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Original Paper

Chrysanthemum flower shape modification by suppression of chrysanthemum-*AGAMOUS* gene

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Abstract Creation of new flower shapes is a major breeding target for ornamental plants. The ABC model on the flower structure is known to be applicable to a broad range of plants. Suppression of the C gene would produce a double flower phenotype with loss of floral determinacy. The flower shape of chrysanthemum (*Chrysanthemum morifolium*) was modified by suppressing the chrysanthemum-*AGAMOUS* (*CAG*) gene, which might be a C gene, with an antisense transgene. We obtained 103 transgenic plants; however, only a single line (951-2) showed modified flower shape. The pistil of each ray floret in line 951-2 was transformed to several corolla-like tissues (secondary corolla) and a pistil-like tissue. Southern blot analysis showed multiple-copy integration of the transgene into the 951-2 genome. The amount of *CAG* mRNA was reduced in line 951-2 compared to the wild-type. On the ray florets, the cell shapes of the adaxial surfaces of the corolla seemed to be almost the same between the proper corolla and the secondary corolla. The stigma was poorly developed and plain structured both on the ray and disk florets. We observed abnormally wide filaments in 951-2. The structure of the surface cells of the abnormal filaments transformed to corolla-like cells. In this study, we demonstrate that suppression of the *CAG* gene converted the stamen and pistil into corolla-like tissues.

Key words: *AGAMOUS*, *Chrysanthemum morifolium*, ornamental plants, transformation.

Flower shape is one of the most important characteristics in ornamental plants. Creation of new flower shapes in these plants is a major breeding target. Key transcriptional factors for the identification of floral organs have been clarified by analyzing model plants such as *Arabidopsis*. The ABC model (Coen and Meyerowitz 1991) and its modified version (Theiβen 2001) are known to be applicable to a broad range of plants (Kim et al. 2005). The ABC model proposed that three functionally different genes i.e., A, B, and C specified the four-whorl structure of the flower. Gene A is responsible for the sepal development in the first whorl (outermost). A and B together specify the petals in the second whorl. B and C determine the stamens in the third whorl and C alone specifies the carpels in the fourth whorl (Coen and Meyerowitz 1991). A mutation on the C gene, *AGAMOUS*, produces a double flower phenotype (no stamens and no carpels) with loss of floral determinacy in *Arabidopsis* (Bowman et al. 1989). In gerbera (*Gerbera hybrida*), the same plant family (Asteraceae) as chrysanthemum, the ray florets in transformants with antisense gerbera-*AGAMOUS* formed corolla-like organs in the third whorl and all floret types formed carpelloid- and pappus-like organs in the fourth whorl. However, they maintained floral determinacy (Yu et al. 1999). Generally, the double flower phenotype has a higher ornamental value than the single one. Therefore, we tried to modify the flower shape of chrysanthemum, one of the most important ornamental plants in the world, by suppressing the chrysanthemum-*AGAMOUS* (*CAG*) gene, which might be a C gene.

Genetic transformation is a powerful tool for breeding ornamental plants. A blue transgenic carnation has been marketed (Tanaka et al. 2005). Breeding of new color chrysanthemums, such as blue, would be valuable for the flower industry and its consumers. For the release of transgenic plants, we should also consider the possible risk of transgene escape into the natural environment. Chrysanthemum cultivars cross easily with their wild species (Jong and Rademaker 1989) and there are many native chrysanthemums in Japan (Huxley et al. 1992). For commercialization of transgenic chrysanthemum, male sterility might be essential; however, female sterility may also be considered for the candidate plants. Suppression of the C gene may cause sterility in both male and female reproductive functions, by converting

stamens and pistils into corollas. In this study, we also aimed at sterilization by suppressing the *CAG* gene in addition to modifying the flower shape.

Materials and methods

Plant materials and transformation

We used the chrysanthemum (*Chrysanthemum morifolium*) cultivar 'Sei-Marine' in our experiment. Transgenic plants were obtained using an *Agrobacterium*-mediated transformation system described previously (Aida et al. 2004).

