Plant regeneration from suspension cells induced from hypocotyls derived from interspecific cross *Alstroemeria pelegrina* × *A. magenta* and transformation with *Agrobacterium tumefaciens*

Yoichiro HOSHINO [1)2)*], Yukiko KASHIHARA [3)], Tomonari HIRANO [2)], Naho MURATA [4)] and Koichi SHINODA [4)]

1) Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, JAPAN
2) Division of Innovative Research, Creative Research Initiative 'Sousei' (CRIS), Hokkaido University, Kita 21, Nishi 10, Kita-Ku, Sapporo 001-0021, JAPAN
3) Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 10, Nishi 5, Kita-Ku, Sapporo 060-0810, JAPAN
4) National Agricultural Research Center for Hokkaido Region, Hitsujigaoka 1, Toyohira-Ku, Sapporo 062-8555, JAPAN

*Correspondence: Yoichiro HOSHINO
Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, JAPAN
Telephone number: +81-11-706-2857 FAX number: +81-11-706-2857
e-mail: hoshino@exfarm.agr.hokudai.ac.jp

Key words: Alstroemeriaceae · Callus · Organogenesis · Ovule culture · Transgenic plants

Abstract

Embryogenic cell suspension cultures were established using the ovule culture of an interspecific cross, *Alstroemeria pelegrina* var. *rosea* × *A. magenta*. Ovules harvested 14 d after pollination were cultured on Murashige and Skoog (MS) medium without plant growth regulators (PGRs); calli were produced on the hypocotyl surface in germinating zygotic embryos. Suspension cells were induced from the calli by using liquid MS media containing 2,4-dichlorophenoxyacetic acid or 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram). Adventitious embryos developed from the suspension cells on half-strength MS medium supplemented with 0.5 mg l^{-1} of both α-naphthaleneacetic acid and N^6-benzylaminopurine; they grew into

plantlets on the same medium. The plantlets formed rhizomes following transfer to half-strength MS medium without PGRs, and acclimatized plants were easily established. Subsequently, *Agrobacterium*-mediated transformation system was applied. The suspension cells were co-cultivated with *A. tumefaciens* strain EHA101/pIG121Hm or LBA4404/pTOK233, both of which contain neomycin phosphotransferase II, hygromycin phosphotransferase and intron-containing ß-glucuronidase (intron-*GUS*) genes. Seven days after co-cultivation, the cells were subjected to GUS assay; staining was most pronounced in the cells subcultured in a picloram-containing liquid medium and co-cultivated with EHA101/pIG121Hm. The co-cultivated cells were transferred to the MS medium containing picloram and 20 mg l^{-1} hygromycin; 1 month later, several hygromycin-resistant callus lines showing GUS activity were obtained. Transgenic plants were obtained through our plant regeneration system, and foreign gene insertion into the regenerated plants was confirmed by polymerase chain reaction.

Abbreviations

BAP	N^6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
HPT	Hygromycin phosphotransferase
intron–GUS	Intron–containing ß-glucuronidase
NAA	α-Naphthaleneacetic acid
NPTII	Neomycin phosphotransferase II
PGRs	Plant growth regulators
picloram	4-Amino-3,5,6-trichloropyridine-2-carboxylic acid
TDZ	Thidiazuron

Introduction

The genus *Alstroemeria* belongs to the family Alstroemeriaceae and includes many

ornamental species (Hoshino et al. 2006). This genus, which includes more than 60 species, originated in South America. Thus far, numerous cultivars of this genus, which are being used worldwide as cut flowers and potted plants, have been produced by interspecific hybridization and mutation breeding. For further improvements in *Alstroemeria* cultivars, biotechnology-based approaches are currently being attempted. Different explants and culture media have been examined for callus induction and plant regeneration. Mature embryo (Gonzalez-Benito and Alderson 1992; Hutchinson et al. 1994, 1997) and immature embryo (Van Schaik et al. 1996) were used for callus induction and subsequent plant regeneration. Moreover, leaf (Lin et al. 1997), leaf axil (Lin et al. 1998, 2000a), stem segment (Lin et al. 2000b), rhizome splitting (Chiari and Bridgen 2000), floral apices (Padraza-Santos et al. 2006) and nodes with axil tissue (Kim et al. 2006) were applied for plant regeneration system. For further application of biotechnology to *Alstroemeria* breeding programs, more efficient plant regeneration systems are required for use in this wide range of plant species.

In the present study, we established plant regeneration systems by using hypocotyls derived from an interspecific hybrid between *A. pelegrina* var. *rosea* and *A. magenta*. The objectives of this study were to establish reliable plant regeneration systems from callus cultures that are suitable for genetic transformation with *Agrobacterium tumefaciens*. In *Alstroemeria*, particle bombardment (Lin et al. 2000c) and *Agrobacterium*-mediated transformation (Kim et al. 2007) procedures have been applied for genetic transformation. In an attempt to demonstrate the application of transformation techniques to other strains and to enhance reproducibility, we showed that using the plant regeneration protocol established in this study, transgenic plants can be obtained by *Agrobacterium*-mediated transformation protocol.

Materials and Methods

Plant materials and callus induction

The ovaries derived from *A. pelegrina* var. *rosea* × *A. magenta* were harvested 14 d after pollination. These ovaries were surface sterilized with sodium hypochlorite solution (1% active chlorine) for 10 min and then washed 3 times with sterilized distilled water. They were cut longitudinally into 3 pieces with a razor blade, and the ovules were detached from the placenta. These ovules were cultured on 2 g l^{-1} gellan gum-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose. In addition to the ovules of *A. pelegrina* var. *rosea* × *A. magenta*, the ovules of *A. magenta*, *A. aurea*, *A. pelegrina* var. *rosea*, *A. pelegrina* var. *alba* and *A. ligtu* were harvested 14 d after pollination for callus induction, and subsequently their genotypes were compared.

Establishment of cell suspension cultures

The calli induced from germinated seedlings were detached and transferred to several combinations and concentrations of plant growth regulators (PGRs) for further proliferation. Three auxins—α-naphthaleneacetic acid (NAA), 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) and 2,4-dichlorophenoxyacetic acid (2,4-D)—and 2 cytokinins—N^6-benzylaminopurine (BAP) and thidiazuron (TDZ)—were tested individually and in combination. All media contained 30 g l^{-1} sucrose and were solidified with 2 g l^{-1} gellan gum.

