A method of transformation and current progress in transgenic research on cucumbers and *Cucurbita* species

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Abstract Cucumber (*Cucumis sativus* L.) and *Cucurbita* species (squashes, pumpkins, and gourds), belonging to the Cucurbitaceae family, are among the major vegetable crops in the world. Transgenic approaches could contribute to the accumulation of new knowledge of these species and to the development of elite cultivars. Despite this, research reports using transformants of these species are very limited so far. One of the reasons for this may be that although there are effective transformation methods, these methods are not well known among researchers. In the present review, we describe efficient protocols for the transformation of cucumber and squash plants and mention possible pitfalls in and advice for following these protocols. In addition, we discuss the current progress of genetic transformation research using cucumbers and squash, including genome editing.

Key words: *Cucumis sativus*, *Cucurbita* species, genome editing, regeneration, transformation.

Introduction

The cucumber (*Cucumis sativus* L.), belonging to the Cucurbitaceae family, is commonly consumed as a vegetable in daily life. In 2017, the global production of cucumbers was 83.8 million tons (FAOSTAT, <http://www.fao.org/faostat/en/#home>), of which China accounted for 77.4%. In Japan, cucumbers are also popular, with 0.56 million tons produced in 2017, making Japan the 10th largest producer of cucumbers in the world. Cucumber plants suffer from several diseases, such as downy mildew, powdery mildew, anthracnose, and cucumber mosaic virus, which limit crop production. Developing resistance to these diseases is a major subject of cucumber plant breeding (King et al. 2012). However, it is difficult to improve the tolerance of cucumbers to pathogen attack by conventional breeding due to the plant's narrow genetic base, with a genetic variability of only 3–8% (Plader et al. 2007). Squash (also pumpkin and gourd; *Cucurbita pepo*, *C. maxima*, and *C. moschata*) is another important vegetable in the Cucurbitaceae family. The global production of *Cucurbita* species reached 27.4 million tons in 2017 (FAOSTAT), primarily

in China and India, which produced 29.1% and 18.7%, respectively, of all squash worldwide. The vegetables are used for food, animal feed, and ornamental purposes. Also, they are used as rootstocks for other cultivated cucurbits, including watermelon, cucumber, and melon, to strengthen tolerance to low temperatures and soilborne diseases (Davis et al. 2008). In addition, they have a unique ability to take up more organic xenobiotics and persistent organic pollutants from soil than the other plant families (Otani et al. 2007).

Next-generation sequencing (NSG) data have been acquired for cucumber and *Cucurbita* species (Huang et al. 2009; Montero-Pau et al. 2018; Sun et al. 2017). The analysis of this data allows for the discovery of new genes and regulatory sequences and confirmation of their positions, and makes available large collections of molecular markers. Genome-wide expression studies provide breeders with an understanding of the molecular basis of complex traits, such as the ripening process, phloem transport, disease resistance, cold tolerance, and fruit quality. Transformation approaches for cucumber and *Cucurbita* species would contribute to both basic science and molecular breeding.

Abbreviations: ABA, abscisic acid; BA, 6-benzyladenine; GA₃, gibberellic acid; GFP, green fluorescent protein; HPT, hygromycin phosphotransferase; NPTII, neomycin phosphotransferase.

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The successful transformation of cucumber plants with *Agrobacterium rhizogenes* was first reported in 1986 (Trulson et al. 1986). A particle bombardment method of transformation was also reported in 1992 (Chee and Slightom 1992). In recent years, *Agrobacterium tumefaciens*-mediated transformation has been primarily used for cucumbers. Transgenic cucumber lines have been produced for the analysis of genes involved in pathogen resistance, stress tolerance, and sex determination (Narusaka et al. 2013; Wang et al. 2014; Zhang et al. 2014). Despite this, to our knowledge, there are few reports on stable transformation of squash to date. Tricoli et al. (1995) first reported the development of transgenic yellow crookneck squash lines (*C. pepo*) that exhibit multiple virus resistance, but the detail of their method remains enigmatic. The second article about the transformation of *C. pepo* was published

in 2008 and reported a transformation efficiency of 0.7%. The successful transformation of *C. moschata* was achieved in 2011 (Nanasato et al. 2011), with a transformation efficiency of 2.7%. The transformation efficiency could be increased to 9.2% by applying vacuum infiltration to wounded explants to enhance *Agrobacterium* access to the cells in deeper layers (Nanasato et al. 2013b). We successfully transformed *C. pepo* and *C. maxima* using the same method.

