

Establishment of an efficient genetic transformation system for *Tanacetum cinerariifolium*

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Abstract

The Dalmatian Daisy *Tanacetum cinerariifolium* is an Asteraceae plant species that produces the natural insecticide “pyrethrum”, which is effective against mosquito disease vectors and household pests. To enhance the content of pyrethrum in flowers, a more detailed understanding of the mechanisms underlying pyrethrum biosynthesis is needed. Even though gene transformation and genome editing techniques are vital for investigating pyrethrin biosynthesis, limited information is available on the transformation of *T. cinerariifolium*. Furthermore, each seedling possesses a distinct genotype with large variations by self-incompatibility. We herein employed *T. cinerariifolium* line #14 with weak self-incompatibility to establish a protocol of efficient regeneration from leaf segments and transformation. Leaf segments formed calli on 1/2 Murashige and Skoog’s basal medium (MS) with naphthalene acetic acid 1 mg L⁻¹ and 6-benzylaminopurine (BAP) 2 mg L⁻¹, regenerated shoots from calli on 1/2 MS with BAP 0.5 mg L⁻¹ and GA₃ 0.2 mg L⁻¹, and elongated shoot stems on 1/2 MS with indole-3-butyric acid 0.5 mg L⁻¹ and BAP 0.5 mg L⁻¹. To establish genetic transformation, *Rhizobium radiobacter* strain EHA105 with the highest infectivity and the *mas1*’2’ bidirectional promoter with the highest expression were used, and the antibiotic G418 was added to medium at a concentration of 10 to 20 mg L⁻¹ to select transformed cells. By using established regeneration techniques, we successfully obtained transformants that highly expressed the transgene *gusA*. This technique allowed us to elucidate the mechanisms underlying pyrethrum biosynthesis in *T. cinerariifolium* and was useful for creating transgenic *T. cinerariifolium* with increased pyrethrum biosynthesis.

Key messages

A reproducible genetic transformation system is not yet available for *Tanacetum cinerariifolium*. We created a transformation technique using a *Rhizobium radiobacter* strain and a vector for high gene expression.

Introduction

The Dalmatian Daisy *Tanacetum cinerariifolium* is an Asteraceae plant species that produces pyrethrins, which are natural insecticides. Its dried flower extract “Pyrethrum” exhibits high knockdown activity against various pest insect species by modulating neural transmission (Matsuda et al. 2005; Lybrand et al. 2020; Varga et al. 2022). Pyrethrum is effective against mosquito disease vectors and household pests. The active components pyrethrins modulate voltage-sensitive sodium channels, thereby inducing repetitive firing and the subsequent blockade of neural conduction (Narahashi 1992). Recent studies reported that pyrethrins interacted with odorant receptors in antennae and induced repellency in flying insects (Liu 2021; Wang 2021). While the sale of pyrethrins is increasing, supply is limited, as is the case with natural products of plant origin that are subject to changes in temperature and rain fall as well as disease infection and herbivory. A more detailed understanding of the mechanisms underlying pyrethrin biosynthesis is needed to increase pyrethrin contents in flowers.

The genome of *Tanacetum cinerariifolium* is principally diploid, but may become triploid and tetraploid by repeated crossing. The genome size is ~ 7 Gb and its sequence has been partially elucidated (Yamashiro et al. 2019). Several biosynthetic enzymes have been identified. However, the roles of most gene products and their regulation remain largely unknown. Self-incompatibility and the difficulties associated with genetically modifying this species are obstacles to elucidating the mechanisms underlying pyrethrin biosynthesis.

Previous studies reported methods for tissue cultivation (Roest and Bokeimann 1973; Aoki et al. 1976; Cashyap et al. 1978; Hitmi et al. 1999; Hedayat et al. 2009) and genetic transformation (Mao et al. 2013). However, these

methods have not been used in the development new transgenic lines of *T. cinerariifolium*, which may be attributed to its self-incompatibility and each seedling possessing a distinct genotype with large variations (Bhat and Menary, 1986; Parlevliet, 1974). Furthermore, shoot regeneration competence varies among genotypes in tissue cultures (Teixeira da Silva, 2003). Therefore, the development of an easy, reproducible and efficient genetic transformation technique is needed. In the present study, we created a protocol for the efficient transformation of *T. cinerariifolium* material #14 via *Rhizobium radiobacter* (*Agrobacterium tumefaciens*).

Materials and Methods

Plant material and preculture conditions

Pyrethrum (*Tanacetum cinerariifolium*) #14 with weak self-compatibility, which was found at Innoshima Island in Hiroshima, Japan, was used in experiments. Seeds were surface-sterilized by briefly dipping in 70% ethanol for thirty seconds and then in a 1% sodium hypochlorite solution (including 1% Tween 20) for 15 min, followed by rinsing 3 times for 10 min in sterile distilled water. Sterilized seeds were sown in plastic Petri dishes (10 cm in diameter) on Murashige and Skoog's (1962) basal medium (Murashige and Skoog, 1962) (MS; containing 3% sucrose). One week later, germinated seeds were replanted in plant culture boxes (Bio Medical Science, Co., Japan) containing four different media: MS (containing 3% sucrose), 1/2 MS medium containing half-strength inorganic nutrients (1/2 MS; containing 1.5% sucrose), White medium containing 2% sucrose (White 1963), and Gamborg's B5 medium containing 2% sucrose (Gamborg et al. 1968). Each medium was adjusted to pH 5.8 and 0.3% Gellan Gum (Gel.) (Wako Pure Chemical Industries, Ltd., Japan) or 0.8% Agar (Wako Pure Chemical Industries, Ltd., Japan) was added prior to autoclaving at 120°C for 15 min. Cultures were kept at 25°C under a 16-h photoperiod using cool white fluorescent lamps or at 25°C in darkness. The lamps provided a photosynthetic photon flux [PPF (400-700nm)] of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After two months, rooting frequency, plant height, and root length were measured. Ten germinated seeds were replanted in plant culture boxes containing the respective media and data were collected on 10 replicates.

Callus induction and regeneration

The effects of the material leaf growth stage and combination of phytohormones on the efficiency of regeneration were examined. Leaves in the following growth stages were used in this experiment: Age#1, leaves in the second week after expanding (leaf length of 1 to 1.5 cm); Age#2, leaves in the first week after expanding (leaf length of approximately 8 mm to 1 cm); and Age#3, leaves just expanding (leaf length of 3 to 5 mm) (See Figure 1). The phytohormone 6-benzylaminopurine (BAP) as a cytokinin and 1-naphthylacetic acid (NAA) as an auxin were used as regulators of regeneration from leaf segments. Leaf segments were inoculated on 1/2 MS solid medium supplemented with 9 combinations of BAP (0.5, 1.0, and 2.0 mg L^{-1}) and NAA (0.1, 0.5, and 1.0 mg L^{-1}). After three weeks, the number of leaf segments that formed calli or shoots was counted. The green callus (length <1 mm) were inoculated on seven different regeneration medium (1/2 MS solid medium + 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA; 1/2 MS solid medium + 0.5, 1.0, and 2.0 mg L^{-1} BAP; and 0.1 and 0.2 mg L^{-1} GA₃) and each shoot length was measured three weeks after replacement. Twelve leaf fragments or green calli were placed in Petri dishes containing the respective media and data were collected on 10 replicates.

Elongated shoots (length >3 mm) were placed in four different stem elongation media: 1/2 MS solid medium + BAP (0.0 and 0.5 mg L^{-1}) and indole-3-butyric acid (IBA; 0.5 and 1.0 mg L^{-1}). Shoots that formed elongated stems

were placed in phytohormone-free 1/2 MS solid medium for rooting. Each medium contained 1.5% sucrose, except for stem elongation and rooting media, which contained 1%. pH was adjusted to 5.8 and 0.3% Gel. was added prior to autoclaving at 120°C for 15 min. Cultures were placed at 25°C under a 16-h photoperiod using cool white fluorescent lamps or at 25°C in darkness. The lamps provided PPF (400-700 nm) of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Four shoots were replanted in plant culture boxes containing the respective media and data were collected on 10 replicates.

Effects of *Rhizobium* strains and promoters on efficient gene insertion, selection, and stable expression of foreign genes.

