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Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*

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Abstract

The transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes* was studied. Almost all roots induced by four bacterial strains, R1000, R1601, A4 and R1205 were putative hairy roots. The effects of bacterial strains, bacterial concentration, acetosyringone, silver nitrate and co-cultivation pH on Torenia transformation were investigated. Strain R1000, co-cultivation for 3 days, 30 μmol L^{-1} acetosyringone, 4 mg L^{-1} silver nitrate and pH 6.5 in the cultivation medium provided the optimal conditions under which transformation frequency approached 90%.

Keywords: *Torenia fournieri* L.; *Agrobacterium rhizogenes*; Hairy root; Genetic transformation; Acetosyringone; Silver nitrate

1. Introduction

Torenia fournieri L., known as Torenia or wishbone flower, is a common bedding ornamental plant during summer and belongs to the Scrophulariaceae family. It has become a model plant for biological research, its culture requirements in vitro are well established (Tanimoto and Harada, 1981). As Torenia is easy to grow in both field and laboratory, and its genetic transformation has potential for epocha cultivar breeding (Aida, 1998), many transgenic plants have been obtained by *Agrobacterium tumefaciens*-mediated transformation. The first transgenic plant of Torenia was reported in the mid-1990s (Aida and Shibata, 1995). Some of these transgenic plants were characterized by modified flowers with various longevities and colors (Aida, 1998; Aida et al., 2000, 2001).

Agrobacterium rhizogenes strains contain a single copy of a large Ri plasmid. In the Agropine Ri plasmid, T-DNA is referred to as left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA). T_R-DNA contains genes homologous to the Ti plasmid tumor-inducing genes and the *tms* loci genes, two morphogenic loci of the T_R-DNA correspond to the tms loci of the Ti plasmids (White et al., 1985), can directly synthesize auxin (Capone et al., 1989). Genes of T_L-DNA, *rolA*, *rolB*, *rolC* and *rolD* direct the synthesis of a substance that reprograms the cells to differentiate into roots under the influence of endogenous auxin. T-DNA is transferred to wounded plant cells and becomes stably integrated into the host genome (Chilton et al., 1982). Transformants are selected by detecting the genes located in T-DNA such as *rolA*, *rolB*, *rolC* and *rolD*.

Hairy roots have been induced in many dicotyledonous plants by transformation with *A. rhizogenes* Ri T-DNA (Costamtino et al., 1994). Hairy roots are able to regenerate whole viable plants with high genetic stability. Most have an altered phenotype including hairy root syndrome, dwarfing, altered flowering, wrinkled leaves and increased branching, which have proven useful in ornamental plant breeding programs (Giovanni et al., 1997). However, the transformation of Torenia mediated by *A. rhizogenes* has not yet been reported.

In this study, we describe the Ri plasmid-mediated transformation of Torenia and investigate the effects of several major physical and chemical factors, on hairy root formation.

2. Materials and methods

2.1. Plant materials

Seeds of *T. fournieri* L. were soaked and sterilized according to a previous report (Li et al., 2001). Seeds were

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germinated on half-strength Murashige and Skoog medium (1/2 MS) (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar or on damp absorbent cotton, with or without light at 25 °C. Cultures were maintained under a 16-h photoperiod regime with fluorescent light at 25 °C.

For transformation experiments, leaves were excised from 4- to 7-cm-tall Torenia plants, with 4 expanded leaves.

2.1.1. Agrobacterium

A. rhizogenes strains R1000, R1601, R1205 and A4 belong to the agropine type and harbor the Ri plasmid with two parts of T-DNA (left T-DNA and right T-DNA) (Table 1). For the transformation experiments, each strain was inoculated into liquid YEB medium (sucrose 5 g L^{-1}, beef extract 1 g L^{-1}, yeast extract 1 g L^{-1}, peptone 5 g L^{-1}) (An et al., 1988) with 100 mg L^{-1} kanamycin and shaken for 30 h at 28 °C. The cultures were then centrifuged at 1500×*g* and resuspended in liquid MS medium containing 20 μmol L^{-1} acetosyringone before dilution to different concentrations for infection.

2.1.2. Kanamycin resistance test of normal roots

Roots excised from normal plants were cultured on selection media containing a range of concentrations from 0 to 300 mg L^{-1} kanamycin. The percentage of roots survived after 15 days culture was determined.

