Morphological Property and Role of Homeotic Genes in Paracorolla Development of *Antirrhinum majus*

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This study aimed to clarify the morphological properties and the role of homeotic genes in the development of paracorolla, a petaloid organ fused with stamens at the base in *Antirrhinum majus*. Two primordia of paracorolla initiated at the lateral and slightly outer (i.e., basal) position of a stamen primordium, suggesting that the paracorolla is a stipule of the stamen. In the developed paracorolla, expression of the A-class gene *SQUAMOSA* (*SQUA*) and the B-class genes *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) were high, while those of the C-class genes *PLENA* (*PLE*) and *FARINELLI* (*FAR*) were very low. In contrast, in the paracorolla primordia, expressions of *SQUA* and *PLE* were not detected. Thus, the expression patterns of homeotic genes in the paracorolla were basically the same as those of the petal, and this pattern was established during paracorolla development. In the stamen, which fused with the paracorolla at the base, *DEF*, *GLO*, *PLE*, and *FAR* were expressed in the anther. However, in the filament, *SQUA* was also expressed at a higher level in addition to these genes, indicating that the expression pattern shifted from the stamen type in the anther, to the petal type in the paracorolla, through the intermediate type in the filament. This gradient of expression pattern among these organs, which apparently belong to the same whorl of the flower, may be essential for the development of the paracorolla into a petaloid organ.

**Key Words:** *Antirrhinum majus*, flower morphology, homeotic genes, paracorolla.

**Introduction**

Flower morphology is undoubtedly one of the most important characteristics that determine the economic success of floricultural plants. Ornamental floral morphologies, such as double flowers and large flower size, have been selected preferentially during the breeding of wild plants to produce floricultural plants. A dicotyledonous flower basically consists of four different organs: sepals, petals, stamens and carpels. Floral organ identity is controlled following the well-known ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). Individual and combined expression of A-, B-, and C-class genes specify the identity of the four organs. Expression of A-class genes alone establishes sepal identity. Combined expression of A- and B-class genes establishes petal identity, and that of B- and C-class genes establishes stamen identity. Carpel identity is established by expression of C-class genes alone. The A- and C-class genes counteractively control their expression. The A-class gene, *APETALA2* (*AP2*) downregulates expression of the C-class gene, *AGAMOUS* (*AG*) in *Arabidopsis thaliana* (Drews et al., 1991), and *AG* down-regulates expression of the A-class gene, *APETALA1* (*AP1*) (Gustafson-Brown et al., 1994). This antagonistic regulation avoids overlapping of A- and C-class gene expressions. Furthermore, it has been found that D-class genes determine ovule identity (Colombo et al., 1995), while E-class genes contribute to the development of petals, stamens and carpels along with the A-, B-, C-, and D-class genes (Ditta et al., 2004; Pelaz et al., 2000; Theissen, 2001). Based on these studies, the ABCDE model is advocated.

Floral organs are transformed to petaloid organs by certain changes in the expression patterns of homeotic genes. Low expression of the C-class genes due to the insertion of a transposon into their intron transforms the stamen of *Ipomoea nil* into a petal (Nitasaka, 2003). In loss-of-function mutants of C-class genes, the carpel is substituted by a new flower (e.g. loss-of-function mutants of *PLENA* (*PLE*) in *Antirrhinum majus*; Bradley et al., 1993, that of *AG* in *A. thaliana*; Yanofsky et al.,...
These transformations are brought about by the heterotropistic expression of A-class genes in whorls 3 and 4, which is induced by the loss of C-class genes (Gustafson-Brown et al., 1994). Such substitution of stamens and carpels with petaloid organs is commonly observed in double flowered cultivars of floricultural plants (Saito, 1969), suggesting homeotic genes may play an important role in generation of double-flowered cultivars.