Cell suspension cultures were induced from calli showing rapid growth on 2,4-D- or picloram-containing media in the above experiments. The calli cultured on 2,4-D- or

picloram-containing media were transferred to 100-ml Erlenmeyer flasks containing 40 ml of MS liquid medium supplemented with 2, 4-D or picloram, respectively. The cultures were maintained at 20 ± 1 °C under 24-h illumination (35 μmol $m^{-1}s^{-1}$) on a gyratory shaker at 100 cycle min^{-1}. Subcultures were performed every 14 d by transferring ca. 1 g fresh weight of cells to flasks containing 40 ml of fresh medium.

Plant regeneration

In order to examine organogenesis, cell suspension cultures were transferred onto half-strength MS media supplemented with NAA in combination with BAP or TDZ at several different concentrations. Regenerated plantlets formed on media containing the PGRs were transferred onto a PGR-free medium for inducing rhizome formation.

Regenerated plants with well-established root systems were washed carefully to remove the gellan gum and then transferred to pots containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20 ± 1 °C under 24-h illumination (45 μmol m^{-2} s^{-1}) with fluorescent lamps in the growth chambers. After 2–4 weeks, the acclimatized plants were transferred to the greenhouse.

Agrobacterium inoculation and GUS histochemical assay

Agrobacterium tumefaciens was inoculated as described by Hoshino et al. (1998). The suspension cells were co-cultivated for 5 d with *A. tumefaciens* strains EHA101/pIG121Hm or LBA4404/pTOK233. The *A. tumefaciens* strain LBA4404 /pTOK233 (Hiei et al. 1994) was provided by Japan Tobacco Inc. The plasmid pTOK233 encoded the inserted *virB*, *virC* and *virG* genes derived from the supervirulent Ti-plasmid pTiBo542 (Watson et al. 1975), which is known to enhance the

virulence of *A. tumefaciens* against plant cells (Jin et al. 1987). The T-DNA region of pTOK233 contained the neomycin phosphotransferase II (*NPTII*) gene under the control of nopaline synthase (NOS) promoter, the hygromycin phosphotransferase (*HPT*) gene under the control of cauliflower mosaic virus (CaMV) 35S promoter and the ß-glucuronidase (*GUS*) gene with an intron fused to the CaMV 35S promoter. Another *A. tumefaciens* strain used was EHA101 (Hood et al. 1986) that harboured the plasmid pIG121Hm (Ohta et al. 1990), which similar to pTOK233, contained the *NPTII*, *HPT* and *GUS* genes in the T-DNA region. The co-cultivated cells were transferred to the MS medium containing 1 mg l^{-1} picloram and 20 mg l^{-1} hygromycin. After 1 month, the cells were transferred onto the regeneration medium established in this study.

GUS gene expressions were examined using the GUS assay according to the procedure described by Jefferson et al. (1987) and Hoshino et al. (1998). For staining, the callus or tissue samples were incubated at 37 °C for 3–5 h in Na-phosphate buffer (50 mM, pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc; Wako Pure Chemical Industries, Ltd., Japan) on 24-well tissue culture plates (FALCON® 3047; Becton Dickinson and Company, New Jersey, USA). In order to remove chlorophyll, the leaf tissues were washed several times with 70% ethanol after incubation. The tissues exhibiting indigogenic staining were considered to express the *GUS* gene. GUS assay was performed after plantlet regeneration and at 7 d after the initiation of co-cultivation.

DNA extraction and PCR analysis

Total genomic DNA was isolated from the leaves of putative transgenic *Alstroemeria* plants by using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to

the manufacturer's instructions. Specific oligonucleotide primers for detecting the *GUS* and *NPTII* gene sequences (Hamill et al. 1991) were used to identify the presence of these genes in the genomic DNA of the above mentioned plants. Template DNA (50 ng) and primers (5 µM) each at 1 µl were mixed with 2.5 µl of 10 × *Taq* DNA polymerase reaction buffer, 100 µM (final concentration) dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP) and 1 unit of *Taq* DNA polymerase (Toyobo, Japan) in a final volume of 25 µl. Forty cycles of PCR were performed in a programmed temperature control system (iCycler; Bio-Rad Laboratories, Inc., Tokyo, Japan). A single cycle consisted of the following steps: denaturation of DNA at 92 °C for 1 min, annealing at 55 °C for 1 min and DNA extension at 73 °C for 1.5 min. Amplified DNA fragments were detected following electrophoresis on a 1.5% (w/v) agarose gel using Tris-acetate EDTA (TAE) as a running buffer at 100 V for 1 h.

Results

Callus induction and establishment of cell suspension cultures

In the preliminary experiments, flower buds, petals, leaves and internodes of *A. pelegrina* were used as explants for callus induction on media containing several different concentrations of auxins and cytokinins. However, no callus formation was observed in these experiments. For callus induction, ovules harvested at 14 d after pollination (Fig. 1a) were examined in several *Alstroemeria* species such as *A. magenta*, *A. aurea*, *A. pelegrina* var. *rosea*, *A. pelegrina* var. *alba* and *A. ligtu* and the interspecific cross *A. pelegrina* var. *rosea* × *A. magenta*

Germination was observed between 3 and 8 weeks at varying frequencies in the range of 11.9–53.6% depending on the species and the interspecific cross (Table 1).

Some seedlings developed normally. On the other hand, in all the species examined, callus formed on the hypocotyls of many seedlings at different frequencies (42.9–100%) (Fig. 1b, Table 1); the callus types differed among these species. The calli induced from *A. magenta*, *A. aurea*, *A. pelegrina* var. *rosea*, *A. pelegrina* var. *alba* and *A. ligtu* were yellowish and compact. These calli did not proliferate easily despite the application of PGRs (data not shown). On the other hand, the calli formed on the hypocotyls derived from the interspecific cross *A. pelegrina* var. *rosea* × *A. magenta* was friable and white (Fig. 1b). These calli grew more rapidly than the calli from other *Alstroemeria* species examined in this experiment. Thus, the explants of interspecific hybrids showed a high growth rate of callus.

For further callus proliferation, these friable calli were transferred to the media containing several different types and concentrations of PGRs (Fig. 2). The addition of cytokinins tended to inhibit callus proliferation primarily on most media supplemented with BAP and kinetin. On the other hand, auxins stimulated callus growth. Among the auxins, 2,4-D and picloram at 1 mg l^{-1} were highly effective in inducing callus proliferation.

