A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Rapid Shoot Propagation and *Agrobacterium*-mediated Transformations from Micro-cross Sections in 'Hayward' Kiwifruit (*Actinidia deliciosa*)

'헤이워드' 참다래(*Actinidia deliciosa*)에서 미세절단 배양에 의한 급속 증식과 아그로박테리움 매개 형질전환

By

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Department of Horticultural Science

GRADUATE SCHOOL

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A dissertation submitted in partial fulfillment of the requirement of the degree of Doctor of Philosophy

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ABSTRACT

This study was conducted to develop a rapid shoot propagation technique using micro-cross sections (MCS) method and increase carotenoid content in 'Hayward' kiwifruit (*Actinidia deliciosa*).

Rapid propagation technique of kiwifruit shoot through a micro-cross sections (MCS) system was established in this study. Optimal culture conditions were determined for different types of explant, section sizes, concentrations of inorganic salts, and plant growth regulators. The rates of survival and callus formation were higher in half-strength MS salts than in full-strength MS media. Similar performance in terms of survival and callus formation was achieved with section sizes of either 800 μm or 1200 μm. The auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) was the most effective in promoting callus formation and shoot production that only occurred with zeatin or TDZ treatments. TDZ, however, resulted in excessive number of abnormal shoots. Therefore, zeatin was used as hormone for shoot regeneration in micro-cross sections protocol. Proliferation efficiency was the greatest when explants from stem tissue were cultured on half-strength MS supplemented with 0.001 mg/mL 2,4-D and 0.1 mg/mL zeatin. The number of shoots averaged 2.61 per explant, representing an efficiency of 94%.

The shoots regenerated by MCS were subjected to RAPD and SSR analysis to detect somaclonal variation. In RAPD analysis, 19 of 24 primers produced 63 bands that ranged from 500 to 3500 bps. In SSR analysis, 17 of 19 primers showed 46 reproducible bands that ranged from 50 to 1500 bps. RAPD and SSR analysis revealed that the regenerated plants from our MCS system were genetically stable. These results show that the culture of micro-cross sections from stem tissue is a powerful method for kiwifruit propagation.

As a molecular breeding to improve the quality of kiwifruit, carotenoid biosynthetic genes were introduced by *Agrobacterium*-mediated transformation method. The used genes are as follows; *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCABMIA1300 and *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121. Each plasmid contained the carotenoid synthesis genes such as *GGPS* that encoded geranylgeranyl diphosphate synthase, *PDS* that encoded phytoene desaturase, *ZDS* that encoded ζ-carotene desaturase, *CHX* that encoded β–carotene hydroxylase, and *PSY* that encoded phytone synthase. Also, Hygromycin phosphotransferase II (*HPT*II) gene and neomycin phosphotrasnferase II (*NPT*II) gene were used as selectable markers.

The transformed plant was selected on half-strength MS medium containing 0.001 mg/mL of 2,4- D and 0.1 mg/mL of zeatin, either 5 μg/mL hygormycin or 25 μg/mL kanamycin and 500 μg/mL cefotaxime. The survival rate for total treated explants were 13.3% in *GGPS*, 4.3% in *PDS*, 16.4% in *ZDS*, 35.3% in *CHX*, 68.4% in *PSY*, respectively. The shoot regeneration efficiency of treated explants were 13.3% in *GGPS*, 4.3% in *PDS*, 16.4% in *ZDS*, 14.11% in *CHX*, and 37.9% in *PSY* transgenic plants. The transformation efficiency was 8% in *GGPS*, 2.9% in *PDS*, 12.3% in *ZDS,* 9.4% in *CHX*, and 22.1% in *PSY* when each target gene was checked and 12% in *GGPS*, 2.9% in *PDS*, 13.7% in *ZDS,* 14.1% in *CHX*, and 24.2% in *PSY* when selection marker gene was checked. The PCR positive for regenerated shoot was 66.7% in *GGPS*, 100% in *PDS*, 90% in *ZDS*, 66.7% in *CHX*, and 87.5% in *PSY* for the target specific genes and indicated also 100% except for 95.8% in *PSY* for the reporter genes.

The RT-PCR results showed that the transcripts of each inserted genes with the expected size were 160 bps for *GGPS*, 163 bps for *PDS*, 269 bps for *ZDS*, 508 bps for *CHX*, and 540 bps for *PSY* transgenic plants. The Southern analysis for *HPT*II

gene showed a single band or more than one band in *GGPS* transgenic plants.

The HPLC analysis revealed that β-carotene contents were not changed in the transgenic plants overexpressing *GGPS*, *PDS*, and *ZDS*. However, *GGPS* clone 8 was increased by 1.2 folds with 0.25 mg/g in lutein content than non-transgenic plants. CHX transgenic plants were increased by 1.6 to 2.0 folds with 0.33 mg/g to 0.39 mg/g in lutein content and by 1.7 to 2.0 folds with 4.3 mg/g to 5.6 mg/g in β-carotene content than non-transgenic plants. *PSY* transgenic plants were also increased by 1.6 to 1.9 folds with 0.3 mg/g to 0.34 mg in lutein content and by 1.35 to 1.7 folds with 3.4 mg/g to 4.3 mg/g in β-carotene content than non-transgenic plants.

In conclusion, we suggest that *CHX* and *PSY* genes are functional and potentially useful to increase carotenoid production than *GGPS*, *PDS*, and *ZDS* genes.

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ABBREVIATIONS

ABA	abscisic acid
AFLP	amplified fragment length polymorphism
BA	benzyladenine
BHT	2,6-Di-tert-butyl-4-methylphenol
CHX	β-carotene hydroxylase
GGPP	geranylgeranyl pyrophosphate
GGPS	geranylgeranyl pyrophosphate synthase
DMAPP	dimethylallyl pyrophosphate
IBA	indole-3-butyric acid
IPP	isopentenyl pyrophosphate
LCYB	lycopene β-cyclase
MCS	micro-cross sections
MeOH	methanol
MS	Murashige and Skoog
NAA	naphthaleneacetic acid
NH_4Cl	ammounium chloride
PDS	phytoene desaturase
PSY	phytone synthase
RFLP	restriction fragment length polymorphism
RAPD	random amplification of polymorphic DNA
TDZ	thidiazuron
2,4-D	2,4-dichlorophenoxyacetic acid
RT-PCR	reverse-transcriptase PCR
SSR	simple sequence repeat
ZDS	ζ-carotene desaturase

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Rapid Shoot Propagation of Micro-Cross Sections of 'Hayward' Kiwifruit (*Actinidia deliciosa*)

I. Introduction

The genus *Actinidia*, commonly known as kiwifruit, includes 66 species and 118 taxa. This species has a distribution from Siberia, Korea and Japan through China to Indo-China, Thailand, India etc., with a remarkably wide natural range extending from the tropics (latitude 0°C) to cold temperate regions (50 °N). These primarily deciduous plants are climbing or scrambling perennial vines. All species also appear to be dioecious and vary in size, shape, hairiness, and external color (Warrington and Weston, 1990; Ferguson, 1999; Huang et al., 2004). The main cultivar in the market is 'Hayward', which was selected from seeds of *Actinidia deliciosa* var. *deliciosa* imported into New Zealand from China in 1904 (Ferguson and Bollard, 1990). This fruit is popular world wide because of its nutritional and medicinal values but it has short history of cultivation. For example, kiwifruit has a higher vitamins C (92.7 mg/100 g) and E (1.46 mg/100 g) content than oranges, apples, bananas, and mangoes (USDA, 2004).

International kiwifruit industry of approximately 100,000 ha has been developed (Hunag et al., 2004) and the worldwide annual production of kiwifruit is approximately one million tons at present (FAO, 2006). This fruit mostly has been cultivated around the southern coastal area as Jeollanamdo, Gyeongsangnamdo, and Jeju since introduced 1970s in Korea. It has been increased to about 982 ha in cultivation area and 15,000 tons of annual production in 2006 (FAO, 2006).

Cultivation area for kiwifruit will also be expanding because of the Free Trade Agreements (FTAs) for Global trading market that requires changing economic crop as citrus, recently.

On the other hand, newer varieties have now been bred those that have a range of flesh colors and high contents of vitamin C and other functional substances. Global kiwifruit breeding programs began in the late 1970s. Several cross-breeding and biotechnology techniques have been investigated for developing more varieties (Janssen and Gardner, 1993; Ferguson, 1999; Kobayashi et al., 2000; Jung et al., 2003; Kim et al., 2003, 2004; Huang et al., 2004; Kim et al., 2007a).

Tissue culture has been the best method for rapid propagation and genetic improvement of kiwifruit, relying mainly on inductions from shoot tips (Marino and Bertazza, 1990) and axillary buds (Shen et al., 1990). Although culturing of the leaf, stem, and petiole have recently been examined, results have varied according to species, cultivar, sex, explant type, and culturing conditions (González et al., 1995; Zhang et al., 1998; Takahashi et al., 2004).