In addition to double flowers, paracorolla is another important factor generating ornamental flower morphology. The paracorolla observed in plant species such as Narcissus, Passiflora, Asclepias, is a petaloid structure formed on or adjacent to the corolla. The embryological origin of the paracorolla has been studied anatomically. The paracorolla of Passiflora originated from the receptacle and that of Asclepias and Narcissus originated from the stipule of the stamen (Troll, 1957). Although the outstanding appearance of the paracorolla characterizes the attractiveness of those species, the mechanism by which organs with non-petaloid origin develop into paracorolla is largely unclear.

Thus, we intended to investigate this mechanism in A. majus. A. majus is not only a commercially-important floricultural plant (Rogers, 1992), but also as a model plant of flower morphogenesis. The function of its homeotic genes is well elucidated as follows. In A. majus, SQUAMOSA (SQU) functions as an A-class gene (Huijser et al., 1992). DEFICIENS (DEF) and GLOBOSA (GLO) function as B-class genes (Sommer et al., 1990; Tröbner et al., 1992), while PLE and FARINELLI (FAR) function as C-class genes (Bradley et al., 1993; Davis et al., 1999). Although loss of PLE gene function caused substantial changes in the flower structure as described above, loss of FAR gene function caused no marked transformation in floral organs, but resulted in partial male sterility (Davies et al., 1999).

Some horticultural cultivars of A. majus have a paracorolla, which develops laterally from the basal end of the stamen. Thus it is predicted that the paracorolla of A. majus originates from the stipule of the stamen as in Narcissus and Asclepias. Further, the mechanism for the development of the paracorolla into a petaloid organ despite its location in the lateral position of the stamens and the likelihood of its belonging to whorl 3 of the flower, remain unknown. Thus, elucidation of this mechanism will provide us not only scientific knowledge regarding why the whorl-3 organ develops into a petaloid organ, but also key information on paracorolla generation, which increases ornamental value in floricultural plants.

In the present study, we investigated the anatomical origin of the A. majus paracorolla. Further, we investigated the expression pattern of homeotic genes to clarify the molecular mechanism underlying the development of the paracorolla into a petaloid organ.

### Plant materials

**Antirrhinum majus**, ‘Madame Butterfly Yellow’ (Takii & Co., Ltd., Kyoto, Japan), which forms a paracorolla, was used. ‘Yellow Butterfly’ (Takii & Co., Ltd.), which does not form a paracorolla, was used as the control. We used this control cultivar which forms a labiate corolla because ‘Madame Butterfly Yellow’ forms the same type of corolla.

### Materials and Methods

**Quantification of homeotic gene expression**

To design the primers for quantitative real-time PCR analysis, we cloned the full-length cDNA of homeotic genes and UBQUITIN (UBQ) from the flower bud (about 1 cm in length) of both cultivars tested, and analysed the nucleotide sequence. Open reading frames (ORFs) of the cDNAs were amplified by PCR using KOD-Plus-DNA Polymerase (Toyobo, Osaka, Japan) and the following primers: forward 5'-AAAGGAAAAA AAATAAAAATT-3' and reverse 5'-GGAAATACTTAC AAAACATA-3' for SQUA, forward 5'-CAAAATCTTAT CACAGTTTAGAAGAA-3' and reverse 5'-AATC ACAATAAACCATT-3' for DEF, forward 5'-TTAT CTTGCAAAAACAAAGAGAA-3' and reverse 5'-TGAGAAACCCGATGCTGGG-3' for GLO, forward 5'-GTGAAAGAGAGTGAATTTGACAAAGG-3' and reverse 5'-TGTGCCTCTAAGATAACTTG-3' for PLE, forward 5'-ACACCTCTCATACTCCATCTCTTTC-3' and reverse 5'-GATAAGCAGTAACACTGTCATCAT-3' for FAR, and forward 5'-AGAGGAGGAGGATGACAT TTTACG-3' and reverse 5'-ACATCATTGAAACC ACCAAGG-3' for UBQ. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Cycle sequencing reactions were performed using a DNA Sequencing Kit (Applied Biosystem, Foster City, CA, USA) and sequences were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem).