Vector plasmids

Derivatives of the binary vector pBI121 (Clontech, Mountain View, CA, USA) were used by replacing the cauliflower mosaic virus 35S RNA promoter and *β-glucuronidase* gene with a tobacco elongation factor (EF)-1α promoter (Aida et al. 2005) and a chrysanthemum-*AGAMOUS* homolog (*CAG1*; accession No. AB354249) in an antisense orientation, respectively, followed by a nopaline synthase (*nos*) terminator. *CAG1* was isolated from the chrysanthemum cultivar 'Shuho-no-Chikara'. The isolated fragment seemed to be a part of the *CAG* gene lacking upper sequences corresponding to the first 13 amino acids, as compared to a reported full length *AGAMOUS*-homolog of chrysanthemum (*CDM37*; accession No. AY173059; Shchennikova et al. 2004).

Southern blot and real-time PCR analyses

Total DNA was extracted from the leaf tissue following the method of Hasebe and Iwatsuki (1990). About 15 μg of DNA was digested with *Hin*dIII, electrophoresed in a 0.8% agarose gel and transferred onto a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). *Hin*dIII was used to cut the plasmid at a single site outside the *CAG1* gene. The *CAG1* sequences were labelled with digoxigenin (DIG) using a PCR DIG probe synthesis kit (Roche Diagnostics) and then used as probes. Primer sequences used for amplification of the probes read as 5′-TGCATACATAATACGATGATCCAAA-3′ (antisense orientation of *CAG1*) in the forward direction and 5′-TTGAACGATCGGGGAAATTC-3′ (beginning sequence of the nos terminator) in the reverse direction with the transformation vector as a template. Southern hybridization was done with the DIG high prime and DIG luminescent detection kit for nucleic acids (Roche Diagnostics). The hybridized blots were finally washed with 0.2×SSC, 0.1% SDS at 68°C.

Total RNA was extracted from the corolla and pistil tissues of the ray florets using the SV Total RNA isolation system (Promega, Madison, WI, USA) and cDNAs were synthesized using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Levels of endogenous *CAG* transcripts were then analyzed using real-time PCR with the SYBR Premix Ex Taq system (Takara Bio, Otsu, Japan). Reactions were performed on the LightCycler system (Roche Diagnostics). Primer sequences for *CAG* were designed according to a full length *AGAMOUS*-homolog of chrysanthemum (*CDM37*; AY173059, Shchennikova et al. 2004) to avoid amplification of *CAG1* transgene transcripts. Chrysanthemum *actin* (AB205087) was used as the respective constitutive control. Primer sequences used for the real-time PCR reaction were as follows: forward 5′-TTCATGGCAAATTCTGATGC-3′ (containing start codon; *CAG1* transgene transcripts does not have these sequences) and reverse 5′-ATTCGTGGTGGTGTTCTCGATCC-3′ for *CAG*, and forward 5′-ACATGCTATCTTGCGTTTGG-3′ and reverse 5′-CTCTCACAATTTCCCGTTCA-3′ for *actin*.

Observation by scanning electron microscopy

We used fresh samples of flowering plants for observation by scanning electron microscopy (VE-7000, Keyence Co., Osaka, Japan) according to the manufacturer's manual.

Results and discussion

Production of a flower-shape modified chrysanthemum (951-2)

We obtained 103 transgenic plants and transferred them to a greenhouse to observe the flower shape. Modification rate was very low; only a single line (951-2) showed a modified flower-shape phenotype and all the rest had normal shapes. The ray florets of 951-2 had additional secondary corollas (Figure 1A) as their pistils changed into several corolla-like tissues and a pistil-like tissue (Figure 1B, C). We used the 951-2 line for further analysis. The possible difference in the *CAG* sequences might be one of the reasons for the low modification rate in flower shape among the transformants. The other possible reason is that the introduced antisense transgene might have lacked the upper sequences corresponding to the first 13 amino acids.

Southern blot and expression analyses on the CAG gene

The 951-2 line showed at least three additional bands compared to the wild-type on Southern blot analysis (Figure 2A), indicating multiple-copy integration of the transgene into the genome. Digestion of the vector with *Hin*dIII cuts the plasmid at a single site outside the antisense *CAG1* gene (before the EF-1α promoter). The additional bands detected might have been cut by *Hin*dIII at sites in both the vector and the genome. The wild-type showed at least four bands, indicating existence of several endogenous *CAG* homolog sequences. The additional bands on 951-2 were higher intensity than the putative endogenous bands. It could have been possible that the Southern probe contained some *nos* terminator region that affected the intensity.

