Ph. D. Thesis

Construction of transformation system and expression of the astaxanthin biosynthesis genes in *Freesia hybrida*

（フリージアにおける形質転換系の構築とアスタキサンチン生合成遺伝子の発現）

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I. ABSTRACT

A method for *Agrobacterium*-mediated transformation of *Freesia* × *hybrida* is described. Cormlet-derived calli of two cultivars, 'Mosera' and 'Ishikawa f3' were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pIG121-Hm, which included hygromycin phosphotransferase gene and an intron-containing β-glucuronidase gene in the T-DNA region. Callus pieces were co-cultivated with *A. tumefaciens* on the callus proliferation medium [Murashige & Skoog (MS) medium containing 1 mg l^{-1} thidiazuron, 1 mg l^{-1} dicamba, 20 mg l^{-1} 3′, 5′-dimethoxy-4′-hydroxyacetophenone, 1% (w/v) glucose, 3% (w/v) sucrose, and 0.2% (w/v) Gelrite]. Then, they were cultured on the callus proliferation medium containing 300 mg l^{-1} cefatoxime and 10 mg l^{-1} hygromycin B. Hygromycin-resistant lines of both cultivars regenerated into plantlets after transfer onto MS medium containing 2 mg l^{-1} 3-indoleacetic acid and 3 mg l^{-1} 6-benzyl aminopurine and/or plant growth regulator-free MS medium. Transgenic plants were identified by β-glucuronidase assay and verified by Southern blot analysis. Two transgenic plant lines were obtained from 475 callus pieces of 'Mosera', and one transgenic plant line was obtained from 290 callus pieces of 'Ishikawa f3'. This is the first report of the genetic transformation of *Freesia*. This method will allow the genetic improvement of this horticulturally important flower.

Secondary, we introduced the astaxanthin biosynthesis genes, which encoded the marine-bacterial CrtW, CrtZ, and Idi, into freesia cultivar 'Ishikawa f6' by Agrobacterium-mediated transformation under the control of the CaMV 35S promoter. The introduced genes were functionally expressed especially in the callus, which produced large amounts of astaxanthin including its esterified forms [63% of total carotenoids (32.2 μg/g fresh weight)] and free form (4.3%) in addition to other ketocarotenoids that contained fritschiellaxanthin (3%), 4-ketoantheraxanthin (1.6%), adonirubin (5.4%), adonixanthin (2%), and canthaxanthin (4.2%). The regenerated freesia plant exhibited bronze (reddish green) color, and its stems and (large) flower buds were found to accumulate astaxanthin esterified forms [50.3% and 35.3% of total carotenoids (83.1 and 21.6 μg/g fresh weight)], respectively. Whereas, the petals contained trace amounts of carotenoids, presumably because of rapid reduction and/or degeneracy of the chloroplasts (plastids).

II. INTRODUCTION

Freesia is a member of the subfamily Ixioideae of the family Iridaceae, and is native to the Cape Province in South Africa. The modern *Freesia*, *Freesia* × *hybrida*, is derived from crosses among several *Freesia* species. It is a popular cut flower because of plant and flower forms, flower fragrance and wide color variations of flower. In the Netherlands, the shipment value of *Freesia* as a cut flower was approximately €5 billion in 2010 (http://www.cbs.nl/en-GB/menu/home/default.htm). Commercially sold *Freesia* corms are sometimes infected by viral and/or fungal pathogens. Viruses are particularly problematic for *Freesia*, because the plants are propagated each year by corms and cormlets that may harbor viruses (Stein 1995). To obtain virus-free plants, there have been some attempts to propagate *Freesia* using tissue culture techniques such as meristem culture (Brants and Vermelen 1965; Brants 1968). However, the problem of viral contamination has been solved neither by culture techniques nor by conventional breeding methods because of the lack of available genetic resources for virus resistance. Therefore, genetic transformation is a promising alternative approach. Genetic transformations of *Gladiolus* and *Iris germanica* in the same family, Iridaceae, were already reported. Transformation of *Gladiolus* was demonstrated by particle bombardments of cormel slices (Kamo et al. 1995a), suspention cells and callus (Kamo et al. 1995b). Transformants of *Iris germanica* were achieved using regenerable suspention cultures via *Agrobacterium*-mediated method (Jeknic et al. 1999). In this report, we describe the *Agrobacterium*-mediated genetic transformation of *Freesia* × *hybrida*. This is the first report of genetic transformation of members of the *Freesia* genus.

Ishikawa prefecture (Department of Agriculture, Forestry and Fisheries, and the Ishikawa Agriculture and Forestry Research Center) has bread new varieties of freesia, 'Ishikawa f1 ～f11' of the series named 'Airy-Flora', which are already distributed in market. The freesia flowers can generate not only pink and purple colors of flavonoids but also yellow and orange colors of carotenoids in addition to white and red colors. Recently, we clarified that the yellow petals of ‘Ishikawa f2’ (and also cultivars ‘Aladin’ and ‘Kayak’) produced crocetin glycosides, crocetin neapolitanosyl ester and crocetin neapolitanosyl diester (Shindo et al. 2022).

We constructed the Agrobacterium-mediated transformation system of freesia using cultivars ‘Mosera’ and ‘Ishikawa f3’ as the hosts. In order to evaluate the productivity of astaxanthin (in freesia), which is the red carotenoid with strong anti-photooxidative activity, we have here tried to produce transgenic plants of 'Airy-Flora' cultivar ‘Ishikawa f6’, which expresses the astaxanthin biosynthesis genes from marine bacteria.

