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Research Article

Production of transgenic cucumber expressing phytoene synthase-2A carotene desaturase gene

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Abstract The objectives of this study were to 1) evaluate the efficiency of the protocol of *Agrobacterium*-mediated transformation of cucumber to introduce phytoene synthase-2a carotene desaturase (PAC genes); 2) demonstrate the integration of PAC genes into the genome of putative transgenic cucumber based on growth on selection medium, PCR and Southern analysis; 3) evaluate the expression of PAC genes in transgenic cucumber based on the analysis of RT-PCR and Northern blot hybridization. Out of 5,945 cotyledonary-node explants inoculated with *Agrobacterium*, 65 (1.1%) explants produced 238 shoots. Integration of PAC genes into the genome of the cucumber was demonstrated based on the analysis of gDNA-PCR, 21 out of the 238 plants regenerated; while 6 plants proved positive for Southern blot hybridization. Transgene expression was demonstrated based on analysis of RT-PCR, 6 plants proved positive out of the 6 plants analyzed; while 4 plants out of 6 proved positive during Northern blot hybridization. This study successfully demonstrated the production of transgenic cucumber, integration, and expression of the PAC gene in cucumber.

Keywords β-carotene, *Cucumis sativus*, cotyledonary-node explant, transformation efficiency

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Introduction

Carotenoids are important natural color in living organisms and precursors of abscisic acid (ABA), which regulates embryo development, plant growth, and stress responses (Goodwin and Britton 1988). Furthermore, some carotenoids, β-carotene, are precursors of vitamin A which is vital for human health. Vitamin A deficiency leads to severe clinical symptoms related to night blindness, xerophthalmia and breakdown of the human immune system. Carotenoids are leading natural pigments of red, orange, yellow synthesized in plants, bacteria, algae, and fungi (Goodwin et al. 1988). In addition, the carotenoids present in plant growth of plants, is a precursor for the embryo development, the stress response, such as to control the ABA (abscisic acid). Several carotenoids, including β-carotene (Vitamin A) are essential for human health worldwide, as the precursors of natural and biological factors (Nambara et al. 2005). Deficiency of Vitamin A can cause blindness; It was estimated that a quarter million children in Southeast Asia were blind due to the deficiency (Sommer 1989).

To increase the intake of β-carotene for human consumption, crop cultivars with high β-carotene content have been developed, i.e., using transgenic technology. Ye et al. (2000) and Paine et al. (2005) reported development of transgenic rice using Agrobacterium-mediated transformation to introduce the entire β-carotene pathway into rice indosperm. Also, 'New Golden Rice' (Ha et al. 2010) and transgenic soybean (Kim et al. 2012) by the introduction of Psy-st2A-Tp-CrtI (PAC) has been developed, respectively. A new gene expression system was applied to the phytoene synthase and

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carotene desaturase genes, thereby encouraging some genes associated with metabolism by a single promoter gene 2A (60 bases) and apply the next six types of synthetic genes as well as a variety of plant biotechnology research that could contribute (Ha et al. 2010). Also, the PAC gene is potentially applicable to the development of other beta-carotene transgenic crops, i.e., cucumber.

Cucumber is an economically important crop, and is widely cultivated throughout the world (Wang et al. 2013). The species belongs to the group of the most popular vegetables worldwide (Nishibayashi et al. 1996). Breeding cucumber to develop superior cultivars have been progressed by the introduction of target genes using transgenic technology. Since transgenic cucumber with neomycin phosphotransferase (NPT II) gene was firstly reported (Trulson et al. 1986), the genetic improvement of cucumber opened new era. Transgenic cucumber resistant to virus has been developed using Agrobacterium-mediated transformation (Gaba et al. 2004). Up to now, the transformation protocols for cucumber have been optimized in various aspects involving in transformation methods, explants types, Agrobacterium strains, Agrobacterium concentration, length of exposure time to Agrobacterium, and selection markers, etc (Chee 1990; Kodama et al. 1993; Kose and Koç 2003; Lin et al. 2011; Kim et al. 2008). Agrobacterium-mediated genetic transformation is proved to be a reliable way for transferring foreign genes into cucumber genome, and a stable transformation system has been improved without chimerical transgenic event (Jang et al. 2011). Using the Agrobacterium-mediated transformation technology, transgenic cucumber expressing TPSP gene or Nit gene related to abiotic stress resistance have recently been developed by our research team (Kim et al. 2010; Jang et al. 2013). Using the stable transformation method (Jang et al. 2011), we report here the development of a transgenic cucumber events expressing PAC gene to enhance beta-carotene content. The objectives of this study were to 1) evaluate the efficiency of the protocol of Agrobacterium-mediated transformation of cucumber to introduce PAC genes; 2) demonstrate the integration of PAC genes into the genome of putative transgenic cucumber based on analysis of Southern blot hybridization and gDNA-PCR; 3) evaluate the expression of PAC genes in transgenic cucumber based on analysis of RT-PCR and Northern blot hybridization.

