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**Original Paper**

# Embryogenic callus induction and *Agrobacterium*-mediated genetic transformation of 'Shine Muscat' grape

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**Abstract** We established a method for embryogenic callus induction and highly efficient *Agrobacterium*-mediated genetic transformation of a table grape cultivar 'Shine Muscat' (*Vitis labruscana*). Embryogenic calli were induced using flower bud filaments from a dormant cane. *Agrobacterium* strain LBA4404 harboring the binary plasmid pBin19-s*gfp*, which contains the s*gfp* and *nptII* genes, was used to infect embryogenic calli. Infected calli were selected on 1/2 MS medium containing 5% maltose and 2% agar supplemented with 15 mg $l^{-1}$ kanamycin. Efficiency of transformation of regenerated plants reached nearly 100% as determined by PCR and Southern blot analyses. The developed method will open a new avenue for genome editing of 'Shine Muscat' and contribute to the advancement of grape breeding.

**Key words:** *Agrobacterium*, embryogenic callus, genetic transformation, 'Shine Muscat' grape.

## Introduction

A table grape cultivar 'Shine Muscat' (*Vitis labruscana*) was released by the Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization in 2006. This cultivar produces large (≥12 g) white seedless berries if streptomycin is sprayed before flowering in combination with two gibberellin applications to flower or fruit clusters (Yamada et al. 2008). The berries have crispy flesh and a muscat flavor, and do not show any cracking. They have thin non-astringent skin and are palatable without peeling, in contrast to berries of 'Kyoho,' one of the most important cultivars in Japan. The fruit shelf life is longer than that of 'Kyoho.' 'Shine Muscat' is moderately tolerant to downy mildew and ripe rot, but is sensitive to anthracnose (Kono et al. 2013; Yamada et al. 2008). The cultivation and management of 'Shine Muscat' are relatively easy; its cultivation has rapidly increased to 1195.6 ha (8.7% of grape cultivation area in Japan) in 2016 (MAFF, https://www.e-stat.go.jp), only 9 years after the first sales of young plants in 2007 (Yamada and Sato 2016).

Yet, some 'Shine Muscat' traits such as pest and disease resistance, environmental stress tolerance, and accumulation of functional components including resveratrol need to be improved. One method to create new cultivars is genetic transformation. Most of the transformation methods of grape rely on *Agrobacterium* infection of embryogenic calli (ECs) (Martinelli and Mandolino 1994; Nakajima et al. 2006; Yamamoto et al. 2000). Flower organs, such as anthers (Hirabayashi and Akihama 1982; Nakano et al. 1997), ovaries (Nakano et al. 1997), unfertilized ovules (Nakajima et al. 2000; Notsuka et al. 1992; Srinivasan and Mullins 1980), and filaments (Nakajima and Matsuta 2003), have been used as explants and source materials to induce somatic embryogenesis. Leaves have also been used for this purpose (Matsuta 1992; Matsuta and Hirabayashi 1989; Nakano et al. 1997; Yamamoto et al. 2003), because they are available all year round. Nakano et al. (1997) used leaves, anthers, and ovaries as explants in 11 *Vitis vinifera* cultivars, 11 *V. labruscana* cultivars, and a *V. rupestris*

Abbreviations: AC, activated charcoal; AEC, aggregated embryogenic cells; 2,4-D, 2,4-dichlorophenoxyacetic acid; EC, embryogenic callus; GFP, green fluorescent protein; Km, kanamycin; Nospro, nopaline synthase promoter; Noster, nopaline synthase terminator; nptII, neomycin phosphotransferase II; sgfp, synthetic GFP (S65T) mutant; 35Spro, 35S promoter.

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cultivar. In some cultivars, somatic embryogenesis was induced only when ovary or anther explants were used, with ovaries being more efficient. Nakano et al. (1997) also demonstrated the importance of species for the induction of somatic embryos or ECs: they were more effectively induced in *V. vinifera* than in *V. labruscana*.

Grapes are transformed mainly by *Agrobacterium*-mediated transformation of somatic embryos or ECs (Hoshino et al. 1998; Iocco et al. 2001; Kandel et al. 2016; Martinelli and Mandolino 1994; Matsuta et al. 1993; Marchive et al. 2013; Nakajima et al. 2006; Perl et al. 1996; Scorza et al. 1996; Yamamoto et al. 2000, 2003). The success of genetic transformation depends on somatic embryogenesis and on the interaction of *Agrobacterium* with a particular cultivar.

Somatic embryogenesis and genetic transformation of 'Shine Muscat' grape have never been reported. A few reports of genetic transformation of grape via organogenesis have been published, but the number of suitable cultivars remains limited (Dutt et al. 2007; Sabbadini et al. 2019; Xie et al. 2016). Field-grown materials have limited use in the experiments on EC induction because grapes flower only once a year in vineyards. Recurrent cycles of secondary embryogenesis of *V. vinifera* cv. Thompson Seedless have been reported (Zhou et al. 2014). Here, we established a new procedure for the induction and maintenance of 'Shine Muscat' ECs using flower buds from dormant canes. We also report a genetic transformation system mediated by *Agrobacterium*, which will be applicable to genome editing.

