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Agrobacterium-Mediated Transformation in Liliaceous Ornamental Plants

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Abstract

Studies on *Agrobacterium*-mediated transformation in 3 Liliaceous ornamental plants, *Lilium formosanum*, *Agapanthus praecox* ssp. *orientalis* and *Muscari armeniacum*, were described. Three different strains of *A. tumefaciens* were used, all of which harbored the binary vector carrying the *nptII*, *hpt* and *gus-intron* genes in the T-DNA region. For *L. formosanum*, no transgenic tissues nor plants were obtained after co-cultivation of organogenic calli with *A. tumefaciens*, although transient expression of the *gus* gene could be detected in the calli during co-cultivation. On the other hand, several hygromycin-resistant (Hyg^r) cell clusters were obtained for both *A. praecox* ssp. *orientalis* and *M. armeniacum* following the transfer of co-cultivated embryogenic calli onto hygromycin (Hyg)-containing media. Hyg^r calli developed into complete plants via somatic embryogenesis, and most of them were confirmed to be transgenic plants based on GUS histochemical assay and PCR analysis. Southern blot analysis revealed the integration of 1 to 5 copies of the transgene into the genome of the transgenic plants of both 2 species, but most of them had 1 or 2 copies. *Agrobacterium*-mediated transformation systems developed for *A. praecox* ssp. *orientalis* and *M. armeniacum* may be useful as a tool for their genetic improvement as well as molecular biology studies.

Discipline: Biotechnology / Horticulture / Plant breeding
Additional key words: agapanthus, embryogenic callus, lily, muscari, transgenic plant

Introduction

The family Liliaceae contains many commercially important ornamental plants, such as *Lilium* spp., *Tulipa gesneriana*, *Muscari armeniacum*, *Hemerocallis hybrida* and *Agapanthus praecox*. Breeding of these plants has so far been carried out by sexual hybridization and sport selection[1,15], but available genetic information in traditional breeding is limited. Recently, genetic transformation has started to be applied for plant breeding in order to overcome some limitations of the traditional breeding methods. The use of genetic transformation paves the way for the use of a wide range of beneficial genes, which are not restricted to a given and related plant species, but may be obtained from any organisms[3,4]. In addition, this method allows the introduction of only desirable traits to pre-existing and desirable genotypes within a short period[1,15]. In the Liliaceous ornamental plants, genetic transformation is also expected to be applied in combination with traditional breeding methods for genetic improvement of ornamental as well as marketable qualities.

Among the genetic transformation techniques, *Agrobacterium*-mediated transformation offers the following advantages: no requirement for special equipment and techniques, no requirement for protoplast culture systems, defined integration of transgenes, potentially low copy number, and preferential integration into transcriptionally active regions of the chromosome[7,9]. For a long period of time, however, since monocotyledonous plants such as Liliaceous ornamentals had not been included in the host range of *Agrobacterium*, their genetic transformation had been carried out exclusively via direct gene transfer. However, substantive confirmation is now accumulating that stably transformed monocotyledonous plants can be produced via *Agrobacterium*-mediated gene transfer[1,7].

In the Liliaceous ornamental plants, *Agrobacterium*-mediated production of transgenic plants has not yet been demonstrated, although only one report on particle bombardment-mediated production of transgenic plants had been published for *Lilium longiflorum*[26]. However, successful application of *Agrobacterium*-mediated transfor-

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mation has recently been reported for some Liliaceous vegetable plants including *Asparagus officinalis*[8], *Allium sativum*[10] and *Allium cepa*[5], indicating that *Agrobacterium*-mediated transformation may be applicable to Liliaceous ornamental plants. Therefore, we examined the development of an efficient and reproducible system for producing transgenic plants via *Agrobacterium*-mediated transformation in several Liliaceous ornamentals.

Plant materials and *Agrobacterium tumefaciens* strains

Three Liliaceous ornamental plants, *Lilium formosanum* Wallace, *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton 'Royal Purple Select' and *Muscari armeniacum* Leichtl. ex Bak. 'Blue Pearl' were used. For all 3 species, we used regenerable calli as a target material for *Agrobacterium*-mediated transformation. Bulb scale-derived organogenic calli of *L. formosanum*[16] and leaf-derived embryogenic calli of *A. praecox* ssp. *orientalis*[21] were maintained on callus proliferation media containing 1 mg L^{-1} picloram (PIC), and leaf-derived embryogenic calli of *M. armeniacum*[19] were maintained on a callus proliferation medium containing 10 mg L^{-1} α-naphthaleneacetic acid (NAA). These calli regenerated numerous plantlets via shoot organogenesis (for *L. formosanum*) or somatic embryogenesis (for *A. praecox* ssp. *orientalis* and *M. armeniacum*) upon transfer to regeneration media lacking plant growth regulators (PGRs). All the callus proliferation and regeneration media used were based on MS medium[12] and were solidified with gellan gum.

