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Research Article

Genetic Analysis of *ele* Mutants and Comparative Mapping of *ele1* Locus in the Control of Organ Internal Asymmetry in Garden Pea

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Abstract

Previous study has shown that during zygomorphic development in garden pea (*Pisum sativum* L.), the organ internal (IN) asymmetry of lateral and ventral petals was regulated by a genetic locus, *SYMMETRIC PETAL 1* (*SYP1*), while the dorsoventral (DV) asymmetry was determined by two *CYC-like* TCP genes or the *PsCYC* genes, *KEELED WINGS* (*K*) and *LOBED STANDARD 1* (*LST1*). In this study, two novel loci, *ELEPHANT EAR-LIKE LEAF 1* (*ELE1*) and *ELE2* were characterized. These mutants exhibit a similar defect of IN asymmetry as *syp1* in lateral and ventral petals, but also display pleiotropic effects of enlarged organ size. Genetic analysis showed that *ELE1* and *ELE2* were involved in same genetic pathway and the enlarged size of petals but not compound leaves in *ele2* was suppressed by introducing *k* and *lst1*, indicating that the enlargement of dorsal petal in *ele2* requires the activities of *K* and *LST1*. An experimental framework of comparative genomic mapping approach was set up to map and clone *LjELE1* locus in *Lotus japonicus*. Cloning the *ELE1* gene will shed light on the underlying molecular mechanism during zygomorphic development and further provide the molecular basis for genetic improvement on legume crops.

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Introduction

Floral zygomorphy (flower with bilateral symmetry), as a specialized form of flower symmetry, is an important trait of flowering plants and plays a key role as an adaptation to enhance the utilization of diverse and reliable pollinators (Endress 1999). The work on the mechanism in the control of floral zygomorphy is always eye-catching and has the advantage of easy perception by people from different disciplines. It has been shown that zygomorphic flowers evolved multiple times independently from the ancestors with radial symmetry (Endress 2001; Cubas 2004; Sargent 2004).

Pea and *Lotus japonicus*, like other species in the subfamily Papilionoideae, possess the typical zygomorphic flower, which have a single large dorsal petal (the standard), two lateral petals (the wings) and two small ventral petals (the two form a keel) (**Figure 1A,C**) (Tucker 2003). The dorsoventral (DV) differentiations among different petals can be defined in aspects of shape, size and epidermal cell types (Feng et al. 2006). However, conspicuous differentiation can also be

