Efficient Genetic Transformation in Lavandin using *Agrobacterium rhizogenes* as Vector

Masato Tsuro*, Hiroyuki Ikedo and Hiroe Kato

Faculty of Agriculture, Meijo University, Shiogamaguchi, Tenpaku, Nagoya 468-8502, Japan

To develop an efficient procedure for the genetic transformation of lavandin, wild-type *Agrobacterium rhizogenes* harboring pIG121-Hm was used for infection. β-Glucuronidase (GUS) expression was compared in leaf segments and leaf-derived calli as inoculation explants after five days of cocultivation, but GUS expression was only observed on calli. Hairy roots with strong GUS expression formed in 27.3% of these calli. When root segments excised from hairy roots were cultured in media containing various concentrations of 6-benzylamino purine (BA), thidiazuron (TDZ), or N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU), adventitious shoots were formed in some media. The adventitious shoot formation rate depended on the plant-growth regulator, and the highest adventitious shoot formation rate was 77.5%, in medium containing 0.02 mg·L⁻¹ CPPU. This medium was not as effective in the formation of adventitious shoots from calli. Shoots from hairy roots were easily rerooted and acclimatized in a temperature- and light-controlled room. Transformation efficiency was more than six times higher with the *Agrobacterium rhizogenes*-infected culture series (20.6%) than with the *Agrobacterium tumefaciens*-infected culture series (3.3%), in terms of the rates of hairy root induction and regeneration from hairy root segments. These results indicate that efficient genetic transformation in lavandin can be achieved with *A. rhizogenes* as the vector.

Key Words: *Agrobacterium rhizogenes*, GUS, hairy root, lavandin, transformation.

Introduction

Lavandin, *Lavandula × intermedia* Emeric, is one of the most useful aromatic plants native to the Mediterranean region. Essential oil obtained from its floral spikes by steam distillation is used in the production of perfumes, cosmetics, and pharmaceuticals. Lavandin produces much more essential oil per plant than other species of lavender because of heterosis when true lavender, *L. angustifolia* Mill., is crossed with spike lavender, *L. latifolia* Medic (Rabotyagov and Akimov, 1987). On the other hand, large quantities of camphor and borneol in lavandin result in lower-quality oil than that of other lavender species (Tsuro et al., 2004); therefore, an important aim of lavandin breeding is to decrease the level of these compounds, although the hybrid sterility of lavandin precludes the use of conventional crossbreeding methods to improve its traits. *Lavandula* plants with mutations in genes affecting essential oil characteristics have been reported: three of 63 regenerated true lavender plants had fragrances that differed from that of the original plant (Tsuro et al., 2001) and four somaclonal lavandin variants among 755 regenerated plants had low levels of camphor and borneol (Tsuro et al., 2004).

Genetic transformation is an efficient method of modifying important agricultural traits in plants. Floricultural plants with improved traits, such as flower color and shape, have been produced using this method (Aida et al., 2008; Katsumoto et al., 2007). *Agrobacterium tumefaciens* has been used to induce genetic transformation in *Lavandula*: genetic transformants of *L. latifolia* have been produced from leaves and leaf-derived calluses (Mishiba et al., 2000; Nebauer et al., 2000) and transgenic lavandin plantlets have been produced from leaf explants (Dronne et al., 1999); however, the rates of transformation are slow, 1.8%–7.7%, when *A. tumefaciens* is used.

*Agrobacterium rhizogenes* is a soil-borne bacterium that causes hairy root disease. It has been used to introduce binary plasmid T-DNA into plant genomes, e.g., petunia and melon (Kiyokawa et al., 1992; Toyoda et al., 1991). Tsuro et al. (2005) suggested that
A. rhizogenes is a more efficient transformation agent than A. tumefaciens in chrysanthemum; however, transformation of Lavandula plants using A. rhizogenes has not been reported.

In this report, we describe a genetic transformation system for lavandin in which A. rhizogenes is used and compare the transformation efficiencies of A. tumefaciens and A. rhizogenes.

