Establishment of an efficient transformation method of garden stock (*Matthiola incana*) using a callus formation chemical inducer

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Abstract  *Matthiola incana* is an important floricultural plant that blooms from winter to spring, and had been desired to be established a transformation system. This study successfully obtained stable transgenic plants from *M. incana*. We used *Agrobacterium tumefaciens* harboring a binary vector containing the β-glucuronidase gene (*GUS*) under the control of cauliflower mosaic virus 35S promoter to evaluate the transformation frequency of *M. incana*. We observed that cocultivation with the *A. tumefaciens* strain GV3101 for 5 days effectively enhanced the infection frequency, assessed through a transient *GUS* expression area in the seedling. Furthermore, the addition of 100 µM acetosyringone was necessary for *Agrobacterium* infection. However, we could not obtain transgenic plants on a shoot formation medium supplemented with 1 mg l⁻¹ 6-benzyladenine (BA). For callus formation from the leaf sections, a medium supplemented with 1–50 µM fipexide (FPX), a novel callus induction chemical, was employed. Then, the callus formation was observed after 2 weeks, and an earlier response was detected than that in the BA medium (4–6 weeks). Results also showed that cultivation in a selection medium supplemented with 12.5 µM FPX obtained hygromycin-resistant calli. Thus, this protocol achieved a 0.7% transformation frequency. Similarly, progenies from one transgenic line were observed on the basis of GUS stains on their leaves, revealing that the transgenes were also inherited stably. Hence, FPX is considered a breakthrough for establishing the transformation protocol of *M. incana*, and its use is proposed in recalcitrant plants.

Key words:  *Agrobacterium*-mediated transformation, FPX, GUS, ornamental plants, transgenic plants.

Introduction

Garden stock (*Matthiola incana* R. Br.) belongs to the family Brassicaceae, one of the most important ornamental plants blooming during winter-spring seasons. Therefore, *M. incana* cultivars are used as cut and pot flowers in Japan. A previous study reported genetic variations at its flowering time, attributed to differential sensitivities to low temperatures and day lengths, in addition to varying juvenile phase lengths (Hisamatsu et al. 2000). The garden stock have several flower colors, ranging from pure white to lavender, purple, pale blue, red, pink, and pale yellow (Dole and Wilkins 2005). Accordingly, our previous studies demonstrated that anthocyanin accumulation was responsible for the varying flower colors in *M. incana* cultivars. Hence, practically all anthocyanin biosynthesis-related genes have been isolated to examine resultant effects (Nuraini et al. 2021, 2020). Most *M. incana* producers selected marketable double-flowered individuals using different leaf characteristics from single flower ones. However, choosing double-flowered individuals during seedling cultivation is laborious. Therefore, we identified two mutation alleles of *AGAMOUS* (*MiAG*) involved in the double-flower trait. Then, we developed DNA markers that discriminate between single- and double-flowered seedlings (Nakatsuka and Koishi 2018). Although previous studies have identified the responsible genes using the difference among cultivation varieties, the production of transgenic *M. incana* plants is necessary to further reveal these gene functions.

We aimed to breed *M. incana* plants to obtain bright yellow- or pure blue-colored flowers, to obtain plants that are resistant against *Sclerotana sclerotiorum*, *Botrytis cinerea*, or turnip mosaic viral diseases, and to develop cyanic double-flowered cultivars that do not require to choose seedlings. However, no natural genetic resource

Abbreviations: BA, 6-benzyladenine; FPX, fipexide; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; X-gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

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exists to achieve the above breeding results. Therefore, we previously developed a transient gene expression system using the turnip mosaic virus vector (Nuraini et al. 2020). The results showed that the overexpression of foreign genes in the infected leaves induced green fluorescence and anthocyanin accumulation. Nevertheless, no report that a stable transformation system was established in M. incana exists.

In Brassicaceae, to which M. incana belongs, some plants with stable transformation systems have been established via *Agrobacterium tumefaciens*. For example, in the model plant *Arabidopsis thaliana*, an in vitro protocol using regeneration from hypocotyl explants was developed (Akama et al. 1992). Also, the in planta protocol using floral dip is widely prevalent (Clough and Bent 1998). *Agrobacterium*-mediated transformation protocols for most Brassicaceae vegetable crops, including *Brassica napus* (De Block et al. 1989; Moloney et al. 1989), broccoli (*B. oleracea var. italica*), Chinese cabbage (*B. rapa ssp. pekinensis*), cabbage (*B. oleracea var. capitata*; Kuginuki and Tsukazaki 2001), komatsuna (*B. rapa ssp. chinensis*; Takasaki et al. 1997), and radish (*Raphanus sativus*; Cho et al. 2008; Muto et al. 2021) have also been established. The genetic transformation of *B. rapa* and *R. sativus* has been difficult because of their low regeneration ability from the cultured tissues and low infection frequency with *Agrobacterium* (Cho et al. 2008; Takasaki et al. 1997).