III. Agrobacterium-mediated Transformation and Regeneration of *Freesia × hybrida*

We used cormlets of two cultivars, 'Mosera' and 'Ishikawa f3' (bred at the Ishikawa Agriculture and Forestry Research Center). The cormlets were kept at 30°C for 8 weeks to break dormancy. After the heat treatment, the cormlets were sterilized as follows: first, they were immersed in 0.5% (w/v) Benlate T (thiuram 20% and benomyl 20%; Hokko Chemical Industry Co., Tokyo, Japan) for 20 min. Next, they were dipped briefly in 70% (v/v) ethanol and then immersed in a 5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) for 20 min. Finally, they were rinsed three times in sterile water. The surface-sterilized cormlets were immediately cut aseptically into ca. 5-mm cubes. The cubes were placed on the callus proliferation medium [MS medium (Murashige and Skoog 1962) containing 1 mg l^{-1} thidiazuron (TDZ) (Wako Pure Chemical Industries, Osaka, Japan), 1 mg l^{-1} dicamba (Wako Pure Chemical Industries), 3% (w/v) sucrose, and 0.2% (w/v) Gelrite (San-eigen FFI Co. Ltd., Osaka, Japan)]. All the media used in the present study were adjusted to pH 5.8 prior to autoclaving at 120°C for 15 min. The cultures were maintained at 22±2°C in the dark. Calli were produced after 6–8 weeks, and were subcultured onto fresh callus proliferation medium every 4–6 weeks. The calli of both cultivars were light yellow and nodular (Figure 1A).

The calli of 'Mosera' produced adventitious buds by 3–4 weeks after transfer to the regeneration medium [MS medium containing 2 mg l^{-1} 3-indoleacetic acid (IAA) (Wako Pure Chemical Industries), 3 mg l^{-1} 6-benzyl aminopurine (6-BA) (Nakalai Tesque, Kyoto, Japan), 3% (w/v) sucrose, 0.6% (w/v) agar (Gao et al. 2010)]. For 'Mosera', the adventitious buds and green spots from nodular calli were transferred onto plant growth regulator (PGR)-free MS medium [MS medium containing 3% (w/v) sucrose and 0.6% (w/v) agar] to induce plantlet regeneration. For 'Ishikawa f3', the calli were cultured on PGR-free MS medium. They produced adventitious buds by 6–8 weeks after transfer to this medium. Plantlets of both cultivars were regenerated by approximately 3–4 months after transferring the calli onto the regeneration medium and/or PGR-free MS medium. For regeneration, cultures were maintained at 22±2°C under a 16-h light/8-h dark photoperiod, and were subcultured onto fresh PGR-free MS medium every 4–5 weeks.

For transformation experiments, we used calli 3-5 weeks after subculture. We used *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993), which harbors the binary vector pIG121-Hm (Ohta et al. 1990). This binary vector contains the neomycin phosphotransferase II gene (*nptII*), under the control of the nopaline synthase gene (*nos*) promoter and the *nos* terminator; the hygromycin phosphotransferase gene (*hpt*), under the

control of the cauliflower mosaic virus (CaMV) 35S promoter and the *nos* terminator; and the intron-containing □-glucuronidase gene (*gus*), under the control of the CaMV 35S promoter and the *nos* terminator (Figure 2A). This bacterial strain was cultured for 2 days at 26°C on LB medium solidified with 1.5% (w/v) agar containing 50 mg l^{-1} kanamycin monosulfate (Wako Pure Chemical Industries, Osaka, Japan) and 50 mg l^{-1} hygromycin B (Wako Pure Chemical Industries, Osaka, Japan). The bacteria were collected and suspended in the co-cultivation medium [MS medium supplemented with 1 mg l^{-1} TDZ, 1 mg l^{-1} dicamba, 3% (w/v) sucrose, 20 mg l^{-1} 3′, 5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone) (Sigma-Aldrich, St. Louis, MO, USA) and 1% (w/v) glucose]. For *Agrobacterium* inoculation, the bacterial suspension was adjusted to an OD_{600} of 0.15–0.20, and then shaken on a reciprocal shaker at 120 rpm for 2.5 h, in the dark, at 25°C. Calli of both cultivars were cut into pieces with a diameter of ca. 1 cm, and then the pieces were soaked in the bacterial suspension for 10 min. The pieces were removed from the suspension and blotted dry with sterile Kimwipes (Nippon Paper Crecia Company, Tokyo, Japan) to remove excess bacteria. Then, they were transferred to the co-cultivation medium solidified with 0.2% (w/v) Gelrite and co-cultivated with *Agrobacterium* at 25°C in the dark for 7 days. After co-cultivation, the calli of both cultivars were transferred onto the callus selection medium, which was the callus proliferation medium with the addition of 300 mg l^{-1} cefotaxime (Claforan) (Sanofi Aventis, Tokyo, Japan) and 10 mg l^{-1} hygromycin B. The calli pieces were cultured at 22±2°C in the dark and subcultured every 3 weeks onto the fresh selection medium, in which the cefotaxime concentration was gradually decreased to 0 mg l^{-1}.

The calli of ‘Mosera’ and ‘Ishikawa f3’ were subjected to a β-glucuronidase (GUS) histochemical assay (Kosugi et al. 1990) 2–3 weeks after co-cultivation with *A. tumefaciens* to identify those harboring the foreign T-DNA. Tissues were incubated for 24 h at 37°C in 150 mM sodium phosphate buffer (pH 7.0), 20% methanol (to eliminate the endogenous GUS activity) and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). Blue spots were observed in calli of ‘Mosera’ (data not shown) and ‘Ishikawa f3’ (Figure 1B), indicating successful delivery of the *gus* gene and its expression. Ten calli of each cultivar were subjected to the GUS histochemical assay, and 0–3 blue spots per callus (average, 1 spot per callus) were detected. There was no blue staining in the non-infected calli of ‘Ishikawa f3’(Figure 1B), but some blue staining was detected in non-infected calli of ‘Mosera’ (data not shown).

