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Review

Breeding of fragrant cyclamen by interspecific hybridization and ion-beam irradiation

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Conventional breeding of cyclamen has relied on crossings among *Cyclamen persicum* cultivars without consideration of the scent of the flowers. *Cyclamen purpurascens* is a wild species with the most fragrant flowers in the genus *Cyclamen.* Allodiploid ($2n = 2x = 41$, AB) and allotriploid ($2n = 3x = 65$, AAB) plants have been produced from crosses of diploid and autotetraploid cultivars of *C. persicum* ($2n = 2x = 48$, AA; $4x = 96$, AAAA) × diploid wild *C. purpurascens* ($2n = 2x = 34$, BB) by embryo rescue, but are sterile. Fertile allotetraploid ($2n = 4x = 82$, AABB) plants have been produced by chromosome doubling of the sterile allodiploids *in vitro*. Autotetraploid *C. purpurascens* ($2n = 4x = 68$, BBBB) has been produced by chromosome doubling of diploid *C. purpurascens*, and other fertile allotetraploids ($2n = 4x = 82$, AABB) have been produced from crosses of autotetraploid cultivars of *C. persicum* × autotetraploid *C. purpurascens* by embryo rescue. Commercial cultivars of fragrant cyclamen have been bred by conventional crosses among the allotetraploids. Mutation breeding using ion-beam irradiation combined with plant tissue culture has resulted in fragrant cyclamens with novel flower colors and pigments. In contrast, allotriploids (AAB) have not been commercialized because of seed sterility and poor ornamental value. The flower colors are determined by anthocyanins and flavonol glycosides or chalcone glucoside, and the fragrances are determined by monoterpenes, sesquiterpenes, phenylpropanoids, or aliphatics. Techniques for the production of fragrant cyclamen and knowledge of flower pigments and volatiles will allow innovation in conventional cyclamen breeding.

Key Words: *Cyclamen persicum*, *C. purpurascens*, interspecific hybrid, ion-beam, flower color and pigments, flower scent and volatiles.

Introduction

The genus *Cyclamen* (*Primulaceae*) has 22 species. All species form a tuber and can be propagated by seed, but they never propagate by natural splitting (Grey-Wilson 2002). Cultivars have been developed as highly popular pot plants through crossing among selected natural mutants of wild *Cyclamen persicum* Miller and are commercially grown in many countries. Although wild *C. persicum* is diploid ($2n = 2x = 48$), *C. persicum* cultivars include spontaneously derived autotetraploids ($2n = 4x = 96$) (Legro 1959). Wild *C. persicum* has small flowers consisting of a deep purple "eye" (the region of the petal base) and a white, pink or purple "slip" (the region excluding the eye). *Cyclamen persicum* forma *albidum*, with a white slip and a white eye, is occasionally found in the wild (Grey-Wilson 2002). *Cyclamen persicum*-derived cultivars can have a purple, pink, red, red-purple, pale yellow, or white slip, but the eye color is always the same as in wild *C. persicum*. They also have various flower shapes, sizes, and patterns. The flowers of most *C. persicum* cultivars emit a weak woody or powdery scent, and thus the scent has not been regarded as an important trait in breeding. Wild *Cyclamen purpurascens* Miller ($2n = 2x = 34$) has small flowers with a purple slip and a deep purple eye, but *C. purpurascens* forma *album*, with a white slip and a white eye, is occasionally found in the wild. Neither form has been developed into major commercial cultivars (Grey-Wilson 2002). However, their flowers emit a sweet fragrance resembling rose, hyacinth, or lily of the valley (Ishizaka *et al.* 2002). The introduction of *C. purpurascens* fragrance would enhance the commercial value of *C. persicum*-derived cultivars.

Wild *Cyclamen* species other than *C. persicum* are not used in major commercial culture, and have not been used in breeding, owing to pre- or post-fertilization barriers with *C. persicum* (Ishizaka 2008). Legro (1959) reported that interspecific hybridization within *Cyclamen* (especially

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between *C. persicum* and *C. purpurascens*) by conventional crossing is difficult. However, several attempts to create a novel fragrant cyclamen with both the color range of *C. persicum* cultivars and the fragrance of *C. purpurascens* have been made using several breeding techniques (Ishizaka 2008). This review describes the use of plant tissue culture and ion-beam irradiation for the production of fragrant cyclamens, along with the analysis of flower pigments and volatile compounds of those cyclamens and their parents.

Production of allodiploids and allotriploids

For the purposes of this review, *C. persicum* has the A genome and *C. purpurascens* has the B genome. Diploid and autotetraploid cultivars of *C. persicum*—'Strauss' ($2n = 2x = 48$, AA, **Fig. 1A**), 'Pure White' ($2n = 2x = 48$, AA, **Fig. 1B**), 'Golden Boy' ($2n = 2x = 48$, AA, **Fig. 1C**), and 'Salmon Scarlet' ($2n = 4x = 96$, AAAA), resembling 'Vuurbaak' ($2n = 4x = 96$, AAAA, **Fig. 1E**)—have been used as seed parents, and diploid wild *C. purpurascens* ($2n = 2x = 34$, BB, **Fig. 1D**) have been used as pollen parents, but reciprocal crossing has not been performed, because *C. purpurascens* plants have very few flowers. Histological observations reveal that ovules fertilized in these cross combinations contain weak hybrid embryos without endosperm, which eventually collapse. This suggests a post-fertilization barrier involved in the abortion of hybrid embryos between *C. persicum* and *C. purpurascens*, which can be overcome by embryo rescue (Ishizaka and Uematsu 1995a). In fact, aseptic culture of placenta-attached ovules containing weak hybrid embryos *in vitro* has produced plantlets, which have grown into mature plants in greenhouse culture. Chromosome analysis of root tip cells and morphological observation confirm that these mature plants are true interspecific hybrids, both allodiploids ($2n = 2x = 41$, AB) and allotriploids ($2n = 3x = 65$, AAB) (Ishizaka and Uematsu 1995a). Thus, ovule culture is a valuable method for creating interspecific *Cyclamen* hybrids (Ewald 1996, Shibusawa 2003, Shibusawa and Ogawa 1997, Yamashita and Takamura 2007).

