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Research Article

Optimization of *Agrobacterium*-mediated transformation procedure for grapevine 'Kyoho' with carrot antifreeze protein gene

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Abstract We report an *Agrobacterium*-mediated transformation procedure optimized for 'Kyoho' that is a major table grapevine cultivar in Korea, and its transgenic plants with antifreeze protein gene of carrot (*DcAFP*). The full length of *DcAFP* coding region in accordance with the previous report was isolated from young leaves of carrot and recombined into a plant transformation vector. Ethylene inhibitors such as silver nitrate and aminoethoxyvinylglycin (AVG) supplemented in a co-cultivation medium distinctly increased frequency of shoot regeneration when explants were sub-cultured in a selection medium: particularly ten-fold higher in treatment with 0.1 mg/L AVG than one without ethylene inhibitor. Among various antibiotics and their concentrations, the combination of 150 mg/L cefotaxime plus 150 mg/L Clavamox^{TM} was selected for elimination of *Agrobacterium* cells in addition to minimization of adverse effect on shoot regeneration, while 50 mg/L kanamycin monosulfate effectively suppressed regeneration of non-transgenic shoots. Applying the elucidated culture condition, we finally obtained a total of 5 transgenic 'Kyoho' plantlets with *DcAFP*, of which integration with the grapevine genome and transcription was confirmed by nucleic acid analyses.

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Keywords Genotype, *Agrobacterium* overgrowth, Antibiotics concentration, Vitis vinifera, Cold-tolerance

Introduction

Grapevine (*Vitis* spp.) is considered to be one of the major fruit crops in the world based on hectares cultivated and economic value (Torregrosa et al. 2015). Recently, various approaches to improve economically important characteristics in this crop has comprised genetic engineering, by which transgenic grapevines have been obtained in a stream (Iocco et al. 2001; Kim et al. 2013; Li et al. 2006). However, few efforts were involved in developing the event with enhanced cold-tolerance that is a primary characteristics in grapevine cultivation (Jin et al. 2009; Sun et al. 2016). Coldness like other abiotic stresses has adverse effects on the growth and development of grapevine plants (Wang and Nick 2017). A table grapevine 'Kyoho' is generally less cold-tolerant than the American grapevine cultivars derived from an interspecific hybridization (Sun et al. 2016), resulting in being cultivated in a greenhouse or buried during winter season in Korea. The former leads to increase cost of production while the latter make the scions be vulnerable to soil-borne diseases, especially to crown gall.

Since the characterization of antifreeze protein (AFP) from Antarctic fishes (DeVries et al. 1970), many other *AFP*s were isolated from Arctic fishes, insects, and carrot at last (Jang et al. 1999; Meyer et al. 1999; Worrall et al. 1998). Functional studies on the *AFP* of carrot (*DcAFP*) further revealed the inhibitory effects of its protein on ice growth and recrystallization, which suggests the potential of this gene for crop improvement. Although genetic transformation technology encourages to express *DcAFP* for cold-tolerance in grapevines, each grapevine genotype crucially demands a deliberate genetic transformation system merged with an efficient *in vitro* regeneration procedure (Fan et al. 2002; Fan et al. 2008). Of

various factors influencing on *Agrobacterium*-mediated transformation and further *in vitro* regeneration of transgenic plant, it has been reported that ethylene inhibitors such as silver nitrate and aminoethoxyvinylglycine dramatically improve the transformation efficiency in several plant species (Chi and Pua 1989; Han et al. 2005; Sgamma et al. 2015). Hence, to ascertain the effect of the ethylene inhibitors on *Agrobacterium* -mediated transformation in grapevine 'Kyoho' become one of the goals of this study. Collectively, we here report an *Agrobacterium*-mediated genetic transformation system optimized for grapevine 'Kyoho' and the transformants with *DcAFP* obtained as applying the improved system.

