1 **Generation of transgenic watermelon resistance to *Cucumber mosaic virus***
2 **facilitated by an effective *Agrobacterium*-mediated transformation method**

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25 **Abstract**

26 Watermelon is an annual vegetable crop that suffers from various diseases during
27 growth. Genetic engineering is an effective tool for improving plant disease resistance.
28 This paper presents an effective transformation protocol designed for a female
29 parentwatermelon line mediated by *Agrobacterium*. In this protocol, 2 times MS Fe-
30 EDTA were supplemented into Murashige and Skoog (MS) medium to suppress the
31 chlorosis during watermelon explants culturing, and the optimal preculture time (5
32 days) and coculture time (5 days) were screened out to improve the frequency of
33 shoots regeneration. Using our protocol, 17 putative transgenic plants expressing
34 artificial microRNAs that target *Cucumber mosaic virus* (CMV) 2a/2b genes were
35 recovered, and seven of them were succeeded in obtaining T1 generation seeds
36 because of the technique difficulties of self-pollination of the transgenic watermelon
37 lines. PCR and Southern blot analyses confirmed that the transferred fragment was
38 successfully imported and integrated into the genome of three of these seven
39 transgenic lines. DAS-ELISA assay indicated that the CMV can be detected in the
40 nontransgenic line but not in the transgenic lines, and the transgenic lines displayed
41 resistance to CMV infection.

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44 **Keywords:** Watermelon, Transformation, Cucumber mosaic virus, resistance

45 Artificial microRNA

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47

48 **Abbreviations**

49	MS	Murashige and Skoog medium
50	1/2 MS	Half-strength MS medium
51	BA	6-Benzyladenine
52	<i>NPTII</i>	Neomycin phosphotransferase II gene
53	IAA	Indoleacetic acid
54	GUS	β-Glucuronidase
55	KT	Kinetin
56	Hpt	Hygromycin phosphotransferase gene
57	DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay

1. Introduction

Watermelon (*Citrullus lanatus* Thunb.) is an important summer crop worldwide and is prone to several virus infections, such as *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), and *Cucumber green mottle mosaic virus* (CGMMV) (Fuchs et al. 1997; Park et al. 2005; Tricoll et al. 1995; Varveri et al. 2002). These viral diseases are responsible for the great losses encountered in watermelon production (Chen et al. 2008; Gaba et al. 2004; Gu et al. 2008). CMV, a type species of the genus *Cucumovirus*, family *Bromoviridae*, is a positive-sense RNA virus with a tripartite genome (Palukaitis et al. 1992). Typical symptoms in cucurbits caused by CMV infection are mosaic, mottled, distorted, curled leaves and plant stunting (Palukaitis et al. 1992; Papayiannis et al. 2005).

Given its importance in the agricultural industry and lack of resistant germplasm in crops, CMV has become one of the most targeted viruses in plant viral resistance engineering (Morrone et al. 2008). Efficient regeneration and transformation protocols are the foundation of plant genetic engineering. The earliest study on watermelon transformation was reported by Choi et al, they successfully obtained a transgenic watermelon plant that expresses the *GUS* reporter gene (Choi et al. 1994). Thereafter, several delivery methods have been utilized for watermelon transformation; these methods include biolistic transformation (Suratman et al. 2010) and pollen tube pathway (Chen et al. 1998), with the *Agrobacterium tumefaciens*-mediated transformation being the predominant method. And few transgenic watermelon plants were successfully obtained, such as expression an untranslatable chimeric construct containing truncated ZYMV coat protein (CP) and PRSV W CP genes to generate ZYMV and PRSV resistance watermelon (Yu et al. 2011b), expressing the yeast

83 *HALI* gene to enhance watermelon salt tolerance (Ellul et al. 2003), expressing the
84 *Cucumber green mottle mosaic virus* coat protein gene (CGMMV-CP) to develop
85 CGMMV resistance in watermelon rootstocks (Park et al. 2005), and so on.

86 Although there are few successful transformation reports on watermelon and its
87 rootstocks, the watermelon is still acknowledged recalcitrant crop for transformation.
88 Several factors would affect watermelon transformation, such as the genotypes of
89 explants, gene escape and chimeric shoots. The transformation frequencies of
90 cultivars can differ even when the same transgenic methods are used. For instance, in
91 one experiment, 23 positive transgenic lines were obtained on the watermelon cultivar
92 ‘Feeling’, 3 and 1 positive transgenic lines were obtained on cultivars ‘China baby’
93 and ‘Quality’, whereas no transgenic lines was obtained on other seven cultivars (Yu
94 et al. 2011b). During the regeneration of transgenic watermelon shoots through *A.*
95 *tumefaciens*-mediated transformation, a high degree of obtained watermelon
96 regeneration plants are nontransgenic, which are called escapes, because
97 nontransformed watermelon cells can easily differentiate, cause adventitious and
98 elongate to shoots even under high selective pressure (Dong et al. 1991; Gaba et al.
99 2004). The higher degree of escapes and chimeric shoots occurred in watermelon
100 transformants leads it is much more difficult to obtain transgenic watermelon plants
101 than other plant.

