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Effective and Comprehensive Chrysanthemum (*Dendranthema X grandiflora*) Regeneration and Transformation Protocols

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Abstract: This study provides detailed reproducible methods for the transformation of *Dendranthema X grandiflora* Shuhou-no-chikara (standard) and Lineker (spray) stem internode Thin Cell Layers (TCLs) and conventional stem internode explants, using intron-containing and intron-less plasmids. Discussed methodologies and results have used GUS reporter, *nptII* selector genes, as well as *pac1/2,5-A/RNaseL* genes coding for virus/viroid resistance. Transformation efficiencies are reported at the kanamycin selection (callus formation and plantlet rooting), GUS, PCR, Southern and Western levels. Notes on regeneration improvement through the use of TCLs, as well as the effective use of sonication for both regeneration and generation of transformants, stimulation of *Agroinfection* and elimination of *Agrobacterium*, are included. Protocols are provided for the use of scanning electron microscopy to confirm developmental processes, as well as flow cytometry to check for cell competence and division stages. The difficulties still experienced in the genetic transformation of chrysanthemum globally by researchers require a detailed protocol that exposes the subtleties in methodology. This study provides that information source.

Key words: 2,5-A-RNaseL, acetosyringone (AS), AGL0, CaMV-35S promoter, *Dendranthema*, flow cytometry, LBA4404, *nptII*, *pac1* PCR, Plant Growth Regulator (PGR), SAAT, SEM, southern hybridization, western blot

INTRODUCTION

Chrysanthemum (*Dendranthema X grandiflora*) is one of the most important global floricultural assets whose cultivation and improvement/modification by traditional breeding dates back over 2,000 years. The latter part of the 21st century was marked by *in vitro* regeneration studies, while the last 15 years have emphasized improvement by genetic transformation^[1]. Due to generally low transformation efficiencies being reported and due to inconsistencies and repeatability of protocols, regeneration studies have once again come to the fore. The intricate interlink between the success of *Agrobacterium*-mediated transformation of chrysanthemum and regeneration will be highlighted in this study. In almost 50 reported cases of *Agrobacterium*-mediated transformation of chrysanthemum, the initial explant used was: leaf (50%), stem (22%), leaf/stem (14%), roots (2%) and flower parts (2%). The choice of selective agent was: kanamycin sulphate (80%), G418 (4%), geneticin (4%), hygromycin (10%), Basta (2%), paramomycin (2%), or none (6%); selection timing was early (76%), late (12%) or unspecified (12%). Furthermore, where kanamycin was the choice of agent, more than half of the studies employed a low (≤ 25 mg L⁻¹) level of initial selection with only 20% of studies increasing the selection level during regeneration, with most (78%) research groups decreasing

the selection pressure^[1]. The choice of *Agrobacterium* strain was: LBA4404 (56%), EHA101/105 (22%), AGL0/1 (20%), A281 (10%) or others (28%); only a single study of *A. rhizogenes* using LBA9402 was reported^[2]. In these studies, during regeneration, the bacterocidal agent used for elimination of *Agrobacterium* was: cefotaxime (64%), vancomycin (26%), carbenicillin (16%), ticarcillin (16%), individually, or in combination. These agents were applied, in most cases (84%), at ≥ 250 mg L⁻¹ initially, with 36% of studies decreasing/halving this concentration at later stages of selection. Only a single study reported the use of Sonication-Assisted *Agrobacterium* Transformation, or SAAT^[3].

Indeed the difficulties facing the genetic transformation of chrysanthemum resulting from the choice of explant, selective agent and/or *Agrobacterium*-eliminating bactericide and the levels and timing of *Agrobacterium* application, as well as the choice of *Agrobacterium* strain is confounded by the reports of the need for wounding^[4], chrysanthemum cultivar and *Agrobacterium* strain dependence^[1], late transgene (GUS) expression^[5], transgene silencing^[6-8], chimerism caused by transgene inactivation, primarily by methylation^[9] and possible transgene truncations leading to smaller proteins^[10].

The protocol within this study aims to address these difficulties and shortcomings. Although the

protocols described are based on *in vitro* plantlet stem internode TCLs, very similar transformation frequencies at the GUS, PCR, Southern and Western levels can be obtained for leaf material, but the ease of explant manipulation and repetitiveness of the protocol is much higher in the case of the former. Moreover these small explants allow for the controlled regeneration of transgenic organs (shoot, root, somatic embryo, friable callus), depending on the chosen medium^[11]. Transformation efficiencies (the number of molecular method-derived plantlets from the number of plantlets formed) range from 62% at the GUS expression level to 0% for certain genes at the Southern and certain proteins at the Western levels. A brief explanation as to possible reasons for fluctuations is provided. This report also provides skeleton protocols for the molecular and cytogenetic analyses conducted with chrysanthemum and aims to provide the reader with detailed methodologies, often not explicit in regular papers.

MATERIALS AND METHODS

Wherever mentioned the following abbreviations mean: Room Temperature (RT); Plant Growth Regulator (PGR). All reagents are Tissue Culture or Molecular Biology grade. All solutions are prepared in sterile, autoclaved double-distilled water (SDW), 18.2 MΩ cm, organic content <5 ppb. Autoclaving is always for 15 min at 121°C and 103 kPa.

Ex vitro explant preparation

1. Protocols were developed with two leading Japanese cultivars, Shuhou-no-chikara, a standard and Lineker, a spray-type chrysanthemum (*Dendranthema X grandiflora* (Ramat.) Kitamura). Both greenhouse and *in vitro* plant material serve equally well as initial explant sources (culture conditions for both provided below), but *in vitro* material is recommended to avoid viral contamination.
2. PGR stocks: 6-benzyladenine (BA) and α -naphthalene acetic acid (NAA) at 100 mg L⁻¹ each. Dissolve 100 mg NAA or BA in 1N NaOH and bring to volume in SDW. Do not pH. Keep both BA and NAA stocks at 4°C for up to 6 months.
3. Optimized greenhouse shoot induction medium (SIM_{GH}): Preparation as SIM_{IV}, but add 1 mg L⁻¹ BA, 1 mg L⁻¹ NAA and 20 g L⁻¹ sucrose.
4. Adjust media to pH 5.7-5.8 with 0.1 M NaOH or 1 N HCl before agar addition and autoclave. All *in vitro* cultures are maintained at 25°C, 16 h photoperiod, light intensity = 45 μmol/m²/s¹ (PGF lamps).

In vitro explant preparation and *in vitro* growth conditions

1. Optimized *in vitro* shoot induction medium (SIM_{IV}): add 2 mg L⁻¹ BA, 0.5 mg L⁻¹ NAA and 40 g L⁻¹ sucrose to full-strength Murashige and Skoog (MS macro-and microelements and vitamins^[12]) medium. pH to 5.7 and autoclave. Commercial MS powder (Sigma-Aldrich) provides the same results.
2. *In vitro* shoot regeneration medium (SRM_{IV}) and greenhouse shoot regeneration medium (SRM_{GH}): 3 g L⁻¹ Hyponex® (Japan) + 20 g L⁻¹ sucrose.

