Mutations Affecting Lateral Petal Development in <i>Lotus japonicus</i>

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Abstract To investigate the genetic loci to regulate the development of petal shape, screening of the mutation affecting lateral petal development was conducted in a model plant of legume, <i>Lotus japonicus</i>. Two independent mutants, <i>kew</i>2 and <i>kew</i>3 with the similar phenotype were obtained, whose shape of lateral petal is altered and resembles the one of ventral petal in the wild type. Genetic analysis showed that <i>kew</i>3 was caused by the same single recessive locus and allelic to the one of <i>kew</i>1, a formal identified mutant in the locus <i>KEW1</i>. <i>KEW1</i> has been shown to be an ortholog of <i>K</i> in pea, but whose mutation at the molecular level is unsolved yet. Furthermore, genetic analyses of <i>kew</i>2 indicate that there are other genetic factors which could interact with <i>kew</i> and involve in the lateral petal development. Analysis of these mutations and cloning the corresponding loci will shed light on the underlying molecular mechanism in the control of floral asymmetry and facilitate our understanding on the evolution of the zygomorphic development.

Keywords <i>Lotus japonicus</i>; Mutagenesis; Keeled-wing locus; Floral symmetry

Background <i>Lotus japonicus</i> belongs to the subfamily Papilionoideae, being named after its unique papilionaceous (butterfly-like) flowers, and provides an excellent experimental system to investigate the molecular mechanisms in the control of zygomorphic flower development. <i>L. japonicus</i> has been adopted as a model legume plant in genomic and the molecular genetic study in the late 1980s, being recognized for its relative simple genome structure, small genome size, feasible for genetic transformation and the other unique characteristics (Arumuganathan et al., 1991; Handberg and Stougaard, 1992; Blondon et al., 1994; Greilhuber et al., 1997; Asamizu et al., 2000; Kawasaki and Murakami, 2000; Sandal N, et al., 2002).

In recent years, there has been a great progress in legume genomics due to the genome sequencing program in a few model legume plants, including the one of <i>L. japonicus</i> (Lamblin et al., 2003; Cannon et al., 2005; Sato et al., 2008; Shoemaker et al., 2008; Grant et al., 2009). Nevertheless, screening mutants is still an efficient research strategy to identify the key genes in the regulatory network controlling important biological process. In the previous study, we have reported several important loci being found in the mutagenesis and a few important key regulators in the control of floral development were cloned from our mutagenized population of <i>L. japonicus</i> (Dong et al., 2005; Chen et al., 2006; Feng et al., 2006).

<i>L. japonicus</i> possesses the flowers which are zygomorphy and display the dorsoventral (DV) asymmetry: there is a DV axis in their floral plane, along which there exist a dorsal, two lateral and two ventral petals; three types of petals are different in shape and size (Figure 1a). In the previous study, a mutation, <i>keeled wing</i> in <i>Lotus 1</i> (<i>kew</i>1), was analyzed (Figure 1b; Feng et al., 2006), which affects the development of lateral petal and is named for its similar phenotype to the mutant <i>keeled wing</i> (<i>k</i>) in pea (Pellwe and Sverdrup, 1923; Ellis et al., 1992). Mutant flowers of both <i>kew</i>1 and <i>k</i> bear the aberrant lateral petals which acquire the same identity as the one of ventral petal in the wild type (Figure 1b). Comparative mapping experiment demonstrated that <i>KEW1</i> and <i>K</i> should be the orthologous regulators and play an important role in the control of lateral petal development in papilionoid legumes (Feng et al., 2006). Recently, <i>K</i> has been cloned and is found to encode a
TCP transcriptional factor (Wang et al., 2008). In *L. japonicus*, *LjCYC3*, the homolog of *K*, was isolated but no detectable mutation has been identified in a 5 kb region containing the *LjCYC3* gene in the *kew1* mutant (Wang et al., 2008).