R. radiobacter viz. EHA105 (provided by Dr. L. S. Melchers, Zeneca Mogen) and LBA4404 (Ooms et al. 1982) were used in experiments. Four different binary vectors were used: pBIK201G (provided by Dr. H. Ichikawa, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization: NARO), pBI121 (Jefferson et al. 1987), and pBE2133 and pBE7133 (provided by Dr. I. Mitsuhashi, NARO) (Mitsuhashi et al. 1996) (see Figure 2). In each vector, the *gusA* gene was controlled by a different promoter. In binary vector pBIK201G, the *gusA* gene and selectable marker gene *npt* were driven by the novel bi-directional promoter fragment (465 bp) for the *mannopine synthase-1'* and *-2'* (*mas1'-2'*) genes from *R. radiobacter* strain AtC1 (MAFF301276) provided by the NIAS Genebank (Tsukuba, Japan). In binary vector pBI121, the *gusA* gene was driven by the promoter region of the *Cauliflower mosaic virus 35S* RNA (CaMV 35S) promoter, which has been widely used as a constitutive strong promoter in plant transformation. In binary vector pBE2133 (Mitsuhashi et al. 1996), the *gusA* gene was driven by the E12 Ω promoter with two tandem repeats of the 5'-upstream sequence of the CaMV 35S promoter (-419 to -90), the G-free sequence (Ω sequence) in the 5'-untranslated region of *Tobacco mosaic virus*. In binary vector pBE7133 (Mitsuhashi et al. 1996), the *gusA* gene was driven by the E7 Ω promoter with seven tandemly repeated enhancer-like elements (-90 to -290, relative to the site of the initiation of transcription as +1) of the CaMV 35S promoter (E7) and the Ω sequence. In pBE2133 and pBE7133, GUS activity levels were 20- to 70-fold higher than those with the 35S promoter in pBI121 in Tobacco (*Nicotiana tabacum* cv. Samsun NN) and rice (*Oryza sativa* cv. Nipponbare) protoplasts (Mitsuhashi et al. 1996). In pBI121, pBE2133-GUS, and pBE7133-GUS, the selectable marker gene *npt* was driven by the nopaline synthase (*nos*) promoter from *Rhizobium* Ti plasmids (Bevan et al. 1983)

Each binary vector was transformed into *Rhizobium* strains EHA105 and LBA4404 using the triparental mating method (Ditta et al. 1980).

***Rhizobium* infection**

The genetic transformation procedure reported by Shinoyama et al. (2002) was used with minor modifications (Table 1). *R. radiobacter* was cultured for 4 to 5 hours in YEP liquid medium on Bio-Shaker BR-3000L (TAITEC, Japan) at 28°C under dark conditions. Leaf segments were prepared by cutting with a scalpel, as shown in Figure 1. They were immersed at room temperature for 15 min in Infection medium [1/2 MS liquid medium containing 5% Tween 20 (Nacalai Tesque, Japan) and 50 μM acetosyringone] with *Rhizobium* (final OD₆₆₀ = 0.1). After immersion, leaf segments were placed onto co-cultivation medium [1/2MS solid medium + 1.0 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP (Callus formation solid medium; CS) containing 1.0 g L⁻¹ casamino acid] and co-cultivated at 25°C in darkness for 2 days.

Optimization of cefotaxime and G418 concentrations for *Rhizobium* elimination and transformant selection

Two days after infection, leaf fragments were transferred to Bacteria elimination liquid medium (Table 1) supplemented with each concentration of cefotaxime sodium salt (250 and 500 mg L⁻¹) to 1/2 MS liquid medium containing 1.0 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP (Callus formation liquid medium; CL) with shaking at 25°C overnight under a 16-h photoperiod (Table 1). They were then placed in Bacteria elimination solid medium (CS + 100 mg L⁻¹ cefotaxime sodium salt). Fourteen days after infection, leaf segments were transferred to Bacteria elimination liquid medium (CL + 100 mg L⁻¹ cefotaxime sodium salt) with shaking at 25°C overnight under a 16-h photoperiod. They were then placed in Selection medium (CS + 100 mg L⁻¹ cefotaxime sodium salt and 10 or 20 mg L⁻¹ G418) for the selection of putative transformed callus. Leaf fragments were transferred to new medium every 14 days. The number of green calli was counted after transplanting to the selection medium twice at an interval of 14 days.

Analysis of *gusA* gene expression

Leaf explants, calli, regenerated shoots, and regenerated plants were confirmed by the GUS histochemical assay according to Jefferson (1987) and Murakami and Ohashi (1992). Calli and regenerated shoots were incubated in 50 mM phosphate buffer (pH 7.2) containing 1 mM 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc), 5 mM dithiothreitol (DTT), 0.3% Triton X-100, 5% methanol, 0.5 mM potassium ferrocyanide, and 0.5 mM potassium ferricyanide at 37°C overnight. After staining, calli and regenerated shoots were decolorized with 99% ethanol for the extraction of chlorophyll. GUS activity in transgenic callus was analyzed by the fluorometric method reported by Kosugi et al. (1990) with 4-methyl-umbelliferyl glucuronide as the substrate. Twelve leaf segments were used in the GUS assay (number of blue spots) and 10 replications were performed. In the GUS activity test, ten calli and shoots were used and data were collected on 10 replications.

DNA extraction and polymerase chain reaction (PCR) analysis of the *nptII* gene and *gusA* gene

Total DNA was extracted from 100 mg of the fresh young leaves of regenerated or control plantlets by the method of Takagi *et al.* (1993). Leaves were homogenized in liquid nitrogen using a ceramic mortar and pestle and suspended in 1 ml of HEPES buffer [0.1 M HEPES (pH 8.0), 0.1% PVP, and 4% 2-mercaptoethanol]. After centrifugation at 15,000 rpm at 4°C for 5 min, the supernatant was discarded and the pellet was suspended in new HEPES buffer. This procedure was repeated 3 times to remove polyphenols and polysaccharides. Total DNA was isolated from the pellet by the sodium dodecyl sulfate extraction method reported by Honda and Hirai (1990). PCR was performed using 200 ng genomic DNA, 1 U recombinant Taq DNA Polymerase (TaKaRa Taq™, Takara Bio, Japan), 0.5 mM of specific forward and reverse primers, 0.2 mM dNTPs, and PCR buffer in a final volume of 20 µL. A PCR analysis was performed using the forward primer 5'-ATGTTACGTCCTGTAGAAAC-3' and reverse primer 5'-TTCATTGTTTGCCTCCCTGC-3' for the *gusA* gene (1,811 bp), and the forward primer 5'-ATGATTGAACAAGATGGATT-3' and reverse primer 5'-TCAGAAGAACTCGTCAAGAA-3' for the *npt* gene (795 bp). Amplification was performed in Mastercycler® ep (Eppendorf SE, Japan) under the following conditions: pre-denaturation at 94°C for 2 min, followed by 30 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 1 min), and finally at 72°C for 5 min followed by an incubation at 4°C. PCR products were separated on 1.0% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV for documentation.

Southern blot hybridization

Total DNA was extracted from 100 mg of fresh young leaves of the transformed lines and non-transformed control plants according to Shinoyama et al. (2002). A 25-µg aliquot of DNA digested with *Xba*I was subjected to

electrophoresis and blotted onto a Hybond N+ nylon membrane (Sativa, USA). Southern blot hybridization (Southern, 1975) was performed using a digoxigenin (DIG)-labeled *nptII* fragment (approximately 800 bp) as a probe (Figure 2) and a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Germany) according to the supplier's instructions.

Statistical analysis

Data obtained from all experiments were presented as the mean $\pm$ SD and separation was performed using Turkey-Kramer's HDS. Percentage data were transformed to arcsine data and statistical analyses were performed.

Results

Plant material and preculture conditions

Ten germinated seeds of *T. cinerariifolium* were replanted in eight different media and the growth of seedlings was examined. The rooting frequency ranged between 28.0 ± 0.4 and $80.0 \pm 0.0\%$ and was the highest in 1/2 MS + Gel. medium. Plant heights ranged between 14.8 ± 0.2 and 27.4 ± 1.6 cm, and were significantly higher on MS + Gel., MS + Agar, and 1/2 MS + Gel. medium than on other media. Root lengths ranged between 31.7 ± 0.7 and 52.4 ± 3.4 mm, with the highest being measured on Gamborg's B5 + Gel. (Table 2).

Green callus induction and regeneration

Age #2 leaves were initially used as plant material for phytohormone combination tests. Green calli formed on the edge of segments after 3 weeks and some adventitious shoots formed on calli after 4 to 5 weeks. The number of green callus/shoot-forming leaf segments was measured and the green callus/shoot-forming leaf segment ratio per number of leaf segments tested was calculated (Table 3). The green callus formation ratio was significantly higher at the 1% level on media containing 1.0 mg L^{-1} NAA + 0.5, 1.0, and 2.0 mg L^{-1} BAP and that containing 0.5 mg L^{-1} NAA + 1.0 mg L^{-1} BAP. Shoots formed on media containing 0.5 mg L^{-1} NAA + 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP, with no significant difference in the formation ratio of both. Differences in the age of the leaves used as material (Age#1, #2, and #3) were examined using the medium with the highest callus formation ratio, namely, 1/2 MS + 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP medium. Formation rates were significantly higher for Age#2 and #3 than for Age#1. No significant difference was observed between Age#2 and #3 (Table 4).

Green calli (length < 1 mm) were transferred to regeneration medium for shoot regeneration. The longest elongated length of 5.8 ± 4.0 mm was observed at 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} GA₃. In the combination of BAP and GA₃, the elongation length slightly decreased with at higher concentrations of BAP and increased at higher concentrations of GA₃. Shoots of approximately 3 mm were replanted in plant culture boxes with air vent membranes (Milliseal Membrane Seal, FWMS01800, Merck KGaA, Germany) and containing four different stem-elongating media (1/2 MS solid medium + BAP 0.0 and 0.5 mg L^{-1} , IBA 0.5 and 1.0 mg L^{-1} , respectively). Two weeks after plating, all shoots on 1/2 MS medium containing only 1.0 mg L^{-1} of IBA turned brown, whereas those placed on other media grew and formed stems (Figure 4). Four weeks after plating, the medium with 1.0 mg L^{-1} IBA + 0.5 mg L^{-1} BAP resulted in the highest green callus formation ratio (Table 4). Shoots with elongated stems placed in phytohormone-free 1/2 MS medium showed rooting (Figure 5).

