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Inheritance of Bluish Flower Color of Transgenic Chrysanthemum by Interspecific Hybrids

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Abstract

Bluish transgenic chrysanthemums have already been produced and are expected to be commercialized. As domesticated chrysanthemum (*Chrysanthemum morifolium*) is known to cross with wild species native to Japan, the risk of transgene flows into the wild population must be considered. However, little research has been conducted on the crossability of transgenic chrysanthemums or on the heritability of the transgenes involved. In this study, we investigated artificial cross-pollination between color-modified chrysanthemums and the wild species *Chrysanthemum japonense* var. *japonense*. Relatively high seed set rates of 28.8%-53.5% were achieved. Based on a comparison between the size and shape of the flowerheads and leaves of the seedlings and the parent plant, most seedlings were assumed to be hybrids. Polymerase chain reaction was applied to amplify the *Campanula F3'5'H* gene fragment. The segregation and inheritance of the transgenes were confirmed. Some transgenic progeny exhibited a modified flower color similar to that of the parent. The progeny were also demonstrated to have accumulated anthocyanins specific to the parent plant. The results confirmed the transmission of the transgenes to the interspecific progeny. The modified anthocyanins then produced petals of similar color. To prevent unintended environmental consequences, techniques are needed to reduce the possibility of transgene flow to native chrysanthemum species. This will be a necessary precursor to the commercialization of bluish chrysanthemums in East Asia, specifically Japan.

Discipline: Horticulture
Additional key words: environmental risk, flower color, ornamental plants, transformation

Introduction

The creation of novel flower colors is an important target when breeding ornamental plants. Recent developments in molecular biology have allowed the creation of bluish roses (Katsumoto et al. 2007), carnations (Tanaka et al. 1998), and chrysanthemums (Brugliera et al. 2013, Noda et al. 2013) by anthocyanin B-ring hydroxylation. However, it remained impossible to produce truly blue versions of these economically important flowers until 2017 when Noda et al. reported the creation of blue chrysanthemums by combining the same process with glucosylation. Bluish roses and carnations have now been commercialized, and chrysanthemums will follow. This new strain is expected to generate new consumer demand and increase chrysanthemum production.

Japan is home to 32 species of *Chrysanthemum* (Ohashi & Yonekura 2004), many of which can be crossed with cultivars (Nakata et al. 1987, Nakata et al. 2001, Ohashi & Yonekura 2004). Before the genetically modified chrysanthemums can be commercialized, concerns about the risk associated with crossing must be addressed. However, little is known about the crossability of the color-modified chrysanthemums or about the heritability of the modified color.

In this study, we examined the crossability of transgenic chrysanthemum with the wild species *Chrysanthemum japonense* var. *japonense*. The heritability of the transgene and modified flower color was demonstrated.

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Materials and methods

1. Plants

The purple flower line 1434-18 and blue flower line 1575-14 are transgenic plants of *Chrysanthemum morifolium* 'Taihei'. The line 1434-18 was transformed using binary vector pB324 (Fig. 1), which includes a *flavonoid 3′,5′-hydroxylase* (*F3′5′H*) gene isolated from *Campanula medium* (GenBank accession number FW570877). This produces purple-colored ray floret petals that contain delphinidin 3-(6″-malonyl)glucoside (A3) and delphinidin 3-(3″,6″-dimalonyl)glucoside (A4) as major anthocyanins (see Fig. 5). The 1575-14 line was transformed using binary vector pB361 (Fig. 1), introducing *Campanula F3′5′H* and *Clitoria ternatea A3′5′GT*(AB115560)thatencodeUDP-glucose:anthocyanin 3′,5′-*O*-glucosyltransferase, producing blue-colored ray floret petals containing delphinidin 3,3′,5′-triglucoside (A5) and delphinidin 3-(6″-malonyl)glucoside-3′,5′-diglucoside (A6) as major anthocyanins (see Fig. 5). Wild-type 'Taihei' plants were used as controls. A laboratory line GU3 of *Chrysanthemum japonense* var. *japonense*, a wild chrysanthemum species native to Japan, was used as one parent in crossing experiments. This species was selected as it is one of the main wild chrysanthemum species and has the same hexaploid chromosome set as that of commercially produced chrysanthemums.