Materials and Methods

The procedure of Agrobacterium-mediated transformation to develop transgenic cucumber (*Cucumber sativus* L. Cv. Eunsung), plants expressing phytoene synthase-2A carotene desaturase gene in this study basically followed the one described by Jang et al. (2011). The estimate of transformation efficiency was based on the percentage of explants producing adventitious shoots growing on selection medium and the average number of plantlets regenerated per explant. Integration of PAC genes on the genome of cucumber was demonstrated based on analysis of gDNA-PCR and Southern blot hybridization. Transgene expression was demonstrated based on analysis of RT-PCR and Northern blot hybridization.

Plant materials

Explants were prepared from cucumber seeds, cv. Eunsung. The cultivar was chosen based on high-efficiency of in vitro regeneration (Cho et al. 2005a). The seed coat of cucumber seeds were removed manually, soaked in 70% ethanol for 1 minute and 1% sodium hypochlorite for 15 min, and then rinsed three times with sterile deionized-distilled water. These seeds were germinated in the dark on $^{1}/_{2}$ MS medium (Murashige and Skoog 1962). The pH of all media was adjusted to 5.8 before autoclaving. Twenty-five ml of medium was dispensed into 90 x 15-mm plastic Petri dishes. Explants of 2 to 3 cm-long cotyledonary nodes were prepared from 7-10 day old seedlings by making a horizontal slice through the hypocotyls region, approximately 3-5 mm below the cotyledon. A subsequent vertical slice was made between the cotyledons, and then the embryonic axis was removed. The cotyledonary-node explants were used as a material for co-cultivation with Agrobacterium (Jang et al. 2011).

Expression vector and preparation of inoculum Agrobacterium tumefaciens

In this study, phytoene synthase (psy) and carotene desaturase (crtI) genes were introduced into genome of cucumber (cv. Eunsung). Genes psy-st2A-Tp-crtI (PAC) (Ha et al. 2009), Korean patent registration number: 10-0905219) were cloned into pMBP-1 vector (Fig. 1). The T-DNA contained two

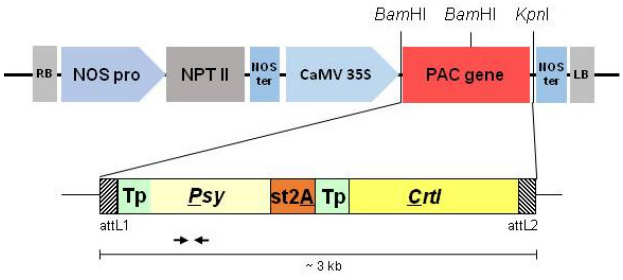

Fig. 1 Schematic map of the pMBP-1-PAC binary vector. attL1, 2: bacterial recombination site, Tp: transit peptide, Psy: phytoene synthase, st2A: sulfo-transferase 2A, CrtI: carotene desaturase gene (Ha et al. 2010)

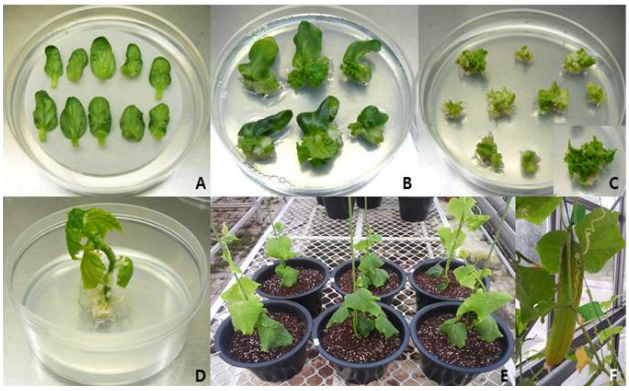

Fig. 2 Putative transgenic cucumber plants regenerated from cotyledonary-node explants. PAC genes were inserted into the genome of cucumber using Agrobacterium-mediated transformation. A. Cotyledonary-node explants on co-cultivation medium. B. Explants produced buds or shoots on selection medium containing 100 mg/L paromomycin at 8 weeks in selection medium. C. Shoot bud or shoots elongated on medium containing 100 mg/L paromomycin. D. Putative transgenic shoots grew on root induction medium. E and F. R_0 transgenic cucumber plants aclimatize grew and produced fruit in the greenhouse, respectively