Three strains of *A. tumefaciens*, EHA101/pIG121Hm, LBA4404/pIG121Hm and LBA4404/pTOK233 were used in this study. All the strains harbored the binary vector carrying the genes for kanamycin resistance (*nptII*), hygromycin resistance (*hpt*) and β-glucuronidase (*gus-intron*) in the T-DNA region. Prior to co-cultivation, each bacterial strain was inoculated into liquid YEP medium containing appropriate antibiotics and incubated for more than 24 h with reciprocal shaking. Bacterial cells were collected by centrifugation and resuspended to a final OD_{600} of 0.2 into liquid inoculation media.

Co-cultivation and transient expression of the *gus* gene in *Lilium formosanum*[22]

Agrobacterium cells were resuspended into MS-based, liquid inoculation media with or without 10, 20, 50, 100 or 200 mg L^{-1} of acetosyringone (AS). Organogenic calli of *L. formosanum* were immersed into the inoculation medium containing bacterial cells for 1, 5, 30 or 60 min, and blotted on sterile filter papers. The calli were then transferred and incubated onto MS-based, co-cultivation medium containing 20 mg L^{-1} AS and solidified with gellan gum.

Histochemical assay for β-glucuronidase (GUS) was carried out every day for 5 days after the initiation of co-cultivation, but cells displaying the *gus* gene expression were never observed on the calli from any inoculation and co-cultivation treatments. However, when the co-cultivation period was extended to 15 to 30 days, some of the calli showed several blue spots, which may have resulted from transient or stable expression of the *gus* gene (Fig. 1A). Such blue spots were observed on the calli co-cultivated with *A. tumefaciens* strains EHA101/pIG121Hm or LBA4404/pTOK233 in the presence of 20 mg L^{-1} or more of AS; in these treatments, 1 to 8 spots were generally observed per 0.1 g fresh weight (FW) of calli. Although no overgrowth of *Agrobacterium* was observed even 30 days after co-cultivation, the calli frequently became brown with the increase of the duration of co-cultivation. However, blue spots were also detected in the calli which showed partial browning (Fig. 1B). There was no apparent relationship between the number and size of the blue spots and various inoculation and co-cultivation treatments.

For selecting transformed tissues, the calli co-cultivated for 15 to 30 days were transferred and successively subcultured onto the callus proliferation medium containing 50 mg L^{-1} Hyg and 500 mg L^{-1} carbenicillin. However, the surviving calli failed to exhibit the *gus* gene expression 4 weeks after transfer. In addition, all of the calli became brown and died by 6 weeks after transfer, presumably due to minimal or the absence of integration of T-DNA into the *L. formosanum* genome.

Regeneration of transgenic plants in *Agapanthus praecox* ssp. *orientalis*[23]

Agrobacterium cells were resuspended into MS-based, liquid inoculation media containing 1 mg L^{-1} PIC with or without 20 mg L^{-1} AS. Embryogenic calli of *A. praecox* ssp. *orientalis* (Fig. 2A) were immersed into the inoculation medium containing bacterial cells for 1 min and blotted on sterile filter papers. They were then transferred and incubated onto the co-cultivation medium which was the same as the inoculation medium but was solidified with gellan gum.

The calli were subjected to the GUS assay every day for 10 days after the initiation of co-cultivation. In the absence of AS, no blue spots were detected in the calli throughout the 10-day co-cultivation period regardless of

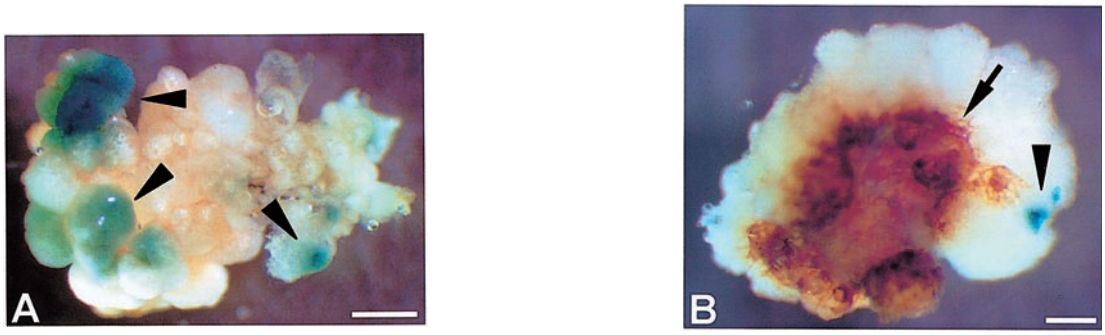

Fig. 1. *Lilium formosanum* calli showing transient expression of the *gus* gene (arrow heads) following co-cultivation with *Agrobacterium tumefaciens* strain EHA101/pIG121Hm

A: Callus clump which was immersed into the bacterial suspension containing 20 mg L^{-1} AS for 5 min and co-cultivated for 27 days. Bar = 1 mm. B: Callus clump with partial browning (arrow) which was immersed into the bacterial suspension containing 200 mg L^{-1} AS for 60 min and co-cultivated for 30 days. Bar = 0.5 mm.