## Materials and methods

### Plant materials

Dormant canes of 'Shine Muscat' were collected in an orchard (Akitsu, Higashihiroshima, Hiroshima, Japan) on January 27, 2014, and kept in a refrigerator at 4°C. The canes were cut into several pieces with 1 or 2 buds per piece on February 17, placed on rock wool, and tap water was poured into the container to about half of the rock-wool height. The canes were incubated at 26°C (16 h light/8 h dark) to induce flower bunches. Flower buds are first stuck to each other, then each bud becomes loose as it develops (here, we designated this status as "slightly apart from each other"). About 1 month after the start of the incubation, flower buds ("slightly apart from each other") were picked and sterilized for 20 min in sodium hypochlorite solution (1% available chlorine) containing Tween-20 (about 0.2 g $l^{-1}$). The buds were then rinsed with sterile distilled water, and the filaments were picked aseptically under a stereo microscope.

### Culture conditions

Filaments were transferred into 100-ml conical flasks (9 or 10 per flask) with 25 ml of liquid 1/2 MS (Murashige and Skoog 1962) medium containing 1×MS vitamins, 1 $\mu$M 2,4-dichlorophenoxyacetic acid (2,4-D), 1 $\mu$M *N*-(1,2,3-thiadiazol-5-yl)-*N*′-phenylurea, and 30 g $l^{-1}$ sucrose (1D1T). The medium was adjusted to pH 5.8 before autoclaving. Flasks were agitated continuously at 60 rpm in a gyro-rotary incubator at 26°C in the dark. After 1 month of culture, individual filaments were transferred to solid medium of the same composition as 1D1T with added 0.85% agar (1D1TS) for 2 months. The medium was renewed once a month. Three months after the culture initiation, filaments (some of them formed calli during the culture) were transferred to 1/2 MS medium containing 1×MS vitamins, 1 $\mu$M 2,4-D, 50 g $l^{-1}$ maltose, and 3% agar (1D3A5M) to maintain and propagate the ECs. Maltose was used at 5% because somatic embryogenesis and genetic transformation of 'Kyoho' grape, recalcitrant to somatic embryogenesis and genetic transformation, were established using 5% maltose in our previous study (Nakajima et al. 2006). Embryogenic ability was checked by transferring some calli on MS hormone-free medium to examine whether they would regenerate somatic embryos.

### Genetic transformation

*Agrobacterium tumefaciens* strain LBA4404, which harbors two chimeric gene expression cassettes, 35Spro-s*gfp*-Noster cassette and Nospro-*nptII*-Noster, in the binary plasmid pBin19-s*gfp* (Ghorbel et al. 1999), was used for transformation. *Agrobacterium* was cultured in YEB medium containing 50 mg $l^{-1}$ kanamycin (Km) and 50 mg $l^{-1}$ rifampicin with shaking overnight at 140 rpm at 28°C. *Agrobacterium* was collected by centrifugation and resuspended at a cell density of $1\times10^8$ cfu $ml^{-1}$ in liquid 1/2 MS medium containing 50 g $l^{-1}$ maltose and 100 $\mu$M acetosyringone. The ECs were immersed in the *Agrobacterium* suspension for 15 min and were then transferred on sterilized filter paper to remove excess *Agrobacterium*-containing medium. The ECs were co-cultured with *Agrobacterium* on solid 1/2 MS medium containing 5% maltose, 0.85% agar, and 100 $\mu$M acetosyringone for 4 days at 26°C in the dark. After co-cultivation, the ECs (about 2 mm each) were rinsed in 1/2 MS medium containing 5% maltose, 400 mg $l^{-1}$ cefotaxime and cultured on the 1/2 MS selection medium containing 5% maltose, 15 mg $l^{-1}$ Km and 200 mg $l^{-1}$ cefotaxime and 0.85% agar in the dark for 4 weeks. Thereafter, the aggregated embryogenic cells (AEC) (aggregate size, 2 mm) were divided into three groups and were cultured for 1 month on the second selection medium, which consisted of 1/2 MS medium containing 5% maltose, 2% agar, and 0, 15, or 25 mg $l^{-1}$ Km. The AEC selected on 25 mg $l^{-1}$ Km were further transferred to the same fresh medium once a month for 2 months. Elongated embryos were transferred to 1/2 MS medium containing 5 $\mu$M zeatin, 3% sucrose, 25 mg $l^{-1}$ Km, and 2% agar to promote shoot elongation under a 16 h light/8 h dark photoperiod. After true leaves appeared, regenerated plants were sub-cultured on 1/2MS medium containing 0.1 $\mu$M $\alpha$-naphthaleneacetic acid, 2.5 $\mu$M 6-benzylaminopurine, 0.85% agar, and 3% sucrose (MSNB medium).

Two nodes of each regenerated plant were cut and cultured on MSNB medium in the dark for more than a month (two nodes were used, because not all nodes of axial buds always elongate), and a white elongated shoot in good condition per line was used to detect green fluorescent protein (GFP) fluorescence. GFP expression was visualized under an MZ FL III or M165FC stereo fluorescence microscope (Leica, Germany) with a 480/40 nm excitation filter and 510 nm barrier filter.