Here, we present some information for researchers who are interested in tissue culture and transformation of cucumber and/or squash but have never worked with such approaches. The information given is based on our protocols for the transformation of cucumber and squash (Nanasato et al. 2011, 2013a, b; Nanasato and Tabei 2012, 2015; Tabei and Nanasato 2012) (Figure 1, Table 1). Even if specific procedures are followed, tissue culture and

Figure 1. Steps for the transformation of cucumber (cv. Shinhokusei No.1) and squash (cv. Heiankogiku) plants via direct-shoot organogenesis from cotyledonary explants. The compositions of shoot induction (SI) medium, inoculation (IN) medium, selection (SE) medium, and elongation (EL) medium are shown in Table 1. The concentration of *Agrobacterium* (OD₆₀₀) is adjusted to 0.5 in INc for cucumber and 0.1 in INs for squash.

Table 1. Composition of culture media used for transformation of cucumber and squash.

Plant species	Designation	Purpose of use	Composition
Cucumber	SIc	Shoot induction	MS salts and vitamins + 2 mg l ⁻¹ BA + 1 mg l ⁻¹ ABA [§] + 3% (w/v) sucrose + 0.8% (w/v) agar, pH 5.7–5.8
	INc	Inoculation	MS salts and vitamins + 2 mg l ⁻¹ BA + 1 mg l ⁻¹ ABA [§] + 10 mM MES + 3% (w/v) sucrose + 200 μM acetosyringone [§] , pH 5.2
	SEc	Selection	SIc medium + 50 mg l ⁻¹ kanamycin [§] + 10 mg l ⁻¹ meropenem [§] , pH 5.7–5.8
Squash	SIs	Shoot induction	MS salts and vitamins + 1 mg l ⁻¹ BA + 3% (w/v) sucrose + 0.8% (w/v) agar, pH 5.7–5.8
	INs	Inoculation	MS salts and vitamins + 1 mg l ⁻¹ BA + 10 mM MES + 3% (w/v) sucrose + 500 μM acetosyringone [§] , pH 5.2
	SEs	Selection	SIs medium + 50 mg l ⁻¹ kanamycin [§] + 10 mg l ⁻¹ meropenem [§] , pH 5.7–5.8
Common in cucumber and squash	EL	Shoot elongation	1/2 MS salts and vitamins + 1 mg l ⁻¹ GA ₃ [§] + 1.5% (w/v) sucrose + 50 mg l ⁻¹ kanamycin [§] + 10 mg l ⁻¹ meropenem [§] + 0.8% (w/v) agar, pH 5.7–5.8

The pH of all media in Table 1 is adjusted with NaOH before addition of agar and autoclave. [§]Add aseptically to sterilized media that has been cooled to approximately 55°C. ABA, abscisic acid; BA, 6-benzyladenine; GA₃, gibberellin A₃; MES, 2-(N-morpholino)ethanesulfonic acid; MS, Murashige-Skoog

transformation often fail. We identify possible pitfalls in documented protocols and provide suggestions to avoid them. Finally, we discuss the current progress of genetic transformation research on cucumbers and squash, including genome editing.

Preparation of explants for efficient regeneration

Various tissues, such as true leaves, hypocotyl, stem nodes, and cotyledons, have been used as explants in the regeneration and transformation of cucumber plants (Nishibayashi et al. 1996; Miao et al. 2009; Trulson et al. 1986; Ziv and Gadasi 1986). In 1994, Colijn-Hootmans et al. reported efficient regeneration of cucumber plants using the proximal end of young seedlings (1 day of preculture) (Colijn-Hootmans et al. 1994). The use of the proximal region of cotyledons as explants is often seen in the literature (Figure 2A, B) (Hu et al. 2017; Selvaraj et al. 2006, 2010; Vengadesan et al. 2005). After 1 day of preculture, cotyledons are detached from seeds using forceps. The distal parts of cotyledon are removed because they have no regeneration ability. The preparation process for squash explants is similar to that for cucumbers: immature cotyledons are excised from 1-day-old seedlings. However, especially in the case of squashes, care should be taken not to remove the

