Genetic control of floral zygomorphy in pea (Pisum sativum L.)

Zheng Wang*,†, Yonghai Luo*,†, Xin Li†, Liping Wang*, Shilei Xu*, Jun Yang*, Lin Weng*, Shusei Sato‡, Satoshi Tabata§, Mike Ambrose¶, Catherine Rameau†, Xianzhong Feng*, Xiaohe Hu*, and Da Luo**

*National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200032, China; †School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China; ‡Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan; ¶Department of Crop Genetics, John Innes Centre, Colney, Norwich NR4 7UH, United Kingdom; and §Station de Génétique et d’Amélioration des Plantes, Institut J.P. Bourgin, UR 254 INRA, Versailles, France

Edited by Enrico Coen, John Innes Centre, Norwich, United Kingdom, and approved May 13, 2008 (received for review April 4, 2008)

Floral zygomorphy (flowers with bilateral symmetry) has multiple origins and typically manifests two kinds of asymmetries, dorsoventral (DV) and organ internal (IN) asymmetries in floral and organ planes, respectively, revealing the underlying key regulators in plant genomes that generate and superimpose various mechanisms to build up complexity and different floral forms during plant development. In this study, we investigate the loci affecting these asymmetries during the development of floral zygomorphy in pea (Pisum sativum L.). Two genes, LOBED STANDARD 1 (LST1) and KEELED WINGS (K), were cloned that encode TCP transcription factors and have divergent functions to constitute the DV asymmetry. A previously undescribed regulator, SYMMETRIC PETALS 1 (SYPI), has been isolated as controlling IN asymmetry. Genetic analysis demonstrates that DV and IN asymmetries could be controlled independently by the two kinds of regulators in pea, and their interactions help to specify the type of zygomorphy. Based on the genetic analysis in pea, we suggest that variation in both the functions and interactions of these regulators could give rise to the wide spectrum of floral symmetries among legume species and other flowering plants.

F

lower development in higher plants gives rise to an enormous variation of flower morphology and immense aesthetic diversification in nature. An important aspect for divergent floral developments is the establishment of floral symmetries, where a few distinct basic forms could be distinguished (1, 2): the monosymmetry (zygomorphy, with one symmetric plane), polysymmetry (actinomorphy, with several symmetric planes), and left–right asymmetry (with no symmetric plane). Among these, zygomorphy is considered the more specialized form and has been the most under investigation for its origin and underlying mechanisms.

Fabaceae (legumes) is one of the largest families in angiosperm, with a range of floral symmetric forms, and its success is thought to be coupled with its predominant zygomorphic flowers (3, 4). Most zygomorphic flowers are found in the subfamily Papilioideae (5, 6), which attracted the attention of researchers since the end of the 18th century (7). Darwin (8) demonstrated the role of this type of zygomorphy in pollination biology, and the special floral shape of papilionoid legumes was an important factor in Mendel’s groundbreaking work on the laws of genetic inheritance in the 1850s. Pea flowers, like most zygomorphic flowers, possess prominent corolla with three petal types, which are arranged along a dorsoventral (DV) axis, and manifest two types of asymmetries: DV asymmetry in the floral plane and organ internal (IN) asymmetry in the floral organ plane (Fig. 1a). It is well documented that DV asymmetry in papilionoid legumes comes in the floral meristem when the asymmetric development of floral organ primordia occurs (Fig. 2a) (5, 6).However, IN asymmetry is variable among petals: one dorsal petal (the standard) is IN symmetric, and two lateral (the wing) and two ventral petals are IN asymmetric (the two ventral petals are united on the lower edge and form a keel). This raises the question of how key regulators generate DV and IN asymmetries and superimpose them during zygomorphic development. The conspicuous zygomorphic flower of pea, for which there is a large collection of mutants, makes it a good model for exploring the key regulators to determine floral symmetry. For example, two loci, KEELED WINGS (K) and LOBED STANDARD 1 (LST1), were identified, respectively, in 1919 and 1985 (9, 10). The mutants at the LST1 locus give rise to the abnormal shape in the dorsal petals (Fig. 1c) (10), whereas the k-1 mutant has been used as a morphological marker in genetic analysis for its homeotic transformation phenotype, i.e., the lateral petals mimic the ventral in size, shape, and color (Fig. 1b). K in pea could be an ortholog of KEELED WINGS in Lotus 1 (KEWI), because their mutants share a similar phenotype by bearing ventralized lateral petals (Fig. 1 b and f), and both are located in the syntenic regions in the pea and Lotus genomes, respectively (12). However, neither LST1 nor K has been cloned, partly because of the difficulty caused by the large genome size of pea.