Using liquid cultures with MS media supplemented with 1 mg l^{-1} 2,4-D or picloram, cell suspension cultures were easily established on a rotary shaker (Fig. 1c). The appearance of cell suspension cultures maintained with 2,4-D and with picloram was similar. The growth rate of the callus cultured in the picloram-containing medium was slightly higher than that cultured in the 2,4-D-containing medium. The calli cultured in the picloram- and 2,4-D-containing media were used for the subsequent regeneration and transformation experiments.

Plant regeneration

The effect of PGRs on plant regeneration was examined using cell suspensions cultures derived from the interspecific cross between *A. pelegrina* and *A. magenta* described above. To examine their effect on organogenesis, the cell suspension cultures were transferred onto half-strength MS media supplemented with NAA in combination with BAP or TDZ at several different concentrations (Fig. 3). Adventitious embryos were derived from suspension cells grown on half-strength MS medium supplemented with NAA and BAP. In particular, the highest regeneration frequency was obtained at 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. No obvious differences were observed between the calli grown on picloram- and 2,4-D-containing media. The plantlets derived from adventitious embryos formed rhizomes following their transfer to half-strength MS medium lacking the PGRs. The regenerated plants with well-established root systems were easily acclimatized. After 2–4 weeks, the acclimatized plants were transferred to the greenhouse. In the greenhouse, the regenerated plants flowered within one year after acclimatization (Fig. 4). No morphological alterations were observed between the regenerated plants and zygotic embryo-derived plants. The flowers showed intermediate characters (Fig. 4a) between *A. pelegrina* and *A. magenta* (Fig. 4b), thus indicating that the callus had been derived from the interspecific zygotic embryo and not the endosperm or nucellar tissue.

Transformation with *Agrobacterium*

An *Agrobacterium*-mediated transformation system was developed using the embryogenic suspension cells. The suspension cells were co-cultivated for 5 d with the *A. tumefaciens* strain EHA101/pIG121Hm or LBA4404/pTOK233. A schematic

procedure for *Agrobacterium* inoculation is shown in Fig. 5. Seven days after initiating co-cultivation, the cells were subjected to the GUS histochemical assay; blue spots were most pronounced in the cells that had been subcultured in a picloram-containing liquid medium and co-cultivated with the *A. tumefaciens* strain EHA101/pIG121Hm (Fig. 6 and Fig. 7a).

The co-cultivated cells were transferred to the MS medium containing 1 mg l^{-1} picloram and 20 mg l^{-1} hygromycin; 1 month later, several hygromycin-resistant callus lines showing GUS activity (Fig. 7b) were obtained (Fig. 7c). After 1 month of culturing on 20 mg l^{-1} hygromycin, the control callus, which was not inoculated with *Agrobacterium*, appeared brown. All callus cultures were subcultured every 2 weeks on the same MS medium supplemented with 20 mg l^{-1} hygromycin and 500 mg l^{-1} Claforan. In order to induce plant regeneration, a callus growing on hygromycin-containing medium was selected and transferred onto regeneration medium.

Approximately 6 months after the *Agrobacterium* inoculation, plantlets had regenerated from *Agrobacterium* inoculated calli grown on hygromycin-containing regeneration medium supplemented with NAA and BAP at 0.5 mg l^{-1} (Fig. 7d). The plantlets continued to survive on the hygromycin-containing medium. The shoots and leaves of these plantlets showed GUS activity (Fig. 7e), suggesting that the insertion of the *GUS* gene into the *Alstroemeria* genome. PCR analyses confirmed that the *GUS* and *NPTII* genes were incorporated into the genome of the regenerated *Alstroemeria* plants (Fig. 8).

Discussion

For inducing the maintenance of callus regeneration ability in *Alstroemeria*, several explants were grown in different culture conditions. In the primary study for callus induction with tissue culture of *Alstroemeria*, Gonzalez-Benito and Alderson (1992) cultured mature embryo of cultivar 'Butterfly' on MS medium supplemented with 2,4-D and picloram. Mature embryos were examined in tetraploid cultivars on a medium comprising MS salts and B_5 vitamin and supplemented with NAA and kinetin (Hutchinson et al. 1994). Lin et al. (1997, 1998, 2000a) reported direct shoot regeneration from excised leaf explants grown on MS medium containing TDZ and indole-3-butyric acid (IBA). Lin et al. (2000b) also induced a friable callus possessing the regeneration ability from a stem segment. Furthermore, rhizome splitting (Chiari and Bridgen 2000), floral apices (Padraza-Santos 2006) and nodes with axial tissue (Kim et al. 2006) were utilized as explants to develop plant regeneration systems. Thus, culture conditions differed among these different studies. It appears that successful plant regeneration systems are considerably genotype dependent. Therefore, the development of new culture systems is required for further research on *Alstoemeria*. In the present study, we demonstrated the availability of hypocotyls as a novel explant for inducing a friable callus with regeneration capability. Moreover, the explants derived from the interspecific cross might exhibit hybrid vigour, such as high callus proliferation rate. These findings provide insights to develop novel tissue culture techniques for application to *Alstroemeria*.

In previous studies on *Alstroemeria* in tissue cultures, the use of BAP was effective for plant regeneration in most cases (Gonzalez-Benito and Alderson 1992; Kim et al. 2006; Lin et al. 1997, 1998, 2000a, b; Padraza-Santos et al. 2006; Van Schaik

et al. 1996). However, in our study, BAP combined with NAA was effective for regeneration. Thus, BAP plays an important role in stimulating regeneration in *Alstroemeria*.

In conclusion, we report the successful regeneration of transgenic *Alstroemeria* plants through callus cultures derived from hypocotyls of interspecific zygotic embryos. Van Schaik et al. (2000) reported the first successful attempt at *Alstroemeria* transformation. They confirmed transient gene expression in embryogenic callus after particle bombardment. Subsequently, Lin et al. (2000c) produced transgenic *Alstroemeria* plants using particle bombardment. Recently, Kim et al. (2007) succeeded in achieving *Agrobacterium*-mediated transformation in *Alstroemeria*. As mentioned above, successful regeneration systems are genotype dependent. To adapt the transformation techniques to a wide range of *Alstroemeria* species, a case study is required. Our procedure would be valuable in further research on genetic transformation with important genes, including those regulating flower colour and viral resistance; moreover, our culture system can also be applied to protoplast cultures.