2. Crossing and seed germination experiments

Plants were raised to produce flowers in a closed greenhouse used for transgenic plant experiments. The flowers of chrysanthemum are aggregate, and the flowerhead is organized into two different types of floret. Ray florets form the decorative edge of the flowerhead and contain only female reproductive organs. Tubular florets within the flowerhead house both male and female organs. As only tubular florets were used in our experiments, the ray florets were cut shortly before flowering, and the heads ware bagged. As chrysanthemum generally has a low self-pollination rate (Wang et al. 2014), pollination was carried out without emasculation. A practical challenge was the unitary stamen/pistil structure, which made it difficult to isolate the stamens from the small tubular florets. To allow the monitoring of self-pollination, six bagged heads within each crossing combination were not artificially pollinated. In each line, 15 (chrysanthemums) or five (*C. japonense* var. *japonense*) flowerheads were used for reciprocal crossing by artificial pollination. Chrysanthemum is protandrous, with pollen appearing before the opening of the stigma. A lot of pollen was therefore supplied repeatedly to the opened stigma of the seed parents by direct application

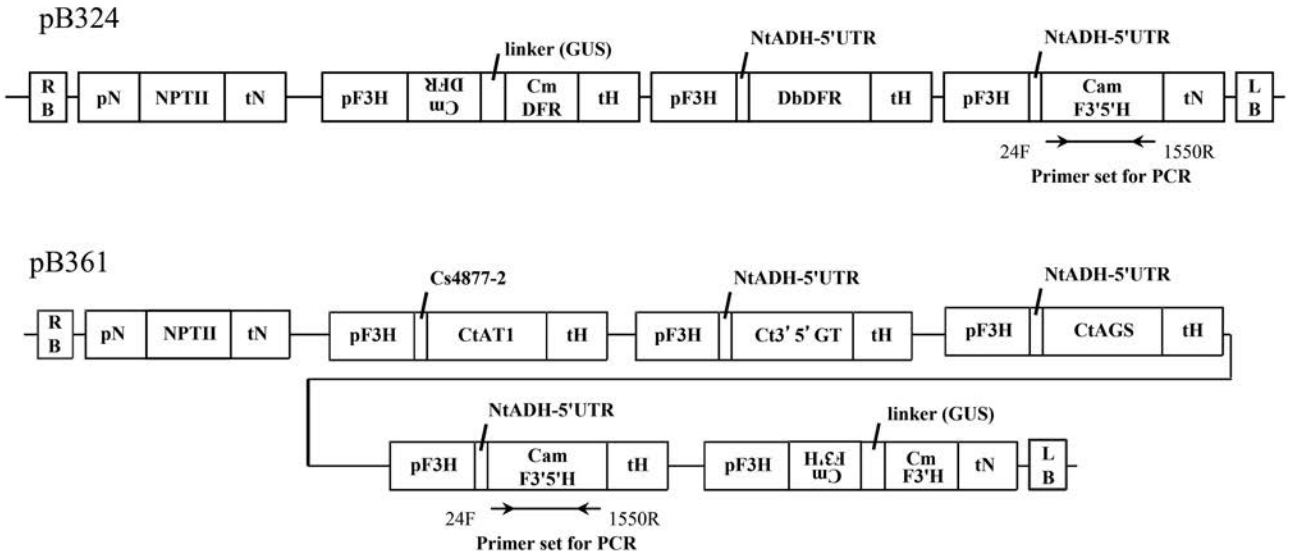

Fig. 1. Binary vectors used in transformation

RB: right T-DNA border; pN: *Agrobacterium NOS* promoter; NPTII: *Escherichia coli neomycin phosphotransferase II*; tN: *Agrobacterium NOS* terminator; pF3H: chrysanthemum *F3H* promoter (GenBank accession number FW570860); linker (GUS): 3′ region of *Escherichia coli β-glucuronidase*; CmDFR: chrysanthemum *dihydroflavonol 4-reductase*; tH: *Arabidopsis HSP* terminator; NtADH-5′UTR: tobacco *ADH-5′*-UTR region; DbDFR: delphinium *dihydroflavonol 4-reductase* (AB221083); CamF3′5′H: *Campanula medium flavonoid 3′,5′-hydroxylase* (FW570877); LB: left T-DNA border; Cs4877-2: *Chrysanthemum seticuspe* EST contig 4877-2, putative endoplasmic reticulum signal sequence; CtAT1: *Clitoria ternatea 1-O-acylglucose:anthocyanin 3′,5′-O-glucoside acyltransferase* (AB267671); CtA3′5′GT: *Clitoria ternatea UDP-glucose:anthocyanin 3′,5′-O-glucosyltransferase* (AB115560); CtAGS: *Clitoria ternatea 1-O-acyl-glucose synthase* (AB185946); CmF3′H: chrysanthemum *flavonoid 3′-hydroxylase*.