102 Fusarium wilt and CMV viruses severely restrict watermelon production, and
103 creating Fusarium wilt and virus resistance germplasm to develop disease-resistant
104 cultivars is an important project in watermelon breeding. Many Fusarium wilt-
105 resistant watermelon germplasms had been created in our group previously. To obtain
106 a watermelon germplasm that resistant to both Fusarium wilt and CMV, a Fusarium
107 wilt resistant germplasm was used to produce the CMV-resistant germplasm by

108 genetically engineer in our laboratory. In this paper, we present an effective *A.*
109 *tumefaciens*-mediated transformation method designed for a *Fusarium* wilt resistant
110 female parent watermelon line. Using this method, we successfully transferred an
111 artificial microRNA that targets CMV 2a/2b genes to this line. PCR assay and
112 Southern blot analysis showed that the transferred fragment was successfully
113 imported and integrated into the watermelon genome. Further, a CMV-resistance
114 assay revealed that the transgenic lines showed stronger virus resistance than the wild-
115 type line after CMV inoculation.

116

117 **2. Materials and methods**

118 **2.1. Plant materials and culture conditions**

119 The *Fusarium* wilt resistant watermelon line, the female parent line of
120 watermelon cultivar ‘Zhengkang No.6’ was used in this study. Basic MS medium
121 (Murashige and Skoog 1962) containing 3% sucrose, 0.3% phytigel, and various
122 growth regulators at pH 5.8 was used. At various stages, explants were cultured under
123 a 16 h/8 h (light/dark) photoperiod at 26 ± 2 °C.

124 **2.2. Watermelon shoots regeneration**

125 Watermelon seeds with coat removed were soaked in 70% ethanol for 1 min,
126 sterilized for 10 min in 0.1% HgCl₂ with two drops of Tween-20, and then rinsed
127 several times with sterile distilled water. Initially, the sterilized seeds were placed onto
128 1/2 MS medium containing 1% sucrose for 2 d in the dark at 30 °C, and then
129 transferred to a 16 h/8 h (light/dark) photoperiod for 3 d at 25 °C. The 5-day-old
130 cotyledons that turned from yellow to green were used as explants. After excising and
131 discarding the hypocotyls and apical portion, the cotyledons were cut into distal and
132 proximal halves. The proximal cotyledon halves were cultured in 90 mm × 15 mm

133 Petri dishes with induction medium for 3 weeks until adventitious shoots emerged.
134 The cotyledons with regenerated adventitious shoots were transferred onto shoot
135 elongation medium (MS medium supplemented with KT 0.2 mg/L) and then cultured
136 for a month. Elongated shoots grown more than 3 cm in height were individually
137 excised, transferred onto rooting medium (MS supplied with IBA 1 mg/L), and then
138 cultured for 2–3 weeks. The plantlets with well-developed roots were taken from the
139 rooting medium, washed to remove root-adhered phytigel, and then transferred to
140 plastic cups containing vermiculite. Each cup was covered with another transparent
141 plastic cup to maintain humidity for 2 weeks and then uncovered for a week.
142 Acclimatized plantlets were transplanted into flowerpots containing compost and
143 grown in a greenhouse.

144 Considering that grafting is an alternative approach to reduce soil-borne diseases,
145 and increase the root system vigor in Asian countries. We developed *in vitro* grafting
146 as an alternative to improve the survival rate of these plants. *In vitro* plantlets that
147 elongated to 6 cm were cut off in wedges at 2–3 cm upper stem on both ends.
148 Seedlings of pumpkin rootstock that developed two cotyledons were cut vertically,
149 and the *in vitro* plantlets were inserted into the rootstock. Thereafter, the grafted
150 seedlings were tied with string and covered with plastic cups. After 2 weeks, the
151 plastic cups were removed, and the plants were transplanted into pots.

