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Research Article

Development of transgenic cucumber expressing TPSP gene and morphological alterations

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Abstract To develop transgenic cucumber tolerant to abiotic stress, a cotyledonary-node explants were co-cultivated with *Agrobacterium tumefaciens* (EHA101) carrying TPSP gene (pHC30-TPSP). After transfer to fresh medium every two week for eight weeks, putative transgenic plants were selected when shoots grown a length greater than 3 cm from the cotyledonary-node explants on selection medium supplemented with 5 mgl^{-1} phospinotricin as selectable agent. The confirmation of transgenic cucumber was based on the Northern blot analysis. Thirty four shoots (5.2%) with resistance to phospinotricin were obtained from 660 explants inoculated. Of them, transformants were only confirmed from 11 plants (1.7%). Transgenic cucumber expressing *TPSP* gene was more synthesized at 3.8 times amounts of trehalose (0.014 mg g fresh wt^{-1}) than non-transformants (0.0037 mg g fresh wt^{-1}). However, all of transgenic plants showed abnormal morphology, including stunted growth (< height 15 cm), shrunken leaves, and sterility as compared with non-transgenic plants (> height 150 cm) under the same growth environment. These results lead us to speculate that the overproduction of trehalose was toxic for cucumber, even though that had known for rice as non-toxic.

Keywords *Agrobacterium*, stunted growth, transgenic cucumber, trehalose

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Introduction

Cucumber is one of the major vegetable crops in Asia and Europe. Recent studies in plant molecular breeding have been progressed by the introduction of specific genes into cells and tissues, regenerated to plant. Using *Agrobacterium*-mediated transformation technology, cultivars with superior agronomic characters such as virus resistance (Gaba et al. 2004) could be developed. Although the cucumber was known as a recalcitrant plant species, a stable transformation system has been improved without chimerical transgenic event (Kim et al. 2008). Abiotic factors such as low temperature, drought, and high salinity are common stress conditions that adversely affect the plant growth and yield. The development of the abiotic stress-tolerance could be bred into genotypes with increasing yield. Trehalose is a non-reducing disaccharide composed of two glucoses that are found in bacteria, algae, fungi, yeast, and some plants (Elbein 1974). This component works as a protective molecule under a variety of abiotic stress conditions because of their high hydrophility and chemical inertness in many organisms (Eleutherio et al. 1993). Thus, trehalose allows plants to tolerate naturally occurring stress during the period of dehydration and rehydration (Drennan et al. 1993).

Transgenic tobacco plants expressing TPS or *otsA* gene were shown the enhancing of dehydration tolerance (Romero et al. 1997), and trehalose-producing tobacco plants showed enhanced tolerance to drought and salinity stresses (Jun et al. 2005). Similar results were also reported in transgenic rice plants increases trehalose accumulation and abiotic stresses tolerance (Jang et al. 2003; Garg et al. 2002). These plants also showed a specific physiological and morphological alterations, including increased water potential and stunted growth (Pilon-Smits et al. 1998). Recently, Jang et al. (2003) reported that transgenic rice plants produced by introduction of a gene encoding a bifunctional fusion (*TPSP*) of the trehalose-6-phosphate (*T-6-P*) synthase (*TPS*) and T-6-P

phosphatase (*TPP*) of *Escherichia coli* increased trehalose accumulation and tolerance to drought, salt, and cold without stunting growth, and that the trehalose levels in leaf and seed extracts of the transgenic rice plants were specially 200-fold higher than that of transgenic tobacco plants with *TPS* or *TPP* gene (Jang et al. 2003). Up to now, there have been no reports on the production of abiotic stress resistance transgenic cucumber and alterations of their morphological characteristics.

This paper described the development of abiotic stress-tolerant cucumber. We report here for a transgenic cucumber events expressing *TPSP* gene and the morphological alterations, including stunted growth.

Materials and Methods

Plant materials

Zygotic embryos of F_1 hybrid cucumber (*Cucumis sativus* L., c.v. Eunchim) were used for the explants of transformation experiments. The embryos were dissected out of the mature seeds and the surface was disinfected with 70% ethanol for 1 min 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 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 node 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 the embryonic axis was removed.