Materials and Methods

Plant materials

For isolation of carrot antifreeze protein gene (*DcAFP*), seeds of carrot (*Daucus carota* cv. Socheon) (Asia Seed Company, Seoul, Korea) were germinated under a 16/8 h (day/night) at 27°C. On the other hand, *in vitro* stock plants of grapevine (*Vitis vinifera*) 'Kyoho' were obtained *via* shoot tip culture using the methods by Kim et al. (2013) and Monette (1985). Briefly, the distal parts about 3 cm long were detached from rapidly growing shoots in the spring and surface-sterilized by submersion in 3.0% sodium hypochlorite solution for 10 minutes, followed by rising them with sterile distilled water. Each shoot tip with several leaf primordia was excised after removing individual leaf scales, then placed on the MS medium (Murashige and Skoog 1962) with 1 mg/L BA (Sigma-Aldrich, USA), 30 g/L sucrose (Duchefa Biochemie, The Netherlands), and 8 g/L Plant Agar™ (Duchefa Biochemie). When the explants developed a shoot about 2 cm long and four to five well expanded leaves, they were transferred to the multiplication medium consisted of MS, 1 mg/L BA, 0.1 mg/L IBA (Sigma-Aldrich), sucrose 30 g/L, 10 g/L Plant Agar™. Leaf discs from the multiplied shoots were excised and used as an explants for *Agrobacterium*-mediated transformation experiments.

Construction of pBI121-*DcAFP* vector and bacterial strain

Total RNA was extracted from carrot leaves using the method for pine trees (Chang et al. 1993) with some modification: use of extraction buffer consisting of 2% cetyltrimethylammonium bromide, 2% polyvinylpyrrolidone, 100 mM Tris-HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid, 2 M NaCl, 0.05% spermidine, and 0.24% DTT. Reverse transcription was performed from 3 μg of total RNA with oligo-dT by *Moloney murine leukemia virus* reverse transcriptase (Promega, Madison, WI, USA). We designed a primer set to amplify *DcAFP* from the synthesized first strand cDNA: forward primer 5'-ACTCGAAAACATAATCCA-3' and reverse primer 5'-TGCACTGCTTGAGCTGCATA-3'. After PCR, the product was separated by electrophoresis in 1.5% agarose gel. Band of the expected size (approximately 1,099 bp) was excised from the gel and purified by using GENECLEAN turbo kit (MPbio). To create pBI121-*DcAFP* binary vector carrying the *CaMV 35S* promoter::*DcAFP*::*NOS* terminator and *NOS* promoter::*nptII*::*NOS* terminator cassettes (Fig. 1), the purified amplicon was combined with pBI121 vector (Genbank AF485783.1) after digesting its *GUS* gene as described previously (Wu et al. 2012). The recombined plasmid was introduced into *Agrobacterium tumefaciens* strain LBA 4404 by an electroporation, and the transformed *Agrobacterium* was selected and used for co-cultivation with grapevine leaf explants.

Ethylene inhibitor treatment during co-cultivation of explants with *Agrobacterium*

To observe the effect of ethylene inhibitors, ethylene action inhibitor silver nitrate ($AgNO_3$: 0.1, 0.5, 1.0, 3.0 mg/L) and ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG: 0.001, 0.01, 0.1, 0.5 mg/L) were additionally supplemented in a co-cultivation medium. Co-cultivation medium was consisted of MS inorganic salts, 0.1 g/L myo-inositol, 0.8 mg/L thiamine-HCl, 30 g/L sucrose, 0.1 mg/L IBA, 10 mg/L acetosyringone (Sigma-Aldrich), 1 mM proline (Sigma-Aldrich), and 8 g/L Plant Agar™, followed by adjusting pH

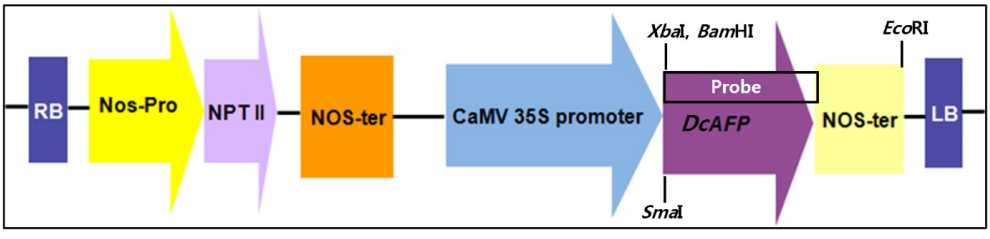

Fig. 1 Linear map of the T-DNA region of the recombinant pBI121-*DcAFP* showing the site of the inserted *DcAFP*. The *Eco*RI restriction site used for Southern blot analysis is shown above the map

to 5.9 before autoclaving at 121°C for 20 min. Culture flasks were sealed with rubber septum stoppers throughout co-cultivation. After co-cultivation, the explants were transferred to a selection medium. For the selection medium, 5 mg/L thidizuron (TDZ; Sigma-Aldrich) and 200 mg/L Clavamox™ (Pfizer, NY, USA) were supplemented in the co-cultivation medium, meanwhile acetosyringone, proline and ethylene inhibitors were removed from the co-cultivation medium. Regenerated shoots were observed five weeks after culturing on the selection medium.