A thin cell layer (TCL) technique, using small and thin explants derived from plant organs, was first introduced with tobacco (*Nicotiana tabacum*; Tran Thanh Van, 1973). This method has been successful for the rapid proliferation of certain plant species, including the common bean (*Phaseolus vulgaris*), large crabgrass (*Digitaria sanguinalis*), lily (*Lilium longiflorum*), oilseed rape (*Brassica napus*), and trifoliate orange (*Poncirus trifoliate*) (Klimaszewska and Keller, 1985; Van et al., 1998, 1999; Cruz de Carvalho et al., 2000; Nhut et al., 2002). Although two sectioning methods – hand or machine – have been tested for TCL culture, explants obtained manually have been irregular and difficult for stable propagation. Therefore, Lee-Stadelmann et al. (1989) have developed micro-cross sections (MCS) technique using a vibratory microtome, and have achieved stable propagation of uniform products from poplar

(*Populus* spp.). This method exhibited a proliferation rate of approximately 25 times than that of the conventional method using 1.0 cm leaf explants.

Although the ultimate goal of tissue culture is rapid multiplication, *in vitro* techniques can lead to genetic mutation, especially somaclonal variation (Larkin and Scowcroft, 1981). This becomes a critical issue when proliferating valuable germplasms that are genetically fixed. Therefore, micro-propagation should be accompanied by a molecular marker approach, e.g., Amplified Fragment Length Polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), or simple sequence repeat (SSR), that can detect somaclonal variants, as has been done with garlic (Al-Zahim et al., 1999), cauliflower (Leroy et al., 2000), oil palm (Jaligot et al., 2002), kiwifruit (Palombi and Damiano, 2002), and pine (Goto et al., 1998; Burg et al., 2007).

This study was conducted to develop a new method for rapid and stable propagation of kiwifruit shoots compared with other tissue culture methods, as verified via RAPD and SSR marker analysis.

II. Materials and Methods

1. Preparation of explants and cultures

Branches from a 10-year-old vine of 'Haywrad' kiwifruit (*A. deliciosa*) were placed in water for 3 weeks. Tips from newly emerged shoots without leaves were immersed in 70% (v/v) ethanol for 1 min, then sterilized for 7 min in 1% sodium hypochlorite solution (v/v) containing one drop of Tween 20. After they were rinsed four times with sterile distilled water, the explants were placed in the test tubes containing MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v) and solidified with 0.8% agar (w/v) at pH 5.8 (adjusted with 1N NaOH before autoclaving at 121°C for 12 min). The cultures were incubated at 25 ± 2°C under a 16 hr photoperiod supplied by fluorescent lamps (45 μmol m^{-2} s^{-1}). The shoots were later transferred to a 500 mL jar containing 100 mL of a proliferation medium with 1 mg/mL benzyladenine (BA) and 0.1 mg/mL indole-3-butyric acid (IBA). All cultures were incubated at 25 ± 2°C, with a 16-h photoperiod under fluorescent lamps (45 μmol m^{-2} s^{-1}).

2. Preparation of MCS

The section segments were prepared with a Vibratory Microtome (Vibratome® Series 1000 Sectioning System, USA). Micro-cross sections of organs were prepared by the modified procedure of Lee-Stadelmann et al. (1989). Briefly, the interior of the Vibratory Microtome was sterilized with 70% (v/v) ethanol for 1 h, then rinsed with sterile distilled water. A Styrofoam block (2×2 cm) and rubber string for fixing the tissues were immersed in absolute alcohol for 1 day, then rinsed with sterilized water. Segments obtained from this transversal micro-cross section system were

stored in 1/2 MS liquid medium prior to the onset of the experiments. The entire micro-cross sectioning process is illustrated in Figure 1.

3. Regeneration of MCS

3.1. Effect of inorganic salt, hormone and segment thicknesses

The effect of inorganic salt concentrations was studied by using either half- or full-strength MS media. Three segment thicknesses such as 400 μm, 800 μm, and 1200 μm were tested for their suitability in regeneration. The optimal concentrations of auxins [naphthaleneacetic acid (NAA), IBA, and 2,4-dichlorophenoxyacetic acid (2,4-D)], cytokinins [benzyladenine (BA), kinetin, zeatin], and a cytokinin-like substance [thidiazuron (TDZ)] were also evaluated.

3.2. Suitable regeneration condition by 2,4-D and zeatin combinations

Tissue segments from petiole, stem, and leaf were placed on half-strength MS supplemented with sucrose (3%, w/v) plus a combination of 2,4-D (0.001, 0.01, 0.1, or 1 mg/mL) and zeatin (0.001, 0.01, 0.1, or 1 mg/mL). The pH was adjusted to 5.8, and plant agar (0.8%, w/v, Duchefa, The Netherlands) was included as a gelling agent. Zeatin was also added to the medium after filter-sterilization. All treated cultures were replicated 3 times in 87 ×15 mm Petri dishes. After 7 weeks, the regeneration characteristics, including survival rate, fresh plant weight, and the number of shoots produced per explant were examined.

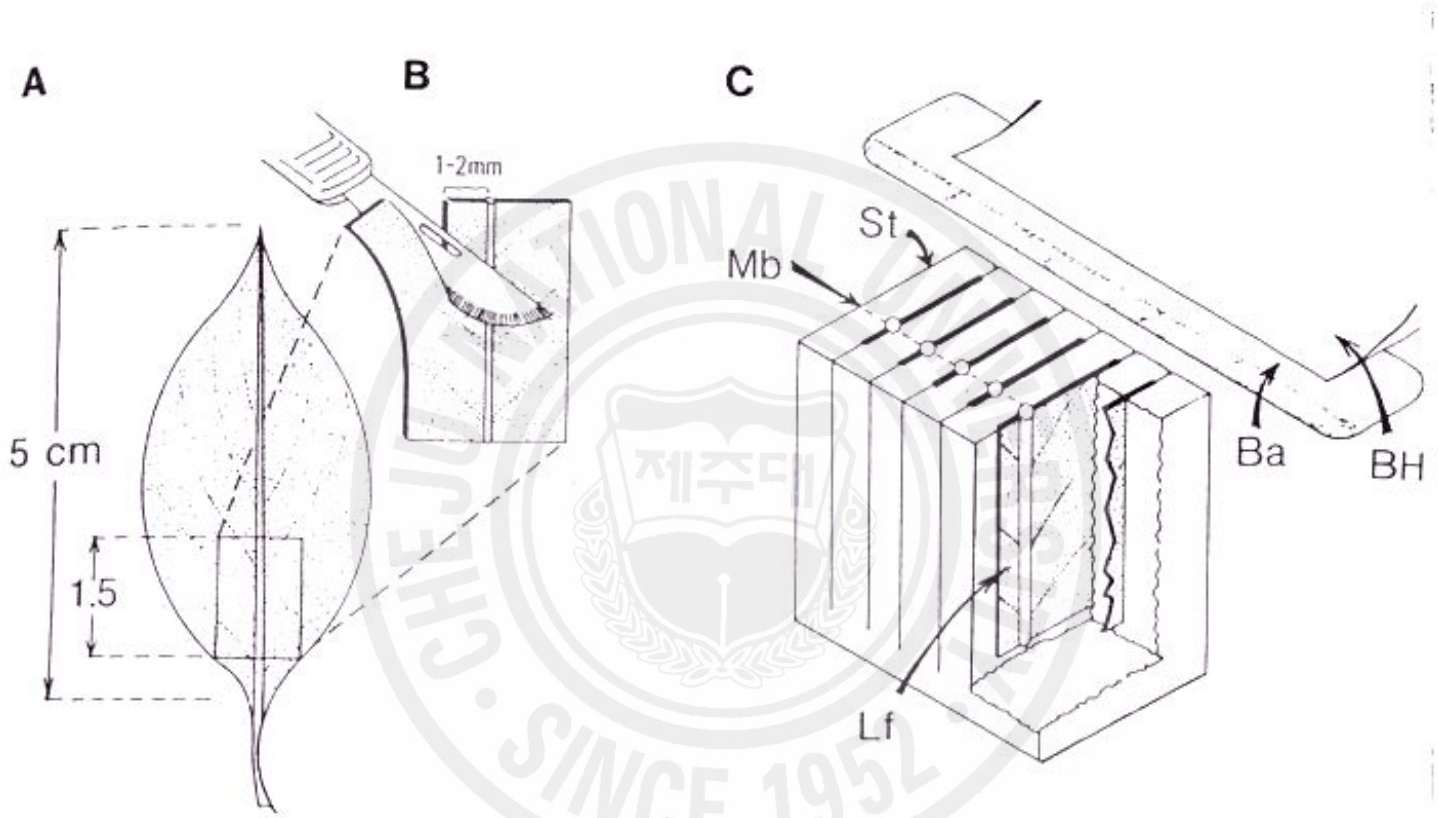


Fig. 1. The total procedure of sectioning leaf segments of 'Hayward' kiwifruit (*A. deliciosa*) using micro-cross section method of Lee-Stadelmann et al. (1989). (A) Leaf segments from the basal portion of the leaf; (B) lamina both sides of the midvein removed except for 1 mm remnat; (C) leaf segments placed into incisions of the syryofoam block. Mb, midveins; St, Styrofoam block; Lf, leaf segements; Ba, brade; BH, blade holder

