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ORIGINAL PAPER

Agrobacterium-mediated transformation of kabocha squash (*Cucurbita moschata* Duch) induced by wounding with aluminum borate whiskers

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Abstract An efficient genetic transformation method for kabocha squash (*Cucurbita moschata* Duch cv. Heiankogiku) was established by wounding cotyledonary node explants with aluminum borate whiskers prior to inoculation with *Agrobacterium*. Adventitious shoots were induced from only the proximal regions of the cotyledonary nodes and were most efficiently induced on Murashige–Skoog agar medium with 1 mg/L benzyladenine. Vortexing with 1% (w/v) aluminum borate whiskers significantly increased *Agrobacterium* infection efficiency in the proximal region of the explants. Transgenic plants were screened at the T_0 generation by sGFP fluorescence, genomic PCR, and Southern blot analyses. These transgenic plants grew normally and T_1 seeds were obtained. We confirmed stable integration of the transgene and its inheritance in T_1 generation plants by sGFP fluorescence and genomic PCR analyses. The average transgenic efficiency for producing kabocha squashes with our method was about 2.7%, a value sufficient for practical use.

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Introduction

The genus *Cucurbita* includes five important cultivated species: *C. argyrosperma* Huber, *C. ficifolia* Bouché, *C. maxima* Duchesne, *C. moschata* Duchesne, and *C. pepo* L. Global production of the *Cucurbita* genus reached 21 million tons in 2009 (http://faostat.fao.org). *Cucurbita* crops are consumed as food, incorporated into processed foods, and used as animal feed. In Japan, some *Cucurbita* species are used as rootstocks for cucumber, melon, and watermelon (Oda 2008) to induce resistance to diseases and insect pests, to provide higher stress tolerance, to provide higher yields, and to improve fruit quality (Edelstein 2004). In addition, the *Cucurbita* genus has the unique ability to take up organic xerobiotics and persistent organic pollutants from the soil (Hulster et al. 1994; Inui et al. 2008; Otani et al. 2007; White et al. 2003), and thus, helps in phytoremediation.

Genetic engineering can improve crops by exploiting useful genes and engineering transgenic plants with desirable properties, such as disease resistance. Several reports of genetic transformation of members of the family Cucurbitaceae, including *Cucumis melo* (Akasaka-Kennedy et al. 2004; Wu et al. 2009), *Cucurmis sativas* (Tabei et al. 1998; He et al. 2006; Vasudevan et al. 2007), *Citrullus lanatus* (Akashi et al. 2005; Choi et al. 1994), and *Lagenaria siceraria* (Han et al. 2005) are present. However, molecular analysis of the *Cucurbita* genus has been hampered by a lack of effective transformation methods. To date, only two transformation methods have been reported (Tricoli et al. 1995; Shah et al. 2008).

Tricoli et al. (1995) obtained transgenic *C. pepo* with virus resistance, but the details of their method or the transformation efficiency remain unknown. Shah et al. (2008) obtained transgenic *C. pepo* via shoot organogenesis, but efficiency of their method was only 0.7%. Regeneration via embryogenesis from cotyledons was reported by Jelaska (1972) and recently, other groups have also succeeded in inducing shoot organogenesis in *C. pepo* (Ananthakrishnan et al. 2003; Kathiravan et al. 2006), *C. maxima* (Lee et al. 2003), and *C. moschata* (Zhang et al. 2008) using cotyledonary explants. Reports of transformation of *C. maxima* or *C. moschata* are absent, although some varieties of C. *moschata* are more tolerant to various abiotic stresses, diseases, and insect pests than other members of the *Cucurbita* genus, and thus, are of great research interest.

Cucurbita species are susceptible to *Agrobacterium* infection (Smarrelli et al. 1986; Toppi et al. 1997), and studies have shown that wounding enhances *Agrobacterium*-mediated transformation efficiency (Curuk et al. 2005; Yamada et al. 2010). Whiskers, needle-like crystals with very high tensile strength, have often been used for direct gene transfer into callus tissues (Asad et al. 2008; Mizuno et al. 2004; Petolino et al. 2000). In this study, we report an efficient method for transformation of *C. moschata* Duch cv. Heiankogiku. We found that wounding explants with aluminum borate whiskers is a critical step for efficiently producing transgenic pumpkins. The average transformation efficiency was increased to about 2.7% with this method, making it suitable for practical use. This is the first report of an efficient transformation via adventitious shoot organogenesis in *C. moschata* Duch.

