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Note

Agrobacterium-mediated transformation and regeneration of *Freesia*×*hybrida*

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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 and 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.

Key words: *Agrobacterium tumefaciens*, cormlet, *Freesia*×*hybrida*, genetic transformation, nodular callus.

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 sales amount of *Freesia* as a cut flower was approximately €50 million 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 Vermeulen 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.

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)

Abbreviations: 6-BA, 6-benzyl aminopurine; CaMV, cauliflower mosaic virus; CTAB, hexadecyltrimethylammonium bromide; DIG, digoxigein; *gus*, *β*-glucuronidase gene; GUS, *β*-glucuronidase; *hpt*, hygromycin phosphotransferase gene; Hyg^r, hygromycin-resistant; IAA, 3-indoleacetic Acid; MS medium, Murashige and Skoog medium; *nptII*, neomycin phosphotransferase II gene; *nos*, nopaline synthase gene; PGR, plant growth regulator; TDZ, thidiazuron; X-gluc, 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide.

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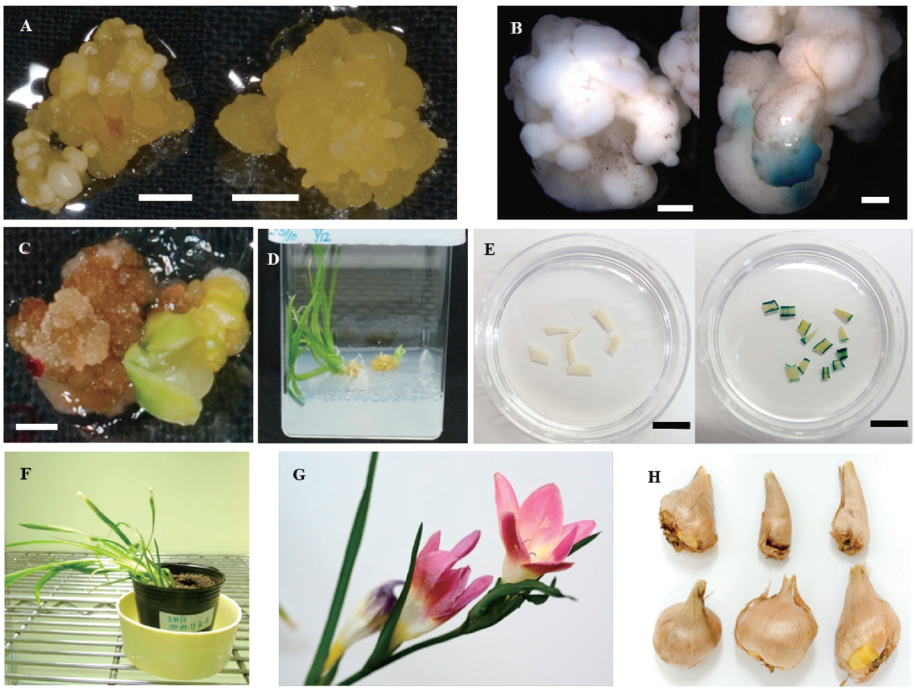

Figure 1. Production of transgenic *Freesia*×*hybrida* plants via *Agrobacterium*-mediated transformation of nodular calli. Nodular calli of 'Mosera' (A, left) and 'Ishikawa f3' (A, right) before inoculation with *A. tumefaciens*. Bar=5 mm in (A). Histochemical assay of GUS activity in 'Ishikawa f3' 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).

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, Ltd., Osaka, Japan), 1 mg l^{-1} dicamba (Wako Pure Chemical Industries, Ltd.), 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, Ltd.), 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 *β*-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, Ltd., Osaka, Japan)

and 50 mg l^{-1} hygromycin B (Wako Pure Chemical Industries, Ltd., 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}.

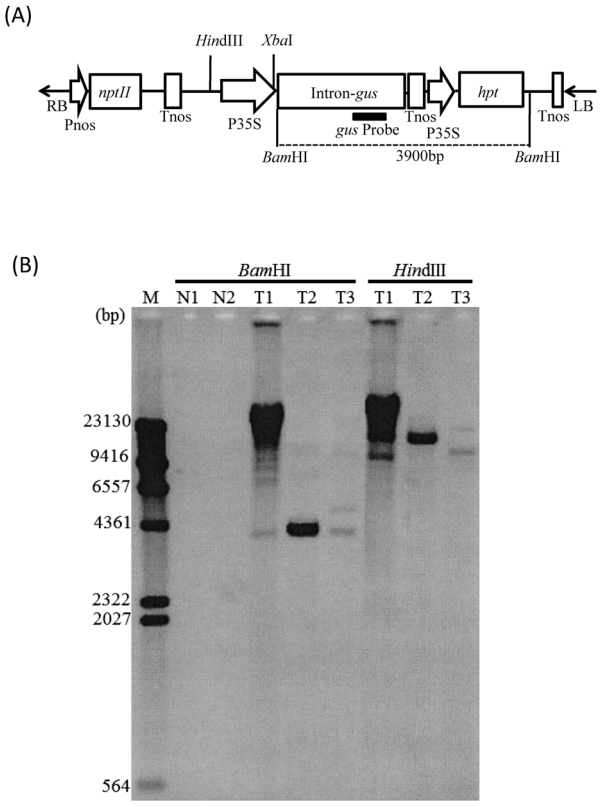

Figure 2. Transformation vector and Southern blot analysis. Schematic diagram of part of T-DNA region of transformation vector pIG121-Hm (A). RB, right border; LB, left border; Pnos, *nos* promoter; Tnos, *nos* terminator; P35S, CaMV 35S promoter; *nptII*, gene for neomycin phosphotransferase II; Intron-*gus*, 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'.

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 (Table 1). The calli on hygromycin-containing media became brown within 1–3 months, but hygromycin-resistant (Hyg^r) lines gradually grew from

Table 1. Results of transformation experiments on two *Freesia* cultivars.

Cultivar	No. of tested calli	No. of regenerated plantlets	No. of GUS-positive[a] plantlets	No. of transformed plantlets
Mosera	475	27	2	2
Ishikawa f3	290	9	1	1

[a] Plantlets showing positive result in β-glucuronidase assay.

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, Table 1).

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′-CTG CTG TCG GCT TTA ACC TC-3′ (forward) and 5′-TGA GCG TCG CAG AAC ATT AC-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 resistance to fungal and viral pathogens.

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