Agrobacterium culture, inoculation and elimination and SAAT

1. *A. tumefaciens* strain LBA4404 harbours pBI121 (Fig. 1^[13], pKT2 or pKT66 (Fig. 1; Kirin Breweries, Inc.^[14]). *A. tumefaciens* strain AGL0 harbours a binary plasmid pKT3, pKT61, pKT2-L-*PacI*, or pKT-MF-*PacI* (Fig. 1; Kirin Breweries, Inc.).
2. LB (Luria-Bertani) medium: 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl. For plating, add 15 g L⁻¹ bactoagar and pH 5.3. Autoclave, store at 4°C.
3. Antibiotics stocks: kanamycin and rifampicin at 10 mg mL⁻¹ each, cefotaxime (Claforan®) at 100 mg mL⁻¹. Filter sterilize. Store at -20°C for up to 6 months.
4. Acetosyringone (AS; 3,5-Dimethoxy-4-hydroxy-acetophenone; Sigma-Aldrich) stock (0.1 M): Dissolve AS in dimethyl sulphoxide (DMSO) or in 100% EtOH. Store at 4°C in foil for up to 6 months. AS crystallizes at 4°C but is liquid at RT. Add 1 μL AS stock to 1 mL *Agrobacterium* medium or 1 mL to 11 co-cultivation medium for 100 mM final concentration.

GUS histochemical staining

1. X-Gluc stock: 5-bromo-4-chloro-3-indolyl-D-glucuronide cyclohexylammonium salt (X-glucuronide, Wako, Japan) 40 mg mL⁻¹ in dimethyl formamide. Store at 4°C in foil for up to 1 year.
2. Staining buffer: 100 mM NaH₂PO₄, 10 mM Na₂EDTA, 0.5 mM K ferrocyanide (MW 422.4), 0.5 mM K ferricyanide (MW 329.3), 20% (v/v) EtOH, 1 mL Triton X-100. Adjust to pH 7.0 with NaOH. Store at 0-4°C.
3. GUS staining: Dilute 100 mL X-Gluc stock into 8 mL staining buffer. Dispense volume as required and in suitable recipient (e.g. glass bottle or plastic wells).

DNA extraction, PCR and electrophoresis

1. Extraction buffer: 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0. Add 0.2% (w/v) β-mercaptoethanol under a fume hood just prior to use.
2. 24 : 1 chloroform : isoamyl alcohol (v/v): keep in foil, at RT.

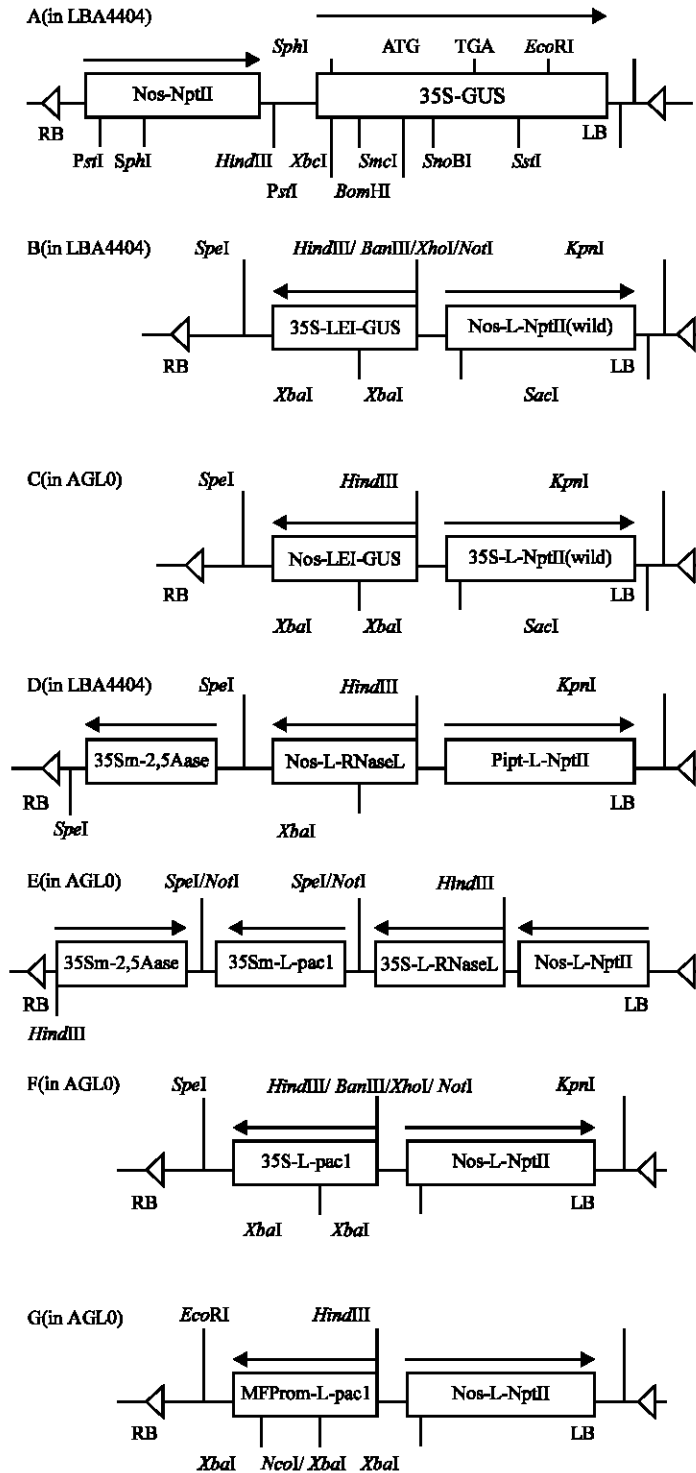


Fig. 1: Structure of plasmids (courtesy of Kirin Breweries, Co., Ltd., Japan) used in transformation experiments. GUS-containing plasmid constructs: (A) pBI121; (B) pKT2; (C) pKT3. Virus/viroid resistance gene-containing plasmids: (D) pKT66; (E) pKT61; (F) pKT2-L-Pac1; (G) pKT-MF-Pac1. Arrows indicate direction of transcription (5'-3'). E=Enhancer; L=leader sequence from soybean β -glucanase

3. TE: 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. Make from 1 M stock Tris-Cl (pH 7.5) and 500 mM stock EDTA (pH 8.0).
4. TaKaRa La Taq[®] Hot Start Version PCR reagent kit: TaKaRa La Taq[®] HS 5 U μL^{-1} , Mg²⁺ buffer, 2.5 mM dNTP mix.
5. Primers (Greiner, Japan, HPLC grade, in TE buffer, kept at 4°C) are: *nptII*-5' = 5-GAGAGGCTATTCGGCTATGA-3, *nptII*-3' = 5-GATGCTCTTCGTCCAGATCA-3, 438 bp fragment; *uidA*-5' = 5-CTGTAGAAACCCCAACCCGTG-3, *uidA*-3' = 5-GCTGTGCGTAATTACCTGACCTAACC-3, 954 bp fragment; *RNaseL*-5' = 5-GACAGAGAGGACATTGTGGA-3', *RNaseL*-3' = 5-TG CACCAGATCCAGTTCTT-3', 3,2 Kbp fragment; *2,5-Aase*-5' = 5'-GAGGAGTTCTCTACCCAAGT-3', *2,5-Aase*-3' = 5'-TGTCCAGTTCTCTCCACCT-3', 548 bp fragment; *pac1*-5' = 5'-TCCTCATCCGCTTCCGACTC-3', *pac1*-3' = 5'-CAAGGACTTCGAGTGCTTGC-3', 1021 bp fragment. Amplification conditions are: *uidA/nptII*: 35X [94°C, 30 s; 60°C, 2 min; 72°C, 50 s]; *2,5Aase*: 94°C, 3 min; 35X [94°C, 30 sec; 62°C, 30 sec; 72°C, 70 sec] 72°C, 5 min; *RNaseL*: 94°C, 3 min; 35X [94°C, 30 sec; 64°C, 2 min; 72°C, 50 sec]; *pac1*: 94°C, 8 min; 30X [94°C, 2 min; 62°C, 1 min; 72°C, 2 min]. In all cases, drop to 4°C until storage or utilization following the final annealing step.
6. 100 bp marker ladder (Biolabs Inc., USA); loading/tracking dye (Takara, Japan).
7. SeaKem[®] GTG[®] agarose: BioWhittaker Molecular Applications, USA.
8. TAE, Tris-Acetate-EDTA (50X): 242 g L⁻¹ Tris base, 5.7% (v/v) glacial acetic acid, 10% (v/v) 0.5 M EDTA (pH 8.0). Autoclave and store up to 1 year at RT.
9. Blocking reagent (DEPC treated): blocking reagent (Boehringer) 10% (w/v), DEPC 0.1% (v/v).
10. DEPC stock: 0.1% (w/v) DEPC. pH 7.0 and autoclave.
11. DEPC-QW: 10% (v/v) 20 X SSC, 20% SDS (w/v), 90% (v/v) DEPC stock.
12. Pre-hybridization buffer (PB): 25% (v/v) 20 X SSC, 10% (v/v) blocking reagent, 65% (v/v) DEPC stock.
13. Hybridization buffer (HB): 10 mL PB + 10 $\mu\text{g mL}^{-1}$ DIG-PCR probe.
14. Buffer 1: 0.1 M maleic acid, 0.15 M NaCl, 0.1% DEPC, pH 7.5.
15. Buffer 2: 10% (v/v) blocking reagent, 90% (v/v) DEPC stock.
16. Buffer 2.5: 0.3% (v/v) Tween-20, 97.7% (v/v) buffer 1.
17. Buffer 3: 0.1 M Tris (pH 9.5), 0.1 M NaCl, 90% (v/v) DEPC-QW.
18. Antibody solution: buffer 2 : Anti DIG AP = 10 : 1 (v/v).

