Flower color is the most important trait in the breeding of ornamental plants. In the floriculture industry, however, bluish colored flowers of desirable plants have proved difficult to breed. Many ornamental plants with a high production volume, such as rose and chrysanthemum, lack the key genes for producing the blue delphinidin pigment or do not have an intracellular environment suitable for developing blue color. Recently, it has become possible to incorporate a blue flower color trait through progress in molecular biological analysis of pigment biosynthesis genes and genetic engineering. For example, introduction of the F3′5′H gene encoding flavonoid 3′,5′-hydroxylase can produce delphinidin in various flowers such as roses and carnations, turning the flower color purple or violet. Furthermore, the world’s first blue chrysanthemum was recently produced by introducing the A3′5′GT gene encoding anthocyanin 3′,5′-O-glucosyltransferase, in addition to F3′5′H, into the host plant. The B-ring glucosylated delphinidin-based anthocyanin that is synthesized by the two transgenes develops blue coloration by co-pigmentation with colorless flavone glycosides naturally present in the ray floret of chrysanthemum. This review focuses on the biotechnological efforts to develop blue flowers, and describes future prospects for blue flower breeding and commercialization.

Key Words: anthocyanins, blue flower, chrysanthemum, co-pigmentation, delphinidin, genetic engineering, ornamental plant.
anthocyanin biosynthesis, have been isolated. In particular, isolation of F3′5′H encoding flavonoid 3′,5′-hydroxylase, a key gene for synthesis of the anthocyanidin delphinidin, which leads to blue coloration in flowers, was reported by Holton et al. (1993). Such genes were expected to lead to blue coloration in various flowers, such as roses and chrysanthemums; however, it has not proved so easy to achieve blue flower coloration.

In this review, I summarize the status of research and development aimed at using genetic engineering technologies to create blue flowers; in particular, I focus on flower color modification of chrysanthemum, in which a truly blue flower color was recently realized for the first time. I also discuss future prospects for molecular breeding and practical application of blue flower coloration.

**Vacuolar pH determinant and metal ion transporter involved in blue color development**

Delphinidin-type anthocyanidins include delphinidin, petunidin and malvidin derivatives in which the 3′ and 5′ positions of the B-ring are hydroxylated and/or methoxylated. Flowers containing these anthocyanins do not always produce a blue color, however, but instead are often purple, mauve, or pink. In such flowers, control of elevation of vacuolar pH and/or coexistence with metal ions is considered to be effective for blue coloration. In this section, I describe recent studies on vacuolar pH determinants and metal ion transporters involved in the color development of blue flowers.

**Manipulation of genes related to vacuolar pH**

In hydrangea, which develops blue coloration under relatively low pH conditions, pH 4 to 5 is suitable for blue color development due to the interaction of delphinidin 3-glucoside, 5-caffeoylquinic acid and Al3+, while a more acidic pH of 3 to 4 leads to red coloration (Yoshida et al. 2003). In petunia, methylated anthocyanins, and petunidin- and malvidin-based anthocyanins accumulate in the corolla. Even for petunia flowers with the same anthocyanin composition, the color can differ greatly depending on the pH of the corolla.

In petunia, the PH1–PH7 gene loci are reported to be involved in determining the pH of petals (de Vlaming et al. 1983, van Houwelingen et al. 1998). PH5 encodes a H+ P3A-ATPase proton pump present in the vacuolar membrane. Mutation of PH5 causes the increased pH in the vacuole, resulting in the petals changing coloration from purplish red to bluish violet (Verweij et al. 2008). In addition, the H+ P3A-ATPase encoded by PH5 physically binds to the F3B-ATPase encoded by PH1, resulting in an increase in proton transport activity and a more acidic vacuolar pH (Faraco et al. 2014). In addition, the expression of PH5 is directly regulated by PH3 and PH4 transcription factors. The PH6 gene, also termed ANTHOCYANIN1 (ANI), encodes a bHLH protein that interacts with PH3, PH4 and WD40 protein AN11. When a transposon is inserted into ANI, anthocyanin is not synthesized and the pH of petals rises (Spelt et al. 2002).