2.1.3. Transformation

Leaf explants were pre-cultivated on MS medium solidified with 0.8% (w/v) agar at a varying pH range (4.5, 5.5, 6.5, 7.5) and concentrations of acetosyringone (0, 10, 20, 30, 40 μmol L^{-1}) and silver nitrate (0, 1, 2, 3, 4 mg L^{-1}) for 2 days. The explants were then transferred to the *Agrobacterium* suspensions containing 20 μmol L^{-1} acetosyringone and incubated for certain time, then blotted dry on sterilized filter paper. The explants were returned to the same pre-cultivation medium for co-cultivation. After 0 to 4 days, these explants were transferred to resting media (1/2 MS) containing 500 mg L^{-1} carbenicillin and 200 mg L^{-1} kanamycin. Every 4 days, the explants were transferred to selection media (1/2 MS containing 250 mg L^{-1} kanamycin and 150 mg L^{-1} carbenicillin) until hairy roots formed. The numbers of explants with roots (20 days after infection) were recorded.

The standard condition referred to here is 15 min infection, 2 days pre- and co-cultivation at pH 5.5 using a bacterial concentration of 1.5 (OD_{600}). To determine the optimum conditions for transformation, one factor of the standard conditions was changed each time and the effects on hairy root formation measured.

Table 1
Bacterial strains and plasmids used in this work and their relevant characteristics

Strains	Ri plasmid	Type of plasmid	References
A4	pRiA4 and pB1121	Agropine	White and Nester, 1980
R1000	pRiA4b and pB1121	Agropine	White et al., 1985
R1601	pRi1500 and pTVK291	Agropine	Pythoud et al., 1987
R1205	Ri (*tms-I* and *tms-2* deletion)	Agropine	White et al., 1985

Table2
The effect of light and media on the germination frequency of *Torenia* seeds (40 seeds per test)

Medium	Treatment	Percentage of germination (%)
1/2 MS	Light	95.0
	Dark	52.5
Absorbent cotton	Light	100.0
	Dark	60.0

2.1.4. PCR amplification of the rolB gene

Total DNA was isolated from hairy roots according to the procedure of Edward et al. (1991). Two primers, 5′-CGCAAGC-TACAACATCATAG-3′ and 5′-CAGTAGATCTCACTC-CAGCA-3′, were used for PCR amplification of the 583 bp fragment of the *rolB* gene. DNA was amplified by 30 cycles of 30 s at 92 °C, 30 s at 52 °C and 1 min at 72 °C. The DNA size marker was DL 2000 (TaKaRa Biotechnology (Dalian) Co., Ltd.).

2.1.5. Southern blot analysis

Genomic DNA was digested with *nar I and ned I* and resolved by electrophoresis on 0.8% agarose gel before transferring to a nylon membrane by the method of Southern (1975). The membrane was pre-hybridized at 65 °C in 7% SDS and 0.25 M $NaHPO_4$ and then hybridized with the *rolB* gene fragment, which was isolated from restricted Ri plasmid with *nar I* and *nde I*, labelled with ^{32}P-dCTP by the method of Sambrook and Russell (2001). The hybridized blot was subjected to three washes in 20 m mol L^{-1} $NaHPO_4$ and 1% SDS. The blot was exposed to X-ray film (Kodak) at −70 °C for 3 days.

2.1.6. Statistical analysis

All data frequency on hairy root formation are the mean of three independent experiments (±S.E.) with a minimum of 20 explants per treatment. All roots that survived in the selection medium for 20 days were designated as hairy roots. All data among different treatment were tested by SSR testing (Duncan, 1955).

3. Results

Seeds were germinated on both 1/2 MS agar medium and damp absorbent cotton with or without fluorescent light after 4 days. Ten days later, the percentage of germinated seeds under light was much higher than that under dark on both media indicating that light strongly affected seed germination (Table 2).

Normal roots were placed on 1/2 MS medium containing a range of kanamycin concentrations (Fig. 1A) to examine their resistance to the antibiotic. All untransformed roots survived at kanamycin concentrations up to 100 mg L^{-1}. At higher concentrations, the survival frequency decreased and no roots survived at 250 or 300 mg/l. On this basis, 250 mg L^{-1}