In *M. incana*, multiple shoots were formed from the cotyledon on MS medium supplemented with 0.1–0.8 mg l−1 6-benzyladenine (BA; Gautam et al. 1983). Efficient shoot regulation was also developed from mature leaves on a medium supplemented with 1–2 mg l−1 BA (Fukuzumi 1994). Moreover, adventitious shoots were induced in 9–16% callus of proplasts when cultured on a medium supplemented with 1 mg l−1 BA or 1 mg l−1 zeatin (Hosoki and Ando 1989). Thus, BA is considered an optimal plant hormone to form adventitious shoots from *M. incana* leaves. Recently, fpixide (FPX), a psychoactive drug used to treat senile dementia and memory impairment in mammals, was isolated as a useful regulatory compound through a chemical biology-based screening method (Nakano et al. 2018), acting as a chemical inducer in callus formation, shoot regeneration, and *Agrobacterium* infection. The function of FPX might reveal unknown metabolic signaling that is involved in plant cell division through direct or auxiliary effects (Nakano et al. 2018). In Arabidopsis, rice, poplar, and model grass (*Brachypodium distachyon*), FPX also improved the regeneration and transformation efficiency compared with the conventional plant hormones (Nakano et al. 2018; Yu et al. 2020).

Hence, this study investigated the effect of FPX on callus formation in leaves. Then, we attempted to establish the *Agrobacterium*-mediated transformation system of *M. incana*.

Materials and methods

**Preparation of explants**

*Mattiola incana* ‘Kiss me Violet’ (Takii seed, Kyoto, Japan) seeds were steriley germinated on an MS basal medium, containing 30g l−1 sucrose and 8g l−1 agar (Murashige and Skoog 1962), after which cultivation was conducted at 25°C under a 16 h photoperiod of 40µmol m−2 s−1, using fluorescent lamps in a plant growth chamber CLE-305 (Tomy Seiko, Tokyo, Japan). Afterward, two-week-old seedlings were used to investigate *Agrobacterium* infection conditions. Then, leaf explants (approximately 8×8 mm) were prepared from the green upper leaves of 2-month aseptic plants (4–5 cm height, with 10–12 leaves) for subsequent experiments. Finally, FPX (Fipixide hydrochloride, Sigma-Aldrich, St. Louis, MO) at optimal concentrations of 0, 1, 5, 10, 12.5, 25, 50, and 100 µg l−1 were added to the MS basal medium, and 20 leaf sections were placed on each medium, followed by further cultivation at 25°C under 16 h fluorescent lamp conditions for a month. A medium supplemented with 1 mg l−1 BA was prepared as the control experiment.

**Sensitivity of leaf sections to antibiotic reagents**

To determine the optimal concentration required to select transgenic cells, we investigated *M. incana* leaf explants’ sensitivity to several antibiotic regents. First, an MS basal medium containing 12.5 µg l−1 FPX was supplemented with 0, 5, 10, and 20 mg l−1 kanamycin sulfate or hygromycin B as antibiotic regents. Then, 32 leaf sections were placed on each medium and cultivated at 25°C under a 16 h light condition for three weeks. The callus formation degrees were evaluated according to the following three categories: none, explants formed no callus and turned white; moderate, explants formed <2 mm sized calli; intense, explants formed >2 mm sized calli.