4. Genetic stability analysis

4-1. Isolation of genomic DNA

The DNA was extracted from 1 g of tissue according to a method modified from that of Dellaporta et al. (1983). Approximately 1.5 g of fresh leaves was poured with aqueous nitrogen, which was suspended and incubated at 60°C for 30 min in isolation buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 2% SDS; 1% PVP; 0.1% β-mercaptoethanol). And then, DNA was immediately precipitated with 4 mL of 5 M potassium acetate (pH 6.5) on ice for 5 min and centrifuged at 13,000 rpm for 10 min at 4°C, which was dissolved by TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). For DNA re-precipitation, which was centrifuged with same revolutions and the clear lysate was stored at -20°C for 2 hr in an equal amount of isopropyl alcohol (contains 1/10 fold of sodium acetate, pH 7.6). The residual RNA and protein were dissolved with RNase A (Promega, USA) and Proteinase K (Promega, USA) in TE buffer. They were re-suspended by same amount of phenol and upper layer was collected by spin down and then re-treated with same amount of phenol : chloroform (1:1). The purified DNA was collected with ethanol precipitation method and recovered by TE buffer.

4-2. RAPD analysis

The RAPD analysis was conducted on 29 plants obtained with our MCS method. Random decamer primers included OPO 01 to OPO 13 and OPP 01 to OPP 20 (Operon Technologies, USA) (Table 1). Ten ng of DNA was used as template in a reaction mixture (total volume 25 μL) that contained 2.5 μL of 10x reaction buffer, 500 μM of each dNTP, 0.25 μM of primer, and 1.5 units i*Taq* DNA polymerase (iNtRON Bio, Korea). The PCR amplifications were performed in a GeneAmp PCR System 9600 Thermal Cycler (Perkin Elmer Cetus, USA), with initial denaturation

Table 1. The primer sequences used in RAPD analysis.

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
A (OPP 01)	GTAGCACTCC	K (OPP 17)	TGACCCGCCT
B (OPP 03)	CTGATACGCC	L (OPP 20)	GACCCTAGTC
C (OPP 04)	GTGTCTCAGG	M (OPO 01)	GGCACGTAAG
D (OPP 05)	CCCCGGTAAC	N (OPO 02)	ACGTAGCGTC
E (OPP 08)	ACATCGCCCA	O (OPO 03)	CTGTTGCTAC
F (OPP 10)	TCCCGCCTAC	P (OPP 08)	CCTCCAGTGT
G (OPP 11)	AACGCGTCGG	Q (OPO 09)	TCCCACGCAA
H (OPP 12)	AAGGGCGAGT	R (OPO 10)	TCAGAGCGCC
I (OPP 13)	GGAGTGCCTC	S (OPO 11)	GACAGGAGGT
J (OPP 14)	CCAGCCGAAC		

for 2 min at 94°C; then 35 cycles of 1 min at 94°C, 1 min at 38°C, and 2 min at 72°C; followed by 5 min of final extension at 72°C.

4-3. SSR analysis

The SSR analysis was conducted on 30 plants obtained with our MCS method. This analysis was carried out by microsatellites consisting of tandem repeats of short sequence (Huang et al., 1998) (Table 2). The PCR amplifications and reactions for SSR were performed in an ABI2700 thermal cycler (Applied Biosystmes, USA) according to Huang et al (1998).

4-4. Electrophoresis of PCR products

The RAPD and SSR products were separated on 1% or 3% Seakem agarose LE (FMC BioProducts) agarose gels at 100 V for 40 min using 1x Tris-borate-EDTA (TBE) buffer, and finally stained with ethidium bromide (Figs. 4 and 5).

5. Statistical analysis

Our study discriminated between normal shoot as morphologically normal and abnormal shoot such as distortions, malformation, and hyperhydricity because abnormal shoot is possible to have a somaclonal variation in regenerated shoots.

This study was designed to distinguish between normal shoots and those with abnormalities that possibly resulted from somaclonal variation. Data were analyzed using SAS (2002) Version 6.12, and significant differences among means were tested using Duncan's multiple range tests at $P \leq 0.05$.

Table 2. The primer sequences used in SSR analysis.

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
A (UDK96-001)	F-GAATCGCGTAATGATTGATGG R-GTTTCCCACTCTGCAAAAGC	K (UDK96-039)	F-GGTTTGATCGGTCTTCGAAA R-ATAAATGTGTGCCAGTGCGA
B (UDK96-015)	F-CCGAGTCATGATCGAGTTGA R-GGCTCAACTTGGAGAAGTGG	L (UDK96-040)	F-TCGAGTTACCTAGCTACTCCGC R-CAAGGGAAGAAAATGTTGAACC
C (UDK96-016)	F-TTAGGTGAAAGACACACCACAC R-ATAAACGTCATGGGCTCAGC	M (UDK97-401)	F-GAGCAAAAAGCTTGACACCC R-ACCTGGTGGTAGATCCTCCA
D (UDK96-018)	F-GTAAACTGCATTTGGTCCTCG R-TTAGTTTTCATCCTTGGTTCCA	N (UDK97-402)	F-ATATGATAGAACGCCATCCCC R-GACGTGCCATCAGTTTCCTT
E (UDK96-020)	F-TTGGCTCGATTAACTGTTTA R-TCTGCTGTCAAACTGGATCA	O (UDK97-403)	F-CTTTTTTGAATTCGTCCCCA R-GCGGCGAAGGTGAATAATCA
F (UDK96-023)	F-CCAAACGAGTCCTAGATCTTGC R-ATCTGGAGCCATACGGAGG	P (UDK97-408)	F-GTGCTCCTCCGTCCATGTAT R-CGTCCTCTCTTCGCCATTTA
G (UDK96-026)	F-CGCTGACCAGATTCTGATGA R-TTGAAAATCACTGAGCACAACC	Q (UDK97-409)	F-ATGACCTATTGCCAAGTGGC R-TTGTGTGTACCCACCACCC
H (UDK96-030)	F-TCATGTTTGTGGTTGAGTTGTG R-AGCAATAAACTCAAGCGCGT	R (UDK97-411)	F-TGATTAAATCCCCAAACCCA R-TCCCAATCTCATCATCCCTC
I (UDK96-033)	F-AGGCAGCAATGCTCTCTCTC R-CTGGTGGACAGGGCTAAAG	S (UDK97-414)	F-GCCATTTCAAGAACATTTTTTG R-TGGCTATATTTTGCAAGCCC
J (UDK96-034)	F-TTATATGGTGCGGCATGCTA R-TGAATGCAGAAGGCAATCAG		

III. Results and Discussion

1. Selection of suitable culture conditions for MCS

1-1. Selection of inorganic salts

To determine how the growth response of kiwifruit cultures was associated with the concentration of inorganic salts, we tested petiole segments (800 μm) were tested on half-strength MS and full-strength MS media, and the survival rates of 100% and 88.9%, obtained respectively. The size of the callus did not differ between them, although some segments gradually died without forming any calli on full-strength MS medium (Table 3), perhaps because of higher osmotic stress than from the half-strength MS salts.

Table 3. The percent survival and callus formation and size of kiwifruit in micro-cross section cultures affected by concentration of inorganic salts in MS media[z].

Medium	Survival (%)	Callus formation (%)	Callus size[x] (cm)
Full-strength MS[y]	88.9	88.9	1.12 ± 0.09
Half-strength MS	100.0	100.0	0.99 ± 0.02

[z]All materials were cultured on inorganic salts medium + 3% sucrose + 0.8% agar + 0.1 mg/mL 2,4-D (pH 5.8).

[y]MS, Murashige and Skoog (1962) medium.

[x]Callus size is given as means ± SE.

1-2. Selection of optimum size

The optimum size for segments was 800 μm (Table 4), which differed from the 400 to 500 μm recommended for poplar leaf sections (Lee-Stadelmann et al., 1989).

Table 4. The percent survival and callus formation and size of kiwifruit in micro-cross section cultures affected by fragment size and type of tissue used[z].