cassettes (nos-nptII-nos and CaMV35S-PAC genes-nos). The gene of nptII (neomycin phosphotransferase II) confering resistance to paromomycin was expressed under the control of both nos (nopaline synthase) promoter and terminator; the PAC genes were under the control of CaMV 35S promoter and nos terminator. The pMBP-1-PAC binary vector was transformed into the cells of Agrobacterium strain GV3101. Agrobacterium inoculum was prepared by culturing on YEP medium containing 50 mg/L kanamycin and 50 mg/L rifampicin for 18 hours at 28°C. The suspension of Agrobacterium was ready for inoculation when the culture was on exponential growth phase (OD_{650} = 0.6 - 1.0).

Cucumber transformation: Explant preparation, inoculation, co-cultivation, and in vitro regeneration

The method of transformation and composition of the culture medium were performed according to Jang et al. (2011). The cotyledonary-node explants were submerged suspension of Agrobacterium carrying pMBP-1 vector (GV3101) for 30 minutes, and then transferred to the co-cultivation medium without antibiotics for co-culture in the dark for three days (Fig. 2A). In this study, 5,945 explants were inoculated. Then, the explants after three times-washing in sterilized water were transferred to the selection medium containing 100 mg/L paromomycin for two-weeks. Adventitious buds growing from the cotyledonary-node fragments during the initial two weeks were removed. Then the explants were sub-cultured on the same selection medium containing 100 mg/L paromomycin for 8 weeks in light, subculterd every two weeks. In the growth chamber is adjusted to 26°C were incubated at 46 μmol $m^{-2}s^{-1}$ light for 16 hours photoperiod, and sub-cultured every 2 weeks to induce shoot. Plantlets obtained from selection medium were transferred to soil in the greenhouse. The R_0 plants were artificially self-pollinated and the R_1 fruits were harvested.

Analysis of gDNA-PCR and RT-PCR

To demonstrate the integration of PAC genes into the genome of putative transgenic cucumber, analysis of gDNA-PCR and RT-PCR were conducted. Genomic DNA was extracted from young leaf using REDExtract-N-Amp™ Plant PCR kit (Sigma-Aldrich) and was used as template in amplification. Presence of the PAC coding region in the genomic DNA was analysed by PCR amplification using forward primer sequence 5'-TTTGTCCGACACAGTTTCCA-3' and reverse primer sequence 5'-CTCCGTTGTTGCCTTTGATT-3'. The PCR reactions were as follow: for 94°C for 4 min hot start, 30 cycles of 94°C for 30 sec denature, 55°C for 30 sec annealing, 72°C for 2 min extension, and 72°C for 10 min final extension. The amplified DNA fragments were then run on 0.8% agarose gel for size analysis. In addition, Total RNAs were isolated from young leaf using Trizol reagent (Invitrogen) according to the manufacturer's instruction. After first-strand cDNA synthesized using Express 1st Strand cDNA Synthesis System (Legene, CA 92121 USA), all cDNA samples were diluted to 1 ng/μl and the cDNA used for reverse transcriptase-PCR (RT-PCR). Each reaction contained 2 ul (2 ng/μl) of cDNA, 0.2 μl (10 pm/μl) of each primer, and 10 μl Premix Ex Taq™ (Takara) in a total volume of 20 μl. The PCR reactions were conducted with above same conditions and primer for gDNA-PCR.