Effects of *Rhizobium* strains and promoters for efficient insertion into the *T. cineraria* genome, selection, and stable expression of foreign genes

To evaluate *Rhizobium* infection ability and promoter activity, a histochemical assay for the *gusA* gene was performed three days after infection. Among the *Rhizobium* lines tested, the number of GUS spots was significantly higher at the 1% level for EHA105 than for LBA4404 for all promoters (Figure 6a). GUS activity levels were analyzed using ten transgenic calli induced on selection medium containing 10 mg L⁻¹ G418. On both calli and shoots, GUS activity was the highest at the *mas1'-2'* promoter carried by pBIK201, followed by the EI2Ω promoter carried by pBE2113 and the E7Ω promoter carried by pBE7133, and was the lowest at the 35S promoter carried by pBI121 (Figure 6b and 6c). GUS activity at the *mas1'-2'* promoter in pBIK201G was approximately 6-fold higher than that at the 35S promoter in pBI121. No significant difference was observed in GUS activity between the EI2Ω promoter and E7Ω promoter. Furthermore, GUS activity did not significantly differ among different *Rhizobium* strains with the same promoter. According to the histochemical GUS assay, chimeric expression was not detected in callus or adventitious shoots (Figure 6d).

Optimization of G418 concentrations for the efficient selection of transgenic lines

The elimination of *Rhizobium* performed 2 days after infection was possible at 250 or 500 mg L⁻¹ of cefotaxime sodium salt in Bacteria elimination medium A and *Rhizobium* did not subsequently appear on the medium. Leaf segments 14 days after *Rhizobium* infection were replanted in selection medium containing 10 or 20 mg L⁻¹ G418, and the number of calli was counted after transplanting to the selection medium twice at an interval of 14 days. The regeneration ratio was slightly higher on 500 mg L⁻¹ than on 250 mg L⁻¹ of cefotaxime sodium salt and higher on 10 mg L⁻¹ than on 20 mg L⁻¹ of G418. The highest regeneration ratio was 9.5% in the area with 500 mg L⁻¹ of cefotaxime sodium salt and 10 mg L⁻¹ of G418 (Table 7). No band was detected in control plants by the PCR analysis. An approximately 800-bp single band of *nptII* and 1,800-bp single band of *gusA* were detected for some regenerated plants, but not for others (Supplementary Figure S1). Selection with 20 mg L⁻¹ of G418 resulted in fewer regenerated plants than that with 10 mg L⁻¹; notably, all of the regenerated plants had transgenes (i.e. escapes) (Table 7). The presence and number of T-DNA copies in individual regenerated plants were confirmed by the Southern blot analysis of *Xba*I-digested genomic DNA probed with a fragment of *nptII*. No hybridization signals were detected in non-transgenic *T. cinerariifolium* controls. Single to multiple bands hybridizing to the *nptII* probe were observed in regenerated plantlets, while no hybridization signals were detected in regenerated plants (Figure 7a), which was consistent with the results of the PCR analysis (Figure S1). The GUS assay on young leaves not fully expanded and the elongated roots of individuals confirmed the expression of the *gusA* gene (Figure 7c1 and c2) only in the transformants (Figure 7b1 and b2).

Discussion

The callus and adventitious shoot formation technique is very useful for clonal propagation and the production of genetically modified plants. In *T. cinerariifolium*, Hedayat et al. (2009) successfully achieved regeneration via direct organogenesis from leaves. In the present study, we used *T. cinerariifolium* line #14 with weak self-compatibility; however, difficulties were associated with the regeneration of this line from tissue due to browning (particularly leaf segments) during the tissue culture and a poor rooting ability from the shoots. Edible plants belonging to the Asteraceae family, such as burdock (*Arctium lappa* L.), Japanese butterbur (*Petasites japonicus*), Japanese mugwort (*Artemisia indica* var. *maximowiczii*), and dandelion (*Taraxacum* genus), undergo significant browning

during cooking and processing (Han 2008). Regarding browning components, Nakamura et al. (1968) identified polyphenols as chlorogens in burdock root, butterbur, and dandelion. Panozzi et al. (1954) isolated an isomer of chlorogenic acid, cynarin (1.4-dicaffeoyl quinic acid), from *Cynarascolymus*. Han (2008) demonstrated that Asteraceae vegetables contained chlorogenic acids as the main polyphenols, but at various contents, and that the enzymatic browning of Asteraceae vegetables was caused by the oxidative reaction of chlorogenic acids. Based on these findings, we successfully reduced the browning of leaf segments using a shaking culture for bacteria elimination and performing fast selection, which was attributable to reduced PPO in tissues by the shaking culture.

Hedayat et al. (2009) successfully elongated shoots using BA-free B5 medium containing NAA only; however, this method did not work for shoot elongation from the #14 line. We found that the #14 line was rooted by the formation of fully elongated stems with IBA and BAP, which were then transferred to phytohormone-free 1/2 MS medium (Table 6 and Figure 5). This rooting ability may also be one of the differences in genetic backgrounds because of their self-incompatibility (Bhat and Menary, 1986; Parlevliet, 1974, Teixeira da Silva, 2003).

Regarding gene transformation efficiency, the number of GUS spots per leaf segment was 3-fold higher in EHA105 than in LBA4404. EHA105 is derived from super virulent strain A 281 (Hood et al. 1993), while LBA4404 is derived from the less virulent strain Ach 5 (Hoekema et al. 1983). EHA105 was previously reported to be more efficient than other strains for the transformation of *Punica granatum* (Terakami et al. 2007), *Saccharum* spp. (Manickavasagam et al. 2004), and *Zea mays* L. (Yu et al. 2013). On the other hand, the elimination of strain LBA4404 from plant tissues was easier using lower concentrations of antibiotics (Maheswaran et al. 1992), while that of strain EHA105 was difficult (Terakami et al. 2007). We compared 250 and 500 mg L⁻¹ cefotaxime sodium salts for regeneration, but did not detect a significant difference between the two conditions. The other concentrations remain to be tested for more efficient regeneration and transformation in the future.

Another important aspect of genetic transformation is the expression level of foreign genes. Since promoters regulated gene expression both quantitatively and qualitatively, we evaluated the expression of the *gusA* marker gene in *T. cinerariifolium* for four types of promoters: the 35S promoter, the 35S promoter combined with an enhancer of El2 Ω or E7 Ω (Mitsuhara et al. 1996), and the bi-directional *mas1'-2'* promoter. The 35S promoter functions in many organs in a large number of higher plants (Benfey and Chua 1990; Terada et al. 1990; Yang and Christou 1990). However, in some plant species, promoters are required to control the high expression of the transgene. In comparisons with the 35S promoter in pBI121, the bi-directional *mas1'-2'* promoter was 5-fold stronger, while El2 Ω and E7 Ω (Mitsuhara et al. 1996) were approximately 4-fold stronger in terms of enhancements in GUS activity levels. Using the bi-directional *mas1'-2'* promoter, we successfully induced the highly resistant transgenic florists' chrysanthemum (*Chrysanthemum morifolium*) for genes, such as insects and diseases (Shinoyama et al. 2015), and male and female sterility transgenic chrysanthemums (Shinoyama et al. 2020). Therefore, the bi-directional *mas1'-2'* promoter is expected to increase the synthesis of foreign gene products in transgenic *T. cinerariifolium* and florists' chrysanthemums.

A challenge associated with genetic transformation technology is the emergence of chimeras, which has been reported for florists' chrysanthemums (Pavingerova et al., 1994; Benetka and Pavingerova, 1995). Shinoyama et al. (2002) demonstrated that regeneration technology via calli suppressed the emergence of chimaeras. The regenerated transformed plants obtained in the present study also showed the whole-body expression of the *gusA* gene (see Figure 6c), which is indicative of the minimized formation of chimeras.

Transformation efficiency markedly varies among florists' chrysanthemum cultivars. Shinoyama et al. (2002) attributed this to differences in shoot regeneration efficiencies from calli and *Rhizobium* infection efficiency. The regeneration and transformation efficiencies of *T. cinerariifolium* genotype #14 were approximately 50% those of the florist's chrysanthemum cultivar 'Yamate-shiro' and similar to those of 'Shuho-no-chikara'. Each *T. cinerariifolium* line exhibits a distinct genotype with large variations by its self-incompatibility (Bhat and Menary, 1986; Parlevliet, 1974; Teixeira da Silva, 2003), which needs to be considered for further improvements in regeneration and genetic transformation techniques in the future.

