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Agrobacterium rhizogenes-mediated Transformation and Regeneration of *Vinca minor* L.

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Hairy roots of *Vinca minor* L. plant, which produces an important indole alkaloid called vincamine, was incited by infection with the *Agrobacterium rhizogenes* strain DC-AR2, a derivative of strain MAFF301724 isolated in Japan. Regenerants could be obtained from the hairy roots which had been cultured on Murashige-Skoog's medium containing 1 mg/*l* naphthaleneacetic acid. Some of *in vitro* cultured regenerants which had revealed the reduced apical dominance indicated *ca.* 10-fold increased shoot number compared with an untransformed plant. Kanamycin-resistant regenerants were also obtained from the hairy roots by infection with strain DC-AR2 carrying binary vector pBI121, which contains a neomycin phosphotransferase II (NPTII) gene and a β-glucuronidase (GUS) gene. Both the hairy roots and the regenerants showed GUS activity in their tissue.

Introduction

Lesser periwinkle (*Vinca minor* L.), a member of Apocynaceae, is not only a horticultural plant with lilac-blue flowers but also a medicinal plant producing an important alkaloid, named vincamine mainly accumulated in the leaves. Vinpocetine, a derivative of vincamine, is a cerebral vasodilator marketed by some pharmaceutical companies in European countries and Japan. In the field, the growth of the plants is comparably slow. Moreover, since the plant is usually propagated by cutting rather than seeds, the number of plants that can be propagated at a time is considerably limited. Stapfer and Heuser[1] have repòrted that an addition of cytokinin such as benzyladenine and kinetin into the medium had the effect of increasing the shoot number on *V. minor* plant culture. In our preliminary reproduction test in which the plants were cultured on medium containing 0-15 mg/*l* of BA, however, no significant effect was observed on the increase of the shoot number[2].

Transformed adventitious roots directed by root-inducing plasmid (pRi) of *Agrobacterium rhizogenes, viz.* hairy roots, are expected to be valuable biocatalysts in biochemical productions[3]. In some cases, furthermore, hairy roots are able to give rise to whole plants (pRi-transformed regenerants). It is generally recognized that the pRi-regenerants display the so-called hairy root syndrome including various morphogenic and physiological alterations[4]. In this syndrome, dwarf phenotype is an important characteristic for breeding flower crops such as *Eustoma grandiflorum*[5] (prairie gentaian) and *Dianthus* spp.[6].

In *in vitro* culture, the pRi-transformed regenerants frequently show rapid growth because of

both increased lateral-bud formation and rapid leaf-development[7]. This trait prompted us to apply it to micropropagation of plants such as *V. minor* which are difficult to multiply. Nevertheless, *Agrobacterium*-mediated transformation and regeneration system for *V. minor* has not been established up to the present.

In this article, we report the establishment of a transformation and regeneration system for *V. minor* by infection with *A. rhizogenes* harboring pRi1724, and simultaneously carrying binary vector pBI121. The characteristics of these regenerants and the foreign gene expression in them are also reported.

Materials and Methods

1. *Bacteria and plasmids*

Agobacterium rhizogenes strain DC-AR2 (kanamycin-sensitive mutant)[8], a derivative of strain MAFF301724 (renamed from MAFF03-01724, harboring mikimopine-type pRi1724) which had been isolated from a diseased melon plant in Japan[9], was used in the present study. A binary vector pBI121[10] (Toyobo Co., Japan) containing genes of both neomycin phosphotransferase II (NPTII) and β-glucuronidase (GUS) was introduced into the strain DC-AR2 by electroporation[8]. Chimeric plasmids, pRTE7.6[11] and pBI221[10] (Toyobo Co., Japan), were used for preparation of probes for Southern-blot hybridization: the former previously constructed by us contains a portion of pRi1724 T-DNA in the 7.6-kb *Eco*RI fragment[11]; the latter contains GUS gene in the 3.0-kb *Hin*dIII-*Eco*RI fragment[10].