Expression vector and preparation of *Agrobacterium* suspension

The transformation of cucumber was performed with the binary vectors pHC30-TPSP, which contained a bifunctional fusion (*TPSP*) of the trehalose-6-phosphate (*T-6-P*) synthase (*TPS*) and T-6-P phosphatase (*TPP*) of *Escherichia coli*, under the control of the maize ubiquitin promoter (*Ubi1*) and the herbicide resistance gene (*bar*) as selective marker, respectively (Fig. 1, provided by Dr. J.K. Kim). Disarmed *Agrobacterium tumefaciens* strains (EHA101) were used as helper strains in a binary vector system. The binary vector was introduced into *A. tumefaciens* strains EHA101 by the freeze-thaw method (An et al. 1987). The Agrobacteria were grown in YEP medium amended with the appropriate antibiotics to an OD_{650} = 0.6 to 0.8 at 27℃. The pellets after being centrifuged at 3,500 rpm for 10 min were resuspended to a final OD_{650} = 0.6 to 0.8 in an 1/10 MS basal medium amended with 3.2 mgl^{-1} BA, 0.5 mgl^{-1} IBA, 200 μM acetosyringone (AS) and 3% sucrose. The medium was buffered with 20 mM MES, pH 5.4. All components including growth regulators, vitaminscomponents and AS filters were sterilized post autoclaving.

Production of transgenic cucumber

Cotyledonary-node explants were immersed in the *Agrobacterium* suspensions for 30 min and then incubated on co-cultivation media (pH 5.4) supplemented with 20 mM MES, 100 mgl^{-1} cysteine, 3.2 mgl^{-1} BA, 0.5 mgl^{-1} IBA, 200 μM acetosyringone and 3% sucrose. Six explants were cultured per 90 x 15 mm Petri-dish and the explants were positioned with the adaxial side on a filter paper laid over the media. After co-cultivation the explants were washed with three times by a sterilized distilled water and then were cultured on shoot induction medium (MS salt, B5 vitamin, 3% sucrose, 3.2 mgl^{-1} BA, 0.5 mgl^{-1} IBA, 5 mgl^{-1} phospinotricin, 50 mgl^{-1} ticarcillin, 50 mgl^{-1} cefotaxime, 50 mgl^{-1} vancomycin, 3 mM MES, pH 5.6, **SI**). After 2 weeks of culture, the hypocotyls region was excised from each of the explants, and the remaining explant, cotyledon with differentiating node, was subsequently subcultured onto fresh SI medium. Following an additional 2 weeks of culture on SI medium, the cotyledons were removed from the differentiating node. The node explant was subcultured to shoot elongation medium (MS salt, 0.1 mgl^{-1} IBA, 0.5 mgl^{-1} GA_3, 3 mgl^{-1} phospinotricin, 50 mgl^{-1} ticarcillin, 50 mgl^{-1} cefotaxime, 50 mgl^{-1} vancomycin, 3 mM MES, pH 5.6, **SE**) solidified with 0.8% agar. Subculture to fresh SE medium was done every two weeks until shoots reached a length greater than 3 cm. Elongated shoots were transferred to root initiation medium (**RI**) comprised of 1/2 MS salts, 3% sucrose, 3 mM MES, 50 mgl^{-1} cefotaxime, 0.8% agar, pH 5.6. The rooted plants were transferred to soil. Plantlets (R_0) were acclimatized and grown to maturity in the

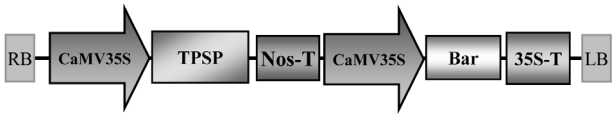

Fig. 1 Plant transformation vector (pHC30-*TPSP*), which consists of the CaMV35S promoter (CaMV35S) linked to the *TPSP* coding region, and a gene expression cassette that contains the 35S promoter, the *bar*-coding region, and the 3' region of nopaline synthase (*nos*). The *TPSP* construct was made by in-frame fusion of the *E. coli otsA* and *otsB* genes, which encode *TPS* and *TPP*, respectively.

greenhouse. The leaf of R_0 plants were also used for molecular level analysis.

Northern-blot analysis

Accordingly with the instructions of the manufacturer (Invitrogen) total RNA was prepared using Tri-Reagent, 4 randomly selected R_0 plants and the non-transgenic control of cucumber (c.v. Eunchim). Approximately 30 μg of total RNA was electrophoresed on 1% agarose gel containing 5.1% (v/v) formaldehyde and then blotted onto nylon membrane (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad) in 20X SSC. The 700-bp TPSP product by PCR analysis (TPSP-F: gaa aat att ccg cta ctc tga c, TPSP-R: gat act gag cga tga ctg tat g) was used and then labeled with [^{32}P]dCTP using the Random Primed DNA Labeling kit (Boehringer Manheim).