Allodiploids (AB) show complete pollen sterility caused by a low frequency of chromosome pairing between the A and B genomes at metaphase I in the pollen mother cells and subsequent abnormal cell division, yielding no fertile seeds by self-pollination or by backcrosses with *C. persicum* cultivars (Ishizaka 1997). Allotriploids (AAB) can be obtained by crossing between allotetraploids (AABB), described in the next section, and *C. persicum* cultivars (AA). Two kinds of allotriploids, obtained from crosses of AAAA × BB and AABB × AA, have 24 bivalent chromosomes, probably originating from the A genome, and 17 univalent chromosomes, probably originating from the B genome, in the pollen mother cells, resulting in abnormal cell division (Ishizaka, unpublished). Consequently, the allotriploids produce few fertile pollen grains, which produce very few viable seeds by self-pollination or by backcrosses with *C. persicum* cultivars (Ishizaka 1997, Ishizaka and Uematsu 1995a, 1995b). These results confirm the difficulty of breeding using allodiploids and allotriploids.

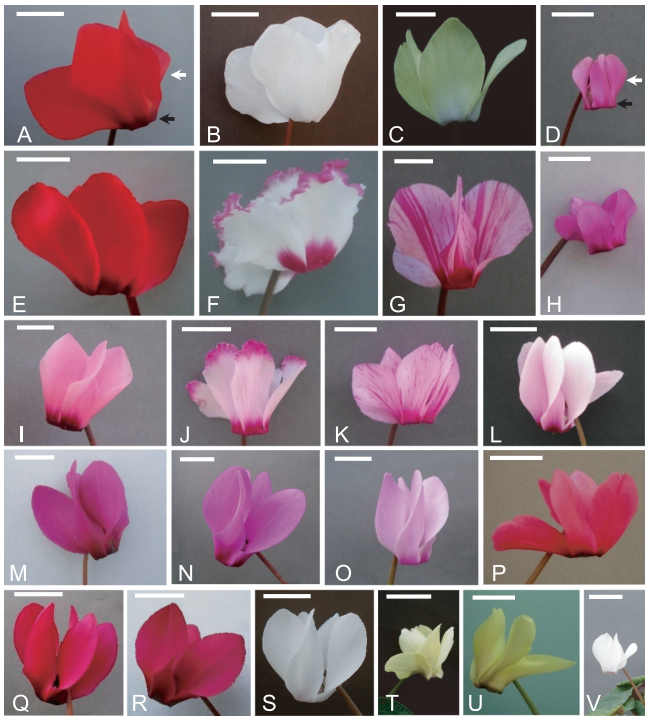

Fig. 1. Characteristics of *Cyclamen persicum* cultivars, wild *C. purpurascens*, their interspecific hybrids, and ion-beam-derived mutants. (A) Diploid *C. persicum* 'Strauss'. (B) Diploid *C. persicum* 'Pure White'. (C) Diploid *C. persicum* 'Golden Boy'. (D) Diploid *C. purpurascens*. (E) Autotetraploid *C. persicum* 'Vuurbaak'. (F) Autotetraploid *C. persicum* 'Victoria'. (G) Autotetraploid *C. persicum* 'Harlequin'. (H) Autotetraploid *C. purpurascens*. (I) Allotetraploid of autotetraploid 'Vuurbaak' × autotetraploid *C. purpurascens*. (J) Allotetraploid of autotetraploid 'Victoria' × autotetraploid *C. purpurascens*. (K) Allotetraploid of autotetraploid 'Harlequin' × autotetraploid *C. purpurascens*. (L) Fragrant 'Uruwashi-no-Kaori'. (M) Fragrant 'Kaori-no-Mai'. (N) Fragrant 'Kokou-no-Kaori'. (O) Allotetraploid "GBCP" derived by chromosome doubling of allodiploid of diploid 'Golden Boy' × diploid *C. purpurascens*. (P) Ion-beam-derived mutant 'Tennyo-no-Mai'. (Q) Ion-beam-derived mutant 'Miyabi-no-Mai'. (R) Ion-beam-derived mutant with a red-purple flower due to delphinidin. (S) Mutant with a white flower derived from fragrant 'Kokou-no-Kaori' irradiated by ion beam. (T) Mutant with a pale yellow flower derived from the mutant of dihaploid of GBCP irradiated by ion beam. (U) Allotetraploid mutant with a pale yellow flower derived from dihaploid of GBCP. (V) Sterile mutant with a white flower derived from dihaploid of GBCP irradiated by ion beam. Bars = 20 mm. Black arrow, "eye"; white arrow, "slip".

Production of allotetraploids and fragrant cyclamens

Seed sterility poses a barrier in the breeding and propagation of cyclamen. In many plants, sterility caused by the lack of affinity between different genomes can be overcome by

chromosome doubling. *In vitro* colchicine treatment of placenta-attached ovules derived from crosses of *C. persicum* 'Strauss', 'Pure White', and 'Golden Boy' (all $2n = 2x = 48$, AA) × *C. purpurascens* ($2n = 2x = 34$, BB) has produced allotetraploids ($2n = 4x = 82$, AABB) owing to chromosome doubling (Ishizaka and Uematsu 1995b, Kameari *et al.* 2010). The allotetraploids produce viable pollen grains through frequent formation of 41 bivalent chromosomes in the pollen mother cells by pairing of homologous chromosomes within the A and B genomes, and produce fertile seeds by self-pollination (Ishizaka 1997).

Allotetraploids (AABB) obtained from allodiploids (AB) of *C. persicum* 'Strauss' (AA) × *C. purpurascens* (BB) have flowers with a pink slip and a deep purple eye. Those obtained from allodiploids of *C. persicum* 'Pure White' × *C. purpurascens* have a pale pink slip and a purple eye. F_1 progeny of crosses between these two allotetraploids also have flowers with a pink slip and a deep purple eye. The F_2 population has various flower colors, with a pink, pale pink, pale purple, purple, or deep-purple slip and a purple or deep-purple eye (Ishizaka, unpublished). Fragrant progeny selected from the F_2 population have been developed into three cultivars: 'Uruwashi-no-Kaori' (**Fig. 1L**), 'Kaori-no-Mai' (**Fig. 1M**), and 'Kokou-no-Kaori' (**Fig. 1N**). Other allotetraploids (AABB) produced by chromosome doubling of allodiploids of diploid *C. persicum* 'Golden Boy' (AA) × *C. purpurascens* (BB), referred to here as "GBCP" (**Fig. 1O**), have not yet been developed into commercial cultivars, but GBCP is useful breeding material for creating fragrant cyclamens with yellow flowers by mutation breeding (Kameari *et al.* 2010).