For quantitative real-time PCR analysis, total RNAs were extracted from the sepal, petal, anther, filament, carpel, and paracorolla of flower buds that were 0.5 and 1 cm in length using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). For RNA extraction, we treated the samples with DNase to avoid potential contamination of genomic DNA using an RNase-Free DNase set (Qiagen). Reverse transcription using RNA from each floral part was performed using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche Applied Science, Indianapolis, IN, USA).

Primers were designed to amplify 216–340 bp fragments of the 3' regions of the ORFs. Primer sequences used for quantitative real-time PCR reaction were as follows: forward 5'-AAAAGAGAGATCAAAGAGA AGGAGAGAA-3' and reverse 5'-TGAAACAGGT GTAATTTATCTCATAAGCC-3' for SQUA, forward 5'- AGATCGACACCAGCAAGAAAAGTT-3' and reverse 5'-CAACCAACATATTTTAGTACAAGACCCTA-3' for UBQ, forward 5'-AAAGGAAAAA AAATAAAAATT-3' and reverse 5'-TTAT CTTGCAAAAACAAAGAGAA-3' for FAR.
AGTTCT-3' for DEF, forward 5'-AATGGGACCTCAGCTCTCAAACAAACACAG-3' and reverse 5'-GTAGGTTTTCTGATTTTTTACTGAATAAAGG-3' for GLO, forward 5'-AGTACTGTGTTGCAGAGATAGAGCACATACTCACG-3' and reverse 5'-CTTTTTACTACACACGATGAACTGCTTTTAC-3' for FAR, and forward 5'-AGAGGAGTGCAGATTTTCG-3' and reverse 5'-ACATACATTGAAACACCAACCCAGG-3' for UBQ. Expression of the genes was quantified using a quantitative real-time PCR machine (LightCycler, Roche Diagnostics, Mannheim, Germany) and SYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan). The samples were then transferred to 100% t-butanol/ethanol (1:hyde) at 4°C overnight, then dehydrated through a graded ethanol series. Hybridization was performed with probes at 37°C in PBS for 15 min and then washed with PBS. The sections were treated with 10 µg·mL⁻¹ proteinase K (Takara Bio) in PBS for 30 min at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, again washed with PBS, and placed in 0.2 M HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 M triethanolamine HCl (pH 8.0) and 0.25% acetic anhydride for 10 min. After washing with PBS, the sections were dehydrated through a graded ethanol series. Hybridization was performed with probes at concentrations of 300 ng·mL⁻¹ in the Probe Diluent (Genostaff) at 60°C for 16 h. After hybridization, the sections were washed in HybriWash (Genostaff), equal to SSC, at 60°C for 20 min and then in 50% formamide, HybriWash at 60°C for 20 min, followed by RNase treatment in 50 µg·mL⁻¹ RNase A in 10 mM Tris-HCl buffer (pH 8.0), 1 M NaCl and 1 mM EDTA for 30 min at 37°C. Then, the sections were washed twice with HybriWash at 60°C for 20 min and once with TBST (0.5 M NaCl, 20 mM Tris-HCl, 0.1% Tween 20, pH 7.5). After treatment with 0.5% blocking reagent (Roche Applied Science) in TBST for 30 min, the sections were incubated with anti-DIG AP conjugate (Roche Applied Science) diluted with TBST for 2 h. The sections were washed twice with TBST and then incubated in 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween 20, and 100 mM Tris-HCl buffer at pH 9.5. Colouring reactions were performed with NBT/BCIP solution (Sigma, St Louis, MO, USA) overnight and then washing with PBS. The sections were counterstained with Kernechtrot stain solution (Mutoh, Tokyo, Japan), dehydrated, and then mounted with Malinol (Mutoh).