It has been shown that zygomorphy has multiple origins and plays a key role in the speciation and diversification in flowering plants (3, 13). Concomitant with their independent origins, variations in petal arrangement manifest in various superimpositions of the DV and IN asymmetries in distinct species. The flower in snapdragon (Antirrhinum majus) of the Asterid family represents another type of zygomorphy: an IN symmetric petal is positioned in the ventral, and two dorsal and two lateral petals with IN asymmetry are in pairs along the DV axis, in contrast to the arrangement in pea flower (12). Thus, the constitution of the two asymmetries may establish the developmental framework for different floral zygomorphies and give a clue as to the divergent actions of the underlying regulators among plant genomes. It has been shown that, in Antirrhinum, two closely related genes, CYCLOIDEA (CYC) and DICHTOTOMA (DICH) (14, 15), are the key regulators that establish DV asymmetry, encode TCP transcription factors, and are expressed in the dorsal region of the floral meristem (16). These two regulators are also responsible for the elaboration of organ IN asymmetry and regulate and/or interact with two distinct MYB proteins, 


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1Z.W., Y.L., and X.L. contributed equally to this work.

**To whom correspondence should be addressed. E-mail: dluo@sibs.ac.cn.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803291105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
DIVARICATA (DIV) and RADIALIS (RAD), respectively, to
determine lateral and ventral identities (17–19). Despite the
prominent difference in the zygomorphies between pea and
Antirrhinum, recent studies in papilionoid legumes show that
CYC orthologs play a key role in determining dorsal identity in
two legume species, Lotus japonicus and Cadinia (12, 20). Never-
theless, the CYC-like TCP genes, like DV regulators in distinct
categories, could have divergent functions apart from their common
one in the control of DV identity: abolishing the activity of DV
regulators in Antirrhinum gives rise to a default ventralized floral
form, with all petals manifesting bilateral symmetry (the cyc dich
flower, ref. 14), demonstrating that both CYC and DICH work
together to determine IN asymmetry (15). However, a default
ventralized form in papilionoid legumes should have all petals,
which mimic the asymmetric shape of the ventral petal in wild
type (12), suggesting that the CYC-like TCP genes might not be a
prerequisite in the control of IN asymmetry in legumes.
Therefore, different types of zygomorphies could be generated
either by the divergent functions of the common key regulators
and/or by the evolution of other distinct regulators.

In this study, we cloned K and LST1 loci and demonstrated
that they are DV regulators in the control of lateral and dorsal
identities, respectively, and that they originate from the dupli-
cation of an ancestral TCP gene during the speciation of
papilionoid legumes. A locus, SYMP (SYMMETRIC PETALS 1,
named for its mutant flowers bearing all symmetrical petals
without normal IN asymmetry), was isolated, whose function is
to establish IN asymmetry of petals. In the genetic analysis, three
default floral forms with absent DV or/and IN asymmetries were
identified in pea, demonstrating that DV and IN asymmetries
can be independently controlled by distinct regulators, and that
their interaction is important for the zygomorphic development.
Taking these together, we propose that variation in both the
entities and interactions of these regulators in the control of DV
and IN asymmetries could give rise to the wide spectrum of floral
symmetries among flowering plants.

