Establishment of Transgenic Lines of Watermelon Rootstock Resistant to CGMMV (Cucumber Green Mottle Mosaic Virus) and Environmental Risk Assessment

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Introduction

In Korea and Japan, rootstock grafting is popularly used in the cultivation of *Cucurbitaceae* crops such as watermelon, cucumber, and melons, because of the poor viability of the crop's root. The watermelon rootstock, *Citrullus lanatus* (Twinser) cv. gongdae is one of the rootstocks used for grafting commercially important watermelon varieties. Although this rootstock is hardier in the soil environment, it is equally vulnerable to virus infection by such as CGMMV (cucumber green mottle mosaic virus), a member of the Tobamovirus genus. CGMMV infects many *Cucurbitaceae* species causing mosaic symptoms, a yellowish leaf, and finally fruit deterioration (Lee et al., 1990; Lee, 1996; Choi, 2001). In fact, CGMMV outbreaks have caused marked losses in the total yields of *Cucurbitaceae* crops nationwide for the past several years in Korea. Since CGMMV is easily transmitted by soil, the development of a virus resistant rootstock provides a viable solution. Unfortunately, there is no genetic source for a resistance gene against CGMMV infection, and therefore, there is no access to a solution via breeding management. The alternative then is to use a viral gene such as coat protein (*CP*) gene, transform it into a watermelon rootstock, and induce resistance to CGMMV.

Watermelon is known to be one of the most recalcitrant plants to transform by *Agrobacterium*. Only a few reports have been published (Choi et al., 1994; Chen et al., 1998; Ellul et al., 2003) on the topic and no reproducible system has been established. Gongdae is a wild type watermelon and is not used for breeding commercial varieties, other than as a grafting stock. In addition, most of watermelon lines are not crossed with the gongdae (Y.S. Shin, personal communication).

Here we present a successful transformation system of the watermelon rootstock (gongdae) by *Agrobacterium*-mediated transformation with the CGMMV coat protein (*CP*) gene. Stable transformation was obtained in the frequency range 0.1-0.3%, and the transformants resistant to CGMMV were identified. The resistant transformants were self-crossed and T_3 generation was established. In order to evaluate the environmental risk assessment, transgenic plants were grown in an isolated field house and several parameters were examined.

Results and Discussion

Transformation rate

A protocol used for transforming gongdae is detailed in Table 1. Generally, use of *Agraobacterium* either LBA4404 or EHA105 did not influence the transformation efficiency, and either kanamycin or hygromysin could be used for selection. For both selection types, shoots formed from the explants 4 weeks after placing them on selection media. Shoots formed directly from the cut region of the explant

and no callus was formed around the cut region. Roots were regenerated 5-6 weeks after placing shoots in rooting media. When shoots had grown to about 10 cm in height, the seedling was acclimated gently on a zippy pot. The developmental processes with kanamycin and hygromycin selection produced no phenotypic differences, and both gave rise to fruits and seeds as did the non-transformed plants.

A total of 9000 explants were prepared by germinating around 1000 seeds for kanamycin selection (Table 2A). To determine the transformation rate, shoots grown with rooting were tested by PCR to confirm the presence of the *CGMMV-CP* insert. Twenty-one shoots of 9000 explants (0.23%) showed a 550 bp band. Eleven To plants were then self-crossed to produce T_1 seeds. For hygromycin selection, 640 explants were transformed by the injection method; 50 shoots contained the *CGMMV-CP* insert (7.8%) (Table 2B). The shoot acclimation process proved difficult and many PCR positive shoots failed to survive in zippy pots. Five plants only grew and these were self-crossed to obtain T_1 seeds.

Southern and Northern blot analysis

Twenty μg of genomic DNA isolated from To plants was digested with *Xba* I, and fractionated on 0.8% agarose gel. The restriction bands produced confirmed the presence of the *CGMMV-CP* gene (Fig. 1). Several distinct To origins were found in terms of copy number: lane 4, 7, 8 vs. 5, 6 vs. 12, 26 vs. 41, 42. The same copy number or band pattern must have been due to the propagation of multishoots. The band patterns of those plants in lanes 4, 12 and 26 showed that they contained one insert copy, while those of lanes 5, 6, 7, 8, 41, and 42 showed the presence of more than one copy. We confirmed by genomic Southern blot that the T_1 plants self-crossed from To containing one copy also contained the identical one copy (data not shown).