**Results**

**Anatomy of paracorolla development**

Mature flowers of ‘Madame Butterfly Yellow’ have two paracorolla at the lateral and slightly basal position of a stamen (Fig. 1A, 1B, 1C). A stamen and its two lateral paracorolla fuse tightly at the base, forming an organ complex (Fig. 1C). In contrast, a paracorolla was not formed in the control cultivar ‘Yellow Butterfly’ (Fig. 1A, 1B, 1C). There was no morphological difference in the other floral organs between the two cultivars.

Paracorolla primordia were not observed in early stage 6 (Fig. 1D). Paracorolla primordia initiated at the lateral position of the filament when the filament became distinguishable from the anther (Fig. 1E, late stage 6).
This arrangement of the stamen and paracorolla primordia resembles that of the leaf and leaf bases, indicating that the paracorolla is the stipule of the stamen as observed in *Asclepias* (Troll, 1957). The paracorolla developed into a petaloid organ fused with filaments at the basal position (Fig. 1F, 1G).

**Morphology of the paracorolla**

The adaxial epidermal cells of petal limbs were a conical shape with yellow pigmentation, while the abaxial epidermis consisted of flat cells without pigmentation (Figs. 1B and 2). The abaxial epidermis had sparsely distributed pubescence. The apical part of the paracorolla was relatively wide with yellow pigmentation, resembling the individual petal limb of the corolla (Fig. 1C). The conical epidermal cells seen on the adaxial surface of the petal limb were seen on the abaxial surface of the paracorolla, while the flat epidermal cells seen on the abaxial surface of petal limb were seen on the adaxial surface of the paracorolla.

![Paracorolla images](image-url)

**Fig. 1.** Paracorolla of an open flower (A–C) and a young flower bud (D–G). Left and right in A–C are the control cultivar ‘Yellow Butterfly’ and the paracorolla-forming ‘Madame Butterfly Yellow’. Photos D–G show the ‘Madame Butterfly Yellow’. (A) Front view of opened flower; (B) Opened flowers bisected longitudinally; (C) Adaxial face of stamen of ‘Yellow Butterfly’ (left) and paracorolla of ‘Madame Butterfly Yellow’ fused with the stamen (right); (D) Paracorolla primordia is not initiated at the stage of stamen primordia formation (early stage 6); (E) Initiation of paracorolla primordium (late stage 6); (F) Development of paracorolla (late stage 6 slightly proceeded than that of this photograph E); (G) Young paracorolla fused with stamen at their base (adaxial face). Bars = 100 µm (D, E, F), 1 mm (G); Pc, Paracorolla; F, Filament.

**Fig. 2.** Scanning electron micrograph of the petal and paracorolla of ‘Madame Butterfly Yellow’ at flower opening. Bars = 100 µm.
(Fig. 2). In contrast, both adaxial and abaxial surfaces of the basal part of the paracorolla had an epidermis consisting of long, flat cells with pubescence (Fig. 2). These characteristics were identical to the epidermis of the petal tube. The morphological features described above clearly indicate that the paracorolla is a petaloid organ.

**Sequence analysis of isolated cDNA**

The nucleotide sequence of the full-length isolated cDNA was the same for the two cultivars tested. The nucleotide sequence of SQUA was identical to the one reported previously (X63701, Huijser et al., 1992), while those of other genes (DEF, AB516402; GLO, AB516403; PLE, AB516404; FAR, AB516405) were slightly (1–3 bp) different from the ones reported previously (DEF, Sommer et al., 1990; GLO, Tröbner et al., 1992; PLE, Bradley et al., 1993; FAR, Davies et al., 1999). However, the deduced amino acid sequences of all the genes were identical to those reported previously.