In the florists' chrysanthemum (*C. morifolium*), one of the Asteraceae plants used as a horticultural species, genetically modified traits with insect and disease resistance (Takatsu et al. 1999; Toguri et al. 2003; Shinoyama et al. 2015), unique flower colors (Noda et al. 2017), and novel shapes (Sasaki et al. 2021) have already been produced. Genome editing technology has recently been used to create new traits in florists' chrysanthemums (Kishi-Kaboshi et al. 2017; Shinoyama et al. 2020). The resulting transgenic or genome-edited organisms are not only useful to agriculture, but also to consumers who seek beauty and healing properties. In the present study, we established a method for the efficient shoot regeneration and genetic transformation of *T. cinerariifolium* genotype #14, which facilitates our understanding of and enhances pyrethrin biosynthesis in *T. cinerariifolium*. Since the environmental safety of the introduced foreign genes is an important issue in transgenic plants, genotype #14 with weak self-compatibility will also be useful for inducing null segregants with genome editing technology.

Declarations

Data Availability Statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Non-financial competing interests

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Author Contributions

K.M., M.H. and H.S. designed the research, performed experiments, and analyzed data together with M.S.; H.S. and M.S. conceived the experiments. The first draft of the manuscript was written by H.S., M.S., and K.M. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Aoki S, Kaneto K, Hashimoto S, Oogai H (1976) Production of pyrethrin by tissue culture. Chem Abstr 89:20589q
2. Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinational regulation of transcription in plants. Science 250:959–966
3. Benetka V, Pavingerova D (1995) Phenotypic differences in transgenic plants of chrysanthemum. Plant Breed 114:169–173. <https://doi.org/10.1111/j.1439-0523.1995.tb00784.x>
4. Bevan MW, Flavell RB, Chilton MD (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187
5. Bhat BK, Menary RC (1986) Genotypic and Phenotypic correlation in Pyrethrum (*Chrysanthemum cinerariaefolium* Vis) and their implication in selection. Pyrethrum Post 16 (2):61–65
6. Cashyap MM, Kueh JSH, MacKenzie IA, Pattenden G (1978) *In vitro* synthesis of pyrethrins from tissue cultures of *Tanacetum cinerariaefolium*. Phytochem 17:544–555
7. Ditta GS, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77:7347–7351
8. Frey L, Janick J (1991) Organogenesis in carnation. J Ame Soc Hortic Sci 116:1108–1112
9. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
10. Han YZ (2008) Studies on polyphenol oxidase (PPO) of compositae plant. Dissertation, Kagoshima University
11. Hedayat M, Abdi Gh, Khosh-Khui M (2009) Regeneration via direct organogenesis from leaf and petiole segments of Pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.]. American-Eurasian J Agric & Environ Sci 6(1):81–87
12. Hitmi A, Barthomeuf C, Sallanon H (1999) Rapid mass propagation of *Chrysanthemum cinerariaefolium* Vis. by callus culture and ability to synthesise pyrethrins. Plant Cell Rep 19:156–160. <https://doi.org/10.1007/s002990050726>
13. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180. <https://doi.org/10.1038/303179a0>
14. Honda H, Hirai A (1990) A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Jpn J Breed 40:339–348

15. Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* 2:208–218
16. Jefferson RA (1987) Assay chimeric gene in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5:387–407
17. Kishi-Kaboshi M, Aida R, Sasaki K (2017) Generation of gene-edited *Chrysanthemum morifolium* using multicopy transgenes as targets and markers. *Plant Cell Physiol* 58:216–226
18. Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β -glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous-glucuronidase activity. *Plant Sci* 70:133–140
19. Liu F, Wang Q, Xu P, Andreazza F, Valbon WR, Bandason E, Chen M, Yan R, Feng B, Smith LB, Scott JG, Takamatsu G, Ihara M, Matsuda K, Klimavicz J, Coats J, Oliveira EE, Du Y, Dong, K (2021) A dual-target molecular mechanism of pyrethrum repellency against mosquitoes. *Nat Commun* 12 (1):2553.
20. Lybrand DB, Xu H, Last RL, Pichersky E (2020) How plants synthesize pyrethrins: Safe and biodegradable insecticides. *Trends Plant Sci* 25 (12):1240–1251
21. Maheswaran GM, Welander M, Hutchinson JF, Graham MW, Richards D (1992) Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. *J Plant Physiol* 139:560–568
22. Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthuriangan S (2004) *Agrobacterium* mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. *Plant Cell Rep* 23:134–143
23. Mao J, Cao LY, Kong LF, Jongsma MA, Wang CY (2013) An *Agrobacterium*-mediated transformation system of pyrethrum (*Tanacetum cinerariifolium*) based on leaf explants. *Sci Hortic* 150:130134
24. Matsuda K, Kikuta Y, Haba A, Nakayama K, Katsuda Y, Hatanaka A, Komai K (2005) Biosynthesis of pyrethrin I in seedlings of *Chrysanthemum cinerariaefolium*. *Phytochem* 66 (13):1529–1535
25. Messeuger J, Arconada MC, Mele E (1993) Adventitious shoot regeneration in carnation (*Dianthus caryophyllus* L.). *Sci Hortic* 54:153–163
26. Mitsuhashi I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* 37:49–59
27. Murakami T, Ohashi Y (1992). Method for histochemical detection of GUS reporter gene expression in transgenic plants. *Plant Cell Tech* 4:281–286
28. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
29. Nakamura T (1968) Studies on the Tannin of Fruits and Vegetables. Part III Polyphenolic compounds and phenol oxidative enzymes of edible burdock. *Jpn Soc Food Sci Tech* 15:199–206 (in Japanese)
30. Narahashi T (1992) Nerve membrane Na⁺ channels as targets of insecticides. *Trends Pharmacol Sci* 13(6):236-241. [http://dx.doi.org/10.1016/0165-6147\(92\)90075-H](http://dx.doi.org/10.1016/0165-6147(92)90075-H)
31. Noda N, Yoshioka S, Kishimoto S, Nakayama M, Douzono M, Tanaka Y, Aida R (2017) Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism. *Sci Adv* 3, e1602785. <https://www.science.org/doi/10.1126/sciadv.1602785>
32. Ooms G, Hooykaas PJJ, Veen RJMV, Beelen PV, Regensburg-Tuinck TJG, Schilperoort RA (1982) Octopine Ti-plasmid deletion mutants of *agrobacterium tumefaciens* with emphasis on the right side of the T-region.

33. Panizzi L, Scarpati ML (1954) Constitution of cynarine, the active principle of the artichoke. *Nature* 174:1062–1063. <http://dx.doi.org/10.1186/1475-2891-3-5>
34. Parlevliet JE (1974) The genetic variability of the yield components in the Kenyan pyrethrum populations. *Euphytica* 23:377–384
35. Pavingerova D, Dostal D, Biskova R, Benetka V (1994) Somatic embryogenesis and *Agrobacterium*-mediated transformation of chrysanthemum. *Plant Sci* 97: 95–101
36. Sasaki K, Yoshioka S, Aida R, Ohtsubo N (2021) Production of petaloid phenotype in the reproductive organs of compound flowerheads by the co-suppression of class-C genes in hexaploid *Chrysanthemum morifolium*. *Planta* 253, 100. <https://doi.org/10.1007/s00425-021-03605-4>
37. Shinoyama H, Kazuma T, Komano M, Nomura Y, Tsuchiya T (2002) An efficient transformation system in chrysanthemum [*Dendranthema × grandiflorum* (Ramat.) Kitamura] for stable and non-chimeric expression of foreign genes. *Plant Biotechnol* 19:335–343. <https://doi.org/10.5511/plantbiotechnology.19.335>
38. Shinoyama H, Mitsuhashi I, Mochizuki A, Kato K, Ichikawa H (2015) Transgenic chrysanthemum (*Chrysanthemum morifolium* Ramat.) carrying both insect and disease resistance. *Acta Hort* 1087:485–497
39. Shinoyama H, Ichikawa I, Yokoi-Nishizawa A, Skaptsov M, Toki S (2020) Simultaneous TALEN-mediated knockout of chrysanthemum DMC1 genes confers male and female sterility. *Sci Rep* 10: 16165, <https://doi.org/10.1038/s41598-020-72356-1>
40. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517
41. Takagi H, Tanaka Y, Tarumoto I, Murata N (1993) Evaluation of genetic diversity of sweet potato germplasm. I. Characterization by restriction polymorphisms analysis. *Jpn J Breed* 43 (Suppl.1):192
42. Takatsu Y, Nishizawa Y, Hibi T, Akutsu K (1999) Transgenic chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) expressing a rice chitinase gene shows enhanced resistance to gray mold (*Botrytis cinerea*). *Sci Hort* 82: 113–123
43. Teixeira da Silva, JA (2003) Anthemideae: advances in tissue culture, genetics and transgenic biotechnology. *Afr J Biotech* 2(12):547–556
44. Terada R, Shimamoto K (1990) Expression of CaMV 35S-GUS gene in transgenic rice plants. *Mol Gen Genet* 220:389–392
45. Terakami S, Matsuta N, Yamamoto T, Sugaya S, Gemma H, Soejima J (2007) *Agrobacterium*-mediated transformation of the dwarf pomegranate (*Punica granatum* L.). *Plant Cell Rep* 26:1243–1251
46. Toguri T, Ogawa T, Kakitani M, Tukahara M, Yoshioka M (2003) *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*) plants with a disease resistance gene (pac1). *Plant Biotechnol* 20:121–127
47. Roest S, Bokelmann GS (1973) Vegetative propagation of *Chrysanthemum cinerariaefolium in vitro*. *Sci Hort* 1:120–122
48. Varga F, Liber Z, Jakse J, Turudic A, Satovic Z, Radosavljevic I, Jeran N, Grdisa M (2022) Development of Microsatellite Markers for *Tanacetum cinerariifolium* (Trevis.) Sch. Bip., a Plant with a Large and Highly Repetitive Genome. *Plants* 11(13):1778. <https://doi.org/10.3390/plants11131778>
49. Wang Q, Xu P, Andreazza F, Liu Y, Nomura Y, Duran P, Jiang L, Chen M, Takamatsu G, Ihara M, Matsuda K, Isaacs R, Oliveira EE, Du Y, Dong K (2021) Identification of multiple odorant receptors essential for pyrethrum