Acknowledgments

This work was supported in part by grants from The Akiyama Foundation, Inamori Foundation and Grant-in-Aid for Scientific Research.

References

Chiari A, Bridgen MP (2000) Rhizome splitting: a new micropropagation technique to increase *in vitro* propagule yield in *Alstroemeria*. Plant Cell Tiss Org Cult 62:39–46

Gonzalez-Benito ME, Alderson PG (1992) Callus induction and plant regeneration in *Alstroemeria*. J Exp Bot 43:205–211

Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. Plant Cell Rep 10:221–224

Hiei Y. Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hood EE, Helmer GL, Fraley RT, Chilton M-D (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291–1301

Hoshino Y, Murata N, Shinoda K (2006) Isolation of individual egg cells and zygotes in *Alstroemeria* followed by manual selection with a microcapillary-connected micropump. Ann Bot 97:1139–1144

Hoshino Y, Zhu YM, Nakano M, Takahashi E, Mii M (1998) Production of transgenic grapevine (*Vitis vinifera* L. cv. Koshusanjaku) plants by co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* and selecting secondary embryos. Plant Biotechnol 15:29–33

Hutchinson MJ, Senaratna T, Tsujita JM, Saxena PK (1997) Somatic embryogenesis in liquid cultures of a tetraploid *Alstroemeria*. Plant Cell Tiss Org Cult 47:293–297

Hutchinson MJ, Tsujita JM, Saxena PK (1994) Callus induction and plant regeneration from mature zygotic embryos of a tetraploid *Alstroemeria* (*A. pelegrina* × *A. psittacina*).

Plant Cell Rep 14:184–187

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jin S, Komari T, Gordon MP, Nester EW (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. J Bacteriol 169:4417–4425

Kim JB, Raemakers CJJM, Jacobsen E, Visser RGF (2006) Efficient somatic embryogenesis in *Alstroemeria*. Plant Cell Tiss Org Cult 86:233–238

Kim JB, Raemakers CJJM, Jacobsen E, Visser RGF (2007) Efficient production of transgenic *Alstroemeria* plants by using *Agrobacterium tumefaciens.* Ann Appl Biol 151:401–412

Lin H-S, De Jeu MJ, Jacobsen E (1997) Direct shoot regeneration from excised leaf explants of in vitro grown seedlings of *Alstroemeria* L. Plant Cell Rep 16:770–774

Lin H-S, De Jeu MJ, Jacobsen E (1998) Formation of shoots from leaf axils of *Alstroemeria*: The effect of the position on the stem. Plant Cell Tiss Org Cult 52:165–169

Lin H-S, De Jeu MJ, Jacobsen E (2000a) The application of leafy explant micropropagation protocol in enhancing the multiplication efficiency of *Alstroemeria*. Sci Hort 85:307–318

Lin H-S, van der Toorn C, Raemakers KJJM, Visser RGF, De Jeu MJ, Jacobsen E (2000b) Development of a plant regeneration system based on friable embryogenic callus in the ornamental *Alstroemeria*. Plant Cell Rep 19:529–534

Lin H-S, van der Toorn C, Raemakers KJJM, Visser RGF, De Jeu MJ, Jacobsen E

(2000c) Genetic transformation of *Alstroemeria* using particle bombardment. Mol Breed 6:369–377

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobbacco cell cultures. Physiol Plant 15:473–497

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a ß-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol 31:805–813

Pedraza-Santos ME, López-Peralta MC, González-Hernández VA, Engleman-Clark EM, Sánchez-García P (2006) *In vitro* regeneration of *Alstroemeria* cv. 'Yellow King' by direct organogenesis. Plant Cell Tiss Org Cult 84:189–198

Van Schaik CE, Posthuma A, De Jeu MJ, Jacobsen E (1996) Plant regeneration through somatic embryogenesis from callus induced on immature embryos of *Alstroemeria spp.* L. Plant Cell Rep 15:377–380

Van Schaik CE, van der Toorn, C, de Jeu MJ, Raemakers CJJM, Visser RGF (2000) Towards genetic transformation in the monocot *Alstroemeria* L. Euphytica 115:17–26

Watson B, Currier TC, Gordon MP, Chilton M-D, Nester EW (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J Bacteriol 123: 255–264

Table 1. Effect of developmental stages on frequency of callus formation from *Alstroemeria* ovules.

	No. of ovaries used	No. of ovules cultured (A)	No. of germinated seedlings (B)	Frequency of germination (B/A × 100%)	No. of callus-forming seedlings (C)	Frequency of callus formation (C/A × 100%)	Frequency of callus formation (C/B × 100%)
A. magenta	8	178	76	42.7a	33	18.5c	43.4c,d
A. aurea	7	118	14	11.9b	6	5.1d	42.9d
A. pelegrina var. *rosea*	5	99	55	55.6a	55	55.6a	100a
A. pelegrina var. *alba*	5	115	49	42.6a	32	27.8b,c	65.3b,c
A. ligtu	9	176	90	51.1a	51	29.0b,c	56.7b,c,d
A. pelegrina var. *rosea* × *A. magenta*	13	265	142	53.6a	105	39.6a,b	73.9a,b

Ovaries were harvested 14 d after pollination.
Data were recorded 5 months after the initial culture.
Values followed by the same letter are not significantly different at the 0.01 level based on an LSD test from data subjected to an arcsin transformation.

Legends to figures

Fig. 1 Plant regeneration from cell suspension cultures derived from the hypocotyls in *Alstroemeria*

(a) Ovules harvested at 14 d after pollination from the cross between *A. pelegrina* and *A. magenta*. Bar = 3 mm

(b) Callus formation on the surface of the hypocotyl of the germinating zygotic embryo. Bar = 5 mm

(c) Cell suspension cultures maintained in the liquid MS medium supplemented with 1 mg l^{-1} picloram. Bar = 3 cm

(d) Adventitious embryogenesis in cell suspension cultures on half-strength MS medium containing 0.5 mg l^{-1} of both NAA and BAP. Bar = 3 cm

(e) Some embryos formed on the half-strength MS medium containing 0.5 mg l^{-1} of both NAA and BAP. Bar = 3 mm

(f) Developing shoots from an adventitious embryo. Bar = 5 mm

(g) A regenerated plantlet cultured on the half-strength MS medium lacking plant growth regulators. The rhizome produced by the plantlet in vitro. Bar = 2 cm

(h) Acclimatized plant grown in the greenhouse. Bar = 5 cm

Fig. 2 Effect of PGRs on the growth of ovule-derived calli (expressed as fresh weight) at 5 weeks after the initial culture

Calli (0.5 g) were cultured on gellan gum-solidified MS media supplemented with PGRs. Data represent the mean ± standard error of three replicates.

