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Regeneration and Characterization of Prairie Gentian (*Eustoma grandiflorum*) Plants Transformed by *Agrobacterium rhizogenes*.

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Hairy roots were obtained from prairie gentian (*Eustoma grandiflorum* (Griseb.) Schinners *cv.* Murasaki-no-mine) infected with *Agrobacterium rhizogenes* strain A13. Plant regeneration occurred spontaneously on Linsmaier-Skoog (LS) medium lacking phytohormones under the light condition. Phenotypic alterations such as dwarfness, wrinkled leaves and cup-shaped corolla were observed in transformed plants. Southern analysis revealed the introduction of Ri-T-DNA in the plant genome. The transformed plants exhibited reduced fertility but some of them set viable seeds.

Introduction

The soil bacterium *Agrobacterium rhizogenes* causes hairy root disease in many dicotyledonous plants[1,2], Ri plasmid present in the bacterium is capable of inducing hairy-roots which can grow as root organs in a phytohormone-free medium. Autonomous proliferation of induced roots is the result of the expression of genes in the T-DNA of the Ri plasmid integrated into the host plants[3]. Regeneration of whole plants from hairy roots has been reported in several plant species including carrot[4], *Catharanthus roseus*[5], cauliflower[6], horseradish[7], morning glory.[4], oilseed rape[8], potato[9] and tobacco[4,10]. In some cases, fertile plants can be regenerated spontaneously from hairy roots on phytohormone-free medium. Some of the regenerated plants possessed an altered phenotype such as wrinkled leaves, plagiotropic roots, short internodes and reduced apical dominance. Such morphological changes are due to the expression of core T-DNA genes inherited by the progeny[4,11].

Prairie gentian (*Eustoma grandiflorum* (GRISEB.) SCHINNERS) is one of the popular cut flowers in Japan and, is becoming popular in the United States and European countries[12]. Micropropagation of *E. grandiflorum* has been developed recently[13,14], but transformation of this species has not been reported yet.

The present article reports fertile plant regeneration from Ri-transformed root lines of prairie gentian and the characterization of the transformed plants.

Materials and Methods

Plant material. Seeds of *E. grandiflorum cv.* Murasaki-no-Mine (Sakata seed Corp.) were surface sterilized with 0.5 % sodium hypochlorite for 20 min and aseptically germinated on a Linsmaier-Skoog (LS) medium without sucrose in 16 h light at 17°C.

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Fig. 1 Hairy roots with spontaneously emerged plantlets (arrow) of *E. grandiflorum*.

Bacterial strain. A virulent *A. rhizogenes* strain A13 (MAFF 02-10266)[15], harbouring mikimopine type Ri plasmid, was used for inoculation. The bacterium was grown at 28°C on solid YEB medium[16].

Inoculation and hairy-root culture. The stems of four week old plantlets were inoculated by needle with freshly grown *A. rhizogenes*. The inoculated seedlings were transferred onto an LS medium with 3 % sucrose and 250 mg/*l* cefotaxime. The culture was kept at 20°C under a 16 h light. Hairy roots that appeared at the inoculation site were excised and transferred to the same medium.

Plant regeneration. Adventitious buds that appeared from the hairy-roots were excised and placed on an LS medium with 1.5 % surcrose and 0.3 % gellan gum in a bottle covered with 0.2 μm membrane filter at 20°C under 16 h light. The developed plantlets were transferred to pots containing a mixture of vermiculite and perlite, and grown at 20°C under a 16 h light.

DNA isolation and Southern blot hybridization. Total DNA was isolated from leaves by SDS extraction method according to Honda and Hirai[17]. DNA digested with EcoRI was electrophoresed and transferred to Amersham's Hybond-N nylon membrane. Southern analysis was carried out with DIG labelling and AMPPD detection system (Boehringer manheim) according to the supplier's instructions. The T-DNA probe, 7.5 kbp EcoRI fragment including the core T-DNA region[18], was constructed from mikimopine type Ri plasmid pRi 1724 of *A. rhizogenes* strain 1724 (MAFF03-01724)[19].