At 8–9 weeks after subculture, the calli of ‘Mosera’ and ‘Ishikawa f3’ were transferred onto the regeneration and PGR-free MS media without hygromycin B, respectively. They were cultured at 22±2°C under a 16-h light/8-h dark photoperiod. Hygromycin B tended to inhibit regeneration from calli. Threfore, the hygromycin B-free media were used for the first

3 weeks and then the media which contained 5 mg l^{-1} hygromycin B were used for 6 weeks. Both 'Mosera' and 'Ishikawa f3' calli were subsequently cultured on PGR-free MS media containing 10 mg l^{-1} hygromycin B. In 'Mosera' and 'Ishikawa f3', the totals of 475 and 290 calli were co-cultivated with *A. tumefaciens* in six and three independent trials, respectively (Table1). The calli on hygromycin-containing media became brown within 1–3 months, but hygromycin-resistant (Hyg^r) lines gradually grew from the surface of the dead calli (Figure 1C). In total, 27 and 9 independent Hyg^r plantlets of 'Mosera' and 'Ishikawa f3', respectively, were regenerated by 10–12 months after *A. tumefaciens* inoculation (Figure 1D, Table1).

When the leaves of Hyg^r plantlets (i.e., putative transgenic plantlets) were subjected to the GUS assay, a GUS- positive response was observed in 2/27 'Mosera' plantlets and 1/9 'Ishikawa f3' plantlets (Figure 1E, Table 1). The GUS-positive plantlets were transplanted into soil and grown at 23°C under a 16-h light/8-h dark photoperiod in a phytotron after acclimatization (Figure 1F).

Southern blot analysis was used to confirm the integration of foreign genes into the genome of the *Freesia* plants. Total genomic DNA was isolated from young leaves of putative transgenic plants (GUS positive plants; two plants derived from 'Mosera' and one from 'Ishikawa f3') and wild-type plants (non-transgenic 'Mosera' and 'Ishikawa f3' plants) using the CTAB method (Murray and Thompson 1980). Genomic DNAs were digested with *Bam*HI or *Hin*dIII. Southern blotting was performed using the DIG non-radioactive nucleic labelling and detection system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. A part of the *gus* coding region was amplified by PCR and labeled using the primer pair 5′-CTGCTGTCGGCTTTAACCTC-3′ (forward) and 5′-TGAGCGTCGCAGAACATTAC-3′ (reverse). The resulting fragment was used as a probe (Figure 2A). Southern blot analysis with the *gus*-specific probe revealed one to two bands when the genomic DNA from three Hyg^r plants was digested with *Hin*dIII (Figure 2B). This result indicated that the transformants had a single copy or two copies of the introduced genes in different patterns, and that they were independent transgenic lines.

The transgenic plants of 'Mosera' flowered after 60 days of a low-temperature flower-inducing treatment at 10°C. The flower shape was normal (Figure 1G) and corms were formed at the subterranean part after flowering (Figure 1H).

In conclusion, this is the first report of the *Agrobacterium*-mediated genetic transformation of *Freesia* × *hybrida*. The methods may be applicable to a wide range of *Freesia* cultivars. This transformation protocol for *Freesia* × *hybrida* could be used to produce varieties with new flower colors, longer flower life, and/or resistance to fungal and viral pathogens.

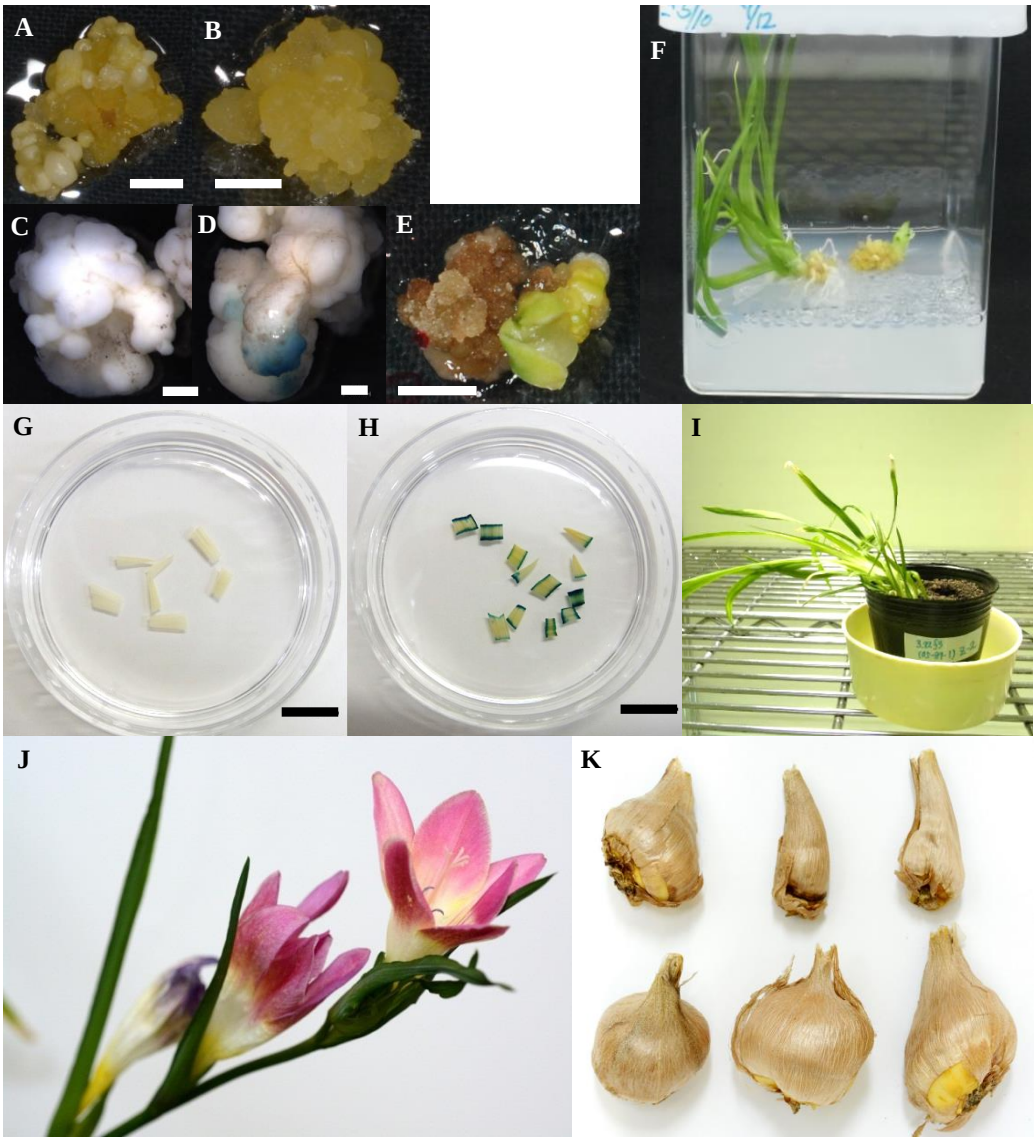
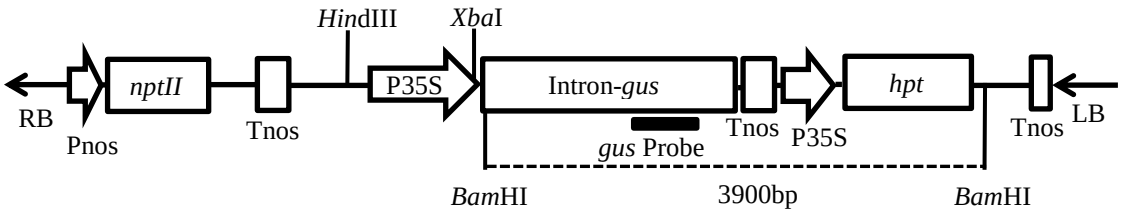