Fig. 3 Effect of PGRs on the frequency of plant regeneration from *Alstroemeria* callus.

Data represent the mean ± standard error of three replicates.

Fig. 4 Comparison of flowers between the regenerated plant and its parents

The flower in the regenerated plant shows intermediate characters between its parents; this proves that the callus used in the present study originated from a zygotic embryo.

(a) The flowering plant regenerated from a cell suspension culture.

(b) Flowers of *A. pelegrina* var. *rosea* (left) and *A. magenta* (right).

All bars = 5 cm

Fig. 5 Scheme for the co-cultivation of suspension cells with *A. tumefaciens*

1. Prior to co-cultivation, *A. tumefaciens* strain EHA101 (pIG121Hm) or LBA4404 (pTOK233) was inoculated in the liquid LB medium in an Erlenmeyer flask.

2. After subculturing for 2 weeks, the suspension cells were placed on a nylon membrane and exposed to a freshly grown suspension culture of bacteria.

3. The inoculated suspension cells were washed several times with distilled water (D.W.) and placed on the MS medium containing picloram or 2,4-D with or without acetosyringone.

4. After 5 d of co-cultivation, the suspension cells were transferred to 500 mg l^{-1} Claforan-containing MS medium supplemented with picloram or 2,4-D.

5. After 7 d of co-cultivation, histochemical localization of *GUS* gene expression was detected in the suspension cells.

6. After 1 month of co-cultivation, the suspension cells were transferred to 20 mg l^{-1} hygromycin-containing medium.

Fig. 6 Effect of bacterial strains and acetosyringone (AS) on GUS activity in the suspension cells following co-cultivation with *A. tumefaciens*

Data were obtained 7 d after inoculation. Data represent the mean ± standard error of three replicates.

Fig. 7 Plant regeneration from hygromycin-resistant callus

a. Suspension cells showing GUS activity 7 d after co-cultivation. Bar = 3 mm

b. Hygromycin-resistant calli showing GUS activity 2 months after co-cultivation. Bar = 5 mm

c. Selection of hygromycin-resistant calli 2 months after co-cultivation. Bar = 3 cm

d. Shoot regeneration from hygromycin-resistant callus. Bar = 1 cm

e. A shoot from the regenerated plant showing GUS activity. Bar = 5 mm

Fig. 8 Detection of *GUS* and *NPTII* genes in regenerated plants

a. Detection of *GUS* gene

Lane M, *λ*/*Hin*d III and *ϕX*174/*Hae* III as the molecular markers (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, plasmid pIG121Hm from *E. coli* (positive control); lane 2, non-transformed plant; lanes 3–5, transgenic plants. Arrow indicates the position of the expected 1.2 kbp fragment that includes the *GUS* gene.

b. Detection of the *NPTII* gene

Lane M, *λ*/*Hin*d III and *ϕX*174/*Hae* III as the molecular markers (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, plasmid

pIG121Hm extracted from *E. coli* (positive control); lane 2, plasmid pIG121Hm extracted from *A. tumefaciens* (positive control); lane 3, non-transformed plant; lane 4–6, transgenic plants. Arrow indicates the position of the expected 0.7 kbp fragment that includes the *GUS* gene.