The ratio of endogenous *CAG* mRNA to *actin* mRNA (relative *CAG* mRNA) is shown in Figure 2B. As mentioned previously, primer sequences for the real-time PCR reaction were designed to avoid amplification of *CAG1* transgene transcripts. In the wild-type, the *CAG* mRNA was slightly detected on the corolla tissue (relative *CAG* mRNA; 0.12) and strongly detected on the

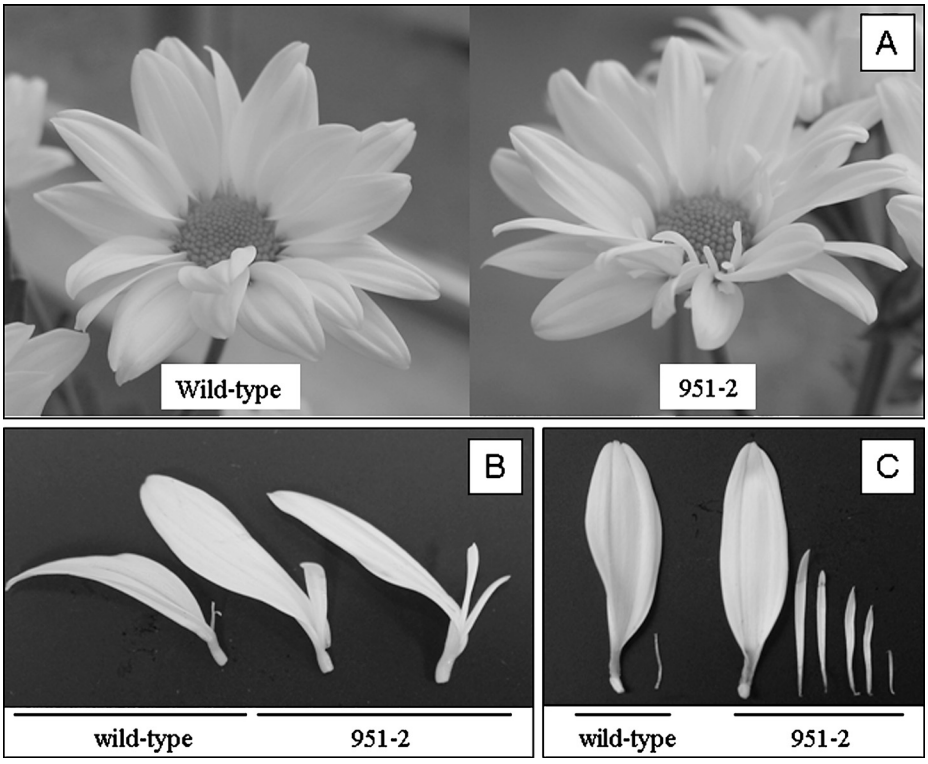

Figure 1. Modified ray florets of a transformant 951-2. Pistils of the ray florets of a transgenic chrysanthemum 951-2 changed into several corolla-like tissues and a pistil-like tissue. (A) Capitulum, (B) intact ray florets, and (C) ray florets divided into each tissue type.