Figure 1. Production of transgenic *Freesia* × *hybrida* plants via *Agrobacterium*-mediated transformation of nodular calli. Nodular calli of ‘Mosera’ (A, left) and ‘Ishikawa f3’ (A, right) before inoculation with *A. tumefaciens*. Bar = 5 mm in (A). Histochemical assay of GUS activity in ‘Ishikawa f3’ calli non-infected (B, left) and infected with *A. tumefaciens* (B, right). Assays were carried out 2-3 weeks after co-cultivation. Bar = 2 mm in (B). Hyg^r adventitious buds (C, right) and dead calli (C, left) of ‘Mosera’ on hygromycin-containing medium (C). Bar = 5 mm in (C). Plantlets regenerated from Hyg^r culture line of ‘Ishikawa f3’ (D). GUS-negative response in leaves of non-transgenic ‘Ishikawa f3’ plant (E, left) and GUS-positive response in leaves of transgenic ‘Ishikawa f3’ plant (E, right). Bar = 1 cm in (E). Transgenic ‘Ishikawa f3’ plant (F). Flowers of transgenic ‘Mosera’ plant (G). Corms of transgenic ‘Mosera’ plant (H)

A



B

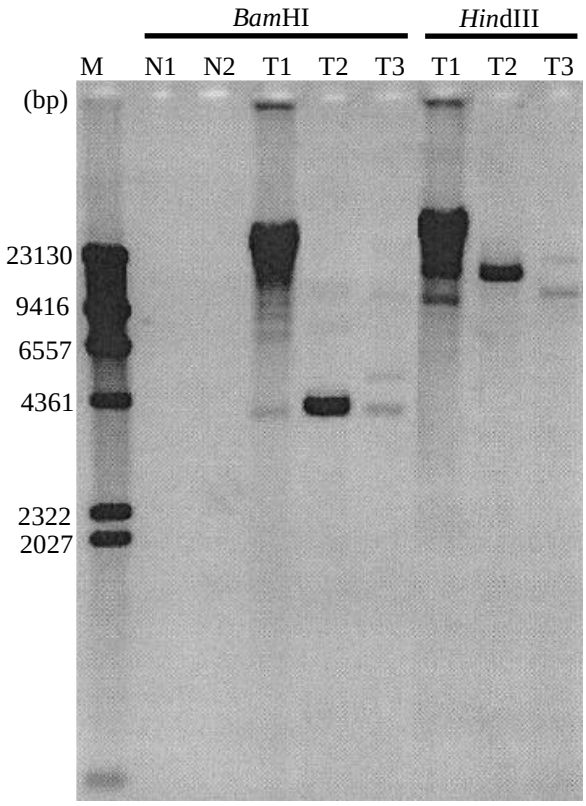


Figure 2. Transformation vector and Southern blot analysis. Schematic diagram of part of T-DNA region of transformation vector pIG121-Hm (A). RB, right border; LB, left border; Pnos, *nos* promoter; Tnos, *nos* terminator; P35S, CaMV 35S promoter; *nptII*, gene for neomycin phosphotransferase II; Intron-*gus*, coding region of *gus* gene with an intron; *hpt*, gene for hygromycin phosphotransferase; *gus* Probe, region of *gus* gene used for Southern blot analysis. Southern blot analysis of transgenic *Freesia* × *hybrida* plants (B). Genomic DNA was digested with *Bam*HI or *Hin*dIII. Lane M, DIG-labeled λ/*Hin*dIII molecular marker; Lane N1, non-transgenic 'Mosera'; Lane N2, non-transgenic 'Ishikawa f3'; Lanes T1 and T2, transgenic 'Mosera' #1 and #2, respectively; Lane T3, transgenic 'Ishikawa f3'.