152 **2.3. Effect of Fe-EDTA concentration in MS medium on watermelon** 153 **regeneration**

154 Different Fe-EDTA concentrations, including 0, 1, 1.5, 2, 3, and 4 fold MS Fe-
155 EDTA (Conventional concentration is 1× Fe-EDTA), were added to the induction
156 medium (MS basal medium plus 3.0 mg/L BA) to prevent the explants from turning
157 slightly brown and losing vigor. The growth response and adventitious shoots-induced

158 rate were recorded after 1 month culture.

159 **2.4. Watermelon Transformation**

160 *A. tumefaciens* strain LBA4404 was used for the transformation. This strain
161 harbors the binary vector pBI-pre-miR-2a/b, which contains an artificial microRNA
162 that targets CMV 2a/2b genes (Zhang et al. 2011). A single colony of *Agrobacterium*
163 was selected from the solid LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L
164 NaCl, pH 7.2), inoculated in 25 mL of LB medium supplemented with 100 mg/L
165 kanamycin, and then incubated at 28 °C overnight with continuous shaking at 200
166 rpm. The bacterial suspension was centrifuged at 4000 rpm, and the harvested
167 bacterial cells were re-suspended in hormone-free liquid MS medium supplemented
168 with 30 g/L sucrose. Prior to *Agrobacterium* infection, the explants were precultured
169 on the induction medium (MS medium containing 3.0 mg/L BA) for 0–7 d. The
170 precultured cotyledons were immersed in liquid MS medium containing
171 *Agrobacterium* cells for 20 min, dried on filter papers, and then returned onto the
172 induction medium. The cotyledon explants were cocultivated in the dark at 26 ± 2 °C
173 for 2–7 d and then placed onto selective induction medium (induction medium
174 supplemented with 150 mg/L kanamycin and 500 mg/L cefotaxime) at 28 °C under a
175 16 h/8 h (light/dark) photoperiod. The produced adventitious shoots were transferred
176 onto fresh selective elongation medium (elongation medium supplemented with 150
177 mg/L kanamycin and 500 mg/L cefotaxime). Well-developed shoots were transferred
178 onto selective rooting medium (rooting medium supplemented with 75 mg/L
179 kanamycin and 500 mg/L cefotaxime).

180 **2.5. DNA extraction and PCR analysis**

181 Genomic DNA was extracted from young leaves of transgenic and nontransgenic
182 watermelon plants through the CTAB method (Fulton et al. 1995). The DNA served as

183 a template for PCR. The forward primer Fw: AGACAATCGGCTGCTCTGAT and the
184 reverse primer Rv: TCATTTCGAACCCCAGAGTC were used to amplify the plant
185 selection marker *NPTII* gene, with an expected product size of 500 bp. PCR
186 amplification with a total reaction volume of 25 µL was performed with an initial
187 denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 58 °C for 45
188 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were
189 separated through electrophoresis on a 1.0% (w/v) agarose gel.

190 **2.6. Southern blot analysis**

191 Genomic DNA (40 µg) extracted from three transgenic watermelon lines and
192 nontransgenic watermelon was digested with *EcoRI* overnight and separated through
193 electrophoresis on a 0.8% agarose gel in 1× TAE buffer and then transferred to nylon
194 membranes. The DNA bound membranes were hybridized with *NPTII* probes in
195 accordance with the instructions of the DIG High Primer DNA Labeling and
196 Detection Starter Kit II (Diagnostics GmbH, Germany).

197 **2.7. CMV resistance evaluation**

198 CMV (CH99/90) was isolated from melon in China in 1999. It was classified
199 into subgroup I and maintained on leaves of pumpkin. For the mechanical inoculation
200 of CMV, the pumpkin leaves with CMV viral symptoms were ground with phosphate
201 buffer of 1:5 (w/v), and the sap was used to inoculate the fully explanted watermelon
202 cotyledons by rubbing. The resistances of three independent transgenic lines, as well
203 as wild-type line, were evaluated. For each transgenic line, a set of ten PCR-positive
204 transgenic plants were mechanical inoculated, and the inoculated plants were
205 monitored for symptom development for 3–5 weeks in a greenhouse.

206 **2.8. CMV accumulation detected by DAS-ELISA**

207 Young leaves (0.1 g) from each 1-month-old virus-infected seedling were used

for CMV assay in accordance with the instructions of the ELISA test kit for CMV (Agdia, Inc., USA). ELISA tests were performed using the DAS method (Clark and Adams 1977). When visual positive control turned yellow after 1 h, the absorbance was read by using a spectrophotometer (Bio-Rad, Model 3550) at 405 nm. To evaluate CMV accumulation, the I/H ratio was calculated as follows:

$$\text{I/H} = (\text{OD}_{405} \text{ value of Sample plant} - \text{OD}_{405} \text{ value of Blank control}) / (\text{OD}_{405} \text{ value of negative control plant} - \text{OD}_{405} \text{ value of Blank control})$$

I/H ratio ≥ 3.0 were considered positive, i.e., the plants were sensitive to the virus.