2. *Induction and culture of hairy roots*

The leaf-disk inoculation method previously reported by us[12] was employed for infection of *A. rhizogenes* to *V. minor* plants with some modifications as follows: the plant materials mainly used were stem-segments with a node (*ca.* 2 cm in length) excised from 30-days cultured plantlets[13]; the inocula used were *A. rhizogenes* strain DC-AR2 harboring pRi1724 or both pRi1724 and pBI121, cultured in LB liquid medium at 28°C for 36 hr. After contact with the bacteria on water solidified with 1% agar for 3 days under light, the inoculated plant materials were cultured on Murashige-Skoog's (MS) medium[14] solidified with 0.2% Gellun Gum (Wako Pure Chemical Industries, Ltd., Japan), supplemented with or without 1 mg/*l* naphthaleneacetic acid (NAA), at 25°C in the dark. For elimination of the inoculated bacteria, 500 μg/m*l* carbenicillin (Sigma) and 500 μg/m*l* vancomycin (Sigma) were also added into the MS medium. The induced hairy roots were cut from the stem-segments, transferred to a fresh MS medium as described above and continuously cultured. For selection of pBI121-transformed hairy roots, 50 μg/m*l* kanamycin monosulfate was added into the MS medium.

3. *Regeneration and culture of shoot*

For shoot regeneration, hairy roots were cultured on MS medium containing 1 mg/*l* NAA in the dark. The obtained regenerants were grown on a hormone-free MS medium at 25°C for 12 hr-photoperiod and maintained by transfer of the apical tips (*ca.* 2 cm in length) every 30 days. For culture of pBI121-transformed regenerants, 50 μg/m*l* kanamycin was added to the MS medium.

4. *Opine assay*

Mikimopine[15], a kind of opine, in hairy roots and their regenerants was detected by quick opine assay method devised by Tanaka[16].

5. *DNA isolation and Southern-blot hybridization*

DNA was isolated from the leaves of regenerants from hairy roots by a small-scale DNA extraction method as described by Dellaporta *et al.*[17], followed by purification with CsCl-ethidium

bromide centrifugation.

An ECL gene detection kit (Amersham) was used for DNA labeling, Southern blot, and detection of target sequences according to the supplier's directions. A nylon membrane (Hybond-N^+, Amersham) was used for blotting. Chemiluminescence signal bands were visualized by exposure for 60 min. to an X ray film (Hyperfilm™-ECL, Amersham).

6. *GUS activity assay*

GUS activity in the plant materials was assayed as described by Uchimiya[18] with some modifications. Plant materials were homogenized in 100 μl of GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0, supplemented with 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl and 10 mM mercaptoethanol), followed by centrifugation at 12,000 rpm for 5 min. The supernatant adjusted to 250 μl with GUS extraction buffer was added to an equal volume of GUS extraction buffer including 1 mM 4-MUG (4-methyl-umbelliferyl-β-D-glucuronide). After incubation for 60 min. at 37°C, 50 μl aliquot of the reaction mixture was added to 950 μl stop buffer (0.2 M Na_2CO_3), and then the fluorescence was measured with spectrofluorometer set the excitation wavelength to 365 nm and the emission detector to 455 nm, or observed under UV light at 360 nm. The protein concentration in the extract was measured with spectrophotometer at 750 nm by using DC protein assay kit (Bio-Rad) according to the supplier's directions.