Table1. Results of transformation experiments in two *Freesia* cultivars

Cultivar	**No. of calli tested**	**No. of regenerated plantlets**	**No. of GUS＋ plantlets**	**No. of transformed plantlets**
Mosera	**475**	**27**	**2**	**2**
Ishikawa f3	**290**	**9**	**1**	**1**

IV. Functional expression of the astaxanthin biosynthesis genes in *Freesia× hybrida*

1. Materials and Methods

1.1 Plant materials

In this study, we used *Freesia* × *hybrida* cultivar 'Ishikawa f6' ('f6') generating white flowers with partially faint pink patterns (Fig. 31a) as the host. The bulb is also shown in Figure 1b (left). The cormlets of 'f6' were sterilized as follows: first, they were immersed in 0.5% (w/v) Benlate T (thiuram 20% and benomyl 20%; Hokko Chemical Industry Co., Tokyo, Japan) for 20 min. Next, they were dipped briefly in 70% (v/v) ethanol and then immersed in a 5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 80) for 20 min. Finally, they were rinsed three times in sterile water. The surface-sterilized cormlets were immediately cut aseptically into ca. 5-mm cubes. The cubes were placed on the callus proliferation medium [MS (Murashige and Skoog) medium containing 1 mg/L of thidiazuron (TDZ) (Wako Pure Chemical Industries, Osaka, Japan), 1 mg/L of 5,6-dichloro-1H-indole-3-acetic acid (Wako Pure Chemical Industries), 3% (w/v) sucrose, and 0.32% (w/v) gellan gum (Nacalai tesque, Kyoto, Japan)] (Uwagaki et al. 2015). All the media used in this study were adjusted to pH 5.8 prior to autoclaving at 120°C for 15 min. The cultures were maintained at 22±2°C in the dark.

1.2 Sensitivity of cormlets to hygromycin

To determine the optimum concentrations of hygromycin for the selection of transformed 'f6' cells, the cubes from the cormlets were placed on the callus proliferation medium containing different concentration of hygromycin (Hygromycin B; Wako Pure Chemical Industries) (0, 10, 25, and 50 mg/L). The cubes were incubated at 22±2°C in the dark. The ratio of callus formation was determined after a month of the cultures.

1.3 Construction of plasmids

The plasmid pUTR-crtZWidi (pZH2B) for plant transformation and expressing the astaxanthin biosynthesis genes were previously constructed as described (Otani et al. 2022, Mortimer et al. 2017). This plasmid contained a binary vector pZH2B(Kuroda et al. 2010), in which three enzyme genes was inserted for β,β-carotenoid 3,3'-hydroxylase (CrtZ) and β,β-carotenoid 4,4'-ketolase (4,4'-oxygenase; CrtW) from a marine bacterium *Brevundimonas* sp. strain SD212, and isopentenyl diphosphate isomerase (type 2 Idi) from a marine bacterium *Paracoccus* sp. strain N81106, under the regulation of the CaMV 35S promoter and the transit peptide sequence of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO)

small subunit from pea. And the 5'-untranslated region (UTR) of the *Nicotiana tabacum* alcohol dehydrogenase gene (*NtADH*) was attached to the ATG of each gene, preceded with the transit peptide sequence.

1.4 Agrobacterium-mediated transformation

We carried out the Agrobacterium-mediated transformation. The cubes from the 'f6' cormlets were placed on the callus proliferation medium for 0 day, 7 day or 14 days. *Agrobacterium tumefaciens* strain EHA101, in which plasmid pUTR-crtZWidi (pZH2B) had been introduced, was cultured for 2 days at 26°C on LB medium solidified with 1.5% (w/v) agar containing 50 mg/L of kanamycin monosulfate (Wako Pure Chemical Industries, Osaka, Japan) and 50 mg/L of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan). This bacterial strain was collected and suspended in the co-cultivation medium [MS medium supplemented with 1 mg/L TDZ, 1 mg/L 5,6-dichloro-1H-indole-3-acetic acid, 3% (w/v) sucrose, 20 mg/L 3′, 5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone) (Sigma-Aldrich, St. Louis, MO, USA), and 1% (w/v) glucose]. For the Agrobacterium inoculation, the bacterial suspension was adjusted to an OD600 of 0.15–0.20. The cubes were soaked in the bacterial suspension for 10 min. The pieces were removed from the suspension and blotted dry with sterile Kimwipes (Nippon Paper Crecia Company, Tokyo, Japan) to remove excess bacterial cells. Then, they were transferred onto the co-cultivation medium solidified with 0.32% (w/v) gellan gum, and co-cultivated at 23°C in the dark for 5 days. The cubes were then transferred onto the callus selection medium, which was the callus proliferation medium with the addition of 50 mg/L of meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, japan) and 15 mg/L of hygromycin B. The 'f6' cubes were cultured at 22±2°C in the dark onto the fresh selection medium, in which the meropenem trihydrate concentration was gradually decreased to 0 mg/L. The cubes were sub-cultured onto the fresh medium with every 3 weeks intervals.

After few months of the culture on the selection medium, the induced calli were transferred onto regeneration medium [MS medium containing 3 mg/L TDZ, 3% (w/v) sucrose, 15 mg/L hygromycin B, 0.32% (w/v) gellan gum], and cultured at 22±2°C under a 16-h photoperiod for a month. Calli and adventitious buds, which were formed from hygromycin-resistant tissues, were transferred onto the plant growth regulator (PGR)-free MS medium [MS medium containing 3% (w/v) sucrose, 15 mg/L hygromycin B and 0.32% (w/v) gellan gum] to induce plantlet regeneration.

1.5 PCR analysis

Genomic DNA was extracted from the transformed and wild-type plants of freesia 'f6'

according to the method of the CTAB method (Murray et al. 1980). PCR was performed using TaKaRa Ex Taq polymerase (Takara, Ohtsu, Japan). The PCR conditions were as follows: 98°C for 10 sec, 66°C for 30 sec, 72°C for 3 min; 35 cycles. Primer pairs were designed at the start codon of *crtZ* (5'-ATGGCTTGGCTTACTTGGATCGCTCTTTTCCT-3') and the stop codon of *crtW* (5'-TCAAGACTCTCCTCTCCAAAGTCTCCACCAAG-3'). PCR products were electrophoresed using a 1% (w/v) agarose gel.

1.6 Analysis of carotenoids

We carried out HPLC-PDA-MS/MS analysis on carotenoids that accumulated in the transformed and wild-type plants of freesia 'f6' based on the methods described(Otani et al. 2022, Maoka et al. 2016).