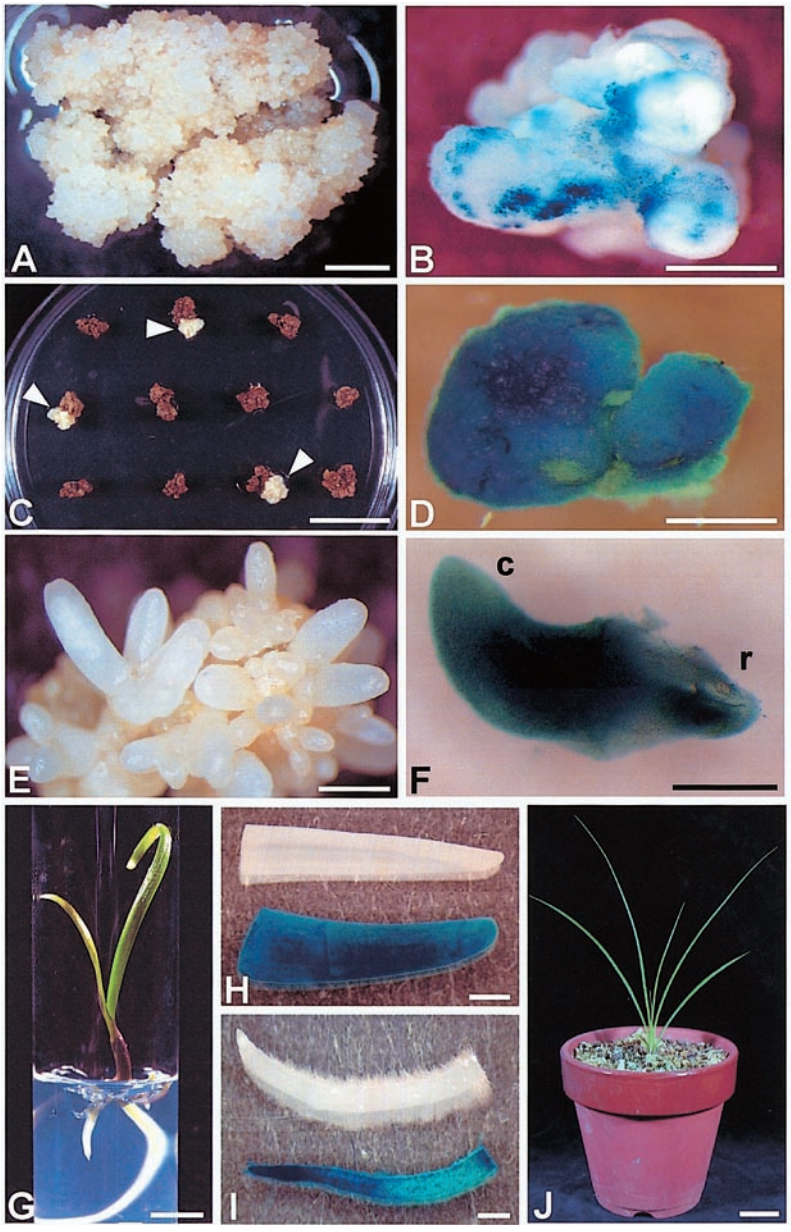

Fig. 2. Production of transgenic plants of *Agapanthus praecox* ssp. *orientalis* via *Agrobacterium*-mediated transformation of embryogenic calli

A: Embryogenic calli used for co-cultivation with *A. tumefaciens*. Bar = 2 mm. B: Embryogenic callus clump showing transient expression of the *gus* gene after 7 days of co-cultivation with *A. tumefaciens* strain EHA101/pIG121Hm in the presence of AS. Bar = 2 mm. C: Hygromycin-resistant (Hyg^r) cell clusters (arrow heads) developing on the selection medium containing 50 mg L^{-1} hygromycin. Bar = 20 mm. D: Hyg^r callus clump showing stable expression of the *gus* gene. Bar = 2 mm. E: Numerous somatic embryos developed from Hyg^r calli on a PGR-free medium containing 50 mg L^{-1} hygromycin. Bar = 2 mm. F: Somatic embryo showing stable expression of the *gus* gene. c, cotyledon; r, radicle. Bar = 2 mm. G: Plantlet regenerated from Hyg^r calli. Bar = 10 mm. H and I: GUS histochemical assay of leaf (H) and root (I) segments of transgenic (lower) and non-transformed, control (upper) plantlets. Bar = 2 mm. J: Transgenic plant established in the greenhouse. Bar = 50 mm.