SDS-PAGE and western blotting

1. Protein extraction buffer: 0.1 M Tris HCl, 3% sodium dodecyl sulphate (SDS), 0.3% glycerine, 0.03% mercaptoethanol.
2. Tris-buffered saline (TBS): 20 mM Tris, 500 mM NaCl, pH 7.5.
3. TTBS: 0.05% Tween-20 (v/v) in TBS, no pH.
4. Blocking solutions: *pac1*: skimmed milk 5% w/v, 10 mM Tris, 150 mM NaCl, 0.05% Tween-20 (v/v), pH 7.2; *2,5-A*, *RNaseL*: 1% gelatin (w/v) in TTBS.
5. DNA labelling: DIG DNA labeling and detection kit (Boehringer).
6. Colour development solution: CDP-Star detection kit (Amersham).

Light Microscopy (LM) and Scanning Electron Microscopy (SEM)

1. FAA: formalin : acetic acid : ethanol, 5 : 5 : 90. Keep in dark bottle at RT.
2. LM series: Place samples for sectioning for >6 h in each of the following: 1, xylene; 2, xylene : 100% EtOH (1 : 1); 3, 100% EtOH; 4, 95% EtOH; 5, 85% EtOH; 6, 70% EtOH; 7, 50% EtOH; 8, 30% EtOH; 9, hematoxylin; 10, 30% EtOH; 11, 50% EtOH; 12, H₂SO₄ : 100% EtOH (1 : 1); 13, H₂SO₄ (brief dip); 14, 85% EtOH; 15, 95% EtOH; 16, 100% EtOH; 17, xylene : 100% EtOH (1 : 1); 18, xylene.
3. SEM alcohol series: Place desired samples for sectioning for at least ½ day in each of the following: 1, 70% EtOH; 2, 85% EtOH; 3, 80% EtOH : *n*-butanol (6 : 4); 4, 90% EtOH : *n*-butanol (1 : 1); 5, 100% EtOH : *n*-butanol (1 : 3); 6, 100% *n*-butanol; 7, 100% *n*-butanol.

Rapid miniprep plasmid extraction

1. Solution I (-lyso): 25 mM Tris-HCl (pH 8.0), 10 mM Na₂-EDTA, 0.9% (w/v) glucose. Add 2 mg mL⁻¹ lysozyme (Takara) to stock solution I, autoclave, store at 4°C.
2. Solution II: 0.2 N NaOH, 1% SDS. Store at RT.

Southern blot hybridization

1. Restriction endonucleases (RE, Takara, Japan): Figure 1 for appropriate RE sites for each plasmid construct.
2. Use the chemiluminescence substrate (CPD star) detection system (Boehringer).
3. NaOH buffer: 0.8% (w/v) NaOH + 0.1% (w/v) SDS.
4. 20 X SSC: 3 M NaCl, 0.3 M trisodium citrate dihydrate (C₆H₅Na₃O₇•2H₂O).

Flow cytometry

1. Partec Buffer A: 2 mg L⁻¹ 4,6-diamidino-2-phenylindole (DAPI), 2 mM MgCl₂, 10 mM Tris, 50 mM sodium citrate, 1% (w/v) PVP K-30, 0.1% (v/v) Triton-X, pH 7.5^[15]. Keep wrapped in foil, use on ice, store at 4°C.

Morphological scoring and transformation efficiencies

1. To test for morphological stability, the following parameters should be measured in both control and transgenic greenhouse plants: number of leaves, number of flower buds (for spray-type), plant height, number of disk and ray florets and flower colour.
2. For any one treatment and for any one plasmid construct, 50 petri dishes with 10 TCLs/stem explants are used, i.e. 500 initial explants. Transformation efficiency is reported at 10 levels (Table 2). Broadly, transformation efficiency is calculated as [(Y/Z) X 100]%, where, Y = the number of actively dividing calli or rooted *in vitro* cuttings on 30 mg L⁻¹ selective medium (SIM_{IV} or SRM_{IV}, respectively), the number of GUS-spot or GUS-stain-containing explants (transient expression) or GUS expressing rooted plants (stable expression) on selective SRM_{IV} (in young, middle-aged and old leaves), the number of plants with correct banding in Southern or in Western analysis; Z = the total number of initial explants (500).

In vitro explant preparation from *ex vitro* material:

In vitro shoot cultures are initially established from greenhouse stem explants. Often chrysanthemum breeders who wish to conduct genetic transformation of a cultivar or variety that they have observed in a greenhouse need to first acclimatize it to *in vitro* conditions. This regeneration protocol is also essential to the success of conversion of transgenic plants from *ex vitro* to *in vitro*.

1. Excise terminal 10-15 cm of greenhouse mother plants with a sterile lamina. Actively-growing mother plants, preferably 30-40 cm high (approximately 2 months old) should be used. Placed cut-ends into SDW.
2. Remove all leaves and wash naked stems in a commercial dishwashing liquid solution for 1 min.
3. Rinse thoroughly under luke-warm SDW and rinse further 3X with SDW.
4. Transfer stems to a 1% NaOCl (1% active Cl) or Ca(ClO₂). Add 1% (v/v) polyoxyethylene (20) sorbitan monolaurate (surfactant), or Tween-20 and leave to stir for 15 min under laminar flow conditions, rinse 3X with SDW.

5. Remove apical 2 cm from sterilized stems, as well as any old, basal tissue (typically exhibiting white, lignified tissue) using sterilized No. 22 stainless steel surgical blades (Feather, Japan).
6. Cut remaining internode tissue transversally into 3-5 mm thick slices, again cut in half (= semi-cylindrical explants, 3-5 mm in length).
7. Place sections adaxial surface down onto SIM_{GH}.
8. All *in vitro* cultures are maintained at 25°C, 16 h photoperiod, light intensity = 45 μmol/m²/s¹ (PGF lamps).