cotyledonary nodes around the shoot apex by breaking off the hypocotyl (Figure 2C), because only this part retains regeneration ability, as reported previously (Ananthakrishnan et al. 2003; Kathiravan et al. 2006; Lee et al. 2003; Zhang et al. 2008). For the purpose of generating more transgenic shoots, we use the proximal parts of cotyledons, cut longitudinally into two pieces, as explants (Figure 2B, D). However, some groups use the whole proximal parts of cotyledons as explants.

Plant hormones for regeneration

Although indirect organogenesis *via* somatic embryogenesis has been reported in cucumber plants (Burza et al. 2006; Chee 1990; Raharjo and Punja 1994), direct organogenesis has an advantage over indirect organogenesis in terms of time for regeneration. Murashige–Skoog (MS) medium (Murashige and Skoog 1962) with 1–2 mg l⁻¹ 6-benzyladenine (BA) and 0.5–1.0 mg l⁻¹ abscisic acid (ABA) has been shown to lead to efficient regeneration of cucumber plants (Tabei et al. 1998; Zhang et al. 2017), whereas in squash, the addition of ABA caused necrosis (Nanasato et al. 2011). For squash cultivars, MS medium with 1 mg l⁻¹ BA was used for regeneration (Kathiravan et al. 2006; Nanasato et al. 2013b; Zhang et al. 2008).

Strains of *Agrobacterium tumefaciens*

The use of particle bombardment for gene transfer was reported in cucumber plants (Kodama et al. 1993; Schulze et al. 1995). Currently, an *Agrobacterium*-mediated transformation method is more commonly used for gene transfer; strains EHA105 and LBA4404 can be used similarly in the transformation of cucumber and squash. In our case, EHA 105 harboring the pBI121-derived binary vector was cultured with Luria–Bertani (LB) medium 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 μ M acetosyringone, pH 5.2 at 28°C until the optical density at 600 nm (OD₆₀₀) reached 0.4–0.8. The bacterial cells were pelleted and resuspended in inoculation medium (IN), and the concentration of *Agrobacterium* (OD₆₀₀) was adjusted to 0.5 in INc for cucumber and 0.1 in INs for squash (Table 1).

Physical treatments performed during *Agrobacterium* infection

Physical treatments, such as vacuum infiltration and wounding, during *Agrobacterium* infection are the most important factors in the successful transformation of cucumber and squash (Figure 3A–C). These treatments contribute to increased *Agrobacterium* inoculation

Figure 2. Preparation of explants. Seed coats are removed using a scalpel and forceps. The peeled seeds are sterilized for 10 min in a 1% (w/v) sodium hypochlorite aqueous solution supplemented with one drop of Tween 20 with occasional agitation and then rinsed five times with sterile distilled water. The sterilized seeds are germinated at 28°C in dark for 1 day on the corresponding shoot-inducing medium. (A) A germinating cucumber seed, incubated in the dark for 1 day at 28°C. Scale bar=5 mm. (B) Detached cucumber cotyledons. The proximal regions marked with arrows are used as explants. Scale bar=5 mm. (C) Proximal region of squash cotyledon. Yellow arrow indicates the junction of the cotyledon and hypocotyl. Black arrowhead indicates shoot apex. Scale bar=1 mm. (D) Detached squash cotyledons. As in cucumbers, the proximal regions are used as explants. Scale bar=5 mm. All pictures are adapted from Nanasato and Tabei (2012) and Tabei and Nanasato (2012), courtesy of Kagaku-Dojin, Kyoto, Japan.

Figure 3. Special treatments for infection with *Agrobacterium*. (A) Vortex apparatus and 50 ml tube with 1% whisker suspension. (B) Desiccator connected to a vacuum pump. (C) 50 ml tube with a vent cap. White arrows indicate vent holes for gas exchange. GUS activity in squash explants without wounding treatment (D) and after wounding treatment by vortexing explants with 20 ml of 1% (w/v) whisker suspension in 50 ml tube for 30 min before *Agrobacterium* infection (E). Scale bars=2 mm. (F) Cocultivation of cucumber explants on three pieces of stacked filter paper moistened with 5.5 ml inoculation medium at 25°C in the dark for 3 day. Scale bar=1 cm. Pictures A–C, and F are adapted from Nanasato and Tabei (2012) and Tabei and Nanasato (2012), courtesy of Kagaku-Dojin, Kyoto, Japan.

