Plant regeneration and Agrobacterium-mediated genetic transformation systems in liliaceous ornamental plants

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Abstract  The family Liliaceae (Cronquist system) contains various important ornamental plants. We have been examining for about 20 years the establishment of plant regeneration and genetic transformation systems in liliaceous ornamental plants for their biotechnological breeding and elucidation of the molecular mechanisms determining ornamental traits. In this review, studies on in vitro plant regeneration in 7 genera and on Agrobacterium-mediated production of transgenic plants in 4 genera are described. Plant regeneration was achieved via callus cultures in Agapanthus, Hemerocallis, Hosta, Lilium, Muscari and Tricyrtis. Auxins (2,4-dichlorophenoxyacetic acid, α-naphthaleneacetic acid and/or picloram) were effective for inducing regenerable calli. Tulipa species and cultivars were very recalcitrant to callus induction and plant regeneration. Agrobacterium-mediated transformation was examined in Agapanthus, Lilium, Muscari and Tricyrtis, and transgenic plants were obtained in all genera by using regenerable calli as a target material for Agrobacterium inoculation, inoculation and co-cultivation with Agrobacterium in the presence of acetosyringone, and selection of transgenic tissues and plantlets on hygromycin-containing media. Among 4 genera, Tricyrtis has several advantages for transformation studies: higher transformation efficiency, relatively small plant size, ease of cultivation, and taking only 1 year from in vitro regeneration to flowering. We are now investigating the molecular mechanisms for determining plant form, flower color and flower form by using Tricyrtis spp. as liliaceous model plants.

Key words: Agapanthus, Lilium, Muscari, regenerable callus culture, Tricyrtis.

Introduction

The family Liliaceae (Cronquist system) contains various important ornamental plants such as Lilium spp., Tulipa gesneriana, Muscari armeniacum and Hemerocallis spp. Breeding of these species has so far been carried out mainly by sexual hybridization and sport selection, and a number of attractive cultivars have so far been produced by these traditional methods (Robinson and Firoozabad 1993). However, traditional breeding is time-consuming, and available genetic information in traditional breeding is restricted. We have been examining for about 20 years the establishment of plant regeneration and genetic transformation systems in liliaceous ornamental plants for their biotechnological breeding such as induction and selection of valuable mutants and production of valuable somatic hybrids and transgenic plants. Efficient genetic transformation systems are also indispensable for elucidation of the molecular mechanisms determining ornamental traits and for applying genome editing technology (Yan et al. 2019).

In this review, studies on in vitro plant regeneration in 7 genera and on Agrobacterium-mediated production of transgenic plants in 4 genera are described. Protocols of plant regeneration and genetic transformation of some liliaceous plants are also described.

Plant regeneration systems

We examined the establishment of efficient plant regeneration systems in various species and cultivars belonging to Agapanthus, Hemerocallis, Hosta, Lilium, Muscari, Tricyrtis and Tulipa (Table 1). For Lilium species and cultivars, plant regeneration could be obtained via adventitious shoot formation from various organs such as young leaves, stems, bulb-scales, tepals and filaments (unpublished). However, for species and cultivars belonging to the other 6 genera, cultured organs showed...
poor regeneration, and only a few explants produced adventitious shoots.

For most species and cultivars belonging to 6 genera except for *Tulipa*, plants were regenerated from organogenic (*Hemerocallis, Hosta, Lilium and Muscari*) or embryogenic (*Agapanthus, Muscari and Tricyrtis*) calli. These regenerable calli were induced from leaves, roots, bulb-scales, scapes and/or immature floral organs on media containing 2,4-D, NAA or PIC. Organogenic calli produced adventitious shoots after transfer to PGR-free, BA-containing or TDZ-containing media. On the other hand, embryogenic calli produced somatic embryos after transfer to PGR-free media. Adventitious shoots and somatic embryos were readily developed into plantlets on PGR-free medium.

Plant regeneration systems via callus cultures in 6 genera are as follows. Some modifications are added to the original method.

**Somatic embryogenesis and plant regeneration from callus cultures of Agapanthus praecox ssp. orientalis (Suzuki et al. 2002)**

1. Use field-grown plants of ‘Royal Purple Select’ as a plant material.

   2. For callus induction, harvest young leaves before anthesis, wash in running water for 1 min, surface-sterilize with a sodium hypochlorite solution containing 1% active chlorine for 10 min, and rinse 3 times with sterile, distilled water. Cut leaves transversely into segments (ca. 5 mm in length), and place on MS medium containing 1 mg l\(^{-1}\) PIC, 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Agapanthus* callus induction medium; AgCIM). Incubate at 25 \(^{\circ}\)C under continuous illumination with white fluorescent lighting (ca. 35 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)).

   In our study, leaf explants started to form yellowish compact calli mainly from the cut end 3 to 4 weeks after culture initiation. Over 30% of the explants produced such calli after 2 months on AgCIM.

3. Isolate the yellowish compact calli from the explants, transfer to fresh AgCIM, and incubate for 2 months at 25 \(^{\circ}\)C in the dark. Pick up the newly-formed, creamy-white and friable calli (embryogenic calli), and further transfer to fresh AgCIM. Maintain them by monthly subculturing to fresh AgCIM under the same conditions.