2.9. Statistical analyses

All data were analyzed using ANOVA and Duncan's multiple range test ($P < 0.05$) with SPSS Statistics 20.

3. Results

3.1. Response of explants to different Fe-EDTA concentrations in MS medium

Fe-EDTA is a key component in MS medium because it supplies iron for plant growth and development. The final concentration of Fe-EDTA in MS medium is 0.5 mM. Cotyledons of watermelon showed different responses to induction media containing different levels of Fe-EDTA. Without Fe-EDTA supply, the cotyledons turned slightly brown and lost vigor when cultured (Fig. 1). Cotyledon explants responded best to the induction medium containing 1.5–2× MS Fe-EDTA; these explants were dark green and became more vigorous than those grown in the media with other concentrations of Fe-EDTA. The cotyledons cultured in induction medium with 1× MS Fe-EDTA showed slighter browning than those cultured in the medium without Fe-EDTA, whereas those cultured in the medium with high Fe-EDTA concentrations (i.e., 3–4× MS Fe-EDTA) became curly, brownish, and withered (Fig.

1). Meanwhile, the higher adventitious shoots regeneration frequencies (75.9-88.9%) were obtained in the induction media containing 1.5–2×MS Fe-EDTA, but only 16.7% and 21.3% regeneration frequencies were produced in the induction media containing 1× and 3×MS Fe-EDTA respectively. And no adventitious shoots were observed when explants grown in the induction medium without Fe-EDTA or with 4×MS Fe-EDTA (Table 1). Thus, supplying an optimal concentration of Fe-EDTA is important for watermelon shoots regeneration.

3.2. Effect of preculture and coculture time on watermelon transformation

As presented in Table 2, without preculture, the frequency of adventitious shoots regeneration was very low (10.7%), no valid shoot or only a few single white bud formed in the explants. Within 5 days, the frequencies of adventitious shoots regeneration increased gradually with the prolonged of preculture time, and it reached the highest (42.9%) at 5 days. After that, the frequencies of adventitious shoots regeneration decreased with prolong of preculture time. These results indicated that the optimal preculture time is important for watermelon transformation, shorter and longer preculture time would significantly suppress the adventitious shoots regeneration in watermelon transformation.

The effect of different coculture time on the frequencies of adventitious shoots regeneration are shown in Table 3. In all tested days, the lowest frequency (13.8%) was presented at the cotyledon explants cocultured with *Agrobacterium* for 2 d. Similar with the effects of preculture time on the watermelon explants, the frequencies of adventitious shoots regeneration also increased gradually with the prolong of coculture time within 5 days, and it reached the highest (39.2%) at 5 days, and this frequency was significant higher than other treatments.

These results indicated that both preculture time and coculture time could

significantly affect the frequencies of adventitious shoot regeneration during watermelon transformation. Basing on these results, 5 days preculture time and 5 days coculture time were used in our following research in order to obtain a high transformation frequency.

3.3. Regeneration of transgenic watermelon plants

Following our cotyledon-cutting method, a few calli and many shoots sprouted around the cut region within one month (Figs. 2A and 2B). After another 3 weeks, these regenerated shoots elongated to approximately 3 cm (Fig. 2C), and they were excised and transferred to the rooting medium for 3 weeks. The rooted shoots were transferred into plastic cups containing vermiculite to acclimate. More than 60 individual kanamycin-resistant plantlets were obtained after acclimation. Upon planting, these plantlets were very difficult to recover because of their weak roots. Grafting was employed to overcome this problem. The grafted plantlets grew well, similar to normal watermelon seedlings (Fig. 2D). Seventeen individual kanamycin-resistant plantlets were recovered through grafting and self-rooting.

3.4. Transgene confirmation in putative transgenic watermelon plants

Seventeen individual kanamycin-resistant watermelon shoots were recovered, but only seven of them succeeded in obtaining T1 generation seeds because of the technique difficulties of self-pollination of the transgenic watermelon lines (Figs. 2E and 2F). To confirm that the target gene was transferred into the putative transgenic watermelon plants, a PCR assay was conducted with *NPTII* fragment-specific primers. Assay results indicated that only three of the seven lines had the expected 500 bp PCR product of the *NPTII* gene (Fig. 3), the false positive frequency was 57%, indicating there were a high degree of transgene escapes. Southern blot was performed to further confirm the integration of the target gene in the three PCR-positive transgenic lines.