Although anthocyanins become unstable, flowers can develop a blue color under alkaline conditions. For example, if polycyclization with aromatic organic acids occurs, anthocyanins remain stable even under weakly alkaline conditions. In the corolla of morning glory, a rise in pH from 6.6 to 7.7 leads to blue coloration due to the presence of polycyclized peonidin glycoside (Yoshida et al. 1995). The genes involved in producing weakly alkaline vacuoles at the time of flowering are InNHX1 and InNHX2, which encode proteins responsible for transporting K+/Na+ into the vacuoles (Fukuda-Tanaka et al. 2000, Ohnishi et al. 2005, Yamaguchi et al. 2001).

Even if delphinidin-type anthocyanin accumulates, rose petals tend to express redness due to high the acidity of their vacuoles (Katsumoto et al. 2007). To express blue coloration, it is important to introduce genes that can create a weakly acidic pH of about 5.6 to 6.2, or to use strains or cultivars that naturally exhibit a relatively higher pH. Cyclamen spp. have several natural flower colors such as white, light yellow, pink, red, magenta, and purple. The anthocyanins responsible for cyclamen flower color include anthocyanidin 3,5-diglucoside; a peonidin-based anthocyanin that is 3′-methylated cyanidin in the reddish flower; and a malvidin-based anthocyanin in the magenta flower (Hase et al. 2012). In 2012, violet cyclamens were developed by Hokko Chemical Industry Co., Ltd., and are currently being sold as “SerenadiaTM” by Suntory Flowers Ltd. Violet cyclamens were produced through the mutation of cultured cells (Terakawa 2012). Takamura et al. (2015) reported that the major anthocyanin responsible for the violet coloration in cyclamen is malvidin 3,5-diglucoside, and that a change toward a blue color can be achieved by a recessive mutation that increases the pH in petal cells. Multi-petaled cyclamens produced by suppression of the AGAMOUS gene using Chimeric REPressor gene-Silencing Technology (CRES-T) have also been reported (Tanaka et al. 2013), and the development of blue cyclamens with multi-petal blooms is expected in the near future.

**Manipulation of genes encoding metal transporters**

Tulip (Tulipa gesneriana) has varieties with purple flowers in addition to white, red and yellow. Delphinidin 3-rutinoside accumulates as a major pigment in the purple perianths (Shoji et al. 2007). No tulips have blue coloration throughout the whole perianth; however, the inner bottom portion may develop a blue color due to a local accumulation of iron ions (Shoji et al. 2007). A vacuolar ion transporter of iron, TgVit1, facilitates the coexistence of iron ions and delphinidin 3-rutinoside (Momonoi et al. 2009).

When TgVit1 is transiently overexpressed in the cells of purple perianths, and the ferritin synthesis gene TgFER is suppressed to prevent an accumulation of ferritin bound to iron ions, the cell color becomes blue (Momonoi et al. 2009, Shoji et al. 2010). There have been attempts to produce a...
blue tulip by expressing TgVit1 in whole perianths under the control of a petal-specific TgMYB1 promoter (Shoji 2015). We expect that the flowering of these blue tulips will be reported in the near future. In addition, attempts have been made to introduce TgVit1 into the mutant of the interspecific hybrid of Cyclamen persicum and C. purpurascens, which accumulates delphinidin 3,5-diglucoside in petals (Kondo et al. 2009), to create blue cyclamens (Kurihara et al. 2015). The blue coloration was observed by transient TgVit1 expression in the petal cells, and blue flower colored cyclamen is expected to bloom due to stable expression.

Via metal complex formation, iron ions are involved in the blue coloration of Nemophila (Yoshida et al. 2015), cornflower (Shiono et al. 2005, Takeda et al. 2005) and blue poppy (Yoshida et al. 2006). A TgVit1 homologue, CeVIT, has been reported in cornflower (Yoshida and Negishi 2013). The HmVALT and HmPALT1 genes, which encode Al3+-transporters localized in vacuolar and cytoplasmic membranes respectively, have been reported in hydrangea, which develops blue coloration through the involvement of Al3+ (Negishi et al. 2012, 2013).