**Plasmid and Agrobacterium preparations**

The pSHG-35SrintronGUS binary vector (35S:GUS), composed of a hygromycin phosphotransferase (*HPT*) gene controlled by the nopaline synthase promoter and the β-glucuronidase (*GUS*) gene, with an intron controlled by a cauliflower mosaic virus 35S promoter in the T-DNA region, was used. First, the binary vector was transformed into *A. tumefaciens* strains EHA101, LBA4404, and GV3101 through electroporation, after which the strains were selected on an LB plate containing 200 µg l−1 spectinomycin and 25 mg l−1 rifampicin. Next, a single *Agrobacterium* colony was transferred to a 3 ml liquid LB medium supplemented with 200 µg l−1 spectinomycin, followed by shaking for 16 h at 28°C. Subsequently, 500 µl of the *Agrobacterium* culture was transferred to a 100 ml liquid YEB medium (5 g l−1 beef extract, 1 g l−1 yeast extract, 1 g l−1 peptone, 5 g l−1 sucrose, 2 mM MgSO4, pH 7.2) supplemented with the above antibiotic regents, after which shaking was conducted again for 16 h at 28°C. Finally, the *Agrobacterium* pellet was collected by centrifugation, and the setup was resuspended in an infection buffer (10 mM MgCl2, 10 mM MES, 100 µM acetoxyringone, pH 5.6) at OD600=1.0.
pShyg-35SMiAG-35SGFP vector (35S::AG-GFP), composed of the MiAG and green fluorescent protein (GFP) controlled by the cauliflower mosaic virus 35S promoter, was transformed into *A. tumefaciens* GV3101, and the *Agrobacterium* infection solution was prepared as described above.

**Transformation of *M. incana***

*Agrobacterium* strain and cocultivation period investigations were evaluated using 25 days seedlings. First, seedlings were immersed in a 50 ml tube containing an *Agrobacterium* suspension. Then, they were vacuumed for 15 min at −97 kPa using a desiccator and aspirator. After rinsing three times with sterile water, seedlings were placed on an MS basal medium covered with a paper filter, after which they were cultivated at 25°C in the dark.

Subsequently, the stable transformation of *M. incana* was conducted according to the procedure below. First, the leaf section explants of ‘Kiss me Violet’ were placed on a cocultivation medium (MS basal medium containing 12.5 µM FPX, 100 µM acetosyringone, 30 g l⁻¹ sucrose, and 8 g l⁻¹ agar, pH 5.7), after which they were cultured for three days under 16 h daylength using a fluorescent lamp at 25°C. Precultured leaf explants were then immersed in the *Agrobacterium* suspension for five minutes. Next, they were placed on a cocultivation medium covered with a paper filter, followed by cocultivation in the dark at 25°C for five days. Additionally, *Agrobacterium*-infected leaf sections were transferred to a selection medium (an MS basal medium containing 12.5 µM FPX, 10 mg l⁻¹ hygromycin, 10 mg l⁻¹ melopenum [Meropen, Sumitomo Dainippon Pharma, Osaka, Japan], 30 g l⁻¹ sucrose and 8 g l⁻¹ agar, pH 5.7) where they were subjected to subcultivation every two weeks. First, however, the hygromycin-resistant callus was transferred to a shoot induction medium (MS basal medium containing 0 or 2 mg l⁻¹ BA, 10 mg l⁻¹ hygromycin, 10 mg l⁻¹ melopenum, 30 g l⁻¹ sucrose, and 8 g l⁻¹ agar, pH 5.7). Extended shoots were also transferred to a plant box containing 50 ml rooting medium [3 g l⁻¹ Hyponex (N-P-K=6-6-19, Hyponex Japan, Osaka, Japan) and 17 g vermiculite, pH 5.8]. Then, transgenic plants with roots were finally acclimated and grown at 20°C under a 16 h photoperiod using a fluorescent lamp (75 µmol m⁻² s⁻¹).

**Histochemical GUS assay**

Hygromycin-resistant calli and plants were subjected to stable histochemical GUS assay (Jefferson et al. 1987). First, each tissue was washed in cold 90% acetone for 15 min. Then, they were immersed in an X-gluc solution [10 mM sodium phosphate buffer (pH 7.0), 40 µM potassium ferrocyanide, 40 µM potassium ferricyanide, 20% (v/v) methanol, 0.5 g l⁻¹ 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc), and 0.3% (v/v) Tween 20] and incubated at 37°C for a day. Finally, blue-stained areas were assessed using ImageJ after bleaching with 70% (v/v) ethanol.