Materials and methods

Preparation of cotyledonary explants

Seeds of *C. moschata* (cv. Heiankogiku) were purchased from Takii & Co., Ltd. (Kyoto, Japan). Seed coats were removed with a scalpel and forceps. The peeled seeds were sterilized for 10 min using 1% (w/v) sodium hypochlorite and one drop of Tween 20 and rinsed five times with sterile distilled water. The sterilized seeds were germinated at 28°C in the dark for 1 day on shoot-inducing (SI) Murashige–Skoog (MS) medium (Murashige and Skoog 1962) with 1 mg/L 6-benzyladenine (BA), and 0.8% (w/v) agar. All media were autoclaved at 121°C for 15 min and their pH was adjusted to 5.7–5.8 prior to the addition of agar. Cotyledons were excised from post-germination seedlings using a stereoscopic microscope, taking care not to remove the cotyledonary nodes (Fig. 1 a, b). The cotyledons were first cut in half crosswise, and the distal regions were discarded. The explants were then cut into two longitudinal sections (Fig. 1c) and placed with their abaxial sides up in a 9-cm petri dish containing SI medium. They were precultured for 1 day at 25°C in the dark before being transformed with *Agrobacterium*.

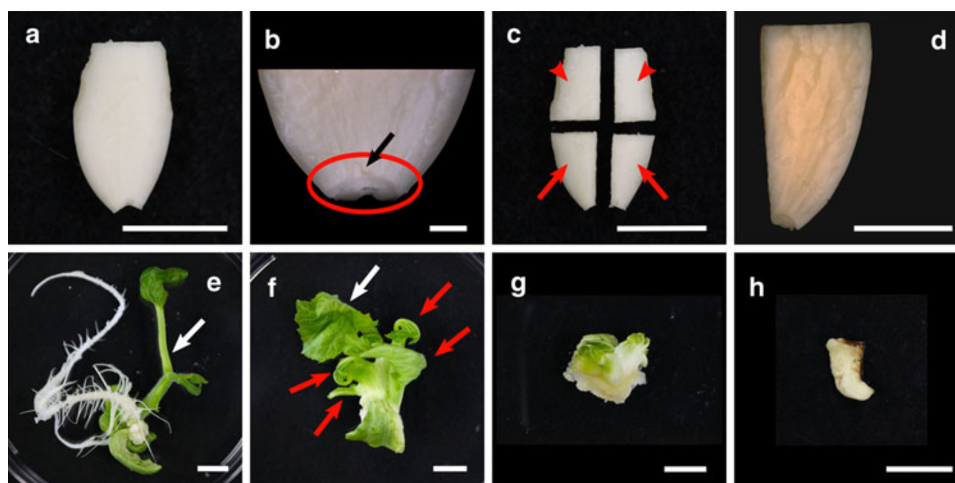

Fig. 1 Shoot regeneration from cotyledonary explants of *C moschata* cv. Heiankogiku **a** A cotyledon detached from a hypocotyl. *Bar*, 5 mm. **b** Proximal region of a cotyledon. The *red circle* and *black arrow* indicate the junction of the hypocotyl and cotyledon, and the shoot apex, respectively. *Bar*, 1 mm. **c** Preparation of cotyledonary explants. *Red arrows* indicate the proximal regions of the cotyledons used as explants; *arrowheads* indicate the distal regions. *Bar*, 5 mm. **d** A magnified explant. Note that the junction between the cotyledon and hypocotyl is intact. *Bar*, 3 mm. **e–h** Explants grown under MS medium with 0.1 mg/L BA (**e**), 1.0 mg/L BA (**f**), 4.0 mg/L BA (**g**), and 2.0 m/L BA plus 1.0 mg/L ABA (**h**). The explants were incubated in the medium described for 37 days. The *white arrows* in (**e**) and (**f**) indicate shoots developed from the shoot apex, and the *red arrows* in (**f**) indicate shoots induced by shoot organogenesis, respectively. *Bars*, (**e–h**) 10 mm

Bacterial strain and binary vector

Agrobacterium tumefaciens strain EHA105 harboring the binary vectors pIG121-Hm (Akama et al. 1992), pIG-sGFP, and pGFP-S65C was used for transformation (Fig. 2). The pIG-sGFP vector carried a *β*-glucuronidase gene (*GUS*) with a castor bean intron and synthetic green florescent protein gene (*sGFP*) with serine at position 65 replaced with threonine (S65T) (Chiu et al. 1996). The pGFP-S65C vector carried a *sGFP* with serine at position 65 replaced with cysteine (sGFP-S65C) by site-directed mutagenesis under the control of the constitutive promoter CaMV35S, and was constructed by replacing *GUS* from pIG121-Hm with *sGFP-S65C*. *Agrobacterium* was cultured with 10 ml of Luria–Bertani medium (pH 5.2) containing 50 mg/L kanamycin, 25 mg/L chloramphenicol, 25 mg/L rifampicin, and 20 μM acetosyringone at 28°C until the optical density at 600 nm reached 0.4–0.8. The bacterial culture was centrifuged and resuspended in inoculation medium (IN) containing SI liquid medium with 500 μM acetosyringone, and final concentration of *Agrobacterium* (measured by optical density at 600 nm) was adjusted to 0.1. Prior to inoculation, the *Agrobacterium* culture was resuspended by gentle shaking at 28°C for 1–2 h to ensure efficient induction of *vir* genes.