repellency in *Drosophila melanogaster*. PLoS Genet 17 (7): e1009677

50. White PR (1963) The cultivation of animal and plant cells, 2nd ed. Ronald Press Co., New York.
51. Yamashiro T, Shiraishi A, Satake H, Nakayama K (2019) Draft genome of *Tanacetum cinerariifolium*, the natural source of mosquito coil. Sci Rep 9 (1):18249
52. Yang NS, Christou P (1990) Cell type specific expression of a CaMV 35S-GUS gene in transgenic soybean plants. Dev Genet 11:289–293
53. Yu GR, Liu Y, Du WP, Song J, Lin M, Xu LY, Xiao FM, Liu YS (2013). Optimization of *Agrobacterium tumefaciens*-Mediated Immature Embryo Transformation System and Transformation of Glyphosate-Resistant Gene 2mG2-EPSPS in Maize (*Zea mays* L.) J Integr Agric 12:213–2142.
[https://doi.org/10.1016/S2095-3119\(13\)60567-5](https://doi.org/10.1016/S2095-3119(13)60567-5)

Tables

Table 1. Timetable for the transformation of *Tanacetum cinerariifolium*

Day	Procedure
0	Culture <i>Rhizobium</i> in liquid YEP medium for 4 to 5 h
	Immerse leaf segments in Infection medium containing <i>Rhizobium</i> for 15 min
	Co-cultivate leaf segments with <i>Rhizobium</i> in infection medium
2	(End of co-cultivation for 2 days)
	Transfer to Bacteria elimination liquid medium A and shake and incubate overnight
3	Transfer to Bacteria elimination solid medium
13	Transfer to Bacteria elimination liquid medium B and shake and incubate overnight
14	Transfer to Selection medium (selection of putative transformed cells)
28	Transfer to fresh Selection medium
42	Transfer to fresh Selection medium
	(Callus induction on the edge of leaf segments)
56	Transfer to Bacteria elimination solid medium
70	Transfer to fresh Bacteria elimination solid medium
84	Transfer green calli to Regeneration medium
98	Transfer to fresh Regeneration medium (shoot regeneration)
	Collect elongating shoots (first collection) and transfer to Stem elongation medium
112	Transfer to Rooting medium
112-150	Transfer rooted plantlets to a greenhouse
150 onwards	Plants available for testing
Medium construction	
Callus formation solid medium (CS medium): 1/2 MS + 1.0 mg L ⁻¹ NAA, 2.0 mg L ⁻¹ BAP, 1.5% sucrose, 0.3% Gel.	
Callus formation liquid medium (CL medium): 1/2 MS + 1.0 mg L ⁻¹ NAA, 2.0 mg L ⁻¹ BAP, 1.5% sucrose.	
Infection medium: CL medium + 5% Tween 20, 50 µM acetosyringone.	
Co-cultivation medium: CS medium +1 g L ⁻¹ casamino acid	
Bacteria elimination liquid medium A: CL medium + 250 or 500 mg L ⁻¹ cefotaxime.	
Bacteria elimination liquid medium B: CL medium + 100 mg L ⁻¹ cefotaxime.	
Bacteria elimination solid medium: CS medium + 100 mg L ⁻¹ cefotaxime.	
Selection medium: CS medium + 100 mg L ⁻¹ cefotaxime, 10 or 20 mg L ⁻¹ G418.	

Regeneration medium: 1/2 MS + 0.5 mg L⁻¹ BAP, 0.2 mg L⁻¹ GA₃, 1.5% sucrose, 0.3% gellan gum.

Stem elongation medium: 1/2 MS+ 0.5 mg L⁻¹ BAP, 1.0 mg L⁻¹ IBA, 1.0% suc., 0.3% gellan gum.

Rooting medium: 1/2 MS + 1.0% sucrose, 0.3% gellan gum.

Table 2. Effect of growing media on germination and seedling growth.

Medium ¹⁾	Sucrose concentration (%)	Solidifier ²⁾	Rooting ratio (%)			Plant height (cm) ³⁾			Root length (mm) ³⁾		
MS	3.0	Gellan Gum	28.0	±	0.4a	27.4	±	0.6a	42.6	±	1.2a
	3.0	Agar	36.0	±	0.5a	25.8	±	1.0a	46.5	±	0.6ab
1/2 MS	1.5	Gellan Gum	80.0	±	0.0b	27.4	±	1.0a	49.8	±	2.8b
	1.5	Agar	56.0	±	1.1c	24.0	±	0.4b	43.9	±	1.0a
White	2.0	Gellan Gum	34.0	±	0.9a	15.1	±	0.5c	41.2	±	0.5a
	2.0	Agar	34.0	±	0.5b	15.8	±	0.2c	34.0	±	1.4c
Gamborg's B5	2.0	Gellan Gum	34.0	±	0.5a	14.7	±	0.2d	52.4	±	3.4d
	2.0	Agar	62.0	±	0.4c	14.8	±	0.2d	31.7	±	0.7c

Ten germinated seeds were put on one plant culture box and performed 10 replications.

Each value represents the mean ± SD.

The mean percentages of rooting rate were arcsine-transformed prior to Tukey-Kramer's HSD.

Values followed by the same letter are not significantly different at 1% level by Tukey-Kramer's HSD.

¹⁾ MS; Murashige and Skoog 1962, 1/2MS; 1/2 MS medium containing half strength of inorganic substances, White; White 1963, Gamborg's B5; Gamborg et al. 1968.

²⁾ Gellan Gum; 0.3%, Agar; 0.8% respectively.

³⁾ Data were measured only for rooted plants, 2 months after replacing to each medium.

Table 3. Effects of the phytohormone combination to induce green calli/adventitious shoots

Phytohormone combination and concentration (mg L ⁻¹)		BAP	Green callus formation ratio (%) ¹⁾	Shoot formation ratio (%) ²⁾
NAA	0.1	0.5	8.3 ± 0.0a	0.0 ± 0.0a
		1.0	0.0 ± 0.0b	0.0 ± 0.0a
		2.0	0.0 ± 0.0b	0.0 ± 0.0a
	0.5	0.5	7.5 ± 0.1a	0.0 ± 0.0a
		1.0	18.5 ± 0.1c	7.8 ± 0.1b
		2.0	6.7 ± 0.1a	0.0 ± 0.0a
	1.0	0.5	16.8 ± 0.1c	0.0 ± 0.0a
		1.0	19.3 ± 0.1c	0.0 ± 0.0a
		2.0	19.4 ± 0.1c	9.2 ± 0.0b

Leaf segments were cut from Age #2 (see Figure 1).

Twelve leaf segments were placed on one Petri dish and 10 replications were performed.

The number of leaf segments that formed green calli/shoots was counted after 1.5 months of plating in each medium.

Each value represents the mean ± SD.

Mean percentage data were arcsine-transformed prior to Tukey-Kramer's HSD.

Values followed by the same letter are not significantly different at the 1% level by Tukey-Kramer's HSD.

¹⁾ number of green callus-forming leaf segments/number of leaf segments tested * 100 (%).

²⁾ number of shoots forming leaf segments/number of leaf segments tested * 100 (%).

Table 4. Effects of leaf ages on callus formation

Leaf age ¹⁾	Green callus formation ratio (%) ²⁾		
#1	5.0	±	4.3 ^b
#2	22.5	±	8.8 ^a
#3	27.5	±	7.9 ^a

Twelve leaf segments were placed on one Petri dish and 10 replications were performed.

The number of leaf segments that formed calli was counted after 1.5 months of plating in each medium.

Each value represents the mean ± SD.

The mean percentages were arcsine-transformed prior to Tukey-Kramer's HSD.

Values followed by the same letter are not significantly different at the 1% level by Tukey-Kramer's HSD.

¹⁾ #1: leaves in the second week after expanding (a leaf length of 1 to 1.5 cm); #2: leaves in the first week after expanding (a leaf length of approximately 8 mm to 1 cm); #3: leaves just expanding (a leaf length of 3 to 5 mm).

²⁾ number of green callus-forming leaf segments/number of leaf segments tested * 100 (%).

Table 5. Effect of phytohormon combination for shoot regeneration and its growth.

