

Comparative Morphology and Anatomy of Floral Symmetry in Legumes (Fabaceae)

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Abstract

Information from phylogenetic trees implies zygomorphic flowers have evolved independently from their actinomorphic ancestors several times. There are various types of zygomorphic flowers in the legume subfamilies Caesalpinioideae and Papilionoideae. To understand the relationship between the cell number and size in the petals of zygomorphic flowers, the comparative morphology and anatomy of flower petals were examined in *Cercis chinensis* and *Caesalpinia decapetala* var. *japonica* in Caesalpinioideae, and *Lespedeza formosa* subsp. *velutina* and *Pueraria lobata* in Papilionoideae. Increased petal size is correlated to a higher number and/or size of the cells of the petal in Caesalpinioideae. The characteristic flower morphology of the Papilionoideae has evolved by increased cell numbers, although the cell size was the smallest. Papilionoideae has characteristic papilionoid flowers consisting of five specialised petals. These characteristic morphologies were generated from the complicated changing pattern of cells in petals in the course of legume diversification.

Keywords: anatomy, cell numbers, cell size, legume, zygomorphic flower

1. Introduction

Flowering plants display unrivalled structural diversity in their reproductive traits, which function to promote reproductive success through their influence on pollination and mating. A particularly striking feature of flowering plants is that the mating patterns vary considerably, often among closely related species. One of the main interests of evolutionary biologists is to understand and explain the evolution of morphological diversity.

Zygomorphic flowers are recognised by asymmetrical development along a symmetrical axis (Coen & Nugent, 1994). They appear in various groups of angiosperms, and thus have likely arisen independently from actinomorphic flowers over the course of evolution of flowering plants (Stebbins, 1974); however, the opposite pattern, from zygomorphy to actinomorphy, seems to have also occurred (Donogue et al., 1998). Zygomorphy of flowers is the rule in several large and well-known angiosperm families such as Leguminosae, Scrophulariaceae, Labiatae, and Orchidaceae. Most of these flowers have particularly conspicuous petals and stamens, which probably serve to promote pollination efficiency (Dafni & Kevan, 1996; Neal et al., 1998; Guifa et al., 1999). Among them, the snapdragon (*Antirrhinum majus*) of Scrophulariaceae is a model plant, and studies on the snapdragon play an important role in the establishment of dorsoventral asymmetry in flowers. In fact, genetic analysis of the snapdragon revealed that the achievement of a fully zygomorphic phenotype in the wild-type plant involves the *cycloidea* (*cyc*) gene, which encodes a putative transcription factor of 286 amino acids and is a nuclear localisation signal (Luo et al., 1996). In the case of a *cyc* mutant of snapdragon, the flower is actinomorphic, and the dorsal side of the flower develops similar to the ventral side (Luo et al., 1996). The usual outcome of this ventralisation process is that the dorsal side bears 3 petals, and the flower has 6 petals in all (Luo et al., 1996). Luo et al. (1996) reported that the *cyc* gene is expressed at a very early stage in the dorsal region of floral meristems, where it affects growth rate and primordium initiation and leads to an increase in the number of cells. *Lcycloidea* (*Lcyc*) in toadflax (*Linaria vulgaris*) is a gene similar to *cyc*. A loss of function of this gene

leads to completely radially symmetrical flowers; therefore, the flower of toadflax is controlled by a system similar to that of the snapdragon (Cubas et al., 1999b). In addition, a species of Gesneriaceae forms zygomorphic flowers with the involvement of a *cyc*-like gene (Möller et al., 1999; Citerne et al., 2000). Furthermore, Gillies et al. (2002) reported that homologues of *cyc* might contribute to the generation of zygomorphic ray florets in asteraceous plants. Therefore, these groups belonging to the phylogenetic group Asteridae exhibit an increased number of cells to form a zygomorphic flower. It is expected that an anatomical differentiation similar to that in the snapdragon would be found in zygomorphic flowers of other phylogenetic lineages.