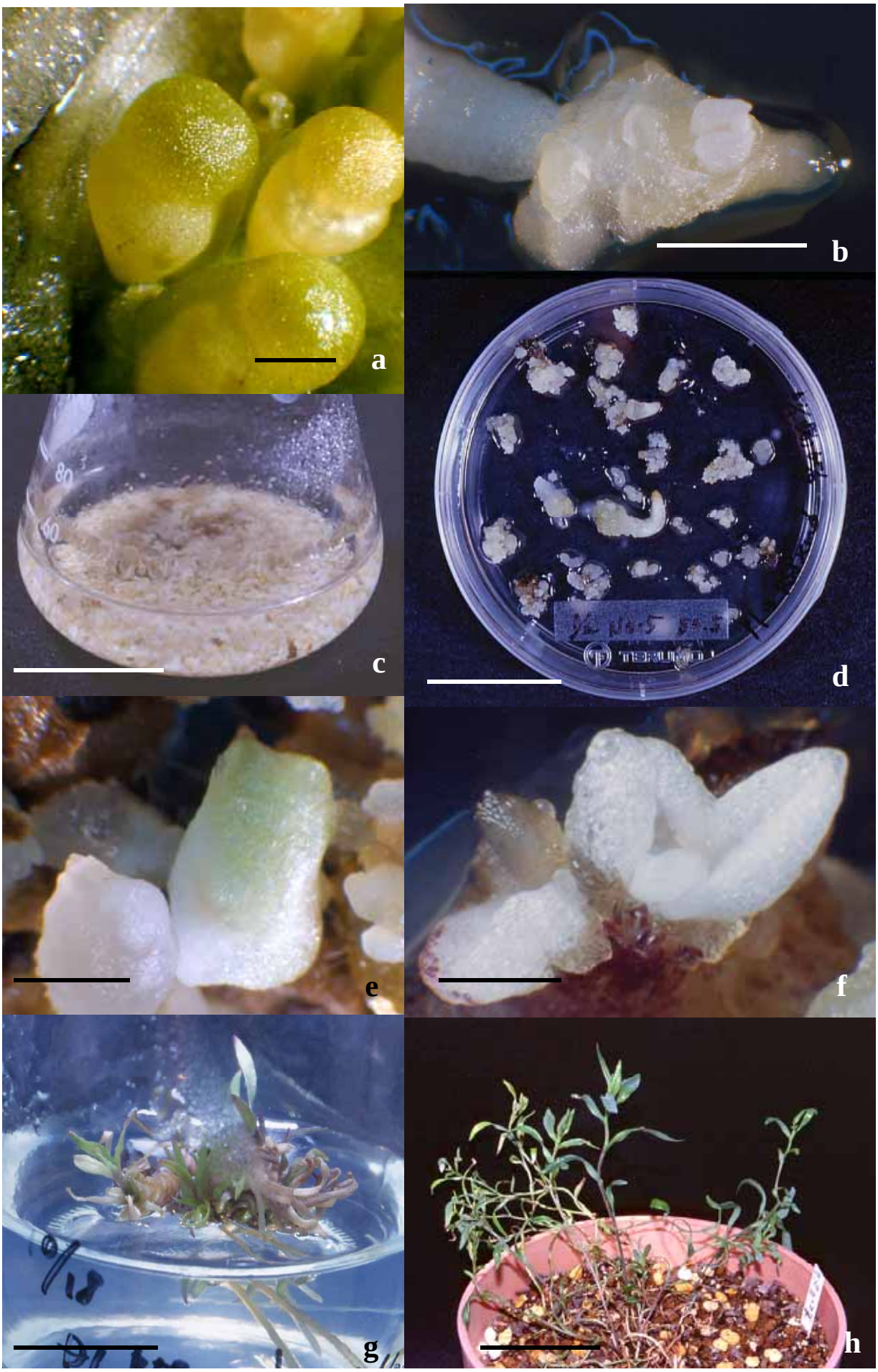


Fig. 1

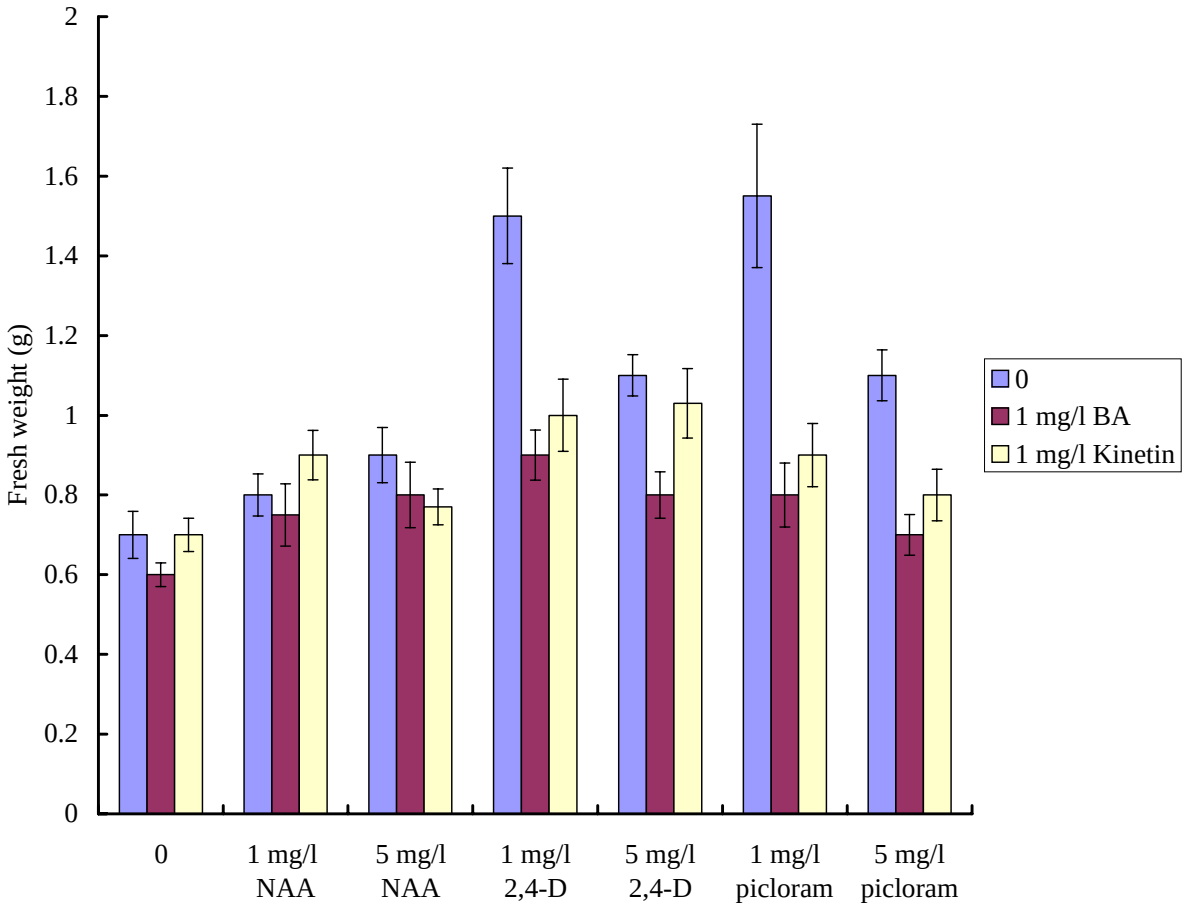

Fresh weight (g)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1 mg/l NAA
5 mg/l NAA
1 mg/l 2,4-D
5 mg/l 2,4-D
1 mg/l picloram
5 mg/l picloram
0
1 mg/l BA
1 mg/l Kinetin