Wounding of explants

Aluminum borate whisker powder (Alborex Y; Shikoku Chemicals Corporation, Kagawa, Japan, Suganuma et al. 1990) was sterilized prior to use by autoclaving. Approximately 100 explants were added to a 50-ml centrifuge tube containing 20 ml of whisker slurry in SI liquid medium under aseptic conditions. The tube was then vortexed for 30 min using a vortex mixer (Vortex Genie 2 with a 29–37 mm Tube Foam Inserts; Scientific Industries, Inc, Bohemia, NY).

Inoculation, co-cultivation with *Agrobacterium*, selection, and regeneration of transgenic plants

Wounded explants were resuspended in the *Agrobacterium* culture and shaken gently for 10 min at room temperature. Excess liquid was removed using sterilized filter papers, and wounded explants were co-cultured on three pieces of filter paper (Ozawa 2009) moistened with 5.5 ml of IN medium at 25°C in the dark for 3 days. After co-cultivation, explants were washed five times with sterilized distilled water and transferred to a selection medium containing SI agar with 10 mg/L meropenem (Ogawa and Mii 2007) and 50 mg/L kanamycin, placed in the growth chamber maintained at 25°C, a 16/8 h (light/dark) photoperiod with white fluorescent lamps. Explants were subcultured on fresh media after a 2-week interval. Following culture on the selection medium for 4–6 weeks, the regenerated shoots were excised and transferred to shoot elongation (SE) medium containing half-strength MS medium with 0.1 mg/L BA, 0.8% agar, 10 mg/L meropenem, and 50 mg/L kanamycin. Non-chimeric transgenic lines were selected via axillary bud culture in SE medium.

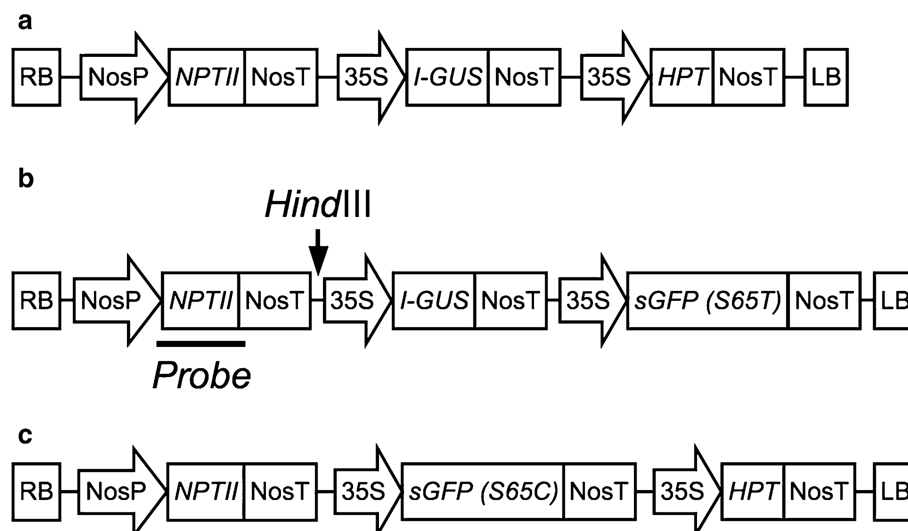

Fig. 2 Schematic diagrams of the T-DNA region of the binary vectors used in this study **a** pIG121-Hm **b** pIG-sGFP **c** pGFP-S65C. A restriction site of *Hind*III and a DNA fragment of *NPTII* used as a probe for Southern blot analysis in pIG-sGFP were indicated, respectively. *RB* right border, *NosP* nopaline synthase promoter, *NPTII* neomycin phosphotransferase, *35S* CaMV 35S promoter, *I-GUS* *β*-glucuronidase containing a castor bean intron, *NosT* nopaline synthase terminator, *HPT* hygromycin phosphotransferase, *LB* left border, *sGFP (S65T)* and *sGFP (S65C)* synthetic green fluorescent protein with serine at position 65 replaced with threonine or cysteine, respectively

Rooting plants were acclimatized and grown in an enclosed greenhouse.

