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Original Paper

Improving the transformation efficiency of *Cucurbita* species: factors and strategy for practical application

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Abstract *Cucurbita* species are refractory to transformation. Hence, the only 2 reports published regarding the transformation of *Cucurbita* species until our successful transformation of *C. moschata* (cv. Heiankogiku) in 2011. The efficiency was 2.7±1.3% using wounded explants vortexed with whisker suspension. To improve transformation efficiency, transformation experiments were carried out with various ages of cotyledonary explants. The highest efficiency was obtained with 1-day-old (3.2±0.9%) and 2-day-old (3.3±0.8%) explants after germination. Histochemical analysis of GUS activity revealed that wounding allowed *Agrobacterium* access to the deeper layer of explants. These results suggested that cells having a high ability of shoot organogenesis exist not on the surface but in the deeper layers of explants. We applied vacuum infiltration to wounded explants to enhance *Agrobacterium* access to the deeper layers, which resulted in improvement of transformation efficiency by 3-fold (9.2±2.9%). To verify the efficacy of our transformation procedure for other *Cucurbita* species, we attempted to obtain transformants with 3 *Cucurbita* species: *C. maxima* (cv. Ebisu), *C. pepo* (cv. Black Tosca), and *C. ficifolia* (cv. Kurotanenankin). The average regeneration efficiencies were 54.2±7.2%, 62.5±19.1%, and 72.2±13.4% under our regeneration system. Although the efficiency for production of tansgenic plants was very low (ca. 0.2–0.3%), a transgenic line was obtained from *C. maxima* and *C. pepo*, respectively. We discuss recent advances that may help in the development of beneficial applications for the transformation of *Cucurbita* species.

Key words: *Cucurbita* species, filter paper wicks, transformation, vacuum infiltration, whiskers.

The genus *Cucurbita* ($2n=2x=40$) consists of 15 species, including *C. pepo*, *C. maxima*, *C. moschata*, and *C. ficifolia*. Areas around Central and South Americas are thought to be the origin of *Cucurbita* species (Sanjur et al. 2002). *Cucurbita* species are commonly used as vegetables in daily life as food, feed, for ornamental purposes. Medicinal properties have also been reported in this species (Caili et al. 2006). Global production of *Cucurbita* species reached 21 million tons in 2009 (http://faostat.fao.org). These species also been shown to have a unique ability to take up organic xenobiotics and persistent organic pollutants from soil (Hulster et al. 1994; Inui et al. 2008; Otani et al. 2007; White et al. 2003) and may thus be helpful in phytoremediation.

Genetic engineering is a powerful way to improve crop quality and an essential procedure in the field of molecular biology. Earlier studies reported regeneration of *Cucurbita* species via embryogenesis from cotyledons (Jelaska 1972). Direct organogenesis has been developed with high efficiency (>50%) using proximal regions of cotyledonary explants (Ananthakrishnan et al. 2003; Kim et al. 2010; Lee et al. 2003; Zhang et al. 2008). However, these species are known to be one of the plants most refractory for transformation. Only 2 reports on transformation in these species (*C. pepo*) existed (Shah et al. 2008; Tricoli et al. 1995) until we established a transformation protocol for *C. moschata* (Nanasato et al. 2011). Wounding before *Agrobacterium* infection was the critical procedure for efficient transformation. The average transformation efficiency was 2.7±1.3%, and an increase in efficiency is necessary for practical use.

In this study, we investigated the effects of explant age and vacuum infiltration on transformation efficiency of *C. moschata*. Then, we tried to produce transformants from other *Cucurbita* species: *C. maxima* (cv. Ebisu), *C. pepo* (cv. Black Tosca), and *C. ficifolia* (cv. Kurotanenankin) using our transformation system. Polymerase chain reaction (PCR)-positive shoots showing green fluorescent protein (GFP) fluorescence were obtained from Ebisu and Black Tosca. We discuss

Abbreviations: AVG, aminoethoxyvinylglycine; BA, 6-benzylaminopurine; GA_3, gibberellin A_3; GFP, green fluorescence protein; GUS, β-glucuronidase; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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future perspectives regarding the establishment of a transformation method for *Cucurbita* species.