without using any specialized tool. Seeds were harvested one to two months after pollination. Germination tests were conducted immediately after harvesting on 30 seeds from each cross combination. These were placed in plastic dishes at 25°C under dark conditions for six days. Twelve seedlings were selected from each cross combination, and transplanted into soil for further cultivation in a closed greenhouse.

3. Polymerase chain reaction (PCR)

For the PCR experiments, total DNA was isolated from the seedling leaves, following the method of Edwards et al. (1991). Two primers—5′-CTTATGTGAACTTGTTGCTGC-3′ (Cam24F) and 5′-ACAGTGTAAGCACTTGGAGGC-3′ (Cam1550R)—were used to amplify a 1527bp fragment of *Campanula F3′5′H* (see Fig. 1). DNA quality was checked by amplifying a 549bp fragment of the endogenous chrysanthemum *actin* gene using two primers—5′-AATGAGCTTCGTGTAGCTCC-3′ (CmActin40F) and 5′-AATACCAGCAGCTTCCATCC-3′ (CmActin588R). DNA was amplified using KOD FX DNA Polymerase (TOYOBO, Osaka, Japan). We performed 32 cycles for 20 seconds at 95°C, followed by 30 seconds at 62°C, and then one minute at 72°C.

4. Characterization of the hybrid plants

The size and shape of the flowerheads and leaves of the hybrids were compared with those of both parents to distinguish hybrid plants from self-pollinated ones. The color of the ray florets was assessed by observers, using the color charts of the Royal Horticultural Society as a guide.

5. Anthocyanin analysis

High-performance liquid chromatography was performed following the method of Noda et al. (2013). The structures of the major anthocyanins detected in the current study were determined and reported by Noda et al. (2017).

Results and discussion

1. Cross fertilization between chrysanthemum 'Taihei' and *C. japonense* var. *japonense*

Table 1 shows the seed set rates of all cross combinations. The maximum rate of 53.5% and minimum rate of 28.8% demonstrated that 'Taihei' crossed readily with *C. japonense* var. *japonense*, including the color-modified transformants. Although the seed set rates of the transformants were slightly lower than those of the 'Taihei' wild type when used as both seed and pollen parents, the effect of the transgenes on fertilization was unclear as few transformants were used in the experiments. Flowerheads that had not been exposed to artificial pollination set no seed, suggesting that little self-pollination occurred in either parental plant. As repeated pollination was performed, the seed set rates were expected to be higher than in the wild state. However, the results demonstrated a significant possibility of crossing between chrysanthemum transformants and *C. japonense* var. *japonense* in the wild. No significant differences in seed size or shape were observed between the wild-type derived seeds and those of the transformants; thus, these results are not shown. Between 25 and 27 seedlings were germinated from each group of 30 seeds, yielding germination rates of 83-90%, six days after sowing. No significant differences were found in the germination rates of the cross combinations.

2. Transmission of transgene in hybrid plants

PCR was used to amplify a specific fragment of the *Campanula F3′5′H* gene. Among the progeny of the purple flowered transformant 1434-18, the transgene was detected in 7 of the 12 plants that were obtained by crossing 1434-18 as the seed parent and in 7 of the 11 plants that were obtained by crossing 1434-18 as the pollen parent (Fig. 2). Progeny No. 49 of 1434-18 (a pollen parent) was removed from the experiment as it was assumed to have been self-pollinated. This is discussed