Thus, it is expected that blue flowers might be produced in other plants by introducing a gene encoding a metal ion transporter known to be involved in the blue color development of petals.

**Breeding of blue flowers by genetic engineering**

In many plants species, cyanidin-based anthocyanins lead to red color development; however, some plants with cyanidin-based anthocyanin as the main anthocyanin may develop blue flowers. As mentioned above, blue color develops due to the formation of metal complexes in cornflowers and blue poppies containing cyanidin, and due to an increase in vacuolar pH during flowering in morning glory containingpeonidin. However, many other flowers develop blue coloration through an accumulation of delphinidin-based anthocyanins.

The key enzyme of delphinidin biosynthesis is F3′5′H, which belongs to the CYP75A or CYP75B subfamily of cytochrome P450 enzymes. cDNAs encoding F3′5′H were initially isolated from petunia (Holton et al. 1993) and eggplant (Toguri et al. 1993) and have now been isolated from various plant species (Tanaka and Brugliera 2013). However, the production of delphinidin-based anthocyanins alone is not sufficient for blue color development. To develop blue coloration, it is necessary to modify the environment (e.g., vacuolar pH) where the delphinidin-based anthocyanin is present, to add aromatic organic acids to the glycosyl moieties of the anthocyanin, or to facilitate an interaction between the anthocyanin and coexisting co-pigments and/or metal ions. In this section, I describe the creation of blue flowers by introduction of the delphinidin biosynthetic pathway and anthocyanin modification gene.

**Carnation and rose**

Violet carnations and roses that accumulate delphinidin-based anthocyanins through the introduction of F3′5′H have been bred by the research group of Suntory and Florigene, and both Florigene Mooncarnation™ and Suntory blue rose Applause™ have been successfully commercialized worldwide (Tanaka and Brugliera 2013).

The anthocyanin responsible for the flower color of carnation is the 3,5-diglucoside-6″-4,6″′-1 cyclic malate of pelargonidin or cyanidin (Bloor 1998, Nakayama et al. 2000). The violet-colored Mooncarnation was initially created by introducing the petunia genes F3′5′H and DFR into the white flower of a DFR mutant carnation. The F3′5′H gene from Viola has also been introduced into Mooncarnations. The violet color is expressed by co-pigmentation, and the bluest Mooncarnation uses C-glucosyl flavones as co-pigments (Fukui et al. 2003).

The main anthocyanin responsible for color development in roses is cyanidin 3,5-diglucoside. In most plant species, anthocyanidin is first glycosylated at the 3-hydroxyl group. In roses, however, the hydroxyl groups at positions 5 and 3 are sequentially glycosylated by a single enzyme: UDP-glucose:anthocyanidin 5,3-O-glucosyltransferase (Ogata et al. 2005).

For a long time, it was considered that blue roses are synonymous with “impossible”. The petals of some roses contain cyanidin-based anthocyanins named rosacynins, in which gallic acid and ellagitannin are combined (Fukui et al. 2002, 2006). Therefore, “blue roses” with a grayish purple or mauve flower color have been produced by cross-breeding roses containing rosacynins. Two Japanese rose breeders, Mr. Moriji Komori and Ms. Junko Kawamoto, have successfully bred blue roses, including ‘Seiryu’ and ‘Misty Purple’, respectively.

By contrast, the research group of Suntory and Florigene developed blue roses by genetic engineering (Katsumoto et al. 2007). Roses that have petals with a high flavonol content and relatively high pH—traits that are considered to be suitable for blue color development—were selected for gene introduction. Among various F3′5′H genes, the pansy F3′5′H gene was found to be effective for producing delphinidin-based anthocyanins in roses. In addition, a Torenia gene encoding anthocyanin 5-acyltransferase was introduced with pansy F3′5′H, which enabled acylation of anthocyanin with an aromatic organic acid, and the world’s first blue rose, Suntory blue rose Applause was created.