In this study, we reported our effort to conduct large scale mutagenesis by using physical-mutagenesis (γ-ray) and chemical-mutagenesis (EMS) in *L. japonicus*. Two new mutants, *kew2* and *kew3*, possessing disrupted shape or identity of lateral petal were isolated and characterized. Genetic analysis indicated that *kew3* was caused by single locus and allelic to the *kew1*. However, *kew2* was a variable mutant and display weaker malfunction on the shape of lateral petals than *kew1* or *kew3*, suggesting another genetic locus might have involved in the lateral petal development. With more mutants and genetic loci were identified, the function of the key regulators in the control of lateral petal development will be elucidated in the coming future.

1 Results

1.1 Physical-mutagenesis and chemical-mutagenesis in *Lotus japonicus*

Physical-mutagenesis (γ-ray) and chemical-mutagenesis (EMS) were used to construct the mutation library in *L. japonicus*, respectively. Different dosages of mutagenesis were evaluated by testing the germination rates, and 50% germination rate was set as the limit dosage for the treatment. Then, 25000 seeds of ecotype Gifu were treated with γ-ray (250Gy) or EMS (0.75%) respectively. The seeds were germinated and sown, giving rise to about 20000 plants and 30000 plants in two M1 populations in two growing seasons respectively. Seeds were collected from each M1 plant and packed separately. 13000 and 25000 M2 families were grown in two separate growing periods in 2002 and 2005 respectively. Individual plants of the M2 generations, were screened for development mutations. Several floral mutations and leaf mutations were obtained and selected for detailed analysis (data not shown).

1.2 Mutations possessing aberrant lateral petals

Two mutants involved in the malfunction of lateral petal development were obtained in the γ-ray mutagenized M2 population. These mutants displayed no other detectable phenotype apart from their common characteristic: the lateral petals have abnormal shapes, while the petals in dorsal and the ventral positions are as normal as ones of the wild type. Since their phenotypes mimic the one of *kew1*, they were designated as *kew2* and *kew3* respectively (Figure 1c; Figure 1d).

When the shapes of lateral petals were compared among *kew1*, *kew2* and *kew3*, the subtle difference between *kew1* and *kew3* could be seen: there are wrinkles at the edge of the *kew3*’s lateral petal; however, the shape of *kew2*’s lateral petal is quite different from the one of either *kew1*/*kew3* or the wild type. It is evident that the identity of lateral petal of *kew1* and *kew3* is altered, which mimics the one of ventral petal in the wild type (Figure 1a; Figure 1b and Figure 1d). In a comparison with the wild type, the lateral petal of *kew2* is narrower and possesses a tip at the top end (Figure 1c).

![Figure 1: Flowers of wild type and *kew* mutants in *L. japonicus*](image)

Note: a: A wild type flower, b-d: Flowers of *kew1*, *kew2* and *kew3* mutants; D: Dorsal petal; L: Lateral petals; V: Ventral petals; Arrows indicate the aberrant lateral petals in the *kew* mutants

1.3 Genetic analysis of *kew2* and *kew3*

When *kew3* was back-crossed with the wild type parent line Gifu B129, all flowers of F1 are wild type, while F2 progenitors gave wild type and mutant flowers in 3:1 ratio (n>1500), indicating *kew3* is caused by a
single recessive locus. After several backcrosses with the wild type in which 3:1 ratios of the wild type and mutant were observed consistently in the segregating populations, kew3 behaved as a stable mutant. However, in the backcross with the wild type, the phenotype of kew2 could disappear in the following generations.

Since the phenotype of kew3 mimicked the one of kew1, allelic testing was conducted. It was found that kew1 and kew3 were allelic, since all the F1 progenies displayed the mutant phenotype as the one of kew1 and kew3. However, in the allelic test of kew2, the F1 progenies of the cross between kew1 and kew2 were segregated as either the wild type or kew1. These suggest that in the kew2 genetic background, the kew1 locus must be mutated but should be in a heterozygous state, suggesting that other genetic locus/loci were contributing to the mutant phenotype in the original kew2 mutant.