Decision of compromised antibiotics strength

Four kinds of antibiotics (cefotaxime, carbenicillin, Clavamox™ and kanamycin) at various concentrations were added to the selection media to decide efficient antibiotics types and their concentrations for suppressing the development of non-transgenic shoot development but minimizing the inhibition of transgenic shoot regeneration (Table 2). Co-cultivation step was skipped for this experiment, and regenerated shoots were investigated.

Agrobacterium-mediated transformation

Agrobacterium cells were grown overnight at 28°C in the YEP medium containing 100 mg/L kanamycin on a rotary shaker (250 rpm) till an OD=1.0 at 550 nm. Leaf explants were inoculated with the *Agrobacterium* culture for 10 min on a rotary shaker (150 rpm), and blotted dry on sterile filter paper. Inoculated explants were placed onto the co-cultivation medium with 0.1 mg/L AVG. The co-cultivation was carried out for 3 days at 27°C in the dark. Following co-cultivation, the explants were rinsed several times with the combination solution of 150 mg/L cefortaxime and 150 mg/L Clavamox™. Further cultivation of the explants was carried out on the selection medium containing 50 mg/L kanamycin. Regenerated shoots were detached from mother explants, and then transplanted on a rooting medium. Rooting medium was consisted of half strength MS, 30 g/L sucrose, and 0.1 mg/L IBA. Rooted plantlets were transferred to plastic pot filled with commercial compost.

Nucleic acid analyses

Genomic DNA was extracted from young leaves using a modification of CTAB method (Torres et al. 1993). Approximately 100 mg of fresh leaves was put into a pre-chilled mortar and grinded in liquid nitrogen. A 700 μL CTAB buffer was added to the powder and the mixture was incubated at 65°C for 30 min. After incubation, equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube, equal volume of chloroform: isoamylalcohol (24:1) was added and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube, equal volume of isopropanol was added and centrifuged at 13,000 rpm for 5 min. The precipitated DNA was washed with 1 mL of 70% ethanol and dried for 1 h. The DNA was dissolved in 100 μL of sterile H_2O with 2 μL of RNase A and incubated at 37°C for 1 h. After determination of DNA concentration and purity, the extracted total DNA was stored at −20°C.

Detection of integrated transgenes was performed by PCR and Southern blot analyses (Southern 1975). Primer sets for PCR analysis were designed to amplify *DcAFP* and *nptII* fragments: primer set for *DcAFP* mentioned above, and forward primer for *nptII*, 5'-ATACTTTCTCGGCAGG AGCA-3' and reverse primer for *nptII*, 5'-ACAAGCCGT TTTACGTTTGG-3'. Southern blot analysis was also performed to confirm stable integration of the *DcAFP*. Briefly, a ten μg of genomic DNA was digested with the restriction enzyme *Eco*RI. Following gel electrophoresis, separated DNA fragments were transferred to Hybond N nylon membrane and hybridized to a PCR-generated DIG-labeled probe. Hybridization was carried out at 68°C for 16 h. DIG Easy Hyb, DIG Wash/Block Buffer set and CDP-Star Ready to Use (Roche) were used for hybridization, washing and detection, respectively. Detected blots were exposed to X-ray films (Amersham Bioscience). In addition, total RNAs were extracted from the newly developing leaves of the plants using Plant RNeasy Mini kit (QIAGEN, USA). First-strand cDNAs were synthesized from 1 μg of total RNA with random hexamers using SuperScript III First-strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. The primer set mentioned above for detection of *DcAFP* was used again for its cDNA amplification.

Results and Discussion

Creation of recombinant pBI121-*DcAFP* vector

A fragment of about 1,099 bp in length was amplified from the cDNA of carrot by RT-PCR, which was recombined into pBI121 binary vector, resulting in creation of pBI121-*DcAFP* (Fig. 1).