Transcript levels of PCR positive To plants were determined by Northern blotting (Fig. 2). Lanes 2, 4, and 25 contained a 550 bp band, but lanes 9 and 29 were of non-transformed gangdae (N) did not. T_1 plants were obtained from To plants corresponding to lanes 4 and 29 by self-fertilization, and the transcripts of T_1 plants were found only in the To plant corresponding to lane 4 (data not shown).

Resistance testing of T_1 to CGMMV

To determine the resistance of the transformed watermelon rootstock, the To plant of the lane 4 was self-crossed, and a total of 140 of the T_1 plants obtained were exposed to CGMMV. Leaves from 4-week-old To plants were inoculated with CGMMV twice with a 2-week interval. Three weeks after the second inoculation, ELISA was performed. A mosaic pattern was identified in the leaves of susceptible plants, whereas no mosaic spot developed in resistant plants. We obtained 10 plants that were resistant to CGMMV infection (Table 3) among these 140 T_1 plants. Non-transformed control plants (40) were all infected by CGMMV. By PCR analysis showed that all of the 10 resistant T_1 plants contained the *CGMMV-CP* insert, and that 89% (116/130) of the susceptible T_1 plants also possessed the *CGMMV-CP* insert; however, 14 T_1 plants did not contain the *CGMMV-CP* insert. The phenotypes of the leaves of susceptible and resistant plants at 7 weeks after CGMMV inoculation were clearly different (data not shown). The susceptible leaves showed a severe mosaic pattern and a discoloration to yellow.

Environmental risk assessment

To assess the environmental risk of released transgenic plants, transgenic gondaes (T_3) were grown in an isolated field house (Fig. 3) and several parameters were evaluated. There was no significant difference in horticultural characteristics between transgenic plants and non-transgenic plants (Table 4). In addition, the *CGMMV-CP* DNA and coat protein in the rootstock did not transfer to scion (data not shown) suggesting that the watermelon rootstock would not necessarily be tested for the health risk assessment. Based on the data, T_3 generation would be a candidate for the F_1 performance test.

References

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Table 1. A protocol for transformation of watermelon rootstock.

Line	Water melon root stock	Duration
Germination	Germination condition under dark did not influence the transformation (1/2MS + 3% sucrose + 0.8% agar, pH5.8)	3 - 5 days
Explant	cotyledon	
Pre-culture	-basic media (MS-B5 + 3.0% sucrose + 0.8% agar, pH 5.8) -1.0 mg/l BA + 0.2 mg/l IAA	1-3 days
cDNA insert	*CGMMV-CP*	
Agobacterium strain	LBA4404, EHA105	
Inoculation	-basic media -Addition of 200 mM acetosyringone -OD (600): 0.7 - 0.9	30 min
Co-culture	-basic media -1.0 mg/l BA + 0.1 mg/l IAA + 200 mM acetosyringone -washing buffer (0.2 M Citric acid + 2.0% sucrose, pH5.2 400 mg/l lilacilline)	3 days in dark
Selection & Shooting	-basic media -40 mg/l kanamycin + 200 mg/l lilacilline with 1.0 mg/l BA + 0.1 mg/l IAA +2 mg/l AgNO3 (7.5 mg/l hgyromycin + 500 mg/l cefotaxim)	-shoot formation: 4 – 5 weeks
Rooting	-basic media -20 mg/l kanamycin + 200 mg/l lilacilline without hormone	-root formation: 5 - 6 weeks -10 cm height: 2 - 3 weeks

Table 2. Transformation efficiency. A: transformation by co-culture (kanamycin selection); B: transformation by injection (hygromycin selection).

A

Seed	Explant	PCR tested	Acclimation	House	Southern (To)
1000	9000	21 (+)	12	11	9
		0.23% (21/9000)			0.1% (9/9000)

B

Seed	Explant	PCR tested	Acclimation	House	Southern (To)
320	640	50 (+)	10	5	2
		7.8% (50/640)			0.3% (2/640)

Figure 1. Southern blot analysis of To plants. Genomic DNA was digested by *Xba* I and the Southern membrane was hybridized with *CGMMV-CP* probe labeled by ^{32}P. N: non-transformed; 4-42: transformed To plants.

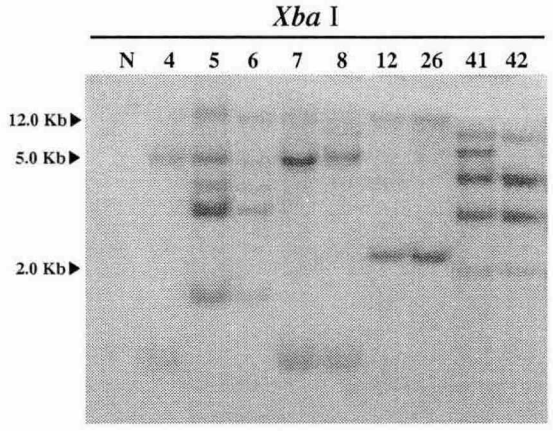

Figure 2. Northern blot analysis of To plants. The membrane was hybridized with *CGMMV-CP* probe labeled by ^{32}P. N: non-transformed; 2-29: transformed To plants.