**Genomic PCR**

This study confirmed the introduction of the transgene to hygromycin-resistant plants through direct genomic PCR. PCR was also conducted to investigate the HPT gene. First, leaf sections from each hygromycin-resistant plant were ground in a 100 µl DNA extraction buffer [100 mM Tris-HCl (pH 9.5), 1 M KCl, and 10 mM EDTA]. The reaction mixture (25 µl) was composed of 1× PCR buffer, 0.4 mM of dNTPs, 0.3 µM of each primer, 0.5 units of KOD FX Neo (Toyobo, Osaka), and 1 µl of crude DNA extract. HPT primer sequences used were as follows: HPT-U97: 5'-GAA TCT GTG TCT TGC AGC TTA G 3' and HPT-L441: 5'-CCA TGT AGT GTA TTT GAT GAT C-3'. Additionally, thermocycling conditions were as follows: denaturation at 94°C for two minutes, followed by 35 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 1 min. Then, PCR products were finally separated on 2% (w/v) agarose gels stained with Midori Green Advance (Nippon Genetics, Tokyo).

**Statistical analysis**

The study data has been presented as mean±SE The statistical comparisons were performed using Tukey–Kramer method (p<0.05).

**Results**

**Optimal FPX concentration**

We evaluated the callus induction ability of *M. incana* ‘Kiss me Violet’ using an MS basal medium of different FPX concentrations (Figure 1A). After a week, we observed that 12.5–50 µM FPX treatments formed undifferentiated cells at the edge of the leaf section. After 2 weeks, white and fragile callus was also formed at all leaf section’s edges. Then, we

![Figure 1](image)
investigated the effect of lower FPX concentrations (1, 5, and 10 µM) on callus formation in *M. incana*. Results showed that 1 µM FPX was enough concentration to induce callus proliferation from the edge of leaf sections (Figure 1B). In the less than 10 µM FPX treatment, the color of the explants did not turn yellow. However, shoot and root formation was not observed during all the FPX concentrations after the two-month cultivation period. Furthermore, 2 mg l⁻¹ BA, a typical plant hormone concentration for *M. incana* regeneration, was not also observed during organogenesis after two weeks (Figure 1A). Nevertheless, after approximately 1.5 months, while the shoot formation was detected in some leaf sections, the shoot regeneration efficiency (number of shoots/number of explants) was 2.7%. Thus, FPX accelerated callus formation more at the edge of *M. incana* explants than BA.

**Evaluation of Agrobacterium-mediated transformation efficiency**

We investigated whether *A. tumefaciens* strains (EHA101, LBA4404, and GV3101) affected the infection efficiency of *M. incana*. Five days after infecting each strain harboring the 35S::GUS, the transformation efficiency was evaluated at the GUS staining area of seedlings. Results showed that while GV3101 had the highest transformation efficiency of 44.0 ± 3.7% among the three strains (Figure 2A, Supplementary Figure S1A), LBA4404 and EHA101 had 29.3 ± 4.9% and 15.4 ± 3.0% transformation efficiencies, respectively. Thus, *A. tumefaciens* GV3101 was suitable to infect *Agrobacterium* of *M. incana*.

Next, we investigated optimal cocultivation periods using *A. tumefaciens* GV3101. While more extended cocultivation periods increased the GUS staining area, the transformation efficiencies for cocultivation achieved after four, five, and six days were 41.3 ± 5.5%, 49.8 ± 5.4%, and 50.2 ± 4.1%, respectively (Figure 2B, Supplementary Figure S1B). However, although the cocultivation after 7 days achieved a 42.9 ± 6.7% transformation efficiency, the GUS staining area of each leaf section varied among leaf sections. In addition, the cocultivation for more than 6 days observed an excessive growth of *Agrobacterium* around the leaf explants. Hence, it revealed that the *A. tumefaciens* GV3101 strain and a cocultivation period for 5 days were suitable for transforming *M. incana*.

**Sensitivity of *M. incana* to antibiotic regents**

To reveal the optimal concentration of antibiotic reagents sufficient for selecting transgenic cells, we

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*Figure 2. Effects of Agrobacterium tumefaciens* strains (A) and the cocultivation period (B) on the transformation efficiency of *Matthiola incana*. Ten seedlings were replicated with each treatment. Different letters in each cultivar indicate significant differences using the Tukey–Kramer method (*p* < 0.05).