2. Results and Discussion

2.1 Resistance experiment of the cormlets to hygromycin

Efficient selection is a necessary prerequisite for successful production of transgenic plants through the Agrobacterium-mediated method. Our result indicated that the formation of the callus from freesia 'f6' explants was extremely sensitive to hygromycin. When the cubes were cultured on the medium containing more than 10 mg/L of hygromycin for 4 weeks, no calli were generated from the cubes (Table 2). Thus, 15 mg/L of hygromycin was used for the selection and maintenance of transformed 'f6' lines.

2.2 Transformation of freesia 'f6'

After five days of the co-cultivation, the 'f6' cubes were transferred onto the callus selection medium. The cultures were incubated at 22±2°C in the dark. After around nine weeks of cultivation, formed hygromycin-resistant orange calli (called #21; indicated with arrow in Figure 1c) were transferred onto the regeneration medium, and the cultures were incubated at 22±2°C under a 16-h photoperiod. After around 30 days of cultivation, calli containing formed adventitious buds were placed on the PGR-free MS medium (Figure 3d) and cultivated. Consequently, three hygromycin-resistant plantlets (called #21-1, #21-2, and #21-3), which exhibited orange color systemically, were regenerated from the totals of 584 cubes [Figure 3e (right), Table 3].

After acclimatization, these three transgenic plants (see ***2.3***) were grown for five months in a biohazard greenhouse to form bulbs. Generated bulbs were kept at 30°C for 8 weeks to break dormancy [Figure 1b (right)]. After this heat treatment, the bulbs were planted and raised in a greenhouse. The transgenic freesia plants exhibited bronze (reddish green) color [Figure 3fg (left)]. Then, the freesia came into flower after the exposure of a low temperature

in winter. According to the growth of the flower buds of the transgenic freesia, their bronze color gradually faded [Figure 3h (right)].

2.3 Confirmation of transgenic plants

Genomic DNA was extracted from the leaves of the non-transgenic plant and the regenerated hygromycin-resistant plants (#21-1, #21-2, and #21-3), which were regenerated from the orange callus. Using these extracts as templates, PCR was performed to detect the *crtZ* and *crtW* genes. Fragments, whose size is anticipated to be 2.9 kb, corresponding to the length from the *crtZ* start codon to the *crtW* stop codon, were detected from all the three putative transgenic plants (Figure 4). It was thus confirmed that they were transgenic plants.

2.4 Analysis of carotenoids produced in the freesia 'f6' transgenic plants

Figure 5 shows the result of HPLC analysis of the hygromycin-resistant callus [#21; callus in Figure 3e (right)] degenerated from the freesia 'f6' plants. Interestingly, this callus was found to produce large amounts of astaxanthin including its esterified forms [63% of total carotenoids (32.2 μg/g fresh weight)] and free form (4.3%) in addition to other ketocarotenoids that contained fritschiellaxanthin (3%), 4-ketoantheraxanthin (1.6%), adonirubin (5.4%), adonixanthin (2%), and canthaxanthin (4.2%) (Table 4). Even in vitro roots [roots in Figure 3e (right)] accumulated high ratio of astaxanthin esters (Table 4).

The regenerated transgenic freesia plant exhibited bronze (reddish green) color, and its stems and (large) flower buds were found to accumulate astaxanthin esterified forms [50.3% and 35.3% of total carotenoids (83.1 and 21.6 μg/g fresh weight)], respectively. Whereas, the petals contained trace amounts of carotenoids, presumably because of rapid reduction and/or degeneracy of the chloroplasts (plastids).

Feasible carotenoid biosynthetic pathway in the freesia 'f6' #21 callus and the transgenic plants regenerated from this callus are shown in Figure 6. Our study indicated that the carotenoid biosynthesis genes were efficiently functionally expressed in freesia plants, except for white color-base petals.