Another way to produce fertile allotetraploids is to cross autotetraploids with different genomes. Autotetraploid *C. purpurascens* has not been found in wild populations, but has been produced by chromosome doubling *in vitro* (Ishizaka and Kondo 2004). Allotetraploids ($2n = 4x = 82$, AABB) were produced by crosses among autotetraploid *C. persicum* 'Vuurbaak', 'Victoria', and 'Harlequin' (all $2n = 4x = 96$, AAAA, **Fig. 1E–1G**) and autotetraploid *C. purpurascens* ($2n = 4x = 68$, BBBB, **Fig. 1H**). The post-fertilization barrier involving the abortion of hybrid embryos in these cross combinations can be overcome by ovule culture. Chromosome analysis of root tip cells, morphological observation, and seed fertility confirm that the resultant plants are allotetraploid (Ishizaka and Kondo 2004). Two allotetraploids (**Fig. 1J**, **1K**) are valuable candidates for developing novel fragrant cyclamens with unique flowers like those of 'Victoria' (**Fig. 1F**) and 'Harlequin' (**Fig. 1G**), but another is very similar to 'Uruwashi-no-Kaori' (**Fig. 1I**, **1L**).

Improvement of fragrant cyclamens by ion-beam irradiation

The diploid cultivars of *C. persicum* described here have desirable flower colors (e.g., **Fig. 1A–1C**: red, white and yellow) that have not appeared in the progeny of allotetraploids of these cultivars × diploid *C. purpurascens* (**Fig. 1L–1O**: pinks and purples). These phenotypes imply that in the allotetraploids, the expression of genes regulating flower colors of *C. persicum* cultivars is suppressed by the presence of genes derived from *C. purpurascens*. Accordingly, the flower colors of *C. persicum* cultivars should appear in the allotetraploids if the *C. purpurascens* genes are inactivated. Since ion-beam irradiation can cause a broad mutation spectrum and a high mutation rate by depositing much more energy in a limited area of the target genome than gamma rays (Tanaka *et al.* 2010), irradiation with 220- or 320-MeV carbon ion beams has been used as a mutagen (Ishizaka *et al.* 2012).

Dihaploids induced from 'Uruwashi-no-Kaori' and GBCP by anther culture and allotetraploids (amphidiploids), including 'Kaori-no-Mai' and 'Kokou-no-Kaori', have been irradiated with ion beams, and M_1 populations have been regenerated from cultures of the irradiated plants. Mutants with a salmon pink flower appeared in M_1 populations derived from a dihaploid of 'Uruwashi-no-Kaori'. Mutants with a pale yellow flower and a white flower appeared in M_1 populations derived from a dihaploid of GBCP (**Fig. 1O**, **1T**, **1V**). The appearance of these mutants suggests that mutated genotypes appear directly as phenotypes in these dihaploids. These three mutants are sterile because of their haploidy. Fertile mutants with a salmon pink flower have been obtained from cultures of the mutants of a dihaploid of 'Uruwashi-no-Kaori' re-irradiated with ion beams (Ishizaka *et al.* 2012), probably owing to chromosome doubling by somaclonal variation, or perhaps to the ion beams. Fertile mutants with pale yellow flowers have been obtained from dihaploid mutants of GBCP by artificial chromosome doubling using colchicine *in vitro*, but those with white flowers have not yet been obtained (**Fig. 1T–1V**; Ishizaka, unpublished). Because no mutants have been obtained among M_1 plants of allotetraploid 'Kaori-no-Mai' and 'Kokou-no-Kaori', M_2 populations were produced by self-pollination of the M_1 plants. The appearance of a mutant with a red-purple flower in M_2 of 'Kaori-no-Mai' and one with a white flower in M_2 of 'Kokou-no-Kaori' suggests that recessive mutated genes suppressed by the alleles in the M_1 plants are homozygous in the M_2 plants.

The fertile mutant with the salmon pink flower has been developed into the commercial cultivar 'Tennyo-no-Mai' (**Fig. 1P**). That with the red-purple flower due to malvidin 3-glucoside has been developed as 'Miyabi-no-Mai' (**Fig. 1Q**), but that with the red-purple flower due to delphinidin 3,5-diglucoside has not yet been developed into a commercial cultivar (**Fig. 1R**). The fertile mutant with the white flower derived from 'Kokou-no-Kaori' and that with the pale yellow flower derived from GBCP are currently under commercial development (**Fig. 1S**, **1U**). However, the sterile mutant with the white flower derived from a dihaploid of GBCP has not been used for further breeding (**Fig. 1V**).

Flower color and pigments

Flowers with various colors, shapes, patterns, and sizes attract pollinators such as insects, ensuring seed set and fruit production. They are also the most important feature in ornamental plants, and new cultivars are always being developed. Major pigments produced in the flowers include flavonoids, carotenoids, chlorophylls, and betalains. Among the flavonoids, anthocyanins are red to purple and flavonol glycosides are colorless or pale compounds, and both types are widely distributed in many organs, notably in cyclamen flowers. Anthocyanin colors in cyclamen flowers change with the presence of flavonol glycosides as co-pigments, resulting in a wide range of colors (Nakayama *et al.* 2012, Takamura and Sugimura 2008).

