Genetic Transformation of ‘Kyoho’ Grape with a GFP Gene

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‘Kyoho’ grapevine (Vitis × labruscana Bailey) was successfully transformed by Agrobacterium-mediated transformation. Embryogenic calli (EC) were co-cultured with A. tumefaciens strain, EHA105 that harbors a green fluorescent protein (GFP) gene with the CaMV35S promoter. Kanamycin at 15 mg·L−1 was suitable for isolating transformed EC from which three plants that express GFP fluorescence were regenerated. DNA analyses, including polymerase chain reaction (PCR) and PCR-Southern hybridization, confirm that these three plants contain sequences comparable to the GFP gene to indicate that it was successfully incorporated into the ‘Kyoho’ grapevine genome.

Key Words: GFP, grapevine, ‘Kyoho’, transformation, Vitis × labruscana Bailey.

Introduction

‘Kyoho’ grape (Vitis × labruscana) is one of the most important cultivars in Japan. It accounts for about 33% of the annual grape production. The cultivar is known for its excellent fruit quality and large-sized berry. However, ‘Kyoho’ has serious problems with cultivation, i.e., insufficient resistance to pests and diseases, inconsistent fruit coloration, and short shelf life. It is important to overcome these genetic traits of ‘Kyoho’ by cross-breeding, but a long juvenile phase of grapevines is a discouraging.

An alternative approach to improve economically important characteristics in plant breeding involves the use of gene transformation by which grapevine plantlets have been obtained from V. vinifera (Iocco et al., 2001; Vidal et al., 2003; Yamamoto et al., 2000), V. × labruscana (Motioike et al., 2002) and other Vitis spp. by using embryogenic calli or somatic embryos via the Agrobacterium method or particle bombardment. There is only one successful transformation of V. × labruscana (Motioike et al., 2002) due to difficulties in inducing embryogenic calli from many of these cultivars. Without a suitable means to regenerate plants from tissue culture, genetic transformation of these cultivars cannot be achieved.

From an embryogenic callus of ‘Kyoho’ that was successfully obtained (Nakajima et al., 2000), we developed a model method for establishing an Agrobacterium-mediated GFP transformation system for ‘Kyoho’.

Materials and Methods

Embryogenic calli (EC)

EC of ‘Kyoho’ were induced from unfertilized ovules as described by Nakajima et al. (2000). The EC were maintained on half strength MS (Murashige and Skoog, 1962) medium (3% agar and 5% maltose) or on NN (Nitsch and Nitsch, 1969) medium (1.5% agar). Both media were supplemented with 1 µM 2,4-D. About 1 g of EC from the modified 1/2MS and 0.4 g of EC from the modified NN medium were used for consecutive Agrobacterium-mediated transformation.

Genetic transformation procedure

Agrobacterium tumefaciens EHA105, which harbors the binary plasmid pBin19-sgfp that contains the Nospro-nptII-Noster cassette and the 35Spro-sgfp-Noster cassette (Ghorbel et al., 1999), was used for transformation. An overnight culture of Agrobacterium was collected by centrifugation and was resuspended in a liquid 1/2MS medium (5% maltose) that contained 100 µM acetosyringone at a cell density of 1×10⁸ cfu/mL.

The EC were immersed in the Agrobacterium MS medium briefly, then co-cultured on solid 1/2MS medium (5% maltose and 0.85% agar) supplemented with 100 µM acetosyringone for 6 days at 26°C in the dark. After co-cultivation, these calli were cultured on 1/2MS selection medium (5% maltose and 0.85% agar) that contained 15 mg·L−1 kanamycin (Km) and 200 mg·L−1 cefotaxime under dark conditions for 2 weeks, then transferred to a similar medium with 3% agar and cultured for 1 month. The calli were divided into 3 blocks and selected on media that contained 0, 15 and 25 mg·L−1 Km for 4 months.

GFP expression was visualized by using an MZ FL III stereo fluorescence microscope (Leica, Germany).
with a 480/40 nm excitation filter and 510 nm barrier filter. Elongated embryos that showed GFP fluorescence were transferred to 1/2MS medium (1% agar), supplemented with 5 µM zeatin to promote shoot elongation in a 16/8-h (day/night) photoperiod. Several nodes of regenerated plants were maintained in the dark to produce etiolated shoots for fluorescence detection.