Fig. 2

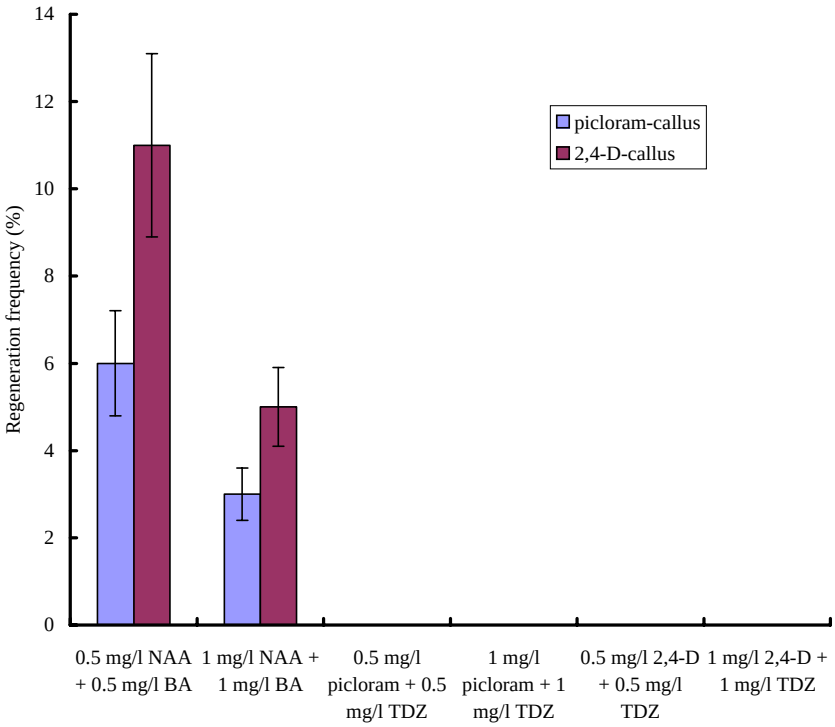

14
12
10
8
6
4
2
0
Regeneration frequency (%)
picloram-callus
2,4-D-callus
0.5 mg/l NAA + 0.5 mg/l BA
1 mg/l NAA + 1 mg/l BA
0.5 mg/l picloram + 0.5 mg/l TDZ
1 mg/l picloram + 1 mg/l TDZ
0.5 mg/l 2,4-D + 0.5 mg/l TDZ
1 mg/l 2,4-D + 1 mg/l TDZ

Fig. 3

Fig. 4

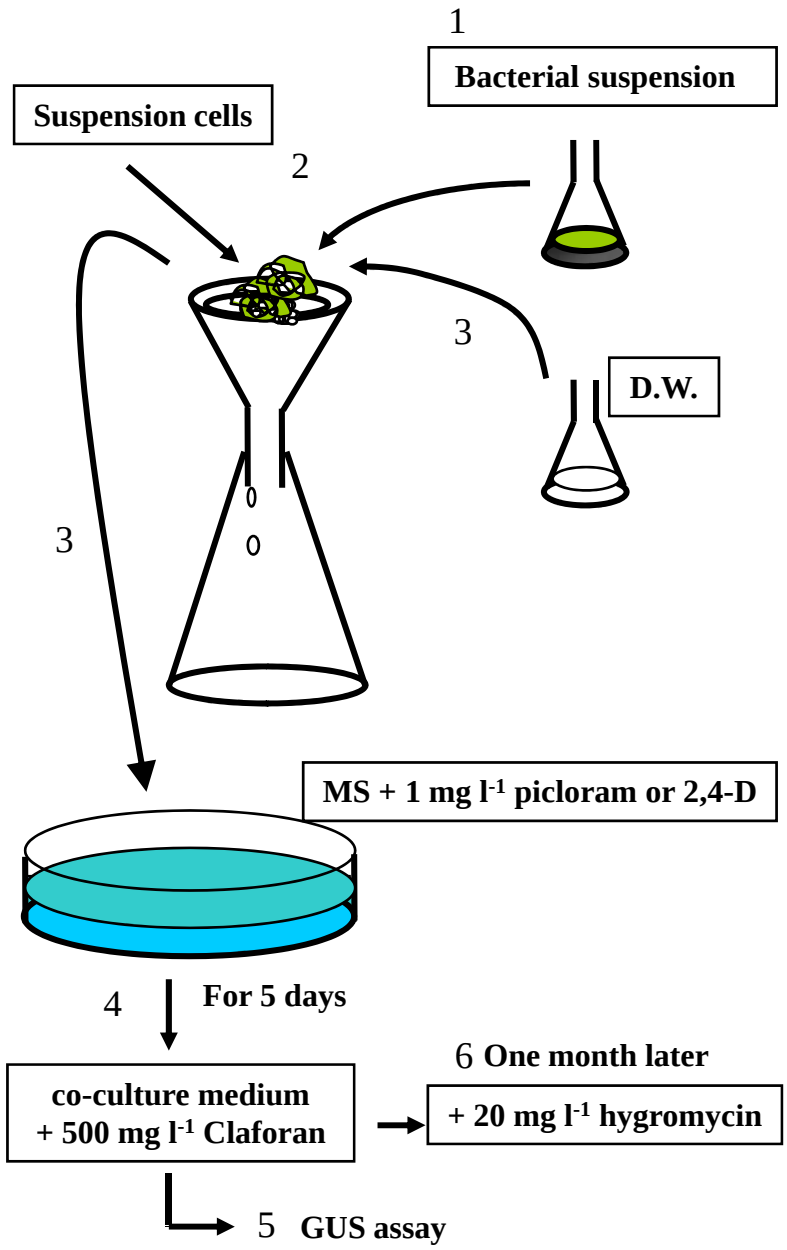

1
Bacterial suspension
Suspension cells
2
3
D.W.
3
MS + 1 mg l-1 picloram or 2,4-D
4
For 5 days
6 One month later
co-culture medium
+ 500 mg l-1 Claforan
+ 20 mg l-1 hygromycin
5 GUS assay