### *DNA analysis*

PCR analysis and Southern hybridization were conducted to detect the GFP gene in transgenic plants. Genomic DNA was extracted from leaves with a DNeasy Plant Mini Kit (Qiagen, Germany) for PCR analysis, and with Genomic-tip 20/G (Qiagen) according to Yamamoto et al. (2006) for Southern blotting.

Primers 5′-GAT GTG ATA TCT CCA CTG ACG TAA G-3′, corresponding to the 35S promoter region, and 5′-GTA TAA TTG CGG GAC TCT AAT-3′, corresponding to the Nos terminator region, were used to amplify an about 1-kb fragment of the 35Spro-s*gfp*-Noster chimeric gene. Primers 5′-GGC TAT TCG GCT ATG ACT GG-3′ and 5′-CAT GTG TCA CGA CGA GAT CC-3′ were used to amplify an about 500-bp fragment of the *nptII* region. PCR amplification was performed in a total volume of 10 $\mu$l containing 5 ng of genomic DNA, 1 $\mu$l each of 10 $\mu$M primers, and 5 $\mu$l of GoTaq DNA polymerase (Promega, USA). Thermal cycler conditions were as follows: 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.

For Southern hybridization, 5 $\mu$g of each genomic DNA sample was digested with EcoRI (Nippon Gene, Japan), separated by electrophoresis on a 1% agarose gel at 30 V for 15 h and blotted onto a nylon membrane (Hybond-N, Amersham, UK). Probes were prepared using a PCR DIG probe synthesis kit (Roche, Germany) and the primers GFP-F 5′-ATG GTG AGC AAG GGC GAG GAG CTG T-3′ and GFP-R 5′-TGA TGC CGT TCT TCT GCT TGT CGG CCA-3′. Hybridization was performed according to the DIG Application Manual (Roche) and signals were detected using a ChemiDoc Touch Imaging system (Bio-Rad, USA).

## Results

### *EC induction and multiplication*

Dormant canes were incubated at 26°C (16 h light/8 h dark) in an incubator. After about 2 weeks, buds began to grow. Flower buds were obtained after about a month of incubation. The induced flower bunches with loose flower buds (“slightly apart from each other”) were used as culture materials (Figure 1A). Flower buds at 5 days before anthesis (2.0–2.5 mm in length) were picked and sterilized, and the filaments were picked aseptically under a stereo microscope. Nine or 10 filaments were cultured in one flask containing 1D1T at 26°C in the dark.

After 1 month of culture, calli were induced from 87 of 98 filaments (88.8%) (Figure 1B). These calli were transferred to 1D1TS medium to induce ECs. Three months after the transfer, somatic embryos were produced from 44 of 98 filaments (44.9%) (Figure 1C). Calli that included somatic embryos were transferred to 1D3A5M to propagate the ECs (Figure 1D, 1E). Part of each EC was transferred to MS hormone-free medium

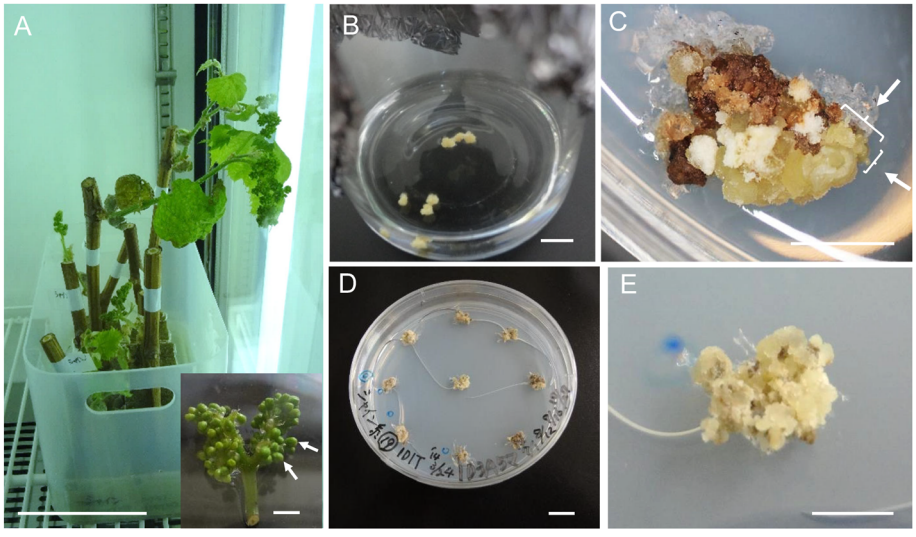


Figure 1. Embryogenic callus induction and propagation. (A) Dormant cane with flower bunches after 1-month incubation (bar=10 cm). Inset shows flower buds that were slightly apart from each other and were used for culture (bar=5 mm). Arrows show the buds at optimal stage for culture. (B) Filament culture 1 month after initiation (bar=1 cm). (C) Callus generated from a filament 4 months after culture initiation. Arrows show somatic embryos (bar=5 mm). (D) Part of embryogenic culture was picked and cultivated on 1D3A5M medium. Calli shown were sub-cultured for about 4 months (bar=1 cm). (E) An EC of ‘Shine Muscat’ (macrograph of D) (bar=5 mm).

to check embryogenic ability. Embryogenic ability was considered confirmed if somatic embryos were induced 1 month after the transfer. Embryogenic ability was maintained for about 4 years by transferring ECs to new 1D3A5M medium once every 1.5 months.