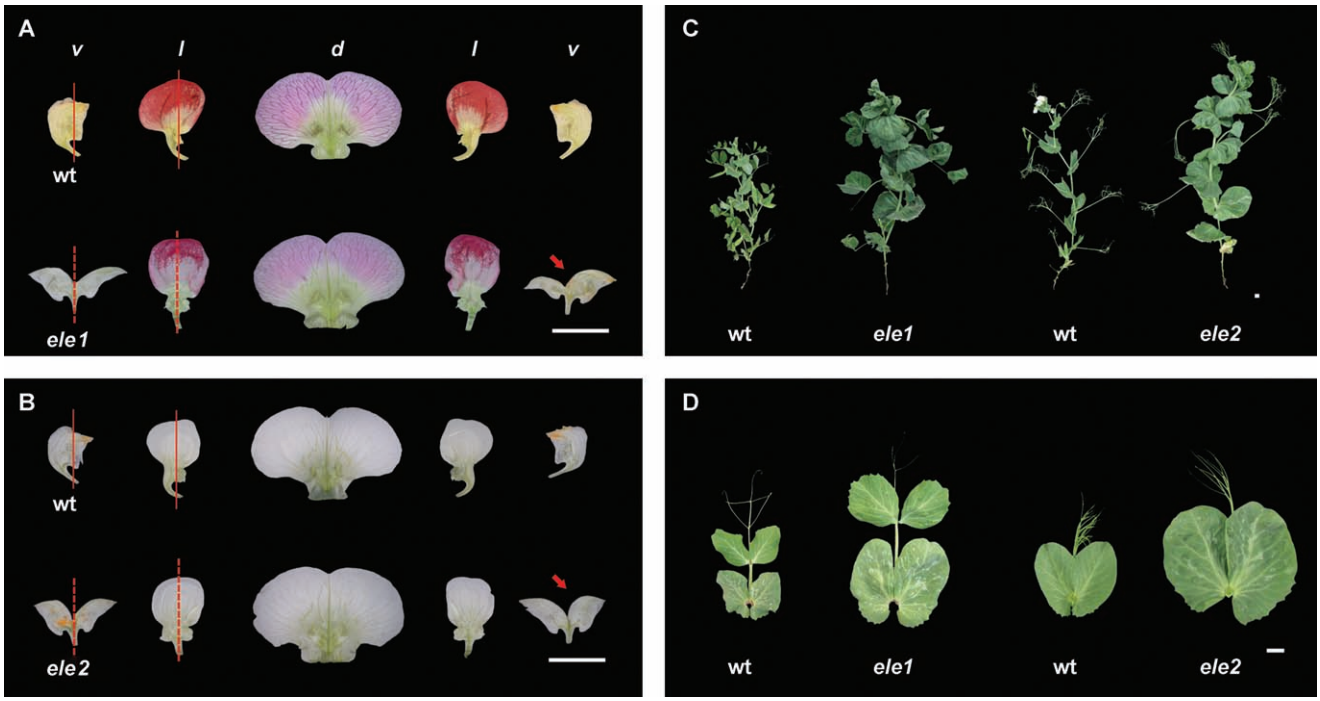

Figure 1. Phenotypes of *ele1* and *ele2* mutants.

(A) Petals of the wild type (wt) and *ele1* possess dorsoventral (DV) differentiation. Flattened petals are shown. Red lines indicate the internal (IN) asymmetry in the lateral and ventral petals of the wild type; the dotted lines indicate the abolishment of the IN asymmetry in *ele1*; the arrow indicates the cutting at the ventral petals so as to flatten the petals which possess a keel structure. *d*, the dorsal petal; *l*, the lateral petal; *v*, the ventral petal.

(B) Petals of the wild type (wt) and *ele2* possess DV differentiation. Red lines indicate the IN asymmetry in the lateral and ventral petals of the wild type; the dotted lines indicate the abolishment of the IN asymmetry in *ele1*; the arrow indicates the cutting at the ventral petals so as to flatten the petals, which possess a keel structure.

(C) Matured plants of the parent lines, *ele1* and *ele2*.

(D) The compound leaves of the parent lines, *ele1* and *ele2*. *ele2* was isolated from the Terese background, which carries the *af* locus and gives rise to a loss of leaflets, which are replaced by branched tendrils.

Scale bar, 1 cm.

expressed in variation of internal (IN) asymmetry among the petals: the lateral and ventral petals in pea and *L. japonicus* possess the IN asymmetry but the dorsal petal does not. Thus, zygomorphic flowers manifest two kinds of asymmetry: the DV asymmetry in the floral plane and IN asymmetry in the organ plane, respectively. Various forms of floral zygomorphy can be found among different species. The flower of *Antirrhinum majus* represents another type of zygomorphy, which has two asymmetrical dorsal petals, two asymmetrical lateral petals and one symmetrical ventral petal. There is thus a prominent difference in the floral zygomorphy between *A. majus* and legumes: the IN asymmetry can occur either in dorsal and lateral petals (in the case of *A. majus*), or in the lateral and ventral petals (in the case of legumes) (Wang et al. 2008). Thus, the IN asymmetry can be variable in different floral organs depending on specific species.