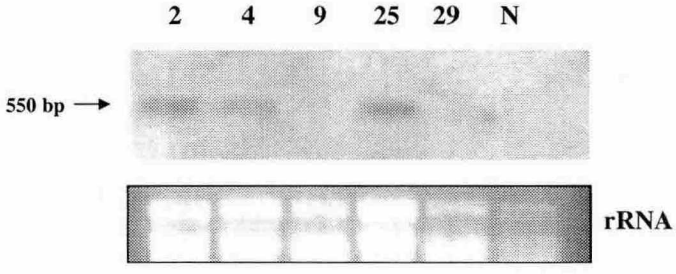

Table 3. Resistance test was performed by exposing T_1 plants to CGMMV twice with a two-week interval. Resistance was determined by ELISA

	Number of plants tested	Susceptible	Resistant
T_1	140	130	10
PCR test		89% (+)	100% (+)
Non-transformed	40	40	0
PCR test		0% (+)	-

Fig 3. An isolated house for the Environmental Risk Assessment

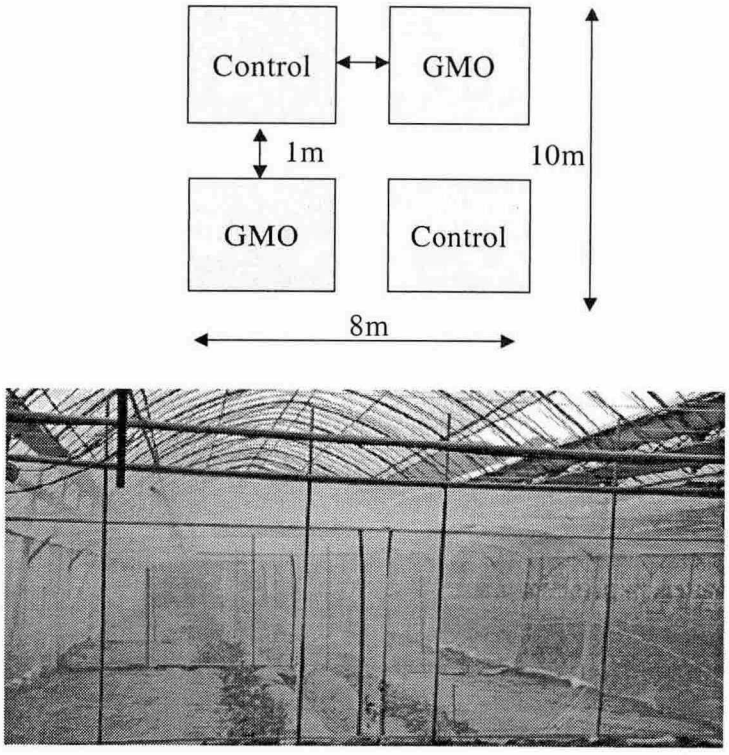

Table 4. Horticultural characteristics of GM rootstock vs. non-GM rootstock

Character	Non-GMO	GMO	Character	Non-GMO	GMO
Growth type	vine	vine	Ovary size	medium	medium
Primary branch length	420.4 ± 6.90	428.8 ± 9.14	Fruit shape	round	round
Hermaphrodite flower	present	present	Fruit skin color	green	green
Internode length	13.9 ± 0.58	13.2 ± 0.40	Fruit stalk length	9.1 ± 0.43	9.1 ± 0.57
Leaf length	17.6 ± 0.50	17.3 ± 0.40	Fruit button shape	round	round
Leaf wide	22.7 ± 0.57	22.1 ± 0.43			
Leaf color	medium	medium	Fruit hilum size	medium	medium
Leaf lobation	medium	medium	Fruit surface convex	non	non
Leaf uneven	medium	medium	Fruit stripes	present	present
Leaf wave shape	medium	medium	Fruit stripes width	medium	medium
Petiole length	18.3 ± 0.91	17.4 ± 0.64	Peel thickness	medium	medium
Female flower size	medium	medium	Flesh color	white	white
			Flesh firmness	hard	hard
Petal number	5	5	Seed size	medium	medium
Ovary fuzz	present	present	Seed number	medium	medium