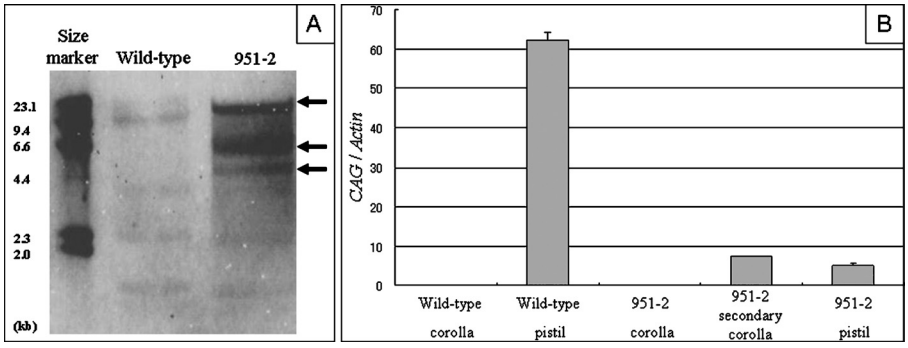

Figure 2. Southern blot and expression analyses of the *CAG* gene on a transformant 951-2. (A) Southern blot analysis showed multiple-copy integration of the transgene into the genome of 951-2. The wild-type showed at least four bands, indicating existence of several endogenous *CAG* homolog sequences. Arrows on 951-2 indicate three additional bands corresponding to *CAG1* transgenes. (B) Expression analysis showed that the level of endogenous *CAG* mRNA was reduced on the secondary corolla and pistil (putative fourth whorl) of 951-2. It seemed that the reduced *CAG* expression on the fourth whorl converted the pistil into a structure of secondary corolla with a pistil.

pistil tissue (62.3). This is a reasonable result because *CAG* is a C function gene, which shows little expression on the corolla and strong expression on the pistil (Coen and Meyerowitz 1991). In 951-2, the *CAG* mRNA was also slightly detected in the proper corolla tissue (0.12); however, considerable *CAG* mRNA was detected on the secondary corolla tissue (7.26). In the pistil tissue of 951-2, a much lower level of the *CAG* mRNA (5.05) was detected than that in the wild-type pistil tissue (62.3), suggesting that the expression of the *CAG* gene was suppressed. The relative *CAG* mRNA level was almost the same between the secondary corolla (7.26) and pistil tissues (5.05) of 951-2. Both tissues were derived from the pistil and located at a putative fourth whorl region on the ABC model. It seemed that the reduced *CAG* expression on the fourth whorl converted a pistil into a structure of several corollas with a pistil.

Surface structure detail of 951-2 flowers

Figure 3 shows the detailed surface structure of flowers of 951-2. On the ray florets, the cell shapes of the adaxial surfaces of the corolla seemed to be almost the same