Delphinidin-derived anthocyanins include petunidin- and malvidin-based anthocyanins in which the hydroxyl groups at the 3′ and/or 5′ positions are methylated. This methylation reaction depends on S-adenosylmethionine:anthocyanin 3′,5′-O-methyltransferase (A3′5′OMT). It has been reported that malvidin-based anthocyanins can accumulate in rose petals through the co-expression of Torenia A3′5′OMT and pansy F3′5′H (Nakamura et al. 2015). The resulting malvidin-producing roses have brilliant magenta flowers as compared with roses that accumulate delphinidin-based anthocyanin.
Dahlia, Phalaenopsis and lily

The major anthocyanins responsible for flower color in dahlia are 3-(6-malonyl)glucoside-5-glucosides of pelargonidin and cyanidin (Takeda et al. 1986, Yamaguchi et al. 1999). The transformation system of the dahlia plant has been reported for the Dahlia x pinnata ‘Yamatohime’ (Otani et al. 2013). In addition, a “blue dahlia” was created in a collaboration between Prof. Mii (Chiba University) and Ishihara Sangyo Kaisha Ltd. The F3′5′H gene derived from dayflower, Commelina communis L., was introduced under the control of the 35S promoter of CaMV to produce delphinidin derivatives, and the resultant transgenic dahlia turned violet (Mii 2013, Nakano et al. 2016). Various blue dahlias have been subsequently created by using the violet transgenic Yamatohime as a parent for hybridization (Mii et al. 2013).

Prof. Mii and collaborators have also succeeded in creating a “blue Phalaenopsis orchid” (Mii 2012). The major anthocyanins of pink-colored Phalaenopsis are polyacylated cyanidin-based anthocyanins in which the 7- and 3′-positions are glycosylated and acylated with aromatic organic acids (Tatsuzawa et al. 1997). For this reason, Phalaenopsis is able to produce delphinidin-based anthocyanin by the introduction of dayflower F3′5′H, and the flowers are the closest to blue that have been achieved by the expression of only F3′5′H.

The F3′5′H gene derived from Phalaenopsis has also been transiently expressed in lily to make the cells of pink petals turn blue (Qi et al. 2013). In addition, purple lilies have been obtained by expressing the Campanula F3′5′H gene under the control of the CaMV 35S promoter (Nakano et al. 2016, Tanaka et al. 2012).

Chrysanthemum

The anthocyanins responsible for color development in chrysanthemums are cyanidin 3-(6″-malonyl)glucoside and cyanidin 3-(3″,6″-dimalonyl)glucoside (Nakayama et al. 1997). To introduce the blue flower color trait into chrysanthemums, it is first necessary to establish a system of regeneration and transformation. Shinoyama et al. (2012) have summarized and reviewed various transformation systems of chrysanthemums. Some promoters, including chrysanthemum ubiquitin extension protein (UEP1) promoter (Annadana et al. 2002) and tobacco elongation factor 1α (EF1α) promoter (Aida et al. 2005), have been reported as effective for the expression of transgenes in chrysanthemum. So far, however, there are no successful examples of chrysanthemums that produce delphinidin through the expression of F3′5′H using these promoters.

The Florigene and Suntory group have obtained purple- and violet-colored chrysanthemums containing a high proportion of delphinidin by suppressing the endogenous F3′H gene and expressing pansy F3′5′H under the control of the rose CHS promoter (Brugliera et al. 2013). When this gene construct was introduced and selected via the NPTII gene, however, the highest delphinidin content achieved was less than 6% (Noda et al. 2013). Noda et al. also examined delphinidin production in chrysanthemum petals using pansy F3′5′H expressed under the control of other promoters, including CaMV 35S, Viola F3′5′H, rugosa rose F3H, Gerbera CHS, and rugosa rose DFR. In all of these cases, however, only individual plants with a low percentage of delphinidin (~1%–2%) were obtained (Noda et al. 2013). Furthermore, delphinidin was not produced when cineraria (Senecio cruentus) F3′5′H (He et al. 2013) or petunia F3′5′H (Seo et al. 2007) was expressed with the CaMV 35S promoter.