Phytohormon combination and concentration (mg L ⁻¹)		BAP	Shoot length (A: mm)			Shoot length after 3 weeks (B: mm)			Expanded length of shoot (B-A: mm)		
NAA	1.0	2.0	2.1	±	0.9	5.1	±	2.0	3.0	±	1.5a
GA3	0.1	0.5	2.1	±	0.6	4.4	±	1.3	2.3	±	1.4ab
		1.0	2.1	±	0.7	3.6	±	0.8	1.5	±	0.6ab
		2.0	2.0	±	0.7	3.2	±	0.6	1.2	±	0.9b
	0.2	0.5	2.1	±	0.9	7.9	±	4.0	5.8	±	4.0c
		1.0	2.1	±	0.6	3.9	±	0.8	1.8	±	0.7ab
		2.0	2.1	±	0.7	3.8	±	0.8	1.7	±	0.9ab

Twelve green callus were put on one petri dish and performed 10 replications.

Each value represents the mean expanded length of shoot ± SD.

Values followed by the same letter are not significantly different at 1% by Tukey-Kramer's HSD.

Table 6. Effect of phytohormon combination for shoot growth and stem elongation.

Phytohormon combination and concentration (mg L ⁻¹)		BAP	Elongated stem formaton ratio ¹⁾		
IBA	0.5	0.0	2.5	±	0.8a
		0.5	22.5	±	0.8b
	1.0	0.0	0.0	±	0.0c
		0.5	55.0	±	0.1d

Four shoots were put on one plant culture box and performed 10 replications.

The number of shoots with elongated stems were counted after 4 weeks of transplanting in each medium.

Each value represents the mean ± SD.

Values followed by the same letter are not significantly different at 1% by Tukey-Kramer's HSD.

¹⁾ number of shoots with elongated stems / number of shoots tested * 100 (%).

Table 7. Effects of antibiotic concentrations on the efficiency of transformed plant selection

Concentration of cefotaxime sodium salt (mg L ⁻¹)	Concentration of G418 (mg L ⁻¹)	Number of leaf segments (A)	Number of regenerated plantlets (B)	Regeneration ratio (%: B/A*100)	Number of transformed plantlets ¹⁾ (C)	Transformation ratio (%: C/A*100)
250	10	196	10	5.1	8	4.1
	20	192	6	3.1	6	3.1
500	10	220	21	9.5	10	4.5
	20	216	7	3.2	7	3.2

Twelve leaf segments were placed on one Petri dish.

¹⁾ Transformed calli were confirmed by the GUS assay, a PCR test, and Southern blot analysis.

Figures



Figure 1

Leaf ages and cutting sites for leaf segments of *Tanacetum cinerariifolium*

Age#1: leaves in the second week after expanding, with a leaf length of 1 to 1.5 cm; Age#2: leaves in the first week after expanding, with a leaf length of approximately 8 mm to 1 cm; Age#3: leaves just expanding, with a leaf length of 3 to 5 mm.

The scale bar indicates 10 mm. Red bars indicate cutting sites for leaf segments.

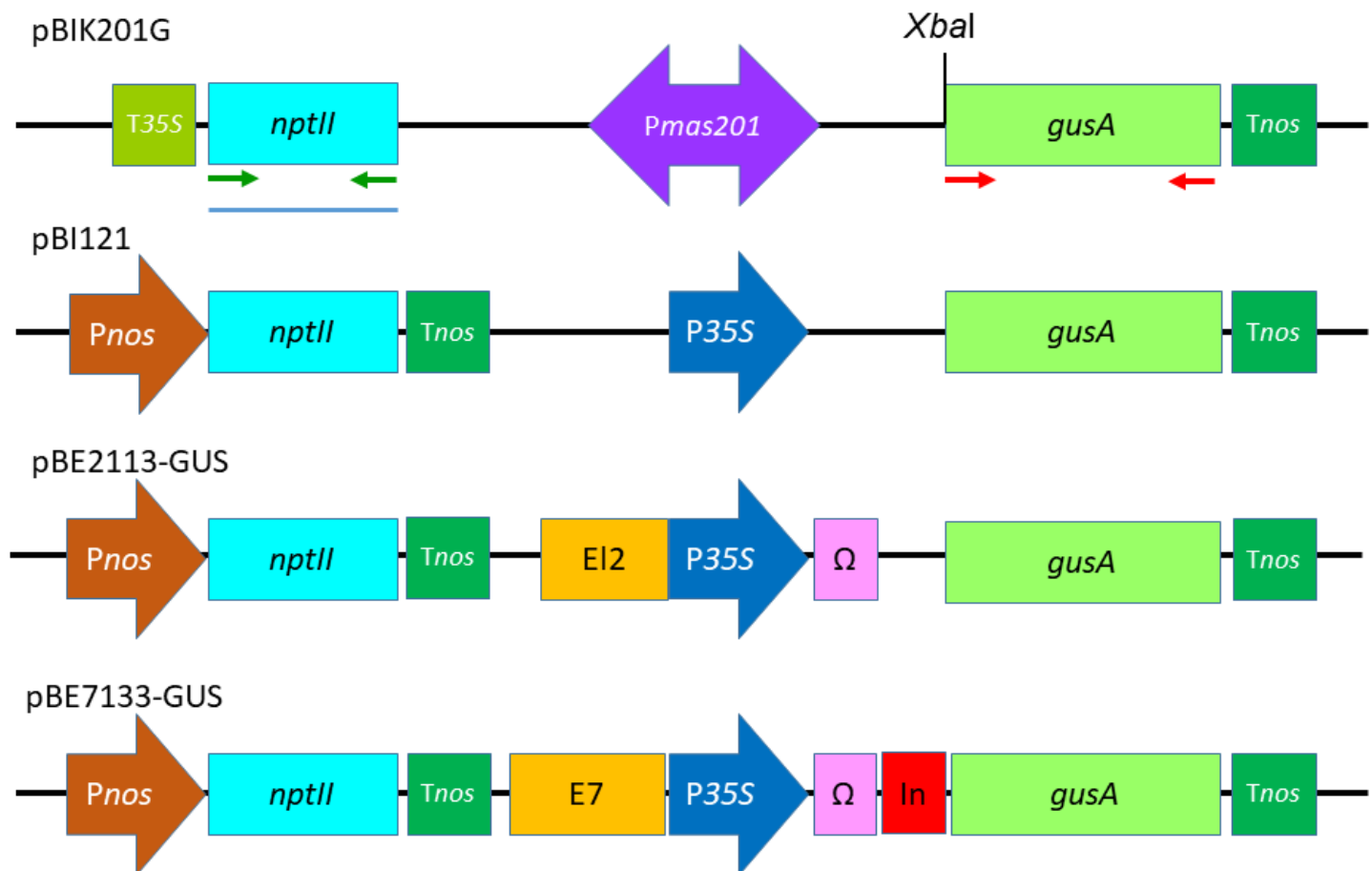


Figure 2

Structures of tested binary vectors

T35S, the polyadenylation signal of the *Cauliflower mosaic virus* (CaMV) 35S transcript; *nptII*, the neomycin phosphotransferase II gene for the selection of transgenic plants; *Pmas201*, the bidirectional promoter cassette from the mannopine synthase 1' and 2' (*mas1'-2'*) genes; *gusA*, the β -glucuronidase gene; *Tnos*, the polyadenylation signal of the gene for nopaline synthase in the Ti plasmid; *Pnos*, the nopaline synthase promoter from *Rhizobium* Ti plasmids; *P35S*, the 5'-upstream sequence of the CaMV 35S promoter (-90 to -1); E7, the 5'-

upstream sequence of the CaMV 35S promoter (-940 to -290) and (-290 to -90) × 7; Ω, the 5'-upstream sequence of Tobacco mosaic virus (TMV); In, the 1st intron of the phageolin gene; E12, the 5'-upstream sequence of the CaMV 35S promoter (-419 to -90) × 2.

Red arrows indicate *gusA*-specific primers to amplify an approximately 1.8-kbp fragment in the *gusA* coding region. Green arrows indicate *npt*-specific primers to amplify an approximately 800-bp fragment in the *npt* coding region. The blue bar indicates the *npt*-specific probe for the Southern blot analysis.