The molecular basis of the control of DV asymmetry was first investigated in *A. majus*. It has been found that two pairs of transcription factors are involved: two closely related TCP transcription factors, *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*) and two MYB transcription factors, *RADIALIS* (*RAD*) and *DIVARICATA* (*DIV*) (Luo et al. 1996, 1999; Galego and Almeida 2002; Corley et al. 2005; Krizek and Fletcher 2005). It has been shown that eudicot plants with zygomorphic flowers recruited ECE-*CYC2* genes in floral asymmetry through parallel evolution (Howarth and Donoghue 2006; Preston and Hileman 2009). In *Iberis amara*, *IaTCP1*, a *CYC* homolog, specifies dorsal identity in the zygomorphic flowers (Busch and Zachgo 2007). In *Gerbera hybrida, GhCYC2* expression patterns and transgenic phenotypes suggest that *GhCYC2* is involved in differentiation among *Gerbera* flower types (Broholm et al. 2008). Recent studies in papilionoid species have shown that

CYC-like TCP genes play key roles in determining dorsal and lateral identity (Feng et al. 2006; Wang et al. 2008; Citerne et al. 2006). Therefore, the molecular basis of DV asymmetry should be well conserved among different species.

The molecular mechanism in the control of IN asymmetry was also investigated in *A. majus*. It has been shown that the interplay of CYC/DICH and DIV/RAD transcription factors is responsible for the elaboration of IN asymmetry in *A. majus* (Luo et al. 1996, 1999; Galego and Almeida 2002; Corley et al. 2005; Almeida and Galego 2005). However, our recent study has shown that a locus in pea, *SYP1*, is responsible for establishing the IN asymmetry in lateral and ventral petals, and furthermore, it was found that the DV and IN asymmetries can be independently controlled during zygomorphic development in pea (Wang et al. 2008). These data suggest that an independent pathway could exist in the genomes of papilionoid legumes, and it is expected that more components in the pathway controlling IN asymmetry could be identified.

The conspicuous zygomorphic flower of pea for which there is a large collection of mutants makes it as a good model for exploring the key regulators to determine floral asymmetry. However, until recently, map-based cloning work has not been a routine in pea partially due to the difficulty caused by its large genome size and the high content of repetitive sequences. Legume ranks third among families of flowering plants, with approximately 700 genera and 19 000 species, consisting of three subfamilies of legumes, Caesalpinioideae, Mimosoideae and Papilionoideae (Doyle and Luckow 2003). Economically, legumes contribute about one-third of human's protein intake and serve as an important source of fodder and forage for animals (Graham and Vance 2003). Two legume species, *Medicago truncatula* and *L. japonicus*, have been used as model systems for studying genomics and the unique biological process in legumes, such as nodulation and zygomorphic floral development. In recent years, there has been big progress in legume genomics due to comparative genomics work and the genome sequencing projects of the model legume**s** and the crop legume soybean. Studies indicated that different species in the Papilionoideae subfamily exhibited extensive genome conservation and shared micro-syntenic blocks (Weeden et al. 1992; Choi et al. 2004; Kaló et al. 2004; Cannon et al. 2006; Cronk et al. 2006). A major challenge for legume comparative genomics is to use the incomplete genome information gained from a few legume species efficiently so as to map and clone the important loci in grain legumes such as pea and peanut, whose genome information is less available (Zhu et al. 2005; Varshney et al. 2009; Young and Udvardi 2009). The recent progress of genome projects in legumes has made it practical to map the genetic loci in pea efficiently by the comparative genomic approach. In recent years, we have tried to set up an experimental framework for virtual map-based cloning in garden pea: mapping of garden pea genes could be carried out in other legume genomes whose genomic information could be available or easy to access. A standard procedure includes the following steps: (i) identify the target genes by screening mutants and mapping the relevant loci in garden pea; (ii) project the loci into other legume genomes; (iii) identify the syntenic regions in different legume genomes; (iv) anchor the target genes in the contigs by constructing the comparative map from the available genomic information; (v) finish the physical maps and move on to confirm the cloning of target genes.