Table 1. Effect of *Agrobacterium* strains on the transformation efficiency of *Agapanthus praecox* ssp. *orientalis*[a)]

A. tumefaciens strains	AS[b)]	No. of Hyg^r callus lines (A)	No. of Hyg^r and GUS$^+$ callus lines (B)	No. of Hyg^r and GUS$^+$ callus lines forming plantlets (C)	No. of Hyg^r and GUS$^+$ callus lines forming GUS$^+$ plantlets (D)	No. of transformed callus lines confirmed by PCR (E)[c)]	Frequency (%)			
							B/A	C/A	D/A	E/A
EHA101/pIG121Hm	–	0	0	0	0	0	0	0	0	0
	+	2	2	2	2	2	100	100	100	100
LBA4404/pIG121Hm	–	0	0	0	0	0	0	0	0	0
	+	0	0	0	0	0	0	0	0	0
LBA4404/pTOK233	–	0	0	0	0	0	0	0	0	0
	+	18	17	14	14	15	94.4	77.8	77.8	83.3

a): In total, 3g FW of embryogenic calli were used for co-cultivation with each *Agrobacterium* strain for 7 days.
b): – and + denote the absence and presence of 20 mg L^{-1} AS in the bacterial suspension and co-cultivation medium, respectively.
c): PCR analysis for the *gus* and *hpt* genes.

the kind of *Agrobacterium* strain. On the other hand, in the presence of AS, blue spots started to appear 3 days after the onset of co-cultivation for all the *Agrobacterium* strains (Fig. 2B). The number of blue spots gradually increased until 7 to 8 days after co-cultivation, when up to 20, 50 and 60 spots per 0.1 g FW of calli were obtained for the *A. tumefaciens* strains LBA4404/pIG121Hm, EHA101/pIG121Hm and LBA4404/pTOK233, respectively. *A. tumefaciens* strain LBA4404/pTOK233 always showed higher numbers of blue spots than the other 2 strains throughout the 10-day co-cultivation period.

After 3, 7 or 10 days of co-cultivation, the calli were transferred and successively subcultured onto the callus proliferation medium containing 50 mg L^{-1} Hyg and 500 mg L^{-1} cefotaxime for selecting transformed tissues[21]. In the absence of AS, all of the co-cultivated calli died on the selection medium regardless of the kind of *Agrobacterium* strain and duration of the co-cultivation period. On the other hand, in the presence of AS, most of the co-cultivated calli also turned brown within 4 to 5 weeks on the selection medium, but creamy-white cell clusters started to develop thereafter (Fig. 2C, Table 1). Such Hyg^r calli were obtained only from embryogenic calli co-cultivated for 7 days in the presence of AS for both *Agrobacterium* strains EHA101/pIG121Hm and LBA4404/pTOK233. Almost all of the Hyg^r callus lines showed a stable expression of the *gus* gene (GUS-positive, GUS$^+$) (Fig. 2D).

All of the Hyg^r callus lines were then transferred to the regeneration medium containing 50 mg L^{-1} Hyg and 500 mg L^{-1} cefotaxime, on which several lines devel-

Table 2. Distribution of the copy number of the transgene in transgenic plants of *Agapanthus praecox* ssp. *orientalis*[a)] and *Muscari armeniacum*[b)] as shown by Southern blot analysis with *hpt* probe

Species	Copy No.	No. of transgenic lines (%)			
		EHA101/pIG121Hm	LBA4404/pIG121Hm	LBA4404/pTOK233	Total
Agapanthus praecox ssp.	1	2 (100)	–	3 (60.0)	5 (71.4)
orientalis	2	0	–	2 (40.0)	2 (28.6)
Muscari armeniacum	1	10 (47.6)	4 (50.0)	8 (40.0)	22 (44.9)
	2	9 (42.8)	3 (37.5)	8 (40.0)	20 (40.9)
	3	0	1 (12.5)	2 (10.0)	3 (6.2)
	4	1 (4.8)	0	1 (5.0)	2 (4.0)
	5	1 (4.8)	0	1 (5.0)	2 (4.0)

a): Analysis of the copy number was performed on 2 and 5 Hyg^r callus lines, which were derived from co-cultivation with *A. tumefaciens* strains EHA101/pIG121Hm and LBA4404/pTOK233, respectively.
b): Analysis of the copy number was performed on 21, 8 and 20 Hyg^r callus lines, which were derived from co-cultivation with *A. tumefaciens* strains EHA101/pIG121Hm, LBA4404/pIG121Hm and LBA4404/pTOK233, respectively.