In vitro regeneration and selection of putative transformants

1. Cut resulting shoots (at least 2 fully-developed leaves) that form from *in vitro* culture (described above) into Magenta-7 plant boxes (4 plants per box) on SRM_{GH} under the same growth conditions (described above). Rooting will occur within 1 week and ~8 cm plants will develop within ~60 days.
2. Cut TCLs (~200-500 μm thick, 1-1.5 mm in diameter) from *in vitro* plantlet stem 2nd or 3rd (from the apex) internode tissue of *in vitro* shoot cultures using sterilized Feather-S blades (0.1 mm thick, Feather, Japan). Place TCLs onto SIM_{IV}. Shoots derived from SIM_{IV} are cut with 3 nodes and placed onto SRM_{IV}. (See Note 1).
3. Cut resulting shoots (at least 3 fully-developed leaves) that form from greenhouse-or *in vitro*-derived explants on selective SIM_{IV} or SIM_{GV} and place on SRM_{IV} under the same growth conditions as described above (See Note 1).

Acclimatization

The most important step in the transformation process is the successful acclimatization and flowering (main target) of *in vitro* plants. Any *in vitro* plantlets (control or transgenic) are placed in a greenhouse, or phytotron, respectively. (See Note 2).

1. Wash rooted *in vitro* plantlets (=2 nodes, 4 leaves) under tap water, place in Metromix[®] (USA) potting soil at 25°C. Alternatively dip terminal 3-4 cm cutting with 3-4 nodes, denuded of leaves, into commercial rooting hormone (IBA) powder (Rooton[®], Japan) and place in Kanuma soil (river sand) at 25°C. The latter method is also ideal for the vegetative propagation of greenhouse material.
2. Acclimatize plantlets indoors for 2 weeks by covering with an aerated plastic bag, watering daily.

3. When plantlets are 6-8 cm tall, transfer to 11 cm diameter plastic pots with autoclaved 7:3 *masa*:compost mix. *Masa* (Jpn.) = decomposed granite. Pot 4 plants per pot (2l, 25 cm diameter, clay). Transplant rooted cuttings 1-2 weeks later and water with Hyponex® medium (6.5:19:3) once a week, all-year round.
4. For both control and transgenic plants, place at 15-25°C under long day conditions (4 h night-break from 22 pm to 2 am) in winter, or natural long days in summer to maintain the vegetative state. Induce flowering of any plant under short day conditions (10 h per day using black out screens or tunnel cover). (See Note 3). Water thoroughly once every 4-5 days in winter, or 1-2 days in summer. Add liquid fertilizer (Hyponex, 6:19.5:3) fortnightly to both vegetative and flowering plants.

Agrobacterium inoculation and SAAT, elimination of Agrobacterium

1. Culture a single colony of *A. tumefaciens* LBA4404 or AGL0 in 20 mL LB medium + 50 mg L⁻¹ kanamycin + 50 mg L⁻¹ rifampicin for 16-20 h at 27°C. Centrifuge 1 mL of broth culture, resuspended in 1 mL of 10 mM glucose + 100 mM AS. Adjust OD₅₄₀ to 0.4-0.5. Replate new colonies on same medium solidified by 7 g L⁻¹ agar (pH 5.7).
2. Preculture TCLs on filter paper overlying non-selective MS_{IV} for 2 days and apply bacteria at 10 µL per TCL (Fig. 3). Co-culture on non-selective MS_{IV} for 3 days co-cultivation (See Note 4). In the case of SAAT (Fig. 2), pre-culture TCLs or longitudinally-bisected stem internode explants on MS_{IV} for 24-36 h.
3. Place 10 TCLs in a 1.5 mL eppendorf tube containing 1 mL 10 mM glucose (or MS_{IV}) + 100 mM AS. Place tubes in a styrofoam float at 27°C in a water bath sonicator (Iuchi® Sonicator, Japan) at 60 Hz, for 2 or 5 min for TCLs or stem explants, respectively.
4. Following sonication, blot-dry TCLs on sterilized filter paper. Place on non-selective MS_{IV} for a 2 day co-cultivation period with *A. tumefaciens*.
5. Following the co-cultivation of any TCL with *A. tumefaciens*, or exposure to SAAT, place on SRM_{IV} + 30 mg L⁻¹ kanamycin + 250 mg L⁻¹ cefotaxime (Claforan®) for 1 week, then transfer onto fresh SRM_{IV} + 30 mg L⁻¹ kanamycin + 125 mg L⁻¹ cefotaxime fortnightly. Any shoot derived from the TCL or stem explant by direct organogenesis or via callus (Fig. 4) is placed on the same level of kanamycin and cefotaxime-supplemented SRM_{IV}.

GUS histochemical analysis

1. To measure β-glucuronidase (GUS) activity, incubate old (basal), middle-aged (mid-positioned) and young (terminal) leaf tissue (3 leaves from each point) from *in vitro* or *ex vitro* shoots in X-gluc + 20% EtOH overnight at 37°C in darkness^[10]. (See Note 5). Fix and bleach leaves in 70% EtOH for light microscopy and GUS transgene visualization and localization.

DNA extraction, PCR and electrophoresis

1. Extract DNA according to the CTAB method^[17] with a slight modification to reduce polysaccharides^[18]. Grind desired analysed tissue in liquid N₂. Transfer powder to 5 mL extraction buffer. Preheat to 65°C. Incubate at 65°C for 1 h with occasional swirling. Mix 2X gently with 5 mL 24:1 chloroform:isoamyl alcohol (v/v). Centrifuge samples at 5000 g for 10 min. Transfer supernatant to a fresh tube. Add 1-1.5X (v/v) isopropanol (-20°C). Precipitate DNA overnight at -20°C to increase yield. Pellet DNA at 1000 g, 10 min, 10°C (Perkin Elmer benchtop centrifuge). Resuspend genomic DNA in 200 µL TE with RNAase A (10 µg mL⁻¹) at 37°C for 1 h. Leave at RT for 30 min. Quantify DNA with a Shimadzu UV-1200 spectrophotometer and establish purity from 260:280 nm ratios. (See Note 6).
2. Positive controls for PCR are plasmid DNA extracted from overnight cultures of different *A. tumefaciens* strains by a small-scale, mini-prep alkali-SDS method. See above.
3. Perform PCR with TaKaRa La Taq® Hot Start Version PCR reagent kit using 0.1 µg DNA template and 5% tracking dye (v/v) in a Perkin Elmer GeneAmp PCR System 2400 thermocycler.
4. Electrophorese 15 µL of PCR products for ~7 cm at 50 V for 90 min or 100 V for 45 min through a 1X TAE, 1.5% agarose gel incorporating EtBr at 0.5 µg mL⁻¹. (See Note 7; Fig. 5 and 6). Repeat 3 times. Compare band sizes with a 100 bp marker ladder. Visualize DNA with a standard 320 nm UV transilluminator and photograph using Kodak 667 Polaroid film.