Major pigments detected in the flowers of *C. persicum* cultivars, *C. purpurascens*, their interspecific hybrids, and ion-beam-derived mutants are listed in **Table 1**, and a schematic outline of their biosynthesis pathways is shown in **Fig. 2**. Cyanidin-, peonidin-, and malvidin-derived anthocyanins and chalcone 2′-glucoside have been detected as major pigments, but pelargonidin-, delphinidin-, and petunidin-derived anthocyanins have not been detected (Boase *et al.* 2010, Miyajima *et al.* 1991, Takamura and Sugimura 2008, Takamura *et al.* 1997, Van Bragt 1962). *Cyclamen persicum* 'Strauss' (AA) and 'Vuurbaak' (AAAA) have flowers with a red slip and a dark red eye (**Fig. 1A**, **1E**). The slip has peonidin 3-glucoside or peonidin 3-neohesperidoside and the eye has malvidin 3-glucoside as major anthocyanins. 'Pure White' (AA), lacking anthocyanins, has a white flower with quercetin and kaempferol glycosides (**Fig. 1B**). 'Golden Boy' (AA), lacking anthocyanins, has a pale yellow flower with chalcone 2′-glucoside as a major pigment (**Fig. 1C**). 'Victoria' (AAAA) and 'Harlequin' (AAAA) have red-purple flowers with unique patterns, containing malvidin 3-glucoside (**Fig. 1F**, **1G**). Flowers of *C. purpurascens* (BB and BBBB) have a purple slip and a deep purple eye containing malvidin 3,5-diglucoside (**Fig. 1D**, **1H**). Flowers of these cultivars and *C. purpurascens* also accumulate quercetin and kaempferol glycosides as major flavonols (Ishizaka *et al.* 2006, 2007, Miyajima *et al.* 1991, Takamura and Sugimura 2008, Takamura *et al.* 2005, Van Bragt 1962, Webby and Boase 1999).

Two kinds of allotetraploids (AABB) have been obtained

Table 1. Flower pigments and volatile compounds detected in flowers of *Cyclamen persicum* cultivars, wild *C. purpurascens*, their interspecific hybrids, and ion-beam-derived mutants

Plant materials[1)]	Major flower pigments[2)]										Major volatile compounds[3)]					
	Kae	Que	Ch2′G	Dp3,5dG	Mv3G	Mv3,5dG	Cy3,5dG	Pn3G	Pn3,5dG	Pn3Nh	AL	PP	MA	SA	SH	RO
Fig. 1 A	4)	4)			5)			6)		6)	7)				7)	
B	4)	4)									7)				7)	
C	4)	4)	4)								7)				7)	
D	4)	4)				4)						7)	7)	7)		7)
E	4)	4)			5)					6)	7)				7)	
F	4)	4)			4)						7)				7)	
G	4)	4)			4)						7)				7)	
H	4)	4)				4)						7)	7)	7)		7)
I	4)	4)				4)	6)		6)			7)	7)	7)		7)
J	4)	4)				4)						7)	7)	7)		7)
K	4)	4)				4)						7)	7)	7)		7)
L	4)	4)				4)	6)		6)			7)	7)	7)		7)
M	4)	4)				4)						7)	7)	7)		7)
N	4)	4)				4)						7)	7)	7)		7)
O	4)	4)				4)						7)	7)	7)		7)
P	4)	4)				4)	6)		6)			7)	7)	7)		7)
Q	4)	4)			4)							7)	7)	7)		7)
R	4)	4)		4)								7)	7)	7)		7)
S	4)	4)											7)	7)		7)
T	4)	4)	4)									7)	7)	7)		7)
U	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[1)] Plant materials indicate **Fig. 1A–1U**.

[2)] Kae, kaempferol glycosides; Que, quercetin glycosides; Ch2′G, chalcone 2′-glucoside; Dp3,5dG, delphinidin 3,5-diglucoside; Mv3G, malvidin 3-glucoside; Mv3,5dG, malvidin 3,5-diglucoside; Cy3,5dG, cyanidin 3,5-diglucoside; Pn3G, peonidin 3-glucoside; Pn3,5dG, peonidin 3,5-diglucoside; Pn3Nh, peonidin 3-neohesperidoside.

[3)] AL, aliphatic compounds: hexanol, 2-ethyl hexanol, methyl nonyl ketone; PP, phenylpropanoids: cinnamic alcohol, cinnamic aldehyde, hydrocinnamic alcohol; MA, monoterpene alcohols: citronellol, geraniol, linalool; SA, sesquiterpene alcohols: farnesol, 2,3-dihydrofarnesol; SH, sesquiterpene hydrocarbons: β-caryophyllene, α-farnesene; RO, rose oxide.

[4)] Flower pigment detected in eye and slip.

[5)] Flower pigment detected in eye.

[6)] Flower pigment detected in slip.

[7)] Volatile compounds emitted from flower.

– Not identified.

