Original Paper

Plant Biotechnology, 19 (5), 365-368 (2002)

Agrobacterium-mediated Transformation of *Oenanthe javanica* (Blume) DC. Plants

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Received 3 July 2002; accepted 25 August 2002

Abstract

Transgenic plants of *Oenanthe javanica* (Blume) DC. 'Shimane-Midori' were obtained by *Agrobacterium*-mediated gene transfer. Embryogenic calli derived from petiole segments were infected with *Agrobacterium tumefaciens* strain EHA101, harboring a binary vector pIG121Hm that included genes for GUS, kanamycin resistance and hygromycin resistance. After four days of co-culture on MS medium containing 0.1 mg l^{-1} 2,4-D, the infected calli were transferred onto hormone-free MS medium containing 500 mg l^{-1} carbenicilin. Seven days after the start of hormone-free MS medium, the calli were transferred onto MS medium containing 50 mg l^{-1} hygromycin for selection. Approximately four month after infection, seventy-one hygromycin-resistant plantlets were formed from about 4 g calli. Expression of the GUS gene was confirmed by histochemical assay. The results of Southern hybridization analysis indicated that at least three independent transgenic plants were obtained, and all of them had a single copy of the GUS gene in their genomes.

Key words: *Agrobacterium tumefaciens*, GUS assay, *Oenanthe javanica*, transformation, transgenic plant.

Introduction

Oenanthe javanica (Blume) DC., called 'Seri' in Japanese, has been used for native vegetable from old times, and cultivated as a crop for approximately two hundred years. Currently, it is cultivated year-round, but mainly harvested between December and January. Though *O. javanica* is a traditional crop, cross breeding has not been conducted, and only the existing domestic strains have been used for cultivation. Since the major commercial strain 'Shimane-Midori' is sensitive to leaf blight (*Septoria apiicola* Spegizzini) and leaf rot (*Pythium afertile* Kanouse *et* Humphrey), breeding of resistant cultivars has been desired.

Recently, disease-resistant transformants by gene manipulation have been reported in many crops such as carrots (Takaichi and Oeda, 2000; Chen and Punja, 2002), cucumbers (Tabei *et al.*, 1998), strawberries (Asao *et al.*, 1997) and grapevines (Yamamoto *et al.*, 2000). The highly-regenerative culture system such as we developed previously (Endo and Shoji, 1994) is useful for genetic transformation. Therefore, genetic transformation is expected to be one of effective methods for improving this crop. However, to our knowledge, there has been only one report on the production of transgenic *O. javanica* plants, which was produced by *Agrobacterium*-mediated gene transfer using *Agrobacterium* strain LBA4404 and kanamycin selection (Been and Kim, 1995). In this paper, we report an effective method for *Agrobacterium*-mediated *O. javanica* transformation using *Agrobacterium* strain EHA101 and hygromycin selection, and confirm the expression of the foreign genes in the transgenic plants by histochemical staining and Southern blotting analysis.

Materials and Methods

Plant materials

'Shimane-Midori', the major commercial strain of *Oenanthe javanica* DC., was used for the experiments. Excised petioles from first and second developing leaves of *O. javanica*, grown aseptically, were

cultured on MS medium (Murashige and Skoog, 1962) containing 0.1 mg l^{-1} 2,4-D, 40 g l^{-1} sucrose and 3 g l^{-1} Gellan Gum (Wako, Tokyo) at 25 ℃ under a 16-h photoperiod (5,000 lux) (Endo and Shoji, 1994). Embryogenic calli, which were formed one to two months after the start of the culture, were used for experiments.

Effect of antibiotic on embryogenesis

The effect of kanamycin and hygromycin on embryogenesis was examined. In a 9 cm-diameter petri dish, 0.1 g embryogenic calli were placed on the medium containing 0-100 mg l^{-1} of either kanamycin or hygromycin, and cultured at 25 ℃ under a 16-h photoperiod. Three weeks after the start of culture, the number of green embryos per plate was counted. Each treatment was carried out in triplicate.