Results and Discussion

1. *Initiation and culture of hairy roots*

As an aseptically preparable plant material, an *in vitro* cultured plantlet of *Vinca minor* was suitable for inoculation of *Agrobacterium rhizogenes* in this study. About 20 days after inoculation, adventitious roots emerged from the edges and nodes of inoculated stem-segments cultured on MS medium containing 1 mg/l NAA (**Fig. 1-A**), whereas no root on hormone-free medium or from leaf disks. However, though new adventitious roots emerged one by one, a continuous culture on this medium suppressed the elongation of roots, consequently directed them to dedifferentiation (**Fig. 1-B**). To confirm the suitable concentration of NAA, the adventitious roots were cultured on MS medium containing 0, 0.01, 0.05, 0.1, 0.5 and 1 mg/l NAA. As shown in **Fig. 1-C**, the adventitious roots vigorously grew and lateral-branched out on the medium containing 0.01 and 0.05 mg/l NAA, while suppressed to elongation on the others. This result suggests that a low NAA concentration allows the growth as well as the initiation of hairy roots on *V. minor*. Indeed, pRi1724 harbored in *A. rhizogenes* strain DC-AR2 is one of the weakly virulent pRis, which possess no auxin-biosynthesis gene strongly stimulating for hairy root induction[19]. Thus, in case of infection with the weakly virulent pRi such as pRi1724, an auxin supplement is indispensable for adventitious root formation on *V. minor*, which probably contains a low amount of endogenous auxin; however, the required concentration of NAA seems to be low. Adventitious roots were also obtained from the stem-segments of *V. minor* by inoculation with the strain DC-AR2 carrying pBI121, showing a resistance to kanamycin in the culture medium (**Table 1**). Mikimopine was detected in the adventitious roots induced by inoculation with *A. rhizogenes* strain DC-AR2 as well as the strain carrying pBI121 (**Table 1**). Therefore, it is clear that the adventitious roots obtained here were the transformed, so-called hairy roots.

2. *Characteristics of regenerants*

Differentiation of shoots from the hairy roots induced by infection not only with the strain DC-AR2 but also the strain carrying pBI121 occurred in the culture on MS medium with 1 mg/l NAA in the dark (**Fig. 1-D**). The obtained shoots with feeble and yellowish wrinkle-leaves initially

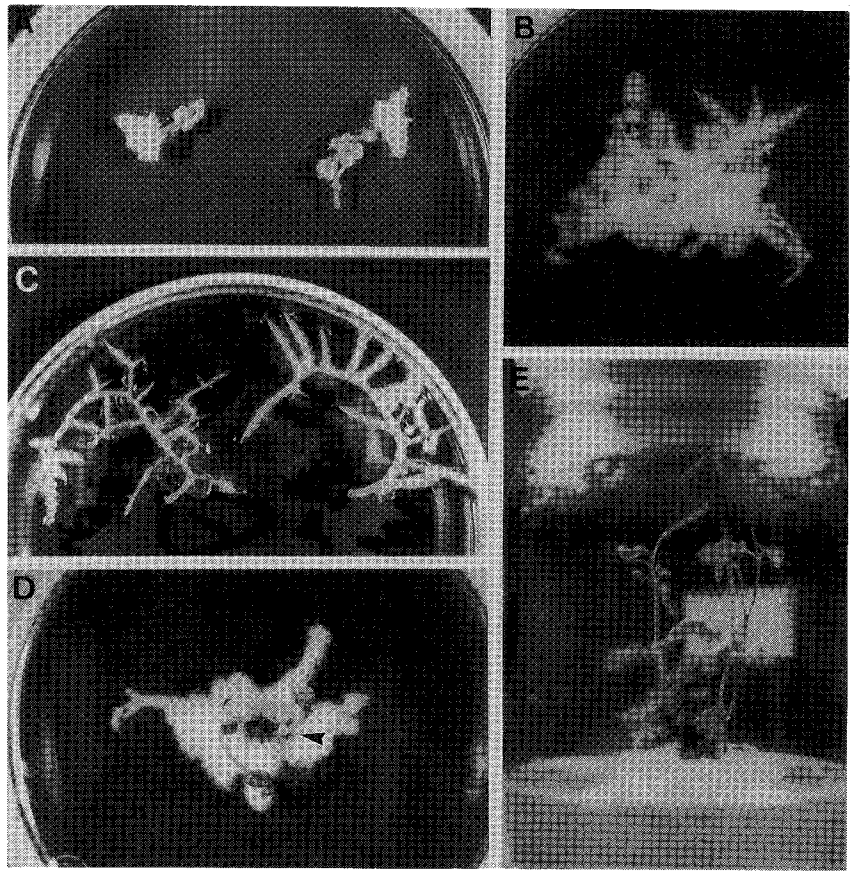

Fig. 1 Induction and culture of hairy roots and their regeneration of *V. minor*.
A, hairy roots induced by inoculation with *A. rhizogenes* strain DC-AR2; **B** and **C**, hairy roots cultured on MS medium supplemented with 1 mg/*l* (**B**) and 0.01 mg/*l* (**C**) NAA; **D** and **E**, shoots regenerated from hairy roots in the dark (**D**) and the continuously cultured plantlets (**E**).