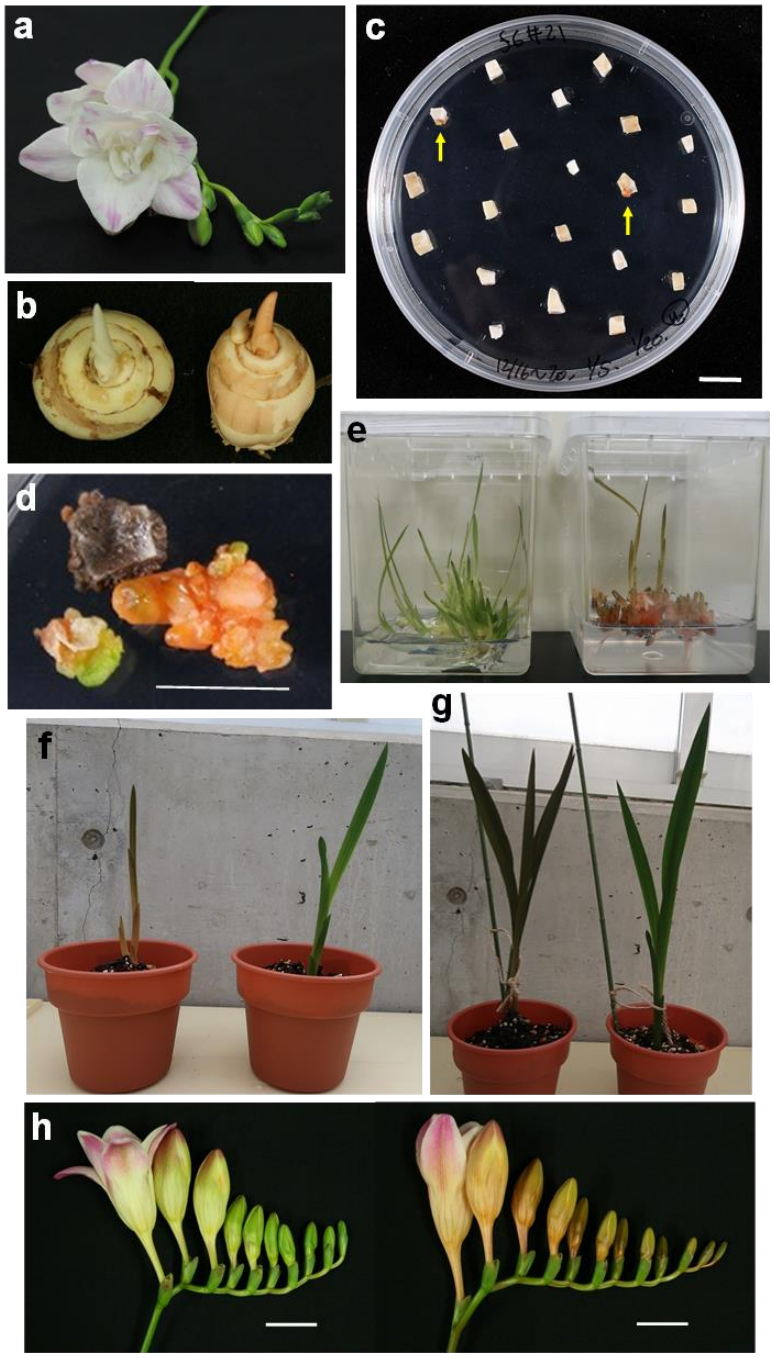


Figure 3. Freesia ‘Ishikawa f6’ and construction of transgenic plants

a, the ‘f6’ flowers (wild type); b, the bulbs of the wild type (left) and transgenic (right) ‘f6’ plants; c, emergence of hygromycin resistant ‘f6’ calli (arrows) (bar =10 mm); d, hygromycin-resistant ‘f6’ calli containing adventitious buds (bar =10 mm); e, *in vitro* plantlets regenerated from the wild type (left) and hygromycin-resistant (right) ‘f6’ calli; f and g, appearance of the wild type (right) and transgenic (left) ‘f6’ plants after 3 weeks (f) and 2 months (g) of cultivation; h, the flowers of the wild type (left) and transgenic (right) ‘f6’ plants (bar =20 mm).

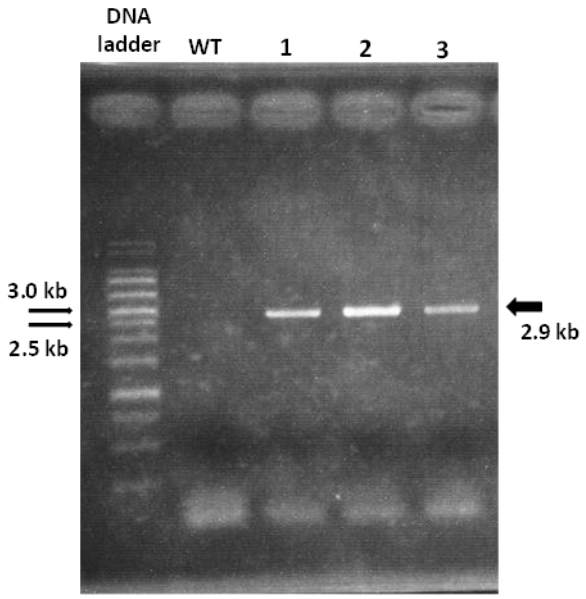


Figure 4. PCR analysis for detection of the crtZ and crtW genes in three independent freesia ’f6’ transformants

lane WT, ‘f6’ wild type; lanes 1~3, independent ‘f6’ transformants #21-1, #21-2, and #21-3.

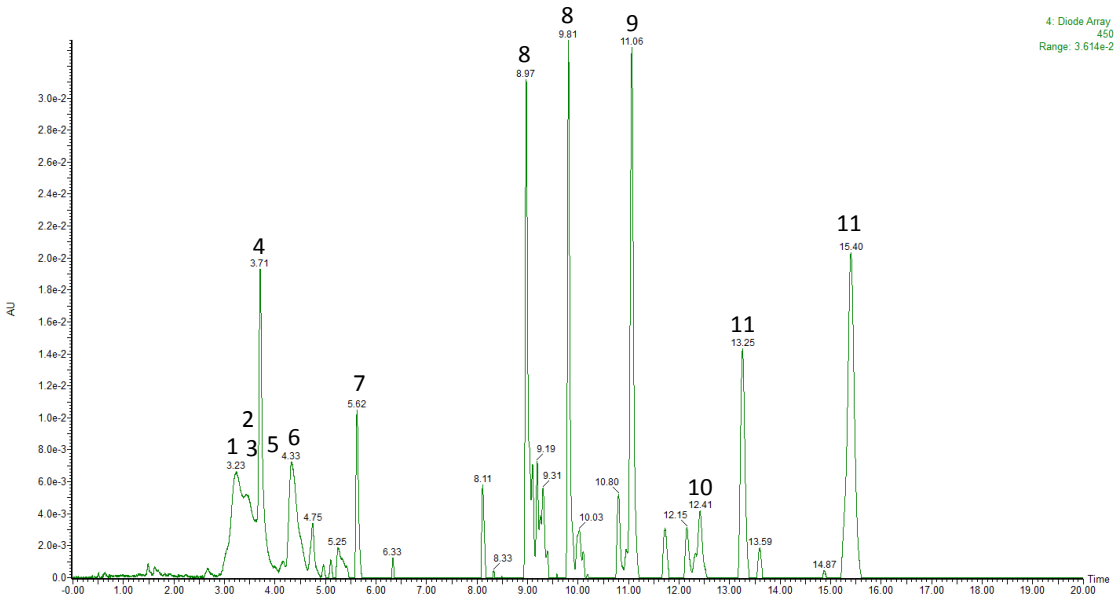


Figure 5. HPLC chromatogram of the extract from freesia ‘f6’ transformant callus 1, astaxanthin (retention time, 3.23 min; per the total carotenoids, 4.3%); 2, fritschiellaxanthin (3.38 min; 3%); 3, adonixanthin (3.45 min; 2.0%); 4, lutein (3.69 min; 4.8%); 5, 4-ketoantheraxanthin (3.82 min; 1.6%); 6, adonirubin (4.33 min; 5.4%); 7, canthaxanthin (5.62 min; 4.2%); 8, astaxanthin monoesters (8.97 and 9.81 min; 32.5%); 9, chlorophyll (11.06 min); 10, β-carotene (12.41 min; 1.2%); 11, astaxanthin diesters (13.25 and 15.40 min; 30.5%).

