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# The Transformation System in Foxglove (*Digitalis purpurea* L.) Using *Agrobacterium rhizogenes* and Traits of the Regenerants

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**Abstract**

Our final goal is to breed dwarf cultivars of foxglove (*Digitalis purpurea* L.) using *Agrobacterium rhizogenes*. For this aim, we examined an effective method for hairy root induction, the phytohormone condition for regeneration from the root and traits of the regenerants. In the case of using a filter paper soaked with 1/2MS liquid medium for co-cultivation of leaf pieces with the bacteria, 30–40 hairy roots per 100 pieces were expected to be induced. Higher effect was given for regeneration from the hairy root through callus in zeatin addition than with BA, kinetin and no phytohormone added. In potted plants, numerical values of plant height, number of leaves, spike length, number of florets and leaf size were lower in a strain of transformants than in non-transformants, and both types of the plants exhibited similar plant forms and leaf morphologies. These traits provide novel possibilities for the breeding of dwarf cultivars of foxglove.

## 1. Introduction

Foxglove (*Digitalis purpurea* L.), one of the most important medicinal plants in the world (Evans, 1989), is also favored as a garden plant or cut flowers. Although a number of ornamental cultivars have been bred by crossing, a good dwarf cultivar for potting is not to be found.

*Agrobacterium rhizogenes* is the causal bacteria for inducing hairy roots from infected plant tissues by introducing T-DNA from the Ri-plasmid in the body into the plant genome (Chilton *et al.*, 1982). In many plants, the hairy roots have been cultured for production of the secondary metabolites (Toivonen, 1993; Bourgaud *et al.*, 1997), or regenerants have been acquired from the roots to breed the dwarf strains (Handa, 1992; Yazawa, *et al.*, 1995; Godo *et al.*, 1997). However, in foxglove, while induction of hairy roots by *A. rhizogenes* and production of cardioactive glycosides in the green hairy root were reported by Saito *et al.* (1990), the efficiency of the hairy root induction, conditions for regeneration from the root and traits of the regenerated plants have not yet been elucidated.

This paper describes, for the first time, an indispensable system for obtaining regenerated transformants in *D. purpurea* L. using *A. rhizogenes* and traits of the regenerants.

## 2. Materials and Methods

### *2.1 Plant material and culture conditions*

To establish plant materials for bacterial inoculation, seeds of *Digitalis purpurea* L. cv. Foxy (Fukukaen Nursery & Bulb Co., LTD.) were surface-sterilized for 15 min with a NaOCl solution (0.5% active chlorine) containing a few drops of Tween 20, rinsed with sterilized water and aseptically germinated on half-strength MS medium (Murashige and Skoog, 1962) solidified with 2% gellan gum (Wako Pure Chem. Instr. Ltd.) (1/2MS solid medium). The medium contained 30 g $l^{-1}$ of sucrose and no phytohormone, and was adjusted to pH 5.8 before autoclaving. The plants and plant tissues were cultured at 25 ℃ under continuous illumination provided with white fluorescent light (FLR40SW/M-B: Hitachi) at 6,000 lux for 16 h throughout the experiments, except during co-cultivation of the plant tissue with bacteria.

### *2.2 Bacterial strain*

The mikimopine type strain A13 of *Agrobacterium rhizogenes* (Daimon *et al.*, 1990) was used for transformation. The bacteria were grown in YEB liquid medium, supplemented with 100 $\mu$M

3', 5'-Dimethoxy-4'-hydroxyacetophenone (acetosyringone) and 5 mM betaine and adjusted to pH 5.2, for 12 h at 28 ℃ with reciprocal shaking.