Table 1. Characteristics of pBI121-transformed hairy roots.

Roots	Kanamycin resistance*1	Mikimopine*2	GUS activity*3
pRi1724-transformed	−	+	−
pBI121-transformed			
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	−	+

*1 Cultured on MS medium added with 50 μg/m*l* kanamycin monosulfate.
*2 Analized by paper-electrophoresis.
*3 Observed under UV light at 360 nm.

revealed slow growth under light (**Fig. 1-E**), but gradually developed firm green-leaves and vigorously grew. In this stage, the independently isolated regenerant-clones showed variations on phenotype such as shape of leaf, internode distance, reduced apical dominance and altered geotropism of root. **Fig. 2-A** to **C** show the views of representative clones of regenerants which displayed the different phenotypes. Among over 10 clones, both Vm-101 (**Fig. 2-A**) and -102 (**Fig.**

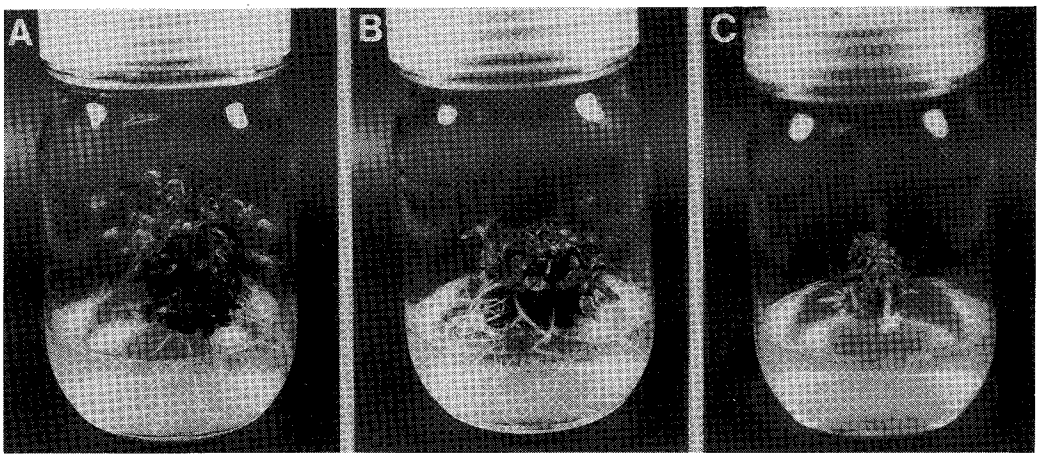

Fig. 2 Three representative pRi1724-transformed regenerants of *V. minor*.
A, clone Vm-101; **B**, clone Vm-102; **C**, clone Vm-103. These plants were cultured for 2 weeks.

