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The development of cold resistance rootstock using *Agrobacterium*-mediated transformation of *Arabidopsis CBF3/DREB1A* in bottle gourd (*Lageneraria siceraria* Standl.)

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ABSTRACT

Plants growth and development are adversely affected by various abiotic stresses. Water and temperature plays important role on plant growth and crop yield. C-repeat binding factor (CBF) acts as transcription factor in plant that involve in cold-response pathway. It has major role in cold acclimation and cold response gene expression. An *Agrobacterium*-mediated transformation system has been established for introducing foreign gene into bottle gourd. We have introduced *Arabidopsis CBF3/DREB1A* gene into bottle gourd (*Lageneraria siceraria* Standl.) using *Agrobacterium*-mediated transformation. Experimental results confirmed that transgenic plants were better cold resistant than non-transgenic plants and *CBF3/DREB1A* gene was not transferred to stem, leaf and fruit of the grafted watermelon (*Citrullus lanatus* var. *lanatus*) from transgenic bottle gourd rootstock. Horticultural traits were similar for transgenic and non-transgenic plants. We concluded that the rootstock of transgenic bottle gourd might be used for growing watermelon at low soil temperature.

1. Introduction

Bottle gourd is Cucurbitaceae family vegetable belongs to *Lagenaria* genus and *Siceraria* species. It is fast growing, climbing or trailing plant, requires enough sunlight for flowering and fruiting. It is commonly cultivated in tropical and subtropical area having various shape and size (Han et al., 2004). Young fruits of bottle gourds are consumed as a vegetable, whereas matured dried fruits are used as container, utensil, or decoration. The fruits have lots of health benefits and also used as drug (Roopan et al., 2016). There is a major loss in crop production worldwide because of water, temperature and soil-borne diseases. Bottle gourd has been exclusively used as a rootstock for cucurbit crop including watermelon in Korea and Japan to reduce incident of soil-borne disease and possesses exceptional tolerance to low soil temperature (Lee and Oda, 2003). It is difficult to develop resistant varieties using traditional plant breeding, however, advancement in gene transformation gives ample of opportunity for crop improvement and breeding (Han et al., 2005). An *Agrobacterium*-mediated transformation has been reported for introduction of foreign genes into Cucurbitacea family like bottle gourd (Han et al., 2005, 2004), cucumber (Chee, 1990; Nishibayashi et al., 1996), melon (Ayub et al., 1996; Fang and Grumet, 1990), and watermelon (Chaturvedi and Bhatnagar, 2001; Compton, 2000; Ellul et al., 2003; Park et al., 2005).

Climate and external environment plays imperative role in growth and development of plants. Particularly, water and temperature plays major role in plant growth and crop yield. Plants are more adaptive in cold acclimation when exposed slowly than that of swiftly at low temperature (Hughes and Dunn, 1996). Many studies were reported that CBF1 (DREB1B), CBF2 (DREB1C), and CBF3 (DREB1A) act as a transcription factor to C-repeat/dehydration-responsive element binding factor (CBF/DREB) in cold-response pathway of *Arabidopsis thaliana* (Liu et al., 1998; Stockinger et al., 1997). *CBF1, CBF2*, and *CBF3* genes are members of the *AP2/EREBP* gene family, playing an important role in the cold-response pathway (Chen et al., 2013; Liu et al., 1998; Novillo et al., 2004, 2007; Stockinger et al., 1997). In this pathway, *CBF* genes are induced by low temperature and bind to CCGAC of C-repeat/dehydration-responsive region in the promoter of the cold responsive (*COR*) gene that increases cold tolerance (Baker et al., 1994; Liu et al., 1998; Yamaguchi-Shinozaki and Shinozaki, 1994).

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Recently, a new plant breeding technique (NBT) has been used to increase crop yield using resistant rootstock. Previously, it was reported that CBF3 of *Arabidopsis* introduced to canola and tobacco, increased stress tolerance of the transgenic plant under drought and cold conditions (Jaglo et al., 2001; Kasuga et al., 2004). Recently, some scientist have focused on NBT to increase crop yield (Lusser et al., 2012). Aim of this study was to introduce CBF3/DREB1A gene into bottle gourd using *Agrobacterium*-mediated transformation to increase cold resistance based on NBT. Thus, transgenic bottle gourd developed could replace commercial rootstock for watermelon that might contribute to harvest watermelon in cold weather.

2. Material and method

2.1. Plant materials

The bottle gourd (*Lagenaria siceraria* Standl) inbred line (G5) was obtained from National Institute of Horticultural and Herbal Science of the Rural Development Administration, Republic of Korea. The seed coat was removed and sterilized with 70% (v/v) ethanol for 3 min, followed by 25% Clorox solution (commercial bleach containing 4% sodium hypochlorite; Yuhan-Clorox, Korea) for 40 min. Seeds were washed for 3 times with sterile distilled water after every step. Seeds were sown on sowing-medium (MS 4.4 g/L, sucrose 30 g/L, plant agar 8 g/L, pH 5.8) for 5 days. After sowing explants were prepared. 5-day old seedlings were cut off and cotyledons pairs were split open. All cotyledons were cut in half across their width and proximal half of cotyledon explants were used as co-cultivation material.