Visible marker assay

Histochemical GUS assays were performed on cotyledonary explants from day 7 after the elimination of *Agrobacterium*. The GUS-staining solution containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM ethylenediaminetetraacetic acid, 0.1% Tween 20, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20% (w/v) methanol, and 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide was introduced into the cotyledonary explants by two sessions of vacuum infiltration for 7.5 min. The tissues were incubated overnight at 37°C. After staining, tissues were rinsed with 70% ethanol to remove chlorophyll.

sGFP fluorescence from transgenic plants was observed using the Leica MZ16FA epifluorescence stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a light source, 100 W mercury bulb, and a FITC/GFP filter set with a 480-nm excitation filter and a 510-nm long-pass emission filter producing blue light.

DNA isolation and polymerase chain reaction (PCR) analysis

For PCR analysis, genomic DNA from leaves of *C. moschata* plants was extracted as described previously (Edwards et al. 1991). Primer pairs used for amplifying *sGFP* were 5′-ctgggtaccatggtgagcaagggcgaggag-3′ and 5′-gcgactagtttacttgtacagctcgtccat-3′; those for amplifying *NPTII* were 5′-ccgcttgggtggagaggctattc-3′ and 5′-ccagccg gccacagtcgatgaat-3′; those for amplifying *HrcA* were 5′-ca tcgtcgaaggttatctcgatacg-3′ and 5′-tataatcgaccatcggtacga tacg-3′; and those for amplifying elongation factor 1-α (*EF1α*: GenBank accession No. AB615454*)* were 5′-agacc accaagtactactgcac-3′ and 5′-ccaccaatcttgtacacatcc-3′. PCR amplification was performed under the following conditions: 94°C for 2 min, 30 cycles at 94°C for 30 s, at 63°C for 30 s, and at 72°C for 1 min, and a final extension step at 72°C for 7 min. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis

Genomic DNA was isolated from young, developing leaves by a DNAs-ici!-P kit (Rizo inc. Tsukuba, Japan), and RNA was degraded using RNase A. Twenty µg of genomic DNA was digested with *Hind*III, separated on a 0.7% agarose gel, and transferred to nylon membranes positively charged (Roche Diagnostics, Indianapolis, IN) with 20x saline sodium citrate buffer. A digoxigenin (DIG)-labeled DNA probe specific for the *NPTII* coding sequence was used for Southern hybridization and detection was performed according to the manufacturer's instructions (Roche Applied Science, Penzberg, Germany).

Results and discussion

Effect of plant hormones on regeneration

In a preliminary examination, we screened 16 varieties of *Cucurbita* species for differences in shoot organogenesis (data not shown) and selected *C. moschata* cv. Heiankogiku. We then examined the effect of each region of the cotyledonary explant detached from the cotyledonary nodes on adventitious regeneration (Fig. 1a, b). Excision of the hypocotyl fragment led to loss of direct shoot development (data not shown), which is in agreement with recent reports of direct regeneration in other *Cucurbita* species (Ananthakrishnan et al. 2003; Kathiravan et al. 2006; Lee et al. 2003; Zhang et al. 2008). We also found that after culturing on SI medium, the proximal region of the cotyledonary node (Fig. 1c, d) was capable of shoot regeneration. Thus, we used proximal regions of the cotyledons with hypocotyl fragments as explants in the following experiments. Tendency of diminishing regeneration efficiency at the distal regions of the cotyledons has also been reported previously (Tabei et al. 1993). We next examined the effect of several plant hormone concentrations on adventitious shoot regeneration (Table 1). Use of 1 mg/L BA significantly increased shoot regeneration with a score of 66.9% (Table 1; Fig. 1f), which is similar to results reported previously (Ananthakrishnan et al. 2003; Lee et al. 2003; Zhang et al. 2008). Furthermore, 0.1 mg/L BA induced shoot elongation, but this elongation appeared to develop because of the tendency of the shoot apex to elongate, and not from shoot organogenesis. Furthermore, a lower BA concentration also stimulated root formation, suggesting that the endogenous cytokinin/auxin ratio was too low to induce shoot organogenesis on such media (Fig. 1e). In contrast, Shah et al. (2008) succeeded in inducing shoot organogenesis from summer squash using 0.05 mg/L BA without root induction. The difference in results may be attributed to the different species characteristics or to the fact that they used shoot tip segments excised from 4- to 5-day-old seedlings. It appeared that elongated shoots were not regenerated adventitious shoots but elongations of axial buds. Using 4 mg/L BA often led to severe vitrification and abnormal shoot formation (Fig. 1g), and in such cases the adventitious shoots were not elongated. Although the combination of BA and abscisic acid (ABA) was efficient in inducing shoot regeneration in cucumber (Tabei et al. 1998), this combination often led to severe growth inhibition and browning

Table 1 The effect of plant growth hormones on organogenesis of cotyledonary explants in *C. moschata* cv. Heiankogiku