Tissue type	Size (μm)	Survival (%)	Callus formation (%)	Callus size[y] (cm)
Leaf	400	6	6	0.02 ± 0.02
	800	100	100	0.53 ± 0.05
	1200	100	100	0.69 ± 0.04
Stem	400	100	100	0.62 ± 0.02
	800	100	100	0.67 ± 0.02
	1200	100	100	0.72 ± 0.02
Petiole	400	100	100	0.61 ± 0.03
	800	100	100	0.62 ± 0.01
	1200	100	100	0.66 ± 0.01

[z]All materials were cultured on half-strength MS medium + 3% sucrose + 0.8% agar + 0.1 mg/mL 2,4-D (pH 5.8).

[y]Callus size is given as means ± SE.

However, the results obtained in this study were similar to previous findings reported that the number of developing shoots increases in proportion to explant size for poplar leaves (Lee-Stadelmann et al., 1989), while the number of shoots diminishes by increment in internode size for native spearmint tissues (Poovaiah et al., 2006).

1-3. Selection of plant growth regulators

All explants formed calli after 10 days of exposure to various plant growth regulators. Among the plant growth regulator tested, 2,4-D was the most effective in

Table 5. The percent survival, callus formation and size, and percent shoots formation in micro-cross section cultures with various plant growth regulator in half-strength MS medium[z].

Plant growth regulator	Survival (%)	Callus formation (%)	Callus size[x] (cm)	Shoot formation (%)
2,4-D	100	100	1.07 ± 0.02	0
NAA	100	100	0.47 ± 0.05	0
IBA	94.44	77.8	0.31 ± 0.04	0
Zeatin	94.44	94.4	0.51 ± 0.05	66.67
BA	44.44	27.8	0.20 ± 0.02	0
Kinetin	66.67	72.2	0.35 ± 0.04	0
TDZ	100	100	0.73 ± 0.03	100

[z]All materials were cultured on half-strength MS medium + 3% sucrose + 0.8% agar (pH 5.8).

[y]MS, Murashige and Skoog (1962) medium.

[x]Callus size is given as means ± SE.

promoting callus formation. Shoot production only occurred with zeatin or TDZ treatments; BA and kinetin were ineffective (Table 5). The positive effect of TDZ on multiple-shoot induction has also been shown in fig (Kim et al., 2007b). Nevertheless, an excessive number of abnormal shoots also appeared from our TDZ-treated tissues. Similar observations have been made with *Arabidopsis* (Gleddie, 1989) and kiwifruit (Kim and Oh, 1998). Not only morphological abnormalities increase with higher TDZ concentrations (Huetteman and Preece, 1993), but even lower levels are associated with numerous malformations and the production of hyperhydric shoots (Fraser et al., 1995). Because Kim and Oh (1998) have also reported that zeatin is

more effective than other cytokinins and cytokinin-like substances for tissue culture of kiwifruit, that hormone was chosen for the micro-cross section protocol of this study.

2. Regeneration of normal shoots in MCS

Tables 6 to 8 present a comparison among various parameters evaluated to determine the most suitable conditions for regenerating normal shoots. Leaf and petiole explants were more successfully utilized on culture media containing 0.001 mg/mL 2,4-D and 1 mg/mL zeatin than other combinations. The frequency of normal shoot formation was also higher from petiole segments (50.0%) than from leaves (11.1%). Although no shoots formed from leaf or petiole explants cultured on media lacking growth regulators, it was possible to produce few shoots from stem tissues under the same condition (Tables 6 and 7). When stem segments were cultured on media containing zeatin, the number of normal shoots and the rate of shoot formation increased in proportion to the concentration of zeatin applied. Moreover, the number and rate of normal shoots increased at higher zeatin concentrations in combination with 2,4-D at a level below 0.001 mg/mL. The number of regenerated shoots averaged 2.61 per explant, at a formation rate of 94% (Table 8 and Fig. 2). Takahashi et al. (2004) have reported that, on a zeatin-supplemented medium, leaf explants from *A. polygama* Miq. show obviously poorer shoot formation than do stem or petiole explants. However, they have also found that particular tissues respond differently to phytohormones. In contrast, Sugawara et al. (1994) have demonstrated that tissue type (leaf, petiole, or stem) is not a significant determining factor when inducing *A. polygama* shoots on media containing BA and NAA. In the current study, that stems were noted to be the best tissue source for kiwifruit micro propagation. In addition, based on the number of shoots that the optimum culture period was

Table 6. The shoot induction and development in kiwifruit leaf micro-cross section segments after 7 weeks of culture in medium with different concentration of 2,4-D and zeatin[zy].

Growth regulator (mg/mL)		Normal shoot				No. of abnormal shoots per explant
2,4-D	Zeatin	Shoot formation (%)	No. of shoots per explant	No. of leaves	Shoot length (cm)	
0	0	0	0b	0b	0b	0c
0	0.001	0	0b	0b	0b	0c
0	0.01	0	0b	0b	0b	0c
0	0.1	5.6	0.12ab	0.06b	0b	0.24c
0	1	0	0b	0b	0b	1.83b
0.001	0.001	0	0b	0b	0b	0c
0.001	0.01	0	0b	0b	0b	0c
0.001	0.1	0	0b	0b	0b	0.11c
0.001	1	11.1	0.17a	0.42a	0.05a	2.72a
0.01	0.001	0	0b	0b	0b	0c
0.01	0.01	0	0b	0b	0b	0c
0.01	0.1	0	0b	0b	0b	0c
0.01	1	0	0b	0b	0b	0.11c
0.1	0.001	0	0b	0b	0b	0c
0.1	0.01	0	0b	0b	0b	0c
0.1	0.1	0	0b	0b	0b	0c
0.1	1	0	0b	0b	0b	0c
1	0.001	0	0b	0b	0b	0c
1	0.01	0	0b	0b	0b	0c
1	0.1	0	0b	0b	0b	0c
1	1	0	0b	0b	0b	0c

[z]Means separation within each column by Duncan's multiple range test at $P \leq 0.05$.

[y]All materials were cultured on half-strength MS medium + 3 % sucrose + 0.8% agar (pH 5.8).

Table 7. The shoot induction and development in kiwifruit petiole micro-cross section segments after 7 weeks of culture in medium with different concentration of 2,4-D and zeatin[zy].

Growth regulator (mg/mL)		Normal shoot				No. of abnormal shoots per explant
2,4-D	Zeatin	Shoot formation (%)	No. of shoots per explant	No. of leaves	Shoot length (cm)	
0	0	0	0c	0c	0c	0d
0	0.001	0	0c	0c	0c	0d
0	0.01	0	0c	0c	0c	0d
0	0.1	5.6	0.06c	0.17c	0.03c	2.17c
0	1	33.3	0.56b	0.77b	0.16b	6.44a
0.001	0.001	0	0c	0c	0c	0d
0.001	0.01	5.6	0.06c	0.06c	0c	0d
0.001	0.1	5.6	0.06c	0.06c	0c	1.39c
0.001	1	50.0	1.06a	1.13a	0.46a	3.67b
0.01	0.001	0	0c	0c	0c	0d
0.01	0.01	0	0c	0c	0c	0d
0.01	0.1	0	0c	0c	0c	0d
0.01	1	0	0c	0c	0c	0.33d
0.1	0.001	0	0c	0c	0c	0d
0.1	0.01	0	0c	0c	0c	0d
0.1	0.1	0	0c	0c	0c	0d
0.1	1	0	0c	0c	0c	0d
1	0.001	0	0c	0c	0c	0d
1	0.01	0	0c	0c	0c	0d
1	0.1	0	0c	0c	0c	0d
1	1	0	0c	0c	0c	0d

[z]Means separation within each column by Duncan's multiple range test at $P \leq 0.05$.

[y]All materials were cultured on half-strength MS medium + 3 % sucrose + 0.8% agar (pH 5.8).

Table 8. The shoot induction and development in kiwifruit stem micro-cross section segments after 7 weeks of culture in medium with different concentration of 2,4-D and zeatin[zy].