The most effective promoter for accumulating delphinidin in chrysanthemum ray florets is the chrysanthemum F3H promoter. Using this promoter to express Campanula F3′5′H, Noda et al. (2013) obtained a delphinidin content of almost 100%. The accumulation of delphinidin can also be effectively improved by using the 5′-untranslated region of the alcohol dehydrogenase gene (ADH) of tobacco, Arabidopsis or rice. Even with the chrysanthemum F3H promoter, the content of delphinidin is about 30% for other F3′5′H genes. Together, these results suggest that selection of the optimal promoter depends on both the host and the transgene used for expression. Synthesis of delphinidin-based anthocyanins via the F3′5′H gene was shown to modify the ray floret color to purple or violet, a color that had not been produced in chrysanthemum previously. However, the flower color was not generally a color that would be said to be “blue”. Further ingenuity was necessary to create truly “blue chrysanthemums”.

In addition to changing the anthocyanidin structure from cyanidin to delphinidin, it was considered that modification with multiple aromatic organic acids or the presence of interactive co-pigments and/or metal ions would be required for blue color development of chrysanthemum. Therefore, Noda et al. (2017) attempted to generate plants that could synthesize delphinidin 3-(6″-malonyl)glucoside 3′,5′-di-p-coumaroylglicoside (ternatin D3), one of blue polyacylated anthocyanins in butterfly pea (Terahara et al. 1998). The resulting F3′5′H-expressing chrysanthemums were found to accumulate delphinidin 3-(6″-malonyl)glucoside. To modify this to ternatin D3, glucosylation at the 3′ and 5′ positions of the B-ring and further acylation with aromatic organic acid were necessary. Therefore, the genes required for glucosylation and acylation were introduced, and blue-colored transformants were obtained. Notably, however, the blue petals of transgenic chrysanthemums did not contain ternatin D3, but rather ternatin C5 glucosylated at the 3′ and 5′-hydroxyl groups. Thus, it seemed that the gene introduced for aromatic acylation did not work well in chrysanthemum and the blue color development was due to an accumulation of ternatin C5. This idea was confirmed by analyzing recombinants in which only two genes were introduced: namely, campanula F3′5′H and butterfly pea A3′5′GT (Fig. 1). This method was found to be applicable to the creation of blue flowers in various chrysanthemums (Fig. 2). Furthermore, it was the first time that such a truly
Fig. 1. Anthocyanins and co-pigments responsible for blue color development in chrysanthemum. A, Pink color of host plant due to the interaction between cyanidin 3-malonylglucoside and flavone 7-malonylglucosides (co-pigments); B, Purple or violet color of transgenic plant due to the interaction between delphinidin 3-malonylglucoside and co-pigments; C, Blue color of transgenic plant due to the interaction between ternatin C5 and co-pigments. FNS, flavone synthase; F3’H, flavonoid 3’-hydroxylase; F3’5’H, flavonoid 3’,5’-hydroxylase; A3’5’GT, UDP-glucose:anthocyanin 3’,5’-O-glucosyltransferase.

Fig. 2. Different types of blue chrysanthemum flower. Host plants are shown on the left; transgenic plants are shown on the right. A, Decorative type of ‘Sei Arabella’; B, Anemone type of S25 line; C, Pompons type of T12 line.
blue color flower has been generated by using genetic engineering technology.

The fact that the blue coloration was developed only through the glycosylation of delphinidin-based anthocyanin was an unexpected discovery. Glycosylation of the hydroxyl group of the B-ring has been reported to be responsible for red color development (Andersen and Jordheim 2010). Furthermore, when purified ternatin C5 was dissolved in a buffer solution of petal juice at pH 5.6, the color of the solution was violet. Thus, other factors were thought to be involved in the blue coloration of chrysanthemum, in addition to ternatin C5 production. Noda et al. (2017) searched for co-pigments that interact with ternatin C5 to develop blue coloration. Analysis of an extract from blue petals by the cross-TLC method revealed that bands of different substances intersected in the blue- and violet-colored parts of the anthocyanin band. The substances contained in these bands were identified as candidates for co-pigments, and their structures were investigated. As a result, flavone 7-malonyl glucosides were identified to interact with ternatin C5 and to develop blue coloration (Fig. 1). In addition, solutions of 3,3′,5′-triglucosyl delphinidins such as ternatin C5 and flavone 7-malonylglucoside mixed at a molar ratio of 1:5 or more were shown to have absorption spectra similar to those of blue petals. Therefore, the blue coloration of the transgenic chrysanthemum was shown to be caused by intermolecular co-pigmentation.