283 All the three lines showed hybridization bands, with lines 2 and 5 showing one band,
284 and line 7 showing two bands (Fig. 4). These results proved that the targeted gene was
285 successfully integrated into the genome of these lines, and the copy numbers of
286 targeted gene in these three lines were 1, 1, and 2, respectively.

287 **3.5. Evaluation of virus resistance in transgenic watermelon lines**

288 The CMV resistance of these three lines was evaluated by mechanical inoculation
289 of the CMV strain with T1 generation plants. The cotyledons of the nontransgenic
290 plants exhibited expanded necrosis at 7 d postinoculation (dpi), and the true leaves
291 showed the typic mosaic disease symptoms at 14 dpi (Fig. 5). Only small necroses
292 were observed in the cotyledons of transgenic plants at 7 dpi, in addition, no
293 expansion necroses were observed at 14 dpi, the true leaves without displaying
294 symptoms (Fig. 5). DAS-ELISA assay indicated that the I/H ratios of transgenic lines
295 2, 5, and 7 were less than 3.0, whereas that of the nontransgenic line was 4.36 (Table
296 4). This result indicates that CMV can be detected in the nontransgenic line but not in
297 the transgenic lines. Therefore, the three transgenic lines are resistant to CMV, with
298 lines 5 and 7 were showing stronger resistance than line 2.

299

300 **4. Discussion**

301 **4.1. Optimal concentration of Fe-EDTA is important for watermelon** 302 **regeneration**

303 High efficient shoot regeneration is important for plant transformation. During
304 the establishment of the regeneration system, we observed that the watermelon
305 explants consistently turned slightly brown and lost vigor when cultured in MS
306 medium supplied with different combinations of shoot-inducing hormones. Thus, we
307 attempted to change the concentrations of different components (NH_4NO_3 , KH_2PO_4 ,

MgSO₄, and Fe-EDTA) in the MS medium to suppress chlorosis and increase vigor. Except for the Fe-EDTA supplemented medium, none of the other induction media with different concentrations of macro-element ingredients inhibited chlorosis (data not shown). Moreover, dark green, vigorous explants and the higher frequencies of adventitious shoots regeneration were presented in the induction media containing 1.5–2× MS Fe-EDTA (Fig. 1). These results illustrate the defection of iron induces the chlorosis of watermelon explants. Iron plays an important role in chlorophyll biosynthesis, and iron deficiency leads to plant chlorosis (Spiller et al. 1982). In the present study, excessive (over 2×) Fe-EDTA levels caused curling and browning in the explants. This result indicates that high iron concentration is toxic to the explants. Thus, supplying optimal concentration of Fe-EDTA is important for watermelon regeneration.

4.2. Optimized transformation protocol of the watermelon line

Cotyledons aged 3–19 d were used as suitable explants for watermelon transformation and regeneration in previous reports (Cho et al. 2008; Choi et al. 1994; Dong and Jia 1991; Ellul et al. 2003; Park et al. 2005; Tabei et al. 1993; Wu et al. 2009). The present results revealed that 5-day-old cotyledons from the watermelon showed the highest shoot regeneration frequency (data not shown). Under this condition, cotyledons turned from yellow to green, indicating a trend of chlorophyll accumulation. Similar to cutting methods used in tetraploid watermelon (Compton and Gray, 1994) and melon (Wu et al. 2009), using proximal halves without shoot tips as explants for watermelon transformation was efficient. Contrary to other reports on watermelon transformation (Compton and Gray 1994; Huang et al. 2011; Park et al. 2005; Yu et al. 2011), the present study revealed that 5-d preculture induced the maximum induction frequency of adventitious buds.

333 To the best of our knowledge, grafting is a common approach to reduce soil-
334 borne diseases, and increase the root system vigor in Asian countries (Edelstein et al.
335 2000; Han et al. 2005; Hassell et al. 2008; Lee and Oda 2002; Pogonyi and Pek 2004).
336 In this research, the *in vitro* grafting of shoots were used for transgenic plant recovery,
337 and our results showed that the grafted transgenic shoots were vigorous and strong.
338 As we know, this is the first report using grafting to recover transgenic plants from
339 their weak roots. This method may also be used to recover regenerated shoots for
340 species with weak roots in tissue cultures.