Materials and Methods

Callus induction

Lateral branches with fully developed leaves were collected from lavandin plants cultivated in a greenhouse. They were immersed in 70% ethanol for 1 min, and soaked in sodium hypochlorite solution (about 2% active chlorine) for 15 min to sterilize them. They were then rinsed three times with distilled sterilized water. The leaves were then cut into small segments (5 mm × 5 mm), and used as explants. For callus induction, the leaf explants were placed on solidified Murashige and Skoog (MS) medium (1962) supplemented with 1.0 mg·L⁻¹ hygromycin, and cultured at 25°C in the dark for two weeks. These two explants, bare leaf segments and leaf-derived calli, were used for Agrobacterium inoculation.

Bacterial strains

Two types of Agrobacterium strains were used for infection. One was wild-type Agrobacterium rhizogenes A-13 (MAFF-02-10266) (Daimo et al., 1990) and the other disarmed Agrobacterium tumefaciens LBA4404 (Hoekema et al., 1983). Both Agrobacterium strains harbor the binary vector pIG121-Hm (Ohta et al., 1990), which contains the β-glucuronidase (GUS) reporter gene containing an intron in the coding sequence. Each bacterium was cultured in liquid yeast-enriched broth (YEB) medium (Vervliet et al., 1974) supplemented with 50 mg·L⁻¹ hygromycin on a rotary shaker (160 rpm) at 28°C for 16 h before infection.

Transformation and regeneration

Leaf-derived calli were immersed in YEB medium containing suspended Agrobacterium (OD₆₀₀ = 0.1) supplemented with 100 μM acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) for 10 min and blotted on sterilized filter paper. They were then cocultivated with Agrobacterium on MS medium supplemented with 1.0 mg·L⁻¹ of 2,4-D at 25°C in the dark. Three days after cocultivation, explants treated with A. rhizogenes were transferred to fresh 1/2 MS medium (MS macro nutrients at half strength) containing 500 mg·L⁻¹ carbenicillin, 100 mg·L⁻¹ kanamycin, and 5 mg·L⁻¹ hygromycin, and cultured at 25°C in the dark, to induce hairy roots and eliminate A. rhizogenes. After six weeks of culture, hairy roots, developed from calli, were cut and divided into 10 to 15 pieces of short segments (about 10 mm in length), and then individually distributed on MS medium containing 200 mg·L⁻¹ carbenicillin and 100 mg·L⁻¹ kanamycin, supplemented with 0, 0.5, 1.0, 5.0, or 10.0 mg·L⁻¹ of benzylamino purine (BA) or thidiazuron (TDZ), or 0.02, 0.03, 0.04, 0.05, or 0.1 mg·L⁻¹ of N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU). For shoot induction, they were cultured at 25°C for three months with continuous exposure to cool white fluorescent light. Shoots with hairy root segments were transferred to 1/2 MS medium to induce rerooting under the same conditions used for shoot induction.

Explants with A. tumefaciens were transferred to MS medium containing 500 mg·L⁻¹ carbenicillin, 100 mg·L⁻¹ kanamycin, and 5 mg·L⁻¹ hygromycin, supplemented with 1.0 mg·L⁻¹ BA or 0.02 mg·L⁻¹ CPPU, and cultured for three months under the same conditions used for the induction of shoots from hairy roots. After one month of culture, hygromycin was eliminated from the shoot-induction medium. Shoots with calli were transferred to 1/2 MS medium to induce root formation under the same conditions used for shoot induction.

The samples were subcultured every two weeks at each culture step.

Transient histochemical GUS assay

β-Glucuronidase (GUS) enzyme assays were performed histochemically according to the method of Jefferson et al. (1987). Cultured tissues were immersed in 5-bromo-4-chloro-3-indolyl β-D-glucuronidase (X-gluc) solution and incubated at 37°C overnight. They were then evaluated visually by the density of the coloration.

Confirmation of transformation

Genomic DNA was extracted from young leaves by the modified cetyltrimethylammonium bromide (CTAB) method (Bousquest et al., 1990; Wagner et al., 1987). Two sets of PCRs were conducted to detect the gus and rolC genes, according to Shinoyama et al. (1998) and Kiyokawa et al. (1992), respectively. For genomic Southern blot analysis, 40 μg of genomic DNA, digested with XbaI or EcoRI, was concentrated by ethanol precipitation and separated on 1.0% agarose gel. The separated DNA was transferred to a Hybond-N nylon membrane (Amersham Bioscience, UK). A DNA probe for the gus or rolC gene was labeled with digoxigenin with the PCR DIG Probe Synthesis Kit (Roche Diagnostics, USA). For gus gene detection, a membrane with XbaI-digested genomic DNA was used for hybridization, according to Shinoyama et al. (2002). The other membrane with EcoRI-digested genomic DNA was employed for hybridization to detect the rolC gene, according to Ohara et al. (2000). Hybridization and detection of the chemiluminescent signal were performed with the DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions.
Results and Discussion