Shoot induction medium[a]	Total no. of explants	Regeneration efficiency (%)[b, e]	Shoot number per explant[c, e]	Root induction efficiency (%)[d, e]
0.1 mg/L BA	107	62.6 ± 6.0	1.27 ± 1.19	34.4 ± 20.7
1.0 mg/L BA	106	66.9 ± 9.4	1.91 ± 1.66	0.3 ± 0.6
4.0 mg/L BA	110	50.2 ± 6.0	1.0 ± 1.17	0
2.0 mg/L BA + 1.0 mg/L ABA	103	35.2 ± 24.2	0.84 ± 1.30	0

[a] All media used in this experiment contained 30 g/L sucrose, MS salts, vitamins, and 0.8% agar

[b] Regeneration efficiency = (Number of explants with one or more shoots/total number of explants) × 100

[c] Average number of shoots induced in each explant

[d] Root induction efficiency = (Number of explants with one or more roots/total number of explants) × 100

[e] Each value represents the mean ± SD from three independent experiments

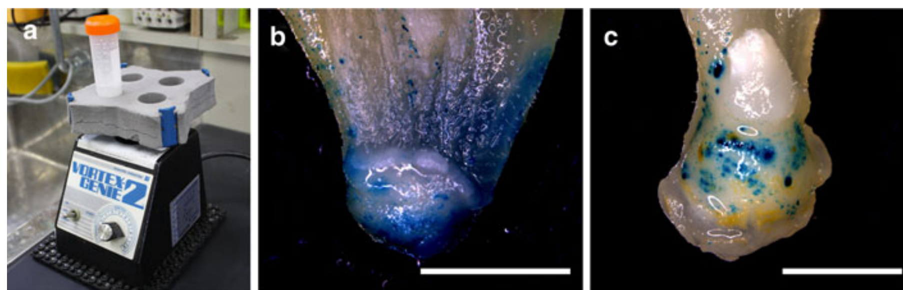

Fig. 3 *Agrobacterium*-mediated transformation **a** vortexing apparatus. Vortex Genie 2 with an adaptor for a 50-ml centrifuge tube. **b**, **c** GUS activity in proximal region of explants wounded using a vortex mixer and whiskers before *Agrobacterium* infection (**b**), and in explants that were not wounded before *Agrobacterium* infection (**c**). *Bars*, 5 mm

of kabocha squash explant edges (Fig. 1h). Therefore, we concluded that 1 mg/L BA was the most effective concentration in a case of using Heiankogiku, and we used this concentration for subsequent genetic transformation experiments.

Improvement of *Agrobacterium* infection efficiency

To optimize conditions for efficient *Agrobacterium* infection, *Agrobacterium* cultures harboring pIG121-Hm (Fig. 2a) were infected and a GUS assay was performed. Strong GUS activity was observed in the cut regions of the explants; however, infection of the proximal regions of the explants was insufficient. Acetosyringone is an effective activator of *vir* genes, and it is frequently used for *Agrobacterium*-mediated transformation in various plant species (Damgaard et al. 1997; Dutt and Grosser 2009; Wang et al. 2009). Therefore, we added 500 μM acetosyringone to the co-cultivation medium in an attempt to increase the infection efficiency. However, transformation efficiency in the proximal region remained insufficient. Antioxidants such as L-cysteine (Olhoft and Somers 2001) and ethylene inhibitors such as aminoethoxyvinylglycine (Ezura et al. 2000) also did not significantly increase the infection efficiency in this region (data not shown). We then examined numerous wounding treatments administered to the proximal regions of the explants, namely (1) cutting the area with a scalpel blade, (2) ultrasonic treatment (150 kW, 1 min, three times), (3) particle bombardment (1,350 psi, 1.6 μm gold particle, two shots), (4) scratching the proximal regions of the explants with a flat file, and (5) scratching the proximal region of the explants with carborandom and a cotton swab. Two independent transgenic pumpkin plants were obtained using method (5) (data not shown), but it was time-consuming and the transgenic efficiency was low (two transformants from 612 explants, 0.33%). No transformants were obtained using other methods.

Production of transgenic plants using aluminum borate whiskers

In an effort to find another method, we used a vortex mixer (Fig. 3a) and "Albolex Y" aluminum borate whiskers (Suganuma et al. 1990), which form white needle-like crystals (10–30 μm in length/0.5–1.0 μm in diameter) with very high tensile strength. Vortexing for 30 min with 1% of the whisker suspension dramatically increased the efficiency

of *Agrobacterium* infection on proximal region of explants (Fig. 3b), compared with unwounded explants (Fig. 3c). Furthermore, this treatment was rapid and not as labor intensive as method (5).