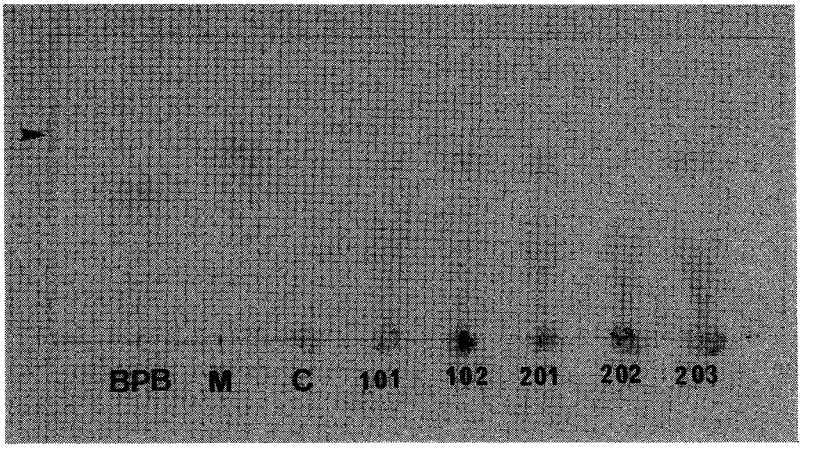

Fig. 3 Detection of mikimopine in the leaves of regenerants by paper-electrophoresis.
Paper-electrophoresis was carried out in 5 g/*l* ammonium bicarbonate buffer (pH 9. 8) at 500 V for 10 min. The spots were visualized with Pauly reagent. An arrowhead indicates authentic mikimopine. Lane BPB, bromophenol blue for tracking dye; lane M, authentic mikimopine; lane C, an extract from leaves of an untransformed plant; lanes 101 and 102, extracts from leaves of regenerants Vm-101 and -102 inoculated with strain DC-AR2; lanes 201 to 203, extracts from leaves of regenerants Vm-201 to -203 inoculated with strain DC-AR2 carrying a binary vector, pBI121.

2-B) showed rapid growth. On 30-day old plantlets, the mean shoot numbers of Vm-101 and -102 were 11. 1 and 8. 8, whereas that of cultured untransfomed-plantlets was 1. 0; accordingly, we can propose that these regenerants are highly propagative plant-materials available for micropropagation of *V. minor*.

Mikimopine was detected in the leaves of all the regenerants examined but not in those of an untransformed plant (**Fig. 3**). Thus, these regenerants seemed to be transformed by pRi1724 T-DNA.

3. *Existence of pRi1724 T-DNA and GUS gene in the regenerants*

To obtain stronger evidence that the regenerants selected here had been transformed by pRi1724 and/or pBI121, we performed Southern-blot hybridization analysis. Total DNAs were isolated

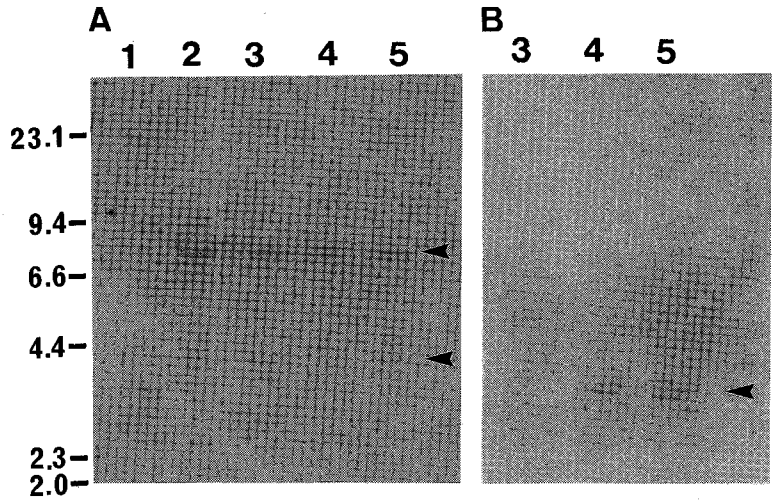

Fig. 4 Detection of T-DNA of pRi1724 and GUS gene of pBI121 in the transformed regenerants by Southern-blot hybridization analysis.
Two μg of each DNA was digested with *Eco*RI (**A**) or *Hin*dIII-*Eco*RI (**B**). **A**: a 7.6-kb fragment, in which *rolABC* genes of pRi1724 exist, isolated from *Eco*RI digests of pRTE7.6 was used as a probe. An upper arrowhead and a lower one indicate the hybridization signal bands corresponding to 7.6 kb of the *Eco*RI fragment and 3.5 kb of an unknown fragment, detected in both transformed and untransformed plants, respectively. **B**: a 3.0-kb fragment, in which GUS gene exists, isolated from *Hin*dIII-*Eco*RI digests of pBI221 was used as a probe. An arrowhead indicates the hybridization signal band corresponding to 3.0 kb of the *Eco*RI-*Hin*dIII fragment. Lane 1, untransformed plant; lanes 2 and 3, regenerants Vm-101 and 102; lanes 4 and 5, regenerants Vm-201 and 203. The numerals on the left are DNA sizes in kb referred from λ/*Hin*dIII digests.