In this study, we screened floral mutants with defects in IN asymmetry in pea. Two mutants with a similar phenotype, *ele1* and *ele2*, were identified in pea. These display predominant deficiency in the asymmetry of both lateral and ventral petals, as well as the enlarged sizes of organs. Genetic analysis demonstrated that the enlargement of the dorsal petal in *ele2* depends on the activities of two *PsCYC* genes, *K* and *LST1*. Genetic analysis showed *ele1* was a single recessive gene located on the long arm of pea linkage group II and was not allelic to *ele2*. The virtual mapping of *ele1* was conducted by projecting the locus into the genome of *L. japonicus*, another model legume plant whose genomic information is available (www.kazusa.or.jp/lotus). The syntenic region containing the *LjELE1* locus was identified and the locus was anchored in a 295-kb contig from the *L. japonicus* genome. Thus the virtual mapping of the *ele1* locus was successful to complete the physical map for cloning the *ELE1* gene, demonstrating that the comparative genomic mapping approach should be a powerful one to conduct virtual map-based cloning in garden pea, as well as in other genomes, whose whole genome information is basically absent.

Results

Characterization of the *ele1* and *ele2* mutants

To investigate the key components in the IN asymmetry pathway in legumes, we screened mutants with defects in IN asymmetry which were isolated from mutagenesis experiments of both pea and *L. japonicus* (Feng et al. 2006; Wang et al. 2008). Two mutants with a similar phenotype in pea, *ele1* and *ele2*, were identified from two genetic backgrounds, respectively. Both mutants displayed significant effects on the asymmetry in both lateral and ventral petals (**Figure 1A, B**). In addition, both *ele1* and *ele2* mutants bore enlarged organs: higher of whole plants and the enlarged size of bulgy compound leaves or stipules were conspicuous although the one of petals was not evident except for the dorsal petal (**Figure 1C, D**). The floral phenotype of *ele1* and *ele2* mimicked the one of *syp1–1* when their petals were compared, except for the enlarged size of dorsal petal in *ele1* and *ele2*: the DV identities of petals were intact since there was no alteration of the epidermal cell types

in the mutant petals (data not shown), and the asymmetry in the lateral and ventral petals was abolished (**Figure 2B, I**).

Genetic analysis of *ele1* and *ele2*

Genetic analysis on *ele1* was conducted by backcrossing the mutants with its parental wild types, JI2822. The phenotype of all F1 plants was as normal as the one of the wild type. In the B2 generation, wild type and mutant plants segregated in a 3:1 ratio (65 wild-type plants and 20 mutants, $\chi^2 = 0.097 < \chi^2_{0.05} = 3.84$). These results indicated that *ele1* was controlled by a single recessive locus. Genetic analysis of *ele2* was conducted by backcrossing the mutant with its parental wild type, Terese. All F1 plants displayed normal phenotype. In the B2 generation, segregation of the wild type and mutant plants (105 wild-type plants and 32 mutants) fitted a 3:1 ratio ($\chi^2 = 0.197 < \chi^2_{0.05} = 3.84$) showing that the *ele2* mutation was controlled by a single recessive gene. The floral phenotype of *ele1* mutant was from the same as that of *ele2* under the same growth conditions, and an allelic test was conducted