### *Genetic transformation*

Immediately after co-culture, GFP spots were observed in about half of the AEC (Figure 2A, B). After 1 month of culture on selection medium containing 15 mg l$^{-1}$ Km, 45 of 120 (37.5%) AEC had GFP fluorescence; 24 of them had pro-embryos and embryos with GFP fluorescence. These 120 AEC were divided into three groups and incubated in the second selection medium containing 0 (39 AEC), 15 (39 AEC), or 25 mg l$^{-1}$ Km (42 AEC). After 1 month of culture, embryo length reached about 1 mm (Figure 2C, D). The percentage of AEC with GFP-fluorescent embryos was highest (43.6%) on 15 mg l$^{-1}$ Km and lowest (33.3%) without Km (Table 1). After 2 months of culture, the percentage of GFP-fluorescent embryos was highest (48.9%) on 25 mg l$^{-1}$ Km and lowest (36.8%) without Km (Table 1). Afterwards, 7 AEC with GFP embryos selected on 25 mg l$^{-1}$ Km were sub-cultured to regenerate transgenic plants. Embryos reached approximately 2 mm (Figure 2E, F) and 5 mm or more (Figure 2G) in darkness 2 and 3.5 months, respectively, after *Agrobacterium* infection. Most embryos showed GFP fluorescence (Figure 2H). In total, 49 plants (7 plants on average per cluster of AEC from 2 to 16 plants; each cluster was designated as no. 1 to no. 7) were regenerated 6 months after *Agrobacterium* infection. Most of the regenerated plants had normal phenotype (Figure 3A), but several had variegated leaves (Figure 3B). GFP fluorescence was observed in three of seven nodes, with different intensity (Figure 3C, D).

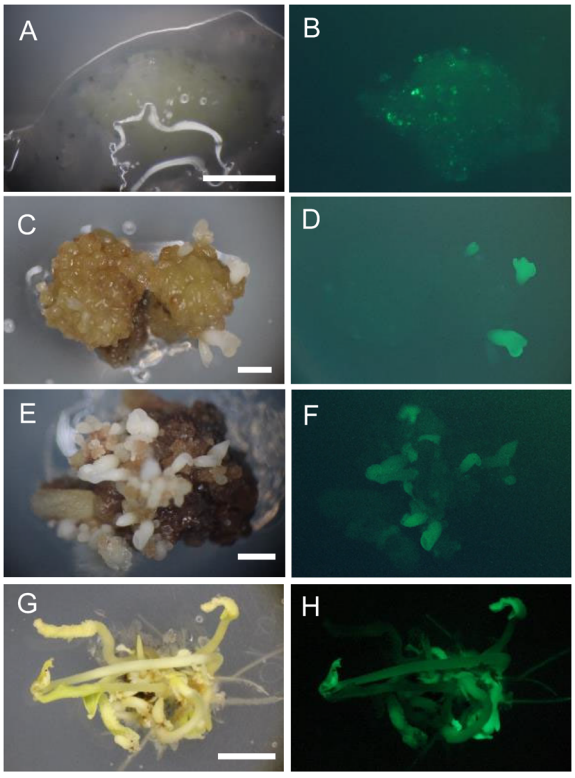


Figure 2. GFP fluorescence of ECs and somatic embryos during *Agrobacterium* co-culture and selection in darkness. (A, B) GFP spots of the AEC after co-culture (bar=1 mm). (C, D) Embryos were 1 mm in length at 1 month after *Agrobacterium* infection (bar=1 mm). (E, F) Embryos were approximately 2 mm in length at 2 months after the infection (bar=1 mm). (G, H) Embryos grown were ≥5 mm at 3.5 months after the infection (bar=5 mm).

### *DNA analysis*

Genomic DNA was extracted from leaves of each regenerated plant. PCR analysis was performed to confirm the integration of *sgfp*. With primers corresponding to the 35S promoter and Nos terminator, all 49 regenerated plants yielded the expected 1-kb

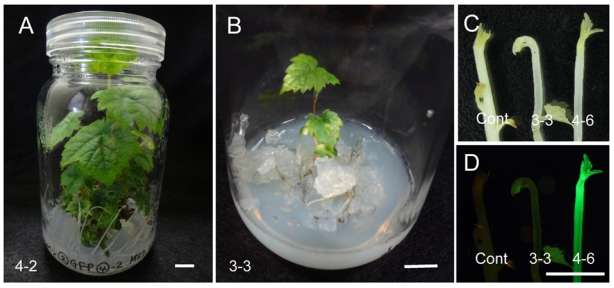


Figure 3. Regenerated plants and their GFP fluorescence. (A) A regenerated plant (no. 4-2) with wild-type morphology and without GFP fluorescence. Bar=1 cm. (B) A regenerated plant (no. 3-3) with variegated leaves. Bar=1 cm. (C, D) GFP fluorescence of representative regenerated plants under a stereomicroscope with white incandescent light (C) and with 480-nm-excitation blue light (D). Cont, wild type (non-transformant). Lines 3-3 and 4-6 exhibited weak and strong fluorescence, respectively. Bar=1 cm.