Determination of trehalose contents and morphological alterations

leaf samples were excised from 4-week-old cucumber plants (R_0) and then were ground in liquid nitrogen, and extracted by boiling 1 g of fresh tissue in 10 ml DW for 15 min. The extract was centrifuged, and the supernatant was filtered through a 0.45-um filter unit. Trehalose contents were determined by HPLC, using Bio-LC DX-600 (Dionex, Sunnyvale, CA, USA). Also, the heights (cm) ere measured from 4-weeks-old transgenic (R_0) after acclimation in soil in the green house. The controls were 4-week-old non-transgenic plants with the same growing condition.

Results and Dicussion

Using the cotyledonary node explants of "Eunchim" cultivar to produce transgenic cucumber plants, the explants inoculated with *Agrobacteria* suspension and then incubated on shoot induction (SI) medium supplemented with 5 mgl^{-1} phospinotricin as selective agents. After 2 weeks of culture, the cotyledonary node explants were gradually expanded and a few shoots were generated from meristematic region with an axillary bud remained. The shoot was removed at two weeks of culture and then node explants were sub-cultured to fresh SI medium until new shoots produced. After four weeks of culture, a few of these node explants showed an adventitious shoots formation with callus. The elongated shoots (> 3 cm) in SE medium containing 3 mgl^{-1} phospinotricin were then separated, transferred to rooting medium, and then acclimated in soil (Fig. 2A-D). The cotyledonary node as explant was only reported for soybean (Hinchee et al. 1988; Zhang et al. 1999) and melon (Cho et al. 2005b) to date, even though cotyledon or hypocotyl explants have been used as plant material for genetic transformation in most studies, including *Cucurbitacea* (Cho et al. 2005a,b; Chee 1990; Dong et al. 1991; Saramento et al. 1992; Nishibayashi et al. 1996). Since it was first report that *Agrobacterium*-mediated transformation of soybean using the cotyledonary node axillary meristem as the target for gene transfer was achieved using kanamycin as a selective agent (Hinchee et al. 1988), the modified transformation system using cotyledonary node explants of melon was developed (Cho et al. 2005b). It may be useful for a recalcitrant species in plant regeneration, since the node explants composed of a meristematic tissue that could be produced multiple shoots.

Table 1 showed the frequency of phospinotricin resistant shoots formed from cotyledonary node explants. The putative transgenic cucumbers were confirmed based on Northern blot analysis, and their morphological characteristics. A total of 34 shoots (5.2%) grew on selection medium containing phospinotricin. The shoots developed, elongated and rooted. Of these plantlets, 11 plants (1.7%) were confirmed as transformants by Northern blot analysis and then were transplanted to potting soil and grown to maturity in a green house. In general, the cucumber was known as a recalcitrant plant species because of non-repeatable, researcher and genotype dependence (Gaba et al. 1996). To solve the problem,

Fig. 2 Plant regeneration from cotyledonary-node explants of cucumber transformed with *TPSP* gene. A: Green callus and adventitious shoot primordia formation on SI medium with 5 mgl^{-1} phospinotricin. B, C: Shoot elongated from the phospinotricin-resistance primordia. D: Putative transgenic cucumber grown in soil. E: Stunted growth of transgenic cucumbers. F: Normal growth of non-transgenic cucumber regenerated from cotyledonary node explants.

Table 1 Analysis of molecular level and morphological characteristics for transgenic cucumber carrying with *TPSP* gene by *Agrobacterium* mediated transformation using cotyledonary-node explants on SI medium supplemented 5 mgl^{-1} phospinotricin

Experiments	Cotyledonary node explants co-cultured	No. of phosphinotricin resistance shoots (%)	Molecular analysis	Morphological characteristics of 4-weeks-old transgenic cucumber in soil
			No. of transgenic cucumber by Northern blot analysis (%)	Average of height (cm), leaf morphology, and reproductivity
I	250	13 (5.2)	5 (2.0)	6.0 (leaf shrinked and sterility)
II	126	4 (3.2)	0 (0.0)	-
III	117	6 (5.1)	2 (1.7)	15.0 (leaf shrunk and sterility)
IV	78	7 (9.0)	3 (3.8)	4.5 (leaf shrunk and sterility)
V	89	4 (4.5)	1 (1.1)	6.0 (leaf shrunk and sterility)
Total	660	34 (5.2)	11 (1.7)	7.9