Growth regulator (mg/mL)		Normal shoot				No. of abnormal shoots per explant
2,4-D	Zeatin	Shoot formation (%)	No. of shoots per explant	No. of leaves	Shoot length (cm)	
0	0	5.6	0.06c	0.06d	0.03e	0.06c
0	0.001	11.1	0.17c	0.14cd	0.05de	0c
0	0.01	38.9	0.39c	0.61c	0.22cd	0c
0	0.1	38.9	0.44c	0.50cd	0.25c	1.06b
0	1	61.1	1.44b	1.46b	0.26c	2.83a
0.001	0.001	72.2	1.22b	1.96ab	0.80a	0.06c
0.001	0.01	88.9	1.28b	1.93ab	0.76a	0c
0.001	0.1	94.4	2.61a	2.16a	0.70a	1.00b
0.001	1	83.3	2.44a	1.54b	0.51b	0.22c
0.01	0.001	0	0c	0d	0e	0c
0.01	0.01	0	0c	0d	0e	0c
0.01	0.1	0	0c	0d	0e	0c
0.01	1	0	0c	0d	0e	0c
0.1	0.001	0	0c	0d	0e	0c
0.1	0.01	0	0c	0d	0e	0c
0.1	0.1	0	0c	0d	0e	0c
0.1	1	0	0c	0d	0e	0c
1	0.001	0	0c	0d	0e	0c
1	0.01	0	0c	0d	0e	0c
1	0.1	0	0c	0d	0e	0c
1	1	0	0c	0d	0e	0c

[z]Means separation within each column by Duncan's multiple range test at $P \leq 0.05$.

[y]All materials were cultured on half-strength MS medium + 3 % sucrose + 0.8% agar (pH 5.8).

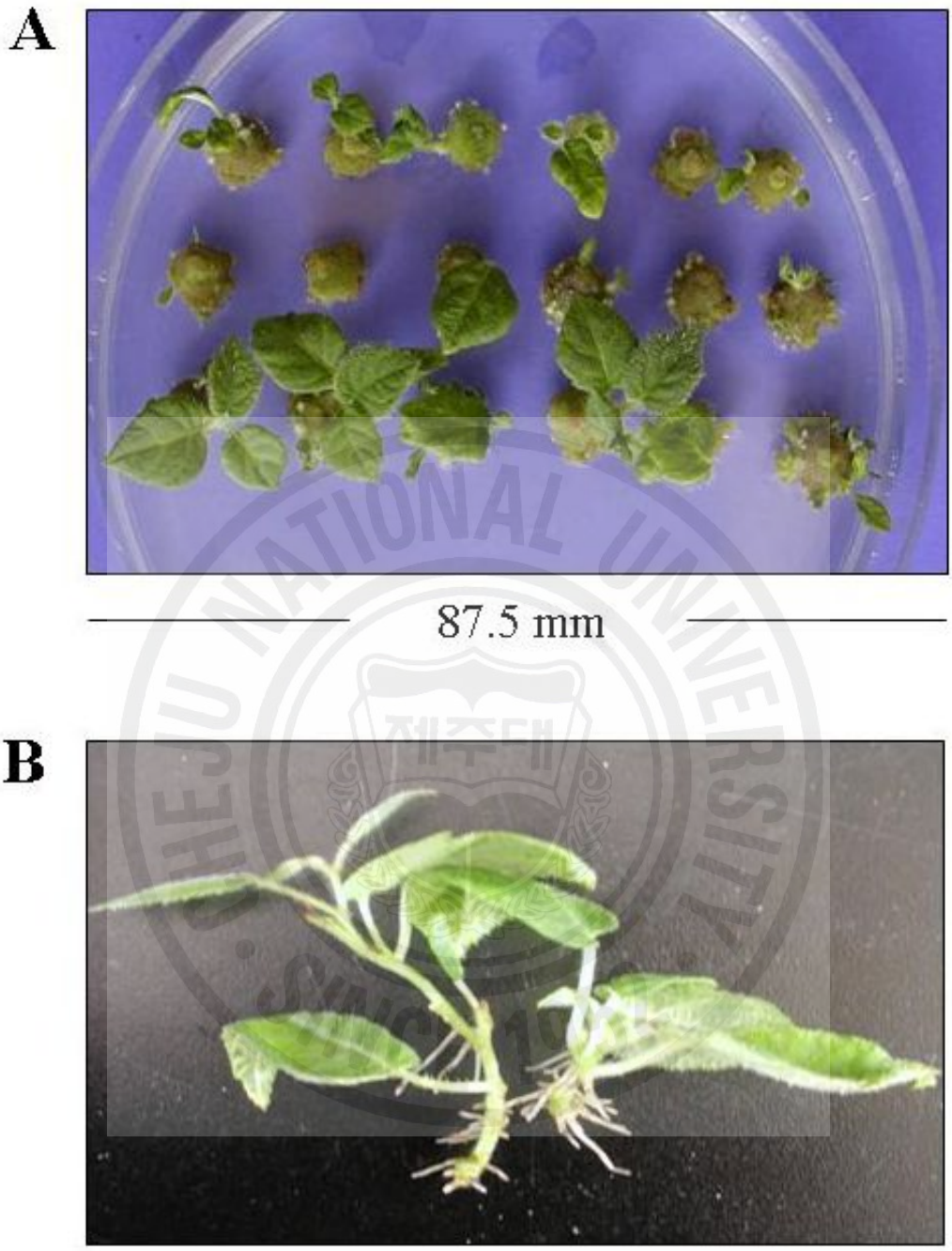


Fig. 2. Organogenesis from micro-cross sections on half-strength MS medium + 0.001 mg/mL 2,4-D + 0.1 mg/mL zeatin + 3% sucrose after 7 weeks (A) and rooted plants after 3 weeks acclimated (B).

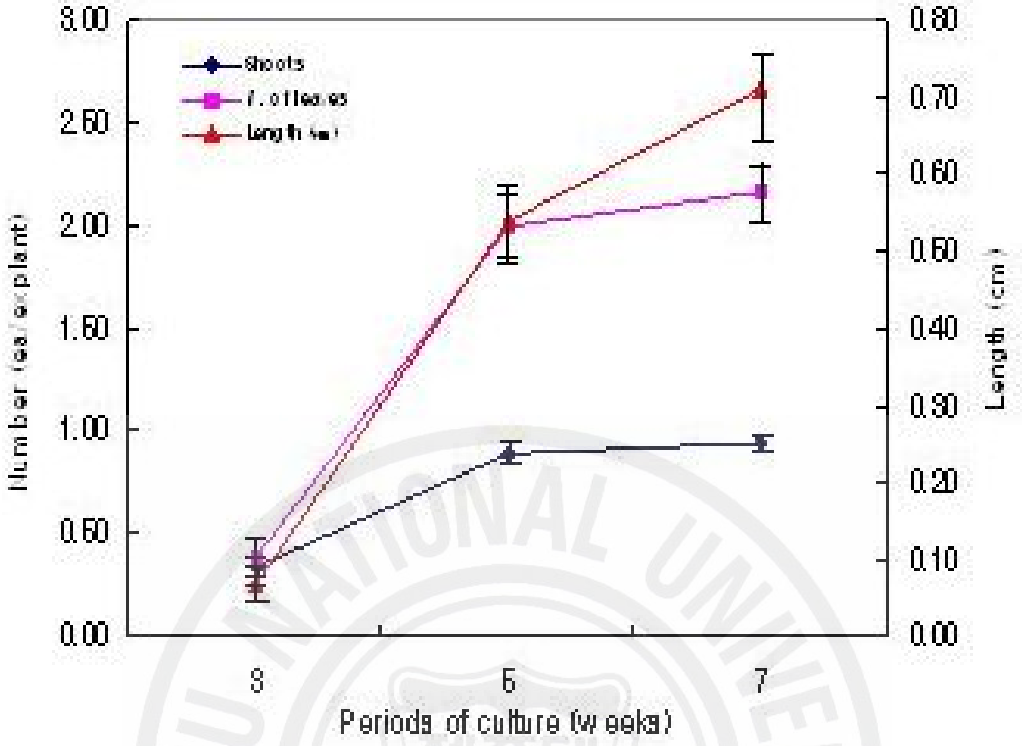


Fig. 3. Growth pattern of stem micro-cross section segments according to periods of culture. Stem segments were cultured on half-strength MS medium containing 0.001 mg/mL 2,4-D + 0.1 mg/mL zeatin. Bars represent means ± SE.

determined to be 5 weeks (Fig. 3). In contrast to traditional nodal culture, which generally requires tissues of at least 1.0 cm, the micro-cross section method utilized in this study, 800 μm thick segments, resulted in producing more than 8 explants from a single 1.0 cm stem. Marino and Bertazza (1990) reported to achieve the best proliferation efficiency with shoot tip cultures from 'Hayward' kiwifruit (*A. deliciosa)*, producing 3.12 normal shoots per explant in a medium containing 1 mg/mL BA. In comparing these culture methods using 1.0 cm nodes, MCS resulted in >20 shoots versus 3.12 shoots from shoot tip cultures. This indicated that the micro-cross section system was approximately seven times more effective for the proliferation of kiwifruit shoots.

3. Demonstration of genetic stability for MCS-regenerated shoots

To determine the genetic stability when employing MCS, 29 regenerated shoots were randomly selected for RAPD analysis and 30 regenerated shoots were selected for SSR analysis. In RAPD analysis, 19 of 24 primers produced 63 bands that ranged from 400 to 3500 bps (Fig. 4). In SSR analysis, 17 of 19 primers showed reproducible bands 46 clear bands that ranged from 50 to 1500 bps (Fig. 5).