Materials and methods

Preparation of cotyledonary explants

Fifteen varieties including *C. pepo*, *C. maxima*, *C. ficifolia*, *C. moschata*, and the *C. maxima*×*C. moschata* hybrid were used. The seeds were purchased as follows: Ebisu, Heiankogiku, Just, Kurotanenankin, No. 8, Sherpa and Utsugiwaseakakuri from Takii (Kyoto, Japan), Gold Rush and Patty Green from Johnny's Selected Seeds (Winslow, ME), Hyuga14 and Kofuki from Nanto Seeds (Nara, Japan), Black Tosca from Sakata Seed Corporation (Kanagawa, Japan), Shirokikuza from Asahi-Noen (Gifu, Japan), Shishigatani from Marutane (Kyoto, Japan) and Ajihei from Mikado Kyowa Seeds (Tokyo, Japan). Seed coats were removed with a scalpel and forceps. After washing with 70% (v/v) ethanol for 10 min, the peeled seeds were sterilized for 10–15 min using 1% (w/v) sodium hypochlorite with 1 drop of Tween 20 and then rinsed 5 times with sterile distilled water. The sterilized seeds were germinated at 28°C in the dark for 1 day in plastic 9-cm Petri dishes containing a shoot-inducing (SI) medium consisting of Murashige–Skoog (MS) medium (Murashige and Skoog 1962) with 1 mg l^{-1} 6-benzylaminopurine (BA) and 0.8% (w/v) agar. The pH was adjusted to 5.7–5.8 before the addition of agar, and all media were autoclaved at 121°C for 15 min. Cotyledonary explants were prepared from post-germination seedlings as described previously (Nanasato et al. 2011). Regeneration efficiency was calculated by counting the number of explants with adventitious shoots from all explants cultured after 1 month of culture. Twenty-four explants were examined in each experiment and were repeated 3 times. Regeneration frequency was averaged in the 3 experiments.

Effects of explant age on transformation efficiency of C. moschata

C. moschata (cv. Heiankogiku) was used for this experiment. For preparation of various ages of explants, 4 combinations of germination period and preculture period of explants were examined (Table 1): germination for 1 to 2 days ("g1" and "g2") and preculture for 0, 1, 2, and 4 days ("p0," "p1," "p2," and "p4"). Wounding and inoculation were performed as described below.

Bacterial strain, binary vector, and culture condition

Agrobacterium tumefaciens strain EHA105 harboring the binary vectors pIG121-Hm and pGFP-S65C (Nanasato et al. 2011) was used for transformation. *Agrobacterium* was cultured in 20 ml of Luria Bertani (LB) medium (pH 5.2) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 50 mg l^{-1} kanamycin, 25 mg l^{-1} chloramphenicol, 25 mg l^{-1} rifampicin, and 20 μM of acetosyringone at 28°C until optical density at 600 nm (OD_{600}) of 0.4–0.8 was achieved. The *Agrobacterium* culture was centrifuged and resuspended in an inoculation (IN) medium containing SI medium buffered with 10 mM MES and pH adjusted to 5.2 and 500 μM of acetosyringone. The final concentration of *Agrobacterium* (measured by OD_{600}) was adjusted to 0.1 for Heiankogiku, Black Tosca, and Kurotanenankin or 0.01 for Ebisu. Prior to inoculation, the resuspended *Agrobacterium* inoculum was gently shaken at 28°C for approximately 2 h for efficient induction of *vir* genes (Hiei et al. 1997).