*2.3 Bacterial inoculation, root induction and opine assay*

The leaves of 6-10 leaf-stage plants were cut into pieces about 5 mm square. The pieces were immersed in a bacterial suspension culture for 10 min and blotted on a sterilized filter paper to remove excessive amounts of the suspension. For co-cultivation of the pieces with the bacteria, two kinds of media (Noda *et al.*, 1987) were prepared. One was a filter paper (No. 2, 90 mm circle: ADVANTEC) soaked with 3 m*l* of 1/2MS liquid medium containing 30 g $l^{-1}$ of sucrose and adjusted to pH 5.8 (1/2MS filter paper medium), and the other was 30 m*l* of 1/2MS solid medium. Plastic dishes (SH90-15: Iwaki Glass) were used as culture vessels for both of the media, and sealed with parafilm (American National $Can_{TM}$). Co-cultivation on the media was performed in the dark for 3 days at 25 ℃. Then the pieces were transferred onto a 1/2MS solid medium containing Claforan (Cefatoxime sodium: Hoechst) to remove the bacteria. The concentrations of Claforan were stepped down from 500 mg $l^{-1}$ to 250, 100 and 0 mg $l^{-1}$ every two weeks. Bacterial inoculation experiments were practiced twice, and adventitious root induction was investigated on the 39th day after the inoculation. After this, induced adventitious roots from the leaf pieces were cut into 10 mm root tips, and each of the root tips was subcultured on the 1/2MS solid medium in a plastic dish at 1-2 month intervals. Four months after inoculation, a root which had filled a dish was judged as an actively grown root. Mikimopine detection was carried out in actively grown roots following the method of Godo *et al.* (1997).

*2.4 Plant regeneration*

Regeneration media, which were, respectively, 1/2 MS media containing zeatin, benzyladenine (BA), or kinetin with concentrations of 0.5, 1.0 or 2.0 mg $l^{-1}$ were dispensed into plastic dishes with a volume of 30 m*l* and solidified with 2% gellan gum. One strain of the mikimopine-producing hairy root was cut into pieces 10 mm in length and applied in each phytohormone condition with 10 pieces per plastic dish containing regeneration medium. The plastic dishes were sealed with Milli Wrap (Millipore). After 34 days of culture, calli induced from the root pieces were cut into pieces of 10 mm in diameter, and transferred to fresh media. Regeneration was investigated on the 73rd day since starting the culture. The experiments were carried out twice, and a total of 50 hairy root pieces were tested in each phytohormone condition.

*2.5 DNA extraction and Southern blot analysis*

Plant DNA was extracted from leaves according to the method using cetyltrimethylammonium bromide (CTAB) (Ausubel *et al.*, 1994). The DNA, digested with *Eco*RI or *Hin*dIII, was electrophoresed and transferred to a nylon membrane (1209 299: Boehringer Mannheim). A DNA probe for the *rol*C gene in the Ri T-DNA region was prepared by the polymerase chain reaction (PCR) method, using PCR DIG probe synthesis kit (Boehringer Mannheim). Template DNA for PCR was extracted from strain A13 of *A. rhizogenes*, and the oligonucleotide primers used for amplification of the DNA fragment in the *rol*C gene were 1724C and 1724D (Kiyokawa *et al.*, 1992). The reaction mixture was prepared according to the supplier's manual with no reduction of the concentration of DIG-dUTP. Thermal cycling was programmed as follows: 95 ℃ for 9 min before the first cycle; 10 cycles at 94 ℃ for 1 min, 55 ℃ for 2 min and 72 ℃ for 3 min; 39 cycles at 94 ℃ for 1 min, 55 ℃ for 2 min and 72 ℃ for 3 min + cycle elongation of 20 sec for each cycle; 1 cycle at 94 ℃ for 1 min, 55 ℃ for 2 min and 72 ℃ for 21 min. The amplified DNA was purified using Wizard PCR preps DNA purification system (Promega). Hybridization and detection of the chemiluminescence signal was performed with DIG luminescent detection kit (Boehringer Mannheim) according to the supplier's instructions.

*2.6 Traits of transformants*

Most of the regenerated shoots vitrificated and also propagated as multiple shoots on the 1/2MS solid media with no phytohormone. The health of the shoots was regained by means of the paper-wick method with 1/2MS liquid media containing no phytohormone. The healthy shoots, derived from one hairy root and confirmed harboring the *rol*C gene, and germinated non-transformed plants were cultured on the 1/2MS solid media with one plant per test tube (40 Φ × 128h mm) for 75 days. On July 22, 1997, the plants were transplanted and acclimatized in plastic containers (60 × 17 × 18h cm), which contained fully water-soaked vermiculite and were wrapped with polyvinylidene chloride films. The containers were placed in the open air, and not exposed to the sun. The films were removed from the containers on August 31. Then, on September 22, the acclimatized plants were transplanted into plastic containers three plants each. The composition of soil in the container was 40% compost

for horticulture (Seishin Instr. Inc.), 40% leaf mold and 20% sand. Seven days after transplanting, 15 g of controlled release fertilizer containing 10% of N, 10% of $P_2O_5$, 10% of $K_2O$ and 1% of MgO (IB S I: Mitsubishi Chem. Inc.) was applied per container. On October 1, the containers were transferred into a plastic film greenhouse with no heating. The number of lateral shoots, the largest leaf and main stem were investigated on Dec. 8, 1997, on May 6, 1998 and at the flowering time in 1998, respectively.