Agrobacterium harboring the pIG-sGFP and the pGFP-S65C binary vectors (Fig. 2b, c) were used for infection, and transgenic plants were screened for sGFP fluorescence. sGFP is a powerful tool for efficiently selecting transgenic plants that develop many escape shoots because GFP-introduced shoots can be easily located without causing any destruction, unlike the GUS staining assay. Initially, we used the pIG-sGFP binary vector containing a *sGFP (S65T)* expression cassette for transformation. However, sGFP (S65T) fluorescence could be scarcely observed in the mature leaves because strong chlorophyll autofluorescence masked it (data not shown). To observe intense GFP fluorescence in regenerated shoots, serine at position 65 of sGFP was replaced by cysteine (GFP S65C, Reichel et al. 1996), and this was successful even in mature green leaves with high chlorophyll autofluorescence. We vortexed the explants with various concentrations of the whisker suspension (0, 0.1, 1, and 10%) to find the optimal strength for obtaining transgenic shoots (Table 2). Vortexing for 30 min with 1% whisker suspension showed the highest transformation efficiency, 2.7%. The 10% whisker suspension often caused serious damage to the explants and decreased transformation efficiency. Kanamycin was effective for selecting transformed shoots (Fig. 4a), and introduction of *sGFP* enabled us to locate transgenic shoots (Fig. 4b, c). Repetition of axially bud culture resulted in selection of non-chimeric shoots. Addition of 0.1 mg/L BA to MS medium stimulated elongation from the axial buds at

Table 2 The effect of whiskers on transformation efficiency

Vortex	Whiskers (%)	Total no. of explants[a]	Total no. of transformed plants	Efficiency (%)[b]
–	0	257	1	0.44 ± 0.77
+	0	246	3	1.33 ± 2.31
+	0.1	253	3	1.20 ± 0.18
+	1	253	7	2.73 ± 1.28
+	10	252	3	1.11 ± 1.03

[a] Cotyledonary nodes from seedlings, germinated for 1 day followed by precultured for 1 day, were used as explants. *Agrobacterium* harboring pGFP-S65C was used for infection

[b] Efficiency = (Number of shoots with GFP fluorescence/total number of explants) × 100. Each value represents mean ± SD from three independent experiments

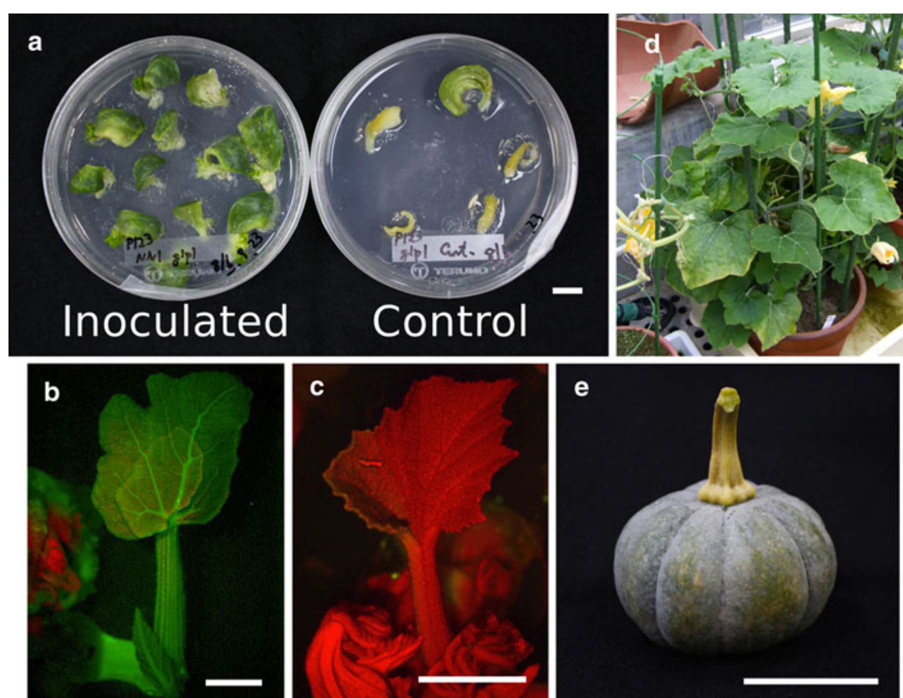

Fig. 4 sGFP-expressing transgenic plants introducing pIG-sGFP **a** Explants on kanamycin selective medium. Inoculated explants remained green on the medium containing kanamycin, whereas control explants without inoculation turned yellow and were finally dead. *Bar*, 10 mm. **b** A GFP-positive shoot appears green under blue light due to GFP fluorescence. *Bar*, 5 mm. **c** A non-transgenic shoot appears red under blue light due to the autofluorescence of chlorophyll. *Bar*, 5 mm. **d** A putative transgenic T_0 plant growing in the greenhouse. **e** A fruit developed from a transgenic plant 40 days after self-crossing. *Bar*, 5 cm