   In our study, about 25% of the yellowish compact calli newly formed embryogenic calli on their

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**Table 1. Summary of in vitro plant regeneration in some liliaceous ornamental plants (Cronquist system).**

| Plant species | Regeneration from organs\(^1\) | Regeneration from calli\(^1\) | Regeneration of somaclonal variants\(^2\) | Note | Reference |
|---------------|-----------------------------|-----------------------------|---------------------------------|------|-----------|
| *Agapanthus praecox ssp. orientalis* | ± | + | ++ | Efficient regeneration from embryogenic calli, frequent appearance of somaclonal variation, large plant size, ease of cultivation, taking 3 to 5 years from *in vitro* regeneration to flowering | Suzuki et al. (2002) Nakano et al. (2003) Mori et al. (2007) |
| *Hemerocallis hybrida* | ± | ± | ± | Regeneration from organogenic suspension cultures, large plant size, ease of cultivation, taking 2 to 3 years from *in vitro* regeneration to flowering | Saito and Nakano (2000) |
| *Hosta sieboldiana* | ± | + | ± | Regeneration from organogenic suspension cultures, large plant size, ease of cultivation, taking 2 to 3 years from *in vitro* regeneration to flowering | Saito and Nakano (2002) |
| *Lilium* species and cultivars | + | + | + | Efficient regeneration from organogenic calli in various species and cultivars, necessity of a cold treatment of regenerated bulblets, large plant size, taking 2 to 3 years from *in vitro* regeneration to flowering | Nakano et al. (2000) Mori et al. (2005) |
| *Muscari* species and cultivars | ± | + | ± | Efficient regeneration from organogenic and embryogenic calli, small plant size, ease of cultivation, taking 2 to 3 years from *in vitro* regeneration to flowering, small flower size | Suzuki and Nakano (2001) Suzuki and Nakano (2003) Mori and Nakano (2004) |
| *Tricyrtis* species and cultivars | ± | + | + | Efficient regeneration from embryogenic calli, appearance of somaclonal variation from old callus cultures, relatively small plant size, ease of cultivation, taking 1 years from *in vitro* regeneration to flowering | Nakano et al. (2004) Nakano et al. (2006) |
| *Tulipa* species and cultivars | ± | — | — | Very recalcitrant to callus induction and plant regeneration | |

\(^{1}\) ++, Good response; +, poor response; ±, very poor response; —, no response. \(^2\) ++, Frequently regenerated; +, sometimes regenerated; ±, rarely regenerated; —, not examined.
surface within 2 months. They contained a few globular somatic embryos, which showed no further development on AgCIM. The embryogenic calli showed 1.5- to 2-fold increases in FW in 1 month.

4. For inducing somatic embryos, transfer the embryogenic calli to PGR-free MS medium containing 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum, pH 5.6 (Agapanthus embryo induction medium; AgEIM), and incubate at 25°C under continuous illumination. In our study, white, club-shaped somatic embryos were formed within 1 month on AgEIM. Almost all of the calli produced somatic embryos, and 30 to 40 embryos were obtained per 0.1 g FW of the embryogenic calli. The calli maintained such potential of somatic embryo production for over 2 years (unpublished).

5. For plantlet induction, isolate the somatic embryos, transfer to PGR-free, half-strength MS medium containing 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum, pH 5.6 (Agapanthus plantlet induction medium; AgPIM), and incubate at 25°C under continuous illumination. In our study, the somatic embryos rapidly produced a green shoot and a primary root. More than 90% of the somatic embryos developed into plantlets on AgPIM.

6. Wash the regenerated plantlets with well-developed roots in running water to remove gellan gum, and transfer to pot containing moist vermiculite. Acclimatize them in a transparent plastic cabinet covered with a polyethylene sheet at 25°C under a 16 h-photoperiod. Punch small holes in the polyethylene sheet after 1 week, and then remove the sheet gradually in a stepwise fashion. After 2 to 3 weeks, transplant the acclimatized plants to pots containing soil (3 : 1 mixture of loam soil and leaf mold), and cultivate in the greenhouse without heating.

In our study, the plantlets were readily transplanted to the greenhouse and produced flowers 2 to 3 years after regeneration (unpublished). Cultivation of the regenerated plants occupied a large space due to large plant size. Most of the regenerated plants showed various somaclonal variations, including chromosome doubling, shorter inflorescence stalks, and decreased numbers of florets per inflorescence (unpublished). Similar somaclonal variations were also observed in plants regenerated from callus-derived protoplasts of *A. praecox* ssp. *orientalis* ‘Royal Purple Select’ (Nakano et al. 2003).

**Adventitious shoot formation and plant regeneration from cell suspension cultures of *Hemerocallis hybrida* (Saito and Nakano 2000)**

1. Use axially bud-derived, in vitro-grown plantlets of ‘Stella d’Oro’ as a plant material.

2. For callus induction, harvest roots from in vitro-grown plantlets 2 weeks after subculture, and cut transversely into segments (ca. 1 cm in length). Place these segments on MS medium containing 10 mg l⁻¹ PIC, 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum, pH 5.6 (*Hemerocallis* callus induction medium; HeCIM), and incubate at 25°C in the dark. In our study, over 50% of root segments produced creamy-white calli within 2 months on HeCIM. No organized tissues such as shoots and roots were formed.

3. For establishing suspension cultures, transfer ca. 1 g FW of the calli to a 100-ml Erlenmeyer flask containing 10 ml of liquid HeCIM, and incubate with rotary shaking (100 cycles min⁻¹) at 25°C in the dark. After stabilization of the growth rate, subculture cell suspensions every 2 weeks by transferring ca. 1 ml PCV of cells to 30 ml of fresh liquid HeCIM. In our study, stably-growing suspension cultures were established within 2 months. These cultures consisted of pale-yellow and fine clumps with 20 to 50 cells, and over 2-fold increases in PCV were obtained within 1 week.

4. For inducing adventitious shoots, place suspension cells initially on HeCIM and culture for 1 month. Then, transfer suspension cell-derived calli to MS medium containing 0.1 mg l⁻¹ NAA, 1 mg l⁻¹ BA, 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum, pH 5.6 (*Hemerocallis* shoot induction medium; HeSIM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 μmol m⁻² s⁻¹). In our study, although adventitious shoots were produced from the calli after 2 to 3 months on HeSIM, its frequency was very low (below 1%). No adventitious shoots were produced from 6-month-old suspension cells (unpublished).

5. For plantlet induction, isolate the shoots from the calli, transfer to PGR-free MS medium containing 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum, pH 5.6 (*Hemerocallis* plantlet induction medium; HePIM), and incubate at 25°C under continuous illumination. In our study, all of the shoots produced roots and developed into plantlets within 1 month.