Formation of metal complexes, another blue color development mechanism, requires the transport of metal ions into vacuoles, as well as the acylation and glycosylation of complex constituents such as anthocyanins and flavones. In addition to controlling multiple glycosylation and acylation, anthocyanin polyacylation requires a vacuolar trafficking system to transport the necessary enzymes to the appropriate cellular organelles (Poustka et al. 2007, Sasaki et al. 2014, Xiang et al. 2013). Notably, the method of creating blue flowers in chrysanthemum does not require these trafficking systems. Thus, introduction of the butterfly pea A3′5′GT gene to synthesize anthocyanin, which then develops blue coloration by interacting with endogenous substances in the host petal, represents an effective method of blue flower generation. Furthermore, it raises the possibility of that bestowing the blue trait on various flower species will be much easier than it had been previously thought.

### Efficient production of blue flowers

Techniques for plant regeneration and transformation have been established for various plant species. However, the efficiency of regeneration and transformation varies depending on the breeding line or cultivar. For many plant species, therefore, it is often labor-intensive to identify lines or cultivars suitable for blue coloration.

In chrysanthemum, for example, only about 30 of 150 breeding lines or varieties tested showed efficient plant regeneration. Of these, only 15 showed altered flower color to violet-blue or blue by transformation; furthermore, the efficiency of blue flower production was understandably varied among them. For some strains, more than 50 transformants can be obtained from 3000 leaflets, while for others only a few are obtained. Individual plants expressing the target blueness are further limited. To efficiently produce blue flowers of high commercial value, therefore, it is necessary to select hosts for which recombinants can be efficiently obtained. Furthermore, optimization of the culture system is required to improve recombination efficiency; in recent years, however, not many researchers are conducting such studies.

Lastly, it is desirable to use a gene construct that can reliably obtain transformants with the target flower color. By combining the appropriate transgene, promoter and terminator, the object metabolite can be synthesized without needing to suppress endogenous competing biosynthetic pathways. In the production of the blue chrysanthemum, for example, the proportion of transformants containing a high proportion of delphinidin-based anthocyanins was increased by using the AtHSP terminator (Nagaya et al. 2010) in the expression cassette of F3′5′H (Noda et al. 2017).

### Commercialization of transgenic blue flowers

Genetically modified flowers have to pass the risk assessments mandated in international protocols (Biosafety Clearing-House 2017) and/or governmental regulations and policies for cultivation, distribution and retail. In order to grow and sell genetically modified flowers in the open environment, it is necessary to prove that the risk to the ecology, including the possibility for gene flow into related wild species and influence on unspecified organisms, is minimized (Chandler et al. 2013, Kikuchi et al. 2008, Nakamura et al. 2011a, 2011b, Shinoyama et al. 2008). In order to use genetically modified flowers for commercial purposes in Japan, it is necessary to obtain approval from the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment and (Japan Biosafety Clearing House 2017). Regulatory approval requires several years of examination and costs. Therefore, any genetically modified flowers aimed at commercialization must have both unprecedented marketability and various agronomic traits suitable for production and distribution.

The transgenic flowers that are so far approved in Japan are roses and carnations. Roses are currently the only genetically modified crops that are cultivated in Japan. To commercialize blue chrysanthemums both domestically and abroad, it will be necessary to obtain approval in accordance with the regulations of each target country. It will be necessary to confirm that there is no competitive advantage or hazardous substance productivity in the blue chrysanthemum. Wild species of chrysanthemums are widely distributed in Eastern Asia including Japan. Thus, it will be necessary to impart traits such as male and female sterility into the transgenic plants to prevent effects on biodiversity. By
Engineering blue flowers

applying “New Plant Breeding Techniques” such as genome editing and other modern biotechnology, blue chrysanthemum varieties with sterility traits will be developed. This will facilitate the commercialization of blue chrysanthemums in Japan, contribute to the development of the flower industry, and meet the demands of farmer, florist and consumer.

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