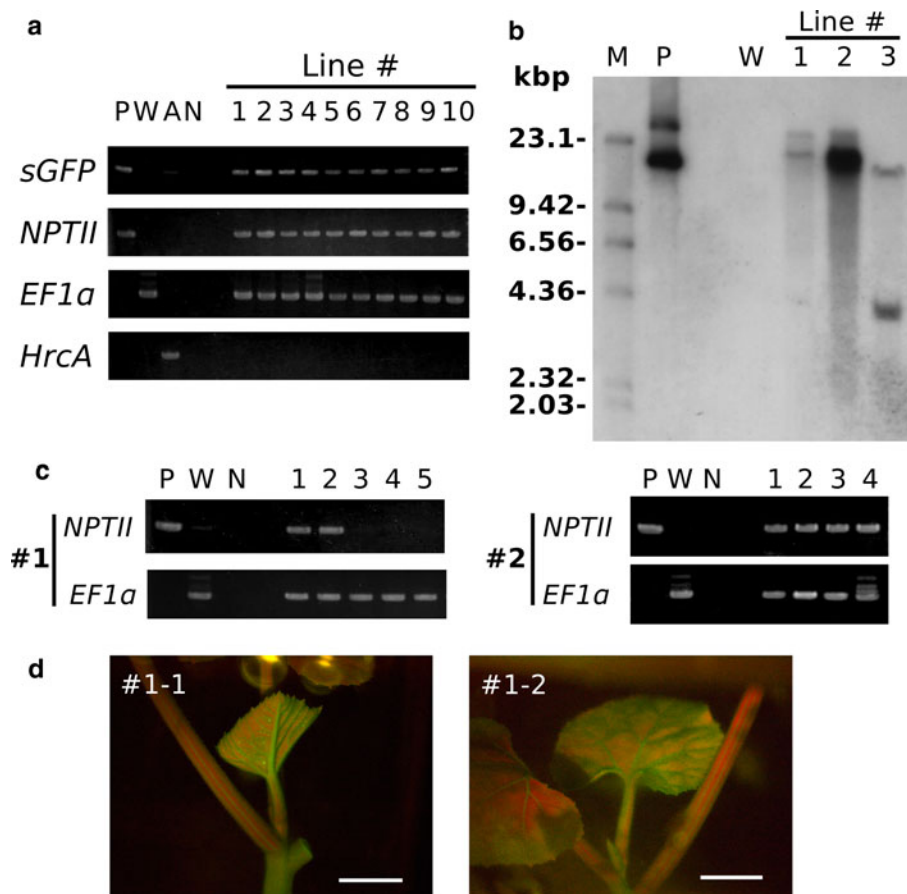

Fig. 5 Molecular analysis of transgenic plants introducing pIG-sGFP **a** 1.5% agarose gel electrophoresis of PCR-amplified DNA from leaf tissue of the regenerated shoots. *Lane W* is the wild-type plant; *Lane P* is pIG121-sGFP (*positive control*); *Lane A* is *Agrobacterium* genomic DNA; *Lane N* is a mock without template DNA (*negative control*); *Lanes 1–10* are different independently regenerated shoots. Size of amplified DNA fragments of *sGFP*, *NPTII*, *HrcA*, and *EF1α* were as follows, 720, 600, 980, and 600. **b** Southern blot analysis of genomic DNA from leaf tissues of selected transgenic plants, a vector, and a non-transgenic plant. Twenty μg of genomic DNA from each line was digested with *Hind*III, separated on 0.7% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with a DIG-labeled *NPTII* probe. *Lane W* is the wild-type plant; *Lane P* is pIG121-sGFP (*positive control*); *Lane M* is DIG-labeled *λ/Hind*III DNA marker (Roche Applied Science). **c** Genomic PCR analysis of *NPTII* in T_1 plants. *Lane W* is the wild-type plant; *Lane P* is pIG121-sGFP (*positive control*); *Lane N* is a mock without template DNA (*negative control*); *Lanes 1–5* in #1 and *lane 1–4* in #2 are independent T_1 progenies from each line. **d** GFP-positive shoots from the T_1 progenies of line #1 appears green under blue light due to GFP fluorescence. *Bar*, 5 mm

the junction of the stem and leaf petioles and did not have a negative effect on vitrification (data not shown). Agar concentration up to 2.0% and an air vent pot were useful for restoring vitrified shoots. Rooting was efficiently induced by adding 0.5 mg/L of indole acetic acid or naphthaleneacetic acid. Transformed plants grew normally in the greenhouse (Fig. 4d), and fruits were obtained by self-crossing (Fig. 4e).