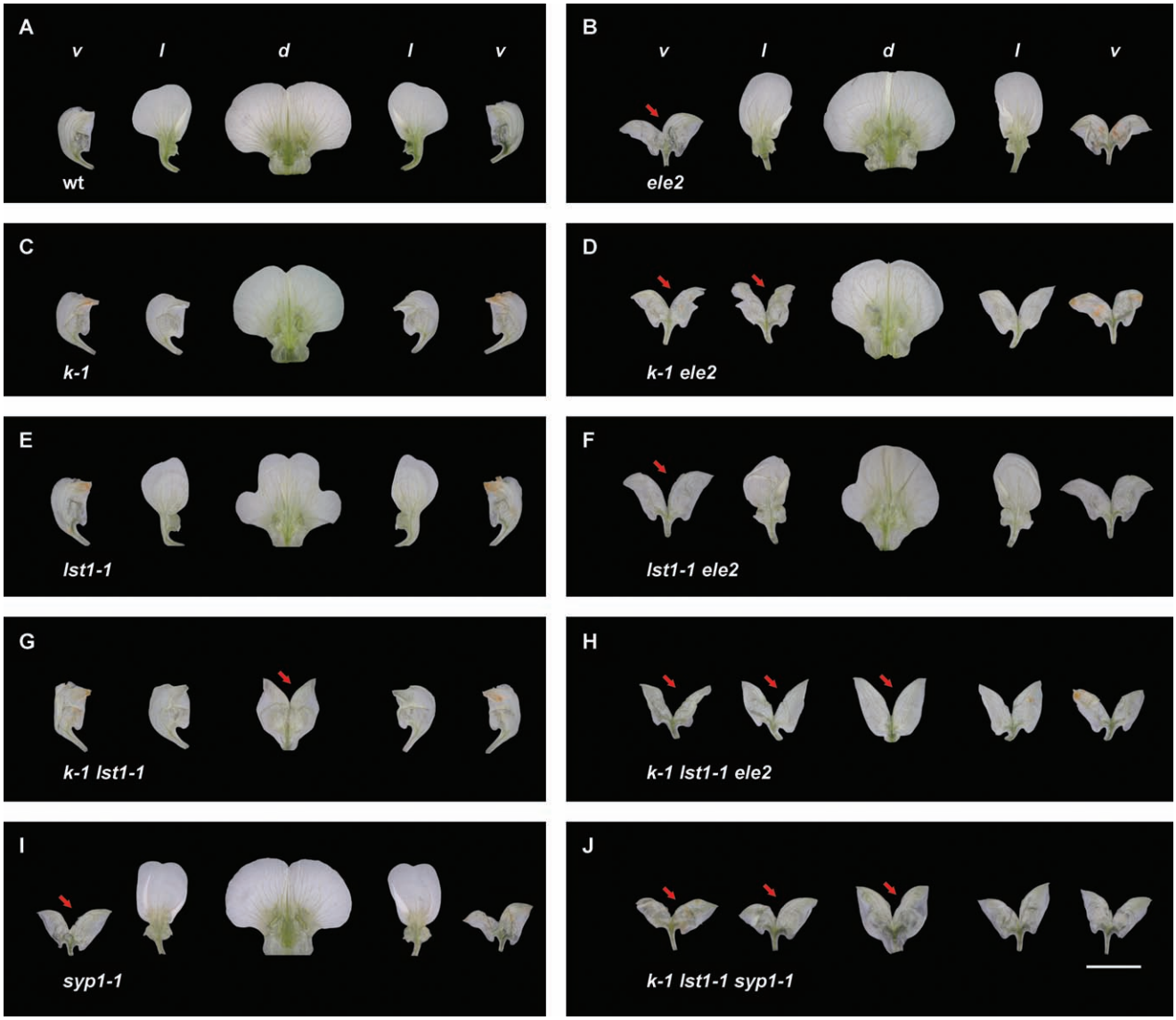

Figure 2. Genetic interactions between the mutants of *PsCYCs* and *ELE2*.

(A–J) Flattened petals of wild type and mutant flowers are shown. Arrows indicate the keeled petals were cut so as to make them flattened. Scale bar,1 cm.

by crossing *ele1* with the heterozygous plants of *ele2*. All of 13 plants from the cross developed normally, indicating that *ele1* was not allelic to *ele2*. The phenotype of the following generation was examined, and the segregation of wild type and *ele* mutants in some F2 populations was fitted into the 9:7 ratio (data not shown), confirming that *ele1* and *ele2* were two separate loci and there is no additive effect in the *ele1 ele2* double mutant. These results indicate that *ele1 ele2* double mutant should look identical to single *ele* mutants, and *ELE1* and *ELE2* act in the same genetic pathway to regulate IN asymmetry and other developmental programs.