The legume family is in the distantly related group Rosidae (Chase et al., 1993; APG, 1998) and comprises three large subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae, and the Caesalpinioideae is a paraphyletic group at the basal position of legume phylogeny (Doyle et al., 1997; Kajita et al., 2001; Doyle & Luckow, 2003). The flowers of legume consist of 5 specialised petals: standard (dorsal), two wings (lateral), and two keels (ventral) and the pattern of petal aestivation distinguishes the three subfamilies (reviewed in Tucker, 2003). The petals of caesalpinoid flowers become imbricate in a pattern called ascending cochleate; the keel margins overlap the adjacent margins of the wings, and wing margins overlap the adjacent margins of the standard or vexillum petal. Mimosoid petal aestivation is predominantly valvate without overlap. Papilionoid petal aestivation is expressed as imbricate in a pattern called descending cochleate, in which the standard petal margins overlap the adjacent wing margins, which overlap the adjacent keel margins and flowers of Papilionoideae has characteristic zygomorphic flowers called papilionoid flowers. From extensive comparisons of the developmental processes of papilionoid flowers in various species, Tucker (1984; 1987) concluded that the differentiation of these petals is correlated and originated in a pattern initiated at an early stage of floral development. The order of petal initiation in legumes is unidirectional, from dorsal to ventral, in its whorl (Tucker, 1987; 1999). Some *cyc*-like genes were also isolated from various legumes, and it has been determined that floral zygomorphy of this group involves, not single, but duplicated *cyc*-like genes (Citerne et al., 2003; 2006; Fukuda et al., 2003; Feng et al., 2006; Wang et al., 2008). Gene duplication is probably the most important mechanism for generating new genes, new biochemical processes, and new functions that have facilitated the evolution of complex organisms from primitive ones (Force et al., 1999; Hughes, 1999; Lynch & Conery, 2000; Lynch & Force, 2000). Therefore, we consider the anatomical differentiation at the cellular level of the legume flower to be more complicated than that of the snapdragon. Here, we compare the number and size of cells in legume petals. We also report that the changing number and size of cells in petals are involved in the evolution of floral symmetry in legumes.

2. Materials and Methods

2.1 Plant Materials

We chose four species in the legume family: *Cercis chinensis* Bunge, *Caesalpinia decapetala* (Roth) Alston var. *japonica* (Siebold et Zucc.) H. Ohashi, *Lespedeza formosa* (Vogel) Koehne subsp. *velutina* (Nakai) S. Akiyama et H. Ohba, and *Pueraria lobata* (Willd.) Ohwi. *Cercis chinensis* and *Cae. decapetala* belong to the subfamily Caesalpinioideae, whereas *L. formosa* subsp. *velutina* and *P. lobata* belong to a different subfamily, Papilionoideae, in the traditional classification (Polhill, 1994) and in phylogenetic groups based on the *rbcL* sequences (Doyle et al., 1997; Kajita et al., 2001; Doyle & Luckow, 2003). Additionally, we used the snapdragon to confirm whether our results are congruent with those of a previous study (Luo et al., 1996). For each species, 30 flowers, representing 10 individuals, were sampled from the field.

2.2 Morphological and Anatomical Analyses

For morphological analysis, individuals were measured for the macromorphological variables of the petal length and width. Measurements were determined using a digital caliper. The petal measurements were taken from the fully expanded petals of each species.

For anatomical analysis, the fully expanded petals were collected from each individual. To count the number of cells on the petals, the surface of each petal was peeled off using Suzuki's Universal Micro-Printing (SUMP) method. The middle part of each petal was analysed to determine the number and size of cells. Thirty copied SUMP images (approximately 1 cm²) of each petal were examined, 15 times for each species, using a light microscope (Figure 1). To avoid pseudoreplication, the average of 15 observation values per petal was used for the analysis.

Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test ($P < 0.05$) as a post-hoc analysis. This test can withstand some deviation from normality, but it is less resistant to heterogeneous variances (Zar, 1999). Thus, homogeneity of variances was examined by Bartlett test before the analysis. If variance was unequal among groups, data were log-transformed.

This procedure sufficiently reduced the heterogeneity of variance and yield normality in most cases (Shapiro-Wilk test, $P > 0.05$).