oped numerous somatic embryos (Fig. 2E). One Hyg^r callus line lacking a stable *gus* gene expression (GUS-negative, GUS$^-$) obtained with the *A. tumefaciens* strain LBA4404/pTOK233 (Table 1) also produced somatic embryos. All of the somatic embryos developed from the Hyg^r-GUS$^+$ callus lines showed a stable *gus* gene expression (Fig. 2F), whereas those from the Hyg^r-GUS$^-$ callus line failed to show the *gus* gene expression (Table 1). More than 90% of the Hyg^r embryos developed into plantlets (Fig. 2G). The plantlets from all of the Hyg^r-GUS$^+$ callus lines showed a stable expression of the *gus* gene in leaves and roots (Fig. 2H, I), whereas stable expression could not be detected in those from the Hyg^r-GUS$^-$ line.

PCR analyses for the *gus* and *hpt* genes allowed the detection of both genes in plantlets from all of the Hyg^r callus lines, although no expression of the *gus* gene had been detected in one line. Southern blot analysis using the *hpt* gene probe was carried out on the plantlets from 7 Hyg^r callus lines: 2 lines transformed with the *A. tumefaciens* strain EHA101/pIG121Hm contained a single copy of the transgene, and 5 lines transformed with the *A. tumefaciens* strain LBA4404/pTOK233 contained a single copy (3/5) or two copies (2/5) of the transgene (Table 2).

The plantlets from all of the Hyg^r callus lines were successfully acclimatized and transferred to the greenhouse (Fig. 2J). At least at early stages of growth, all of them exhibited a normal phenotype in terms of leaf shape and growth habit.

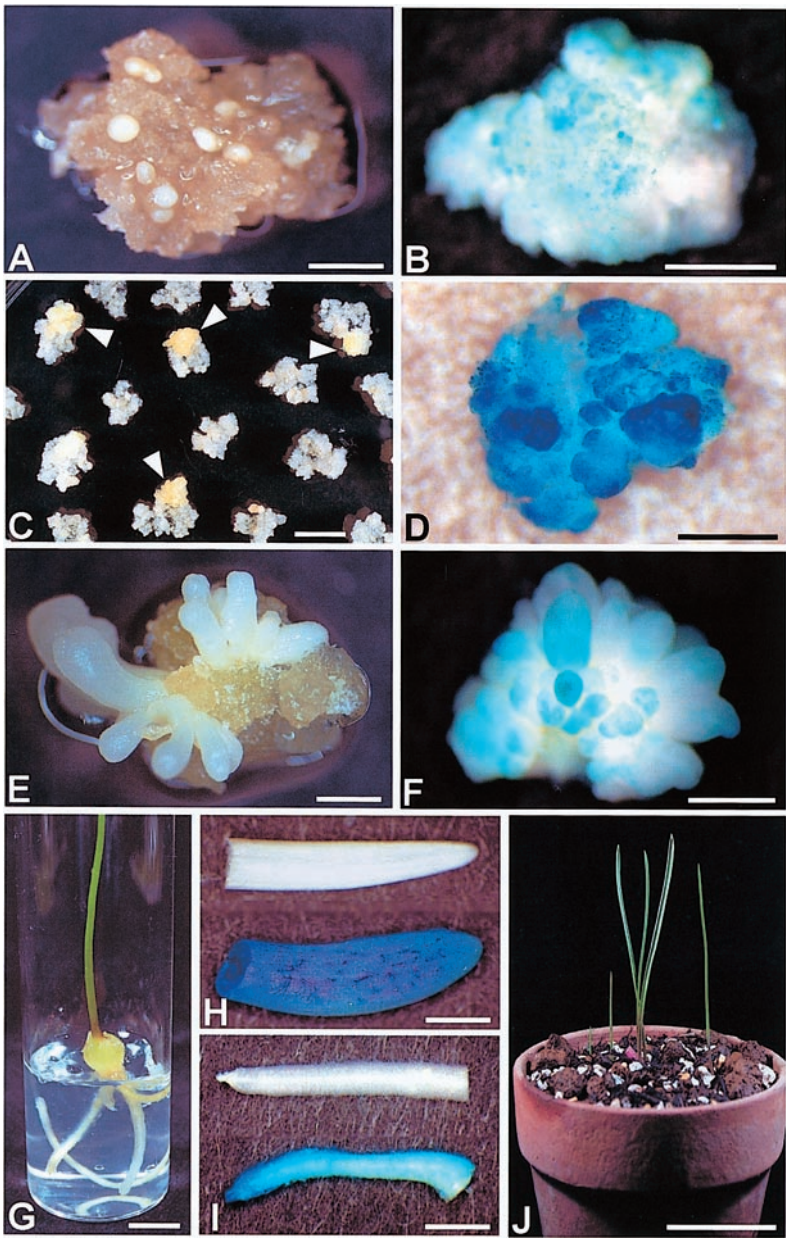

Fig. 3. Production of transgenic plants of *Muscari armeniacum* via *Agrobacterium*-mediated transformation of embryogenic calli