**Results**

**k and LST1 Display Deficiency in the Development of DV Asymmetry.**

The floral morphogenesis of pea mutants at two loci, K and
LST1, was characterized by using SEM in the eight develop-
mental stages according to previous studies (20, 21). k-1, k-2, and
k-3 display the same phenotype, and no other detectable phe-
notype was observed apart from the ventralized petals at the lateral
position (Fig. 1b). In lst1-1, lst1-2, and lst1-3, flowers bear abnormal
standard petals with lobes whose sizes could be sensitive to growth
conditions (Fig. 1c). The visible differences between wild type and
k or lst1 commence at the same stage (stage 6), when the vascular
tissues are developed in petals, and the asymmetric shape of both
lateral and ventral petals becomes obvious (Fig. 2a). At this stage,
the lateral petals in K mimic the ventral in shape, whereas the dorsal
petal in lst1 is retardad in comparison with their wild-type coun-
terparts (Fig. 2d). In mature flowers, alteration of lateral and dorsal
identities in both k and lst1 is evident, as judged by the abnormal
appearance of the lateralized and ventralized epidermal cell types
of the lobed dorsal petals of lst1 and the lateral petals of k,
respectively (Fig. 2c).

When lst1-1 was introduced into k-1 backgrounds, a high
percentage of flowers displayed variable organ numbers in the
dorsal region (Fig. 2b), with absent dorsal petal or more sepal
being the most common (data not shown). In a comparison,
5% lst1-1 flowers displayed a similar phenotype. SEM analysis
showed that primordium initiation was affected in the dorsal
region (Fig. 2a), and the ventralized epidermal cell could be
found in all petals (Fig. 2c). However, the asymmetric develop-
ment of the early floral meristem was not altered in the double
mutant (Fig. 2c). In the double mutants, not only were the
numbers of stamen and sepal at the dorsal region affected, but
also the length of the dorsal stamen could be longer than that of
wild type (data not shown). Although most lateral petals dis-
played perfect ventralized forms, occasionally some lateral pet-
als with conspicuous shape could be found (Fig. 1d). In Lotus,
~3–5% flowers (often under stressed conditions) bear
repetitive morphs of the lateral petal, rather than the most
mirror-image forms in kew1, the putative ortholog of k (Fig. 1g),
which manifest an abnormal left–right asymmetry. However, the
majority of dorsal petals of k lst1 in pea failed to fully expand and
therefore were smaller in size, displaying IN asymmetry to
varying degrees [Fig. 1d; supporting information (SI) Fig. S1].
Nevertheless, a small portion of flowers bore the dorsal petals,
which mimicked the shape of the ventral petal (Fig. 1d), represen-
ting a default state without DV asymmetry. Thus, during
zygomorphic development, K and LST1 play a key role in establishing lateral and dorsal identities, respectively, and their interaction for the development of dorsal organs is revealed in the double mutant.