Inoculation condition and screening transgenic plants

Wounding was performed by vortexing with 1% whisker suspension followed by *Agrobacterium* infection as previously described (Nanasato et al. 2011). Vacuum infiltration treatment was performed as previously described (Nanasato et al. 2013). Wounded explants were placed in a bioreactor tube (TPP TubeSpin; 50 ml; TPP Cell Culture Plastics, St. Louis, MO) containing 20 ml of the *Agrobacterium* inoculum. After addition of 10 μl of Silwet S-77 (final concentration: 0.05%), 2 sessions of vacuum infiltration were applied for 5 min at −0.094 MPa in a desiccator. The vacuum was released slowly to prevent damage from sudden pressure change. Excess *Agrobacterium* suspension was removed using sterilized filter paper. Infected explants were placed in plastic 9-cm Petri dishes containing 3 pieces of sterilized filter paper moistened with 5.5 ml of IN medium. Dishes were sealed with Parafilm (Pechiney Plastic Packaging Inc., Chicago, IL) and placed in the dark at 25°C for 3 days. After cocultivation, explants were washed 5 times with sterilized distilled water, blotted dry, and transferred to a selection medium containing SI agar medium with 10 mg l^{-1} meropenem and 50 mg l^{-1} kanamycin. Explants were subcultured onto fresh media after 2 weeks. After culturing on the selection medium for 4–6 weeks, the regenerated shoots were excised and transferred to a shoot elongation (SE)

Table 1. Effect of explant age on transformation efficiency in *C. moschata* (cv. Heiankogiku).

Explant age	Germination period (day)	Preculture period (day)	Total no. of explants	Total no. of transformed plants[1)]	Efficiency (%)[2)]
g1p0	1	0	352	11	3.2±0.86
g1p1	1	1	406	14	3.3±0.83
g2p1	2	1	331	5	1.74±0.69
g2p4	2	4	353	0	0

[1)] Number of rooting plants with GFP fluorescence. [2)] (Number of rooting plants with GFP fluorescence/total number of explants)×100. Each value represents the mean±SD of 3 independent experiments.

medium containing half-strength MS medium with 1 mg l^{-1} gibberellin A_3 (GA_3), 0.8% agar, 10 mg l^{-1} meropenem, and 50 mg l^{-1} kanamycin. Non chimeric transgenic lines were selected via axillary bud culture in SE medium.

Visible marker assay

Histochemical β-glucuronidase (GUS) assay was performed on cotyledonary explants from day 7 after the elimination of *Agrobacterium*. GUS activity was visualized as previously described (Nanasato et al. 2011). Transverse sections were cut with scalpels by freehand sectioning.

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

DNA isolation and PCR analysis

For PCR analysis, genomic DNA from leaves of *Cucurbita* species was extracted as described previously (Edwards et al. 1991). Primer pairs used for amplifying the synthetic GFP protein gene (*sGFP*) were 5′-ctgggtaccatggtgagcaagggcgaggag-3′ and 5′-gcgactagtttacttgtacagctcgtccat-3′, those for amplifying *HrcA*, distributed in *Agrobacterium* (Nakahigashi et al. 1999), were 5′-catcgtcgaaggttatctcgatacg-3′ and 5′-tataatcgaccatcggtacgatacg-3′. PCR amplification was performed as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension of 72°C for 7 min. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Southern blotting

To confirm inheritance of introduced gene, genomic DNA was isolated from developing young leaves of GFP-florescent observed T_2 lines from transgenic kabocha squash by isoplant II (NIPPON GENE, Tokyo, Japan) and RNA was removed with RNase A. Genomic DNA (20 μg) was digested with *Hin*dIII, separated on a 0.8% agarose gel, and transferred onto nylon membranes, positively charged (Roche Diagnostics, Indianapolis, IN) with 20× saline-sodium citrate (SSC) 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 explant age on transformation efficiency