Increase of shoot regeneration efficiency by using ethylene inhibitors in co-cultivation medium

Wounded plant tissues are able to produce significant amounts of ethylene during *in vitro* culture (Beyer 1979; Pua 1993). Besides wound ethylene obstructs adventitious shoot regeneration from explants, the production of this gaseous phytohormone is promoted by *Agrobacterium* inoculation, resulting in a reduction in the efficiency of foreign gene transfer (Ezura et al. 2000; Han et al. 2004; Han et al. 2005; Nonaka et al. 2008). To ascertain the beneficial effects of $AgNO_3$ and AVG in grapevine transformation, 0.001 ~ 0.5 mg/L AVG or 0.1 ~ 3.0 mg/L $AgNO_3$ were supplemented in the co-cultivation medium. The application of 0.1 mg/L AVG or $AgNO_3$ increased the regeneration frequency by 5% or 4.3%, respectively (Table 1). It has been known that wound ethylene production varies among species, genotypes and tissue types (Ezura et al. 2000). Our result implies that ethylene inhibitors such as AVG and $AgNO_3$ at *Agrobacterium* co-cultivation step can facilitate regeneration of putative transgenic shoots in grapevine as well, although we did not verify whether the regenerated shoots were transgenic or not. Thus, 0.1 mg/L AVG was added in the co-cultivation media during the following experiments.

For this experiment, 50 mg/L kanamycin and 200 mg/L Clavamox™ were also supplied in the selection medium. The data represent the mean values nine replicates. Values followed by the same letter within the last column are not significantly different according to Ducan's multiple range test at the 5% level.

Table 1 Effects of ethylene inhibitors in co-cultivation medium on shoot regeneration from 'Kyoho' leaf explants sub-cultured in selection medium

Ethylene inhibitor (mg/L) in co-cultivation medium		Shoot regeneration (%) in selection medium
AVG	0.0	0.5 d
	0.001	2.9 c
	0.01	3.3 c
	0.1	5.0 a
	0.5	4.1 b
$AgNO_3$	0.1	4.3 b
	0.5	3.2 c
	1.0	3.0 c
	3.0	3.3 c

For this experiment, 50 mg/L kanamycin and 200 mg/L Clavamox™ were also supplied in the selection medium. The data represent mean values of 9 replicates. Values followed by the same letter within the last column are not significantly different according to Duncan's multiple range test at the 5% level

Effects of antibiotics on shoot regeneration

In almost *Agrobacterium*-mediated transformation trials, antibiotics such as carbenicillin, cefotaxime and Clavamox™ are widely used to eliminate bacterial cells that have completed their mission (Alsheikh et al. 2002; Mathias and Boyd 1986). Kanamycin is also applied for selecting out any non-transgenic regenerants if *nptII* is inserted into T-DNA as a selectable marker gene. However, plant regeneration can be disturbed by the antibiotics, especially severely at high concentration (Holford and Newbury 1992; Ling et al. 1998). We tested various antibiotics for application in the subsequent *DcAFP* transformation experiments. The higher concentrations of the antibiotics tendentially showed lower regeneration efficiency, while kanamycin that is the selectable antibiotics in current study drastically suppressed shoot regeneration despite the low concentrations at 25 mg/L or 50 mg/L (Table 2). As results, we chose 150 mg/L cefotaxime plus 150 mg/L Clavamox™ as the compromised condition for controlling feasible *Agrobacterium* over-growth in a selection medium and 50 mg/L kanamycin as the concentration for suppressing the regeneration of non-transgenic shoots.

The data represent the mean values six replicates. Values followed by the same letter within the last column are not significantly different according to Ducan's multiple range test at the 5% level.

Table 2 Effects of antibiotics on shoot regeneration from leaf explants in grapevine 'Kyoho'

Antibiotics (mg/L)		Shoot regeneration (%)
	0	30.9 a
Cefotaxime	150	26.7 b
	250	16.4 c
Carbenicillin	300	25.8 b
	500	18.5 c
Clavamox™	100	20.7 bc
	200	17.5 c
Cefotaxime/Clavamox™	100/100	18.9 c
	150/150	15.7 c
Kanamycin	25	8.4 d
	50	2.0 e

The data represent mean values of 6 replicates. Values followed by the same letter within the last column are not significantly different according to Duncan's multiple range test at the 5% level