6. Acclimatized and transplant the plantlets with the same method as *A. praecox* ssp. *orientalis*. In our study, the plantlets were readily transplanted to the greenhouse and produced flowers 2 to 3 years after regeneration (unpublished). Cultivation of the regenerated plants occupied a large space due to large plant size.
Adventitious shoot formation and plant regeneration from cell suspension cultures of *Hosta sieboldiana* (Saito and Nakano 2002)

1. Use field-grown plants of *H. sieboldiana* as a plant material.
2. For callus induction, harvest scapes 5 to 7 days before anthesis, wash in running water for 1 min, surface-sterilize with 70% ethanol for 30 s followed by with a sodium hypochlorite solution containing 1% active chlorine for 10 min, and rinse 3 times with sterile, distilled water. Cut scapes transversely into segments of ca. 5 mm square, place on MS medium containing 1 mg l\(^{-1}\) PIC, 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Hosta* callus induction medium; HoCIM), and incubate at 25°C in the dark. In our study, about 20% of scape explants produced calli mainly along the cut end within 2 months on HoCIM. They were pale-yellow in color and consisted of nodular cell clusters.
3. For establishing suspension cultures, transfer ca. 0.5 g FW of the calli to a 100-ml Erlenmeyer flask containing 10 ml of liquid HoCIM, and incubate with rotary shaking (100 cycles min\(^{-1}\)) at 25°C under continuous illumination (ca. 35 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). Add 5 ml of liquid HoCIM to the flask every week. After stabilization of the growth rate, subculture cell suspensions every 2 weeks by transferring ca. 1.5 g FW of cell clusters to 20 ml of fresh liquid HoCIM. In our study, stably-growing suspension cultures were established after 1 month on HoCIM. These cultures consisted of pale-yellow and nodular cell clusters of 1 to 5 mm in diameter, and 2- to 2.5-fold increases in FW were obtained in 2 weeks.
4. For inducing adventitious shoots, transfer cell clusters to MS medium containing 0.1 mg l\(^{-1}\) NAA, 1 mg l\(^{-1}\) TDZ, 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Hosta* shoot induction medium; HoSIM), and incubate at 25°C under continuous illumination. In our study, pale-yellow clusters turned green within 2 weeks, and started to produce adventitious shoots and/or roots 2 to 3 weeks later. About 20 shoots per 0.3 g FW of cell clusters were obtained after 2 months on HoSIM. Shoot growth was promoted by subculturing to fresh HoSIM. The calli maintained such potential of somatic embryo production for over 2 years (unpublished).
5. For plantlet induction, isolate the shoots from the calli, transfer to PGR-free MS medium containing 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Hosta* plantlet induction medium; HoPIM), and incubate at 25°C under continuous illumination. In our study, the shoots readily produced roots and developed into plantlets within 1 month on HoPIM.
6. Acclimatize and transplant the plantlets with the same method as *A. praecox* ssp. *orientalis*. In our study, the plantlets were readily transplanted to the greenhouse and produced flowers 2 to 3 years after regeneration (unpublished). Cultivation of the regenerated plants occupied a large space due to large plant size.

Adventitious shoot formation and plant regeneration from callus cultures of *Lilium* spp. (Mori et al. 2005)

1. Use potted plants or leaf-derived, *in vitro*-grown plantlets of various *Lilium* species and cultivars as plant materials.
2. For callus induction from filaments, harvest flower buds 5 to 7 days before anthesis from potted plants, wash in running water for 1 min, surface-sterilize with a sodium hypochlorite solution containing 1% active chlorine for 10 min, and rinse 3 times with sterile, distilled water. Isolate stamens from flower buds and remove anthers. Cut filaments transversely into segments (ca. 1 cm in length), and place on MS medium containing 1 mg l\(^{-1}\) PIC, 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Lilium* callus induction medium; LiCIM). For inducing callus induction from bulb-scales, isolate them from *in vitro*-grown plantlets, transversely cut into halves, and placed on LiCIM. Incubate these cultures at 25°C in the dark. In our study, over 80% of both bulb-scale and filament explants produced calli within 2 months on LiCIM in most species and cultivars. The calli were generally yellow in color and had a nodular appearance.
3. Isolate the calli from the explants, and transfer to fresh LiCIM. Maintain them by monthly subculturating to fresh LiCIM at 25°C in the dark. In our study, bulb-scale- and filament-derived calli showed over 2-fold increases in callus mass in 1 month. No apparent effect of initial explant type was observed on callus growth.
4. For inducing adventitious shoots, transfer the calli to PGR-free MS medium containing 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (*Lilium* shoot induction medium; LiSIM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). In our study, over 80% of calli produced adventitious shoots within 2 months on LiSIM in most species and cultivars. The number of shoots per callus cluster ranged from 2 to 30 depending on the genotype. No apparent effect of initial explant type was observed on shoot regeneration. The calli kept such potential of adventitious shoot production for over 2 years (unpublished).
5. For plantlet induction, isolate the shoots from the calli, transfer to PGR-free, half-strength MS medium.
containing 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (\textit{Lilium} plantlet induction medium; LiPIM), and incubate at 25°C under continuous illumination.

In our study, almost all of the shoots produced roots and developed into plantlets within 1 month on LiPIM. These plantlets subsequently formed a small bulblet at the basal region.

6. Subject the plantlets with a well-developed bulblet in culture vessels to a cold treatment at 4°C in the dark for 2 to 3 months. After that, take out the plantlets from culture vessels, remove scale leaves and roots from the plantlets, and wash the remaining bulblets in running water to remove gellan gum. Transplant the bulblets to pots containing soil (3 : 1 mixture of loam soil and leaf mold), and cultivate in the greenhouse without heating.

In our study, over 90% of the bulblets developed new scaly leaves within 1 months in the greenhouse irrespective of genotype and callus origin. Regenerated plants produced flowers 2 to 3 years after regeneration (unpublished). Cultivation of the regenerated plants occupied a large space due to large plant size.