**Both K and LST1 Encode CYC-Like TCP Proteins.** The comparative genomics approach was conducted to clone K and LST1 in pea. Previous genetic analysis has mapped the K locus in linkage group II (23). In a parallel mapping experiment in *Lotus*, kew1 was located into a 295-kb region, where a complete contig and DNA sequence were subsequently obtained (Fig. 3a). However, no recombination haplotype was found in the large mapping population within the 200-kb region containing KEW1, and sequence analysis revealed a candidate gene within the contig, which is predicted to encode a CYC-like TCP protein with 370 aa and has been reported as *LjCYC3* (12). However, there is no sequence alteration in a 4-kb region of *kew1* containing *LjCYC3*, but whose expression level was found to be decreased (data not shown). Thus, three CYC homologs, designated PsCYC1, PsCYC2, and PsCYC3, were cloned in pea (GenBank accession nos. EU574913, EU574914, and EU574915). Phylogenetic analysis indicated PsCYC3 is the ortholog of *LjCYC3* (Fig. 3b). When the gene structure of PsCYC3 was analyzed, deletions were found in both *k* and *k-3* (Fig. 3c), and a single base deletion was detected at position 350 of PsCYC3 ORF in *k-2*, which should cause a frame-shift and disrupt the conserved TCP domain (Fig. S2a). However, *LST1* was mapped and located in the linkage group VI of pea between two SSR markers, AD51 and AA200 (24). We noticed that *lst1* exhibits a similar phenotype to the *LjCYC2* mutant, *square standard 1* (*squ1*) in *Lotus* (12): both mutants have the dorsal petal phenotype and can interact with *k* or *kew1*, respectively, by giving rise to the ventralized character in all petals. Thus, the ortholog of *LjCYC2* or *PsCYC2*, was subject to detailed analysis, and an STS marker for *PsCYC2* was found to cosegregate with *lst1* in our mapping population (data not shown). When the sequence of *PsCYC2* was analyzed, a single base substitution (C-437-T) was found in *lst1*-1, which would cause an amino acid substitution (S-146-L) in the conserved TCP domain (Fig. S2a). In *VigS-PsCYC2* flowers with weak *k* phenotype (arrow) and strong *k* phenotype (arrow). (j) VIGS-GFP control. (k) VIGS-PsCYC2 in wild-type, dorsal petal with lobes (arrow). (l) VIGS-GFP control in *k-1*. (m) VIGS-PsCYC1 in *k-1*, dorsal petal is retarded (arrow).
primordium initiation, presumably modifying DV orientation. The interaction variation of (PsCYC possesses DV asymmetry. In (H11002 DV) default forms, all petals of pea flower manifest IN asymmetry, whereas (g–f) petals possess a keel structure. Yellow arrow: the additional ventral petal, syp1 flower has radial symmetry with five ventralized symmetrical petals. In d–f, all ventralized petals were cut to make them flat. (Scale bar, 10 mm.) (g) Floral diagrams of wild type and various default states in Antirrhinum and pea. In wild type, both flowers have three distinct petals and possess DV (red lines with arrow) and IN asymmetry (broken lines). In the ventralized (–DV) default forms, all petals of pea flower manifest IN asymmetry, whereas those of Antirrhinum are symmetric. In syp1, the (–IN) default form still possesses DV asymmetry. In k lst1 syp1, the (–DV–IN) default form displays radial symmetry. Based on work on Lotus, another radial symmetry form is expected when PsCYC1 are ectopically expressed, and all petals mimic the dorsal. Furthermore, flowers with left-right asymmetry could be found as the variation of (–DV) default form. (h) Functions and interactions of DV and IN regulators in pea. DV and IN asymmetries are separately controlled by PsCYCs and SYP1. PsCYCs can suppress the manifestation of IN asymmetry and couple IN with DV asymmetry. SYP1 can interact with PsCYCs to regulate organ primordium initiation, presumably modifying DV orientation. The interaction of SYP1 and PsCYCs is important for zygomorphic development in legumes.

displayed lobed or retarded standards (Fig. 3k). VIGS-PsCYC3 silenced lst1 and VIGS-PsCYC2 silenced k plants phenocopied the k lst1 double mutant to various extents (data not shown). When VIGS-PsCYC1 was tested, no phenotype was observed in VIGS-PsCYC1 silenced wild-type plants. No additive or novel phenotypes were found in the VIGS-PsCYC1 silenced lst1 or k lst1 plants. However, in VIGS-PsCYC1 silenced k plants, a portion of flowers with retarded standards were found (Fig. 3l), suggesting PsCYC1 could have a redundant function with LST1 in the interaction with K to control dorsal petal development. Thus, we concluded that both K and LST1 are cloned and encode CYC-like TCP proteins.

The expression pattern of these DV regulators was analyzed, and all were found to express in the apex of wild type during floral development, sharing an asymmetric expression pattern in the dorsal organs of the flower. Both PsCYC1 and LST1 are expressed in standards, whereas the transcripts of K can be found in both dorsal and lateral petals (Fig. 3d). As expected, no transcript of K and LST1 was detected in the deletion mutants of k or lst1, respectively; however, the transcription levels of K and LST1 bearing the point mutation were not down-regulated (Fig. 3e and f). In RNA in situ hybridization experiments, it was found that both PsCYC1 and LST1 are expressed first in the dorsal region of the floral meristem before floral organ initiation and then inside dorsal petals (Fig. S2 b–i). We failed to detect the transcripts of K by RNA in situ hybridization, presumably because of its low expression level. CYC homologs in legumes have been characterized into two major groups, LEGCYC groups I and II (26, 27), and phylogenetic analysis placed PsCYC1 and LST1 into LEGCYC group I and K into group II (Fig. 3b). Thus, the overlapped expression domains and different expression levels are consistent with the functional divergence and phylogenetic relations of these DV regulators.