A: Embryogenic calli used for co-cultivation with *A. tumefaciens*. Bar = 2 mm. B: Embryogenic cell cluster showing transient expression of the *gus* gene after 3 days of co-cultivation with *A. tumefaciens* strain EHA101/pIG121Hm in the presence of 0.1% Tween20 and AS. Bar = 2 mm. C: Hyg^r cell clusters (arrow heads) developed on the selection medium. Bar = 5 mm. D: Hyg^r cell cluster showing *gus* gene expression. Bar = 2 mm. E: Somatic embryos developed from a Hyg^r callus line. Bar = 2 mm. F: Somatic embryos showing *gus* gene expression. Bar = 2 mm. G: Plantlet regenerated from a Hyg^r callus line. Bar = 10 mm. H and I: GUS histochemical assay of leaf (H) and root (I) segments of transgenic (lower) and the non-transformed, control (upper) plantlets. Bar = 2 mm. J: Transgenic plant established in the greenhouse. Bar = 50 mm.

Regeneration of transgenic plants in *Muscari armeniacum*[18]

Agrobacterium cells were resuspended into MS-based, liquid inoculation media with or without 20 mg L^{-1} AS. In some of the experiments, 0.01, 0.1 or 1% of a surfactant, Tween20 was added to the inoculation medium. The inoculation medium containing bacterial cells was gently dropped onto the embryogenic calli of *M. armeniacum* on sterile filter papers. The calli were then transferred onto the callus proliferation media with or without 20 mg L^{-1} AS and co-cultivated for 3 days (Fig. 3A).

After the co-cultivation, the calli were subjected to the GUS assay. For all of the 3 *A. tumefaciens* strains, the addition of AS to both inoculation and co-cultivation media increased the efficiency of transient expression of the *gus* gene; especially, more than 4-fold higher numbers of blue spots (60 spots per 0.1 g FW of calli) were observed in the presence of AS for the *A. tumefaciens* strains EHA101/pIG121Hm and LBA4404/pTOK233 compared with the absence of AS. In the presence of AS, *A. tumefaciens* strain EHA101/pIG121Hm gave the highest number of blue spots (Fig. 3B). The presence of Tween20 in the inoculation medium increased the number of blue spots at all of the concentrations tested, among which 0.1% Tween20 gave the highest number of blue spots, more than 2-fold higher than the control without Tween20.

The calli co-cultivated for 3 days were cultured on the callus proliferation medium containing 500 mg L^{-1} cefotaxime. Seven days later, they were transferred and successively subcultured onto the same medium but with 75 mg L^{-1} Hyg for selecting transformed tissues[20]. Four to 5 weeks following the initiation of selection, Hyg^r cell clusters started to develop among the embryogenic calli that had undergone browning (Fig. 3C). Production of such Hyg^r embryogenic calli was affected by the kind of *Agrobacterium* strain and AS treatment (Table 3). Although some Hyg^r calli were obtained even in the absence of AS for the 3 *Agrobacterium* strains, the number of Hyg^r callus lines markedly increased by the addition of AS. In the presence of AS, *A. tumefaciens* strain EHA101/pIG121Hm produced the highest number of Hyg^r callus lines. More than 87% of the Hyg^r callus lines were GUS^+ regardless of the kind of *Agrobacterium* strain and the addition of AS (Fig. 3D).

After transfer to the regeneration medium containing 25 mg L^{-1} Hyg and 500 mg L^{-1} cefotaxime, almost all of the Hyg^r callus lines developed numerous somatic embryos (Fig. 3E), whereas some lines failed to produce embryos (Table 3). All of the somatic embryos developed from the Hyg^r-GUS^+ callus lines also exhibited the *gus* gene expression (Fig. 3F). More than 85% of the Hyg^r embryos developed into plantlets (Fig. 3G). The plantlets obtained from all of the Hyg^r-GUS^+ callus lines exhibited the *gus* gene expression in both leaves and roots (Fig. 3H, I), whereas no *gus* gene expression could be detected in those from the Hyg^r-GUS^- lines.