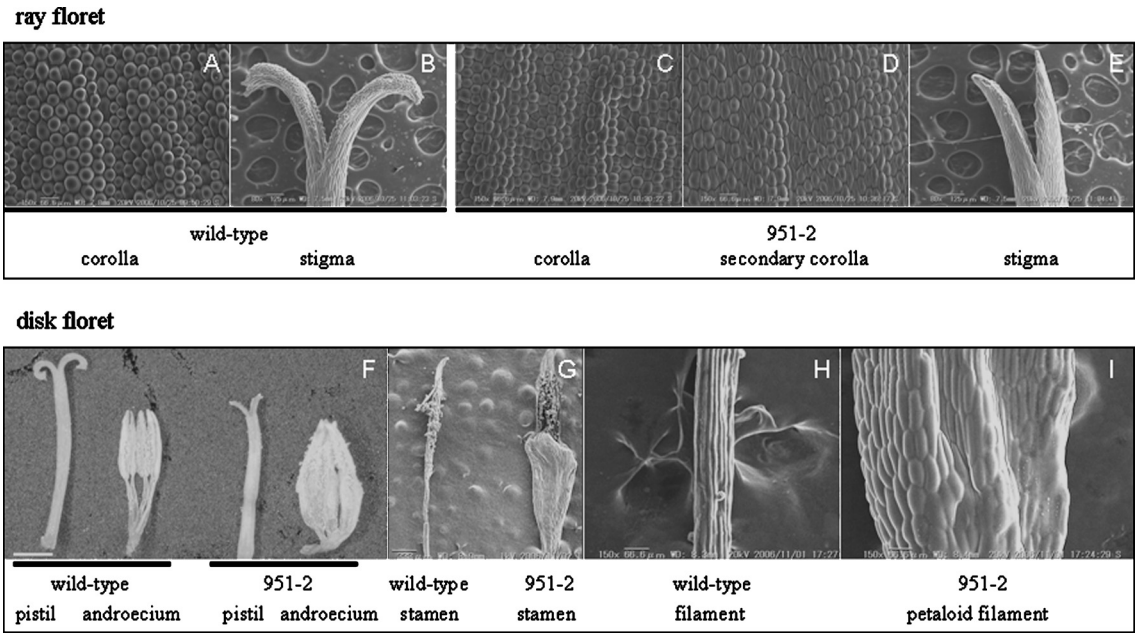

Figure 3. Detailed surface structure of flowers of a transformant 951-2. (A) Corolla and (B) stigma of a ray floret of wild-type, (C) corolla, (D) secondary corolla and (E) stigma of a ray floret of 951-2, (F) pistil and androecium of the disk floret of wild-type and 951-2, (G) stamen of wild-type and 951-2, (H) filament of wild-type, and (I) petaloid filament of 951-2. On the ray florets, cell shapes of adaxial surfaces of corollas seemed to be almost the same between the proper and secondary corollas (A, C, D). On the pistils, the stigma was poorly developed with plain structure both on the ray (B, E) and disk florets (F). We observed abnormally wide filaments on 951-2 (F, G), and structure of the surface cells of the abnormal filaments were transformed to corolla-like cells (H, I). Scale bar stands for 66.6 μm (A, C, D, H, I), 125 μm (B, E), 333 μm (G), and 1 mm (F).

between the proper and secondary corollas (Figure 3A, C, D). This suggests that the secondary corolla is substantially similar to the proper corolla. In pistils, the stigma was transformed to a poorly developed and plain-structured form both on the ray (Figure 3B, E) and disk florets (Figure 3F). Suppression of the *CAG* gene might affect the develpoment of pistils, especially the structure of stigmas. In chrysanthemum, the ray florets lack androecium tissue unlike the disk florets, which have stamens. We observed abnormally wide filaments on 951-2 (Figure 3F, G), the surface cells of which were petaloid (Figure 3H, I). The formation of petaloid filaments strongly suggests that the suppression of the *CAG* gene partially changed the androecium to corolla.

Introduction of the antisense *CAG1* transgene suppressed the *CAG* gene expression on the 951-2 line, which changed the structure of the ray florets to multiple-corollas with a pistil. In *Arabidposis*, suppression of the *AGAMOUS* gene changed the flower to a flower-within-a-flower structure, in other words a (sepal, petal, petal)$_n$ structure, by suppressing the formation of stamens/carpels and the termination of flower development (Bowman et al. 1989). In tomato (*Solanum lycopersicum*), a similar flower structure with loss of floral determinacy was obtained by suppressing the tomato-*AGAMOUS* gene (Pnueli et al. 1994). On the other hand, gerbera transformants with antisense gerbera-*AGAMOUS* showed modified structures in the third and fourth whorls; however, they maintained floral determinacy (Yu et al. 1999). In chrysanthemum, the ray florets consisted of only a corolla and a pistil (lacking calyx and androecium). If the formation of a pistil and termination of flower development were suppressed in the ray florets, a (corolla)$_n$ structure might be produced, similar to the (corolla)$_n$ and pistil structure observed in 951-2.

Disk florets of chrysanthemum consist of a corolla, an androecium, and a pistil (lacking calyx). If a class C gene was suppressed completely, the disk florets of chrysanthemum might have changed to a (corolla, corolla)$_n$ structure; however, the disk florets of 951-2 had a corolla, petaloid-androecium, and poor-pistil structure. Suppression of the *CAG* gene might have also been incomplete on the disk florets compared to the ray florets, which resulted in the imperfect altered pattern.

Conclusion

In this study, we demonstrated that the suppression of the *CAG* gene would modify the androecium and gynoecium to corolla-like tissues; however, we only obtained an incomplete *CAG*-suppressed line. Complete suppression of *CAG* could be achieved by chimeric repressor silencing technology, which can convert a transcription factor into a dominant repressor (Hiratsu et al. 2003). In the future, we should be able to obtain "multiple-corolla-type chrysanthemums", which might be attractive for ornamental use and might have a high horticultural

value. The lack of function of androecium and gynoecium would create male and female sterile chrysanthemums. The complete sterile phenotype would be useful as it would prevent the escape of transgenes into the natural environment, which would foster commercialization of transgenic chrysanthemums.

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