Results and Discussion

Transformed hairy-root culture and plant regeneration.

Within 3 weeks after inoculation, several adventitious roots appeared at the inoculation site. In the control experiments, no roots were observed after stabbing with a needle. Some adventitious roots displayed a typical hairy-root phenotype, characterized by fast growth and lateral root branching on an LS medium without phytohormones.

One to four adventitious buds emerged directly from each hairy root clone within 5 weeks on a hormone-free medium. Most of them appeared at the base of hairy roots (**Fig. 1**). They reproduced several secondary buds, and all of the buds could to be grown to plantlets. Thus approximately 5 -20 plantlets could be obtained from each hairy root clone.

The plantlets often showed a vitrificated appearance when they grew on LS medium with 3 % sucrose and 0.2 % gellan gum. The following modification of the culture improved the growth and overcame vitrification; 0.3 % gellan gum, 1.5 % surcrose and covering with a membrane filter which had the ability to reduce humidity in a bottle (**Fig. 2**).

Acclimation of the regenerated plantlets was difficult, but several plantlets grew into mature

Fig. 2 *In vitro* growth of Ri-transformed *E. grandiflorum* plantlet.

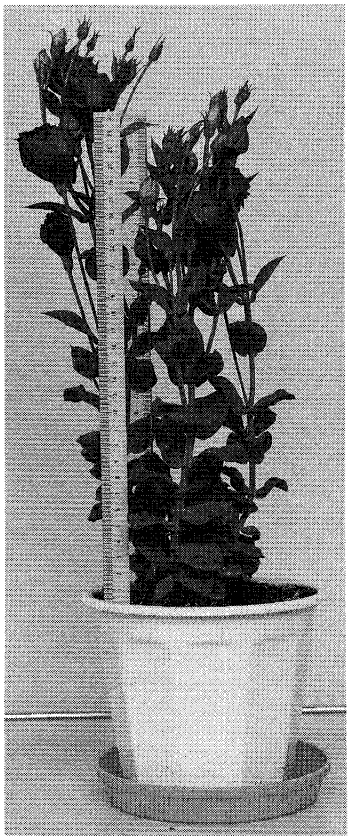

Fig. 3 Ri-transformed phenotype of *E. grandiflorum* plant. Numbers in the scale indicate centimeters.

plants after being transplanted to soil (**Fig. 3**).

Phenotype of regenerated plants

All of the regenerated plantlets exhibited morphological changes. Aerial parts showed dwarf phenotype with short internodes. Some regenerants showed an abundant root system (**Fig. 2**). The final heights of regenerants after transplantation to soil were about 30-40 cm (**Fig. 3**). They had short internodes, wrinkled leaves and cup-shaped corolla, whereas the control plants were 50-70 cm in height and had flat-surface leaves and flat-form collora under the same growth conditions. Pollen of the regenerants showed reduced viability, but some flowers set seeds by self-fertilization. The seeds obtained had enough viability, 84 % (519 of 612) of the seeds germinated (**Fig.4**).

An altered phenotype in the Ri-transformed plants, such as wrinkled leaves and short internodes, induced by Ri-T-DNA has been described for several plant species like cauliflower[5], oilseed rape[8], potato[9] and tobacco[4,10]. The dwarf phenotype observed in this experiment may be a useful horticultural characteristic for improving prairie gentian plants.

T-DNA analysis

To confirm the transformation with Ri-T-DNA, Southern analysis was performed (**Fig. 5**). Leaf DNAs from an untransformed plant and a regenerated plant were analyzed. No hybridization signal was observed between the T-DNA probe and DNA from an untransformed plant. On the contrary, DNA from a regenerated plant hybridized to the same probe. The altered phenotypes exhibited in the regenerants would be due to the expression of *rol* genes in the integrated Ri-DNA.