**Mutant syp1 Gives Rise to a Defect in IN Asymmetry in Petals.** The default state without DV asymmetry in the k lst1, in which all petals are ventralized and manifest IN asymmetry (Fig. 1d), suggests other regulators exist, which should control IN asymmetry independent of K/LST1 function in pea. To target the hypothetical IN regulators, we conducted the screen for mutants bearing petals with altered IN asymmetry. In two large-scale mutagenesis experiments, we identified the SYP1 locus with two mutated alleles (Fig. 4 b and c). In syp1-1, nearly all petals are bilaterally symmetrical but maintain their DV identities (Fig. 4b), and the most conspicuous characteristic is that the symmetric ventral petals possess a keel structure, in contrast to the wild-type flower, whose two ventral petals form a keel (Fig. 4a and b). However, syp1-2, presumably a weaker allele, has a highly variable effect on IN asymmetry of the lateral but not the ventral petals (Fig. 4c). The SYP1 locus has been located in linkage group II of pea, and its syntenic region is anchored in both Lotus and Medicago genomes, to conduct the cloning work (data not shown).

In syp1-1, approximately one-third of the flowers have increased organ numbers in the ventral region (Fig. 2b), where abnormal primordium initiation was found during early development of the floral meristem (Fig. 2a). In syp1-1, the abnormal bilaterally symmetric shapes of the lateral and ventral petals can be observed at petal developmental stage 6, when the malfunction of petal development also occurs in k and lst1 (Fig. 2d). However, the whole floral meristem became more symmetrical than in wild type from the beginning of floral organ primordium development (Fig. 2a). When the epidermal cells of the syp1-1 petals were analyzed, both cell size and type were found to be the same as their wild-type counterparts (Fig. 2c). Therefore, the syp1-1 flower represents a default state without IN asymmetry in all petals, in contrast to the one without DV asymmetry in the k lst1.

**SYP1 and K/LST1 Are Antagonistic at the Early Stage of Floral Development.** To investigate how DV and IN regulators interact with each other during zygomorphic development, syp1-1 was introduced into k, lst1, and k lst1 genetic backgrounds, respectively. The lst1 syp1-1 double mutant displayed an additive phenotype: lobed standards and petals in the lateral and ventral positions without IN asymmetry (Fig. 4d). However, k syp1 flowers possess abnormal standards with an altered shape apart from the expected keels in both lateral and ventral regions (Fig. 4e), revealing a hidden function of SYP1 during the development of the dorsal petal. In the k lst1 syp1-1 triple mutant, the flowers display a radial symmetry (Fig. 4f), and all petals possess ventralized identity with a keel structure. Thus, a default form without DV and IN asymmetries was identified.

In the triple mutant, no other detectable phenotype was found.

Fig. 4. Interaction between PsCYCs and SYP1, floral diagrams, and the establishment of floral zygomorphy. (a–f) syp1 mutants and syp1-1 in different genetic backgrounds. Front and side views of flowers and flattened petals from wild type and mutants are shown. (a) Wild-type pea flower. (b) syp1-1 flower bears symmetrical lateral and ventral petals (white arrow). The ventral petals possess a keel structure. Yellow arrow: the additional ventral petal, which is cut along the edge of the keel. (c) syp1-2 flower with symmetrical lateral petals (white arrow). (d) lst1 syp1-1 flower displays an additive phenotype. (e) k-1 syp1-1 flower has abnormal dorsal petals (white arrow). (f) k-1 lst1 syp1-1 flower has radial symmetry with five ventralized symmetrical petals. In d–f, all ventralized petals were cut to make them flat. (Scale bar, 10 mm.) (g) Floral diagrams of wild type and various default states in Antirrhinum and pea. In wild type, both flowers have three distinct petals and possess DV (red lines with arrow) and IN asymmetry (broken lines). In the ventralized (−DV) default forms, all petals of pea flower manifest IN asymmetry, whereas those of Antirrhinum are symmetric. In syp1, the (−IN) default form still possesses DV asymmetry. In k lst1 syp1, the (−DV−IN) default form displays radial symmetry. Based on work on Lotus, another radial symmetry form is expected when PsCYC1 are ectopically expressed, and all petals mimic the dorsal. Furthermore, flowers with left-right asymmetry could be found as the variation of (−DV) default form. (h) Functions and interactions of DV and IN regulators in pea. DV and IN asymmetries are separately controlled by PsCYCs and SYP1. PsCYCs can suppress the manifestation of IN asymmetry and couple IN with DV asymmetry. SYP1 can interact with PsCYCs to regulate organ primordium initiation, presumably modifying DV orientation. The interaction of SYP1 and PsCYCs is important for zygomorphic development in legumes.
apart from floral symmetry. However, there was little variation in floral organ numbers, in contrast to a high portion of k lst1 flowers with variable organ numbers, in the dorsal region, whereas in the ventral region is notably reduced in comparison with the one in syp1-1, indicating that the malfunction of organ primordium initiation in k lst1 and syp1-1 is completely or partially suppressed in the triple mutant. The expression patterns of K, LST1, and PsCYC1 were analyzed in syp1-1 by RT-PCR, and no detectable alteration was found (data not shown). Thus, the antagonistic interaction of DV and IN regulators is found at the early stage during floral development when floral organ primordia initiate.