Figure 4. GFP-expressing transgenic squash lines. A transgenic shoot under visible light (A) and excitation light for GFP fluorescence (D). Strong GFP fluorescence was observed in the solid transgenic shoot indicated by a white arrow in D, while autofluorescence in red was observed in nontransgenic shoots, indicated by white arrowheads. A sectorial chimeric individual under visible light (B, C) and excitation light for GFP fluorescence (E, F). White arrows in E and F indicate transgenic cells exhibiting GFP fluorescence, and white arrowheads in E and F indicate nontransgenic cells. Scale bar=5 mm.

efficiency in deeper layer cells, which seem to have the potential of organogenesis (Nanasato et al. 2013b). The effectiveness of vacuum infiltration has been confirmed in reports from other research groups (Hu et al. 2017; Zhang et al. 2017). Whiskers, which are white needle-like crystals with very high tensile strength, can be used to wound squash explants (Figure 3D, E) (Nanasato et al. 2011, 2013b). In addition, the cocultivation of explants on filter paper moistened with coculture medium can significantly contribute to improved transformation efficiency (Figure 3F).

Selection and rooting of nonchimeric transgenic lines

A combination of the neomycin phosphotransferase II (*NPTII*) gene and kanamycin has been frequently used for the selection of transformants. The regeneration of escape shoots is almost unavoidable, even under stringent selection conditions (Figure 4A, D). Therefore, the use of visual markers, such as green fluorescent protein (GFP), is the key for obtaining cucumber and squash transformants. Furthermore, transgenic shoots developed *via* direct organogenesis are often chimeric. Chimeric individuals are composed of a mixture of transformed and nontransformed cells. The use of visual markers is effective for the discrimination of

sectorial chimeras (Figure 4B, C, E, F). Root elongation in chimeric individuals is frequently inhibited in media containing antibiotics. Multiple rounds of axillary bud induction in the selection medium are a powerful way to generate solid transformants from chimeric individuals.

Supplementation of the growth media with low concentrations of auxins, such as indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), to promote rooting is recorded in some research papers (Sarmiento et al. 1992; Vasudevan et al. 2007; Zhang et al. 2008). However, auxin-induced chlorosis of the regenerated leaves was also reported (Hu et al. 2017). In many cases, it seems unnecessary to add auxins to promote rooting. After shoots with vigorous roots grow four to five nodes (about 10 cm in height), the shoots should be transplanted to soil as described previously (Nanasato and Tabei 2012, 2015; Tabei and Nanasato 2012).

Application of genome editing technology in cucumber and squash

Since the announcement of CRISPR/Cas9 technology in 2012 (Jinek et al. 2012), genome editing technology has been spreading rapidly in plant functional genomics research and crop improvement. To date, two research groups have reported successful genome editing in cucumber plants using the CRISPR/Cas9 system (Hu et al. 2017; Sherman et al. 2016). Both groups succeeded in producing transgene-free null segregant lines *via* crossing. The CRISPR/Cas9 system was also used for functional analysis of a gene encoding NADPH oxidase in *C. moschata* (Huang et al. 2019).

Conclusion

Research on cucumber and squash has been improved due to the development of genome information. Cucumber is one of the potential candidates for a model plant of the Cucurbitaceae family and would provide new scientific knowledge through molecular analysis. In addition, genome editing technology could contribute to the rapid breeding of cucumber and squash. Transformation techniques will play an increasingly important role in the progress of basic science and molecular breeding. In this review, we describe methods of transformation of cucumber and squash plants based on reports we published previously. We emphasize that the concentration of plant hormones for regeneration and conditions for *Agrobacterium* infection should be adjusted according to the plant cultivar treated. We hope that this review will contribute to the development of Cucurbitaceae plant research and molecular breeding.

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