Agrobacterium-mediated transformation and regeneration of transgenic plants

Agrobacterium tumefaciens strain EHA101 harbors the binary vector pIG121Hm (Ohta *et al.*, 1990); pIG121Hm contains a β-glucuronidase (GUS) gene with a modified intron, a neomycin phosphotransferase II gene (*npt II*) and a hygromycin phosphotransferase gene (*hpt*) in the T-DNA region (**Fig. 1**). The *Agrobacterium* were pre-cultured for two days at 28 ℃ on solid LB medium containing 50 mg l^{-1} kanamycin, 50 mg l^{-1} hygromycin and 15 g l^{-1} agar. A bacterial colony was transferred to liquid LB medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin with shaking at 100 rpm at 28 ℃ overnight. About 2 g embryogenic calli were soaked in ten-fold diluted suspension of the bacteria for twenty minutes, and blotted with sterile filter paper to remove excess bacteria. Then the calli were transferred onto MS medium containing 0.1 mg l^{-1} 2,4-D, 40 g l^{-1} sucrose and 3 g l^{-1} Gellan Gum. Co-culture was carried out at 25 ℃ in the dark. After four days of co-culture, the infected calli were transferred onto hormone-free MS medium containing 500 mg l^{-1} carbenicilin, 30 g l^{-1} sucrose and 3 g l^{-1} Gellan Gum for eliminating *Agrobacterium* and stimulating embryogenesis. Seven days after the start of hormone-free MS medium, the calli were transferred onto the fresh medium of the same composition as mentioned above, except for containing 50 mg l^{-1} hygromycin for selection. The cultures were carried out at 25 ℃ under a 16-h photoperiod. The regenerative yellow calli and green embryos were selected and subcultured onto the fresh medium for one month. Regenerated hygromycin-resistant plantlets were transplanted to moist soil. The experiment was carried out twice.

Assay of GUS activity

A histochemical GUS assay was performed at 37 ℃ as described previously (Ohshima *et al.*, 1990) with a modified reaction mixture solution: 50 mM phosphate buffer (pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc), 5% methanol, 10 μg ml^{-1} cycloheximide and 1 mM dithiothreitol.

GUS activity in crude extracts of the leaves, the stems and the roots were assayed by fluorometric quantification of 4-methylumbelliferone (4-MU) produced from the glucuronide precursor as reported (Kosugi *et al.*, 1990).

Southern hybridization analysis

Total DNA was isolated from leaves with a plant DNA extraction kit (Phytopure™; Amersham Biotech). Southern hybridization was performed using the DIG non-radioactive nucleic acid labeling and detection system (Roshe) according to the manufacturer's instructions. The GUS coding region was PCR-amplified and -labeled using the sense primer 5'-GCAACGTCTGGTATCAGC-3' and the anti-

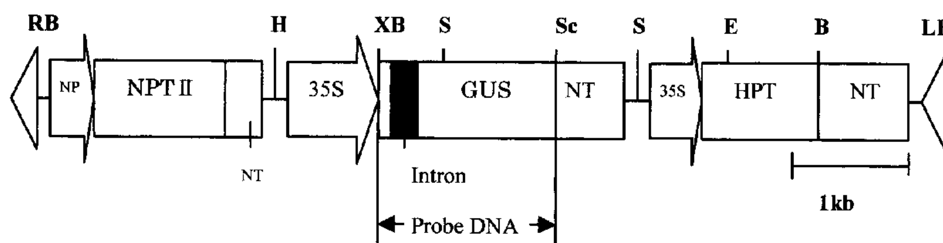

Fig. 1 Schematic diagram of a part of the T-DNA region of transformation vector pIG121-Hm. RB, right border; LB, left border; NP, nopaline synthase promoter; NT, nopaline synthase terminator; 35S, 35S promoter of cauliflower mosaic virus; Intron, the first intron of catalase gene of castor bean; NPT II, gene for neomycin phosphotransferase; GUS, gene for β-glucuronidase; HPT, gene for hygromycin phosphotransferase. Cutting sites of restriction enzymes are indicated; *Bam*HI(B), *Eco*RI(E), *Hind*III(H), *Sal*I(S), *Sac*I(Sc), *Xba*I(X).

sense primer 5'-ACGGTTTGTGGTTAATCAGG-3'; the resulting fragments were used as probes.