Genetic interactions between mutants of *PsCYC* genes and *ELE2*

Our previous study has shown that the DV and IN regulators interact with each other during development of the zygomorphic flower in pea (Wang et al. 2008). To investigate whether there are interactions between *PsCYCs* and *ELE* and because of *ELE1* and *ELE2* acting in the same genetic pathway and *being ELE1* tightly linked with *PsCYC3* (see below), *ele2* was introduced into *k-1*, *lst1-1*, and *k-1 lst1-1* genetic backgrounds, respectively (Wang et al. 2008). In all cases, there was no detectable alteration of the enlarged leaf phenotype in *ele2* when *k-1* and *lst1-1* were introduced. This is consistent with the previous observation that the malfunction of *k* and *lst1* could only be found in the flower. The *k-1 ele2* double mutant seemed to display an additive phenotype: the flower bore symmetric lateral and ventral petals with a keel structure; however, the enlarged size of the dorsal petal was subtle (**Figure 2A–D**). The *lst1-1 ele2* double mutant could also be considered as an additive phenotype: the lateral and ventral petals lost IN asymmetry, but the enlargement of the lobed standard is less evident (**Figure 2E, F**). In the *k-1 lst1-1 ele2* triple mutant, the flower lost both DV and IN asymmetries and all petals possessed a keel structure, which looked the same as the ones in *k-1 lst1-1 syp1-1* triple mutant (**Figure 2H, J**). Particularly, the size of the dorsal petal in the triple mutant was not very different from that in *k-1 lst1-1 syp1-1* triple mutant, indicating that the enlargement effect of *ele2* on the dorsal petal size is suppressed when the *PsCYC* genes were mutated.

Comparative mapping of *ele1*

Mapping *ele1* in garden pea was conducted using 123 individuals who displayed typical *ele1* phenotype in a F2 mapping population containing 511 plants. Primarily, 12 mutant plants from mapping population were randomly selected to comprise DNA pool, and more than 200 molecular markers in seven linkage groups of *P. sativum* were used for linkage analysis with *ele1* (Loridon et al. 2005; Aubert et al. 2006). Simple sequence repeats (SSR) markers AA205 and AB40, and sequence-tagged site (STS) marker *PsSut1* on linkage group II were found to be linked with *ele1* (**Figure 3A**).

To project the *ele1* locus in the genome of *L. japonicus*, genomic information was analyzed. *LjSut1*, the homolog of *PsSut1*, was found to be located in chromosome V of *L. japonicus*, and linked with *LjCYC3* (*KEW1*), the homolog of *PsCYC3* (*K*) (Feng et al. 2006; Wang et al. 2008). To confirm the linkage relationship of *ele1* and *K*, the genomic sequence of *PsCYC3* in JI2822 and JI992 were sequenced, respectively, and an SNP was identified to develop a dCAPS marker. Then it was found that there was one recombinant between *K* and *ele1* in the mapping population. This result showed that *ele1* is tightly linked with *PsCYC3*. It has been shown that the region containing *k* in *P. sativum* was syntenic to the region containing *kew1* in *L. japonicus* and therefore, the syntenic regions in the two genomes were identified (Feng et al. 2006; Wang et al. 2008).

A comparative map of the syntenic regions containing *ele1* locus was constructed by integrating the marker data with the genomic sequence data in the regions containing *k* and *kew1*, respectively (**Figure 3A, B**). Consequently, the positions of two molecular markers based on the gene sequences (*PsPM25* and *PsRUG3*) in *P. sativum* were found to match the ones of the two homologous genes (*LjPM25* and *LjRUG3*) in a contig from the genome of *L. japonicus*. Therefore, the *LjELE1* gene has been anchored in a 295-kb contig from *L. japonicus*. The putative genes in the contig have been annotated and compared with the ones in the duplicated syntenic contigs from the genomes of soybean (**Figure 3C**). At present, the comparative genomic analysis has given a useful clue about the candidate gene for *ELE1* and a confirmation experiment is being conducted.