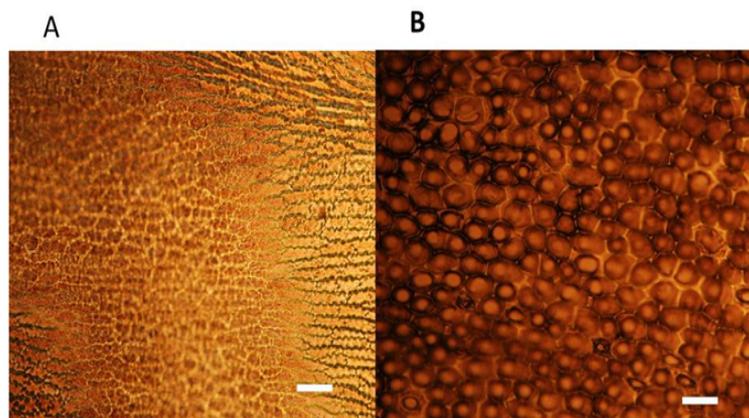


Figure 1. The SEM replicas of *Caesalpinia decapetala* var. *japonica* in Caesalpinioideae and *Pueraria lobata* in Papilionoideae. (A) *Cae. decapetala*; (B) *P. lobata*. Bar = 50 μm

Table 1. Morphological size and size and number of cells in flower petals used in this study

| Species | Position | Morphological size (mm^2) | | Cell size (μm^2) | Number of cells | |
|--|----------|--------------------------------------|------------|-------------------------------|--------------------|-----------------------|
| Scrophulariaceae | | | | | | |
| <i>Antirrhinum majus</i> | Dorsal | 1138.3 | ± 72.3 | a ¹⁾ | 1799.3 ± 203.0 | a 639078 ± 70715 |
| | Lateral | 649.7 | ± 76.9 | b | 1917.5 ± 199.1 | a 340774 ± 51295 |
| | Ventral | 612.4 | ± 44.8 | b | 1831.7 ± 231.9 | a 338382 ± 43146 |
| Fabaceae | | | | | | |
| Caesalpinioideae | | | | | | |
| <i>Cercis chinensis</i> | Dorsal | 79.6 | ± 12.3 | a | 1416.7 ± 170.6 | a 56733 ± 967 |
| | Lateral | 62.0 | ± 10.1 | b | 1127.3 ± 167.5 | b 55755 ± 962 |
| | Ventral | 58.6 | ± 11.5 | b | 1111.1 ± 136.2 | b 52953 ± 937 |
| <i>Caesalpinia decapetala</i> var. <i>japonica</i> | Dorsal | 158.9 | ± 42.8 | a | 628.4 ± 135.4 | a 255114 ± 52873 |
| | Lateral | 162.3 | ± 48.0 | a | 608.5 ± 124.6 | ab 265723 ± 50367 |
| | Ventral | 102.4 | ± 25.8 | b | 548.4 ± 104.3 | b 187608 ± 35653 |
| Papilionoideae | | | | | | |
| <i>Lespedeza formosa</i> subsp. <i>velutina</i> | Dorsal | 26.6 | ± 3.1 | b | 1028.0 ± 150.8 | a 26484 ± 5297 |
| | Lateral | 20.2 | ± 3.8 | c | 615.6 ± 119.6 | b 33659 ± 7892 |
| | Ventral | 77.1 | ± 12.4 | a | 470.6 ± 65.2 | c 165130 ± 24154 |
| <i>Pueraria lobata</i> | Dorsal | 101.2 | ± 8.1 | b | 2331.6 ± 327.1 | a 44173 ± 6639 |
| | Lateral | 80.5 | ± 12.2 | c | 1125.1 ± 179.0 | b 72506 ± 11326 |
| | Ventral | 236.6 | ± 35.2 | a | 934.2 ± 126.0 | c 254253 ± 29553 |

Average \pm standard deviation.