*Figure 3. Sensitivity of leaf sections to antibiotic reagents. Leaf sections were cultivated on a 12.5 µM FPX medium supplemented with each antibiotic reagent for 21 days. A, callus formation ratio for 0–20 mg l⁻¹ kanamycin; B, callus formation ratio for 0–20 mg l⁻¹ hygromycin. Thirty-two leaf sections were placed on each media. After cultivation for 21 days, they were classified into three callus formation degrees (none, moderate, and intense).*
investigated the inhibition degree of callus formation using a callus induction medium containing kanamycin or hygromycin. When the explants were cultured for 3 weeks on a medium containing more than 10 mg l\(^{-1}\) kanamycin, practically all leaf sections were bleached and did not form any calli (Figure 3A). Culturing on a medium containing 5 mg l\(^{-1}\) kanamycin observed intense callus formation in 31% leaf sections. Therefore, *M. incana* leaves had high sensitivity to kanamycin, and the optimal concentration range of kanamycin selection was narrow. Alternatively, although 10 mg l\(^{-1}\) hygromycin inhibited callus formation, the explants were slightly expanded and formed moderate callus (Figure 3B). Besides, more than 20 mg l\(^{-1}\) hygromycin had bleached leaf sections and suppressed callus formation. Complete callus formation was suppressed by 30 mg l\(^{-1}\) hygromycin (data not shown). Hence, based on our results, we proposed 10–20 mg l\(^{-1}\) hygromycin as the optimal selection strength.

**Agrobacterium-mediated transformations using *M. incana* leaf sections**

*M. incana* transformation was conducted using a cocultivation and selection medium supplemented with 12.5 \(\mu\)M FPX. The results showed that 425 leaf sections were infected with *A. tumefaciens* GV3101 harboring 35S::GUS. Then, the leaves were co-cultivated for five days on a selection medium supplemented with 10 mg l\(^{-1}\) hygromycin. At three to four weeks after transfer to the selection medium, leaf sections with frangible callus were formed (Figure 4A). Subsequently, formed calli were excised from leaf sections and transferred to a fresh medium of the same composition. Histochemical GUS stains were observed in practically all hygromycin-resistant calli (Supplementary Figure S2). Afterward, the proliferated callus was transferred to the MS basal medium for a month, after which it was subcultured on a shoot induction medium with 1 mg l\(^{-1}\) BA. A few calli turned green on the shoot induction medium and formed leaf primordia (Figure 4B). When we conducted three independent transformation events, the PCR-positive shoots were obtained from 2, 1, 2 individuals, resulting in a total transformation frequency of 0.7% (Table 1). Next, elongated shoots were excised from the callus and included in the root induction medium after applied rooting induction regent. After one month, transgenic plants were acclimatized.

**Evaluation of transgenic *M. incana* plants**

We evaluated two independent 35S::GUS lines (#1 and #2). Generally, the single- and double-flowered plants of *M. incana* are in a 50:50 ratio. Since the hosts for preparing the explants were selected randomly, lines #1 and #2 bloomed single and double flowers, respectively (Figure 4C and D). Except for flower shape, no change in the morphogenesis of both lines compared with nontransgenic plants was observed. Histochemical GUS stains were also observed in the floral buds and leaves of both lines, with line #1 having a higher intensity of blue staining than line #2 (Figure 4E and F). These results strongly supported that both lines were transgenic plants. Furthermore, approximately 350 bp amplified fragments were detected in both lines during the genomic PCR, implying the introduction of the *HPT* gene (Figure 4G).

Furthermore, this study showed that 35S::GUS line #1 was a single-flowered individual after obtaining its progeny by self-pollination. Results showed that virtually all seeds aseptically germinated on the MS basal medium with 10 mg l\(^{-1}\) hygromycin. Additionally,
they had amplified fragments, indicating the existence of the HPT gene through genomic PCR analysis (data not shown). GUS staining was also observed in the leaves of all seedlings (Supplementary Figure S3). These results implied that line #1 accepted at least two copies of T-DNA in its genome.

Furthermore, we obtained two independent transgenic shoots carrying 35S::AG-GFP (Table 1). In 35S::AG-GFP line #1, strong green florescence under blue light was observed (Figure 5). This result demonstrated that the transgene other than the GUS gene was also able to be overexpressed.

Discussion

Before conducting this study, we hypothesized three reasons why *M. incana* did not establish a genetic transformation system. One is its low *Agrobacterium* infection frequency, the second is its high sensitivity to antibiotic reagents, and the third is its slow shoot formation on a medium supplemented with BA.