Discussion
Zygomorphic development, like other pattern formations in plants and animals, involves the establishment of different body planes with distinct developmental axes, where various asymmetries are generated and superimposed under the control of different regulators. The pea possesses a conspicuous zygomorphic flower with an abundant mutant collection and provides an ideal experimental system to analyze the key regulators in control of floral symmetry. However, its complex large genome (5,000 Mb, 10× the size of either Lotus or Medicago, with >90% repetitive sequence) had obscured progress in molecular analysis (28). By adopting a comparative genomics approach and other molecular genetic tools, we successfully cloned two genes in pea, K and LST1, demonstrating that the limited sequence information, large size, and complex genome are no longer impassable barriers for the molecular study of pea.

In this work, the functions of K and LST1 and PsCYC1 are characterized, which comprise a small CYC-like TCP gene cluster and originate from two duplication events of an ancient TCP gene (26, 27). Because the deletion alleles at both K and LST1 loci show no other detectable malfunction apart from the floral symmetry, it is evident that these TCP genes have been destined for the control of zygomorphic development. Their expression patterns were found to be overlapped but differ spatially and quantitatively, which is consistent with the divergent functions of LST1 and K to determine the dorsal and lateral identities of petals, respectively. It is also consistent with previous reports that alteration of expression pattern and level of TB1, a CYC-like TCP gene in maize, could lead to novel morphogenesis (29, 30). In a comparison, CYC and its close duplicate DICH in Antirrhinum determine DV asymmetry and regulate two distinct small MYB proteins, which are involved in the control of lateral and ventral identities (14, 15, 17–19). These indicate that increasing the copy number of TCP genes should be a key step for elaborating their roles in the control of floral DV asymmetry, and then different copies acquire their divergent functions as different DV regulators during zygomorphic evolution. Thus, the independent duplication events giving rise to divergent DV regulators in different species could account for the molecular basis on the development of different types of floral zygomorphies.

The conspicuously different types of zygomorphic flowers in Antirrhinum and pea demonstrate how different origins of regulators could have resulted in distinct flower forms. When DV regulator function is abolished, the default state without DV asymmetry in both species appears to be a ventralized form, and all petals acquire ventral identity (Fig. 4g). However, ventral identities in the two types of zygomorphic flowers have distinct properties: the ventral petal in Antirrhinum displays a bilaterally symmetric shape, whereas that in pea possesses IN asymmetry (Fig. 4g). An IN regulator, SYP1, should be responsible for IN asymmetry of petals when DV regulators are mutated in pea. In syp1-1, where IN asymmetry is abolished, all petals are bilaterally symmetric, but their DV identities are maintained (Fig. 4b), revealing another default state without IN asymmetry. Therefore, DV and IN regulators can separately generate different asymmetries in pea (Fig. 4g). In contrast to Antirrhinum, two DV regulators, CYC and DICH, interplay to modify IN asymmetry of the dorsal petal. Thus, our findings offer evidence to support the notion that organ IN asymmetry also has multiple origins. It is likely that the independent genetic control of DV and IN asymmetry may not be unique in pea, and two relevant pathways could have evolved independently in other species as well. Therefore, recruiting only one or both two kinds of regulators in the control of DV and IN, respectively, could give rise to various floral forms (Fig. 4g). Furthermore, variation or modification of the interaction between DV and IN regulators could generate floral forms with different asymmetries, such as the left-right asymmetric flowers in k lst1 in pea or kewl in Lotus (Fig. 1d and g) and the radial symmetrical flower in Cadia, where the expanding expression pattern of LegCYC was found (20).

