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

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ABSTRACT

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

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 (Morroni 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 resistence watermelon (Yu et al., 2011), expressing the yeast *HAL1* gene to

Abbreviations: MS, Murashige and Skoog medium; 1/2 MS, half-strength MS medium; BA, N6-benzyladenine; *NPTII*, Neomycin phosphotransferase II gene; IAA, 3-indoleacetic acid; GUS, β-glucuronidase; KT, kinetin; Hpt, hygromycin phosphotransferase gene; DAS-ELISA, double-antibody sandwich enzyme-linked immunosorbent assay.

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enhance watermelon salt tolerance (Ellul et al., 2003), expressing the CGMMV CP gene to develop CGMMV resistance in watermelon rootstocks (Park et al., 2005), and so on.

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

Fusarium wilt and CMV viruses severely restrict watermelon production, and creating Fusarium wilt and virus resistance germplasm to develop disease-resistant cultivars is an important project in watermelon breeding. Many Fusarium wilt-resistant watermelon germplasms had been created in our group previously. To obtain a watermelon germplasm that resistant to both Fusarium wilt and CMV, a Fusarium wilt resistant germplasm was used to produce the CMV-resistant germplasm by genetically engineer in our laboratory. In this paper, we present an effective *A. tumefaciens*-mediated transformation method designed for a Fusarium wilt resistant female parent watermelon line. Using this method, we successfully transferred an artificial microRNA that targets CMV 2a/2b genes to this line. PCR assay and Southern blot analysis showed that the transferred fragment was successfully imported and integrated into the watermelon genome. Further, a CMV-resistance assay revealed that the transgenic lines showed stronger virus resistance than the wild-type line after CMV inoculation.

2. Materials and methods

2.1. Plant materials and culture conditions

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

2.2. Watermelon shoots regeneration

Watermelon seeds with coat removed were soaked in 70% ethanol for 1 min, sterilized for 10 min in 0.1% $HgCl_2$ with two drops of Tween-20, and then rinsed several times with sterile distilled water. Initially, the sterilized seeds were placed onto 1/2 MS medium containing 1% sucrose for 2 days in the dark at 30 °C, and then transferred to a 16 h/8 h (light/dark) photoperiod for 3 days at 25 °C. The 5-day-old cotyledons that turned from yellow to green were used as explants. After excising and discarding the hypocotyls and apical portion, the cotyledons were cut into distal and proximal halves. The proximal cotyledon halves were cultured in 90 mm × 15 mm Petri dishes with induction medium for 3 weeks until adventitious shoots emerged. The cotyledons with regenerated adventitious shoots were transferred onto shoot elongation medium (MS medium supplemented with KT 0.2 mg/L) and then cultured for a month. Elongated shoots grown more than 3 cm in height were individually excised, transferred onto rooting medium (MS supplied with IBA 1 mg/L), and then cultured for 2–3 weeks. The plantlets with well-developed roots were taken from the rooting medium, washed to remove root-adhered phytagel, and then transferred to plastic cups containing vermiculite. Each cup was covered with another transparent plastic cup to maintain humidity for 2 weeks and then uncovered for a week. Acclimatized plantlets were transplanted into flowerpots containing compost and grown in a greenhouse.

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

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

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

2.4. Watermelon transformation

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

2.5. DNA extraction and PCR analysis

Genomic DNA was extracted from young leaves of transgenic and nontransgenic watermelon plants through the CTAB method