The transformation efficiency is dependent on the organogenic potential of the explant. In species belonging to the family Cucurbitaceae, a proximal region of cotyledon with a cotyledonary node is required for regeneration and transformation because regeneration is observed only in a tiny area between cotyledon and hypocotyl (Ananthakrishnan et al. 2003; Han et al. 2004; Lee et al. 2003; Mohiuddin et al. 1997; Nanasato et al. 2011; Tabei et al. 1993; Zhang et al. 2008). One of the important factors in organogenic potential is explant age. We examined the effect of explant age on transformation efficiency (Table 1) using explants of 4 different ages (Figure 1A). Young explants (g1p0 and g1p1) showed cream color (Figure 1B, C). In contrast, g2p1 and g2p4 explants expanded to larger size and acquired a cream/green color. In addition, shoot apices developed in the proximal parts (Figure 1D, E). After wounding by vortexing with a whisker suspension, explants were immersed with the *Agrobacterium* suspension in a bioreactor tube. We obtained on average 3% transformation efficiency with g1p0 or g1p1 explants (Table 1), whereas g2p1 explants having a long preculture period showed decreased transformation efficiency (1.7%) and no transformants were obtained from g2p4 explants. These results indicate that very young explants are required for obtaining transgenic shoots of *C. moschata* (cv. Heiankogiku). It was reported that organogenic potential may disappear in older explants in melon (Tabei et al. 1991), watermelon (Tabei et al. 1993), bottle gourd (Han et al. 2004), and winter squash (Lee et al. 2003). Development of the shoot apex may be inversely proportional to direct organogenesis. To determine the stability of transgenes in the genome, Southern blot analysis was performed in a GFP-florescent observed T_2 line (Figure 1F). This result revealed the stable integration of T-DNA in the genome and confirmed inheritance of transgenes to progenies.

Effect of vacuum infiltration on Agrobacterium *after wounding*

In our preliminary examination, we hardly obtained transgenic shoots, because *Agrobacterium* infection was insufficient in the proximal region of explants (Nanasato et al. 2011). Wounding by vortexing with whisker suspension dramatically improved the transformation efficiency (Nanasato et al. 2011). This result led to 2 hypotheses: 1) wounding allowed *Agrobacterium* to access deeper layer cells of explants, and 2) deeper layer cells had the potential of organogenesis. To verify these hypotheses, histochemical GUS assay was performed on cotyledonary explants. GUS activity was higher on the surface of wounded explants than on the surface of those that were not wounded (Figure 2A, C). Moreover, transverse sections showed that wounding enhanced *Agrobacterium* infection in the deeper layer cells (Figure 2B, D).

To facilitate *Agrobacterium* infection into the deeper layer cells of explants, vacuum infiltration was performed after explant wounding. We confirmed that vacuum infiltration had no marked negative effects on regeneration (data not shown). Transgenic shoots