341 **4.3. Genetic engineering on virus-resistant watermelon**

342 Only a few virus-resistant transgenic watermelons were reported because of the
343 low efficiency of watermelon transformation (Park et al. 2005; Yu et al. 2011; Lin et
344 al. 2012). All of these efforts were focused on manipulating virus coat protein through
345 RNA silencing induced by siRNA. Artificial microRNAs (amiRNAs), which were
346 developed using miRNA, facilitate efficiently gene silencing. As a powerful tool, it
347 has been used to produce transgenic antiviral plants (Zhang et al. 2011; Niu et al.
348 2006; Duan et al. 2008; Qu et al. 2007). Our previous research showed that transgenic
349 tomato expressing amiRNA that targets CMV 2a/2b genes or the highly conserved 3'-
350 untranslated region also exhibit effective resistance to CMV infection (Zhang et al.
351 2011). In this study, we also found that the transgenic watermelon expressing
352 amiRNA targeting CMV 2a/2b gene also displayed CMV resistance. As far as we
353 know, this is the first report on apply amiRNA in cucurbitaceous crop virus resistance
354 research. The miRNA technique proved to be an effective strategy for genetic
355 engineering in plant virus resistance.

356

357

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References

- Chen TC, Lu YY, Cheng YH, Chang CA & Yeh SD (2008) Melon yellow spot virus in watermelon: a first record from Taiwan. *Plant Pathology* 57(4):765-765
- Chen WS, Chiu CC, Liu HY, Lee TL, Cheng JT, Lin CC, Wu YJ & Chang HY (1998) Gene transfer via pollen - tube pathway for anti - fusarium wilt in watermelon. *International Union of Biochemistry and Molecular Biology* 46(6):1201-1209
- Cho MA, Moon CY, Liu JR & Choi PS (2008) *Agrobacterium*-mediated transformation in *Citrullus lanatus*. *Biologia plantarum* 52(2):365-369
- Choi PS, Soh WY, Kim YS, Yoo OJ & Liu JR (1994) Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumefaciens*. *Plant Cell Reports* 13(6):344-348
- Clark MF & Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *The Journal of general virology* 34(3):475-483
- Compton ME & Gray D (1994) Adventitious shoot organogenesis and plant regeneration from cotyledons of tetraploid watermelon. *HortScience* 29(3):211-213
- Dong JZ & Jia SR (1991) High efficiency plant regeneration from cotyledons of watermelon (*Citrullus vulgaris* Schrad). *Plant Cell Reports* 9(10):559-562
- Dong JZ, Yang MZ, Jia SR & Chua NH (1991) Transformation of melon (*Cucumis melo* L.) and expression from the cauliflower mosaic virus 35S promoter in transgenic melon plants. *Nature biotechnology* 9(9):858-863
- Duan CG, Wang CH, Fang RX & Guo HS (2008) Artificial MicroRNAs highly accessible to targets confer efficient virus resistance in plants. *Journal of virology* 82(22):11084-11095
- Edelstein M, Tadmor Y, Abo-Moch F, Karchi Z & Mansour F (2000) The potential of *Lagenaria* rootstock to confer resistance to the carmine spider mite, *Tetranychus cinnabarinus* (Acari: Tetranychidae) in Cucurbitaceae. *Bulletin of entomological research* 90(02):113-117
- Ellul P, Rios G, Atares A, Roig L, Serrano R & Moreno V (2003) The expression of the *Saccharomyces cerevisiae* HAL1 gene increases salt tolerance in transgenic watermelon [*Citrullus lanatus* (Thunb.) Matsun. & Nakai.]. *Theoretical and Applied Genetics* 107(3):462-469
- Fuchs M, McFerson JR, Tricoli DM, McMaster JR, Deng RZ, Boeshore ML, Reynolds JF, Russell PF, Quemada HD & Gonsalves D (1997) Cantaloupe line CZW-30 containing coat protein genes of *cucumber mosaic virus*, *zucchini yellow mosaic virus*, and *watermelon mosaic virus-2* is resistant to these three viruses in the field. *Molecular Breeding* 3(4):279-290
- Fulton TM, Chunwongse J & Tanksley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Molecular Biology Reporter* 13(3):207-209
- Gaba V, Zelcer A & Gal-On A (2004) Cucurbit biotechnology-the importance of virus resistance. *In Vitro Cellular & Developmental Biology-Plant* 40(4):346-358
- Gu QS, Bao WH, Tian YP, Prins M, Yang HX, Lu J, Liu LF & Peng B (2008) *Melon necrotic spot virus* newly reported in China. *Plant Pathology* 57(4):765-765
- Han J-S, Kim C, Park S, Hirschi KD & Mok I-G (2005) *Agrobacterium*-mediated transformation

