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Genetic Transformation in *Vaccaria pyramidata* Using *Agrobacterium rhizogenes*

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Abstract

To establish a transformation system and contribute to breeding of dwarf cultivars in *Vaccaria pyramidata* using *Agrobacterium rhizogenes*, regeneration from a hairy root and traits of the regenerant were examined. The hairy roots were induced and actively grew on phytohormone-free 1/2MS media solidified with 2% gellan gum. Shoots regenerated from the root through callus on the 1/2MS media supplemented with 1.0-2.0 mg l^{-1} of zeatin and with 0.5 mg l^{-1} of IBA and 1.0 mg l^{-1} of zeatin. Plant height of a potted transformant was lower than those of non-transformants, and leaves of the transformant curled. Differences in morphologies and sizes of flowers in both types of the plants were little, although the starting date of flowering was 44 days later in the transformant than non-transformants. The results throw a new light on genetic engineering and the breeding of dwarf cultivars in *Vaccaria*.

The species belonging to *Vaccaria* and *Saponaria*, included in Caryophyllaceae, have been used as ornamental and medicinal plants. *Vaccaria* is so close to *Saponaria* that the former is sometimes included in the latter (Fall *et al.*, 1979). In spite of recent advances in technology of genetic transformation in plants (Draper *et al.*, 1988) or floriculture crops (Hutchinson *et al.*, 1992; Robinson and Firoozabady, 1993), no paper dealing with transformation of these genera have appeared. Therefore, this report describes the first investigation of the transformation in these genera.

We selected *Vaccaria pyramidata* for the plant material, and *Agrobacterium rhizogenes* as the causal bacterium for transformation. *V. pyramidata* has a straight stem and sparse branches in the upper part, and contains some pharmacological components such as saponins, terpenic- and flavonoidal-glycosides, xantones (Kazmi *et al.*, 1989), cyclic peptides (Morita *et al.*, 1996) and ribosome-inactivating proteins (RIPs) (Bolognesi *et al.*, 1995). *A. rhizogenes* is the causal bacterium for inducing hairy roots from infected plant tissues by the transfer of T-DNA of the Ri-plasmid into plant genome (Chilton *et al.*, 1982). The roots have been cultured for production of the secondary metabolites, and some of the roots produced the materials in high efficiency (Toivonen, 1993; Bourgaud *et al.*, 1997). The plants regenerated from the roots exhibit morphological and physiological alterations, such as smaller wrinkled leaves, decreased apical dominance, shortened internodes (Tepfer, 1984). Therefore, the resulting regenerants in the present experiment are expected to become breeding sources for cultivars having dwarf characteristics or high concentrations of pharmacological components.

In vitro plants were aseptically established from seeds of *V. pyramidata* cv. Pink Beauty (Fukukaen Nursery & Bulb Co., LTD.) according to a method of another report (Koga *et al.*, 2000). Leaves were excised at their base from the seedlings and cut in lengths of about 5 mm. Leaf pieces were immersed in a suspension culture of a mikimopine type strain A13 of *A. rhizogenes* (Daimon *et al.*, 1990) for 10 min, and blotted on a sterilized filter paper to remove any excessive suspension. Then, the pieces were put onto a half-strength MS medium (Murashige and Skoog, 1962) solidified with 2% gellan gum (Wako Pure Chem. Instr. Ltd.) (1/2MS solid medium). For the following culture, the above medium was used, and the temperature was adjusted at 25 ℃. During the first three days for co-cultivation of the leaf pieces with the bacteria, the light condition was kept dark, and thereafter it was continuous illumination at 6,000 lux for 16 h. The methods for growth and removal of the bacteria followed methods contained in another report (Koga

et al, 2000).

Each root induced from inoculated leaf pieces was put onto phytohormone-free 1/2MS solid medium in a plastic dish (SH90-15: Iwaki Glass). Two months after the transplantation, a root filling the dish was judged as an actively growing root. Mikimopine production in the actively growing roots on the media was examined following the method of Godo *et al.* (1997).

Regeneration media, which were 1/2MS solid media containing plant growth regulator(s) were dispensed to plastic dishes with a volume of 30 m*l* or to test tubes (25 $\phi \times$ 100h mm) with a volume of 10 m*l*. When using the plastic dishes, the plant growth regulator(s) supplemented in the media were independent cytokinins or those with combinations of auxins and benzyladenine (BA). The cytokinins supplemented independently were 0.1, 0.5 or 1.0 mg l^{-1} of BA, or 1.0 mg l^{-1} of zeatin or kinetin. The combined auxins were 0.05, 0.1 and 0.5 mg l^{-1} of indole-3-butyric acid (IBA) and 1-naphthalene-acetic acid (NAA), and concentrations of the combined BA were 0.1, 0.5 and 1.0 mg l^{-1}. When using the test tubes, supplemented plant growth regulators were composed of 1.0 or 2.0 mg l^{-1} of zeatin only, and those with a combination of 0.5 mg l^{-1} of IBA and 1.0 mg l^{-1} of zeatin. One strain of mikimopine-producing hairy root was cut into 10 mm-length pieces. The numbers of the pieces put onto a regeneration medium were 10 per dish and 1 per test tube. The sample numbers per condition were 20 and 47-50 in using the plastic dishes and test tubes, respectively. The dishes were sealed with Milli Wrap (Millipore) and the tubes were covered with plastic caps. When using the dishes, the calli induced on the regeneration media were cut into pieces 5 mm in diameter and transferred onto fresh media on the 47th day after starting the culture. Investigation of regeneration was conducted on the 115th day from the start. The same procedure was followed in the case of using the tubes on the 104th and 221st day, respectively.