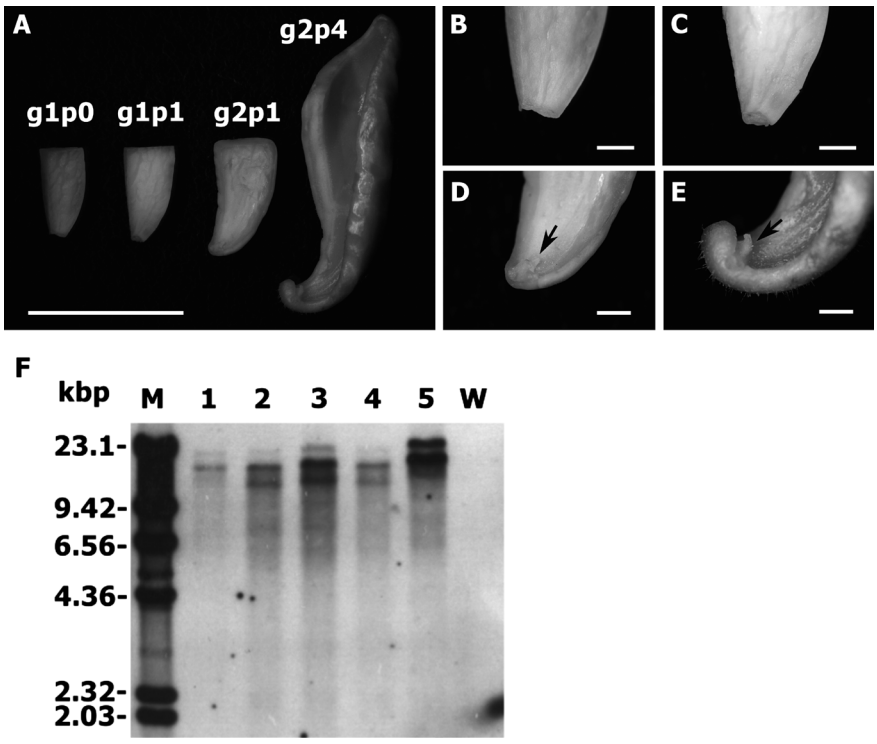

Figure 1. (A–E) Cotyledonary explants of various explant ages. (A) g1p0, germination period for 1 day; g1p1, germination period for 1 day and preculture period for 1 day; g2p1, germination period for 2 days and preculture period for 1 day; g2p4; germination period for 2 days and preculture period for 4 days, respectively. Bars: 10 mm. (B–E) Proximal parts of explants from g1p0 (B), g1p1 (C), g2p1 (D), and g2p4 (E), respectively. Black arrows indicate shoot apices developed. Bars: 1 mm. (F) Southern blot analysis of genomic DNA from selected T_2 plantlets and a non-transgenic plant. Genomic DNA (20 μg) from each line was digested with *Hin*dIII, separated on a 0.8% agarose gel, and transferred onto a nylon membrane. The membrane was hybridized with a DIG-labeled *NPTII* probe. Lane M is DIG-labeled λ-*Hin*dIII DNA marker (Roche Applied Science); lanes 1–5 are independent T_2 progenies from a GFP-introduced T_1 line; lane W is the wild type plant of Heiankogiku.

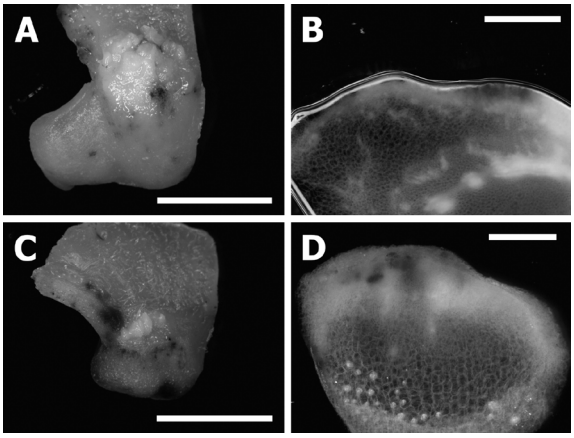

Figure 2. Histochemical GUS assay in cotyledonary explants: proximal region (A, C), transverse section (B, D). Infection without wounding (A, B) and with wounding (C, D). Bars: 5 mm (A, C), 1 mm (B, D).

with GFP fluorescence were counted for evaluation of transformation efficiency. Vacuum infiltration increased transformation efficiency by 3-fold (9.2± 2.9%) than by wounding alone (3.2±1.8%) (Table 2). These results revealed the efficacy of vacuum infiltration in improving transformation efficiency of *C. moschata* (cv. Heiankogiku). Vacuum infiltration has been used

Table 2. The effects of vacuum infiltration of cotyledonary explants on transformation efficiency in *C. moschata* (cv. Heiankogiku).

Treatment	Total no. of explants[1)]	Total no. of transformed plants[2)]	Efficiency (%)[3),4)]
Whisker+vortex[5)]	194	7	3.2±1.8 b
Whisker+vacuum[6)]	174	16	9.2±2.9 a

[1)] Cotyledonary nodes from seedlings, germinated for 1 day followed by preculture for 1 day, were used as explants. [2)] Number of shoots with GFP fluorescence. [3)] Efficiency=(number of shoots with GFP fluorescence/total number of explants)×100. Each value represents the mean±SD of 3 independent experiments. [4)] Means within columns followed by the same letter series are not significantly different by Tukey's test at $p<0.05$. [5)] Described by Nanasato et al. (2011). [6)] After wounding treatment using vortexing with 1% whisker suspension, 2 rounds of vacuum infiltration were performed for 5 min in *Agrobacterium* suspension with 0.05% (v/v) of Silwet L-77.

in other refractory plants, such as cowpea (Bakshi et al. 2011), pine (Charity et al. 2002), and radish (Park et al. 2005).