Results and Discussion

Effect of antibiotic on embryogenesis

The effect of kanamycin and hygromycin on embryogenesis was estimated based on the number of embryos formed after 3 weeks of culture (**Fig. 2**). In control (non-antibiotic medium), 495 green embryos per 0.1 g calli were formed. The number of embryos decreased with high concentrations of kanamycin or hygromycin. Been and Kim (1995) used 50 and 100 mg l^{-1} kanamycin to select transgenic secondary embryos of *O. javanica* . In our result, on the medium containing 100 mg l^{-1} kanamycin, embryogenesis from embryogenic callus was not completely suppressed; averages of 75 embryos (15% of control) were formed. On the other hand, embryogenesis was completely suppressed on the medium containing 50-100 mg l^{-1} hygromycin. Therefore, 50 mg l^{-1} hygromycin contained medium was used to select transgenic cells.

Agrobacterium-mediated transformation and Southern blot analysis

Approximately four month after infection, seventy-one hygromycin-resistant plantlets were formed from about 4 g calli. Fifty-one randomly selected plantlets were stained with X-Gluc; of these, seventeen plantlets expressed the GUS gene. Whole leaves of fifteen plantlets were stained in deep blue, but two plantlets were stained partly and faintly.

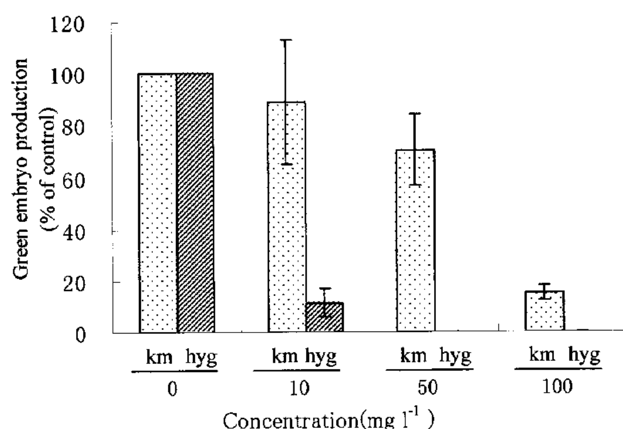

Fig. 2 Effects of hygromycin (hyg) and kanamycin (km) on embryogenesis of *Oenanthe javanica* plants. 0.1 g per plate of embryogenic calli were placed on MS hormone-free medium containing either kanamycin or hygromycin. Three weeks after the start of culture, the number of green embryos per plate was counted. Each treatment was carried out in triplicate. Data consist of percentage of control. 495 green embryos per plate were formed at control.

To confirm the presence of the GUS transgene in the transgenic plants, Southern blot analysis was conducted with the GUS probe prepared by PCR. Total DNA of non-transgenic and transgenic plants was digested with *Hin*d III, since the plasmid pIG121Hm used in this study contained a unique *Hin*d III restriction site. Single hybridized bands appeared in all six of the transgenic plants tested, indicating that a single copy of T-DNA was integrated into the genome (**Fig. 3**). The 8.6 kb fragments observed in plants T1 and T3 were evidence that they might have originated from the same cell. Similarly, the 7.8 kb fragments observed in plants T39, T41 and T42 suggested that they also might have originated from a single cell. Consequently, the presence of three independent transgenic plants was confirmed. Been and Kim (1995) produced transgenic *O. javanica* plants, using *Agrobacterium* strain LBA4404 and kanamycin selection. Our results showed that *Agrobacterium*-mediated *O. javanica* transformation using *Agrobacterium* strain EHA101 and hygromycin selection was also effective.