Rapid miniprep plasmid extraction

1. Transfer 1-1.5 mL of the culture (see above) to a 1.5 mL eppendorf tube and spin for 1 min at 12,000 rpm. Discard the supernatant. Remove the excess supernatant completely with a strip of sterile filter paper or micropipette.
2. Add 200 µL solution I (lysozyme) to each tube. Resuspend pellet completely by vortexing until cell

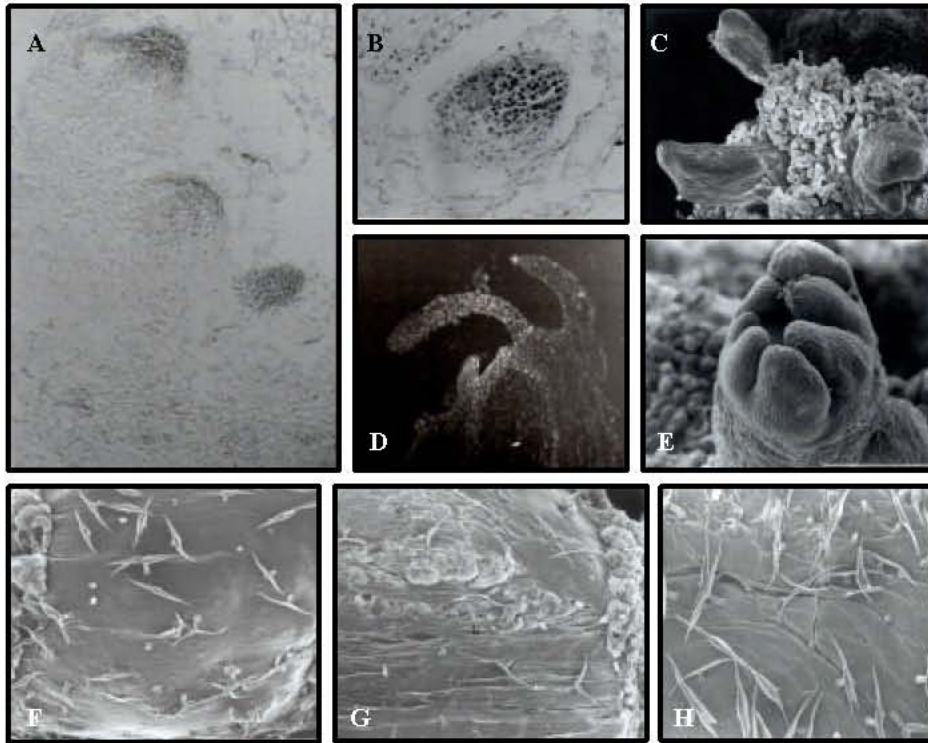


Fig. 2: SEM and light microscope pictures of chrysanthemum development *in vitro*. (A) cross-section and close-up (B) of somatic embryos forming from sub-epidermal tissues, eventually rupturing the epidermis and emerging from among the callus (C). Shoots similarly emerge from the sub-epidermal layers (D,E). A control stem explant prior to sonication (F), 5 min SAAT (G) and 20 min SAAT (H) showing progressive surface damage, but also increased access to inner, sub-epidermal cells by *Agrobacterium*

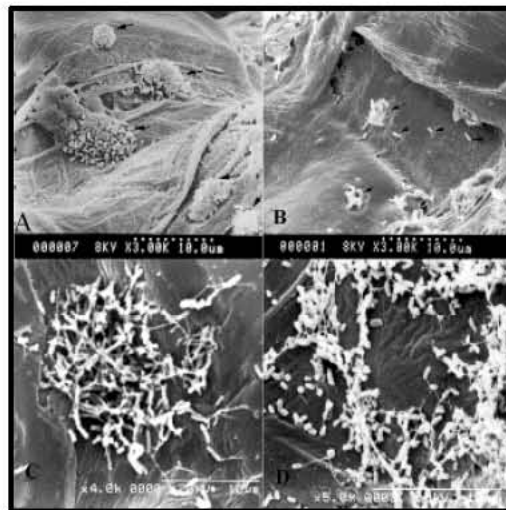


Fig. 3: SEM observation of *Agrobacterium tumefaciens* LBA4404 harbouring pKT3. *Agrobacterium* cluster on explants cocultivated on filter paper soaked with water (A) and water plus acetosyringone (B): many packed *Agrobacterium* clusters are observed and they are bound to the explant surface in a polar orientation. (C and D) *Agrobacterium* clusters on explants cocultivated on phytohormone-rich medium with acetosyringone. Only a few clusters are observed on the explant surface; the clusters aggregate and *Agrobacteria* cohere by cellulose fibrils in all directions

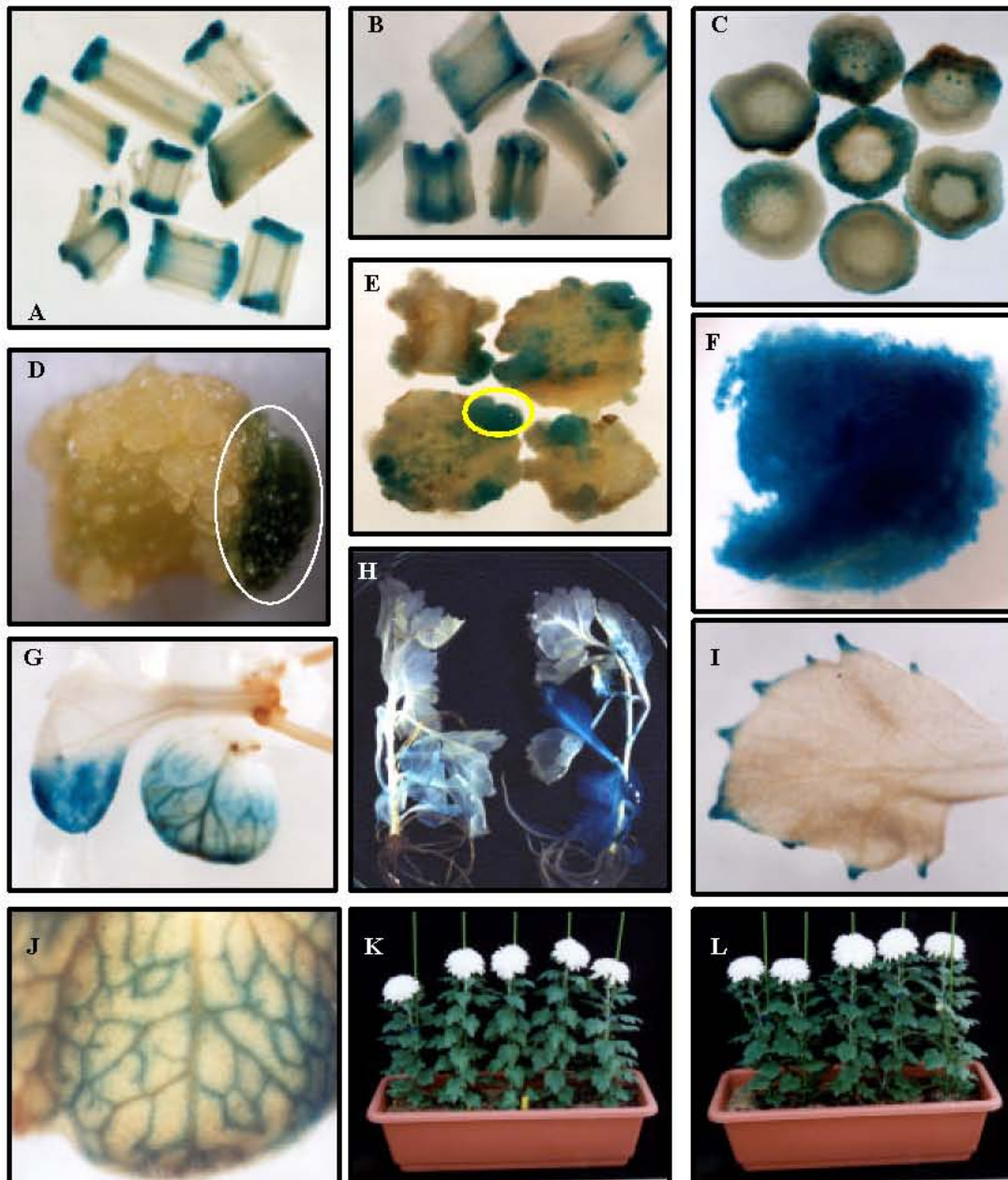


Fig. 4: *Agrobacterium* (AGL0-pKT3) *Shuhou-no-chikara* stem explants and subsequent regeneration of transgenic shoots from callus. *In vitro* stem explants (A) *Agrobacterium* infected or exposed to 5 min SAAT (B). *Agrobacterium* infected *ex vitro* stem explants (C). Green sector (transgenic callus circled in yellow; D) surviving on 30 mg/l kanamycin medium derived from *Agrobacterium* infection, confirmed by GUS (E). Transgenic sectors isolated from callus clusters, once isolated on selective SIM_{TY} form solid transgenic callus clusters (F). If by chance chimeric callus is isolated, chimeric transgenic plantlets arise (G). (H) chimeric transgenic (left) as opposed to full transgenic (right). Despite transgene (*uidA*) expression in all tissue ages, tissue-specific expression can be detected: leaf tips (I) and venation (J), the latter being characteristic of the CaMV-35S promoter. There are no visible differences in flowering or plant quality between control (K) and transgenic (L) *Shuhou-no-chikara* plants