Comparison of regeneration ability across a wide spectrum of Cucurbita *species*

We compared the regeneration ability in various *Cucurbita* species. Regeneration has been reported in various *Cucurbita* species (Ananthakrishnan et al. 2003;

Kim et al. 2010; Lee et al. 2003; Zhang et al. 2008). However, to our knowledge, regeneration efficiencies have not been compared between these species under the same conditions. Such a comparison is critical for screening *Cucurbita* varieties suitable for transformation. We attempted to characterize the ability to regenerate by direct organogenesis from cotyledon explants in 15 varieties of *Cucurbita* species, including *C. maxima*, *C. pepo*, *C. moschata*, *C. ficifolia*, and *C. maxima*×*C. moschata* hybrid (Table 3). Shoot regeneration was observed only in the proximal regions of explants, as described in previous reports using 1 mgl^{-1} BA (Figure 3A–E). The efficiency ranged from 39 to 75%. Shoot regeneration was highest in Heiankogiku and lowest in Gold Rush. Cultivars belonging to *C. pepo* and *C. maxima* grew rapidly compared with those belonging to *C. moschata* and *C. ficifolia*; however, vitrification was often observed and vitrified shoots tended to wilt.

Transformation trials for other Cucurbita *species*

To verify the efficacy of the improved method combining wounding and vacuum infiltration, we selected 3 cultivars, Black Tosca, Ebisu, and Kurotanenankin for experimental trials on transformation. Ebisu is a leading commercial variety in Japan and Kurotanenankin is used as a rootstock in Japan. Moreover, Black Tosca is a popular zucchini variety and is reported to take up persistent organic pollutants (POPs) efficiently from soil (Murano et al. 2010). Infection efficiency differed among the 3 cultivars as estimated by observation of GFP fluorescence in infected explants (Figure 4A–G). Ebisu was most susceptible to *Agrobacterium* (Figure 4D, E). Because *Agrobacterium* infection often reduced the regeneration efficiency accompanied by severe vitrification of explants, the concentration of *Agrobacterium* suspension was subsequently decreased to 0.01 and the agar concentration increased to 1.2% in transformation of Ebisu. Ethylene gas produced by infected explants reduces efficiency of *Agrobacterium* infection (Nonaka et al. 2008b); therefore, in this case, regulation of ethylene is thought to be one of the critical conditions for efficient transformation. Addition of 1 μM of aminoethoxyvinylglycine (AVG), an ethylene inhibitor (Ezura et al. 2000), to the IN medium was highly effective for increasing infection efficiency in the proximal region of Black Tosca explants (Figure 4A, D, G). Kurotanenankin was the most recalcitrant to *Agrobacterium* infection (Figure 4C, F), and GFP signal was very weak in infected explants despite the addition of AVG to IN medium (data not shown). Kurotanenankin is often used as a rootstock of cucumber because of its high tolerance to biotic stress such as from *Fusarium oxysporum* (*Fusarium* wilt-causing bacteria). This may account for its high potential to tolerate biotic stress. We obtained transgenic shoots from selected 2 cultivars, Ebisu and Black Tosca. Two transgenic shoots were resulted from Ebisu (Figure 4H, I) and 1 from Black Tosca (Figure 4J) among more than 500 explants, corresponding to an efficiency of 0.34% and 0.19%, respectively (Table 4). A GFP-expressed shoot from Black Tosca was confirmed transgene by PCR, and this result showed our transformation procedure could apply to *C. pepo*. Two regenerated shoots from Ebisu were also PCR positive (Figure 4H). Plantlets of Black Tosca showed vitrification and wilting, whereas plantlets of Ebisu grew in vitro and then in a closed greenhouse. This is the first report on transformation in *C. maxima* species. Because transgenic T_0 adventitious shoots of *Cucurbita* species tend to be chimeric (Nanasato et al. 2011), axillary bud culture with a selection agent is important for developing

Table 3. Regeneration in diverse cultivars of *Cucurbita* species.