Extraction of DNA from the regenerated plants and Southern blot analysis were carried out with methods described in another paper (Koga *et al.*, 2000).

The regenerated shoots, derived from one hairy root and verified as having the *rol*C gene, and germinated non-transformed plants were cultured by means of the paper wick method with 1/2MS liquid media. On September 24, 1997, the plants were transplanted into fully water-soaked vermiculite and acclimatized in plastic containers (60 $\times$ 17 $\times$ 18h cm) wrapped with polyvinylidene chloride films. After forty-five days, the films were removed, and the plants were cultivated just as the plants in another report (Koga *et al.*, 2000). Plant height was measured on March 15, 1988, in the flowering period of non-transformants, and flower size was investigated in the flowering time of each plant type in 1988.

As the result of several bacterial inoculation experiments, 5-10 roots were induced from 100 pieces of the leaf segments. Percentages of the actively growing roots on the phytohormone-free 1/2 MS solid media were 77-100%. The mikimopine production was examined in five of the actively growing roots, and positive signals were detected in four roots (data not shown).

The first experiment of regeneration was carried out using the plastic dishes. Shoots were regenerated through a green callus derived from the hairy root in only the case of 1.0 mg l^{-1} of zeatin added to the media, at a rate of 1/20. Rate of green callus induction was higher in 1.0 mg l^{-1} of zeatin than other cases where cytokinins only had been added. Additionally, in combinations of IBA or NAA and BA, the rates of green callus induction were higher in IBA than in NAA in most of the comparisons between the same concentrations of the plant growth regulators (data not shown).

Therefore, in the second regeneration experiment using the test tubes, the regeneration media containing 1.0 and 2.0 mg l^{-1} of zeatin only and those with a combination of 0.5 mg l^{-1} of IBA and 1.0 mg l^{-1} of zeatin were compared, and the result was shown in **Table 1**. Four and two percent of the pieces of the hairy roots regenerated through green calli on the media supplemented with 2.0 mg l^{-1} of zeatin and with 0.5 mg l^{-1} of IBA and 1.0 mg l^{-1} of zeatin, respectively. No significant difference was detected in the percentages of green callus induction among the conditions of plant growth regulators (χ^2-test, $P \leq 0.05$). From these results, it was judged that 1.0-2.0 mg l^{-1} of zeatin only or the combination of 0.5 mg l^{-1} of IBA and 1.0 mg l^{-1} of zeatin were the most effective for regeneration from the hairy roots under the present conditions.

Integration of T-DNA in Ri-plasmid of *Agrobacterium* into plant genome was investigated by the Southern blot analysis using the fragment in the *rol*C gene as a hybridization probe (**Fig. 1**). In a non-transformed plant, no hybridization signal was detected in either case where *Eco*RI or *Hin*dIII digested DNA was applied (Lanes 2 and 3). In one transformant, a single hybridization signal was observed in each of the cases (Lanes 4 and 5). In the other transformant, the numbers of hybridization signals detected were one in *Eco*RI digestion (Lane 6) and two in *Hin*dIII digestion (Lane 7). These

Table 1. The effect of plant growth regulators on regeneration from a hairy root.

IBA (mg l^{-1})	Zeatin (mg l^{-1})	No. of hairy root pieces tested	No. of the pieces giving rise to shoot(s)	Regeneration percentage	No. of the pieces giving rise to green calli	Percentage of green callus induction
0.5	1.0	47	2	4	39	83
0	1.0	50	0	0	46	92
0	2.0	50	1	2	41	82

1/2MS solid media in test gubes covered with plastic caps were used for the test.
Transference of the induced calli onto fresh media and investigation were conducted on the 104th and 221st day after starting the culture, respectively.

results demonstrate that the transformants harbor the *rol*C gene of T-DNA in the genomes. The results in the former transformant also indicate that the copy number of the *rol*C gene in the genome is one, and those in the latter give the possibility that the copy number of the gene is two.