## 3. Results and Discussion

*2.1 Hairy root induction*

The efficiencies in root induction from leaf pieces inoculated with *Agrobacterium rhizogenes* were compared between the two cases using different co-cultivation media, referring to the method prescribed by Noda *et al.* (1987) (**Table 1**). On the 39th day after inoculation, the percentage of leaf pieces giving rise to root(s) and the number of induced roots per leaf piece were higher in the case of using a 1/2MS filter paper medium than a 1/2MS solid medium for co-cultivation in each of the two experiments. Most of the roots were induced from the leaf pieces preserving a green color. Further, the percentage of leaf pieces preserving green color was also higher in the case where a 1/2MS filter paper medium was used than in the other case (data not shown). These results suggest the positive correlation between the preservation of the health of the plant tissue and the efficiency of root induction.

Four months after inoculation, the percentages of actively grown roots on the phytohormone-free 1/2MS solid media were 94% in the case of using a 1/2MS filter paper medium and 85% in the other case. Mikimopine production was assayed in the growing roots induced in the case of using a 1/2MS filter paper medium. In eight of eleven roots, mikimopine was detected (data not shown), confirming that these eight were hairy roots.

From these data and subsequent calculation, it is expected that 30-40 hairy roots may be induced from 100 leaf pieces in using a 1/2MS filter paper medium.

No. of hairy roots / 100 leaf pieces
$= (83/154) \times 100 \times 0.94 \times (8/11)$
$= 37$

This expected value is higher than in case of using the other co-cultivation medium, even if 100% mikimopine production was assumed in the growing roots of this case. Therefore, it is concluded that a 1/2MS filter paper medium is more suitable than a 1/2MS solid medium as a co-cultivation medium for the hairy root induction in the present culture conditions.

*2.2 Plant regeneration*

For plant regeneration from the hairy root, we examined adventitious bud formation through callus with different kinds and concentrations of cytokinins (**Table 2**). In all cases where zeatin was added to regeneration media, the regeneration percentages were considerably higher than in all other cases of BA, kinetin and no cytokinin additions. In particular, adding 0.5 mg $l^{-1}$ of zeatin gave a significantly higher regeneration percentage in comparison with the results in all of the cytokinin conditions other than zeatin addition (Fisher's exact probability test, $P \leqq 0.05$). Moreover, most strains of the hairy roots were able to regenerate on the 1/2MS medium containing 0.5 mg $l^{-1}$ of zeatin (data not shown).

In adventitious bud formation of *Digitalis*, previously reported effective cytokinins were no phytohormone or kinetin for non-transformed haploid calli of *D. purpurea* (Corduan and Spix, 1975), or BA for hairy roots of *D. lanata* (Pradel *et al.*, 1997). It is interesting to note that zeatin has a higher effect than those of BA, kinetin and no phytohormone for

**Table 1.** The effects of co-cultivation media on root induction from leaf pieces.

| Co-cultivation medium | No. of leaf pieces tested A | No. of leaf pieces giving rise to root(s) B | Percentage of leaf pieces giving rise to root(s) (B/A) × 100 | No. of induced roots C | No. of induced roots per leaf piece C/A |
|---|---|---|---|---|---|
| 1/2MS filter paper medium[a] | 154 | 26 | 16.8**[c] | 83 | 0.54**[d] |
| 1/2MS solid medium[b] | 163 | 6 | 3.7 | 15 | 0.09 |

[a] A filter paper soaked with 3 m*l* of 1/2MS liquid medium.

[b] Volume of the medium was 30 m*l*.

[c,d] Significant at 1% level in accordance with Fisher's exact probability test and Student's t-test, respectively. The experiments were conducted twice, and investigation was conducted on the 39th day after inoculation.