Southern and Northern blot analysis

For Southern blot analysis, genomic DNA was isolated from approximately 2 g of young leaves of putative transgenic cucumber plants (Dellaporta et al. 1983). About 50 μg DNA was digested with BamHI for 16 hours at 37°C, and then run electrophoresis on 0.8% agarose gel. The DNA in agarose gel was blotted onto Zeta^R-Probe nylon membrane (Bio-Rad, catalog #162-0196) in 20X SSC. The 202-bp PAC gene-PCR product was labeled with ^{32}P-dCTP using labeling mix (Amersham, catalog #RPN1633). The primer pairs for PAC gene 5'-TTTGTCCGACACAGTTTCCA-3' as forward primer and 5'-CTCCGTTGTTGCCTTTGATT-3' for reverse primer (Southern 1975). For Northern blot analysis, total RNAs were isolated from the four-transgenic plants, using Tri-reagent (Molecular

Table 1 Molecular analysis of transgenic cucumber R_0 plants carrying PAC genes. The transgenic plants were developed using Agrobacterium-mediated transformation

Expression vector & Gene	No. of explants inoculated	No. of explants producing shoots (%)	No. of plantlets regenerated	gDNA-PCR analysis (%)	Southern blot analysis (%)	RT-PCR analysis (%)	Northern analysis (%)
pMBP1 -PAC	5,945	65 (1.1%)	238	21 (0.35)	6 (0.1)	6 (0.1)	4 (0.06)

Research Center Inc., catalog # TR-118). Approximately, 30 μg of total RNAs from each sample was electrophoresed on 1% agarose gel containing 5.3% (v/v) formaldehyde, and then blotted onto Zeta-Probe nylon membrane (Bio-Rad, catalog #162-0196) in 20 X SSC. The PCR products for PAC gene (202 bp) was labeled with ^{32}P-dCTP using labeling mix (Amersham, catalog #RPN1633) for RNA-blot hybridization.

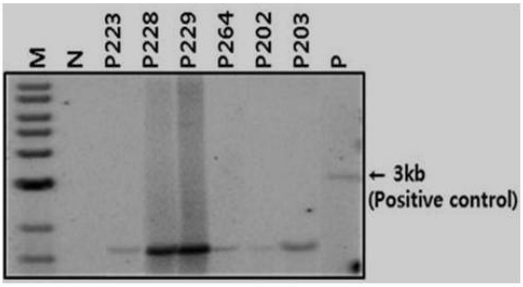

Fig. 3 Anaysis of Southern blot hybridization of transgenic cucumber carrying PAC gene. M: molecular size marker; N: nontransformed control plants; P223, P228, P229, P264, and P203 were transgenic plants. The probe used in this analysis was 202-bp PAC gene-PCR product

Results and Discussion

The efficiency of the protocol of Agrobacterium-mediated transformation of cucumber to introduce PAC genes

After being co-cultivated for 3 days (Fig. 2A) and cultured on selection medium containing 100 mg/L paromomycin for 2 - 8 weeks, buds and shoots were produced and developed from meristematic region of cotyledonary-node explants (Fig. 2B). Shoots rapidly generated from meristematic region were removed from the cotyledonary-node explants, and then the explants were freshly re-cultured on selection medium (Fig. 2C). Shoots then were cultured on shoot-elongation medium supplemented with 100 mg/L paromomycin. Finally the shoots longer than 3 cm were acclimated in soil (Fig. 2E) as our previous study (Kim et al. 2010; Jang et al. 2013).

In this study, the introduction of the PAC genes into the genome of cucumber was conducted using Agrobacterium-mediated transformation (Jang et al. 2011). The estimate of efficiency of transformation was based on the percentage of explants producing shoots on selection medium containing 100 mg/L paromomycin (Table 1). Out of 5,945 explants inoculated with Agrobacterium, 65 explants (1.1%) produced a shoots. In average, out of 5,945 explants inoculated with Agrobacterium, 238 paromomycin-resistant plants (0.04 plants per explants) were regenerated on selection medium containing 100 mg/L paromomycin; and 21 putative transgenic plants (0.0035 shoot per explants) showed positive based on PCR analysis. Of these 21 plantlets, 6 plants (0.1%) were finally confirmed as transformants (P202, P203, P223, P228, P229, P264) by Southern blot (Fig. 3) and RT-PCR analysis (data not shown), respectively. The transgenic plants were transplanted to soil, and plants were grown under greenhouse condition until produce the fruit (Fig. 2F).