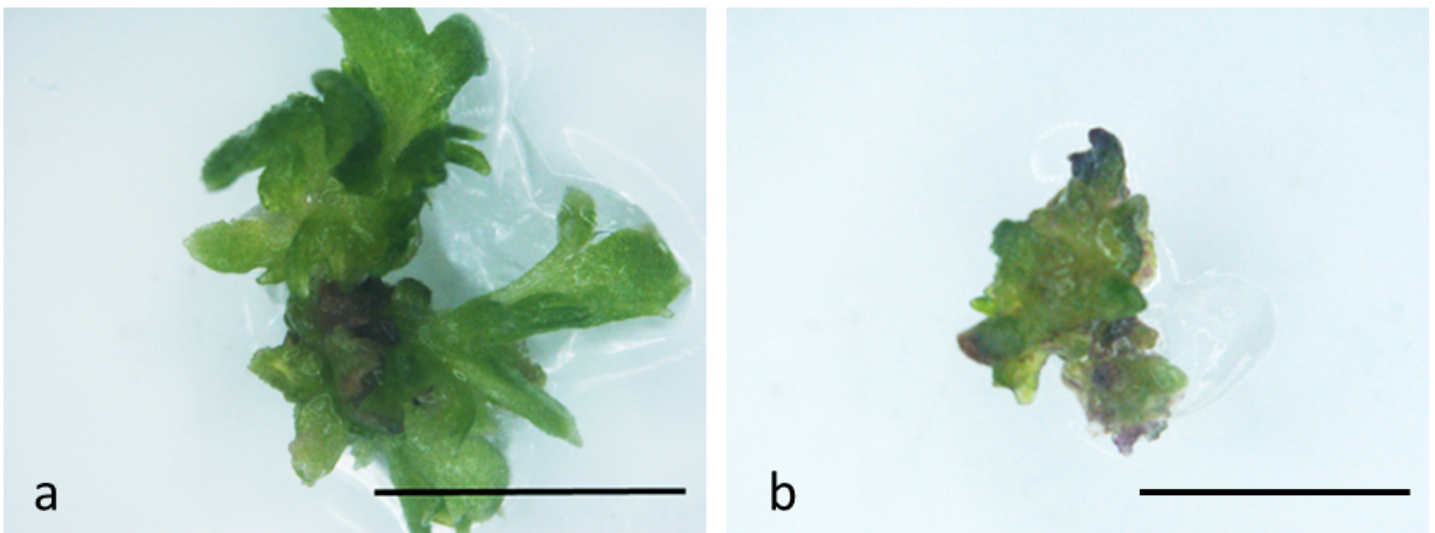


Figure 3

Effects of the phytohormone combination on shoot regeneration

a: 1/2 MS+ 0.5 mg L⁻¹ BAP, 0.2 mg L⁻¹ GA₃; b: 1/2 MS + 1.0 mg L⁻¹, NAA, 2.0 mg L⁻¹ BAP.

Shoots three weeks after plating. Scale bars indicate 1.0 cm.

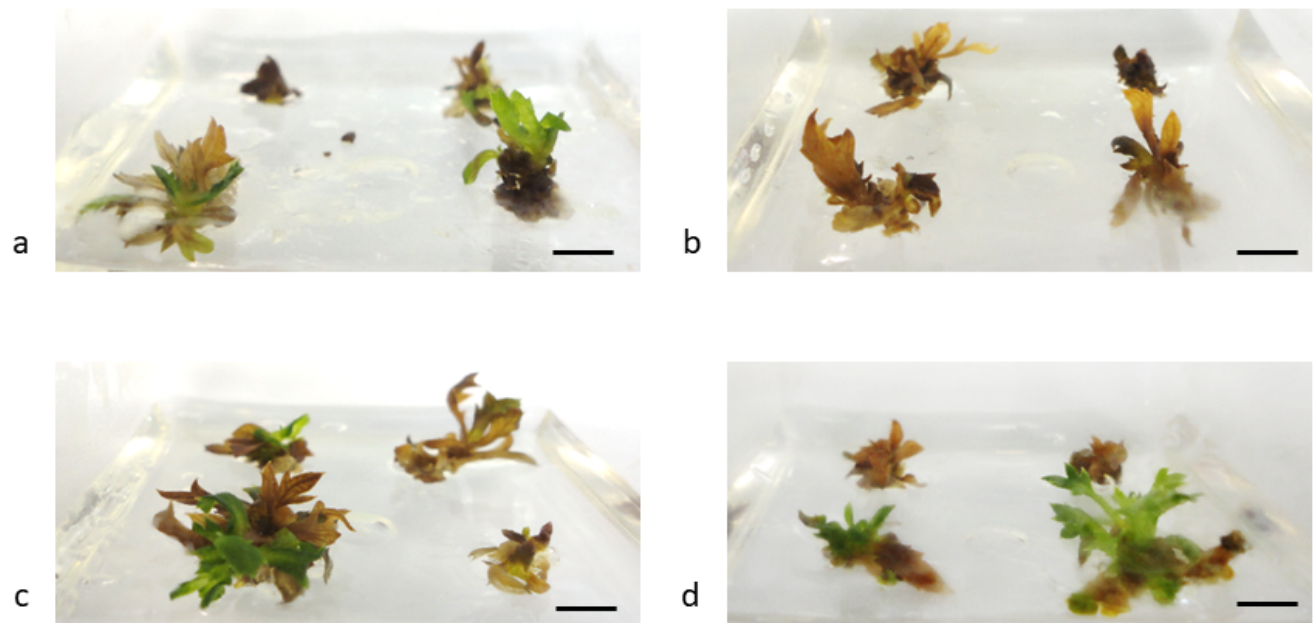


Figure 4

Effects of the phytohormone combination in 1/2 MS basal medium on shoot growth and stem elongation

a: 0.5 mg L⁻¹ IBA; b: 1.0 mg L⁻¹ IBA; c: 0.5 mg L⁻¹ IBA + 0.5 mg L⁻¹ BAP; d: 1.0 mg L⁻¹ IBA + 0.5 mg L⁻¹ BAP. Shoots two weeks after plating. Scale bars indicate 1.0 cm.

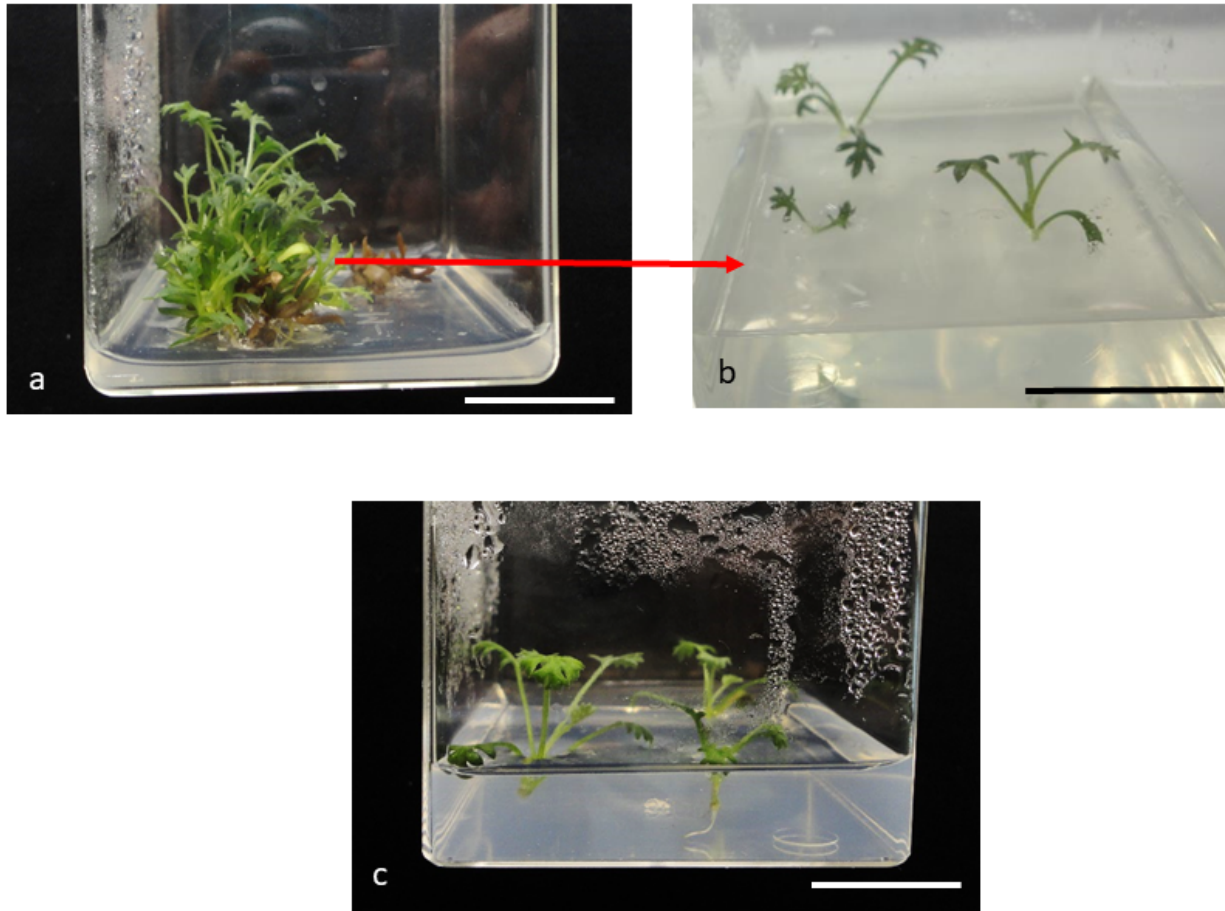


Figure 5

Rooting from elongated shoots

Shoots with elongated stems on 1/2 MS medium containing 1.0 mg L⁻¹ IBA and 0.5 mg L⁻¹ BAP were replanted in 1/2 MS phytohormone-free medium.

a: Shoots on 1/2 MS medium containing 1.0 mg L⁻¹ IBA and 0.5 mg L⁻¹ BAP; b: replanting shoots with elongated stems in 1/2 MS phytohormone-free medium; c: rooting shoots three or four weeks after replanting. Scale bars indicate 2 cm.