Both *gus* and *hpt* genes were detected by PCR analysis in plantlets from all of the Hyg^r callus lines, although no expression of the *gus* gene had been detected in 4 lines. Southern blot analysis using the *hpt* gene probe was carried out on the plantlets from 49 Hyg^r callus lines (Fig. 4) : a single copy of the transgene was carried by 45% of the plants (22/49), and the percentages of

Table 3. Effect of *Agrobacterium* strains on the transformation efficiency of *Muscari armeniacum*[a)]

A. tumefaciens strains	AS[b)]	No. of Hyg^r callus lines (A)	No. of Hyg^r and GUS^+ callus lines (B)	No. of Hyg^r and GUS^+ callus lines forming plantlets (C)	No. of Hyg^r and GUS^+ callus lines forming GUS^+ plantlets (D)	No. of transformed callus lines confirmed by PCR (E)[c)]	Frequency (%)			
							B/A	C/A	D/A	E/A
EHA101/pIG121Hm	–	8	7	7	7	8	87.5	87.5	87.5	100
	+	36	34	32	32	33	94.4	88.9	88.9	91.7
LBA4404/pIG121Hm	–	4	4	3	3	3	100	75.0	75.0	75.0
	+	10	9	9	8	9	90.0	90.0	80.0	90.0
LBA4404/pTOK233	–	9	9	8	8	8	100	88.9	88.9	88.9
	+	31	30	28	26	27	96.8	77.8	77.8	83.3

a): Embryogenic calli were co-cultivated with each *Agrobacterium* strain for 3days in the presence of 0.1% Tween20 in the bacterial suspension medium. In total, 5g FW of embryogenic calli were used for each treatment.
b): – and + denote the absence and presence of 20 mg L^{-1} AS in the bacterial suspension and co-cultivation medium, respectively.
c): PCR analysis for the *gus* and *hpt* genes.

lines containing 2, 3, 4 and 5 copies were 41% (20/49), 6% (3/49), 8% (2/49) and 8% (2/49), respectively (data were pooled for all of the *Agrobacterium* strains) (Table 2). Two plantlets containing 5 copies of the transgene were derived from co-cultivation with each of the *Agrobacterium* strains EHA101/pIG121Hm and LBA4404/pTOK233 (Table 2).

The plantlets regenerated from all of the Hyg^r callus lines were successfully transplanted to the greenhouse (Fig. 3J). No apparent phenotypic alterations were observed at least at early stages of growth.

Conclusion and prospects

We have succeeded in the production of transgenic plants via *Agrobacterium*-mediated transformation of embryogenic calli in *A. praecox* ssp. *orientalis* and *M. armeniacum*: in the former species, the highest transformation efficiency was obtained when the calli were co-cultivated with the *A. tumefaciens* strain LBA4404/pTOK233 for 7 days in the presence of 20 mg L^{-1} AS, whereas in the latter species, the highest efficiency was obtained when the calli were inoculated with the *A. tumefaciens* strain EHA101/pIG121Hm in the presence of both 20 mg L^{-1} AS and 0.1% of a surfactant Tween20 followed by co-cultivation for 3 days in the presence of 20 mg L^{-1} AS. Although neither transformed tissues nor plants could be obtained for *L. formosanum*, we showed the possibility of *Agrobacterium*-mediated transformation of this species through the detection of transient expression of the *gus* gene in co-cultivated calli.

Efficient production of transgenic plants via *Agrobacterium*-mediated transformation in the Liliaceous ornamentals was affected by several factors such as target material for *Agrobacterium* inoculation, kind of *Agrobacterium* strain, duration of the co-cultivation period, and surfactant (Tween20) and AS treatment during co-cultivation[18,22,23], among which the target material and AS treatment appeared to be critical. For both *A. praecox* ssp. *orientalis* and *M. armeniacum*, transgenic plants were produced via transformation of embryogenic calli, whereas our preliminary experiments indicated that neither transformed tissues nor plants could be obtained from any organized tissues. Embryogenic calli have generally been found to have high proliferation and regeneration abilities[25] and have been successfully utilized as a target material for *Agrobacterium*-mediated production of transgenic plants in both monocotyledonous[3] and dicotyledonous plant species[13]. For *L. formosanum*, no transgenic plants could be obtained following co-cultivation of organogenic calli with *Agrobacterium*, which may partly be due to the apparently slower growth of these calli than that of the embryogenic calli from the other 2 species. Low cell division activities in the target calli may result in an inefficient integration of T-DNAs as suggested by Gheysen et al.[6] and Potrykus[14]. In this case, further experiments should be conducted for the production of transgenic *L. formosanum* plants to prepare other target materials with higher proliferation abilities as well as to examine the culture conditions for increasing the proliferation activity of the materials.