Palombi and Damiano (2002) have reported that SSR and RAPD analyses indicated few somaclonal variants that occurred in the tissue-culture of kiwifruit. SSR especially showed approximately 1.6 times polymorphism than RAPD marker, indicating that SSR marker to be more detectable than RAPD marker for genetic variation. In this present study polymorphic bands could not be detected among the primers used (Tables 9 and 10), suggesting that MCS-regenerated plants should be analyzed by several methods, e.g., chromosome-counting and morphological assessments.

In summary, the optimum condition for normal shoot regeneration from kiwifruit using MCS method includes the following components: 1) a half-strength MS

medium with in- organic salts; 2) 800 μm segments from petioles, stems, or leaves; and 3) a combination of 2,4-D (0.001 to 0.1 mg/mL) and zeatin (0.001 to 0.1 mg/mL) in the culture medium. Shoots can be formed in 20 days, with 100% survival rates achieved from petiole and stem explants. These results demonstrated that micro-cross sectioning is not only a good method for rapid propagation of rare kiwifruit cultivars, but is also useful to biotechnology research efforts that require numerous samples for greater success.

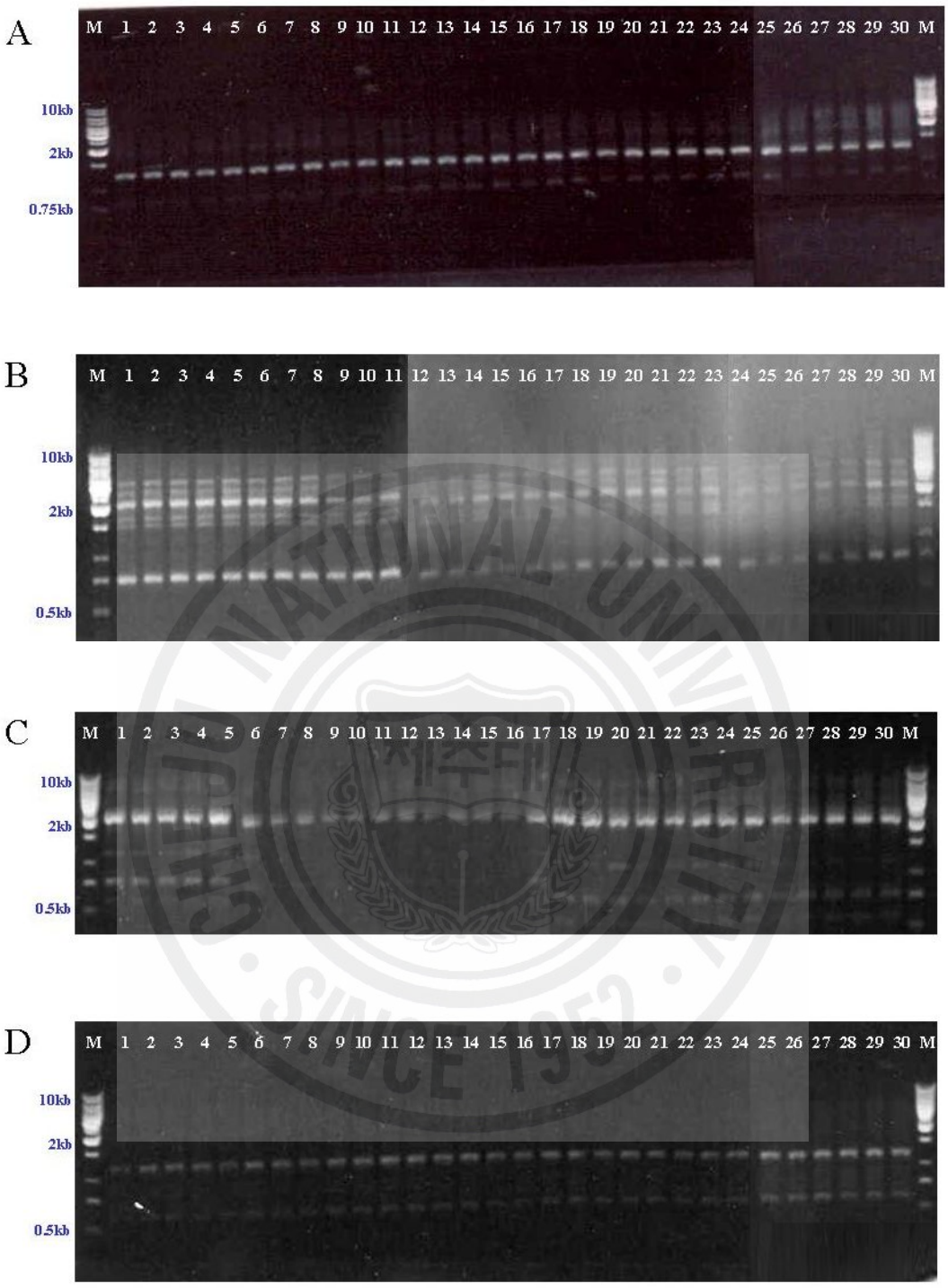


Fig. 4. Agarose gel electrophoresis of amplified fragments from MCS regenerated shoots and *in vitro* shoot using random decamer primers. A to S were showed in Table 1. Lane 1-29, micro-cross sectioned shoots of 'Hayward' kiwifruit (*A. deliciosa*); Lane 30, *in vitro* shoot; M, DNA size marker (GeneRular^{TM} 1kb ladder)

