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Research Note

Improvement of ornamental characteristics in *Rehmannia elata* through *Agrobacterium rhizogenes*-mediated transformation

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Abstract

An attempt was initiated to develop short stature bushy transgenic ornamental plant *Rehmannia elata* from hairy roots culture. Transgenic plants were regenerated by infecting leaf explants with *Agrobacterium rhizogenes* R1000 from hairy roots culture. PCR amplification confirmed the presence of root loci (*rol*) genes from the root-inducing (Ri) plasmid in the transgenic *R. elata*. Three transgenic lines were characterized in the greenhouse trial. Several morphological traits such as the plant height, the size and number of leaves and flowers of these tranformants had changed in terms of ornamental value. The transgenic had different phenotype from that of the wild-type plants: the internodes were clearly shorter in the transformants, with a short, bushy, and compact growth with plenty of flowers. Among the transformants, T2 transformant contained 8-fold higher stem number, 5-fold higher flower number and 3 fold higher leaf number compared to wild-type. Such compact plants with numerous flowers can be used effectively for further breeding.

Keywords: *Agrobacterium rhizogenes*; *Rehmannia elata*; hairy root culture; ornamental plant; transgenic plant.
Abbreviations: *rol*, root loci; Ri, root-inducing; MS, Murashige and Skoog; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction.

Introduction

Rehmannia elata is a perennial plant of the Scrophulariaceae family. The genus *Rehmannia* has been named after the Russian physician Joseph Rehmann, and species name *elata* means “tall” or “lofty”. The plant resembles a foxglove with large, bright, pink/red, and bell-shaped flowers with yellow-spotted throats. *R. elata* is cultivated for ornamental purposes. A good plant quality is imperative for the production of ornamental potted plants (Albach et al., 2007). An important quality required in these plants is compact growth. However, most potted plants like *R. elata* exhibit an elongated natural growth and are susceptible to lodging. Compact growth in ornamental plants is commonly achieved through the application of chemical growth retardants (Rademacher, 2000). During recent years, growth retardants have been increasingly criticized because they are hazardous to human health and to the environment (Andersen et al., 2002; Fujimoto et al., 1997). The use of some growth retardants, such as paclobutrazol and daminozide, has been banned in many countries, and more will probably be banned in the near future. Compact genotypes can be alternatively achieved by inserting the root loci (*rol*) genes of the soil-borne bacterium *Agrobacterium rhizogenes* into the wild-type plants. This bacterium has a natural DNA transferring property, which is among the most widely used approaches of introducing DNA into plant cells. *A. rhizogenes* causes hairy root disease in various plant species, which is characterized by stunted plant growth and abnormal roots called hairy roots (Guillon et al., 2006; Taylor et al., 2006). This bacterium has large plasmids, >100kb in size, called the root-inducing (Ri) plasmids (Tzfira et al., 2004; Zambryski, 1992). This plasmid enables *A. rhizogenes* to induce growth of hairy roots at the site of infection; it induces natural transformation by the insertion of 4 *rol* genes that are present in the T-DNA of the Ri plasmid. *A. rhizogenes* strains are classified on the basis of the type of opine catabolism genes encoded in their T-DNA. The T-DNA of the mannopine-, cucumopine-, and mikimopine-metabolizing strains contains the *rolA*, *rolB,* and *rolC* genes, whereas that of the agropine-metabolizing strains contains the *rolA*, *rolB*, *rolC*, and *rolD* genes and also *aux1* and *aux2*, the auxin biosynthesis genes (Bulgakov, 2008; Petit et al., 1983). Plants regenerated from the hairy roots of several infected plants exhibit morphological alterations such as reduced apical dominance, shortened

internodes, wrinkled leaves, reduced leaf size, and an extremely abundant and plagiotropic root system (Tepfer, 1984). The altered phenotype of the regenerants from hairy roots can be useful in plant breeding programs of some crops and ornamental plants (Casanova et al., 2005; Christey, 2001). Further improvements in the morphological characteristics of *R. elata* are required to be to be used as an ornamental plant. In the present study, we report the production of *R. elata* transgenics that were regenerated from hairy roots produced by *A. rhizogenes* infection showing a huge phenotypic variation.