Table 1. Efficiency of transgenic embryo selection at different concentrations of kanamycin.

| Kanamycin concentration (mg l$^{-1}$) | No. of AEC (A) | No. of AEC with GFP-fluorescent embryos (B)* | Percentage of AEC with GFP-fluorescent embryos (B/A×100) | No. of embryos developed (C)** | No. of GFP-Fluorescent embryos (D)** | Percentage of GFP-fluorescent embryos (D/C×100) |
|---|---|---|---|---|---|---|
| 0 | 39 | 13 | 33.3 | 95 | 35 | 36.8 |
| 15 | 39 | 17 | 43.6 | 166 | 65 | 39.2 |
| 25 | 42 | 17 | 40.5 | 180 | 88 | 48.9 |

AEC: Aggregated embryogenic cells. * After a month of culture. **After 2 months of culture.

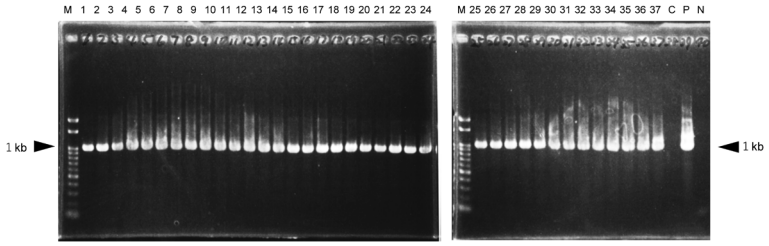


Figure 4. PCR analysis of the 35Spro-s*gfp*-Noster chimeric gene in regenerated plants. Lanes 1–37, regenerated plants. 1–5, plants regenerated from AEC no. 1; 6–9 from no. 2; 10–13 from no. 3; 14–17 from no. 4; 18–31 from no. 5; 32–35 from no. 6; 36 and 37 from no. 7. C, wild type; P, pBin19-s*gfp* plasmid; N, no DNA (negative control). M, 100-bp ladder.

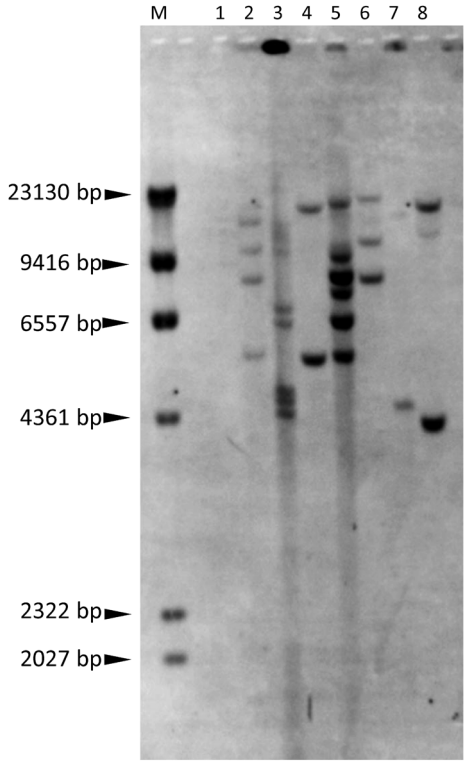


Figure 5. Southern blot analysis of regenerated plants. Genomic DNA was digested with EcoRI and probed with the s*gfp* fragment. Lane 1, wild type. Lanes 2–8, regenerated plants: nos. 1-6, 3-2, 3-3, 4-2, 4-6, 5-5, and 6-6 (from left to right). M, λ-HindIII digest.

Table 2. Number of DNA fragments detected by Southern blot analysis in Figure 5.

| Lane | DNA sample | No. of bands |
|---|---|---|
| 1 | WT | 0 |
| 2 | no. 1-6 | 4 |
| 3 | no. 3-2 | 8 |
| 4 | no. 3-3 | 2 |
| 5 | no. 4-2 | 6 |
| 6 | no. 4-6 | 3 |
| 7 | no. 5-5 | 2 |
| 8 | no. 6-6 | 3 |

fragment (Figure 4). With *nptII* primers, 48 of the 49 regenerated plants yielded the expected 0.5-kb fragment (data not shown).

In Southern hybridization with the s*gfp* gene probe, 2 to 8 bands were detected after cutting genomic DNA with EcoRI (Figure 5, Table 2), showing that the regenerated plants were transgenic. The band sizes differed among regenerated plants, even among plants regenerated from the same AEC (nos. 3-2 and 3-3, nos. 4-2 and 4-6), showing that plants were regenerated from different cells. Of 6 transgenic plants analyzed (nos. 1-6, 3-2, 3-3, 4-2, 4-6, 6-6), GFP fluorescence was detected only in nos. 3-3 and 4-6 (Figure 3C, D). One of the reasons for strong GFP fluorescence in no. 4-6 could be a low copy number (2 copies) of the *gfp* gene. No detection of GFP fluorescence in other plants could be partly ascribed to the relatively high copy number (4–8 copies), which may easily cause gene silencing (Schubert et al. 2004; Tang et al. 2007).

