used to detect ta-siRNA and miRNA, respectively.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FJ617269 to FJ617274.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phylogenetic tree of protein sequences.

**Supplemental Figure S2.** Complementation of *rel1* mutants.

**Supplemental Figure S3.** Expression analysis of *LjYAB1* in wild-type and *rel* mutants leaves.

**Supplemental Table S1.** Oligonucleotide primers used in our experiments.

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