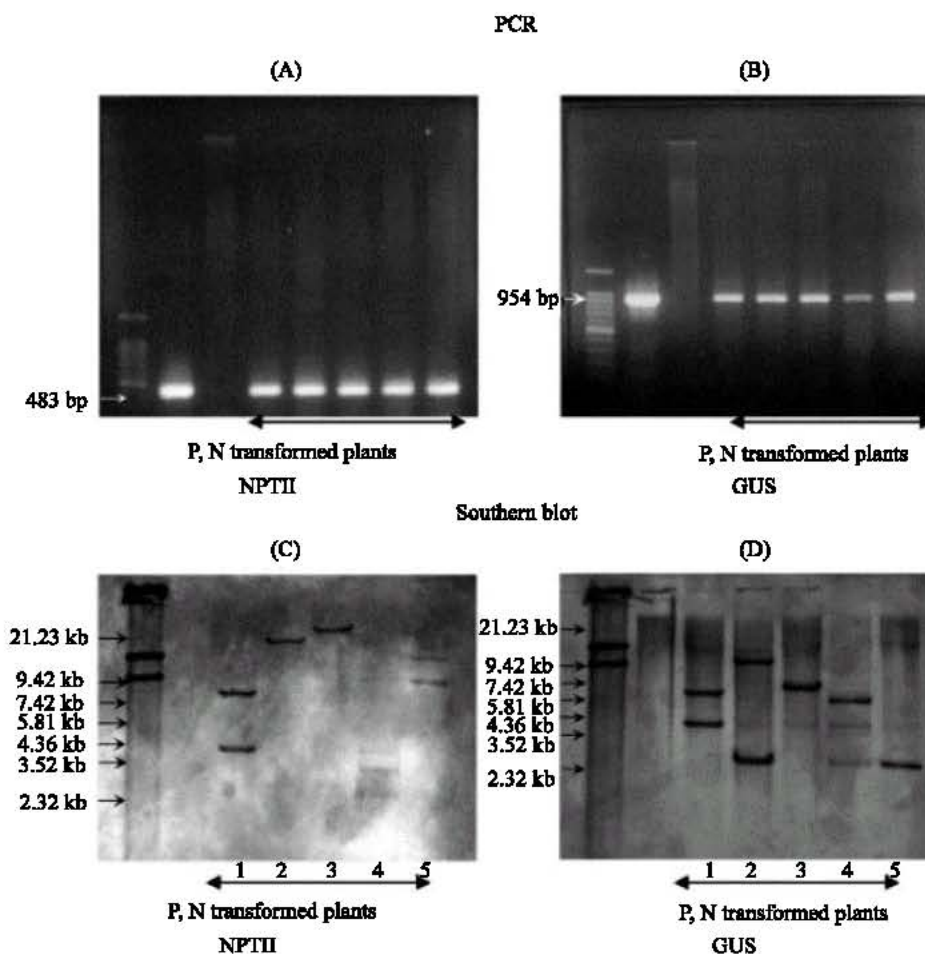


Fig. 5: (A) PCR amplification of a 438 bp fragment of the *nptII* gene. (B) A 954 bp fragment of the *uidA* gene. (C and D) Southern blot of DNA digested with *XbaI* and using the NPTII and GUS probe respectively (P = positive control: pKT3, N = negative control (non-transformed *in vitro* plant leaf)). Different transformed plants show different size fragments, indicating the insertion of different sized DNA by *A. tumefaciens*. Moreover, the same plant (e.g. #4) shows insertion of the *uidA* gene and not the *nptII* gene, highlighting that truncation of the inserted genes can occur, or other mechanisms such as transgene inactivation or silencing

suspension is homogeneous (critical). Incubate for 10-15 min at RT. Add 400 μ L solution II (alkali-SDS) and invert 20X to mix (do not vortex); the suspension will become clear and viscous. Incubate exactly for 5 min on ice. Add 300 μ L of 7.5 M $\text{CH}_3\text{COONH}_4$. Invert to mix well; flocky white precipitate should form. Maintain the tube at 0°C for at least 10 min to overnight.

3. Spin for 10 min at RT. Pour the clear supernatant into a clean 1.5 mL eppendorf tube. Delaying too long will allow chromosomes to resuspend. Add 500 μ L of isopropanol and incubate at RT for 10 min. Spin for 10 min at 12,000 rpm. Pour off supernatant and remove remaining supernatant with micropipette.

4. Add 1 mL cold 80% EtOH and spin for 3 min. Discard the supernatant (see above). Dry at 37°C to vaporize EtOH.
5. Resuspend pellet with 50 μ L TE. Store at -20°C for >1 year.

Southern blot hybridization

1. Concentrating DNA: if DNA needs to be concentrated, then add DNA to 0.3 M CH_3COONa 3H₂O. Mix well, place at -20°C for 2 h, then centrifuge at 15,000 rpm for 15 min, 4°C. Remove about 95% of the contents, never touching the sides of the eppendorf tube, which is where most of the DNA has sedimented. Invert tube gently for 3 min with 70%