Table 1. Seed set rate of each crossing combination with artificial pollination

Crossing combination	Number of tubular florets	Number of seed sets	Seed set rate (%)
'Taihei' wild type × *C. japonense* var. *japonense*	1821	975	53.5
'Taihei' transformant 1434-18 × *C. japonense* var. *japonense*	889	317	35.7
'Taihei' transformant 1575-14 × *C. japonense* var. *japonense*	937	366	39.1
C. japonense var. *japonense* × 'Taihei' wild type	823	355	43.1
C. japonense var. *japonense* × 'Taihei' transformant 1434-18	690	259	37.5
C. japonense var. *japonense* × 'Taihei' transformant 1575-14	465	134	28.8

further in the following section. Among the progeny of the blue flowered transformant 1575-14, the transgene was detected in 11 of the 12 plants that were obtained by crossing 1575-14 as the seed parent, and in 9 of the 12 plants that were obtained by crossing 1575-14 as the pollen parent (Fig. 2). The *Campanula F3′5′H* gene-specific fragment was not amplified on either parental wild-type plant. In DNA quality checking, amplification of the endogenous chrysanthemum *actin* gene was confirmed for every *Campanula F3′5′H* gene-negative plant. These results demonstrated the presence of the transgene in some of the progeny, and further suggested that the PCR-positive plants were hybrids, at least when the pollen parents were transgenic.

3. Characterization of putative hybrid plants and confirmation of hybridism

Twelve seedlings of six cross combinations (total of 72 plants) were grown to produce flowers in a closed greenhouse. Of these, 71 plants were judged to be hybrids based on a comparison of their flowerheads and leaves with those of chrysanthemum and *C. japonense* var. *japonense* (Fig. 3). Only a single plant (No. 49) from the crossing of *C. japonense* var. *japonense* and 'Taihei' transformant 1434-18 was assumed to result from self-pollination, based on its close similarity to the parental *C. japonense* var. *japonense* (images not shown).

4. Inheritance of color modification

Table 2 lists the colors of the ray florets of the

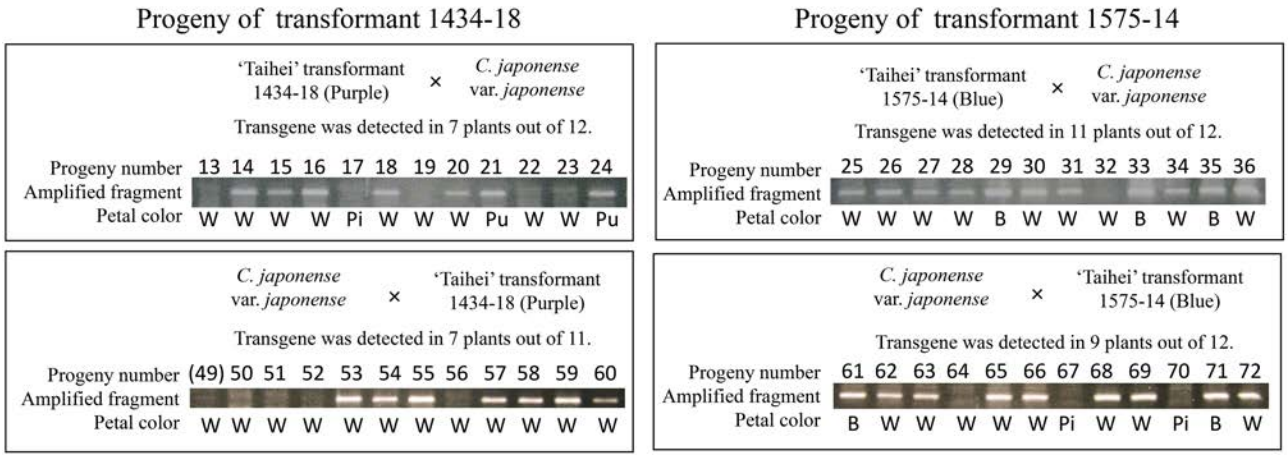

Fig. 2. PCR detection of transgene (*flavonoid 3′,5′-hydroxylase*) in progeny of transformants
Progeny No. 49 was assumed to be self-pollinated. Petal colors: W: white, Pi: pink, Pu: purple, B: blue.