2 Discussion

Floral zygomorphy (flower with bilateral symmetry), as a specialized forms of flower symmetry, is an important evolutionary trait in flowering plants with multiple origins. The molecular analysis on the mechanism in the control of zygomorphic development was first conducted in a model plant, Antirrhinum majus, that was commenced from the screening of floral mutants from mutagenesis experiments (Carpenter and Coen, 1990). Combination of precise genetic analyses and transposon-tagging approach, a few key regulatory genes were identified and cloned. In the past ten years, we have explored the model papilionoid legume, Lotus japonicus, which has the advantages for molecular genetic studies due to its relative smaller size, simple genome structure, feasible for genetic transformation and other characteristics. In this study, we reported our effort to conduct large scale mutagenesis in L. japonicus.

Ionizing radiation, such as γ and X–ray, are the efficient physical mutagens, which produce deletions leading to chromosome break and chromosome aberrations. On the other hand, most chemical mutagens normally cause base pair substitutions, especially from GC to AT in the case of EMS. With the difference in the mechanism, the different mutagenesis, such as, physical and chemical mutagens have different hotspots, produced different spectrum (Van der Veen. 1966; Feldmann, 1991). In our mutagenesis in L. japonicus, a few kew mutants were identified in the γ-ray mutagenized M2, but EMS did not produce the kew mutation yet in our experiment. This could either be a by-chance case or alternatively indicates that different type of muta- tions might be obtained through different mutagenesis.

In Antirrhinum majus, two TCP genes, CYCLOIDEA (CYC) and DICHTOMATA (DICH), and two MYB genes, RADIALIS (RAD) and DIVARICATA (DIV) play key role in the development of zygomorphic flower, and their interplay determine the petal differentiation (Carpenter and Coen 1990; Luo et al., 1996; 1999; Almeida et al., 1997; Galego and Almeida, 2002). It has been shown that both CYC and DICH control the dorsal and lateral petal identity during zygomorphic development. In cyc dich double mutant, all petals resemble the ventral petals of wild type. RAD and DIV interplay with CYC and DICH, and two pairs of key regulators control the asymmetry of the whole flower, as well as the one of lateral and dorsal petals in Antirrhinum majus (Corley et al., 2005).

L. japonicus belongs to Papilionoideae, whose floral asymmetry is thought to have an independent origin, different from the one in Antirrhinum majus. In our recent study on the floral zygomorphy in pea, another well known model plant in papilionoid legumes, we identified two kinds of key regulatory genes in the control of zygomorphic flower development, and proposed that they determine the two kinds of asymmetries independently: the dorsoventral (DV) and organ internal (IN) asymmetries in the floral plane and organ plane respectively (Wang et al., 2008). K and KEW play the important role in the control of lateral petal development and thus are the key regulatory genes in the DV pathway. As a comparison, no single locus which controls the lateral petal development has been identified in Antirrhinum majus.

In this study, we characterized two kew mutants. Genetic analysis indicated that they were allelic to kew1, a mutant locus being identified as the homolog one of kew1 based on the comparative genomic data in the previous study (Feng et al., 2006). K has been cloned and encodes a TCP factor PsCYC3 (Wang et al., 2008). The most closely homolog of PsCYC3 in L. japonicus is LjCYC3. However, in the genomic sequence of LjCYC3, no mutation has been identified yet. With more alleles of kew1 were isolated, the molecular base for the kew
mutation should be addressed and solved soon. Furthermore, genetic analysis of kew2 indicated that the original kew2 mutant should be heterozygous at the kew1 locus. In the analysis of kew1 and kew3, it has been shown that kew mutant is single recessive one. These data suggest that there are other genetic factors in the genome, which could interact with kew locus and determine the lateral petal development. Cloning and functional analysis of K/KEW and other unique loci in legumes genome should provide a good chance to dissect the molecular base on the evolution diversity between different zygomorphic developments.

3 Materials and Method
Lotus japonicus (Gifu B–129) was used in this experiment. Before planting, the seeds were imbibed in liquid N2 for 1 Min to break the tough capsule. M1 plants were grown in greenhouse and maintained under 16 h light, 8 h darkness using supplemental light. M 2 plants, 20~30 plants for each family, were grown in fields, Zhonggu Farm, Shanghai.

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