First, we employed whole in vitro seedlings to evaluate the infection efficiency of *Agrobacterium*. This method is labor-efficient since it omits the preparation of leaf cut sections. In case of a transformation protocol using *M. incana* in vitro seedlings, both acetosyringone supplementation and vacuum treatments were necessary for infecting *Agrobacterium*. On the other hand, in case of a transformation protocol using *M. incana* leaf cut sections, acetosyringone supplementation was necessary for *Agrobacterium* infection, but the presence or absence of the vacuum treatment did not affect the infection efficiency of the leaf sections (data not shown). Acetosyringone availability has previously been reported to induce the vir gene expression of the Ti plasmid (James et al. 1993). Similarly, in *R. sativus* and *B. rapa*, supplementation with acetosyringone improved *Agrobacterium*’s infection efficiency (Cho et al. 2008; Muto et al. 2021; Takasaki et al. 1997). Hence, to transform *M. incana*, adding acetosyringone to the *Agrobacterium* infection solution is necessary.

*A. tumefaciens* GV3101 had significantly higher infection efficiency for *M. incana* than EHA101 and LBA4404 (Figure 2A and Supplementary Figure S1A). Radish also showed that GV3101 is a suitable strain (Cho et al. 2008; Muto et al. 2021). However, EHA101 had the best transformation efficiency in *A. thaliana* (Akama et al. 1992) and *B. rapa* (Takasaki et al. 1997). Thus, the preference of the *A. tumefaciens* strain was proposed to depend on plant species. A cocultivation period of 5–6 days showed the highest GUS stain area in *M. incana* (Figure 2B and Supplementary Figure S1B). Furthermore, since cocultivation for six days was observed during the hyperproliferation of *Agrobacterium*, we decided five days was the optimal cocultivation period. Previous studies reported that the cocultivation period with *Agrobacterium* was two or three days in most Brassicaceae plants (Akama et al. 1992; Cho et al. 2008; Takasaki et al. 1997). Due to the fact that *M. incana* was considered recalcitrant for the *Agrobacterium* infection, a longer cocultivation periods might be required to overcome it.

We subsequently selected transgenic cells by investigating the concentration of antibiotics. First, more than 10 mg l⁻¹ kanamycin, a well-used plant transformation antibiotic, inhibited callus formation completely (Figure 3A). However, since 5 mg l⁻¹

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**Table 1. Transformation frequency of *Matthiola incana* ‘Kiss me Violet’**

|                | Number of explants | Number of Hm-resistant calli | Number of PCR-positive shoots | Transformation frequency (%) |
|----------------|--------------------|------------------------------|-------------------------------|-------------------------------|
| 35S::GUS Exp. 1| 200                | 10                           | 2                             | 1.0                           |
| 35S::GUS Exp. 2| 225                | 1                            | 1                             | 0.4                           |
| 35S::AG-GFP Exp. 1| 265             | 8                            | 2                             | 0.8                           |
| Total          | 690                | 19                           | 5                             | 0.7                           |

Transformation frequency (%) = no. of GUS positive shoots/no. of explants × 100

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Figure 5. Phenotypes of 35S::AG-GFP transgenic plants. The plantlets of NT (non-transformant, left) and 35S::AG-GFP (right) were observed under visible light (A) and under blue light (B). Scale bar indicates 1 cm.
kanamycin did not inhibit callus formation, a consensus regarding the optimal kanamycin concentration to select transgenic cells could not be reached. Thus, the leaves of *M. incana* also had high sensitivity to kanamycin. Although 10 mg l\(^{-1}\) hygromycin moderately inhibited callus formation, explants were slightly expanded, and more than 20 mg l\(^{-1}\) hygromycin bleached the leaf explants (Figure 3B). Firstly, we attempted to select transgenic cells using 20 mg l\(^{-1}\) hygromycin; however, no transgenic *M. incana* callus was obtained (data not shown). Therefore, we propose 10 mg l\(^{-1}\) hygromycin as the optimal concentration for the selection reagent.

During preliminary experiments, we attempted to develop a transformation method for *M. incana* using the MS basal medium supplemented with 1–2 mg l\(^{-1}\) BA. We failed to obtain any hygromycin-resistant callus and shoots, the transient expression of *GUS* was detected immediately after co-cultivating with *Agrobacterium*. Furthermore, when *Agrobacterium*-infected leaf sections were cultivated on a selection medium without hygromycin, some pieces of calli were detected with the chimeric histochemical *GUS* stain after 2 months. However, they were transferred to the selection medium with hygromycin, making it challenging to obtain any hygromycin-resistant callus and shoot again. Therefore, we improved this situation by employing FPX, acting as a chemical inducer during callus formation, shoot regeneration, and *Agrobacterium* infection (Nakano et al. 2018). We observed that 1–50 \(\mu\)M FPX induced callus formation at 2 weeks after the explants were placed on the medium (Figure 1). Additionally, the response of FPX to differentiation was remarkably earlier than the ones by any plant hormone. In soybean and cucumber, callus induction was observed over several FPX concentrations of 15–105 \(\mu\)M (Nakano et al. 2018). However, although 15 \(\mu\)M FPX induced callus formation in tomatoes, higher FPX concentrations inhibited callus formation. Similarly, *M. incana* is proposed to respond to callus formation’s several FPX concentrations.