¹⁾ Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

3. Results

To compare our anatomical analyses using the SUMP method with the results of previous research, we also examined the petal morphology of the snapdragon. In the snapdragon, the dorsal, lateral, and ventral petal sizes had significant differences between the ventral and lateral, and dorsal petals (Figure 2A, Table 1). The dorsal, lateral, and ventral petal cell sizes were no significant differences between them (Figure 2B). The dorsal, lateral, and ventral petal cell numbers had a significant difference between the ventral and lateral, and dorsal petals (Figure 2C). These results indicate that an increased number of cells contributes to the larger size of the dorsal petal compared to that of the ventral and lateral petals. This supports the hypothesis of Luo et al. (1996), which was based on molecular development analyses using loss of *cyc* function.

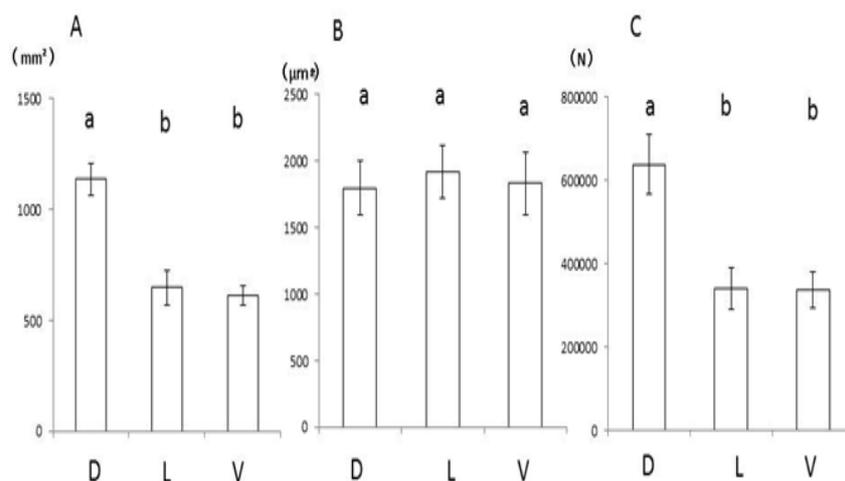


Figure 2. Comparisons of petal size (A), cell size (B), and cell number (C) of the snapdragon
D: dorsal petal; L: lateral petal; V: ventral petal. Error bars indicate standard deviation. Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

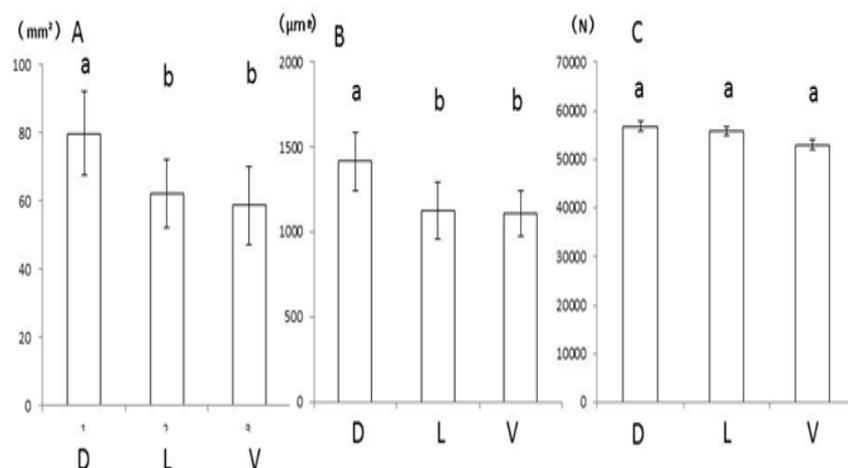


Figure 3. Comparisons of petal size (A), cell size (B) and cell number (C) of *Cercis chinensis*
D: dorsal petal (standard); L: lateral petal (wing); V: ventral petal (keel). Error bars indicate standard deviation. Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