Subspecies	Cultivar	Regeneration efficiency (%)[1), 2)]
pepo	Black Tosca	62.5±19.1
	Gold Rush	38.9±2.41
	Patty Green	70.8±11.0
maxima	Ebisu	54.2±7.22
	Kofuki	54.2±4.17
	Ajihei	65.3±6.36
	Utsugiwaseakakuri	69.4±6.36
ficifolia	Kurotanenankin	72.2±13.4
moschata	Shishigatani	61.1±2.41
	Hyuga14	68.1±31.3
	Shirokikuza	70.8±26.0
	Heiankogiku	76.4±12.7
	No. 8	59.7±8.67
maxima×*moschata*	Just	75.0±8.33
	Sherpa	65.3±22.9

All media used in this experiment contained 30 gl^{-1} sucrose, MS salts, vitamins, and 0.8% agar. Total number of explants was 72 for each species. [1)] Regeneration efficiency=(number of explants with 1 or more shoots/total number of explants)×100. [2)] Each value represents the mean±SD of 3 independent experiments.

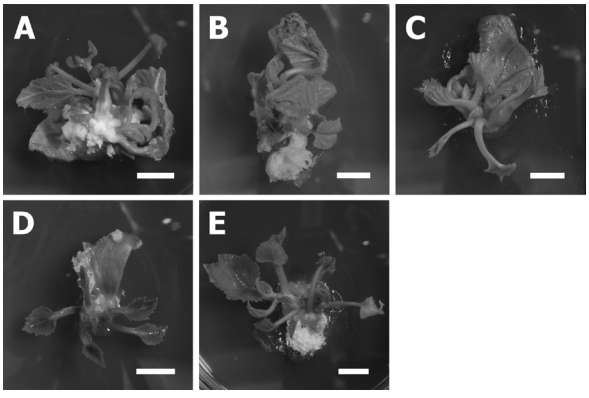

Figure 3. Adventitious shoot regeneration in various *Cucurbita* species. Explants after 1 month on SI medium. (A) *C. pepo* (cv. Black Tosca) (B) *C. maxima* (cv. Ebisu) (C) *C. ficifolia* (cv. Kurotanenankin) (D) *C. moschata* (cv. Heiankogiku) (E) *C. maxima*×*C. moschata* (cv. Just). Bars: 10 mm.