By using this system, organogenic calli could be obtained in various \textit{Lilium} genotypes including species belonging to the sections Archelirion, Daurolirion, Leucolirion, Liriotypus, Martagon, Pseudolirion and Sinomartagon, and cultivars of Asiatic hybrid, LA hybrid, Longiflorum hybrid, Oriental hybrid and Trumpet hybrid. No apparent genotypic difference in regenerable callus production was observed (Mori et al. 2005).

For \textit{L. formosanum}, we established suspension cultures from bulb-scale-derived calli. Suspension cell clusters produced somatic embryos and adventitious shoots after transfer to PGR-free and BA-containing media, respectively. Although the regeneration potential of the cell clusters decreased after long-term culture for 4 years, it could be restored by treating with the auxin transport inhibitor TIBA (Nakano et al. 2000).

**Somatic embryogenesis and plant regeneration from callus cultures of \textit{Muscari armeniacum} (Suzuki and Nakano 2001)**

1. Use hydroponically grown plants of 'Blue Pearl' as a plant material.
2. For callus induction, harvest young leaves when flower stalks are 4 to 5 cm in length, wash in running water for 1 min, surface-sterilize with 70% ethanol for 30 s followed by with a sodium hypochlorite solution containing 1% active chlorine for 15 min, and rinse 3 times with sterile, distilled water. Cut leaves transversely into segments (3 to 5 mm in length), and placed on MS medium containing 10 mg l\(^{-1}\) NAA, 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (\textit{Muscari} callus induction medium; MuClM). Incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)).

In our study, leaf explants started to form creamy-white and soft calli mainly from the cut end 4 to 5 weeks after culture initiation. Over 50% of the explants produced such calli after 2 months on MuClM.

3. Isolate the creamy-white and soft calli from the explants, transfer to fresh MuClM, and incubate for 2 months at 25°C under continuous illumination. Pick up the newly formed, white and friable calli (embryogenic calli), and further transfer to fresh MuClM. Maintain them by monthly subculturing to fresh MuClM under the same conditions.

In our study, about 60% of the creamy-white and soft calli newly formed embryogenic calli within 2 months. They contained a few globular somatic embryos, which showed no further development on MuClM. The embryogenic calli showed about 2-fold increases in FW in 1 month.

4. For inducing somatic embryos, transfer the embryogenic calli to PGR-free MS medium containing 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum, pH 5.6 (\textit{Muscari} embryo induction medium; MuEIM), and incubate at 25°C under continuous illumination.

In our study, about 90% of the embryogenic calli produced a number of somatic embryos within 1 months on MuEIM. The calli maintained such potential of somatic embryo production for over 2 years (unpublished).

5. For promoting plantlet induction, isolate the somatic embryos and transfer to fresh MuEIM, and incubate at 25°C under continuous illumination.

In our study, almost all of the somatic embryos rapidly produced a green shoot and roots. Plantlet thus obtained subsequently formed a small bulblet at the basal region.

6. Remove leaves from the plantlets, and wash the remaining bulblets with roots in running water to remove gellan gum. Transfer the bulblets with roots to pot containing moist vermiculite, and incubate at 25°C under continuous illumination. Following emergence of new leaves, transplant the plants to pots containing soil (3 : 1 mixture of loam soil and leaf mold), and cultivate in the greenhouse without heating.

In our study, the plantlets were readily transplanted to the greenhouse and produced flowers 3 to 4 years after regeneration (unpublished).

By using this system, embryogenic calli were also obtained in several \textit{M. armeniacum} cultivars and \textit{M. neglectum} (Mori and Nakano 2004). In addition, we succeeded in plantlet regeneration from protoplasts...
isolated from embryogenic callus cultures of *M. armeniacum 'Blue Pearl'* (Nakano et al. 2005). However, embryogenic calli were hardly obtained in other *Muscari* species such as *M. azureum*, *M. botryoides*, *M. comosum*, *M. latilolium*, *M. macrocarpum*, *M. moschatum*, *M. paradoxum* and *M. tubergenianum* (Mori and Nakano 2004).

For *M. armeniacum 'Blue Pearl,'* organogenic callus cultures could also be induced from leaf explants on 2,4-D-containing media (Suzuki and Nakano 2001). The organogenic calli produced adventitious shoots on BA-containing media.

**Somatic embryogenesis and plant regeneration from callus cultures of *Tricyrtis* sp. (Nakano et al. 2004)**

1. Use potted plants of ‘Shinonome’ as a plant material.
2. For callus induction, harvest flower buds (12 to 20 mm in length) 3 to 5 days before anthesis, wash in running water for 1 min, surface-sterilize with a sodium hypochlorite solution containing 1% active chlorine for 10 min, and rinse 3 times with sterile, distilled water. Isolate tepals and filaments, transversely cut into halves, and place on MS medium containing 1 mg l$^{-1}$ 2,4-D, 0.1 mg l$^{-1}$ TDZ, 30 g l$^{-1}$ sucrose and 2 g l$^{-1}$ gellan gum, pH 5.6 (*Tricyrtis* callus induction medium; TrCIM). Incubate at 25°C in the dark.

In our study, tepal and filament explants started to form white to yellowish and friable calli (embryogenic calli) mainly from the cut end 3 to 6 weeks after culture initiation. Over 60 and 90% of tepal and filament explants, respectively, produced embryogenic calli after 2 months on TrCIM. The calli occasionally contained a few globular somatic embryos.

3. Isolate the embryogenic calli from the explants, transfer to half-strength MS medium containing 1 mg l$^{-1}$ 2,4-D, 30 g l$^{-1}$ sucrose and 2 g l$^{-1}$ gellan gum, pH 5.6 (*Tricyrtis* callus proliferation medium; TrCPM), and incubate at 25°C in the dark. Maintain them by monthly subculturing to fresh TrCPM under the same conditions.

In our study, the embryogenic calli showed about 3-fold increases in FW in 1 month. They contained a few globular somatic embryos, which showed no further development on TrCPM.

4. For inducing somatic embryos, transfer the embryogenic calli to PGR-free, half-strength MS medium containing 30 g l$^{-1}$ sucrose and 2 g l$^{-1}$ gellan gum, pH 5.6 (*Tricyrtis* embryo induction medium; TrEIM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35µmol m$^{-2}$ s$^{-1}$).