Transient GUS expression in two types of explants

To evaluate the initial transformation efficiency, GUS expression was analyzed in two types of explants, bare leaf segments and leaf-derived calli, after five days of cocultivation. With \textit{A. rhizogenes} infection, no bare leaf segments expressing GUS spots were observed (Fig. 1A). In contrast, strong GUS expression was observed at callus-formed sites in 92 of 100 leaf-derived calli (92.0\%) (Fig. 1B). Similar results were observed in \textit{A. tumefaciens}-infected explants (data not shown). Mishiba et al. (2000) reported that the use of leaf segments as the targets for \textit{Agrobacterium} infection gave much lower transformation efficiency than that achieved with the calli of \textit{L. latifolia}; this result is consistent with our result. There are numerous glandular trichomes on the surface of the leaf, which produce essential oils containing components that differ from those of oil from the flowering part of lavandin (Hikawa, 1998). Some antimicrobial activity has been observed in plant essential oils (Ratanapitigorn et al., 2006). From these reports, successful infection might be prevented by secreted essential oils; therefore, leaf-derived calli were used for \textit{Agrobacterium} infection in subsequent experiments.

Hairy root formation and regeneration

In \textit{A. rhizogenes} infection, adventitious roots differentiated from the surface of the calli 10 days after inoculation. These roots grew vigorously on medium without plant-growth regulators and showed unique phenotypes, e.g. lateral branching and fast elongation (Fig. 2A). Conversely, no roots were observed on the

Table 1. Effects of \textit{Agrobacterium} strain and plant growth regulators on adventitious shoot formation from hairy root segments or leaf-derived calli, and transformation efficiencies in \textit{A. rhizogenes}- and \textit{A. tumefaciens}-inoculated culture.

<table>
<thead>
<tr>
<th>\textit{Agrobacterium} strain</th>
<th>No. calli inoculated</th>
<th>No. hairy root formed calli</th>
<th>No. root segments cultured for shoot induction</th>
<th>Plant-growth regulator for shoot induction</th>
<th>Concentration (mg·L(^{-1}))</th>
<th>No. adventitious shoots formed from explants</th>
<th>Adventitious shoot formation rate (%)(^z)</th>
<th>No. regenerated plantlets with rolC</th>
<th>No. regenerated plantlets with gus</th>
<th>Transformation efficiency for gus (%)(^y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{A. rhizogenes} A-13</td>
<td>750</td>
<td>205 (27.3%)</td>
<td>200</td>
<td>BA</td>
<td>0.5</td>
<td>3</td>
<td>1.5e</td>
<td>2</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>197</td>
<td></td>
<td></td>
<td>1.0</td>
<td>6</td>
<td>3.0e</td>
<td>3</td>
<td>3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>5.0</td>
<td>3</td>
<td>1.5e</td>
<td>3</td>
<td>3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>10.0</td>
<td>2</td>
<td>1.0e</td>
<td>2</td>
<td>2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>TDZ</td>
<td>0.5</td>
<td>1</td>
<td>0.5e</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>5.0</td>
<td>1</td>
<td>0.5e</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>10.0</td>
<td>1</td>
<td>0.5e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td>CPPU</td>
<td>0.02</td>
<td>155</td>
<td>77.5a</td>
<td>150</td>
<td>151</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>135</td>
<td>67.5ab</td>
<td>113</td>
<td>113</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>108</td>
<td>54.0bc</td>
<td>72</td>
<td>72</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>33</td>
<td>16.5d</td>
<td>30</td>
<td>30</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>\textit{A. tumefaciens}</td>
<td>760</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBA4404</td>
<td>466</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^z\) Different letters within the column indicate significantly different at 1\% level, based on \(\chi^2\) value.