Results and discussion

We evaluated the complete and stable transformation of 3 typical plants regenerated from hairy roots by determining integration of the *rol* genes into the genome and their subsequent expression, which was detected from the presence of its transcripts. It is well known that each *rol* gene of the Ri-plasmid of *A. rhizogenes* is responsible for the induction of hairy roots in plants. In order to determine the insertion of *rol* genes, PCR was performed using primers specific for the *rol*A, B, and C genes. The DNA of the control (WT: wild type) roots were negative for the *rol* genes, whereas that of the regenerated plants (lanes 1–3) gave the expected bands for *rolA* (304 bp), *rolB* (797 bp), and *rolC* (550 bp) genes (Fig. 1). The *A. rhizogenes* R1000 strain lysates were used as positive controls. These results showed that the regenerated plants contained the *rol* genes of the Ri-plasmid. One wild-type and 3 transgenic *R. elata* plants were tested for the presence of *rolA*, *rolB*, and *rolC* transcripts (Fig. 2). The analysis of RT-PCR using gene-specific primers revealed that all the genes (*rol*A, B, and C) were expressed in the transgenic plants. No signal was detected in the wild-type plant extract. Regenerated plantlets were successfully established in the greenhouse after acclimatization. The plants exhibited altered phenotypes such as reduced apical dominance with highly branched stems and short internodes (Fig. 3). Three transformant lines characterized in a greenhouse trial revealed that several morphological traits with respect to ornamental value, such as plant height/shoot length, number of lateral shoots/number of stems, leaf size, leaf number, flower size, and number of flowers, had changed in the transformants as compared to that in the control plants (Table 1). At the flowering stage, the plant height was significantly less by 2.18-, 2.74-, and 4.11-fold in the transformant lines 1 (T1), 2 (T2), and 3 (T3), respectively, relative to the control plants (Fig. 3a), and there were significant differences among the transformant lines. The number of lateral shoots (stems) increased significantly in the transformant lines. There was no branching in the control plants (wild type), whereas there were 7, 8, and 4 stems in T1, T2, and T3, respectively. The mean size of the leaves was significantly reduced in all the transformant lines (Fig. 3b), with the leaf of the wild-type plants being 1.5, 1.4, and 1.8 times larger than that of T1, T2, and T3, respectively. The mean number of leaves per plant varied widely among the transformants and the wild-type plants. The number of leaves per plant was increased by 2.2 (n = 106), 2.95 (n = 142), and 1.31 (n = 63) times in T1, T2, and T3, respectively, as compared to the wild-type plants (n=48). The transformants displayed slightly wrinkled leaves. Flower length was slightly higher in the wild-type plants (5.2 cm) than in the transformants (Fig. 3c). The flower length did not differ significantly among the transformants. Flower number was varied quite significantly between the transformants and wild-type plants. The number of flowers per plant was significantly higher in the transformants than in the wild type plants (11 flowers per plant in the wild-type plants, as compared to 51 in T1, 54 in T2, and 18 in T3). The transgenic plants displayed a marked reduction for plant height, because of reduced internode length and an increased number of lateral shoots. Similar types of findings have been reported in scented geranium (Pellegrineschi and Davolio-Mariani, 1997), *Angelonia salicariifolia* (Koike et al., 2003), and *Kalanchoe blossfeldiana* (Christensen et al., 2008) where they found compact growth in *Agrobacterium rhizogens*-mediated transformation. A general feature of the *rol* genes is that they reduce the apical dominance, resulting in increased lateral branching (Godo et al., 1997; Hosokawa et al., 1997; Pellegrineschi and Davolio-Mariani, 1997). Similarly, reduced apical dominance was observed in the present study, as the number of lateral shoots per plant was much higher as compared to the control plants. Increase in the number of flowers was also recorded in the *R. elata* transformants. The higher number of flowers was due to higher inflorescences and more flowers per inflorescence. In contrast to our results, the number of flowers in *Kalanchoe blossfeldiana* has been reported to be lower in the Ri-lines as compared to that in the control plants (Christensen, et al., 2008). However, similar to our results, the size of the flowers in their study was reduced when transformed with *A. rhizogenes*. Our study demonstrated successful transformation and regeneration of the transformant lines in the economically important ornamental plant *R. elata*. Phenotypic analyses showed that many morphological traits with relevance to ornamental value had changed in the transformants as compared to those in the wild-type plants. The most striking change was the growth retardation of all studied plant organs, showing that the genes of *A. rhizogenes* are very effective in producing short *R. elata* plants with plenty of flowers. Therefore, transformation with *A. rhizogenes* and its *rol* genes can be considered a promising method in molecular breeding for creating new diversity in *R. elata* and other ornamental species, particularly for the production of short bushy plants. Finally, we successfully produced transgenic plants from the hairy roots of *R. elata.* Detailed studies on the characterization and evaluation of the phenotypes of the transformants, such as shoot and root morphology, and nodulation on hairy roots are in progress.