**Expression patterns of homeotic genes in the mature flower bud**

The expression pattern of the homeotic genes of the sepal, petal, filament, anther and carpel in ‘Madame Butterfly Yellow’ and ‘Yellow Butterfly’ were almost the same. There were no large differences in the expression pattern in flower buds 0.5 and 1 cm long (Fig. 3). The expression pattern in sepal, petals and anthers was the same as reported earlier (SQUA, Huijser et al., 1992; DEF, Schwarz-Sommer et al., 1992; GLO, Tröbner et al., 1992; PLE, Bradley et al., 1993; FAR, Davies et al., 1999), which followed the rule of the ABC model. In the sepal, expression of SQUA was high. Expression of the other genes was much lower than that of SQUA. In the petal, expressions of DEF, GLO, and SQUA were high, although that of SQUA was lower than that of DEF and GLO. SQUA expression in the petal was slightly lower than that in the sepal. There was no PLE and FAR expression in the petal. In the anther, expression of DEF, GLO, PLE, and FAR was high, although expression of PLE and FAR was lower than that of DEF and GLO. No expression of SQUA was observed in the anther.

In the paracorolla, the expression pattern was almost the same as that in the petal; expression of DEF, GLO, and SQUA was high, although that of SQUA was lower than that of DEF and GLO. Expression of PLE and FAR was very low. These expression patterns clearly showed that the paracorolla had petal identity. In the filament, the expression pattern was intermediate between that of the anther and paracorolla. Expression of DEF and GLO was high, and substantial expressions of SQUA, PLE, and FAR were observed. This expression pattern was different from the one expected from the ABC model. In the carpel, expression of PLE and FAR was high, while that of GLO was very low, as reported earlier (PLE, Bradley et al., 1993; FAR, Davies et al., 1999; GLO, Tröbner et al., 1992), which followed the rule of the ABC model. However, DEF and SQUA were also substantially expressed in the carpel in contrast with earlier reports (DEF, Schwarz-Sommer et al., 1992; SQUA, Huijser et al., 1992) and the expectation from the ABC model.

**Expression of SQUA, PLE, and FAR at paracorolla formation**

Since the developed paracorolla had petal identity as described above, we intended to investigate whether petal identity is established in the primordial stage or the later developmental stage. Therefore, we conducted in situ hybridization of A- and C-class genes which are expressed and not expressed, respectively, in petals. SQUA was expressed in parenchymatous petal cells (Fig. 4). In addition, SQUA was expressed at the basal part of stamen, which develops into the filament. In contrast, SQUA was not substantially detected in the paracorolla primordia. Expression of PLE was mainly observed in the anther and the basal part of the carpel.
while it was very low in the paracorolla primordia. FAR was also expressed mainly in the anther and carpel, although the expression localization was obscure (Fig. 4). FAR was expressed in the paracorolla primordia, in contrast with PLE.

**Discussion**

The expression patterns of the homeotic genes in the developed paracorolla of *A. majus* were similar to those in the petal (Fig. 3). SQUA was highly expressed and the PLE expression was not detected. However, in the paracorolla primordia of *A. majus*, SQUA was not substantially expressed, while PLE expression was very low compared to the anther and basal part of the carpel (Fig. 4). Although we did not conduct in situ hybridization of B-class genes, lack of sufficient expression of A- and C-class genes suggests that the paracorolla was not identified either as a petaloid or a staminoid organ at the primordial stage. The petal identity of the paracorolla may have been obtained at a later stage of development. Expression of FAR was detected in the paracorolla primordia, although it was low in the developed paracorolla. It may be assumed that FAR expression has little effect on the formation of the petaloid paracorolla because the loss of FAR activity had little effect on floral organ identity (Davies et al., 1999).

It should be noted that the expression pattern of homeotic genes was different between the anther and filament in both cultivars tested. Homeotic genes expressed in the typical ‘stamen pattern’ in the anther, but their expression in the filament was intermediate between those of the anther and the petal. SQUA substantially expressed itself in both the developed filament and the basal part of stamen primordia, which developed into the filament. Thus, the intermediate expression pattern of the filament may be consistent from the primordial to the mature stage. Although the function of the intermediate expression pattern is not known, the possibility that it is involved in giving rise to morphological differences between the two organs cannot be excluded.