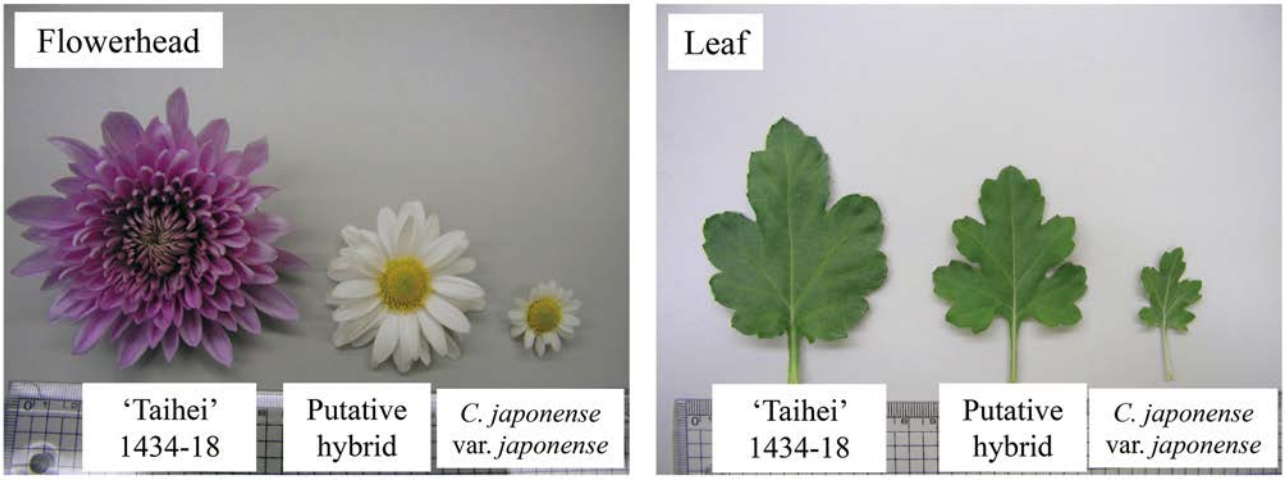

Fig. 3. Flowerheads and leaves of putative hybrids of 'Taihei' and *C. japonense* var. *japonense* with intermediate size and shape relative to those of the parents
Note that 71 of 72 progeny plants were intermediate between 'Taihei' and *C. japonense* var. *japonense*, suggesting that they were hybrids of the two parents. One offspring (No. 49), obtained by crossing *C. japonense* var. *japonense* with 'Taihei' transformant 1434-18, had almost the same shape as *C. japonense* var. *japonense* and was regarded as a product of self-pollination.

hybrids. When crossed with the wild-type chrysanthemum, 3 of the 12 plants obtained by crossing the wild type as the seed parent and 4 of the 12 plants obtained by crossing the wild type as the pollen parent were pink, and the rest were white. The segregation ratio between white and pigmented flowers was close to 3:1, suggesting that anthocyanin accumulation is required for the transmission of two gene loci from 'Taihei'. The progeny of the pink wild-type 'Taihei' only had pink petals, not purple or blue of the transformants. When a purple transformant (1434-18) was used as the seed parent, nine white, one pink, and two purple petaled plants were produced. PCR analysis demonstrated that the two purple offspring carried the transgene, whereas the pink one did not (Fig. 2). The blue transformant (1575-14) produced three blue hybrids when used as the seed parent, and two when used as the pollen parent. PCR analysis showed that all carried a fragment of the transgene (Fig. 2), suggesting that the transgene coding for blue petals was active in the hybrid offspring. The flower pigmentation of all offspring in these experiments was lighter than that of the parental 'Taihei' (Fig. 4). The cause of this was unclear, but we assumed that hybridization with *C. japonense* var. *japonense*, which naturally produces white flowers, reduced the amount of anthocyanin precursors present, resulting in a smaller accumulation of anthocyanins.

Figure 4 shows the inheritance of the modified flower colors by the hybrids plants. An example is shown of a progeny whose flower is similar in color to that of its 'Taihei' parent. Figure 4 shows pink, purple, and blue offspring obtained from a pink wild-type plant (A), purple transformant (B), and blue transformant (C), respectively. Based on the color charts, parents and offspring were judged to have the same color hue. The petals of the offspring were shown to contain the same plant specific anthocyanins as the parents (Fig. 5). The offspring of purple transgenic plants carried anthocyanins A3 and A4, and those of blue transgenic plants carried A5 and A6 (Fig. 5). None of these were present in the wild-

Table 2. Variation of flower color among hybrid plants

Crossing combination	Petal color of ray florets					
		Pigmented				
	White	Pink	Purple	Blue	(Total pigmented)	Total
'Taihei' wild type × *C. japonense* var. *japonense*	9	3	0	0	(3)	12
'Taihei' transformant 1434-18 × *C. japonense* var. *japonense*	9	1	2	0	(3)	12
'Taihei' transformant 1575-14 × *C. japonense* var. *japonense*	9	0	0	3	(3)	12
C. japonense var. *japonense* × 'Taihei' wild type	8	4	0	0	(4)	12
C. japonense var. *japonense* × 'Taihei' transformant 1434-18	11	0	0	0	(0)	11
C. japonense var. *japonense* × 'Taihei' transformant 1575-14	8	2	0	2	(4)	12