from the leaves of untransformed plant, those of regenerants in clones Vm-101 and -102 transformed by pRi1724 (pRi1724-transformed regenerants), and those of regenerants in clones Vm-201 and -203 transformed by both pRi1724 and pBI121 (pBI121-transformed regenerants). First, the hybridization analysis was carried out between the plant DNAs digested with *Eco*RI and the 7.6-kb *Eco*RI fragment in pRi1724 T-DNA: the fragment includes rooting locus genes A, B and C (*rolABC*) responsible for induction of hairy roots[11]. As shown in **Fig. 4-A**, the hybridization signal bands corresponding to 7.6 kb were detected in all the DNAs isolated from the regenerants but not from the untransformed plant. Moreover, less-intense hybridization signal bands corresponding to 3.5 kb were detected in all the DNAs examined (**Fig. 4-A**), although we repeated two independent hybridizations. It has been demonstrated that the untransformed *Nicotiana glauca* genome includes sequences that have more than 80% homology to the TL-DNA of Ri plasmid, the so-called Ng *rol*[20,21]. It seems that such pseudo-*rol* genes exist in the genome of *V. minor*.

Besides, the 3.0-kb *Hin*dIII-*Eco*RI fragment, which includes a complete GUS gene, was also detected in the DNAs isolated from the pBI121-transformed regenerants (**Fig. 4-B**). From these results, we can conclude that an *A. rhizogenes*-mediated transformation and regeneration system, which includes a binary vector, has been established on *V. minor*.

4. *Analysis of GUS activity*

Owing to the fact that plant cells in many species scarcely possess intrinsic GUS activity, GUS gene is a reporter gene available for the observation of gene expression in the cells[10]. We also used it for a model gene expressed in *V. minor* cells in the present study. Under UV light, fluorescence

Table 2. GUS activity in pBI121-transformed regenerants.

Regenerant No.	GUS activity*1 (pmol/min/mg protein ± standard deviation)
101*2	5.4± 1.4
201*3	825.1±236.0
202*3	9.2± 1.3
203*3	35.9± 17.5

*1 An aerial part of three plantlets cultured for 30 days were used for analysis.
*2 A pRi1724-transformed regenerant.
*3 pBI121-transformed regenerants.

exhibiting the GUS activity was observed in the reaction mixture including the extracts from pBI121-transformed roots 1-4, while no fluorescence was observed in the pRi1724-transformed root extract (**Table 1**). On the other hand, the pBI121-transformed root 5 showed kanamycin resistance and GUS activity but not mikimopine accumulation (**Table 1**). Root 5 could have been transformed only by pBI121, thus being a spurious hairy root.

The pBI121-transformed regenerants had GUS activity. As listed in **Table 2**, the activity in each clone was different from the other. The reason the activity differed among the regenerants is unknown, but it is likely that a copy number of integrated GUS gene or the vicinal nucleotide sequence of integrated site regulated the expression. We must carry out more experimentation to understand the reason.

In the present study, we demonstrated the auxin-dependent induction of *V. minor* hairy roots by infection with *A. rhizogenes* strain DC-AR2, which contains no auxin-biosynthesis gene. If the GUS gene in pBI121 carried in DC-AR2 is exchanged with auxin-biosynthesis ones, we will be able to induce adventitious roots without the addition of auxin to the culture medium.

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《和文要約》

Agrobacterium rhizogenes によるヒメツルニチニチソウ(*Vinca minor* L.)の形質転換と植物体再生

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ビンカミン生産植物 *Vinca minor* L. に *Agrobacterium rhizogenes* MAFF301724 株由来の km^S 変異株 DC-AR2 株(pRi1724 保有) を接種し，1 mg/*l* の NAA を添加した MS 培地で培養することによって毛状根が誘発できた．さらに培養を続けると，本毛状根から植物体が再生し，これらは pRi1724 によって形質転換されていることが確認された．再生個体の幾つかは，非形質転換培養植物に比べ茎数が 10 倍以上増加した．km^R 遺伝子および GUS 遺伝子を持つバイナリーベクター pBI1121 を導入した DC-AR2 株接種によっても，毛状根の誘発ならびに再生個体の取得ができ，これらはカナマイシン耐性および GUS 活性を示し，GUS 遺伝子の組み込みも確認された．以上より，*A. rhizogenes* 感染によって *V. minor* から形質転換個体が得られ，その顕著な茎数増加の特徴はクローン繁殖に有用であることが示唆された．