Analysis of transgenic plants

To verify gene integration, genomic PCR analysis was performed for randomly selected putative transgenic plants for which sGFP fluorescence was observed (Fig. 4b). *EF1α*, one of the famous housekeeping genes, was isolated (GenBank accession No. AB615454) from *C. moschata* and used as a loading control. *sGFP* was successfully amplified from ten randomly selected transgenic plants introducing pIG-sGFP (Fig. 5a). *HrcA*, which is widely distributed in *Agrobacterium* (Nakahigashi et al. 1999), was not amplified from the isolated genomic transgenic plant DNA, indicating no *Agrobacterium* contamination. To determine stable integration of the transgene into the plant genome, Southern blot analysis was performed on three randomly selected transgenic plants using a DIG-labeled *NPTII* probe (Fig. 5b). The *NPT II* probe hybridized to digested DNA from transgenic plants, but not to digested DNA from wild-type plants. Transgene inheritance was confirmed in T_1 generation plants using genomic PCR analysis. Amplification of *NPTII* was observed in the

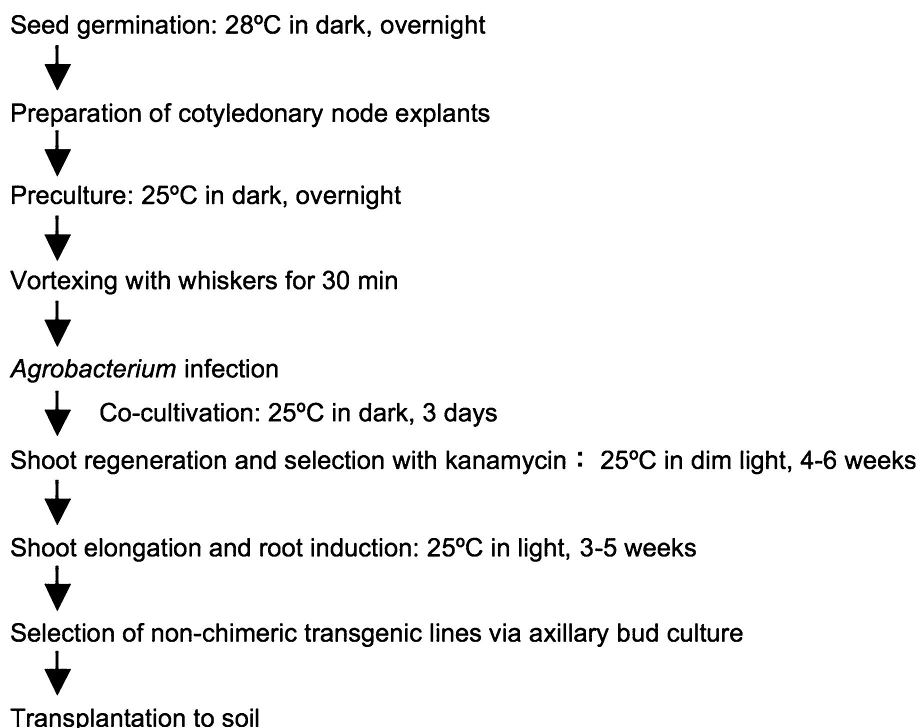

Fig. 6 Steps in the transformation of *C. moschata* Duch cv. Heiankogiku via direct shoot organogenesis from cotyledonary explants

T_1 generation plants (Fig. 5c), indicating successful transgene inheritance in the next generation. T_1 generations of line #3 showed no transgene inheritance (data not shown), suggesting that line #3 was a surface chimera plant (Schmulling and Schell 1993). Such plants were also observed in genetic transformations of cucumber (Konagaya et al., unpublished data). Finally, sGFP fluorescence was observed in T_1 generation plants (Fig. 5d).

Conclusion

We established an efficient regeneration and transformation system for *C. moschata* (Fig. 6). This is the first report of efficient transformation via adventitious shoot organogenesis in *C. moschata* Duch. The average transformation efficiency was about 2.7% (Table 2). Wounding with a whisker suspension was the critical factor for producing transgenic kabocha squash plants. These results suggest that regenerated shoots may be developed in the lower cell layers of explants (Fig. 3c). The whiskers may help in the introduction of *Agrobacterium* into these lower cell layers. Despite it being over 7 years since the report of direct organogenesis of *C. pepo* using cotyledonary explants (Ananthakrishnan et al. 2003), to the best of our knowledge there has only been one report of successful transformation of a *Cucurbita* species (Shah et al. 2008). Whisker treatments may be applied for other important *Cucurbita* species, such as *C. pepo* and *C. maxima* because these species can regenerate via direct organogenesis using cotyledonary nodes (Ananthakrishnan et al. 2003; Lee et al. 2003). The transformation efficiency may remain a matter of improvement; however, the method we established will be applicable as a practical use. Progress in the molecular biology of *Cucurbita* species has been limited because of difficulties in genetic transformation, and this report should aid in molecular breeding experiments involving *Cucurbita* genera.

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