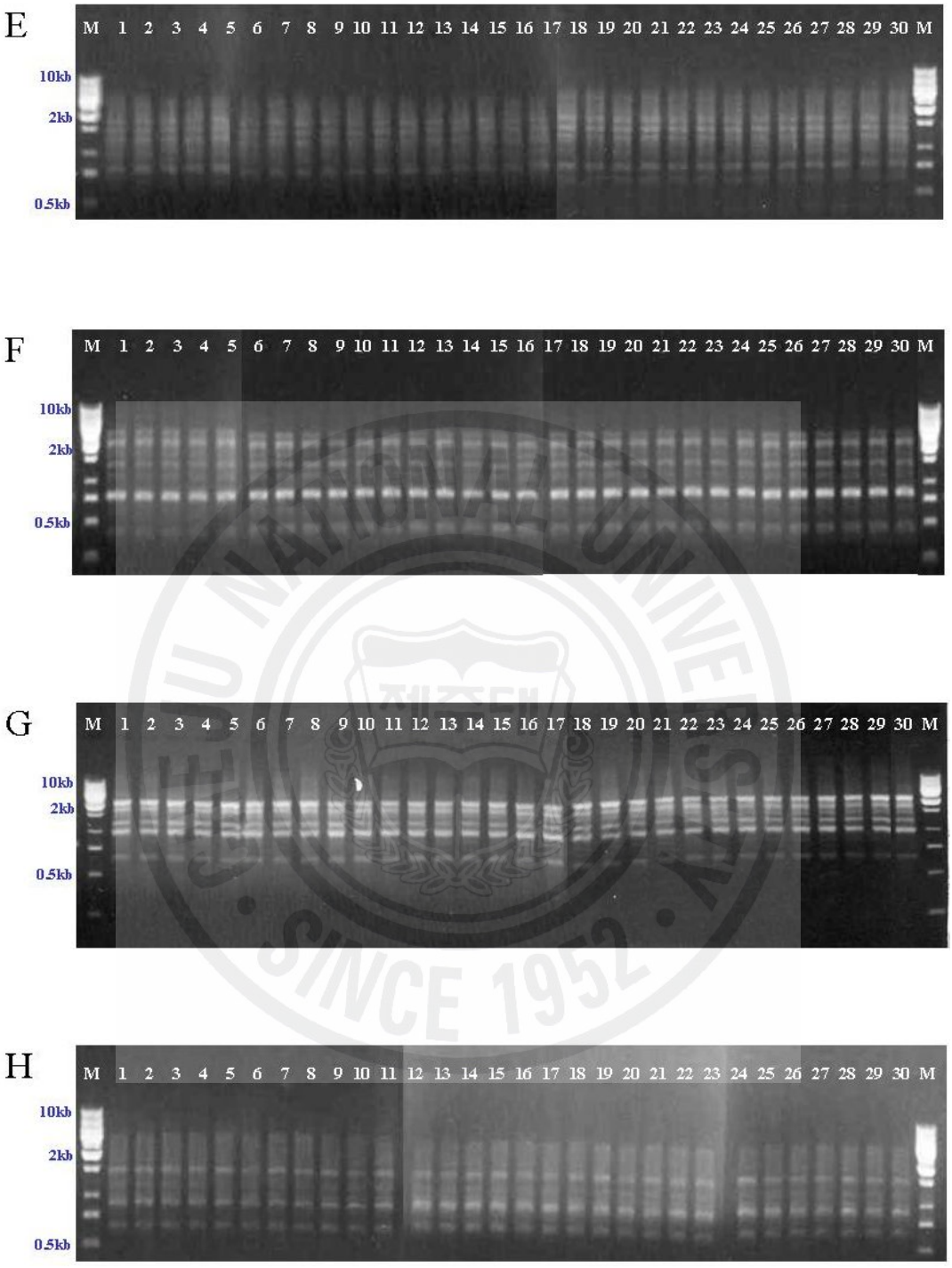
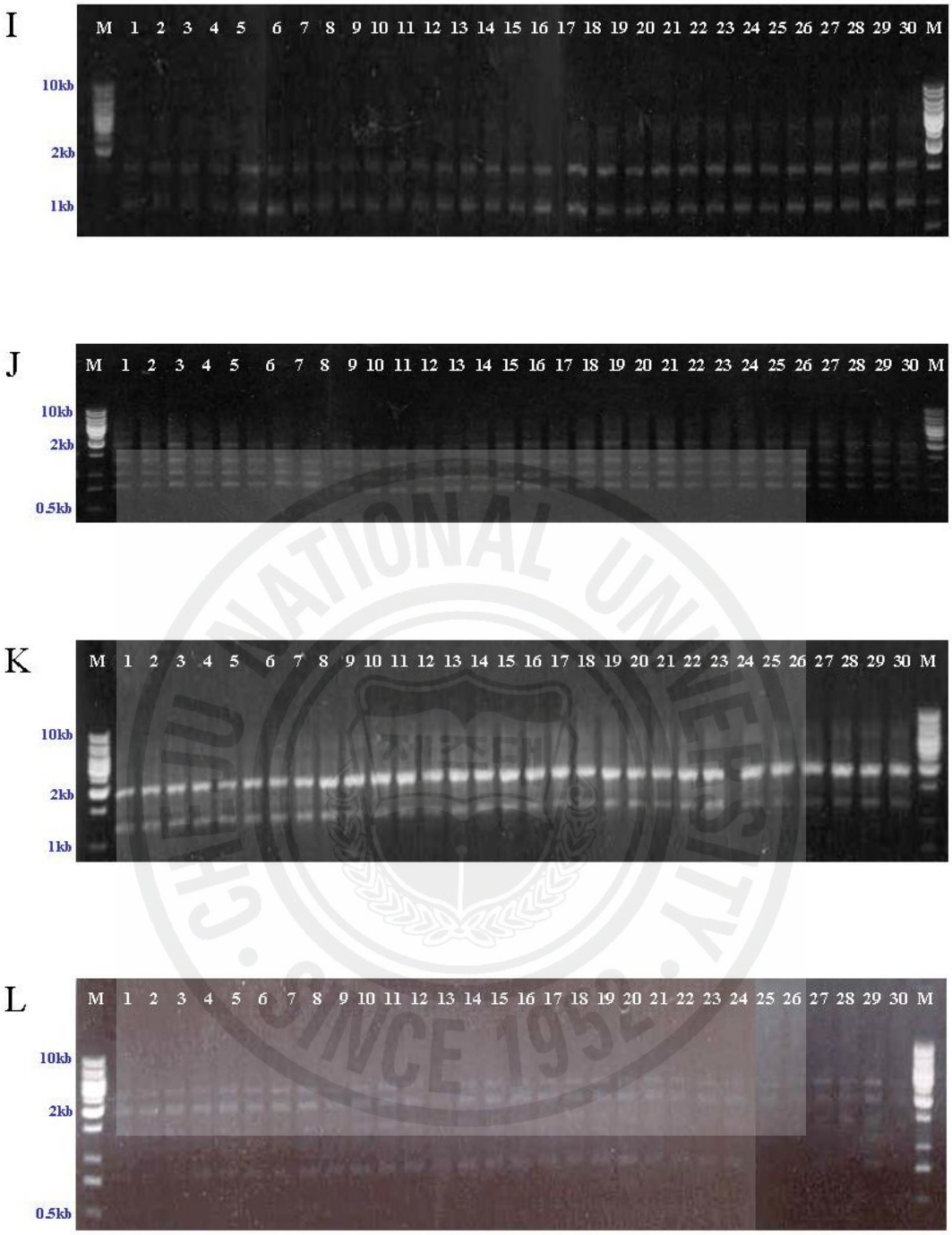


Fig. 4. (Continued)



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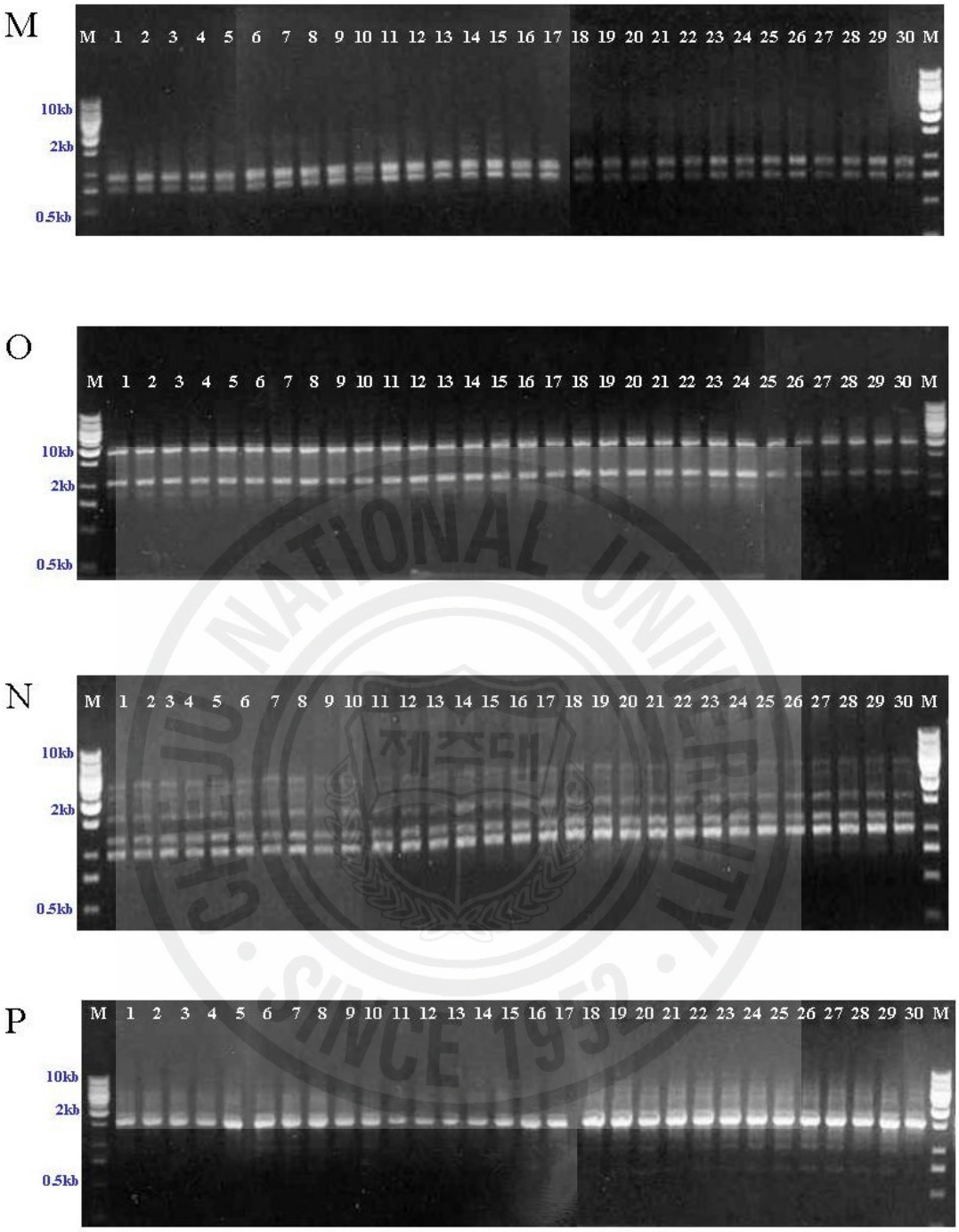


Fig. 4. (Continued)