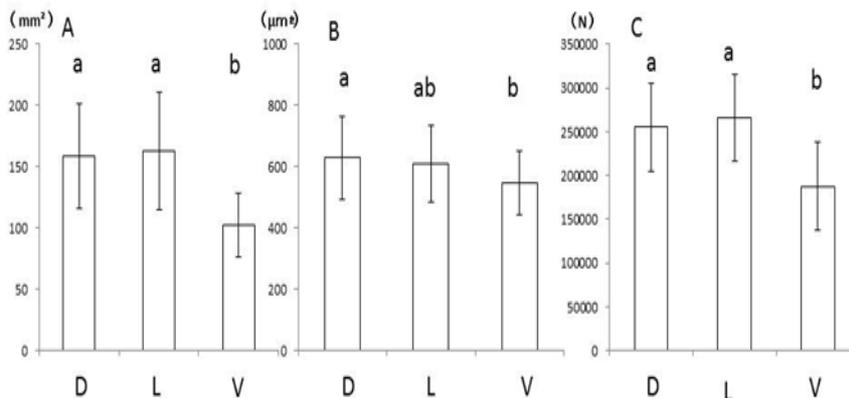


Figure 4. Comparisons of petal size (A), cell size (B) and cell number (C) of *Caesalpinia decapetala* var. *japonica*. D: dorsal petal (standard); L: lateral petal (wing); V: ventral petal (keel). Error bars indicate standard deviation. Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

In the *Caesalpinioideae*, the dorsal, lateral, and ventral petal sizes of *Cercis chinensis* (given as mean \pm SD) were 79.6 ± 12.3 , 62.0 ± 10.1 , and 58.6 ± 11.5 mm², respectively; there was a significant difference between lateral and ventral, and dorsal petals (Figure 3A). The dorsal, lateral, and ventral petal cell sizes of *Cer. chinensis* were $1\,416.7 \pm 170.6$, $1\,127.3 \pm 167.5$, and $1\,111.1 \pm 136.2$ µm², respectively; there was a significant difference between ventral and lateral, and dorsal petals (Figure 3B). The dorsal, lateral, and ventral petal cell numbers of *Cer. chinensis* were no significant differences between them (Figure 3C). These results indicated that an increased cell size contributes to the larger size of the dorsal petal compared to that of the lateral and ventral petals of *Cer. chinensis*. In *Caesalpinia decapetala* var. *japonica*, the dorsal, lateral, and ventral petal sizes were 158.9 ± 42.8 , 162.3 ± 48.0 , and 102.4 ± 25.8 mm², respectively; there was a significant difference between ventral, and dorsal and lateral petals (Figure 4A). The dorsal, lateral, and ventral petal cell sizes were 628.4 ± 135.4 , 608.5 ± 124.6 , and 548.4 ± 104.3 µm², respectively; there was a significant difference between ventral and dorsal petal cell sizes (Figure 4B). The dorsal, lateral, and ventral petal cell numbers were approximately 255 000, 266 000, and 188 000, respectively; there was a significant difference between ventral, and dorsal and lateral petals (Figure 4C). These results indicated that both an increased number and size of cells contribute to the larger size of the dorsal and lateral petals compared to that of the ventral petal.

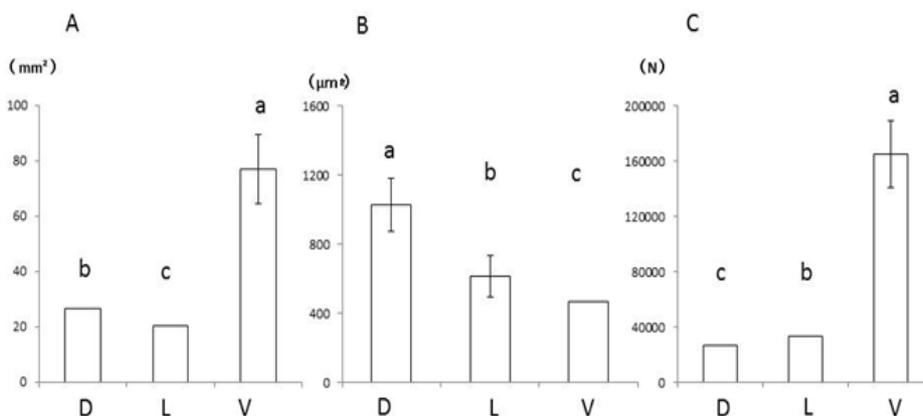


Figure 5. Comparisons of petal size (A), cell size (B) and cell number (C) of *Lespedeza formosa* subsp. *velutina*. D: dorsal petal (standard); L: lateral petal (wing); V: ventral petal (keel). Error bars indicate standard deviation. Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