In our study, the embryogenic calli started to produce somatic embryos, which were oval to club-shaped and cream to yellow in color, 2 weeks after transfer to TrEIM. Over 500 somatic embryos per 0.5 g FW of the embryogenic calli were obtained in 1 month on TrEIM. The calli maintained such potential of somatic embryo production for over 2 years (unpublished). Incidentally, somatic embryo production was promoted by heavy-ion beam irradiation at low doses (Nakano et al. 2010b).

5. For promoting plantlet induction, isolate the somatic embryos and transfer to fresh TrEIM, and incubate at 25°C under continuous illumination.

In our study, almost all of the somatic embryos rapidly produced a green shoot and roots, and plantlet were established from them.

6. Acclimatize and transplant the plantlets with the same method as *A. praecox* ssp. *orientalis*.

In our study, the plantlets were readily transplanted to the greenhouse and produced flowers within 1 year after regeneration (unpublished).

By using this system, embryogenic calli could be obtained in other *Tricyrtis* species such as *T. hirta*, *T. hirta* var. *albescens*, *T. formosana*, *T. flava* and *T. macrantha*, but with lower frequencies than *Tricyrtis* sp. ‘Shinonome’ (unpublished). Embryogenic calli of *T. flava* and *T. macrantha* showed poor growth and poor somatic embryo production compared with other *Tricyrtis* species.

Since some somaclonal variations, such as chromosome doubling and dwarffness, appeared in regenerated plants from 1-year-old embryogenic callus cultures (Nakano et al. 2006), the cultures immediately after establishment should be used for micropropagation and genetic transformation.

By using embryogenic callus cultures of *Tricyrtis* sp., we succeeded in efficient induction of valuable mutants by heavy-ion beam irradiation (Nakano et al. 2010a).

**Agrobacterium-mediated transformation systems**

Among a number of genetic transformation methods, we selected *Agrobacterium*-mediated one for the following advantages: no need of both special equipment and techniques, no need of protoplast culture systems, defined integration of transgenes, potentially low copy number, and preferential integration into transcriptionally active regions of the chromosome (Hiei et al. 2000). It may be possible to transfer foreign gene(s) to various species and cultivars belonging to *Agapanthus*, *Hemerocallis*, *Hosta*, *Lilium*, *Muscari*, *Tricyrtis* and *Tulipa* by *Agrobacterium*-mediated methods, since transient expression of the GUS reporter gene was detected in various organs and/or calli after inoculation and co-
cultivation with *Agrobacterium* (Table 2). However, in order to obtain a number of transgenic plants, it is necessary to use highly regenerable tissues as a target material for *Agrobacterium* inoculation. Therefore, we examined the establishment of efficient *Agrobacterium*-mediated transformation systems by using the above-mentioned highly organogenic calli (*Lilium*) or highly embryogenic calli (*Agapanthus, Muscari* and *Tricyrtis*).

For all genera, addition of AS to *Agrobacterium* inoculation and co-cultivation media was essential for obtaining stably transformed tissues. Efficient selection of transgenic tissues and plantlets was achieved by using hygromycin-containing media. In *Agapanthus*, *Lilium* and *Muscari*, callus growth and development were completely inhibited by hygromycin and bialaphos, whereas the calli showed high levels of natural tolerance to kanamycin and G418 (Suzuki and Nakano 2002; Suzuki et al. 1998, 2002).

Established systems of 4 genera are as follows. Some modifications are added to the original method.

**Agapanthus praecox ssp. orientalis** (Suzuki et al. 2001)

1. Use leaf-derived embryogenic calli of ‘Royal Purple Select’ as a target material for transformation. Maintain them by monthly subculturing to AgCIM at 25°C in the dark.
2. Use *A. tumefaciens* EHA101/pIG121Hm or LBA4404/pTOK233 for transformation. The T-DNA region of both binary vectors, pIG121Hm and pTOK233, contains the NPTII gene under the control of the NOS promoter, the intron-containing GUS gene under the control of the CaMV35S promoter, and the HTP gene under the control of the CaMV35S promoter (Hiei et al. 1994; Ohta et al. 1990). Pre-culture each *Agrobacterium* strain in liquid YEP medium (An et al. 1988) containing 50 mg l⁻¹ kanamycin (kanamycin monosulfate) and 50 mg l⁻¹ hygromycin (hygromycin B) for more than 24 h at 28°C with reciprocal shaking (200 cycles min⁻¹). Collect *Agrobacterium* cells by centrifugation (2,000 g for 10 min), and re-suspend them to a final OD 600 of 0.2 in liquid AgCIM containing 20 mg l⁻¹ of AS (*Agapanthus* inoculation medium; AgIM).
3. Immerse the calli into the bacterial suspension for 1 min, blot on sterile filter papers, and co-cultivate for 7 days with *Agrobacterium* on AgCIM containing 20 mg l⁻¹ AS (*Agapanthus* co-cultivation medium; Table 2. *Agrobacterium*-mediated transformation in some liliaceous ornamental plants (Cronquist system).