Materials and methods

Plant materials

The shoot tips of *R. elata* were surface-sterilized with 0.5% (v/v) sodium hypochlorite solution for 10 min, and then rinsed 3 times in sterilized water. Three shoot tips were placed on 25 mL Phytagar-solidified culture medium in a culture vessel (100 × 150 mm). The basal MS medium (Murashige and Skoog, 1962) with 3% sucrose was adjusted to pH 5.8 and then solidified using 0.8% (w/v) phytagar. The medium was sterilized by autoclaving at 121°C for 20 min. The shoot tips were cultured in a growth chamber at 25°C under standard cool-white fluorescent tubes with 35 µmol s^{-1} m^{-2} flux rate for 16-h photoperiod.

Preparation of A. rhizogenes

A. rhizogenes R1000 was grown to mid-log phase (OD A_{600} = 0.5) at 28°C on a gyratory shaker at 180 rpm in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0). The bacterial cells were collected by centrifugation for 10 min at 2000 rpm, and resuspended at a

Table 1. Phenotype characterization of the wild-type (WT) and the 3 transformed plants (Tn) (n; number of transgenic line)

Characterization of phenotype	WT	T1	T2	T3
Plant height (cm)	74.3± 2.5	34.7 ±3.1	27.0± 1.0	18.3±1.5
Number of stems	1.0±0.0	7.3±0.6	8.0±1.0	4.3±0.6
Leaf length (cm)	7.2 ± 0.8	4.9 ± 0.5	5.1 ± 0.6	3.9 ± 0.4
Number of leaves	48.3±1.5	106.3±4.5	142.0±6.0	63.7±2.1
Flower length (cm)	5.2 ± 0.6	3.3 ± 0.3	3.2 ± 0.4	3.6 ± 0.4
Number of flowers	11.0±1.0	51.0±2.6	54.0±2.0	18.3±1.5

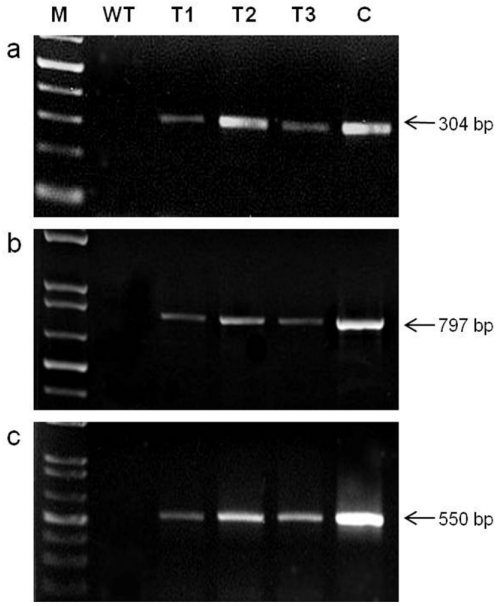

Fig 1. PCR amplification of *rol* genes from non-transformed and transformed plants. *rolA* (a), *rolB* (b), and *rolC* (c) transcripts. [M, molecular size marker; WT, wild type; Tn, transgenic plants; C, positive control; n, number of transgenic line]

Table 2. Primer sequences used to amplify *rol* gene fragments.

Name	Sequences (5′ to 3′)
rol A	CAT GTT TCA GAA TGG AAT TA AGC CAC GTG CGT ATT AAT CC
rol B	TCA CAA TGG ATC CCA AAT TG TTC AAG TCG GCT TTA GGC TT
rol C	ATG GCT GAA GAC GAC CTG TGT TTA GCC GAT TGC AAA CTT GCA

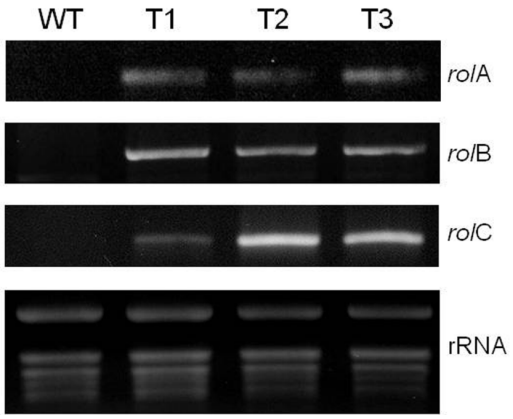

Fig 2. RT-PCR analysis of the 3 *rol* genes in *Rehmania elata* transgenics.