## Discussion

We established a procedure for somatic embryogenesis using flower organs from dormant canes of 'Shine Muscat' grape and *Agrobacterium*-mediated genetic transformation using ECs. Genetic transformation can be carried out all year round because ECs can be maintained for 4 years by sub-culturing them on fresh medium every 1.5–2 months. In PCR analysis, the transformation ratio of regenerated plants was close to 100% (all 49 regenerated plants were PCR-positive for the s*gfp* cassette, and 48 were PCR-positive for the *nptII*, Figure 5). In Southern blot analysis, positive s*gfp* signals were detected in all 7 plants analyzed (Figure 6). Our new procedure enables further physiological functional analysis of target genes through over-expression or knockdown experiments.

To induce ECs from vineyard-grown 'Kyoho' grape, an unfertilized ovule culture needed to be made from flower buds at 19–21 days before anthesis; at this stage, the flower buds are "slightly apart from each other" (see the *Plant materials* subsection for an explanation of this term) and have translucent yellow anthers (Nakajima et al. 2000). At a later stage, such as 7 days before anthesis, EC induction was not successful (Nakajima et al. 2000). Placing cut dormant canes in an incubator accelerated

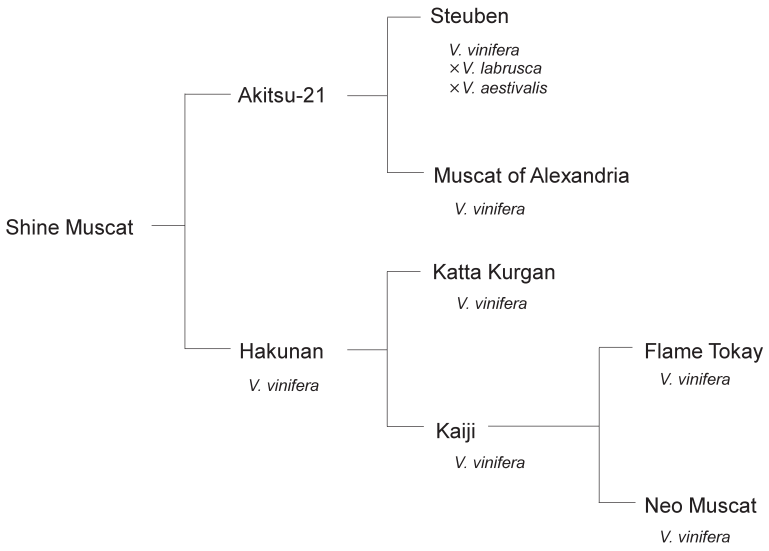


Figure 6. Pedigree of 'Shine Muscat' grape. Pedigree was modified from Yamada et al. (2008).

the development of anther filaments and unfertilized ovules (flower buds from dormant canes bloomed about 1 week after sampling) in comparison with that in the vineyard-grown trees. When the buds were "slightly apart from each other", they tended to be small. Therefore, we have to pay attention to the developmental status of buds on the dormant cane incubated in a growth chamber because being "slightly apart from each other" could be important for EC induction. Another point to consider is the temperature of dormant cane incubation, which seems to be cultivar-specific: 'Shine Muscat' induces flower bunches at 26°C, whereas 'Kyoho' flower bunches die at 26°C before attaining the best stage for culture, and incubation at 22°C solves the problem.

Matsuta and Hirabayashi (1989) compared three embryogenic callus induction media (NN, B5, and MS), and concluded that NN medium (without vitamins, myo-inositol or glycine, and supplemented with 1 $\mu$M 2,4-D) was optimal. The ingredients and composition of 1/2 MS medium (Matsuta and Hirabayashi 1989) are similar to those of NN medium. Moreover, proliferation of ECs was improved to two- to threefold by using 1×MS vitamins in our previous study (Nakajima et al. 2000). Accordingly, 1/2 MS medium was used instead of NN medium. The use of a high concentration (3%) of agar seems to prolong the embryogenic ability of ECs, especially in *V. vinifera* cultivars. On 3% agar medium, ECs were compact and very hard. For maintaining ECs, it could be recommended to select compact and hard calli under a stereo microscope. If cultured on 0.85% agar (normal concentration), calli became friable and lost embryogenic ability faster than on 3% agar. The concentration of agar to maintain ECs seemed to depend on the cultivar, especially among *V. labruscana* cultivars. When many somatic embryos or non-ECs grow on 3% agar medium (1D3A5M) during the maintenance of ECs, the use of lower agar concentrations (e.g., 2%, 1.5%, or 0.85%) helps maintain ECs (Nakajima et al., unpublished observation) and around 0.8% agar (or 0.2% gellan gum) was used in many cases (Das et al. 2002; Hoshino et al. 1998; Perl et al. 1995; Scorza et al. 1996). *Vitis vinifera* originated in Europe, most likely between the Black and Caspian Seas (Einset and Pratt 1975), and is adapted to dry and moderately warm weather during its growing season (Yamada and Sato 2016). *Vitis labruscana* was bred in North America in a region with considerable rainfall (Yamada and Sato 2016). In the 19th century, *V. vinifera* and *Vitis* species native to North America, including *V. labrusca* L. (which provided large fruit, cold resistance, and distinctive flavor) were crossed and many new cultivars were developed. L. H. Bailey defined *V. labrusca*-like cultivars as *V. labruscana*; this species combines vineyard varieties that show strong *V. labrusca* likeness and are its derivatives or hybrids (Bailey and Bailey 1930). Therefore, the different origin might affect the optimal maintenance of ECs. The use of 3% or 2% agar medium had a good effect on somatic embryogenesis and plant regeneration, because it avoided vitrification and abnormal somatic embryogenesis, and steadily produced normal somatic embryos, which became normal regenerated plants.