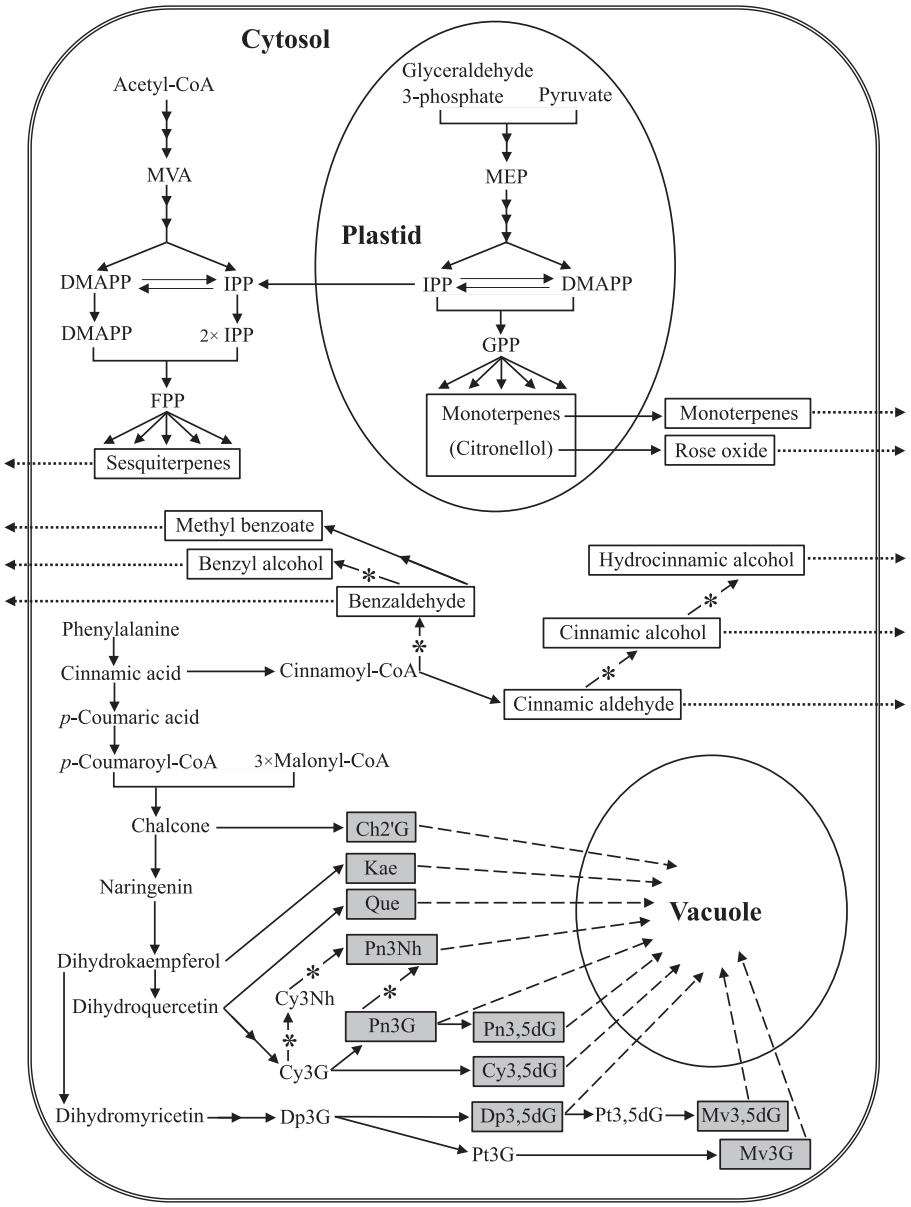

Fig. 2. Schematic outline of biosynthesis of flower pigments and volatile compounds in petal cells of *Cyclamen persicum* cultivars, wild *C. purpurascens*, their interspecific hybrids, and ion-beam-derived mutants. White boxes, end products of volatile compounds; gray boxes, end products of flower pigments. Solid arrows, pathways of volatile compound and flower pigment biosynthesis from precursors indicated by unboxed abbreviations. Solid arrows with asterisks, possible steps not yet identified. Dotted arrows, emission of major volatile compounds from petal cells. Dashed arrows, accumulation of major flower pigments in the vacuole. DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid; Ch2′G, chalcone 2′-glucoside; Cy3G, cyanidin 3-glucoside; Cy3,5dG, cyanidin 3,5-diglucoside; Cy3Nh, cyanidin 3-neohesperidoside; Dp3G, delphinidin 3-glucoside; Dp3,5dG, delphinidin 3,5-diglucoside; Kae, kaempferol glycosides; Mv3G, malvidin 3-glucoside; Mv3,5dG, malvidin 3,5-diglucoside; Pn3G, peonidin 3-glucoside; Pn3,5dG, peonidin 3,5-diglucoside; Pn3Nh, peonidin 3-neohesperidoside; Pt3G, petunidin 3-glucoside; Pt3,5dG, petunidin 3,5-diglucoside; Que, quercetin glycosides.

by chromosome doubling of allodiploids (AB) derived from *C. persicum* ‘Strauss’ (AA) × *C. purpurascens* (BB) and *C. persicum* ‘Pure White’ (AA) × *C. purpurascens* (BB). The former flowers have a pink slip with cyanidin 3,5-diglucoside, peonidin 3,5-diglucoside, and malvidin 3,5-diglucoside, and a deep purple eye with malvidin 3,5-diglucoside. The latter flowers have a pale pink slip and a purple eye, both with malvidin 3,5-diglucoside as a major anthocyanin. Flowers of these allotetraploids also contain quercetin and kaempferol glycosides. Their F_1 plants have the same flower color and pigments as the allotetraploid progeny of *C. persicum* ‘Strauss’ (AA) × *C. purpurascens* (BB) (Takamura *et al.* 2004). Fragrant cyclamens (‘Uruwashi-no-Kaori’, ‘Kaori-no-Mai’, and ‘Kokou-no-

Kaori') have been bred from crosses between these allotetraploids, as described in the previous section.

The fragrant 'Uruwashi-no-Kaori' (AABB) and its dihaploid (AB) have flowers with a pink slip with cyanidin 3,5-diglucoside, peonidin 3,5-diglucoside, and malvidin 3,5-diglucoside, and a dark purple eye with malvidin 3,5-diglucoside, both with quercetin and kaempferol glycosides (Ishizaka *et al.* 2012). From these pigments and the breeding process described in the previous section, it can be determined that 'Uruwashi-no-Kaori' and its dihaploid have the *C. persicum* 'Strauss' (AA) and *C. purpurascens* (BB) genomes. On this basis, in the slip, the biosynthesis of anthocyanidins (cyanidin and peonidin) is probably due to genes from the *C. persicum* 'Strauss' genome, and their glycosylation is probably due to genes from the *C. purpurascens* genome. The biosynthesis of malvidin 3,5-diglucoside in the slip and the glycosylation of the anthocyanidins in the slip and eye are probably due to genes from the *C. purpurascens* genome. Future studies could investigate which genome controls the biosynthesis of malvidin 3,5-diglucoside in the eye and of flavonol glycosides in the slip and eye. 'Vuurbaak' has similar flower color and pigments to those of 'Strauss' (**Fig. 1A**, **1E**), and allotetraploid (AABB) progeny of 'Vuurbaak' (AAAA) × *C. purpurascens* (BBBB) have color and pigments similar to those of 'Uruwashi-no-Kaori' (**Fig. 1I**, **1L**). Thus, the discussion of the color and pigments of 'Uruwashi-no-Kaori' also applies to this allotetraploid.