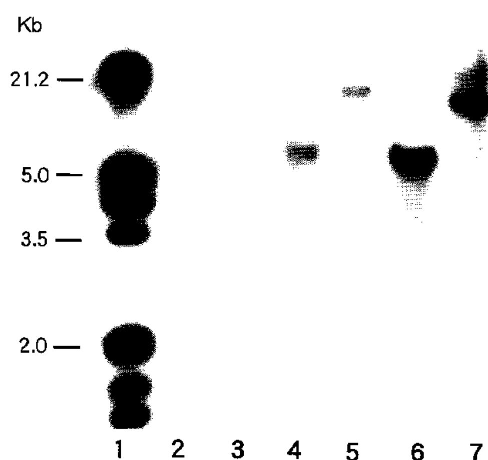

Fig. 1 Southern blot analysis of DNA extracted from a non-transformant and two transformants. Applied DNA was extracted from a non-transformant (Lanes 2 and 3) and two transformants (Lanes 4, 5 and lanes 6, 7), and digested with *Eco*RI (Lanes 2, 4 and 6) and *Hind*III (Lanes 3, 5 and 7). DNA size markers were applied on Lane 1.

Acclimatization of the transformants having the *rol*C gene (Lanes 4 and 5 in **Fig. 1**) was difficult. Although more than one hundred plants were transplanted in the plastic containers, the number of plants which grew and flowered was only one. Traits in potted plants of a transformant and non-transformants were investigated, and represented in **Table 2**. Plant height was lower in a transformant than in non-transformants **(Fig. 2A)**. Leaves of the transformant curled and were smaller than those of non-transformants **(Fig. 2B)**. The starting date of flowering was 44 days later in the transformant than in the non-transformants. Flower morphologies of both types of plants resembled each other, and no significant difference was detected in flower diameter between both types of the plants, although petals were slightly more slender and the length of the calyx was somewhat shorter in the transformant than the non-transformants **(Fig. 2C)**.

Present research throws a new light on the genetic engineering of *Vaccaria* and *Saponaria*. Using the transformants for the breeding of dwarf cultivars of *V. pyramidata* and investigating the pharmacological components in the transformants are awaited.

Table 2. Comparison of traits in potted plants between a transformant and non-transformants.

	Transformant (n[a])	Non-transformant (n)
Plant height[b] (cm)	45 (1)	71 (3)
Starting date of flowering	April 25 (1)	March 7-16 (3)
Flower diameter (mm)	20.3 ± 2.0[c] (30)	19.1 ± 2.0 (20[d])
Length of calyx (mm)	10.3 ± 0.9**[e] (30)	12.1 ± 0.6 (20[d])

[a] Sample number.
[b] Measurement was conducted on March 15, 1998.
[c] Mean ± S. D.
[d] Samples were selected from three plants.
[e] Significant at 1% level in accordance with t-test.

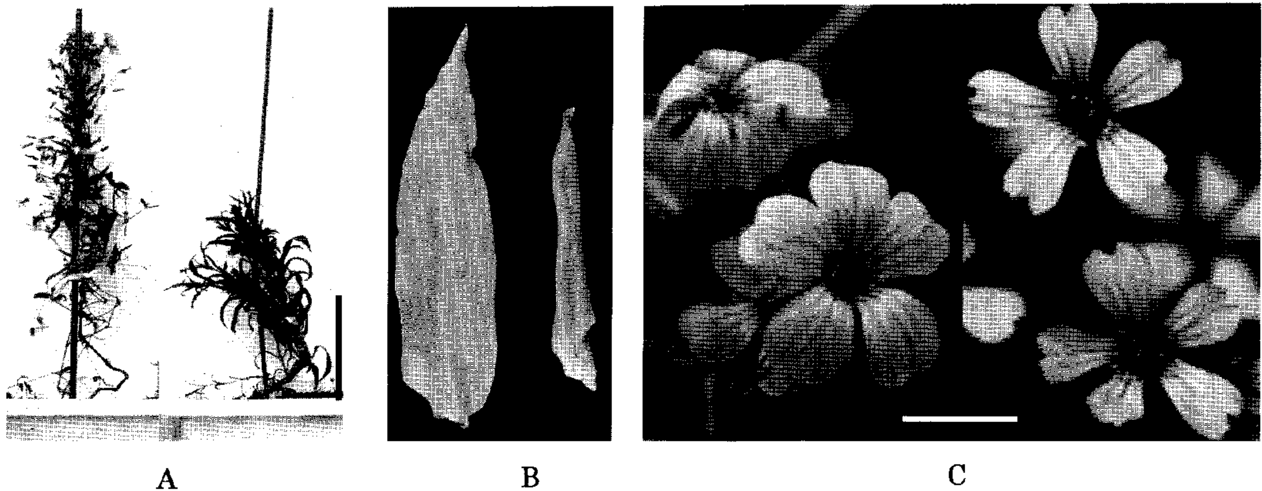

Fig. 2 Comparative morphology between a non-transformant (left) and a transformant (right). (A) Whole plants in flowering time of non-transformants (Bar = 20 cm). (B) Leaves. The leaf of a transformant curled and was smaller than that of a non-transformant. (C) Flowers. Flower morphologies of both the plants resembled each other, although petals were slightly more slender in the transformant than in the non-transformant (Bar = 10 mm).

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