Based on the results (Table 1), the efficiency of transformation of this study was 0.1%; this result was lower than our previous study (0.68 ~ 2.42%) (Jang et al. 2013). Although all of transformation procedure, explants type, and medium of this study were similar to Jang et al.' (2013), the difference in efficiency may be due to the difference in expression vector (pMBP1-PAC) and Agrobacterium strain (GV3101). It has been reported that the efficiency of transformation varied among protocols and plant species. Based on the number of independent transformation events recovered per 100 explants in maize, Frame et al. (2002) and Utomo (2005) reported transformation efficiency was 5.5 and 2.2% respectively. Hinchee et al. (1988), Olhoft and Sommer (2001), and Utomo (2004a) reported transformation eficiency 0.3 ~ 2.2%, 16.4%, and 2.6 ~ 6.5% respectively. Previous studies also reported that the efficiency of transformation depended on Agrobacterium strains (Simmonds and Donaldson 2000; Utomo 2004b), selection marker (Cho et al. 2005), addition of antioxidant L-cysteine (Olhoft and Somers 2001; Frame et al. 2002; Utomo 2005).

The cucumber plants were generally known as a recalcitrant plant species because of non-repeatability and genotype dependence (Gaba et al. 2004). To improve the efficiency of cucumber transformation, new stable transformation system have been developed by using of an alternative selectable marker (Cho et al. 2005a) or a regeneration system via somatic embryogenesis (Kim et al. 2008). However, a few problems in

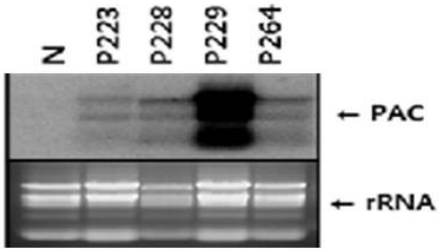

Fig. 4 Northern blot hybridization analysis of transgenic cucumber carrying PAC gene. Northern blothybridization analysis of transgenic cucumber R0 plants carrying PAC genes. 30μg of total RNA was separated in 1% agarose gel in each lane and subjected to Northern blot hybridization. The 202bp PAC PCR product was labeled with [32P]dCTP and then used as a probe. N: Non-transformed control plants; P223, 228, 229, 264: transgenic plants

the low efficiency of transformation, in the detection of chimeric transgenic, and in the low frequency (%) of regeneration have still remained as problem (Cho et al. 2005a; Kim et al. 2008). Since it was first reported that Agrobacterium-mediated cotyledonary-node transformation was achieved in soybean (Hinchee et al. 1988), the cotyledonary-node explants were suitable for target gene in recalcitrant plant transformation, such as soybean (Zhang et al. 1999) and melon (Cho et al. 2005b); allthough cotyledon or hypocotyl explants in many species have been used (Chee 1990; Dong et al. 1991; Saramento et al. 1992; Nishibayashi et al. 1996). Recently, transgenic cucumber was produced by using of cotyledonary-node explants (Jang et al. 2011), and a putative transgenic cucumber could be easily obtained by a rigid procedure according to the previous study (Kim et al. 2010; Jang et al. 2011, 2013). It may be supposed that the cotyledonary-node explants composed of a meristematic tissue, produced multiple shoots, and also shown that Agrobacterium mediated cotyledonary-node transformation is useful for a recalcitrant species such as cucumber.

Total RNA were isolated from leaf tissue of 4 events (P223, P228, P229, P264) (R_0) randomly selected from the 6 transgenic plants, and then subjected to Northern hybridization assay. All of 4 transgenic plants tested were constitutively accumulated amounts of PAC mRNA in contrast with non-transformed plant (Fig. 4). The P229 event specially showed for possibility of utilization for developing of new cultivars because the PAC gene was highly expressed in the transgenic cucumber. To increase β-carotene content in the plants, many genes encoding carotene synthesis have been cloned (Ha et al. 2009) and have been introduced into crop genome, i.e., tomato (Fraser et al. 2002), rape seed (Ravanello et al. 2003), potato (Morris et al. 2006), rice (Ye et al. 2000; Ha et al. 2010), soybean (Kim et al. 2012). In particular, Ha et al (2010) had been reported that the PAC gene related on the biosynthesis of carotenoids was accumulated beta-carotene in transgenic rice endosperm, and in transgenic soybean seed (Kim et al. 2012). In this study, we have developed a transgenic cucumber expressing PAC gene through cotyledonary-node transformation system, and then obtained P229 event with highly and stably expressing the PAC gene.

Acknowledgments

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