- cold EtOH. Spin at 15,000 rpm for 3 min. Vacuum dry DNA. Add SDW to desired volume and loading dye.
2. Digest 10 µg of genomic DNA with desired RE at 37°C for 20 h and constant shaking. Electrophorese DNA products on a 0.8% SeaKem® GTG® agarose, 1X TAE gel at 4V cm⁻¹. Incorporate EtBr at 0.2 µg mL⁻¹.
 3. Probe preparation: Run PCR as described above, but substitute regular dNTPs with 4 X (v/v) DIG-labelled dNTPs.
 4. DNA blot: Assemble the capillary blotting apparatus using 1 N NaOH saline buffer (transfer buffer). Allow the DNA to transfer overnight (best 20 h) onto Hybond N+ nylon membranes (Amersham, UK). Wash the membrane for 1 min in SDW.
 5. DNA hybridization: dry membrane in oven at 80°C for 2 h and place in glass tube. Take care to place DNA side facing inwards. Add 30 mL pre-heated (60°C) HB into glass tube, rotate at 40 rpm for 3 h. Drain off PB (re-usable 2-3X).
 6. Digoxigenin (DIG) labelling: add probe (10 mL HB + 10 µg mL⁻¹ DIG PCR probe). Boil for 10 min, drain off HB and add to tube for 20 h at 68°C.
 7. Membrane wash: wash in 10 mL DEPC-QW for ~30 sec (2X); wash in 10 mL 20 X SSC : DEPC-QW (1 : 1) for 15 min at 68°C (3X); wash in 10 mL buffer 1 for 1 min at 25°C (2X); wash in 10 mL buffer 2 for 1 h at 25°C (1X); wash in 10 mL antibody solution for 30 min at 25°C (1X); wash in 10 mL buffer 2 for 15 min at 25°C (3X); in 10 mL buffer 3 for 1 min at 25°C (1X).
 8. Hybridization detection: Soak membrane in CDP-Star solution (1% in buffer 3) and develop (Fig. 5).
 9. Testing more probes: If the membrane is to be used to test other probe(s), wash membrane in 100 mL SDW for 5 min at 37°C (2X) to remove CDP-Star; wash in 100 mL 0.2 N NaOH buffer for 20 min at 37°C (2X); wash in 100 mL 2 x SSC for 5 min at 37°C (2X); prehybridize for 30 min in HB at 68°C; hybridize with new probe for 20 h at 68°C (after boiling probe for 10 min).
3. Transfer resolved proteins to an immunoblotting PVDF membrane (BioRad) using a transblot apparatus (Hoefer TE22 Mini Tank Transphor Unit, February 17, 2005 Amersham) at 200 mA for 1.5 h.
 4. Immerse membrane in blocking solution. Incubate for 1 h at 28°C. React transfer membrane overnight in a 1:500 dilution of rabbit *pac1*, 2,5-Aase or RNaseL monoclonal antibody (1° antibody) produced against its respective antigen^[20,21]. Wash membrane in TTBS and incubate in a streptavidin-biotinylated goat anti-rabbit alkaline phosphatase (GAR-AP, BioRad) 2°-antibody solution.
 5. Visualize banding with a colour development solution (10 min-3 h) until purple bands became visible. Compare band size against a Precision Prestained Standard (BioRad). Positive controls are: *pac1*-transformed *D. grandiflora* Regan and 2,5-A/RNaseL-transformed tobacco, *Nicotiana tabacum* Xanthi (Fig. 6).

Light Microscopy (LM) and Scanning Electron Microscopy (SEM)

1. For LM, leave samples in 70% EtOH for at least 2-3 days, pass through an ethanol/butanol series, embed in paraffin. To 100 µm using a microslicer (DTK1500, Dosaka Em), stain and view.
2. For SEM, fix explants in 30% FAA, 2 days, 50% FAA, 2 days, 70% FAA, 2 days. Dehydrate the fixed specimens through an EtOH/acetone series, critical point dry, coat with platinum and examine by scanning electron microscopy (SEM, Hitachi S-2150, Japan).

Flow cytometry

1. Isolate nuclei from 0.5 cm² of material (stem, TCL, shoot, callus) derived from 45-day-old shoot leaves (youngest two leaves) by chopping in a few drops of Partec Buffer A. Measure nuclear fluorescence at RT using a Partec® Ploidy Analyzer (Germany) after filtering the nuclear suspension through 30 µm mesh size nylon filter (CellTrics®) and adding five times of Buffer A for 1 min. Measure 3 samples and relative fluorescence intensity of the nuclei is analyzed when the CV<4%. A total of 2500 nuclei are counted for any sample.

Morphological scoring and transformation efficiencies

1. The percentage and position of GUS expression was recorded as the number of GUS focal points (GFPs) and blue-staining areas (BSAs) (Table 1).
2. Score transformation efficiency at the kanamycin-resistant (callus formation and rooting of shoots), GUS, PCR, Southern and Western (See Note 8) levels (Table 2).

SDS-PAGE and Western Blotting (adapted from Kirin Biotech, Japan):

General guidelines were followed^[19]:

1. Homogenize leaf tissue (equal mix of young (apical), middle-aged and old (basal) in protein extraction buffer + 1 mM phenylmethylsulphonyl fluoride (PMSF).
2. Centrifuge the homogenate at 15,000 g for 5 min at 4°C following boiling (denaturation) for 5 min. Separate supernatants by SDS-PAGE at 40 mA for ~1.5 h with 4-20% (RNaseL, *pac1*) or 10-20% (2,5-A) pre-prepared acrylamide gels (PAG-Mini, Daiichi, Japan) after the addition of loading buffer (TaKaRa®).

Table 1: Transient GUS transgene expression after SAAT (pKT3) for different time intervals of *D. grandiflora* Lineker and Shuhou-no-chikara stem TCLs

n=60 Sonication time ^{d)}	Lineker			Shuhou-no-Chikara		
	%GUS ^{a)}	%SRC ^{b)}	%ES ^{c)}	%GUS ^{a)}	%SRC ^{b)}	%ES ^{c)}
0s(1)	0	40	100	0	10	100
1s(1)	0	40	100	0	5	100
2s(1)	0	20	100	0	10	100
5s(1)	0	25	100	0	5	100
10s(1)	0	20	100	0	5	100
30s(1)	40	25	100	20	20	100
1m(1)	40	15	100	40	20	100
2m(1)	60	15	100	40	20	100
5m(1)	100	15	100	80	0	100
10m(1)	100	5	100	100	0	100
20m(1)	100	0	0	100	0	10
30m(1)	100	0	0	100	0	0
1m(6)	100	0	10	100	0	10
1m(12)	100	0	10	100	0	20
2m(6)	100	0	0	100	0	0
2m(12)	100	0	10	100	0	10
5m(6)	100	0	0	100	0	0
5m(12)	100	0	0	100	0	0
10m(6)	100	0	0	100	0	0
10m(12)	100	0	0	100	0	0
20m(6)	100	0	0	100	0	0
20m(12)	100	0	0	100	0	0

a) %GUS = Percentage of Lineker (spray-type) and Shuhou-no-Chikara (standard-type) explants exhibiting blue staining (to any degree). b) %SRC = shoot regeneration capacity i.e. the percentage of explants forming shoots. c) %ES = Percentage of explant survival after 2 weeks on selective (kanamycin 30 mgL⁻¹) medium. d) Sonication time has two parameters: x(y) where x = sonication time (s = sec; m = min) and y = co-cultivation period in days following sonication. GUS was measured after 48h

Table 2: Transformation efficiencies (%) at different stages of the transformation process from an initial explant number, n = 500

Plasmid	Gene	Growth reaction		GUS		PCR		Southern		Western	
		Callus ¹	Rooting ²	IV ³	GH ⁴	IV ⁵	GH ⁶	IV ⁷	GH ⁸	IV ⁹	GH ¹⁰
Control	None	0	0.03±0.01	-	0	0	0	0	0	0	0
pBI121	<i>nptII</i>	6	0.63±0.12	-	-	48	4	6	1	-	-
	<i>uidA</i> (GUS)			18	2	62	6	4	0	-	-
pKT2	<i>nptII</i>	18	1.21±0.22	-	-	16	8	6	1	-	-
	<i>uidA</i> (GUS)			16	6	12	7	6	0	-	-
pKT3	<i>nptII</i>	31	2.02±0.31	-	-	22	8	8	2	-	-
	<i>uidA</i> (GUS)			21	8	21	8	8	1	-	-
pKT66	<i>nptII</i>	13	1.86±0.41	-	-	8	2	4	4	-	-
	<i>RNase-L</i>			-	-	6	1	3	1	0	0
	<i>2,5-A</i>			-	-	6	1	3	1	0	0
pKT61	<i>nptII</i>	18	1.43±0.26	-	-	15	3	14	2	-	-
	<i>RNase-L</i>			-	-	15	3	14	2	0	0
	<i>pac-1</i>			-	-	15	3	14	2	14	14
	<i>2,5-A</i>			-	-	14	2	14	2	2	2
pKT2-L- <i>Pac 1-1</i>	<i>nptII</i>	21	1.37±0.28	-	-	12	3	12	3	-	-
	<i>pac-1</i>			-	-	11	1	10	1	18	14
pKT2-MF- <i>Pac 1-1</i>	<i>nptII</i>	7	0.71±0.16	-	-	10	2	8	1	-	-
	<i>pac-1</i>			-	-	10	2	6	0	6	2