| Plant species                  | Transient expression of the GUS gene¹ | Regeneration of transgenic plants² | Note                                                                                                                                  | Reference                  |
|-------------------------------|----------------------------------------|----------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| *Agapanthus* sp.              | +                                      | +                                | Use of embryogenic calli as a target material for transformation, long-term expression of a transgene in transgenic plants, large plant size, ease of cultivation, taking 3 to 5 years from *in vitro* regeneration to flowering | Suzuki et al. (2001)         |
|                               |                                        |                                  |                                                                                                                                        | Mori et al. (2007)          |
| *Hemerocallis hybrida*        | +                                      | —                                | Transient expression on organogenic calli                                                                                             | Hoshi et al. (2004)         |
| *Hosta sieboldiana*           | +                                      | —                                | Transient expression on organogenic calli                                                                                             | Hoshi et al. (2005)         |
| *Lilium* species and cultivars| +                                      | +                                | Use of organogenic calli as a target material for transformation, promotion of both transient expression and transgenic plant regeneration by scratching-treatment of organogenic calli, necessity of a cold treatment of regenerated bulblets, large plant size, taking 2 to 3 years from *in vitro* regeneration to flowering | Hoshi et al. (2004)         |
|                               |                                        |                                  |                                                                                                                                        | Suzuki and Nakano (2002)    |
|                               |                                        |                                  |                                                                                                                                        | Suzuki et al. (2005)        |
| *Muscari armeniacum*          | +                                      | +                                | Use of embryogenic calli as a target material for transformation, small plant size, ease of cultivation, taking 3 to 4 years from *in vitro* regeneration to flowering | Suzuki and Nakano (2002)    |
|                               |                                        |                                  |                                                                                                                                        | Suzuki et al. (2005)        |
| *Tricyrtis* species and cultivars| + +                                  | ++                               | Use of embryogenic calli as a target material for transformation, very efficient regeneration of transgenic plants, long-term expression of a transgene in transgenic plants, relatively small plant size, ease of cultivation, taking 1 years from *in vitro* regeneration to flowering | Adachi et al. (2005)        |
|                               |                                        |                                  |                                                                                                                                        | Mori et al. (2008)          |
| *Tulipa generiana*            | ±                                      | —                                | Transient expression on immature flower stalk segments                                                                               |                            |

¹Transient expression of the GUS gene after inoculation and co-cultivation with *Agrobacterium* was evaluated by the number of blue spots via histochemical assay. ++, Many blue spots; +, several blue spots; ±, a few blue spots. ++, Very efficient regeneration; +, efficient regeneration; —, not examined.
AgCM) at 25°C in the dark.
In our study, transient expression of the GUS gene in the co-cultivated calli was detected only in the presence of AS in AgIM and AgCM for both Agrobacterium strains.
4. Transfer of the co-cultivated calli to AgCIM containing 50 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime (Agapanthus callus selection medium; AgCSM), and incubate at 25°C in the dark. Subculture them every 2 weeks to fresh AgCSM under the same conditions.
In our study, most of the co-cultivated calli turned brown on AgCSM, but creamy-white cell clusters started to develop thereafter. Such hygromycin-resistant calli were obtained only when the calli were co-cultivated for 7 days in the presence of AS for both Agrobacterium strains. The efficiency of producing hygromycin-resistant calli of A. tumefaciens LBA4404/pTOK233 was higher than EHA101/pIG121Hm.

5. Pick up and transfer the hygromycin-resistant calli to AgEIM containing 50 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime (Agapanthus embryo selection medium; AgESM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 μmol m⁻² s⁻¹).
In our study, most of the hygromycin-resistant calli produced somatic embryos within 1 month on AgESM.
6. Transfer the somatic embryos to AgPIM, and incubate at 25°C under continuous illumination for promoting their growth.
In our study, more than 90% of the somatic embryos developed into plantlets.
7. Acclimatize and transplant the transgenic plantlets with the same method as non-transgenic, embryogenic callus-derived plantlets of A. praecox ssp. orientalis. Cultivate transgenic plants in a growth chamber.
In our study, the transgenic plantlets were readily transplanted to the growth chamber and produced flowers 3 to 5 years after regeneration. Cultivation of the transgenic plants occupied a large space due to large plant size. Most of the transgenic plants showed various somaclonal variations as in the case of non-transgenic, embryogenic callus-derived plantlets. Stable expression of the GUS reporter gene was observed even after 5 years of cultivation of the transgenic plants (Mori et al. 2007).

**Lilium sp. (Hoshi et al. 2004)**
1. Use filament-derived organogenic calli of ‘Acapulco’ as a target material for transformation. Maintain them by monthly subculturing to LiCIM at 25°C in the dark.
2. Use A. tumefaciens EHA101/pIG121Hm for transformation. Pre-culture Agrobacterium in liquid YEP medium containing 20 mg l⁻¹ AS, 50 mg l⁻¹ kanamycin and 50 mg l⁻¹ hygromycin for more than 24 h at 28°C with reciprocal shaking (200 cycles min⁻¹). Collect Agrobacterium cells by centrifugation (2,000 g for 10 min), and re-suspend them to a final OD₆₀₀ of 0.3 in NH₄NO₃-free MS medium containing 700 mg l⁻¹ L-asparagine monohydrate, 700 mg l⁻¹ L-glutamine, 1 mg l⁻¹ PIC, 20 mg l⁻¹ AS and 30 g l⁻¹ sucrose (Lilium inoculation medium; LiIM).
3. Prior to inoculation, apply a scratching-treatment to the calli. Make tubes for the scratching-treatment by attaching a sandpaper (grit number 150; Fuchioka, Japan) to the inside of 50-ml plastic centrifuge tubes (IWAKI, Japan). Transfer the calli to the tubes containing LiIM, and scratch the calli by stirring the tubes at 2,900 cycles min⁻¹ for 10 s with a test tube mixer (TME-21; Advantec, Japan). After the scratching treatment, the surface of the calli become smoother.
4. Immerse the scratched calli into the bacterial suspension for 5 min, blot on sterile filter papers, and co-cultivate for 7 days with Agrobacterium on NH₄NO₃-free MS medium containing 1 mg l⁻¹ PIC, 20 mg l⁻¹ AS, 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum (Lilium co-cultivation medium; LiCM) at 25°C in the dark.
In our study, the surface of the scratched calli partly became brown during co-cultivation. Transient expression of the GUS gene in the co-cultivated calli was promoted by applying the scratching-treatment to the calli and by using the NH₄NO₃-free, co-cultivation medium.
5. Transfer the co-cultivated calli to MS medium containing 0.1 mg l⁻¹ PIC, 0.01 mg l⁻¹ BA, 50 mg l⁻¹ hygromycin, 300 mg l⁻¹ cefotaxime, 30 g l⁻¹ sucrose and 2 g l⁻¹ gellan gum (Lilium shoot selection medium; LiSSM). Subculture them every 3 weeks to fresh LiSSM at 25°C in the dark.
In our study, most of the co-cultivated calli turned brown, but creamy-white cell clusters started to develop after 4 to 5 weeks on LiSSM. These cell clusters produced adventitious shoots 1 to 2 months later.
6. Isolate the shoots from the calli, transfer to LiPIM containing 50 mg l⁻¹ hygromycin and 300 mg l⁻¹ cefotaxime (Lilium plantlet selection medium; LiPSM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 μmol m⁻² s⁻¹).
In our study, all of the shoots produced roots and developed into plantlets within 1 month on LiPSM. These plantlets subsequently formed a small bulblet at the basal region.
7. Subject the transgenic plantlets to the cold treatment, and transplant the transgenic bulblets with the same method as non-transgenic, callus-derived plantlets of *Lilium* spp. Cultivate transgenic plants in a growth chamber.