In contrast, 'Tennyo-no-Mai', with a salmon pink flower, has been derived from a dihaploid of 'Uruwashi-no-Kaori' with a pink flower by ion-beam irradiation (**Fig. 1L**, **1P**). Flowers of both have the same anthocyanins and flavonol glycosides, but 'Tennyo-no-Mai', with a deeper color, has more anthocyanins and less flavonol glycosides than 'Uruwashi-no-Kaori' (Nakayama *et al.* 2012). From the flavonoid biosynthesis pathway (Boase *et al.* 2010, Rausher 2006) and types of anthocyanins and flavonol glycosides in both fragrant cyclamens, it can be determined that flavonoid 3′-hydroxylase, flavonoid 3′,5′-hydroxylase, dihydroflavonol 4-reductase, and flavonol synthase use dihydroflavonols (dihydroquercetin and dihydrokaempferol) as common substrates and that dihydroflavonols are ultimately converted into anthocyanins and flavonol glycosides (quercetin and kaempferol glycosides). Alteration from pink to salmon pink suggests that ion-beam irradiation suppresses genes related to the biosynthesis of flavonol glycosides from dihydroflavonols, which are instead converted into anthocyanins.

'Kaori-no-Mai' (AABB) has a purple slip and a deep purple eye, and 'Kokou-no-Kaori' (AABB) has a light purple slip and a purple eye (**Fig. 1M**, **1N**). Both accumulate malvidin 3,5-diglucoside, quercetin glycosides, and kaempferol glycosides in the slip and eye (Kondo *et al.* 2009a, 2009b). From these pigments and the breeding process described in this section, it can be determined that both have the *C. persicum* 'Pure White' genome (AA) and the *C. purpurascens* (BB) genome. The biosynthesis of malvidin 3,5-diglucoside in the flowers is probably due to genes from the *C. purpurascens* genome. Future studies could clarify which genome controls the biosynthesis of flavonol glycosides.

Two mutants derived from 'Kaori-no-Mai' have a red-purple flower with malvidin 3-glucoside (**Fig. 1Q**) or delphinidin 3,5-diglucoside (**Fig. 1R**), as well as quercetin and kaempferol glycosides (Ishizaka *et al.* 2012, Kondo *et al.* 2009a, 2009b). The former mutant is probably induced by deletion of the 5-glucosyltransferase gene. *Cyclamen persicum* 'Pure White', which provided the A genome of 'Kaori-no-Mai' (AABB), has a white flower owing to inactivation of the gene encoding 3-glucosyltransferase (Okada *et al.* 2011, Takamura *et al.* 2017). The accumulation of malvidin 3-glucoside in the former mutant can be explained by comparing the expression of the 5-glucosyltransferase gene in each genome among 'Pure White', *C. purpurascens*, 'Kaori-no-Mai', and the mutant. The latter mutant is the first identified plant to accumulate delphinidin glucoside as the major anthocyanin in the genus *Cyclamen*. In it, one of the *O*-methyltransferase genes has been deleted by ion-beam irradiation and the enzyme encoded by the other has lost the ability to methylate anthocyanins. Both genes are expressed in the petals of 'Kaori-no-Mai' and mediate methylation of anthocyanins (Akita *et al.* 2011). It remains to be determined which gene is derived from which genome, and why one enzyme lacks the ability to methylate anthocyanins. Delphinidin glucosides color petals purple or blue, and occasionally red-purple, and the color is affected by metal ions (Shoji *et al.* 2007, Yoshida *et al*. 2009). This effect might allow the creation of a fragrant cyclamen with a blue flower.

A white-flower mutant has been induced from 'Kokou-no-Kaori' (AABB), which is probably derived from *C. persicum* 'Pure White' (AA) and *C. purpurascens* (BB). The mutant lacks malvidin 3,5-diglucoside but has quercetin and kaempferol glycosides, whereas 'Kokou-no-Kaori' has malvidin 3,5-diglucoside, quercetin glycosides, and kaempferol glycosides (Ishizaka *et al.* 2012, Kondo *et al.* 2010). 'Pure White' cannot synthesize anthocyanins owing to inactivation of the gene encoding 3-glucosyltransferase, and instead accumulates quercetin and kaempferol glycosides (Okada *et al.* 2011, Takamura *et al.* 2017), whereas *C. purpurascens* has malvidin 3,5-diglucoside, quercetin glycosides, and kaempferol glycosides. Thus, the white flower of the mutant is possibly caused by the inactivation of genes related to malvidin 3,5-diglucoside synthesis derived from *C. purpurascens*. *Cyclamen graecum* Link, which has flowers with a pink or purple slip and a dark purple eye, accumulates malvidin 3,5-diglucoside and flavonol glycosides. *Cyclamen graecum* var. *album* has a white slip and eye caused by the accumulation of flavonol glycosides and the lack of malvidin 3,5-diglucoside, which results from a defect in expression of the dihydroflavonol 4-reductase gene (Akita *et al.* 2010). Accordingly, it is necessary to search for genes underlying the white-flower mutant, with

attention to those for 3-glucosyltransferase and dihydroflavonol 4-reductase as candidates.

'Golden Boy' (AA) has a pale yellow flower owing to the accumulation of chalcone 2′-glucoside. Inactivation of the chalcone isomerase gene by insertion of an unknown sequence leads to a deficiency of chalcone isomerase and consequent surplus chalcone, which is subsequently converted to chalcone 2′-glucoside by glycosylation, which is accumulated in the vacuole (Matsufuru *et al.* 2008, Miyajima *et al.* 1991). GBCP (AABB) and its dihaploid (AB), derived from 'Golden Boy' (AA) and *C. purpurascens* (BB), have a light purple flower with malvidin 3,5-diglucoside. Surplus chalcone is converted to malvidin 3,5-diglucoside, as well as quercetin and kaempferol glycosides, by a series of enzymes, including chalcone isomerase derived from *C. purpurascens*. A pale yellow mutant (**Fig. 1T**) derived from the dihaploid accumulates chalcone 2′-glucoside, probably because of the accrual of surplus chalcone owing to radiation damage to the chalcone isomerase gene derived from *C. purpurascens* after glycosylation, as in 'Golden Boy' (Kameari *et al.* 2012). The discussion of the biosynthesis of chalcone 2′-glucoside in the pale yellow mutant also applies to the allotetraploid mutant (**Fig. 1U**). On the other hand, from the analysis of pigments in the white-flower mutant of 'Kokou-no-Kaori', it is reasonable to assume that the white mutant (**Fig. 1V**) derived from the dihaploid of GBCP lacks malvidin 3,5-diglucoside and accumulates flavonol glycosides. These steps can be clarified by analyzing genes for the biosynthesis of flavonol glycosides and malvidin 3,5-diglucoside, with attention to those for 3-glucosyltransferase and dihydroflavonol 4-reductase as candidates.