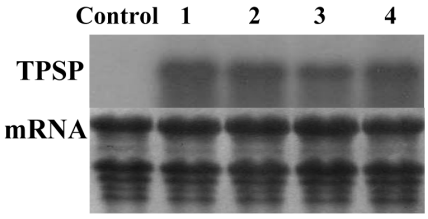

Fig. 3 Northern blot analyses of total RNA extracted from transgenic cucumber plants (R_0 generation). The RNA (30 μg) was separated in 1% agarose gel in each lane and subjected to Northern hybridization. The 700-bp *TPSP* PCR product was labeled with [32P]dCTP and then used as probe. Control, 1: EC2-2, 2: EC3-1, 3: EC3-2, 4: EC22-1

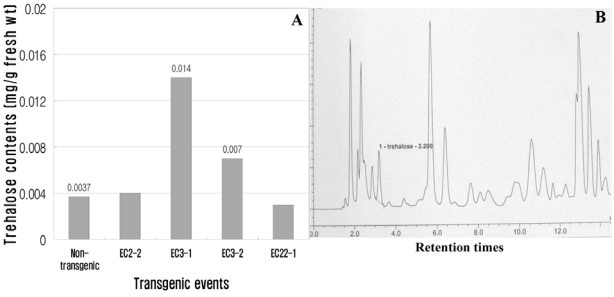

Fig. 4 Determination of trehalose contents (A) of transgenic cucumber events expressed *TPSP* gene (EC2-2, EC3-1, EC3-2, EC22-1) and non-transgenic plant, and trehalose peak (1-trehalose-3.200 marked) of transgenic event (EC3-1) (B)

a new stable transformation system have been developed by using of selection marker gene such as phospinotricin agent (Cho et al. 2005a) or by using of regeneration system via somatic embryogenesis (Kim et al. 2008). However, a few problems in these transformation system have caused difficulty such as low frequency (%) of gene transfer to target cells, transgenic cucumber with chimeric, and not easily regenerated from somatic embryogenesis (Cho et al . 2005a; Kim et al. 2008). In our study, it was still showed at a high frequency (3.5%) of chimeric plants. Accordingly, a modified transformation system to reduce these problems will be developed in further studies.

Of 11 transgenic plants, total RNA of 4 events (R_0) randomly selected (EC-2, EC3-1, EC3-2, EC22-1) was extracted from each leaf tissue and subjected to Northern hybridization assay. Results showed that all of transgenic lines tested constitutively accumulated high amounts of TPSP mRNA, while non-transformed plant was not expressed (Fig. 3). The trehalose levels on carbohydrate content extracted from leaves of these transgenic events expressing *TPSP* gene were analyzed. Quantitative carbohydrate analysis by HPLC showed significant changes in the carbohydrate content of the leaves. In particular, the trehalose concentrations (0.014 mg g fresh wt^{-1}) in EC3-1 event were especially 3.8-fold higher than that of non-transgenic cucumber (0.0037 mg g fresh wt^{-1}) (Fig. 4A, B). However, all of 11 transgenic plants expressed the *TPSP* gene showed abnormal morphology, including stunted growth (<15 cm for height) and leaves shrunk, and they also failed to harvest of R_1 seeds from the transformants because of sterility (Table 1, Fig. 2E), whereas non-transformants grown well under the same growth condition (Fig. 2 F).

In previous reports, constitutive expression of *TPS* and or *TPP* from either *E. coli* or yeast in tobacco or potato plants resulted in undesirable pleiotropic effects, including stunted growth and altered root system under normal growth conditions (Holmstrom et al. 1996; Goddijn et al. 1997; Romero et al. 1997). These pleiotropic growth phenotypes were present even in the absence of bulk accumulations of trehalose (Muller et al. 1999). Whereas, Jang et al. (2003) was reported that construct of a bifunctional fusion (*Ubi1::TPSP*) of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase was stable expressed in transgenic rice plants that showed neither growth inhibition nor visible changes in appearance, but hardly grew with altered phenotypes and died prematurely. These results

are demonstrate that the over expression of trehalose was as toxic in the transgenic cucumber as reported by Jang et al. (2003).

In conclusion, we could be obtained a transgenic cucumber with morphological abnormality and sterility, which are not useful for breeding programs, although the *TPSP* gene in the cucumber genome were stable expressed. We will be analyzed for the structure of cucumber genomic DNA region integrated with the *TPSP* gene for academic research.

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