The paracorolla may be a stipule of the stamen, forming an organ complex with the stamen as described above. Thus, the paracorolla presumably belongs to whorl 3. Within this organ complex, the expression pattern of homeotic genes shifts from the ‘stamen pattern’ in the anther to the ‘intermediate pattern’ in the filament and then to the ‘petal pattern’ in the paracorolla. Although the cause of the shift is unknown, it may be attributable to the position of paracorolla formation. Paracorolla primordia are located in an almost lateral, but slightly basal, position from the filament (Fig. 1). Thus, the boundary of the expression pattern of homeotic genes between ‘petal’ and ‘intermediate’ patterns may be located between the paracorolla and filament, as shown in Figure 5. These shifts in the expression pattern within whorl 3 may be essential for the paracorolla to develop into a petaloid organ. It remains to be confirmed if this shift is common in other cultivars and plant species. The filament of ‘Yellow Butterfly’, which does not form a paracorolla, also had the ‘intermediate’ expression pattern (Fig. 3). This result suggests that the shift in the expression pattern within whorl 3 exists across cultivars of *A. majus*. Future research concerning paracorolla with the same anatomical origin as that of *A. majus* (i.e., *Narcissus* and *Asclepias*) will elucidate the universality of this phenomenon across plant species.

Expression of DEF was relatively high in the carpel (whorl 4) of *A. majus*, although it was lower than that in the petal and filament. High expression of DEF in the carpel was different from the result reported by Schwarz-Sommer et al. (1992), in which no DEF expression was detected in the carpel. However, in our experiment the B-function was also largely absent from whorl 4, where GLO is rarely expressed. This is because

(Fig 4), while it was very low in the paracorolla primordia. FAR was also expressed mainly in the anther and carpel, although the expression localization was obscure (Fig. 4). FAR was expressed in the paracorolla primordia, in contrast with PLE.

**Fig. 4.** *In situ* hybridization of SQUA, PLE, and FAR in a longitudinal section of a flower bud of ‘Madame Butterfly Yellow’ at paracorolla initiation (late stage 6). Arrows indicate paracorolla primordia. P, Petal; A, Anther; C, Carpel.
expression of both DEF and GLO are required for B-function (Tröbner et al., 1992).

Expression of the A-class gene was suppressed by that of the C-class gene in the carpel and stamen in A. thaliana (Theissen et al., 2000). However, SQUA expression was high in the carpel of Antirrhinum (Fig. 3). This result may be due to the analysis being performed on a developed flower bud. There are other examples where the AP1 homolog was expressed in the developed carpel of Amborella and Nupher, which are basal angiosperms (Kim et al., 2005). The reason why the A-class gene is highly expressed in the developed carpel is not known and should be the focus of future studies.

It was reported that the formation of extra floral organs such as the paracorolla, which is not a basic floral organ, was induced with the application of gibberellin or cytokinin in some species (Blahut-Beatty et al., 1998; Mchughen, 1982; Nishijima and Shima, 2006; Sawhney, 1981). Furthermore, cytokinin application affects the expression of homeotic genes (Estruch et al., 1993; Li et al., 2002). However, homeotic genes do not seem to be involved in organ generation of paracorolla in A. majus, but are solely involved in determination of organ identity. This is because ‘Yellow Butterfly’ and ‘Madame Butterfly Yellow’ showed similar expression patterns to the homeotic genes in each floral organ. In Torenia spp., which belongs to the same order as Antirrhinum spp. (Lamiales), paracorolla were induced by application of forchlorfuron, an inhibitor of cytokinin inactivation through oxidation of the isopentenyl side chain (Nishijima and Shima, 2006). Thus, it is possible that a physiological function related to cytokinin, e.g. stimulation of meristematic activity through regulation of meristematic genes (Lindsay et al., 2006; Rupp et al., 1999), regulates paracorolla differentiation.

Although paracorolla develop only in a limited number of plant species, this extra floral organ has a quite outstanding appearance. Thus, if a practical method of paracorolla induction can be exploited based on basic research, it will effectively enable growers to expand variations in flower morphology of floricultural plants.

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Literature Cited