Discussion

In this study, we investigated two mutants, *ele1* and *ele2*, in garden pea. Our detailed analysis showed that *ele1* and *ele2* mutants displayed pleiotropic phenotype: the deficiency of IN asymmetry in lateral and ventral petals; enlarged size of leaflets/stipules and other organs. Recent study demonstrates that the development of petal shape and size in papilionoid legume can be separately controlled by two categories of regulators, whose functions are found not to depend on each other: the DV differentiation of petals is governed by the *CYC*-like TCP genes and the organ IN asymmetry of lateral and ventral petals could be independently controlled by *SYP* gene (Luo et al. 1996, 1999; Feng et al. 2006; Wang et al. 2008). To identify the key components in the control of IN asymmetry, we screened mutants in large scale mutagenesis in garden pea. So far, two types of mutants displaying defects of IN asymmetry in lateral and ventral petals have been isolated. One has the

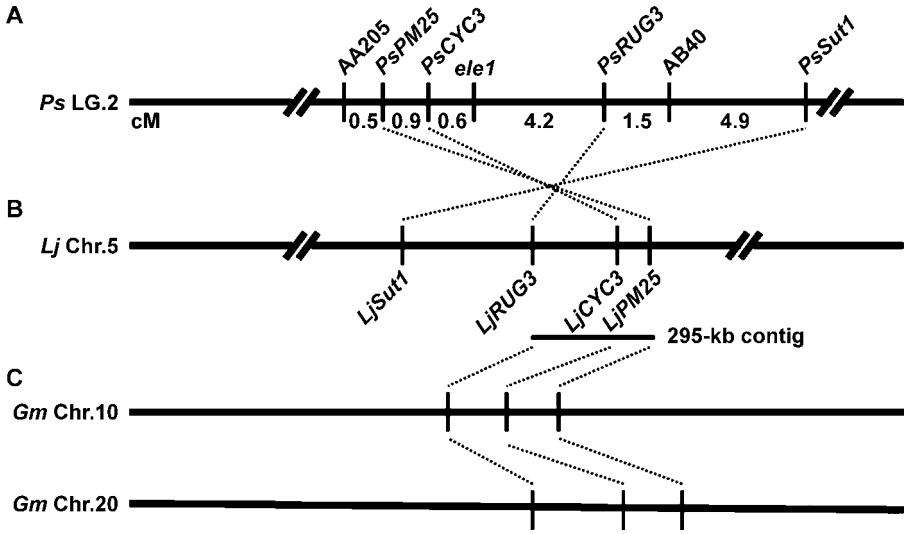

Figure 3. Comparative genomic mapping of *ele1* locus.

(A) The *ele1* locus was mapped in garden pea and found to locate in the long arm of linkage group II. Dotted lines indicate the homologous makers or genes.

(B) Then *ele1* was projected to the genome of *Lotus japonicus* and the syntenic region was identified. The comparative map was developed and *ELE1* was anchored in a 295 kb contig of *L. japonicus*.

(C) The duplicated syntenic contigs in *Glycine max*.

specific effect on the IN asymmetry, such as *syp1*, which has no other visible malfunction except for the floral phenotype (Wang et al. 2008). The other type consists of *ele1* and *ele2* mutants, which have defects of IN asymmetry in petals and also display the pleiotropic effect of enlarged organs. These data demonstrate that multiple components in the control of IN asymmetry have been identified.

Unlike *syp1,* which displays a specific malfunction in petal asymmetry, both *ele1* and *ele2* mutants also give rise to the pleiotropic effect of enlarged organ size in compound leaf. On the other hand, the interaction between *SYP1* and *PsCYCs* has the effect on the organ number or organ initiation but the interplay between *ELE2* and *PsCYCs* affects the organ size and shape (Wang et al. 2008). This raises the question about the role of *ELE1* and *ELE2* in the IN asymmetry pathway, as well as their relationship with *SYP1*. It may not be unexpected that the components that regulate IN asymmetry could somehow be involved in the regulation of organ size and shape, since the mechanism in the control of IN asymmetry might couple the organ size and organ shape at the cellular level during the zygomorphic flower development. In fact, the enlarged leaves of *ele* mutants, also display alteration of leaf shape. Recently, several key pathways to monitor organ size have been identified in *Arabidopsis*, which does not possess zygomorphic flower (Anastasiou and Lenhard 2007; Gonzalez et al. 2009; Krizek 2009). From the putative orthologous gene in the syntenic region containing *ELE1*, there is an indication that *ELE1* would be a homologous component of the identified pathways in *Arabidopsis*.