**Table 2.** The effects of kinds of cytokinins with different concentrations on regeneration from hairy root.

| Cytokinin | Conc. ($mgl^{-1}$) | No. of hairy root pieces tested A | No. of pieces giving rise to bud(s) B | Regeneration percentage (B/A) × 100 |
|---|---|---|---|---|
| No cytokinin | | 50 | 0 | 0 |
| zeatin | 0.5 | 50 | 8 | 16 |
| | 1.0 | 50 | 6 | 12 |
| | 2.0 | 50 | 7 | 14 |
| BA | 0.5 | 50 | 1 | 2 |
| | 1.0 | 50 | 1 | 2 |
| | 2.0 | 50 | 1 | 2 |
| Kinetin | 0.5 | 50 | 0 | 0 |
| | 1.0 | 50 | 2 | 4 |
| | 2.0 | 50 | 2 | 4 |

The calli induced from hairy roots were transferred to fresh media on the 39th day after culture, and investigation was conducted on the 73rd day from starting culture.
The data were collected from two tests.

adventitious bud formation in the hairy roots of *D. purpurea*.

Although the regenerated shoots were able to propagate on the 1/2MS solid medium with no phytohormone, most of them vitrified. To recover their health, the shoots were cultured by means of the paper-wick method with 1/2MS liquid media. As a result of this treatment, a large number of healthy shoots were acquired.

*2.3 Southern blot analysis*

To examine the integration of T-DNA in Ri-plasmid of *Agrobacterium* into plant genome, Southern blot analysis was carried out using the fragment in the *rol*C gene as a hybridization probe (**Fig. 1**). In a non-transformed plant, no hybridization signal was observed in the case where either *Eco*RI or *Hin*dIII digested DNA was tested (Lanes 2 and 3). On the other hand, in a regenerated transformant, two and three hybridization signals were detected when *Eco*RI and *Hin*dIII digested DNA were examined (Lanes 4 and 5). These results demonstrate that the transformant harbors the *rol*C gene of T-DNA in the genome. The result also provides the possibility that two or three copies of the *rol*C gene were introduced into the genome because a single signal was detected in each of the same tests in a transformant of other species (Koga *et al.*, 2000).

*2.4 Traits of transformants*

The traits of the strain of transformants verifying the harboring the *rol*C gene in the previous section were compared with those of non-transformants, and the results are shown in **Table 3** and **Fig. 2**. In the main stem, the numerical values in plant height, number of leaves, spike length and number of florets were lower in the transformants than those of the non-transformants. However, no significant difference was detected from the calculated internode length in the main stem. The flowering date of the main stem was about one month later in the transformants than in the non-transformants.

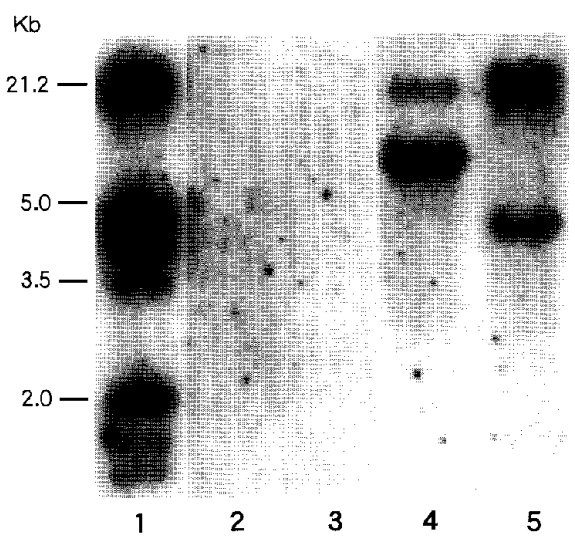



**Fig. 1** Southern blot analysis of DNA extracted from a non-transformant and a transformant. Applied DNA was extracted from a non- transformant (Lanes 2 and 3) and a transformant (Lanes 4 and 5), and digested with *Eco*RI (Lanes 2 and 4) and *Hind* III (Lanes 3 and 5). DNA size markers were applied on Lane 1.