After the establishment of a system for the induction of somatic embryos or ECs, one might consider the next step, *Agrobacterium*-mediated transformation. ECs were thought to be better than somatic embryos as explants for *Agrobacterium* infection. Matsuta et al. (1993) used somatic embryo mass, in which embryos showed various stages of development, for *Agrobacterium* infection. Though most embryos did not grow in the presence of Km, some survived and were white. These embryos did not germinate, suggesting that they were chimeras composed of transgenic and non-transgenic tissues.

They were sub-cultured several times, and secondary or tertiary embryos developed and grew to transgenic plants (Matsuta et al. 1993). In our previous study (Nakajima et al. 2006), we used ECs for genetic transformation of 'Kyoho' grape, which was recalcitrant to genetic transformation, and obtained transformants. Zhou et al. (2014) also considered a proembryo mass to be a more appropriate tissue for genetic transformation than somatic embryos (transformation through secondary embryogenesis), because it took the least time (about 20 weeks) to develop transgenic somatic embryo lines. In our current study, the high transformation efficiency in a relatively short time could be attributed to the use of ECs for *Agrobacterium* infection.

*Vitis vinifera* tends to be relatively easy to transform in comparison with other grape species. Iocco et al. (2001) reported generation of transgenic plants of seven *V. vinifera* cultivars: 'Cabernet Sauvignon,' 'Shiraz,' 'Chardonnay,' 'Riesling,' 'Sauvignon Blanc,' 'Chenin Blanc,' and 'Muscat Gordo Blanco' (synonym of 'Muscat of Alexandria'). In contrast, *V. labruscana* has low transformation efficiency or is impossible to transform (Motioike et al. 2002; Nakajima et al. 2006). The genetic background of 'Shine Muscat' is about 75% *V. vinifera* and 25% *V. labruscana* (Figure 6). 'Muscat of Alexandria' (*V. vinifera*), one of the ancestors of 'Shine Muscat,' shows high transformation efficiency (Nakajima et al., unpublished observation), suggesting a reason for the high transformation efficiency of 'Shine Muscat.'

For the selection of transformants, the optimal Km concentration was 15 mg $l^{-1}$ for 'Kyoho' (Nakajima et al. 2006) and 50 mg $l^{-1}$ for 'Neo Muscat' (*V. vinifera*) (Yamamoto et al. 2000). Low Km concentrations may be more suitable for *V. labruscana* than for *V. vinifera*. Thus, the optimum Km concentration is assumed to be cultivar dependent.

The *Agrobacterium* strain LBA4404 used in this study allowed us to successfully establish a genetic transformation method in 'Shine Muscat.' In our previous report, a supervirulent strain EHA105 (Hood et al. 1993) was used for the transformation of recalcitrant 'Kyoho' grape (Nakajima et al. 2006). However, EHA105 tended to overgrow compared to LBA4404 when the antibiotic cefotaxime was used in 'Kyoho' transformation. According to Ogawa and Mii (2007), meropenem (at 25 mg $l^{-1}$) completely suppresses overgrowth of *Agrobacterium* in the transformation of tobacco, tomato, and rice using LBA4404 and EHA101. Meropenem might also be useful instead of cefotaxime to suppress *Agrobacterium* overgrowth in grape transformation. Another *Agrobacterium* strain GV3101 has been used for grape transformation (Mezzetti et al. 2002; Zhao et al. 2017). Zhao et al. (2017) compared the efficiency of transformation of petiole segments of *Vitis amurensis* with EHA105, GV3101, and LBA4404. GV3101 showed the highest transformation efficiency (29.91%), but it did not differ significantly from that of EHA105 (25.31%). In contrast, the transformation efficiency of LBA4404 was 3.14%. *Vitis amurensis* produced transformed calli, but generation of transgenic calli was almost negligible when the procedure optimized for *V. amurensis* and EHA105 was used to transform *V. vinifera* 'Muscat Hamburg' and 'Centennial Seedless.' Zhao et al. (2017) concluded that the genetic background is key for successful petiole segment transformation. Thus, the choice of the *Agrobacterium* strain used to transform grape seems to depend on the cultivar.