In our study, the transgenic plantlets were readily transplanted to the growth chamber and produced flowers 2 to 3 years after regeneration. Cultivation of the transgenic plants occupied a large space due to large plant size. No apparent morphological alterations were observed in the transgenic plants at the flowering stage (unpublished).

Transgenic plants of *L. longiflorum* 'Georgia' were successfully obtained by increasing the gellan gum concentration of LiCSSM to 10 g l\(^{-1}\) and decreasing the hygromycin concentration in LiSSM and LiPSM to 35 mg l\(^{-1}\) (Hoshi et al. 2005).

**Muscari armeniacum** *(Suzuki and Nakano 2002)*

1. Use leaf-derived embryogenic calli of 'Blue Pearl' as a target material for transformation. Maintain them by monthly subculturing to MuCIM at 25°C under continuous illumination with white fluorescent lighting (ca. 35 μmol m\(^{-2}\)s\(^{-1}\)).

2. Use *A. tumefaciens* EHA101/pIG121Hm, LBA4404// pIG121Hm or LBA4404//pTOK233 for transformation. Pre-culture each *Agrobacterium* strain as in the case of *A. prae cox* ssp. *orientalis*. Collect *Agrobacterium* cells by centrifugation (2000 × g for 10 min), and re-suspend them to a final OD600 of 0.2 in liquid MuCIM containing 20 mg l\(^{-1}\) of AS and 0.1% of the surfactant Tween20 (*Muscari* inoculation medium; MuIM).

3. Blot the calli on sterile filter papers to remove excess culture medium, and then transfer to new sterile filter papers. Drop gently the bacterial suspension (1 ml per 1 g FW of the calli) onto the calli. Transfer the calli immediately to MuCIM containing 20 mg l\(^{-1}\) of AS (*Muscari* co-cultivation medium; MuCM), and co-cultivate for 3 days with *Agrobacterium* at 25°C in the dark.

In our study, transient expression of the GUS gene in the co-cultivated calli was promoted by adding AS to MuIM and MuCM and by adding Tween20 to MuIM for all 3 *Agrobacterium* strains.

4. Transfer the co-cultivated calli to MuCIM containing 500 mg l\(^{-1}\) cefotaxime (*Muscari* callus pre-selection medium; MuCPSM), and incubate for 1 week at 25°C under continuous illumination. Transfer the calli again to MuCIM containing 75 mg l\(^{-1}\) hygromycin and 500 mg l\(^{-1}\) cefotaxime (*Muscari* callus selection medium; MuCSM). Subculture them every 2 weeks to fresh MuCSM under the same conditions.

In our study, hygromycin-resistant cell clusters, which were white to light-yellow in color, started to produce among browned calli after 4 to 5 weeks on MuCSM. Production of hygromycin-resistant calli was markedly promoted by adding AS to MuIM and MuCM for all 3 *Agrobacterium* strains. Among *Agrobacterium* strains, EHA101//pIG121Hm and LBA4404//pTOK233 gave much higher efficiencies of producing hygromycin-resistant calli than LBA4404// pIG121Hm.

5. Pick up and transfer the hygromycin-resistant calli to MuEIM containing 25 mg l\(^{-1}\) hygromycin and 500 mg l\(^{-1}\) cefotaxime (*Muscari* embryo selection medium; MuESM), and incubate at 25°C under continuous illumination with white fluorescent lighting.

In our study, almost all of the hygromycin-resistant calli produced numerous somatic embryos within 1 month on MuESM.

6. Transfer the somatic embryos (ca. 3 mm in length) to MuEIM, and incubate at 25°C under continuous illumination for promoting their growth.

In our study, over 85% of the somatic embryos developed into plantlets after 5 to 6 weeks on MuEIM.

7. Acclimatized and transplant the transgenic plantlets with the same method as non-transgenic, embryogenic callus-derived plantlets of *M. armeniacum*. Cultivate transgenic plants in a growth chamber.

In our study, the transgenic plantlets were readily transplanted to the growth chamber and produced flowers 3 to 5 years after regeneration. No apparent morphological alterations were observed in the transgenic plants at the flowering stage (unpublished). Efficient selection of transgenic tissues and plantlets of *M. armeniacum* 'Blue Pearl' was also achieved by combining the PAT gene as a selectable marker and bialaphos as a selective agent (Suzuki et al. 2005).

**Tricyrtis sp.** *(Adachi et al. 2005)*

1. Use tepal- or filament-derived embryogenic calli of 'Shinonome' as a target material for transformation (Figure 1A). Maintain them by monthly subculturing to TrCPM at 25°C in the dark. Don’t use the calli from old cultures in order to avoid the regeneration of somaclonal variants.

2. Use *A. tumefaciens* EHA101/pIG121Hm for transformation. Pre-culture *Agrobacterium* as in the case of *A. prae cox* ssp. *orientalis*. Collect *Agrobacterium* cells by centrifugation (2000 × g for 10 min), and re-suspend them to a final OD600 of 0.2 in liquid TrCPM containing 50 mg l\(^{-1}\) of AS (*Tricyrtis* inoculation medium; TrIM).