**Table 3.** Comparison of traits in potted plants between transformants and non-transformants.

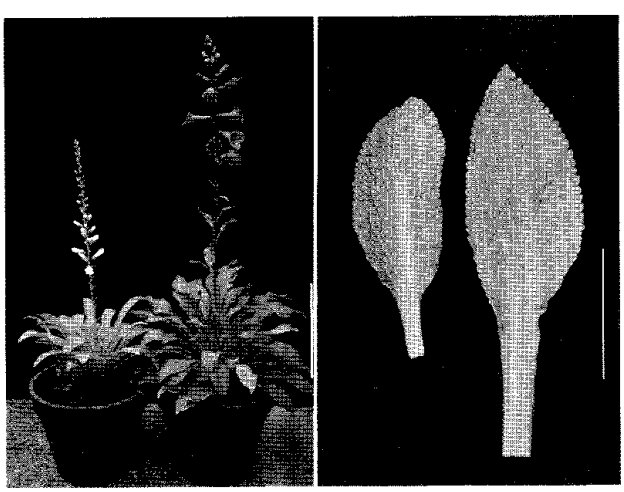
| | | | Transformant | Non-transformant |
|---|---|---|---|---|
| Main stem[a] | | | | |
| Plant height | (cm) | A | 47.6 ± 8.8[d]**[e] | 71.4 ± 15.1 |
| No. of leaves | | B | 33.1 ± 9.6** | 45.3 ± 10.6 |
| Spike length | (cm) | C | 21.9 ± 6.7** | 28.2 ± 8.7 |
| Internode length | (cm) | (A-C)/B | 0.92 ± 0.44 | 1.09 ± 0.53 |
| No. of florets | | | 22.6 ± 7.3** | 32.9 ± 11.7 |
| Flowering date | | | June 20 ± 15.6day** | May 19 ± 27.9day |
| Largest leaf[b] | | | | |
| Blade length | (cm) | | 10.8 ± 2.9** | 13.7 ± 1.8 |
| Width | (cm) | | 4.4 ± 1.1** | 5.8 ± 1.2 |
| Blade length/width | | | 2.61 ± 0.58 | 2.42 ± 0.44 |
| No. of lateral shoots[c] | | | 4.1 ± 3.2*[f] | 1.2 ± 1.2 |
| No. of flower stalks | | | 2.1 ± 1.8 | 1.3 ± 0.9 |

Transformants originated from one hairy root and regeneration event, and non-transformants derived from independent germination. Twelve transformants and 11 non-transformants were investigated.

[a,b,c] Measurements were conducted at the flowering time, on May 6, 1998 and on Dec. 8, 1997, respectively.

[d] Mean ± S. D.

[e,f] Significant at 1% and 5% leve, respectively, in accordance with Welch's t-test, Student's t-test or Mann-Whitney's U test.



A B

**Fig. 2** Comparative morphology between a transformant (left) and a non-transformant (right). (A) Whole plants. Size is smaller in the transformant than the non-transformant, although both plant forms resemble each other. The plants have been transplanted into pots for photographing. (B) Leaves. The leaves of both plants show quite similar morphology, and wrinkles are not seen in either of the leaves.
Leaf positions are different for both. Bar = 20 cm (A) and 5 cm (B).

Just before the flowering period of the non-transformants, the largest leaf of the transformants was smaller than that of the non-transformants, although the morphologies of leaves in both types of the plants resembled each other. In other words, blade length and width of the largest leaf were smaller in the transformants than in the non-transformants, but no difference in the ratios of both traits between both plant types was detected (**Table 3**), and no wrinkled leaf was seen in either plant type (**Fig. 2B**). Wrinkles were observed in leaves of many other plants transformed by *A. rhizogenes* (Tepfer, 1984; Handa, 1992; Sevón *et al.*, 1997). However, neither these potted transformants nor all four strains of in vitro transformants had a wrinkled leaf. In addition, plant forms of both types resembled each other in potted (**Fig. 2A**) and *in vitro* plants. It was also reported in *D. lanata* that the transformants showing the wrinkled leaves were only half the number of the strains. The wrinkling was moderate, and most of the transformants resembled non-transformants (Pradel *et al.*, 1997).

More numbers of lateral shoots were seen in the transformants than the non-transformants on Dec. 8, 1997. However, in this study, no significant difference was detected in the number of flower stalks between both plant types. The cause of these inconsistent results is unclear. Flower stalks from lateral shoots developed later than those from the main stem in both types of plants (data not shown).

Low plant height, numerous lateral shoots and the

original plant form and leaf morphology in transformants provide novel possibilities for breeding dwarf cultivars of *Digitalis purpurea*. Regrettably, no success has been attained so far in the acquirement of transformant seeds. And, the variation of traits among strains of the transformants is unknown. Therefore, investigations into these points and into utilization of the transformants for breeding are the next problems to be solved.

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