Antioxidants may be needed to prevent the deterioration of *Agrobacterium*-infected explants. Motioike et al. (2002) found that *Agrobacterium*-infected 'Niagara' (*V. labruscana*) ECs showed browning that increased during the incubation, and most ECs died even in a medium containing an antioxidant (1% polyvinylpolypyrrolidone); in contrast, the browning was less severe in another *V. labruscana* cultivar, 'Fredonia,' and transformed cells could be selected on the same medium. Perl et al. (1996) assumed that necrogenesis after exposure to *A. tumefaciens* was caused by a hypersensitive response of the grape ECs to the bacterium. They found that a combination of polyvinylpolypyrrolidone and dithiothreitol improved plant viability and inhibited tissue necrosis of the *V. vinifera* cultivar 'Superior Seedless' (Perl et al. 1996). Activated charcoal (AC) was used in grape tissue culture (Oláh 2017). Though AC proved to be effective to reduce browning of calli in transformation experiments, it could also strongly modify the effect of the plant growth regulators added to the medium, probably because of adsorption of the chemicals by AC (Mozsár et al. 1998). Bouquet et al. (2006) proposed the use of 2.5 g $l^{-1}$ AC for induction of somatic embryos and for co-cultivation on solid medium, while other researchers chose medium without AC. In our experiments, AC has not been used so far.

An increase in the copy number of transgene(s) in a transgenic plant tends to promote gene silencing. Tang et al. (2007) analyzed *gfp* transgene expression in eastern white pine transformants carrying different numbers of copies of T-DNA insertions. Post-transcriptional gene silencing was mostly observed in transgenic lines with four or more copies of T-DNA, but not in those with one copy. Schubert et al. (2004) reported that *GFP* transcript levels were high in Arabidopsis plants harboring up to four copies of the *GFP* gene, whereas five or more transgene loci under the control of the CaMV 35S promoter resulted in silencing. In our study, most of the elongated embryos (3.5 months after infection) showed GFP fluorescence (Figure 2H), demonstrating that Km effectively selected transformed embryos. Then, regenerated plantlets were maintained on medium

without Km, implying that the lack of Km might be the reason for the high ratio of silencing of the s*gfp* gene. Further study will be needed to confirm whether silencing of *nptII* also occurred; maintaining regenerated plants on Km-containing medium might select transformants with low T-DNA copy number. Another cause of gene silencing might be the use of the 35S promoter. Mishiba et al. (2010) found transgene silencing in gentian in which the introduced 35S promoter region was methylated irrespective of the transgene copy number and integration loci, whereas transgenic tobacco bearing the same T-DNA showed no silencing. In our study, s*gfp* silencing depended on transgene copy number, although the 35S promoter was used. Therefore, there may be species-specific mechanisms of transgene silencing in plants. In addition, Schubert et al. (2004) reported that the expression of highly similar genes is additive unless transgene transcript levels exceed a gene-specific threshold and consequently trigger gene silencing, and the use of a weaker promoter (e.g., Nos promoter) should reduce variability of transgene expression in populations of transformants. Future studies would be required to examine levels of methylation and to suppress silencing of transgenes in grape.

RNA-guided genome editing using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system has been applied successfully in many plant species, such as rice (Miao et al. 2013), wheat (Shan et al. 2013; Wang et al. 2014), poplar (Fan et al. 2015), tomato (Nonaka et al. 2017), apple (Nishitani et al. 2016), kiwifruit (Wang et al. 2018b), and banana (Naim et al. 2018). CRISPR/Cas9 was developed to induce DNA double-strand breaks at specific sites in the genome. These breaks have a high propensity to induce site-directed mutations through error-prone genome repair via non-homologous end joining. Generating such pinpoint mutations in a specific gene via CRISPR/Cas9 would have advantages over the strategies used in crossbreeding and Targeting Induced Local Lesions In Genomes (TILLING), especially because CRISPR/Cas9 often results in frame-shift mutations that inactivate the target genes. In grape, there have been four reports of genome editing using CRISPR/Cas9. In three of them, genome-edited plants were obtained using transformation with *Agrobacterium* (Nakajima et al. 2017; Ren et al. 2016; Wang et al. 2018a). Another one was the first report of direct delivery of purified CRISPR/Cas9 ribonucleoproteins to grape protoplasts by PEG-mediated transformation (Malnoy et al. 2016). Indel mutagenesis efficiency of the treated protoplasts was 0.1%. According to Malnoy et al. (2016), protoplast isolation, transfection, and transient gene expression in grape have not been sufficiently explored and most of the available methods have not been updated for two decades. Thus, Malnoy et al. (2016) updated and optimized the method that ensures protoplast viability, yield, and efficient transfection. However, plant regeneration from gene-edited protoplasts has not been achieved. Therefore, *Agrobacterium*-mediated genetic transformation combined with CRISPR/Cas9-mediated genome editing will be a promising method for targeted modification of genes in 'Shine Muscat.' An experiment aimed at changing the color of grape pericarp by combining *Agrobacterium*-mediated genetic transformation using ECs and a CRISPR/Cas9 vector in 'Shine Muscat' grape is now underway.

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