It is possible that *ELE1* and *ELE2* have a general function to monitor the organ size during development, but their function in the control of IN asymmetry in petals depends on other downstream factors. Cloning *ELE1*, *ELE2*, *SYP1* and other key components in the IN asymmetry pathway and analyzing their functions in the future should shed light on the molecular basis of the interaction and superimposition of DV and IN asymmetries, as well as the developmental homeostasis between organ size and shape.

In this study, we conducted the comparative genomic mapping of *ele1*, and the physical map for cloning *ele1* was achieved by the virtual mapping in *L. japonicus*. Genetic analysis showed *ele1* was a single recessive locus on the linkage group II in *P. sativum*. By conducting the comparative mapping, the *ELE1* comparative map was constructed and *LjELE1* was anchored in a 295 kb contig of *L. japonicus*. At present, further bioinformatics analysis of the sequences in the syntenic regions among different legume genomes, such as the ones of *M. truncatula* and soybean, is being conducted to identify the candidate gene for *ELE1*. Due to its mutant phenotype, it is expected that *ELE1* should play an important role in the control of organ size and shape in the development and thus should be a conserved locus among legume genomes. Therefore, the

homologous putative genes in the syntenic regions among different legumes have been focused to identify the *ELE1* gene. Other approaches, such as the pea early browning virus (PEBV) induced gene silencing, would be powerful reverse-genetics tools for functional confirmation of cloning *ELE1* in *P. sativum* (Constantin et al. 2004).

Materials and Methods

Plant material and growth conditions

The *ele1* and *ele2* mutants were identified from two fast neutron mutagenesis M2 populations in JI2822 and Terese genetic background, respectively. All plants were grown at 20 °C to 22 °C with a 16:8 h light : dark (L:D) photoperiod at 150 μmol/m^2 per s.

DNA extraction and marker analysis by polyacrylamide gel electrophoresis

DNA samples were prepared from fresh leaves using the cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The polymerase chain reaction (PCR) reaction system (20 μL) consists of 20 ng of DNA, 2 μL 10× buffer, 0.2 mmol/L of dNTP, 0.1 μmol/L of primers and 1 U of Taq polymerase. The PCR program for molecular markers was performed by a PTC-100 PCR machine, programmed for an initial 3 min at 94 °C, then followed by 35 cycles for 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and finally 10 min at 72 °C. The restriction endonuclease digestion for CAPS or dCAPS markers was conducted in a 20 μL reaction mixture containing 2 μL 10× buffer, 0.2 μL 100× bovine serum albumin (BSA) when necessary, 0.1–0.5 μg of PCR products and 15–20 U of restriction enzyme at the optimal temperature for 2–3 h. The polymorphisms of these markers were analyzed on 6% polyacrylamide gel stained with 0.1% silver nitrate.

Genetic analysis and gene mapping

For genetic analysis of *ele1* and *ele2*, *ele1* and *ele2* mutants were backcrossed with wild type parent lines JI2822 and Terese, respectively, and genetic analysis was conducted in the B2 population. Allelic test of *ele1* and *ele2* were conducted by crossing *ele1* (pollen donor) and F1 plants from the cross between *ele2* and JI992.

The *ele1* mapping populations developed from the cross between *ele1* and JI992. The mutant plants were identified after phenotype screening for compound leaves and flowers in F2 population. To map the *ele1* locus, polymorphism markers between the parental line JI2822 and JI992 from pea seven linkage groups (Loridon et al. 2005; Aubert et al. 2006) and the DNA pools were used for whole genome scanning. dCAPS marker of *PsCYC3* (primers: TTATGCGAGATGGCGTCATCT-TCTTAAGCT and GGATATTAGGAATTAGGTTTTGTTGAT) was designed according to the *PsCYC3* genomic sequences of JI992 and JI2822 by dCAPS Finder 2.0 (Neff et al. 2002).

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