The existence of the two reciprocal default forms in pea with only one DV or IN asymmetry raises the question of how the two asymmetries are superimposed and coordinated during zygomorphic development. In this study, the dorsal petal morphogenesis of pea provides a unique example to examine the interaction between IN and DV factors, because it possesses bilateral symmetry. It has been shown that ectopic expression or the altered expression domain of CYC-like TCP genes in papilionoid legumes can suppress the manifestation of IN asymmetry in lateral and ventral petals by altering their identity (12, 20). Consistently, the bilaterally symmetric shape of dorsal petal in pea can become asymmetric when both K and LST1 are mutated (Fig. 1d), indicating that SYP1 action is suppressed in the dorsal petal, where DV regulators are expressed. Other data also support this interaction: The abnormality of dorsal primordium initiation in the k lst1 is suppressed by introducing syp1-1 (Fig. 2a), and the morphology of the dorsal petal is altered in the k syp1, whereas its symmetric shape is otherwise maintained (Fig. 4e). The interaction is not limited to the dorsal petal. For example, in syp1-1, floral organ initiation in the ventral region is affected and can be partially suppressed in the k lst1 syp1 triple mutants, suggesting that both DV and IN regulators participate in organ initiation in the two polar regions of the floral meristem. Previous studies show that a TCP protein in Arabidopsis should be involved in the regulation of cell division (31). It is most likely that both DV and IN regulators could participate in similar biological function to regulate cell division and differentiation in floral meristem and floral organ primordia. Thus, both the divergent functions and the delicate interaction between the two kinds of regulators are essential to regulate zygomorphic developmental process in pea (Fig. 4b).

Using examples from a range of pea floral mutants (Fig. 4g), we suggest that both DV and IN regulators could have arisen through independent evolutionary routes in Papilionoideae and even in other species. Consequently, possession of only one or two types of regulators involved in the control of floral symmetry and the variation or modification of the interaction between them have the potential to generate the wide spectrum of floral forms with varied symmetries found in Fabaceae and among other flowering plants as well.

Materials and Methods
Plant Material and Growth Conditions. All pea lines used in this study were obtained from the John Innes Pismum Germplasm Collection, except syp1-1, k-2 arose by x-ray mutagenesis, and k-3 was derived from fast neutron mutagenesis in J116 and J2296, respectively. lst1-2, lst1-3, and syp1-2 were obtained from fast neutron mutagenesis in J2822. syp1-1 was identified from fast neutron mutagenesis in Tereze. Alleles were confirmed by crosses between k-1 and k-2, k-1 and k-3, lst1-1 and lst1-2, lst1-1 and lst1-3, and syp1-1 and syp1-2, respectively. All plants were grown at 18–20°C with a 16-h light/8-h dark photoperiod at 150 μM·m−2·s−1.
Microscopy. Nonradioactive in situ hybridization was performed essentially as described (32). SEM for mature petals was performed on plastic replicas as described (33). SEM for floral buds was prepared as described (34). Samples were examined in JEOL JSM-6360LV (JEOL).

Gene Cloning and RT-PCR. Primer sets used for amplifying PsCYC1 (SL0842/G3848), LST7 (SL0773/SLO970), and K (SL1254/SLL103) genes were designed by sequence analysis of ESTs from Medicago truncatula and CYC homologs in Lotus. Homologous alignments were performed by using the ClustalX program (version 1.83), and phylogenetic trees were computed by using the Phylip program (version 3.6). Primer pairs SL1098/SLO109, SL1100/SLL101, and SL1102/SLL103 were used for amplification of the PsCYC1, LST7, and K transcriptions, respectively. Histone H4 (GenBank accession no. U10042) was amplified with primers SL1815/SLO363 as an internal control. For in situ probes, PsCYC1 and LST7 transcripts were coamplified with primer sets SL0892/SLO933 and SL0868/SLO970, respectively, from cDNA fragments. Semiquantitative RT-PCR was performed as described (12). Genomic DNA was digested with HindIII before the genomic Southern blot analysis.