2.2. Agrobacterium stain and plasmid

The supervirulent *Agrobacterium* tumefaciens strain EHA 101 (Hood et al., 1986) carrying the binary vector pPAT-GFP-CBF3 (Fig. 1) was used. The binary vector contained the cauliflower mosaic virus (CaMV) 35S promoter-CBF-35S terminator and 35S promoter-GFP-nos (nopaline synthase) terminator located between the left and right borders and hygromycin resistance gene. *Agrobacterium* was maintained on YEP medium supplemented with rifampicin 50 mg/L, spectinomycin 50 mg/L (An et al., 1989).

2.3. Transformation and plant regeneration

Cotyledon explants were inoculated with *Agrobacterium* in the presence of co-cultivation medium (MS 4.4 g/L, sucrose 30 g/L, MES 0.5 g/L, BA 3 mg/L, plant agar 8 g/L, Acetosyringone 50 μM, pH 5.2) for 7 days followed by transferred to selection medium (MS 4.4 g/L, sucrose 30 g/L, MES 0.5 g/L, BA 3 mg/L, plant agar 8 g/L, AgNO3 0.5 mg/L, cefotaxime 500 mg/L, hygromycin 10 mg/L, pH 5.8). When shoots were regenerated from explants, explants were transferred to regeneration medium (MS 4.4 g/L, sucrose 30 g/L, plant agar 8 g/L, cefotaxime 500 mg/L, IAA 0.1 mg/L, pH 5.8) for root generation. The regenerated plants were transferred to soil in a greenhouse at National Institute of Horticultural and Herbal Science of the Rural Development Administration (RDA), Jeonju, Republic of Korea.

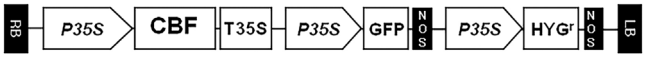

Fig. 1. Vector construct for *Agrobacterium*-mediated transformation for CBF3 into bottle gourd.

2.4. PCR and genetic analysis

2.4.1. PCR analysis and Southern blot

Young leaves were collected for genomic DNA extraction. 100 mg of leaves was crushed into fine powder using liquid nitrogen. High quality DNA was extracted by using CTAB extraction method with slight modification (Doyle and Doyle, 1987). The CBF3 specific primers were as follows; CBF3 forward, 5′-tgtttggctccgattacgag-3′ and CBF3 reverse, 5′-aaaagcatcccttctgccat-3′. The CBF3 specific primers and *AccuPower*® PCR PreMix (BIONEER, Korea) were used for PCR analysis. PCR amplification condition was maintained at 95 °C (5 min) followed by 35 cycles of 95 °C (30s), 58 °C (30s) and 72 °C (30s). For southern blot analysis, genomic DNA (40 μg) was digested by *EcoRI* and separated on 1% agarose gel by electrophoresis. Capillary transfer method was used to digest blot DNA onto nylon HybondTM-N+ membrane (Amersham Life science, UK). *CBF3* specific PCR products were labeled with RedyprimeII™ Random Prime labeling System (Amersham Life science, UK) followed by hybridization and washing were performed using normalization method (Sambrook et al., 1989). Labeled signals were detected by BAS-1800II Bio-Imaging analyzer (FUJIFILM).

2.4.2. qRT-PCR

RNA was extracted from young leaf using the Trizol (Invitrogen, USA). QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used to synthesize cDNA from extracted RNA. The following sets or primers were used to perform qRT-PCR; *CBF3* forward, 3′-cgagtcttcggtttcctcag-5′ and *CBF3* reverse, 3′-accaacgtctcctccatgtc-5′; *Actin* forward, 3′-tcgagactgcaaagagcagttcct-5′ and *Actin* reverse, 3′-tggctggaatagaacttctgggca-5′. The qRT-PCR reaction mixture contains 2 μl cDNA, 10 μl 2X SYBR Premix Ex TaqII (Takara, Japan), 2 μl primer (500 nM) in total volume of 20 μl. Real time PCR amplification reactions were performed with Eppendorf Mastercycler EP Gradient S Thermal Cycler and amplification was started at 95 °C (3 min) followed by 28 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (30 s). *Actin* RNA was used as an internal control. The relative change in gene expression from real-time quantitative PCR was analyzed using 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

2.5. Grafting and gene transfer analysis of watermelon

Control line (G5) and CBF3/DREB1A induced transgenic bottle gourd rootstocks (lane 1) were used for this study. Commercial watermelon (*Citrullus vulgaris* Schrad.) 'Wonderful' (Nongwoo Bio Co., Ltd., Suwon, Korea) were used for grafting with bottle gourd. When seedling had developed cotyledons, seedling of watermelon were grafted onto transgenic and G5 bottle gourd using splice-grafting method (Lee and Oda, 2002). We have examined the fidelity of the graft union after 2 weeks. RT-PCR analysis was performed to detect gene transfer to grafted watermelon from transgenic bottle gourd rootstock. The total RNA was extracted from stem, leaf and fruit of grafted watermelon with transgenic line and control line. PCR was performed using CBF3 primers, maintaining same experimental condition.

2.6. Cold resistance test

To evaluate the cold resistance of the wild-type bottle gourd, 2-week-old plants (i.e., 2 weeks after sowing) were incubated at 8 °C for 7 days. Based on the results of cold-resistance tests of wild-type bottle gourd, 2-week-old *CBF3/DREB1A* transgenic and non-transgenic plants were incubated at 8 °C for 5 days, and then transferred in pots filled with soil and cultivated in the greenhouse. Plants were grown on the mixture of soil and pearlite in a ratio of 3:1. Control group was not treated with the low temperature but