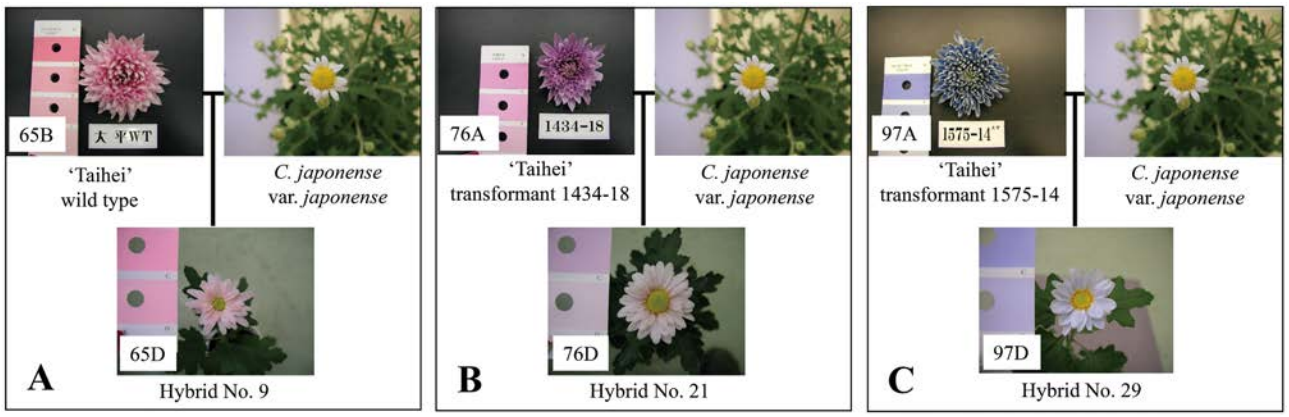

Fig. 4. Modified flower color of chrysanthemum transformants inherited by progeny
Numbers shown at the lower left are the color numbers from the charts of the Royal Horticultural Society. The figure shows examples of hybrid plants with flower color hues similar to those of the 'Taihei' parent: pink wild type (A), purple transformant (B), and blue transformant (C).

type chrysanthemums. One progeny of the purple transformant carried proper anthocyanins A1 and A2, as well as transformant-specific A3 and A4, suggesting that the transgene was weakly active. These results demonstrated that the transgene was inherited by the progeny, and that sufficient modified anthocyanin was accumulated to reproduce the parental flower color.

As shown in Figure 2, most transgene-carrying progeny in this experiment bore white flowers. As noted above, this was attributed to a lack of biosynthesis genes for anthocyanin production. These latent transgenes would reappear after re-crossing with chrysanthemums, including both wild-type and transgenic plants that carry the biosynthesis genes for anthocyanin. If transgenes enter the wild chrysanthemum population, it will be difficult to identify transgene-bearing plants by flower color alone.

5. Future commercialization of bluish transgenic chrysanthemums

Chrysanthemums cross naturally with wild species (Nakata et al. 1987) and some of the resulting hybrids have been classified as independent species (Ohashi & Yonekura 2004). Hybrids of *Chrysanthemum wakasaense* and a chrysanthemum cultivar were shown to have survived in the wild for more than 14 years (Nakata et al. 2001). In this study, we demonstrated the ability of transgenic flower color-modified chrysanthemums to cross with the wild species *C. japonense* var. *japonense*, and showed the progeny to inherit the transgene. To prevent unintended environmental outcomes, it would be wise to reduce the possibility of transgene transfer to wild species. We are currently investigating techniques by which the flow of transgenes into wild *Chrysanthemum* can be reduced, as this will support the future commercialization of bluish strains.