\(^y\) Transformation efficiency with \textit{A. rhizogenes} infection = Hairy root formation rate (27.3\%) \times (No. regenerated plantlets with gus/No. root segments cultured for shoot induction). Transformation efficiency with \textit{A. tumefaciens} infection = No. regenerated plantlets with gus/No. infected calli cultured for shoot induction.
surfaces of *A. tumefaciens*-infected calli. In many plants, transformed hairy roots show typical characteristics, such as branching and rapid growth, which are induced by the expression of *rol* genes in Ri-T-DNA (Hoshino and Mii, 1998; Ohara et al., 2000); therefore, we identified these roots as hairy roots with *A. rhizogenes*. Strong GUS expression was also confirmed in these hairy roots (Fig. 2B); thus, 205 of 750 calli (27.3%) formed hairy roots during culture in medium without plant-growth regulators (Table 1).

In the shoot induction culture, almost all root segments began to form calli just after their transfer to each shoot induction medium. Adventitious shoots differentiated from the surfaces of calli after eight weeks of culture in some media. The adventitious shoot formation rates after 12 weeks of culture are shown in Table 1. The significantly highest adventitious shoot formation rate was 77.5%, observed in medium containing 0.02 mg·L\(^{-1}\) CPPU. In a previous study, few differences in the shoot formation rates, 93.3% and 86.7%, of leaf-derived calli were observed in medium containing BA or CPPU (Tsuro et al., 2004). In fact, in *A. tumefaciens* infection, no significant differences among media containing BA or CPPU were observed in adventitious shoot induction from leaf-derived calli (Table 1). Estruch et al. (1991) showed that *rolC* encodes a cytosolic β-glycosidase that releases free forms of cytokinin from their inactive glucoside conjugates. Our results suggested that dramatic changes in the proportion of phytohormones might have occurred in hairy roots, leading to differences in the efficiency of shoot induction between BA and CPPU.

The developed shoots were excised from the calli and transferred to root induction medium. A clear difference in root formation was observed between shoots transformed with *A. rhizogenes* and those transformed with *A. tumefaciens* (Fig. 3A, 3B). In *A. rhizogenes*-transformed shoots, many roots differentiated and elongated vigorously. Moreover, they showed branching similar to that of hairy roots. Shoots transformed with *A. tumefaciens* also formed roots easily on root induction medium; however, the number of roots, the elongation rate, and the degree of branching were limited compared with those of *A. rhizogenes*-transformed shoots. When root-formed shoots were randomly selected, all showed GUS expression (Fig. 3C). Both *Agrobacterium*-transformed regenerants acclimatized easily and continued to grow well in a temperature- and light-controlled room.

PCR analyses were conducted to confirm the integrated genes. The *gus* gene was amplified from all regenerated plantlets infected with either *A. rhizogenes* or *A. tumefaciens* (Fig. 4A). Furthermore, the *rolC* gene was detected in all plantlets regenerated from hairy roots, except for one plant (Fig. 4B). Interestingly, in the one plant with no detectable *rolC*, no hybridization signal was observed for Ri plasmid T-DNA by Southern blot analysis despite the detection of pIG121-Hm T-DNA (Fig. 5A, lane 6 and Fig. 5B, lane 6). After co-transformation with wild-type *A. rhizogenes*, plantlets regenerated from hairy roots on selective medium always had two kinds of T-DNAs, Ri-T-DNA and binary plasmid-derived genes (Handa, 1996). Why one plant without *rolC* regenerated from hairy roots is unclear. Tsuro et al. (2005) found that wild-type *A. rhizogenes* (with pIG121-Hm) transported T-DNA of binary plasmid more efficiently than Ri-T-DNA alone in chrysanthemum. From our results and the above report, a transformant without *rolC* might have been regenerated.
achieved using wild-type *A. rhizogenes* as a vector. All regenerated plantlets with the rolC gene (150 plantlets), obtained through the most efficient regeneration culture series, grew normally, and no phenotypic variations, such as hairy root syndromes, were recognized in a temperature- and light-controlled room (Fig. 6). We are now evaluating their growth habit and morphological characteristics in a non-temperature controlled greenhouse under natural day-length and light intensity.

**Acknowledgements**

The authors thank Dr. H. Daimon of Osaka Prefecture University for the gift of *A. rhizogenes* A-13.

**Literature Cited**


Mishiba, K., K. Ishikawa, O. Tsuji and M. Mii. 2000. Efficient...