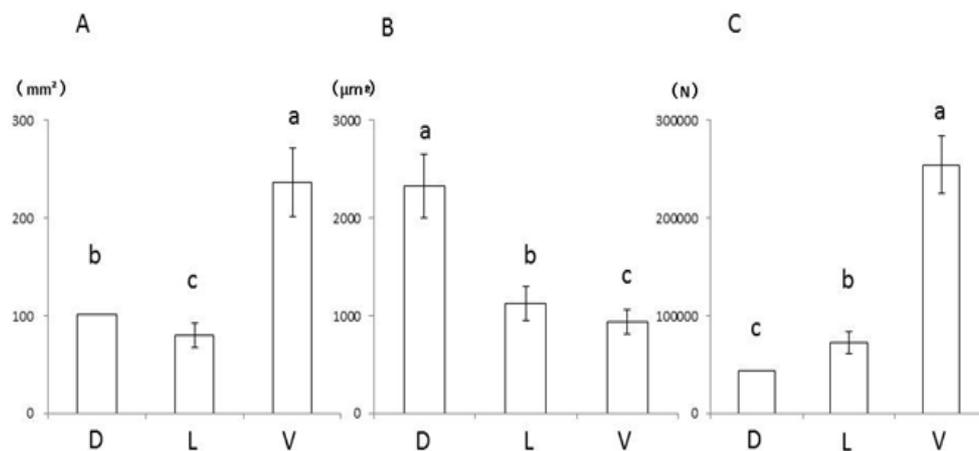


Figure 6. Comparisons of petal size (A), cell size (B) and cell number (C) of *Pueraria lobata*

D: dorsal petal (standard); L: lateral petal (wing); V: ventral petal (keel). Error bars indicate standard deviation. Columns marked by different letters differ significantly based on the Tukey's HSD test ($P < 0.05$).

In the Papilionoideae, dorsal, lateral, and ventral petal sizes of *Lespedeza formosa* subsp. *velutina* were 26.6 ± 3.1 , 20.2 ± 3.8 , and 77.1 ± 12.4 mm², respectively; there were significant differences among them (Figure 5A). These results indicated that the dorsal petal is the largest and the lateral petal is the smallest of all. The dorsal, lateral, and ventral petal cell sizes of this species were $1\,028.0 \pm 150.8$, 615.6 ± 119.6 , and 470.6 ± 65.2 µm², respectively; there were no significant differences between them (Figure 5B). The petal cell sizes in this species increased from the dorsal to ventral petals. The dorsal, lateral, and ventral petal cell numbers of this species were approximately 26 000, 34 000, and 165 000, respectively; there were significant differences among them (Figure 5C). The petal cell numbers of this species decreased from the dorsal to ventral petals. These results indicated that an increased number of petal cells contributes to the larger size of the dorsal petal compared to that of the lateral and ventral petals. In *Pueraria lobata*, the dorsal, lateral, and ventral petal sizes were 101.2 ± 8.1 , 80.5 ± 12.2 , and 236.6 ± 35.2 mm², respectively; there were significant differences among them (Figure 6A). The dorsal, lateral, and ventral petal cell sizes were $2\,331.6 \pm 327.1$, $1\,125.1 \pm 179.0$, and 934.2 ± 126.0 µm², respectively; there were significant differences among them (Figure 6B). The dorsal, lateral, and ventral petal cell numbers were approximately 44 000, 73 000, and 254 000, respectively; there were significant differences among them (Figure 6C). The patterns of cell size and number in petals of this species were similar to those of *L. formosa* subsp. *velutina*. These results indicate that the increased number of cells contributes to the larger size of the dorsal petal compared to that of the lateral and ventral petals.