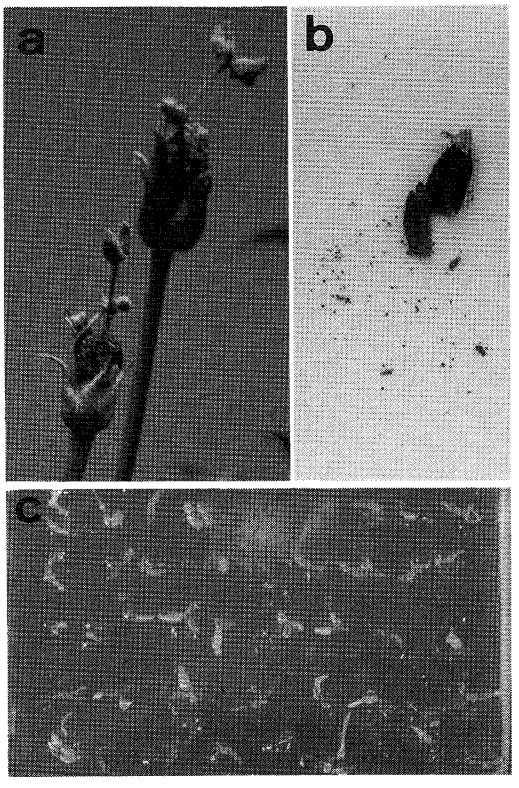

Fig. 4 Seed set of Ri-transformed *E. grandiflorum* plant.
a. seed set of the transformant; b. seed; c. germination of seed.

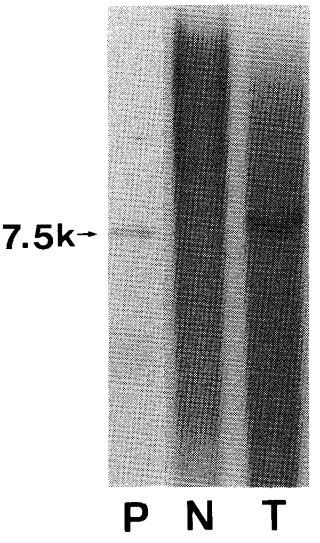

Fig. 5 Southern blot analysis of EcoRI digested DNA from untransformed (N) and transformed (T) plants. P, 7.5 kb T-DNA probe.

Evaluation for breeding use of the transformants should be judged by the same conditions as the common cultivation plants. In general, the transformed plant with a foreign gene must be cultured in the restricted area according to the guidelines for recombinant DNA. In this experiment, however, the domestic wild strain of *A. rhizogenes* possessing wild Ri plasmid was used for the transformation of prairie gentian cultivar. Thus the obtained transformants and their progeny are able to be cultured out of the restricted area such as a greenhouse or field. Some transformants set viable seeds, so further analysis to confirm the inheritance of the Ri-T-DNA and the Ri-derived phenotype by the progeny will be carried out when the progeny have grown enough to be available for analysis.

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《和文要約》

Agrobacterium rhizogenes によるトルコギキョウの形質転換と植物体再分化

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トルコギキョウにおける形質転換系の作成と，再分化体の Ri T-DNA による形態変化を観察する目的から，*Agrobacterium rhizogenes* を用いた実験を行った．トルコギキョウ'紫の峰'実生へ *A. rhizogenes* 国内菌系統 A13 株を接種したところ，接種部位に毛状根が発生した．発生した毛状根を切取り，ホルモンフリーの LS 培地上で培養したところ，毛状根基部に不定芽が複数発生した．これらの不定芽は植物体へと再分化し，馴化したところ開花に到った．毛状根からの再分化体は非形質転換体に比べ，節間が短くなり，葉が波打ち，花が杯状になり，花粉稔性が低くなるといった形態的な特徴がみられた．Ri プラスミドの core T-DNA をプローブにしたサザン分析により，Ri T-DNA の植物ゲノムへの導入が証明された．また，発芽能のある種子を結実した個体も得られ，本法がトルコギキョウの形質転換体作出に有効であることが示された．

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