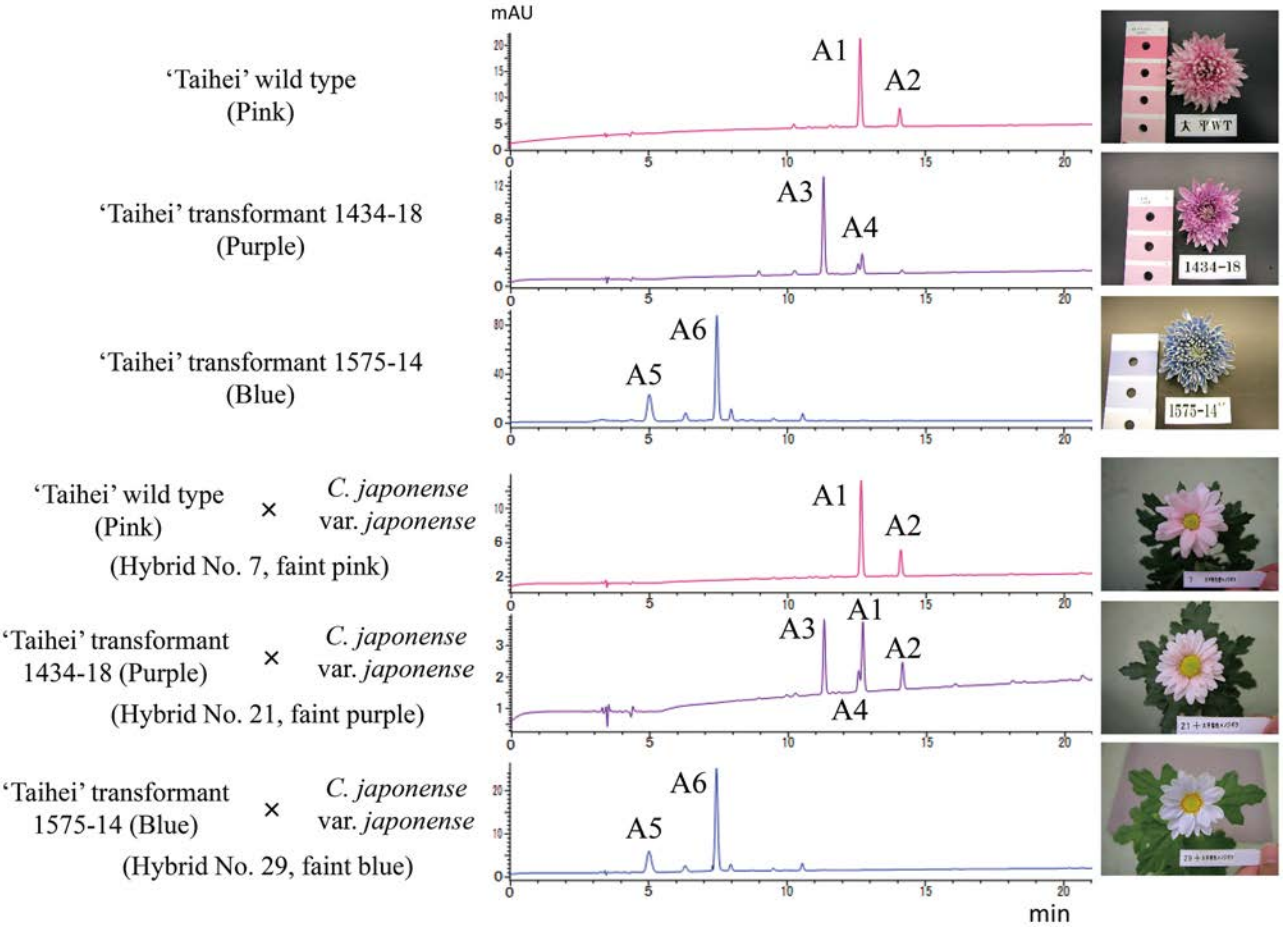

Fig. 5. Modified anthocyanins detected in the petals of the progeny of transformants
A1-A2: Cyanidin-based anthocyanins (proper chrysanthemum anthocyanins); A3-A6: Delphinidin-based anthocyanins (modified anthocyanins accumulated only in transformants); A1: cyanidin 3-(6″-malonyl)glucoside; A2, cyanidin 3-(3″,6″-dimalonyl)glucoside; A3, delphinidin 3-(6″-malonyl)glucoside; A4: delphinidin 3-(3″,6″-dimalonyl)glucoside; A5: delphinidin 3,3′5′-triglucoside; A6: delphinidin 3-(6″-malonyl)glucoside-3′,5′-diglucoside.

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