Expression pattern of GUS gene in transgenic plants

GUS activity in crude extracts of the leaves, stems and roots of transgenic plants T1, T3, T4 and T39 was assayed by fluorometric quantification. The highest activity was observed in the leaf of plant T4. In plant T3, slight activity was observed in the leaf, but none was observed in the stem or root. The GUS activity of plants T1 and T39 was between that of

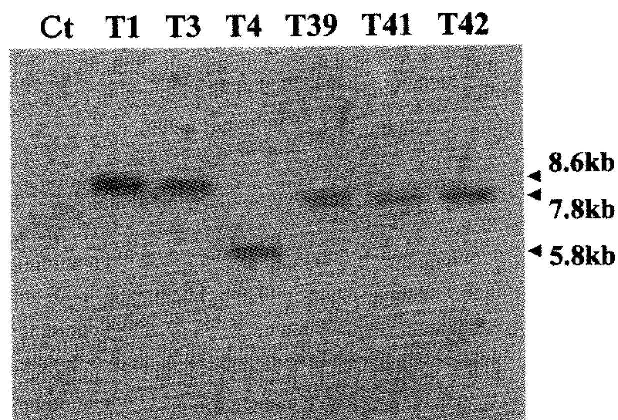

Fig. 3 Southern hybridization analysis of transgenic *Oenanthe javanica* plants. Digested total DNA (10 μg) with *Hin*dIII was hybridized with the PCR-amplified GUS region as a probe. Ct, non-transgenic plant; T1, T3, T4, T39, T41, T42, GUS-positive transgenic plant.

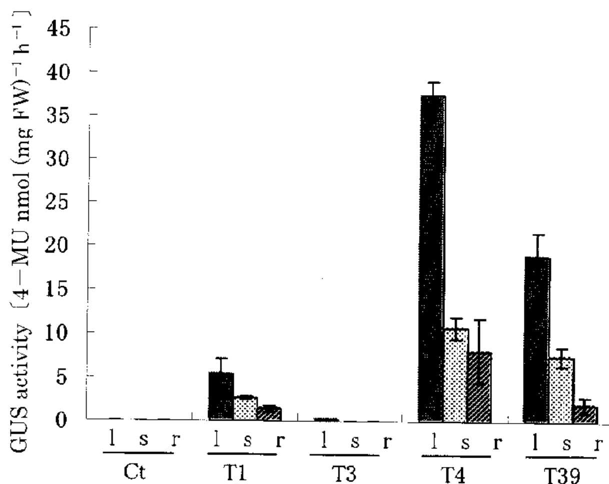

Fig. 4 GUS activity in transgenic *Oenanthe javanica* plants. Fluorometric GUS activity was analyzed in leaf (l), stem(s) and root(r) of non - transgenic (Ct) and transgenic (T1, T3, T4, T39) plants.

T3 and T4. All four transgenic plants showed the highest activity in the leaves and the lowest in the roots (**Fig. 4**).

A histochemical GUS assay of plants T3 and T39 was performed. In plant T3, leaf tissue (**Fig. 5A**), vascular bundles of stem (**Fig. 5B**), and the tip of the root were weakly stained (**Fig. 5C**). In plant T39, the leaf, stem and root were thoroughly and deeply stained (**Fig. 5D - F**). In the flower of plant T39, the anther and flower stalk were stained precisely, while ovary and petals were stained faintly (**Fig. 5G**).

The irregular GUS expression of plant T3, which was posited as a clone of plant T1 but showed lower activity than plant T1, suggests the occurrence of 'gene silencing' (Matzke *et al.*,1995) of the transgene.

No morphological difference was observed between the transgenic plants and non - transgenic plants (**Fig. 5H**).

In the present study, we have established a method for transformation of *O. javanica*. This protocol should be useful to develop *O. javanica* strains by introducing genes of interest such as disease - resistant genes.

Acknowledgement

The authors thank Dr. Kenzo Nakamura, Laboratory of Biochemistry, School of Agriculture, Nagoya University, Japan, for providing the plasmid pIG121Hm.

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