4. Discussion

4.1 Comparison of Number and Size of Cells in Legume Petals

We analysed the petal morphologies of *Cercis chinensis*, *Caesalpinia decapetala* var. *japonica*, *Lespedeza formosa* subsp. *velutina*, and *Pueraria lobata*. Our results indicate that in species of Caesalpinioideae, the number and/or size of cells increases from the dorsal to ventral petals. In addition, in species of Papilionoideae, the cell size increases but the number of cells decreases from the dorsal to ventral petals. Both species of Papilionoideae, i.e., *P. lobata* and *L. formosa* subsp. *velutina*, exhibit a similar changing pattern of cell size and number between the petal types, suggesting that this pattern is conserved in this subfamily. However, our results indicate that species of Caesalpinioideae, i.e., *Cer. chinensis* and *Cae. decapetala*, exhibit different changing patterns of the cell size and number between petal types; in the former species, only an increased cell size contributes to the larger petal size, and in the latter, an increase in both the cell size and number contributed to the size of the larger petals. A suggested reason for this is that Papilionoideae is a monophyletic group but Caesalpinioideae constitutes paraphyletic assemblages, according to the molecular phylogenetic studies of legumes, and phylogenetic trees show that *Cer. chinensis* occupies a more basal position than *Cae. decapetala* in Caesalpinioideae (Doyle et al., 1997; Kajita et al., 2001; Doyle & Luckow, 2003). Our result plotting against phylogenetic relationships of legume is hypothesized that the modified symmetry seen in the petals of the

legume may be generated from by either an increase in petal cell size alone or by an increase in both the size and number of cells of caesalpinioideae flowers into by the complicated changing pattern of the cell size and number of papilionoid flowers. Our results clearly indicate that in the future, it will be necessary to examine not only species of Caesalpinioideae but also the related taxa of legumes, such as species of Polygalaceae, to support this hypothesis. In particular, Bello et al. (2010) indicated that petal development of the keeled flower of Polygalaceae involved organ suppression rather than heterochrony. Interestingly, our study found that Papilionoideae had a more complicated changing pattern of cells in petals than that of Caesalpinioideae; this is discussed below.

4.2 Complicated Changing Pattern of Petal Cells of Papilionoid Flowers

As mentioned above, the snapdragon has a zygomorphic flower with an increased cell number in its dorsal petal (Luo et al., 1996), and we reconfirmed this finding using our SUMP method (Figure 1). *Cyc*-like genes are involved in the symmetry of snapdragon and papilionoid flowers, but it is not yet understood how different cell patterns are generated in papilionoid flowers. This may be related to the gene duplication of *cyc*-like genes in legumes. Gene duplication is one of the most powerful means to achieve a gain of function in the course of plant diversification (Cronk, 2001). For example, the MADS-box gene family is gaining more attention from an evolutionary perspective (Purugganan, 1997), and recent phylogenetic analyses of the MADS-box gene family revealed that most developmental systems evolved by repeated gene duplication and divergence of paralogous genes from ferns and gymnosperms to angiosperms (Kramer et al., 2004). Phylogenetic reconstructions demonstrated that the MADS-box gene family is composed of several well-defined gene clades and that most clade members share highly related functions. Therefore, it has been suggested that changes in MADS-box gene structure and function by gene duplications are a major cause of the overwhelming diversity in floral architecture in nature today (Theissen et al., 2000). Duplicated *cyc*-like genes in legumes have more complicated functions than those of the snapdragon. Moreover, some mutants of floral symmetry, such as the peloric mutant in the snapdragon and toadflax, have been reported (Gustafsson, 1979; Luo et al., 1996; Cubas et al., 1999), and there have been some studies on developmental genetics using floral mutants (Yaxley et al., 2001; Benlloch et al., 2003), which have supported the idea that several genes control floral zygomorphy in papilionoid flowers. In addition, Ojeda et al. (2009) indicated that epidermal cell shape of petals was different among standard, wing and keel of papilionoid flowers. Considering these results, we hypothesised that the changing number, size and shape of the cells in petals of papilionoid flower implies that the genetic factors involved are more complicated than those in the snapdragon. Further comparative analyses using actinomorphic flowers, such as *Cadia* in Papilionoideae, *Gleditsia* in Caesalpinioideae, and species of Mimosoideae, will help clarify the detailed evolutionary histories of zygomorphic flowers in legumes.

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