DNA analysis
PCR analysis and PCR-Southern hybridization were conducted to detect the GFP gene in transgenic plants. Genomic DNA was extracted from leaves by using the DNeasy plant mini kit (Qiagen, Germany).

Nucleotide sequences of the primer sets used for PCR amplification were 5'-GATGTGATATCCTCAGCTGAC GTAAG-3' and 5'-GTATAATTGCCGGACTCTAAT-3' corresponding to the 35S promoter region and the Nos terminator region, which amplifies about a 1 kb fragment of the 35S-gfp-Nos chimeric gene. PCR solutions that contain the genomic DNAs were heated to 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, then 7 min at 72°C. The primer pair 5'-AGCTGACCCTGAAGTTCA and 5'-GTATAATTGCGGGACTCTAAT-3' that correspond to gfp coding regions were also used to confirm the integration of the gfp gene into transgenic plants. The expected size of the amplification product of the gfp gene in using this primer pair is about 0.4 kb. Reaction vials for PCR were heated to 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, then 7 min at 72°C.

The amplified fragments were transferred to a nylon membrane (Hybond-N+, Amersham, UK) and hybridized with a digoxigenin-labeled probe of the gfp gene that was derived from the plasmid pBin19-sgfp by using the DIG DNA Labeling Kit (Roche, Germany). The hybridized bands were visualized by DIG Luminescent Detection Kit (Roche) in accordance to the manufacturer’s instructions.

Results and Discussion
Production of transgenic plants expressing GFP
A preliminary experiment indicated that high concentrations of Km (50 mg·L⁻¹) could be inhibitory for plant regeneration (data not shown). Although successful regenerations have been reported by using 50 mg·L⁻¹ Km in V. vinifera ‘Neo Muscat’ (Yamamoto et al., 2000), we used 0–25 mg·L⁻¹ Km with which 53 elongated embryos were obtained after 4 months of cultivation on the three different Km concentrations (Table 1). Among them, a total of 11 elongated embryos showed GFP fluorescence. Five plants were regenerated from 1/2MS maintenance medium, and three plants from NN maintenance medium from the GFP positive embryos, respectively. Eight plants were derived from independent transformation events, because each resulted from different callus lines. One plant which was regenerated from callus maintained on MS modified medium and isolated by 15 mg·L⁻¹ Km, showed GFP fluorescence on the margin of true leaves. Two plants from the calli maintained on NN modified medium and isolated by 15 mg·L⁻¹ Km (Fig. 1A) also showed GFP fluorescence. The etiolated shoots of these three plants showed green fluorescence to indicate the expression of the GFP gene (Fig. 1B, C). No morphological difference was observed between the transformants and non-transformants. The intensity of GFP fluorescence was slightly different among the three transformants.

DNA analysis
Three regenerated ‘Kyoho’ plantlets that express GFP fluorescence produced PCR amplification products of 1 kb for the primer set designed for the 35S promoter and Nos terminator regions (Fig. 2A), and 0.4 kb for the primer set designed for the gfp coding region (Fig. 2B). Amplification products were transferred to a membrane and hybridized with the gfp DNA probe by using the PCR-Southern hybridization procedure (Fig. 2C). All bands from the three transformed plants showed positive signals at the predicted size. These results indicate that the GFP gene was incorporated into the genomes of all three plants.

Iocco et al. (2001) obtained GUS or GFP-expressing grapevine embryos of ‘Chardonnay’ at a regeneration frequency of about 13% by isolating with 100 mg·L⁻¹ Km, whereas Vidal et al. (2003) used sequentially

<table>
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<th>Embryogenic callus maintenance medium</th>
<th>Kanamycin concentration (mg·L⁻¹)</th>
<th>Inoculated callus (g)</th>
<th>No. of elongated embryos</th>
<th>No. of embryos with GFP fluorescence</th>
<th>No. of germinated plants</th>
<th>No. of plants with GFP fluorescence</th>
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10 mg·L\(^{-1}\) Km and then 15 mg·L\(^{-1}\) Km for isolation after biolistic transformation; they observed a 54% regeneration rate for the same cultivar. Our study suggests that a lower Km concentration (15 mg·L\(^{-1}\)) is more suitable for the isolation and regeneration of ‘Kyoho’. Further study will be necessary to improve the efficiency of genetic transformation of ‘Kyoho’.

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