When 12.5 \(\mu\)M FPX replaced 1 mg l\(^{-1}\) BA in the cocultivation and selection medium, we obtained a lot of hygromycin-resistant calli (Figure 4A). However, although we also transformed using a medium supplemented with 1 \(\mu\)M FPX, no hygromycin-resistant calli were obtained (data not shown). No difference in callus formation efficiency between 1 and 12.5 \(\mu\)M FPX was observed (Figure 1), but there might be a few physiological differences between these coculturations. Adventitious shoots were formed when the obtained hygromycin-resistant calli were transferred to a shoot regeneration medium supplemented with 1 mg l\(^{-1}\) BA (Figure 4B).

The transformation frequencies after three independent experiments were 0.4%, 0.8%, and 1.0%, and indicated 0.7 ± 0.2%, in all (Table 1). Previous studies reported that *R. sativus* and *B. rapa* obtained transgenic shoots at frequencies of 0.26% (Cho et al. 2008) and 5.0% (Takasaki et al. 1997), respectively. Moreover, transformation system improvements for radish were based primarily on the transformation of *B. rapa* (Takasaki et al. 1997), achieving a transformation frequency of 13.5% (Muto et al. 2021). Although we also supplemented AgNO\(_3\) and glucose for their improvement, they remained unaffected by the enhanced transformation efficiency of *M. incana* (data not shown). As observed, the hygromycin-resistant callus formation ratio was 2.8% (19 calli / 690 explants), whereas the transformed shoot formation ratio was 0.7% (Table 1). Thus, because shoot formations from all transgenic calli was not observed, this step was considered a bottleneck to the transformation efficiency of *M. incana* transformations. Nevertheless, further studies should improve the transformation efficiency protocol.

We obtained two independent 35S::GUS transgenic line, #1 and #2, showing single- and double-flowered phenotypes, respectively (Figure 4C and D). Generally, the commercial seeds of *M. incana* are single- and double-flowered individuals in a 50:50 ratio. We randomly selected the host plantlets for the preparation of explants in this study, but further studies can preselect single- and double-flowered individuals with DNA markers that can identify the *MiAG* alleles (Nakatsuka and Koishi 2018).

The selfed progenies of single-flowered transgenic line #1 were obtained (Figure 4C). The investigated progenies were indicated through hygromycin resistance, after which their *HPT* fragments were amplified using genomic PCR (data not shown). Additionally, GUS staining was observed in the leaves of all seedlings (Supplementary Figure S3). Thus, it was revealed that the transgenes were inherited and expressed stably in *M. incana* transgenic plants. Furthermore, we introduced another transgene a GFP under the control of a 35S promoter, and hygromycin-resistant shoots were obtained. The GFP fluorescence was detected in obtained hygromycin-resistant shoots obtained (Figure 5), revealing that transgene other than *GUS* could be expressed in *M. incana*. Since flower color- and floral shape-related genes have been identified from *M. incana* (Nakatsuka and Koishi 2018; Nuraini et al. 2021, 2020), this transformation system can be used to further elucidate these gene functions. Major ornamental plants, including chrysanthemum (Noda et al. 2013; Sasaki et al. 2021), rose (Katsumoto et al. 2007), lily (Otani et al. 2020), lisianthus (Tomizawa et al. 2021), and gerbera (Zhang et al. 2017; Zhao et al. 2020), have also been established in transformation systems, leading to the development of novel genetic resources and characterization of gene functions for floral traits. Similarly, this study showed that *M. incana* established a stable transformation system and should be developed as a valuable transgenic plant in the future.
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Author contributions
YT and KY produced and confirmed the transgenic plants. KK investigated Agrobacterium infection conditions. YT and YA evaluated antibiotic regent sensitivity. YT, KY, and TN wrote the manuscript.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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