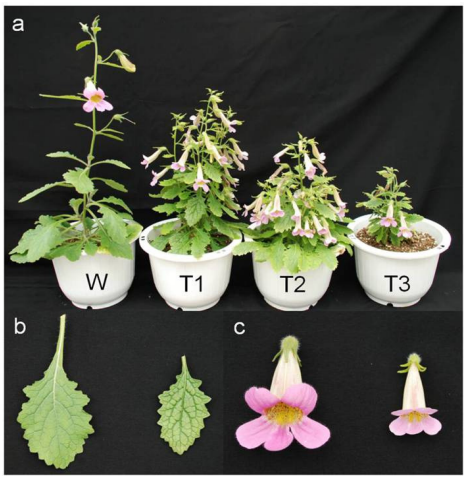

Fig 3. Transformed plants from hairy root cultures of *Rehmania elata.* (a) Plant phenotypes of the wild-type (W) and transformed plants (Tn) of *R. elata* ; (b, c) Leaves and flowers of wild type (W) and transformed (T) plants (n; number of transgenic line).

cell density of OD A_{600} = 1.0 in the liquid inoculation medium (MS salts and vitamins containing 30 g/L sucrose).

Establishment of hairy root cultures

Excised leaves of *R. elata* from *in vitro* grown seedlings were used as explant material for co-cultivation with *A. rhizogenes* R1000. The excised leaves (0.7 × 0.7 cm) were dipped into the *A. rhizogenes* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, placed on the sterilized phytagar-solidified MS medium, and incubated in the dark at 25°C. After 2 days of co-cultivation, the explant tissues were transferred to hormone-free medium containing MS salts and vitamins, 30 g/L sucrose, 200 mg/L timentin, and 8 g/L phytagar. Within 3–4 weeks, numerous hairy roots emerged at the wound sites. The hairy roots were separated from the explant tissue and subcultured in the dark at 25°C on phytagar-solidified MS medium containing 200 mg/L timentin. After repeated transfers to fresh medium, rapidly growing hairy root cultures were obtained.

Plant regeneration from hairy roots

The matured hairy roots were generally thicker and exhibited more prolific branching after repeated transfer to fresh medium for 2–3 months. During culture, we observed that shoots regenerated spontaneously from hairy roots. Various stages of shoot organogenesis from the hairy roots cultured on MS solid medium in absence of plant growth regulators were observed. Initially (2 weeks after incubation), small differentiated shoots were formed. The regenerated shoots developed within 3–4 weeks. Average of 2–3 fully developed shoots, at least 1.2 cm in length, were produced from the hairy root explant at 6 weeks after culture.

Rooting of regenerated shoots

Microshoots (approximately 1cm long) were placed on MS medium. The medium was solidified with 3 g/L gelrite and dispensed at 70 mL per magenta box. Four shoots were cultured in each box. Microshoots were incubated at 25 ± 1°C in a growth chamber with a 16h/8h light and dark under standard cool-white fluorescent tubes for 30 days. After 30 days, the shoots were washed with sterile water to remove agar from their roots and transferred to the pots containing autoclaved vermiculite, covered with polythene bags (to maintain high humidity) and maintained at 25 ± 1°C in a growth chamber for one week. After one week, the bags were perforated. These plants were then transferred to the green house.

Confirmation of transgenic plants

We evaluated the complete and stable transformation of 3 typical plants with three replications regenerated from hairy roots by determining integration of the *rol* genes into the genome and their subsequent expression, which was detected from the presence of its transcripts.

PCR analysis for rol genes

For PCR analysis, genomic DNA was extracted from regenerated plants from hairy roots as described by the manufacturer's protocol (DNeasy Plant mini kit; Qiagen, Valencia, CA). The sequences of a pair of primers used to amplify the *rol* gene fragments are shown in Table 2. The amplification cycle consisted of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 1 min. After 30 cycles and final extension at 72°C for 5 min, the amplified products were analyzed on 1% agarose gels. Gels were stained with ethidium bromide and visualized under UV light.

RT-PCR analysis

To see the rol genes (*rol*A, B, and C) expression in the transgenic plants RT-PCR analysis was done using gene-specific primers. Total RNA was isolated from the leaves using the RNase plant mini kit (Qiagen, Valencia, CA). For RT-PCR, 5 µg of total RNA was reverse-transcribed according to the manufacturer's protocol (Superscript II First strand synthesis kit; Invitrogen, Carlsbad, CA), using an oligo$(dT)_{20}$ primer. The resulting cDNA mixtures were used as templates for RT-PCR analysis, as described above. The gene-specific primer was identical to that used in the PCR analysis.

Phenotypic measurement of transgenic plants

Regenerated plantlets were successfully established in the greenhouse with three replications after acclimatization. The following phenotypic characters i.e., plant height, number of stems, leaf and flower length, length of stems and flower were measured at flowering stage. Plant height was the average height of three transgenic plants of each group. The rest phenotypic parameters were measured from taking all of each treatment and finally averaged from three.

Statistical analysis

Data are expressed as mean ± standard deviation.

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