- of bottle gourd (*Lagenaria siceraria* Standl.). *Plant Cell Reports* 23(10-11):692-698
- Hassell RL, Memmott F & Liere DG (2008) Grafting methods for watermelon production. *HortScience* 43(6):1677-1679
- Huang YC, Chiang CH, Li CM & Yu TA (2011) Transgenic watermelon lines expressing the nucleocapsid gene of *Watermelon silver mottle virus* and the role of thiamine in reducing hyperhydricity in regenerated shoots. *Plant Cell, Tissue and Organ Culture* 106(1):21-29
- Lee JM & Oda M (2002) Grafting of herbaceous vegetable and ornamental crops. *Horticultural reviews-westport then New York* 28:61-124
- Lin CY, Ku HM, Chiang YH, Ho HY, Yu TA & Jan FJ (2012) Development of transgenic watermelon resistant to *Cucumber mosaic virus* and *Watermelon mosaic virus* by using a single chimeric transgene construct. *Transgenic research* 21(5):983-993
- Morroni M, Thompson JR & Tepfer M (2008) Twenty years of transgenic plants resistant to *Cucumber mosaic virus*. *Molecular plant-microbe interactions* 21(6):675-684
- Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum* 15(3):473-497
- Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD & Chua NH (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nature biotechnology* 24(11):1420-1428
- Palukaitis P, Roossinck MJ, Dietzgen RG & Francki RI (1992) *Cucumber mosaic virus*. *Advances in virus research* 41:281-348
- Papayiannis L, Ioannou N, Boubourakas I, Dovas C, Katis N & Falk B (2005) Incidence of viruses infecting cucurbits in Cyprus. *Journal of Phytopathology* 153(9):530-535
- Park SM, Lee JS, Jegal S, Jeon BY, Jung M, Park YS, Han SL, Shin YS, Her NH & Lee JH (2005) Transgenic watermelon rootstock resistant to CGMMV (*cucumber green mottle mosaic virus*) infection. *Plant Cell Reports* 24(6):350-356
- Pogonyi A & Pek Z (2004) Grafting of vegetables. *Hajtatás, Korai Termesztes Hungary*
- Qu J, Ye J & Fang R (2007) Artificial microRNA-mediated virus resistance in plants. *Journal of virology* 81(12):6690-6699
- Spiller SC, Castelfranco AM & Castelfranco PA (1982) Effects of Iron and Oxygen on Chlorophyll Biosynthesis : I. In vivo observations on iron and oxygen-deficient plants. *Plant physiology* 69(1):107-111
- Suratman F, Huyop F, Wagiran A, Rahmat Z, Ghazali H & Parveez G (2010) Biolistic Transformation of *Citrullus vulgaris* Schrad (Watermelon). *Biotechnology* 9(2):119-130
- Tabei Y, Yamanaka H & Kanno T (1993) Adventitious shoot induction and plant regeneration from cotyledons of mature seed in watermelon (*Citrullus lanatus* L.). *Plant Tissue Culture Letters* 10(3):235-241
- Tricoll DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML & Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to *cucumber mosaic virus*, *watermelon mosaic virus 2*, and *zucchini yellow mosaic virus*. *Nature biotechnology* 13(12):1458-1465
- Varveri C, Vassilakos N & Bem F (2002) Characterization and detection of *Cucumber green mottle mosaic virus* in Greece. *Phytoparasitica* 30(5):493-501
- Wu HW, Yu TA, Raja JA, Wang HC & Yeh SD (2009) Generation of transgenic oriental melon resistant to *Zucchini yellow mosaic virus* by an improved cotyledon-cutting method. *Plant Cell Reports* 28(7):1053-1064
- Yu TA, Chiang CH, Wu HW, Li CM, Yang CF, Chen JH, Chen YW & Yeh SD (2011) Generation of transgenic watermelon resistant to *Zucchini yellow mosaic virus* and *Papaya ringspot*

virus type W. Plant Cell Reports 30(3):359-371 Zhang X, Li H, Zhang J, Zhang C, Gong P, Ziaf K, Xiao F & Ye Z (2011) Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. Transgenic research 20(3):569-581

Note:

Table 1. Influence of Fe-EDTA concentration on the induction of adventitious shoots

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 2. Influence of preculture time on the induction of adventitious shoots

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 3. Influence of cocultivation time on the induction of adventitious shoots

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 4. CMV DAS-ELISA detection in transgenic watermelon

Note: I/H ratio ≥ 3.0 is considered positive. Ten PCR-positive seedlings of transgenic lines 2, 5, and 7 at I/H ratio < 3.0 show different resistance levels to CMV; nontransgenic plant with I/H ratio = 4.36 shows sensitivity to CMV.