Pink or purple flowers of diploid and autotetraploid cultivars of *C. persicum* have malvidin 3,5-diglucoside in the slip and eye, whereas some cultivars with red-purple flowers have malvidin 3-glucoside, probably owing to the deletion of the 5-glucosyltransferase gene (Takamura and Sugimura 2008, Van Bragt 1962). Diploid wild *Cyclamen hederifolium* Aiton and *C. purpurascens* consistently accumulate malvidin 3,5-diglucoside (Van Bragt 1962). Interspecific hybrids among malvidin 3-glucoside cultivars and these diploid wild species accumulate malvidin 3,5-diglucoside produced by glycosylation of malvidin 3-glucoside at the 5 position by 5-glucosyltransferase originating from the wild species (Takamura and Aizawa 2007, Takamura *et al.* 2005). Autotetraploid *C. persicum* 'Victoria' and 'Harlequin' have malvidin 3-glucoside, and autotetraploid *C. purpurascens* has malvidin 3,5-diglucoside. Allotetraploids produced by crosses among them exclusively accumulate malvidin 3,5-diglucoside, probably by the mechanism reported by Takamura *et al.* (2005) and Takamura and Aizawa (2007).

Flower fragrance and volatile compounds

More than 1700 low-molecular-weight volatile compounds have been identified in flowers, leaves, and fruits. They are classified as terpenoids, phenylpropanoids / benzenoids, and aliphatic-, nitrogen-, sulfur-, and miscellaneous cyclic compounds, with multiple functional groups, including acids, aldehydes, ketones, alcohols, esters, and ethers (Knudsen and Gershenzon 2006). These compounds are synthesized in specialized gland cells on the surface of leaves and stems or in unspecialized epidermal cells of floral organs, especially petals. They are stored temporarily and released through rupture of the gland cells, or directly from the epidermal cells, to be recognized by the olfactory receptors of humans, animals and insects (Dudareva and Pichersky 2000, Iijima *et al.* 2004, Vainstein *et al.* 2001). These volatile compounds attract pollinators, repel herbivorous insects, and attract natural predators of herbivorous insects. The attraction of pollinators has been widely studied. Several plant species cyclically change their emission of floral volatiles under circadian control, whereas others continuously emit volatiles at a constant level. Cyclic emission by day or by night can be related to the diurnal or nocturnal behavior of pollinators (Dobson 2006, Dudareva and Pichersky 2008, Kolosova *et al.* 2001, Vainstein *et al.* 2001). For humans, these volatiles add flavor or aroma to foods and cosmetics, and add commercial value to ornamental plants, including cyclamen.

Allotetraploid-derived fragrant cyclamens and their parents emit many kinds of volatiles from their petals, with a peak at between 10:00 and 14:00 at 20°C, but the relationship between *Cyclamen* and its pollinators is unclear (Ishizaka, unpublished). Volatile compounds collected over 1 h around noon by the headspace method have been analyzed by gas chromatograph combined with mass-selective detector (Kurihara *et al.* 2004a, 2004b). Major volatile compounds detected from the flowers of *C. persicum* cultivars, *C. purpurascens*, their interspecific hybrids, and ion-beam-derived mutants are listed in **Table 1**, and a schematic outline of their biosynthesis pathways is shown in **Fig. 2**.

Diploid *C. persicum* 'Strauss', 'Pure White', and 'Golden Boy' and autotetraploid *C. persicum* 'Salmon Scarlet', 'Vuurbaak', 'Victoria', and 'Harlequin' emit sesquiterpene hydrocarbons (α-farnesene and β-caryophyllene) and aliphatic compounds (2-ethyl hexanol and methyl nonyl ketone), which have a woody or powdery scent, probably due to the sesquiterpene hydrocarbons (Ishizaka *et al.* 2002, 2006, 2007, Kameari *et al.* 2010). Most cyclamen cultivars emit similar volatile compounds and fragrances, but a few emit a floral scent rich in monoterpenes, as also observed among some wild *C. persicum* (Ishizaka 2011, Kato *et al.* 1995). Recently released *C. persicum* cultivars with various morphological characteristics emit a woody or powdery scent, characteristic of sesquiterpene hydrocarbons. Breeding for flower colors, shapes, sizes, and patterns could have resulted in the unintended selection of cultivars rich in sesquiterpene hydrocarbons. In contrast, flowers of diploid and autotetraploid *C. purpurascens* emit monoterpene alcohols (citronellol, geraniol, linalool), sesquiterpene alcohols

(farnesol and 2,3-dihydrofarnesol), phenylpropanoids (cinnamic aldehyde, cinnamic alcohol, hydrocinnamic alcohol), benzenoids (benzaldehyde, benzyl alcohol, methyl benzoate), and rose oxide, and smell of rose, hyacinth, or lily of the valley. Among aromatic compounds, phenylpropanoids are major volatiles and benzenoids are minor volatiles (Ishizaka *et al.* 2002, 2006, 2007, Kurihara *et al.* 2004a). Nohara *et al*. (1996) reported that a *C. purpurascens* lacked phenylpropanoid volatiles but emitted a few benzenoid volatiles. Natural variants with diverse volatile compounds may have accrued among wild populations of *C. purpurascens* without human involvement.