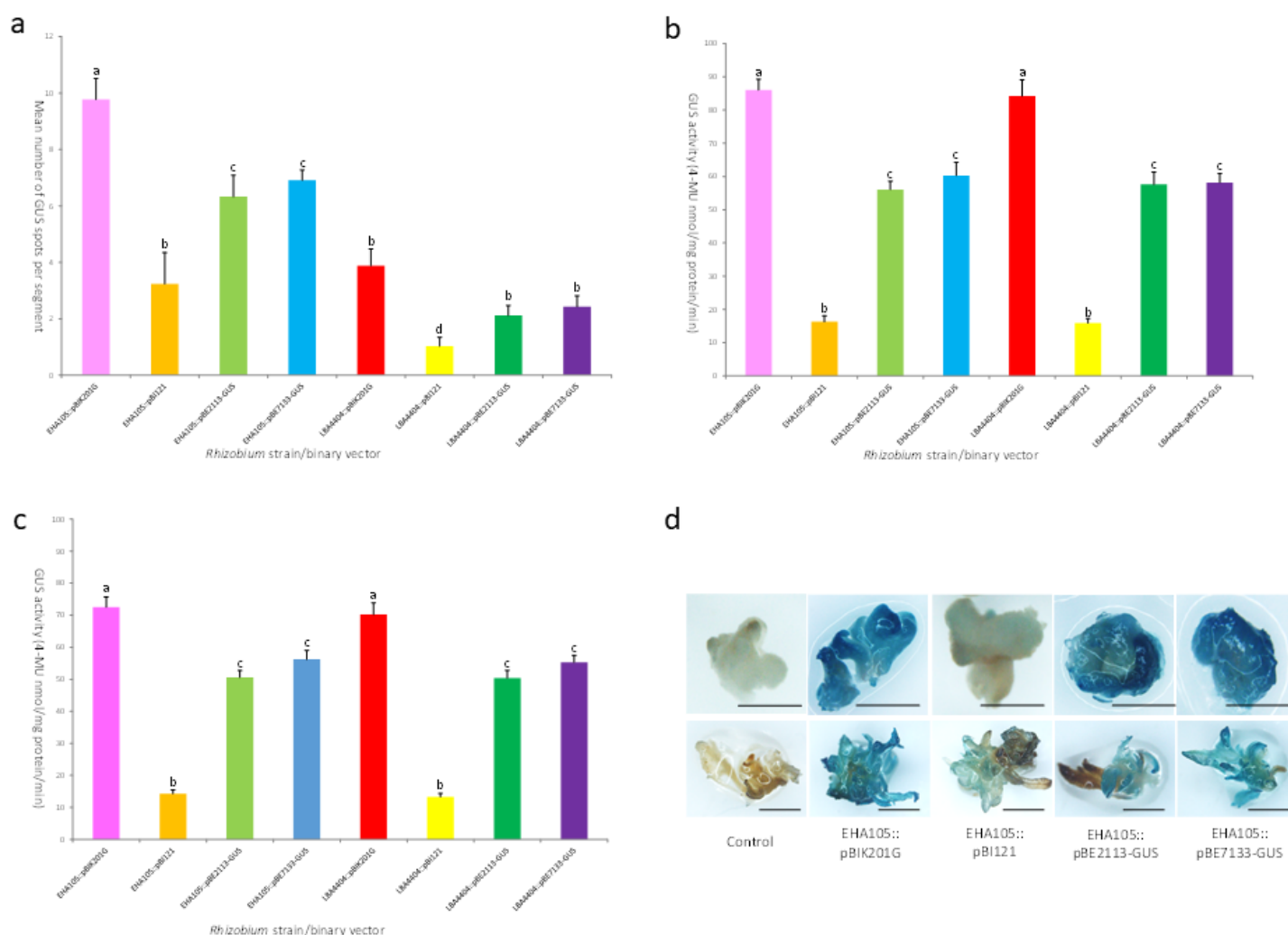


Figure 6

Effects of *Rhizobium* strains and promoters for the *gusA* gene on insertion and expression

a: Effects of *Rhizobium* strains on efficient gene insertion. The GUS spot numbers of 12 leaf explants were measured with 10 replications. Each value represents the mean of GUS spots per leaf explant $\pm$ SD.

b: Effects of promoters on the efficient expression of foreign genes. The GUS activity of calli was measured with 10 replications. Each value represents mean GUS activity $\pm$ SD.

c: Effects of promoters on the efficient expression of foreign genes. The GUS activity of shoots was measured with 10 replications. Each value represents mean GUS activity $\pm$ SD.

Values in b and c with the same letter were not significantly different at the 1% level by Tukey-Kramer's HSD.

d: Effects of promoters on *gusA* gene expression in calli and regenerated shoots.

The upper and lower figures represent expression in calli and regenerated shoots, respectively.

Scale bars indicate 5 mm.

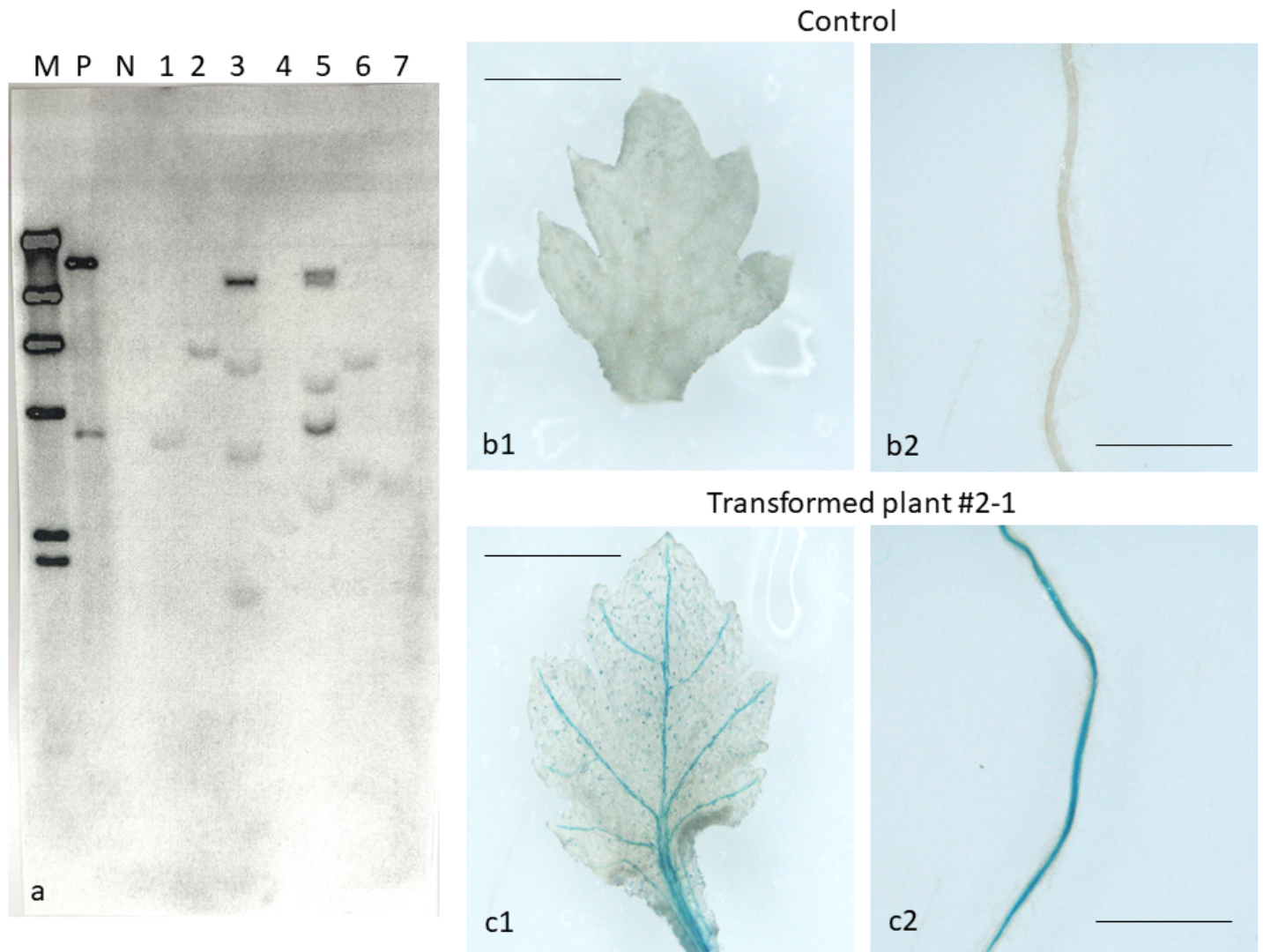


Figure 7

Integration of the *gusA* gene into genomes of transgenic plants

a: Southern blot analysis to detect transgene integration into the genomes of regenerated plants. A 25- μ g aliquot of DNA was digested with *Xba*I and hybridized with the *npt*-specific probe. M: λ -Hind digest, P: the binary vector pBIK201G, N: non-transformed control, 1 to 7: regenerated plants with specific bands of *gusA* and *npt* detected by PCR. b1 to c2: *gusA* gene expression in regenerated plants; b1 and c1: young leaves not fully expanded; b2 and c2: roots. Scale bars indicate 5 mm.

Supplementary Files

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- [ShinoyamaetalPCTOCSupplementarydataset.pdf](#)