Mapping and Cosegregation Analysis. kewl was mapped as described (12). Two SSR markers, ADS1 and AA200 (24), were used to locate LST7 in linkage group VI of pea. A STS marker (LSSR1084 and LSSR1085) for PsCYC2 was used to conduct the cosegregation test for kewl in a kewl−/− × J992 population (n = 280). The cosegregation of PsCYC3 with K was performed in a 140-k−/− × J992 population by using primers SL1102/SL1103.

Virus-Induced Gene Silencing (VIGS) Assay. The VIGS assay in pea was carried out as described (25). The fragments being used for the constructs, VIGS-PsCYC1, -2, and -3, are marked in Fig. 52a. Fifteen 2-week-old plants for each construct were agroinoculated and repeated three times independently.

ACKNOWLEDGMENTS. We thank Dr. Ida Elisabeth Johansen (Danish Institute of Agricultural Sciences, Frederiksberg, Denmark) for providing the pCAPE1, pCAPE2-PDS, and pCAPE2-GFP constructs for VIGS. We acknowledge H. Xia, H. Yin, S. Hao, Z. Xu, J. Yan, and C. Li for critical comments on the manuscript. We also thank Z. Xu, J. Li, Y. Xue, G. Wang, Y. Tian, H. Lin, L. Zhuang, and Y. Liu for encouragement and support for this experiment. This work was supported by the National High Technology Research and Development Program of China (Grant nos. 2006AA10A110 and 2007AA102113), the National Nature Science Foundation of China (Grant nos. 30430330 and 30528016), and FP6-2002-FOOD-1-506223 (Grain Legumes) from the European Commission.

Mapping and Cosegregation Analysis. Figures 52A and 52C show the cosegregation of PsCYC1 with LST7 and K, respectively, from cDNA fragments. Figure 52B shows the cosegregation of PsCYC1 with LST7 and K, respectively, from cDNA fragments. Semiquantitative RT-PCR was performed as described (12). Genomic DNA was digested with HindIII before the genomic Southern blot analysis.

Wang et al.
Fig. S1. Dorsal petals in k lst1 double mutant. In k-1 lst1–1 double mutant, the dorsal petals displayed asymmetrical shape in various extents. White triangle: the most common ones. All petals are without flattening.
Fig. S2. (a) Sequence alignment of PsCYC homologs. Black line marks the conserved TCP domain. Dashed line indicates R domain. Red panes indicate the regions where the relevant DNA sequence was used to construct VIGS-PsCYC. Red pentacle: mutation site in k-2 alleles. Red triangle: mutation site in lst1–1 alleles. (b–i) Expression patterns of PsCYC1 and LST1. These two genes have overlapped expression pattern with slightly difference. PsCYC1 is firstly detected in the regions between I1 and I2 (b), whereas LST1 dispersed in I2 (f). With floral development, both genes were expressed in the dorsal region of flower primordial (c–d and g–h) and finally restricted to the dorsal petals (e and i). I1, primary inflorescence; I2, secondary inflorescence; F, floral meristem; dSe, dorsal sepal; dPe, dorsal petal. (Scale bar, 50 μm.) Primer sequences: LjSSR1084: 5′-ACAATGCGATAATAGGTTAGTG-3′ LjSSR1085: 5′-AGTTGCTTTAAATCTTACATCTT-3′ G3848: 5′-CCACACTTTAAATATTGGTC-3′ SL0363: 5′-GCCAAATCCGTAAAGAGTTC-3′ SL0773: 5′-CAATGCTTTAGCAGGTCGACGAC-3′ SL0842: 5′-TGAGTCAATCTTCAATGTT-3′ SL0868: 5′-GCAAAGAACAATAGTGAAG-3′ SL0932: 5′-CAAGCTTTAGCTTCGGGTGACGAC-3′ SL0933: 5′-CGGATCCTGATCTCAGAATATGCTG-3′ SL0970: 5′-AATACTAACTTTTCAACC-3′ SL1254: 5′-ATGTTTGAAGCTCCAGATCTTTC-3′ SL1815: 5′-AAAGATCAACTCAAATACATCAC-3′.