Transformation efficiency = (Y/Z) X 100%, where Y = number of initial explants (500) and superscripts are Y, where Y, having the following meanings: 1 = % explants forming callus on 30 mg L⁻¹ kanamycin on SIM_{IV}; 2 = number of shoots (average±SE) rooting on 30 mg L⁻¹ kanamycin on SRM_{IV}; 3 = % GUS positive *in vitro* plants (old, middle-aged and young leaves staining positive for GUS) growing on 30 mg L⁻¹ kanamycin on SIM_{IV}; 4 = % GUS positive plants (old, middle-aged and young leaves staining positive for GUS) 2 months after growing in the greenhouse; 5 = % *in vitro* plants on 30 mg L⁻¹ kanamycin on SRM_{IV} (mix of old, middle-aged and young leaf DNA giving positive PCR band); 6 = % greenhouse plants (mix of old, middle-aged and young leaf DNA giving positive PCR band); 7 = % *in vitro* plants on 30 mg L⁻¹ kanamycin on SRM_{IV} (mix of old, middle-aged and young leaf DNA giving positive Southern band); 8 = % greenhouse plants (mix of old, middle-aged and young leaf DNA giving positive Southern band); 9 = % *in vitro* plants on 30 mg L⁻¹ kanamycin on SRM_{IV} (mix of old, middle-aged and young leaf DNA giving positive Western band); 10 = % greenhouse plants (mix of old, middle-aged and young leaf DNA giving positive Western band). (See Note 9)

Notes

1. TCLs have been shown to be useful in controlling desired organogenesis, more than conventional explants where multiple organogenic programmes (shoot, root, callus, somatic embryo) may arise^[22-24].

These programmes (Fig. 2) may be induced by numerous media with single or multiple PGR applications. Only the ideal medium is presented here. TCLs also eliminate genotype-dependence, as seen with several spray and standard-type cultivars.

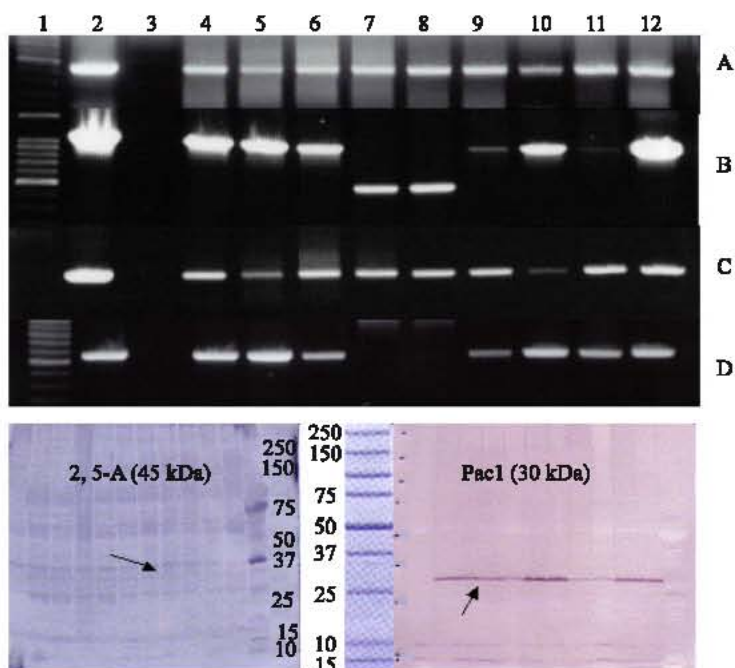


Fig. 6: PCR amplification of genes from either LBA4404 (pBI121, pKT2, or pKT66) or AGL0 (pKT3, pKT61, pKT2-L-*Pac1*, or pKT-MF-*Pac1*) *Agro*infected TCLs. A 438 bp fragment of the *nptII* gene (A), a 1021 bp fragment of the *pac1* gene (B), a 3.2 Kbp fragment of the *RNaseL* gene (C), a 548 bp fragment of the *2,5-A* gene (D). Lanes: 1 = 100 bp Ladder, 2 = pKT61 *Pac1*+ (plasmid, positive control), 3 = -ve control chrysanthemum Shuhou-no-chikara (SNC), 4 = tobacco Xanthi *Pac1*+ (plant, positive control). Lanes 5-8 = Shuhou-no-chikara *in vitro* transformants: 5 = MF-*Pac1*-1, 6 = MF-*Pac1*-2, 7 = pKT61-30, 8 = pKT61-31. Lanes 9-12 = Shuhou-no-chikara greenhouse transformants, 9 = pKT-MF-*Pac1*-1, 10 = pKT-MF-*Pac1*-2, 11 = pKT61-30, 12 = pKT61-31. Two lowest gels indicate Western blots of 2,5-A (left) and *Pac1* (right) in pKT61 showing the 45 and 30 KDa band for transgenic expression, whose levels clearly fluctuate (1-4-fold) in *Pac1*

2. Transgenic plants are placed in isolation in a phytotron since there may be the risk of pollen transfer to wild or other cultivated plants.
3. No additional lighting is required in summer but long days are provided in winter by high pressure sodium lamps (Philips HPS 400W) resulting in an average PAR of 50-200 $\mu\text{mol m}^{-2}\text{s}$ (depending on transmission percentage), although ideally the daily PAR light integral (measure with a Kipp solarimeter) should be 15.42 MJ m^{-2} . To aid spray development interrupt short days by exposing the plants to 10 long days, then place them back under short day conditions for normal flower development.
4. In the case of AGL0, co-cultivation should not exceed 3 days but can be for 4 days for LBA4404. *A. tumefaciens* shows different patterns of growth on TCLs cultured with supplementary AS on filter paper and in the presence or absence of PGRs: the presence of PGRs strongly enhances *Agro*infection (Fig. 3) while the presence or absence of filter paper affects both the effectiveness of regeneration and *Agro*infection^[25]. There exists a fine balance between explant survival, shoot regeneration capacity and % blue staining areas depending on the sonication time (Table 1 and Fig. 2). Pre-and co-culture of TCLs should be on non-selective MS_{IV} .
5. 20% EtOH is added to remove background, intrinsic plant GUS activity.
6. Commercial kits, DNeasy® (Qiagen, USA), Plant DNAzol® Reagent (GibcoBRL, USA) or Isoplant II® (Wako, Japan) are all effective, but differ in purity and recovery.
7. Equally good band resolution is achievable at 0.8-3% agarose.
8. Transformation frequencies (Table 2) are calculated as described above.
9. A decrease in the explant induction or shoot regeneration capacity of explants may reflect the inhibitory nature of *Agrobacterium* on shoot induction. T-DNA inserts from the left border to the right border into the host genome by preferential attachment to A-T-rich regions and by forming a

short DNA duplex^[26]. Consequently, higher *nptII* transformation efficiencies (relative to other transgenes within the same cassette) at most levels (PCR, Southern), reflect a partial or incomplete insertion of the transgene cassette into the plant genome and subsequent lower translation efficiencies (Western). The inverse is observed in pBII 21, where the *nptII* gene is near the right border, thus lower transformation efficiencies are reported for *nptII*. In the case of *RNaseL* and *2,5-A* genes, it appears as if partial insertions resulting in smaller than expected size fragments in PCR (Fig. 6) are the cause of lower transformation efficiencies at the Southern and Western levels. Another viable explanation, although not tested, is that transgene silencing mechanisms at the post-translational levels may be operating^[8,10] for these two genes, since the *pacI* gene, sandwiched between them, continues to have high Western expression levels (Fig. 6).

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