Figure 1. Influence of Fe-EDTA concentrations on the induction of adventitious shoots

Adventitious shoots developed from cotyledon explants in induction medium supplemented with different concentrations of Fe-EDTA: 0, 1, 1.5, 2, 3, and 4 fold MS Fe-EDTA. Cotyledons turned yellow when grown in the medium without Fe-EDTA. Cotyledon explants remained dark green and developed adventitious shoots in 2 $\times$ Fe-EDTA, but the shoots turned yellow and curled in 4 $\times$ Fe-EDTA (A to F: 0, 1, 1.5, 2, 3, 4 $\times$ Fe-EDTA, respectively).

Figure 2. Growth stages of transgenic watermelon

A. Adventitious shoots regenerated from the cut edge of the cotyledon after 3 weeks. B. Enlarged adventitious shoots. C. Elongated plantlets. D. Regenerated shoot grafted onto rootstock. E. Watermelon fruit set in

transgenic T0 plant. F. Watermelon fruit set from self-pollinated T1 plant.

Figure 3. PCR analysis of transgenic plants

Genomic DNA isolated from putative transgenic plants was subjected to PCR amplification with NPTII primers. Lane M: DL5000 DNA Marker (5000, 3000, 2000, 1500, 1000, 750, 500, 250, and 100 bp); Lane P: positive control (plasmid); Lanes 2, 5, and 7: putative transgenic watermelons; Lane N: Nontransgenic watermelon.

Figure 4. Southern blot analysis of transgenic watermelon

Genomic DNA was digested with EcoRI and hybridized with the NPTII probe. Lanes 2, 5, and 7: transgenic plants; Lane N: Nontransgenic watermelon.

Figure 5. Leaf character of pBI-pre-miR-2a/b transgenic watermelon after CMV inoculation

Four-week-old leaves' character of transgenic and nontransgenic lines after inoculation with Cucumber mosaic virus (CMV). No disease symptoms were presented by pBI-pre-miR-2a/b transgenic plant leaves (left), but chlorotic mosaics were presented in wild-type plant leaves (right).

Figure 1

Figure 2

Figure 3

Figure 4

2

5

7

N

Figure 5

Table 1. Influence of Fe-EDTA concentration on the induction of adventitious shoots

Fe-EDTA level	No. of explants	No. of explants with adventitious shoots	Frequency of shoots regeneration (%; mean $\pm$ standard error)
0	108	0	0.0 $\pm$ 0.0 d
1×	108	23	21.3 $\pm$ 0.9 c
1.5×	108	82	75.9 $\pm$ 2.4 b
2×	108	96	88.9 $\pm$ 3.2 a
3×	108	18	16.7 $\pm$ 1.6c
4×	108	2	1.9 $\pm$ 0.9 d

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 2. Influence of preculture time on the induction of adventitious shoots

Preculture days	No. of explants	No. of explants	Frequency of shoots regeneration
		with adventitious shoots	(%; mean $\pm$ standard error)
0	130	14	10.7 $\pm$ 1.6 d
2	172	22	14.6 $\pm$ 2.2 cd
3	247	35	17.2 $\pm$ 2.6 c
4	132	35	27.4 $\pm$ 3.4 b
5	168	72	42.9 $\pm$ 0.0 a
6	140	38	23.8 $\pm$ 4.8 b
7	153	27	17.7 $\pm$ 2.1 c

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 3. Influence of cocultivation time on the induction of adventitious shoots

Co-cultivation days	No. of explants	No. of explants with adventitious shoots	Frequency of shoots formation (%; mean $\pm$ standard error)
2	130	8	13.8 $\pm$ 3.3 c
3	132	35	27.4 $\pm$ 3.4 b
4	152	36	25.3 $\pm$ 4.5 b
5	182	56	39.2 $\pm$ 9.4 a
7	115	30	26.2 $\pm$ 3.5 b

Values followed by the same letter in the last column are not considered significant in Duncan's new multiple range test at the 5% level.

Table 4. CMV DAS-ELISA detection in transgenic watermelon

Transgenic Line	No. of PCR-positive plants	I/H (mean $\pm$ standard error)
2	10	2.28 $\pm$ 0.42 b
5	10	0.59 $\pm$ 0.41 c
7	10	0.87 $\pm$ 0.58 c
Nontransgenic	10	4.36 $\pm$ 0.13 a

Note: I/H ratio $\geq$ 3.0 is considered positive. Ten PCR-positive seedlings of transgenic lines 2, 5, and 7 at I/H ratio $<$ 3.0 show different resistance levels to CMV; nontransgenic plant with I/H ratio = 4.36 shows sensitivity to CMV.