Among interspecific hybrids, allodiploids (AB) and allotetraploids (AABB) emit monoterpene alcohols, sesquiterpene alcohols, phenylpropanoids / benzenoids, and rose oxide and smell similar to *C. purpurascens* (Ishizaka *et al.* 2002). In addition, 'Uruwashi-no-Kaori', 'Kokou-no-Kaori', and 'Kaori-no-Mai' have volatiles and perfumes similar to their allotetraploid parents or *C. purpurascens* (Kurihara *et al.* 2004b). Other allotetraploids obtained from 'Vuurbaak', 'Victoria' and 'Harlequin' (AAAA) × *C. purpurascens* (BBBB) and from 'Golden Boy' (AA) × *C. purpurascens* (BB) have volatiles and perfumes similar to *C. purpurascens* (Ishizaka *et al.* 2006, 2007, Kameari *et al.* 2010). In contrast, allotriploids (AAB) have volatiles rich in sesquiterpene hydrocarbons and smell like *C. persicum* 'Salmon Scarlet' (Ishizaka *et al.* 2002).

The terpenoid biosynthesis pathway plays an important role in the life cycle of plants through the synthesis of a variety of compounds, including monoterpenes and sesquiterpenes (volatiles), carotenoids (pigments), and gibberellins and abscisic acid (plant hormones). The biosynthesis of monoterpenes and sesquiterpenes, the most important volatiles in the fragrance of cyclamen, has been elucidated (**Fig. 2**). First, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), common precursors in the terpenoid biosynthesis pathway, are synthesized through respectively the mevalonic acid (MVA) pathway in the cytosol and the methylerythritol 4-phosphate (MEP) pathway in the plastid. Second, geranyl diphosphate (GPP) is synthesized by the condensation of one molecule each of IPP and DMAPP by GPP synthase. Farnesyl diphosphate (FPP) is synthesized from two molecules of IPP and one molecule of DMAPP by FPP synthase. Third, GPP and FPP are converted to various monoterpenes and sesquiterpenes by their respective synthases (Aharoni *et al*. 2003, Iijima *et al.* 2004, Knudsen and Gershenzon 2006, Lücker *et al.* 2006, Poulter 2006).

In basil, the ratio of total monoterpenes to total sesquiterpenes was weakly correlated with the ratio of the activities of monoterpene and sesquiterpene synthases (Iijima *et al.* 2004). Transgenic tobacco with multiple monoterpene synthase genes derived from lemon emitted more monoterpenes and less sesquiterpenes than wild-type tobacco, which emits more sesquiterpenes, especially caryophyllene (Lücker *et al.* 2004). In interspecific allotetraploid hybrids of cyclamen, monoterpenes may be increased owing to enhancement of the activities of GPP synthase and monoterpene synthase by the presence of the *C. purpurascens* genome; these enzymes may catalyze biosynthesis from IPP, DMAPP, and GPP more effectively than do FPP synthase and sesquiterpene synthase. Allotriploids (AAB) emit α-farnesene (a sesquiterpene), which is suppressed in allotetraploids (AABB); this difference suggests that the dominance of the A genome in the allotriploid enhances the expression of genes encoding FPP synthase and sesquiterpene synthase (Ishizaka *et al.* 2002). These differences between the allotriploids and allotetraploids can be clarified by comparison of the expression of genes for monoterpene and sesquiterpene synthases and for GPP and FPP synthases and their enzymatic activities.

The ion-beam-derived mutants 'Tennyo-no-Mai', 'Miyabi-no-Mai', and a pale yellow mutant of GBCP have volatiles and perfumes similar to their originators ('Uruwashi-no-Kaori', 'Kaori-no-Mai', and GBCP), despite their great alteration in flower color (Kameari *et al.* 2012, Kondo *et al.* 2009a, 2011). Flowers of 'Kokou-no-Kaori' accumulate malvidin 3,5-diglucoside, quercetin glycosides, and kaempferol glycosides as pigments, and emit volatiles similar to those of 'Uruwashi-no-Kaori' and 'Kaori-no-Mai', including monoterpenes, sesquiterpenes, and phenylpropanoids / benzenoids (Kameari *et al.* 2011, Kondo *et al.* 2010). In the white-flower mutants derived from 'Kokou-no-Kaori' by ion-beam irradiation, quercetin and kaempferol glycosides appear owing to the lack of malvidin 3,5-diglucoside. In addition, emission of cinnamic aldehyde, cinnamic alcohol, and hydrocinnamic alcohol (phenylpropanoid volatiles) is remarkably suppressed (Kameari *et al.* 2011, Kondo *et al.* 2010). Zuker *et al.* (2002) reported that in a carnation cultivar with a pink flower due to pelargonidin glucoside, inhibition of pigment synthesis by antisense suppression of a gene related to flavonoid biosynthesis produced a recombinant with a white flower and greater methyl benzoate (benzenoid volatile) content. They suggested that the flavonoid biosynthesis pathway for pigment production and the phenylpropanoid / benzenoid biosynthesis pathway for volatile production share phenylalanine and cinnamic acid as common precursors, the levels of which are cooperatively regulated. Phenylpropanoids and benzenoids are assumed to be synthesized from phenylalanine via cinnamic acid and cinnamoyl-CoA (**Fig. 2**; Anthony *et al.* 2012, Palmer *et al.* 2014). The genome of 'Kokou-no-Kaori' is assumed to consist of the *C. persicum* 'Pure White' and *C. purpurascens* genomes. The ion-beam irradiation could have affected genes in the flavonoid and phenylpropanoid biosynthesis pathways derived from the *C. purpurascens* genome, but this remains to be resolved.

Conclusions

Since its adoption in Europe as an ornamental plant around 1700, numerous diploid and autotetraploid cultivars of

C. persicum have been developed commercially. Fertile allotetraploids were produced by interspecific crosses between *C. persicum* cultivars and wild *C. purpurascens* around 2000, forming the basis for the development of fragrant cyclamens. Ion-beam irradiation has resulted in the creation of novel flower colors in the allotetraploids and fragrant cyclamens. Characteristics of flower color and fragrance of the fragrant cyclamens and their parents have been clarified through breeding. The fragrant cyclamens have been registered in the Register of Plant Varieties under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries of Japan, and have been distributed to Japanese cyclamen growers. The fragrant cyclamen group is currently small compared with *C. persicum*-derived cultivars. Large-scale cultivation of the fragrant cyclamen by breeders and growers will likely reveal natural mutants. In addition, crosses among the fragrant cyclamens and natural mutants will increase the variety of fragrant cyclamens, which have potential for major development.

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