For *A. praecox* ssp. *orientalis*, neither transient expression of the *gus* gene nor stable transformants could be obtained without AS treatment, indicating that AS

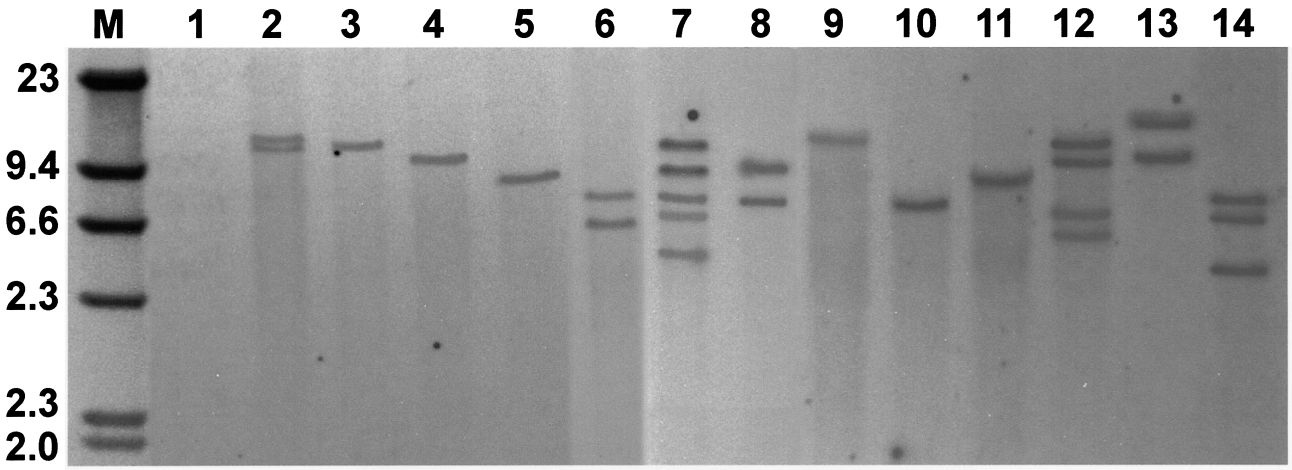

Fig. 4. Southern blot analysis of transgenic plantlets of *Muscari armeniacum*

*Hin*dIII-digested DNA samples were hybridized with *hpt* probe. Lane M: λ/*Hin*dIII DNA as molecular markers. Lane 1: non-transformed, control plantlet. Lanes 2–7: transgenic plantlets obtained with *Agrobacterium tumefaciens* strain EHA101/pIG121Hm. Lanes 8–9: transgenic plantlets obtained with *A. tumefaciens* strain LBA4404/pIG121Hm. Lanes 10–14: transgenic plantlets obtained with *A. tumefaciens* strain LBA4404/pTOK233. Numeral indicates kbp.

treatment is essential for *Agrobacterium*-mediated transformation. On the other hand, for *M. armeniacum*, the AS treatment drastically increased the transformation efficiency, although both transient expression of the *gus* gene and transgenic plants could be obtained in the absence of AS. AS is a phenolic compound activating the *vir* genes of *Agrobacterium*, and production of AS has been demonstrated to be low or lacking in monocotyledonous plant tissues[24]. Therefore, AS treatment has so far been found to be effective for *Agrobacterium*-mediated transformation of several monocotyledonous species[7]. The effectiveness of the AS treatment was also confirmed for 2 Liliaceous ornamental plants.

Compared to direct DNA transfer techniques, *Agrobacterium*-mediated transformation generally offers potentially a low copy number of the transgene[2,7]. A large part of the transgenic plants obtained for *A. praecox* ssp. *orientalis* and *M. armeniacum* also had 1 or 2 copies of the transgene. It had generally been recognized that multiple copies of the transgene with tandem or inverted repeats can easily induce DNA methylation and can trigger homology-dependent gene silencing[11,17]. Therefore the predominantly low copy numbers obtained in the present study may be advantageous for stable expression of the transgene. Since several callus lines, which were resistant to hygromycin but did not express the *gus* gene (Hyg^r-GUS^-), were obtained for both species, further experiments should be carried out to clarify the correlation between the transgene copy number and the activity of transgene expression in the Liliaceous ornamental plants.

We have established efficient systems for *Agrobacterium*-mediated production of transgenic plants for 2 Liliaceous ornamental plants, *A. praecox* ssp. *orientalis* and *M. armeniacum*. Although, in both species, at least at the early stages of growth, all the transgenic plants exhibited the same phenotype as the non-transformed, control plants, their detailed characterization as well as the stability of the introduced genes in their progeny and vegetatively-propagated materials is needed for the practical application of this system for genetic improvement. Hereafter, several valuable genes for herbicide tolerance, resistance to diseases, insects or viruses, or alteration of flower color or plant form should be introduced by using the established systems into *A. praecox* ssp. *orientalis* and *M. armeniacum* for their genetic improvement. In addition, these systems should be utilized as a tool for plant molecular biology such as the identification and function analysis of genes via T-DNA insertional mutagenesis as well as enhancer, promoter and gene trapping.

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