Fig. 5

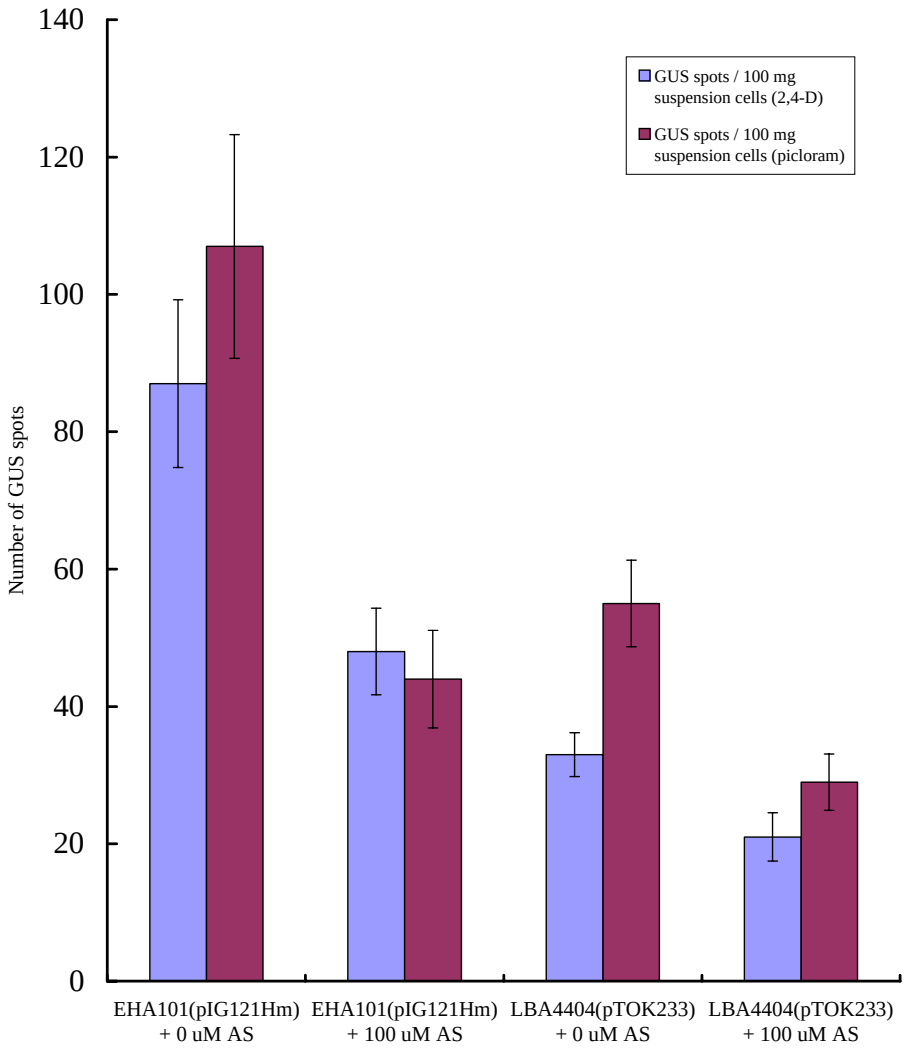

140
120
100
80
60
40
20
0
Number of GUS spots
GUS spots / 100 mg suspension cells (2,4-D)
GUS spots / 100 mg suspension cells (picloram)
EHA101(pIG121Hm) + 0 uM AS
EHA101(pIG121Hm) + 100 uM AS
LBA4404(pTOK233) + 0 uM AS
LBA4404(pTOK233) + 100 uM AS

Fig. 6

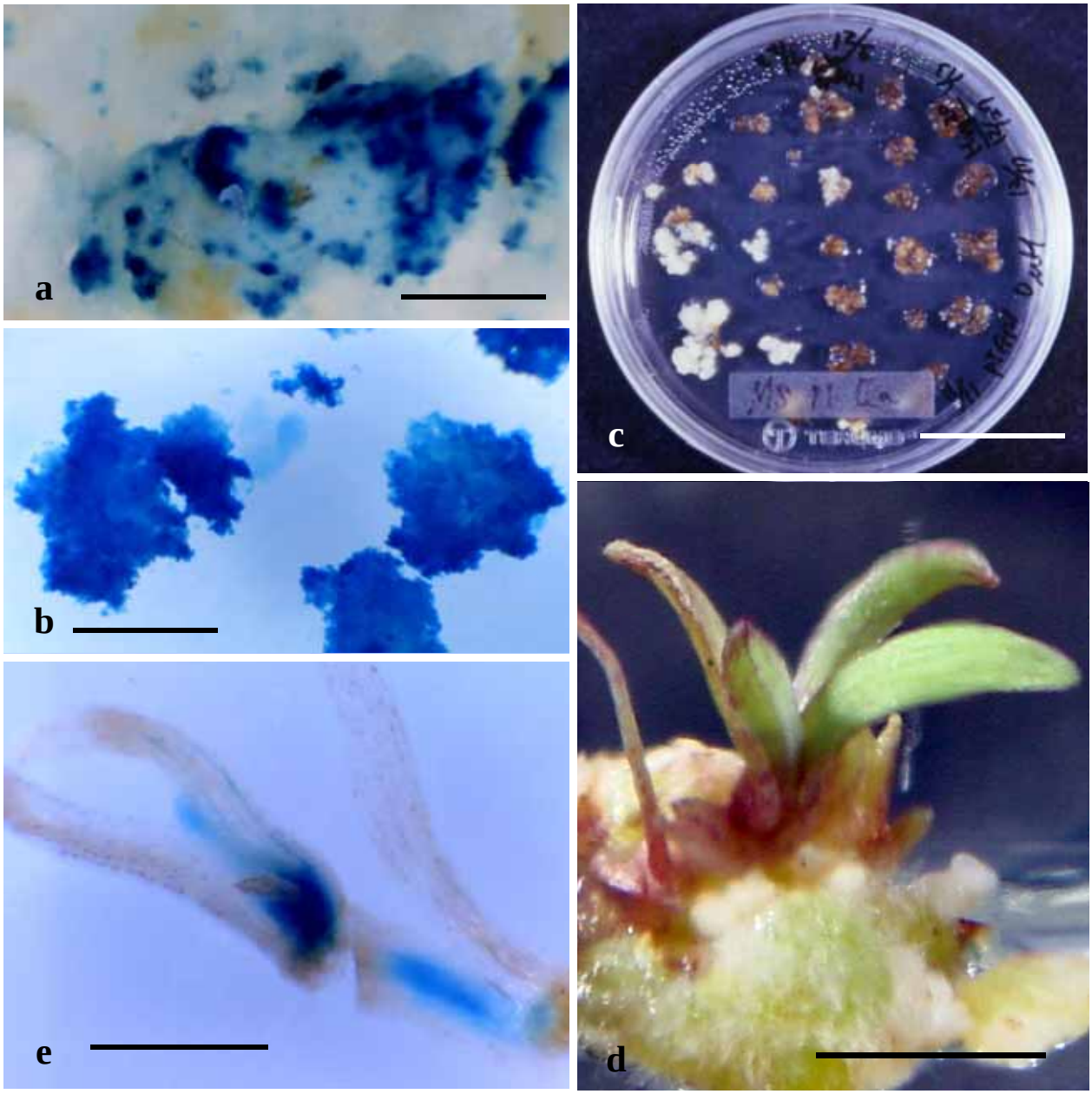
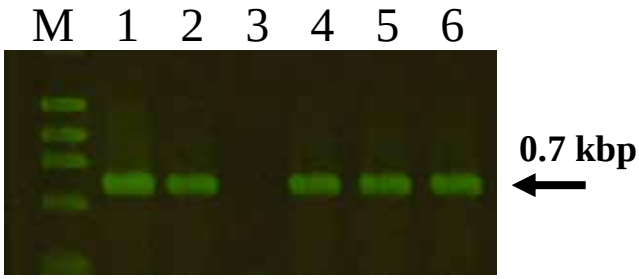


Fig. 7

A. Detection of *GUS* gene



B. Detection of *NPTII* gene

Fig. 8