3. Blot the calli on sterile filter papers to remove excess culture medium, and then immerse into the bacterial...
suspension for 3 min, and blot again on sterile filter papers. Co-cultivate the calli for 7 days with Agrobacterium on TrCPM containing 20 mg l\(^{-1}\) AS (Tricyrtis co-cultivation medium; TrCM) at 25°C in the dark.

In our study, transient expression of the GUS gene in the co-cultivated calli was detected only in the presence of AS in TrIM and TrCM.

4. Transfer the co-cultivated calli to TrEIM containing 40 mg l\(^{-1}\) hygromycin and 300 mg l\(^{-1}\) cefotaxime (Tricyrtis embryo selection medium; TrESM), and incubate at 25°C under continuous illumination with white fluorescent lighting (ca. 35 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). Subculture them every 2 weeks to fresh TrESM under the same conditions.

In our study, most of the co-cultivated calli turned brown within 4 weeks on TrESM, hygromycin-resistant calli and somatic embryos started to develop thereafter (Figure 1B). The hygromycin-resistant calli and somatic embryos were obtained only when the calli were co-cultivated for 7 days in the presence of AS.

5. Pick up and transfer the hygromycin-resistant somatic embryos to TrEIM containing 20 mg l\(^{-1}\) hygromycin and 100 mg l\(^{-1}\) cefotaxime (Tricyrtis plantlet selection medium; TrPSM), and incubate at 25°C under continuous illumination. Transfer germinated somatic embryos to TrEIM, and incubate under the same conditions.

In our study, more than 90% of somatic embryos germinated (Figure 1C) within 4 weeks on TrPSM. Growth of the germinated somatic embryos was promoted on TrEIM (Figure 1D).

6. Acclimatize and transplant the transgenic plantlets with the same method as non-transgenic, embryogenic callus-derived plantlets of Tricyrtis sp. Cultivate transgenic plants in a growth chamber.

In our study, the transgenic plantlets were readily transplanted to the growth chamber and produced flowers within 1 year after regeneration. When the calli from 6-month-old cultures were used for transformation, no apparent morphological alterations were observed in the transgenic plants at the flowering stage (unpublished). Stable expression of the GUS reporter gene was observed even after 2 years of cultivation of the transgenic plants (Mori et al. 2008).

By using this system, transgenic plants were also obtained efficiently in T. hirta, T. hirta var. albescens and T. formosana. No apparent genotypic difference in the transformation efficiency was observed (unpublished).

**Conclusion and prospects**

From a series studies, we found the following critical factors for in vitro plant regeneration and Agrobacterium-mediated transformation in liliaceous ornamental plants (Cronquist system). (1) Regeneration via callus cultures is more efficient than regeneration from cultured organs.
Regenerable calli are induced from young leaves, roots, bulb-scales, tepals and/or filaments on auxin (2,4-D, NAA and/or PIC)-containing media. (3) Depending on the plant species and kind of auxins, organogenic or embryogenic calli are induced. Organogenic calli produce adventitious shoots on PGR-free or BA-containing media. On the other hand, embryogenic calli produce somatic embryos on PGR-free media. (4) For avoiding regeneration of somaclonal variants, newly established callus cultures should be used. (5) Transgenic plants are obtained by using regenerable calli as a target material for Agrobacterium inoculation. (6) Transformation efficiency is much increased by adding AS in inoculation and co-cultivation media. (7) Transgenic tissues and plantlets are efficiently selected on hygromycin-containing media. By examining these factors, we have established plant regeneration systems on hygromycin-containing media. By examining these factors, we have established plant regeneration systems in *Agapanthus*, *Hemerocallis*, *Hosta*, *Lilium*, *Muscaria* and *Tricyrtis*, and Agrobacterium-mediated transformation systems in *Agapanthus*, *Lilium*, *Muscaria* and *Tricyrtis*. However, neither efficient plant regeneration nor genetic transformation has succeeded in *Tulipa* yet. Drastic modification of culture conditions and/or searching non-recalcitrant genotypes are necessary for *Tulipa*. Recently in planta transformation systems have been developed and applied to many plant species (Niazian et al. 2017). Since transgenic plants can be obtained without tissue culture processes by in planta transformation, the applicability of in planta transformation should be examined in liliaceous ornamental plants including *Tulipa*.

When comparing the transformation efficiency among 4 genera, *Tricyrtis* showed a much higher efficiency than *Agapanthus*, *Lilium* and *Muscaria*. Under the optimum conditions, about 10 independent transgenic plants could be obtained from 1 g FW of co-cultivated calli for *Tricyrtis* ‘Shinonome’, whereas about 15 independent transgenic plants from 3 g FW of co-cultivated calli for *A. praecox* ssp. *orientalis* ‘Royal Purple Select’, 5 to 10 independent transgenic plants from 200 co-cultivated callus clumps (50 to 80 g FW of calli before the scratching treatment) for *Lilium* ‘Acapulco’, and about 30 independent transgenic plants from 5 g FW of co-cultivated calli for *M. armeniacum* ‘Blue Pearl’. In addition, *Tricyrtis* has several advantages for transformation studies as follows: no requirement for special techniques for producing transgenic plants, relatively small plant size, ease of cultivation, and taking only one year from in vitro regeneration to flowering. For these advantages, we have been using *Tricyrtis* ssp. as liliaceous model plants for basic and applied researches for over 10 years. Transgenic *Tricyrtis* ssp. plants with altered flower forms (Nakano et al. 2007), altered flower colors (Kamiishi et al. 2012), and altered plant form (Otani et al. 2013, 2014a, 2014b) have already been produced. Wide hybrid plants have also been produced via ovule culture between transgenic and non-transgenic plants in *Tricyrtis* (Otani et al. 2019). Furthermore, a direct evidence supporting the modified ABCE model for the molecular mechanism of two-layered petaloid tepal development has successfully been obtained by suppression of B function in transgenic *Tricyrtis* sp. (Otani et al. 2016). We are now investigating the molecular mechanisms for determining the inflorescence architecture and for regulating tepal spot formation using the genetic transformation system of *Tricyrtis* described here.

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