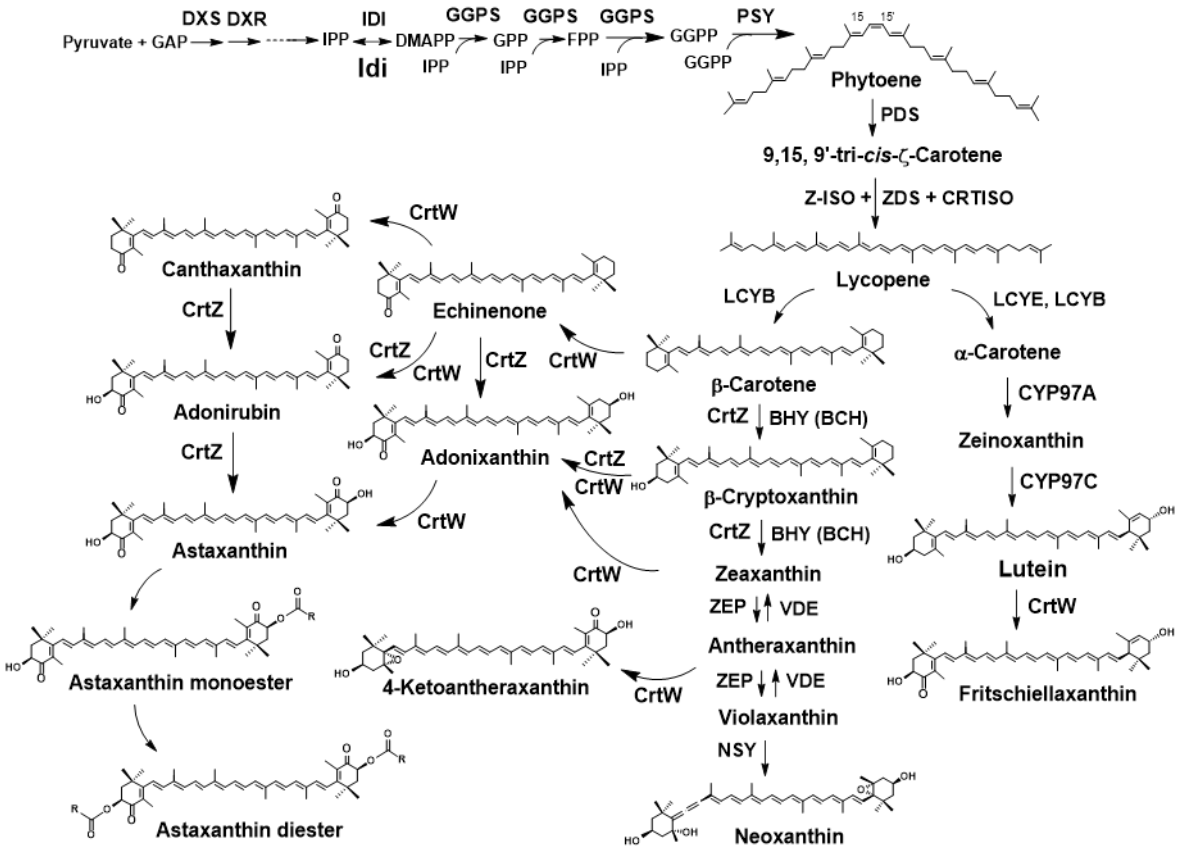


Figure 6. Proposed carotenoid biosynthetic pathway in freesia ‘f6’ transformant callus and the transgenic ‘f6’ plants

Table 2. Hygromycin sensitivity of freesia callus

Hygromycin concentration (mg/L)	0	10	25	50
Callus formation ratio (%)*	93	0	0	0

*Thirty cubes from freesia cormlets were provided for each test.

Table 3. Transformation efficiency of freesia ‘f6

Test number	Pre-culture period	Number of tested callus	Number of hygromycin resistant plantlet	Number of PCR-positive plantlet	Transformation frequency (%)
1	7 days	123	1	1	0.8
2	14 days	128	1	1	0.8
3	14 days	111	1	1	0.9
4	—	222	0	0	0.0

Table 4. Carotenoid content and composition of the transgenic #21 and wild type plants

freesia plant	transgenic #21 plant						wild type plant					
plant organ	callus	*in vitro* root	stem	infant flower bud	large flower bud	petal	callus	*in vitro* root	stem	infant flower bud	large flower bud	petal
total carotenoid content (μg/g FW)	32.2	2.3	83.1	17.9	21.6	trace*	0.89	trace*	111.2	112.4	81.1	trace*
carotenoid composition (%)												
β-Carotene	1.2		24.2	20.2	26.3		38.9		20.4	26.6	33.2	
Lutein	4.8	16.1	15.5	5.2	18.5		45.5		57.1	56.1	46.6	
β-Cryptoxanthin			2.1	2.2	4.1				7.2	7.5	2.1	
Neoxanthin		1.0					10.4					
Epoxy-carotenoids**			2.1	1.2	5.2				6.7	2.5	13.6	
Echinenone			1.5	0.5	0.5							
Canthaxanthin	4.2	2.0	3.2	2.2	2.2							
Adonixanthin	2.0	4.5										
Adonirubin	5.4	2.0										
Astaxanthin	4.3	4.3										
Astaxanthin monoester	32.5	30.2	15.2	25.5	5.2							
Astaxanthin diester	30.5	30.0	35.1	39.8	30.1							
Fritschiellaxanthin	3.0											
4-Ketoantheraxanthin	1.6											
others	10.5	9.8	1.1	3.2	7.9		5.2		8.6	7.3	4.5	

*trace, trace amount of carotenoids; **Epoxy-carotenoids, mean Neoxanthin, Violaxanthin and/or Antheraxanthin.

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