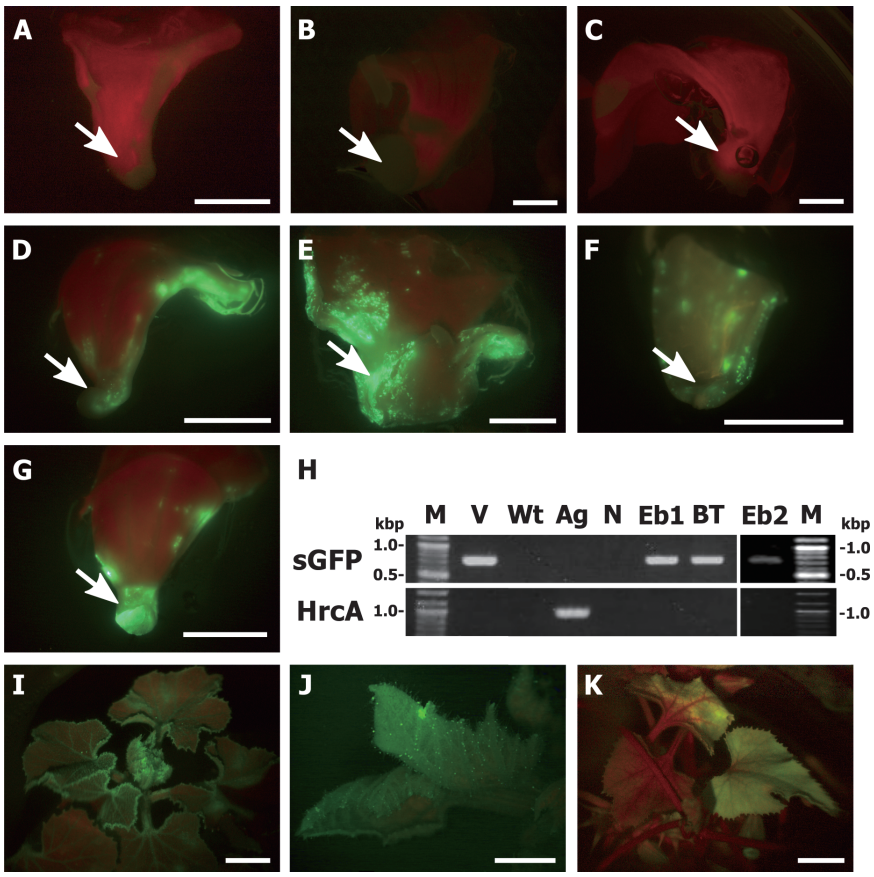

Figure 4. *Agrobacterium*-mediated transformation in cultivars of 3 *Cucurbita* species: Ebisu, Black Tosca, and Kurotanenankin. (A, D, G) represent explants of Black Tosca: control explant (A), infected explants in coculture with 500 μM acetosyringone (D), and with 500 μM acetosyringone and 1 μM AVG (G). (B, E) represent explants of Ebisu: control explant (B) and an infected explant (E). (C, F) represent explants of Kurotanenankin: control explant (C) and an infected explant (F). All explants shown are the 7th day explants after *Agrobacterium* elimination and white arrows indicated proximal regions. Bars: 5 mm. (H) represents PCR analysis of *sGFP* and *HrcA* in putative transgenic plantlets. Lane M is 100-bp DNA ladder (NEB); lane V is pGFP-S65C vector; lane Wt is wild-type plant of Ebisu; lane Ag is *Agrobacterium* genome DNA; lane N is without template DNA (negative control); lane Eb1 and Eb2 are GFP-introduced transgenic Ebisu #1 and #2, respectively; lane BT is GFP-introduced transgenic Black Tosca. (I, J) represent GFP-expressing plants of Ebisu #1 and #2, respectively. Bars: 5 mm (I), 2 mm (J). (K) represents GFP-expressing plant of Black Tosca. Bar: 5 mm.

Table 4. Transformation of 3 cultivars of *Cucurbita* species.

Cultivar	Total no. of explants[1)]	Total no. of transformed plants[2)]	Efficiency (%)[3)]
Ebisu	594	2	0.34
Black Tosca	530	1	0.19
Kurotanenankin	476	0	0

[1)] Values are the sum of at least 3 independent experiments. [2)] Number of PCR-positive transgenic shoots with GFP fluorescence. [3)] (Number of PCR-positive transgenic shoots with GFP fluorescence/total number of explants)×100.

non chimeric transgenic lines. Plantlets of Ebisu were propagated by axillary bud culture.

Conclusions and future perspectives for transformation of Cucurbita *species*

In this study we investigated the improvement of transformation efficiency for *C. moschata* (cv. Heiankogiku) using vacuum infiltration. We also produced a transformant of *C. maxima* using our transformation system. However, several parameters will be required in *C. maxima* for improvement of transformation efficiency. One remaining problem is vitrification induced by *Agrobacterium* infection. Ethylene is the primary cause of vitrification (Kevers et al. 1984), and also inhibits *vir* gene induction of *Agrobacterium* (Nonaka et al. 2008b). pSuperAgro (Inplanta Innovations Inc. Kanagawa, Japan) is a plasmid for *Agrobacterium* containing a 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene that suppresses ethylene evolution during co-cultivation (Nonaka et al. 2008a). Selective agent is another factor in efficient transformation. Kanamycin was used in this experiment as a selective agent. But in other plant species, for example *Pisum sativum*, *Cucumis sativus*, *Jatropha curcus* and *Brassica rapa*, some herbicides such as glyphosate, bialaphos, and bispyribac are more effective

for producing transgenic plants (Bean et al. 1997; Kajikawa et al. 2012; Konagaya et al. 2013; Vengadesan et al. 2005). An efficient transformation system for other *Cucurbita* species may be established by optimization of the factors described above. Genetic and genomic tools for *Cucurbita* species are advancing (Blanca et al. 2011; Esteras et al. 2012). We hope that this transformation study contributes to molecular breeding and basic knowledge.

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