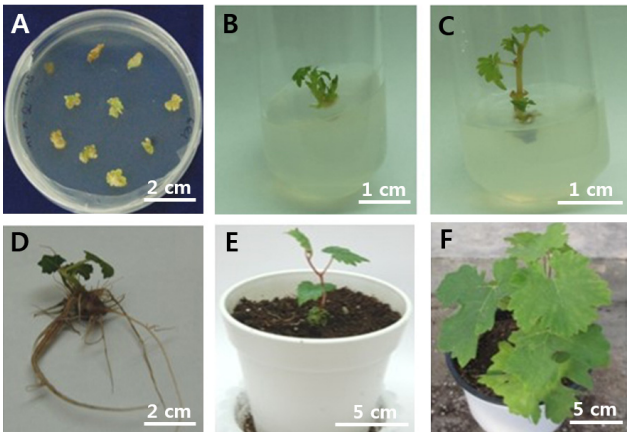

Fig. 2 Exhibition on generation of *DcAFP*-transformant in grapevine 'Kyoho'. A: Induction of putative transgenic shoot from leaf explants on a selection medium, B-C: Detached and subsequently sub-cultured shoot on a rooting medium, D: Plantlet with vigorous roots just before transplanting to a pot, E-F: Acclimatization and growth of the plantlets ex vitro

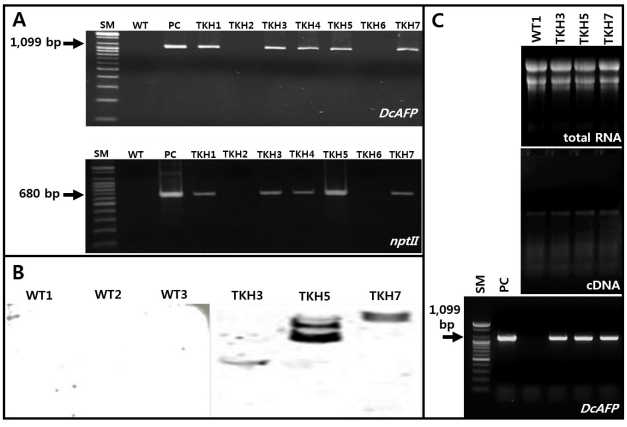

Fig. 3 Detection of transgenes and transcript of *DcAFP* through PCR, Southern blot, and RT-PCR analyses. A: PCR analysis of 7 acclimatized plantlets, B: Southern blot analysis for *DcAFP* of randomly selected three PCR-positive plantlets, C: RT-PCR analysis for detecting transcript of *DcAFP*, SM: 100 bp size marker, WT1-WT3: wild type plants, PC: positive control (plasmid DNA), TKH1-TKH7: putative transgenic plantlets

Generation of *DcAFP*-expressing plants

With five batches of *DcAFP* transformation experiment, a thousand of leaf explants were co-cultivated with the *Agrobacterium* in the medium with 0.1 mg/L AVG and then transferred on the selection medium with antibiotics. Sixteen adventitious shoots with a little bit of calli were regenerated (approximately 1.6%) on the selection medium (Fig. 2A). When the detached shoots were transplanted onto a rooting medium, all of sixteen shoots vigorously rooted (Fig. 2B-D). However, nine plantlets failed in *ex vitro* acclimatization while seven successfully adapted to a greenhouse condition (Fig. 2E-F). This result also implies that the acclimatization is an important step for grapevine in terms of transformation efficiency.

Completely acclimatized plantlets were subjected to PCR, Southern blot, and RT-PCR analyses to detect the transgenes and the transcript of D*cAFP*. Among the seven plantlets, five plantlets contained *DcAFP* and *nptII* at the size of 1,099 bp and 680 bp, respectively (Fig. 3A). This result shows that the transformation efficiency of current study based on the PCR result was about 0.5%. Southern blot analysis also showed the *DcAFP* insertion into the 'Kyoho' genome and the number of T-DNA copy ranging from one to four (Fig. 3B). In addition, the result of RT-PCR analysis indicated that the *DcAFP* gene(s) in each transgenic plant transcribes normally.

Together, we generated transgenic grapevine 'Kyoho' plants with carrot antifreeze protein gene (*DcAFP*) as simultaneously improving *Agrobacterium*-mediated transformation procedure, especially as applying ethylene inhibitors and compromised selection pressure. The final transgenic products are going to be tested for cold tolerance after vegetative propagation. In addition to current study, we are making to effort for enhancing the cold tolerance of a rootstock that is an important factor in grapevine cultivation.

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