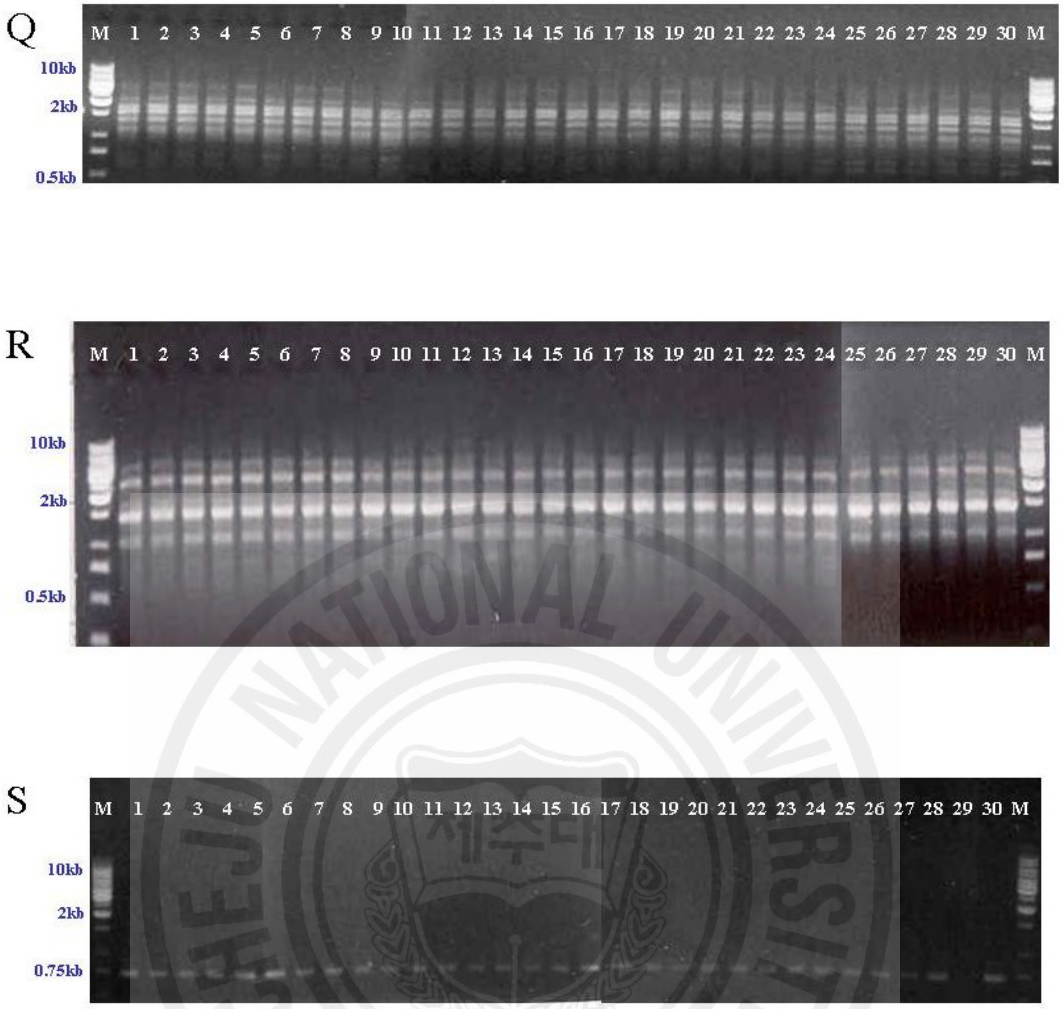


Fig. 4. (Continued)

Table. 9. The number of countable bands, band size, and the number of polymorphic bands for RAPD analysis.

Primer	No. of bands	Band size (base pairs)	No. of polymorphic bands
A (OPP 01)	2	800, 1300	0
B (OPP 03)	6	750, 1500, 1700, 2100, 2500, 3000	0
C (OPP 04)	2	600, 1300	0
D (OPP 05)	4	650, 800, 1250, 2500	0
E (OPP 08)	4	750, 1300, 1400, 1800	0
F (OPP 10)	5	400, 750, 1400, 2300, 3000	0
G (OPP 11)	5	600, 1000, 1200, 1800, 2300	0
H (OPP 12)	3	600, 900, 1500	0
I (OPP 13)	2	1000, 1500	0
J (OPP 14)	4	750, 1000, 1400, 2000	0
K (OPP 17)	2	1200, 2000	0
L (OPP 20)	3	750, 2000, 2500	0
M (OPO 01)	2	750, 900	0
N (OPO 02)	5	1000, 1100, 1700, 3000, 3500	0
O (OPO 03)	3	850, 1000, 2000	0
P (OPP 08)	1	1500	0
Q (OPO 09)	5	1100, 1300, 1500, 1800, 2000	0
R (OPO 10)	4	1000, 1400, 2500, 3500	0
S (OPO 11)	1	750	0

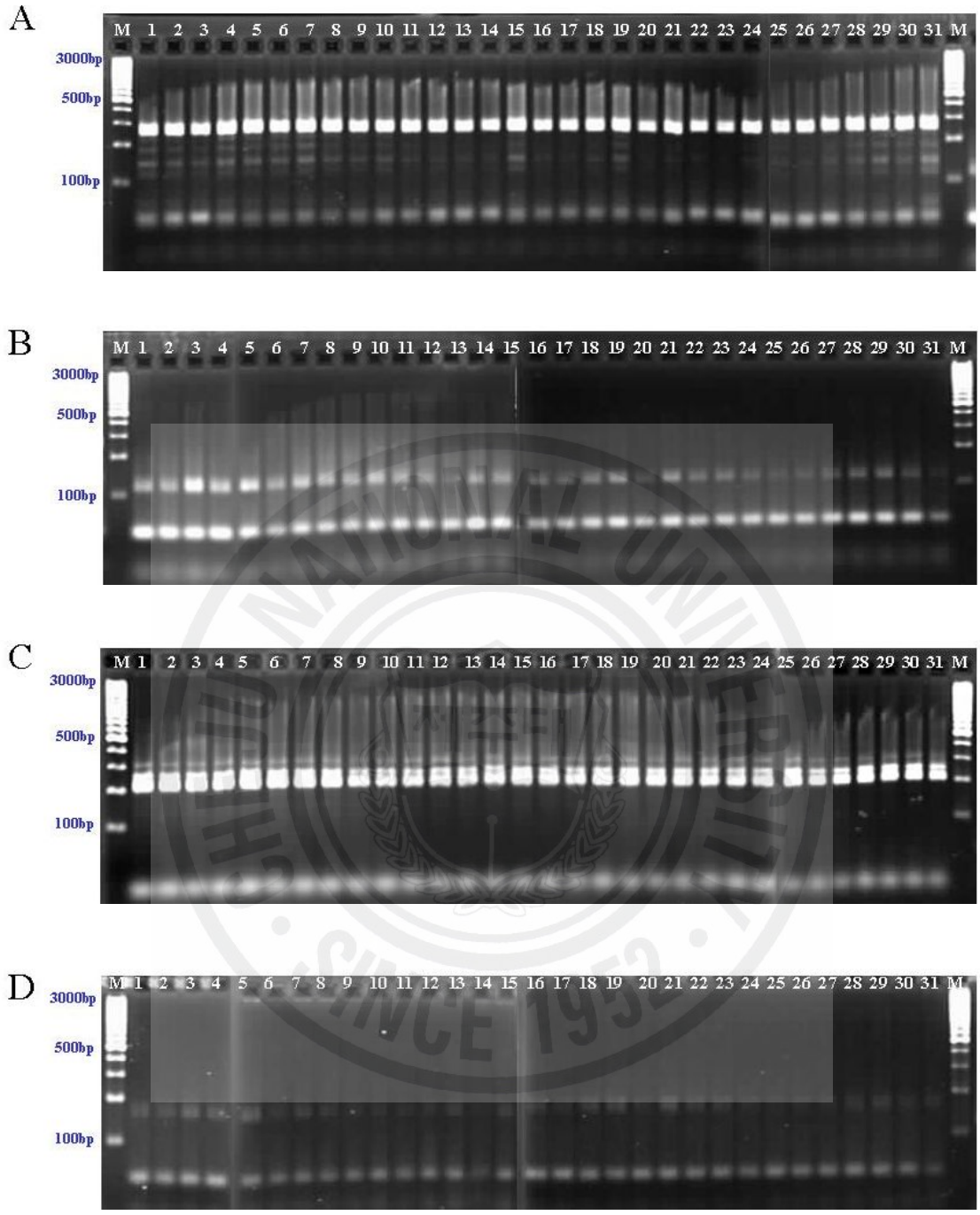


Fig. 5. Agarose gel electrophoresis of amplified fragments from MCS regenerated shoots and *in vitro* shoot using simple sequence repeat primers. A to S were showed in Table 2. Lane 1-30, micro-cross sectioned shoots of 'Hayward' kiwifruit (*A. deliciosa*); Lane 31, *in vitro* shoot; M, DNA size marker (GeneRular^{TM} 100bp ladder plus)

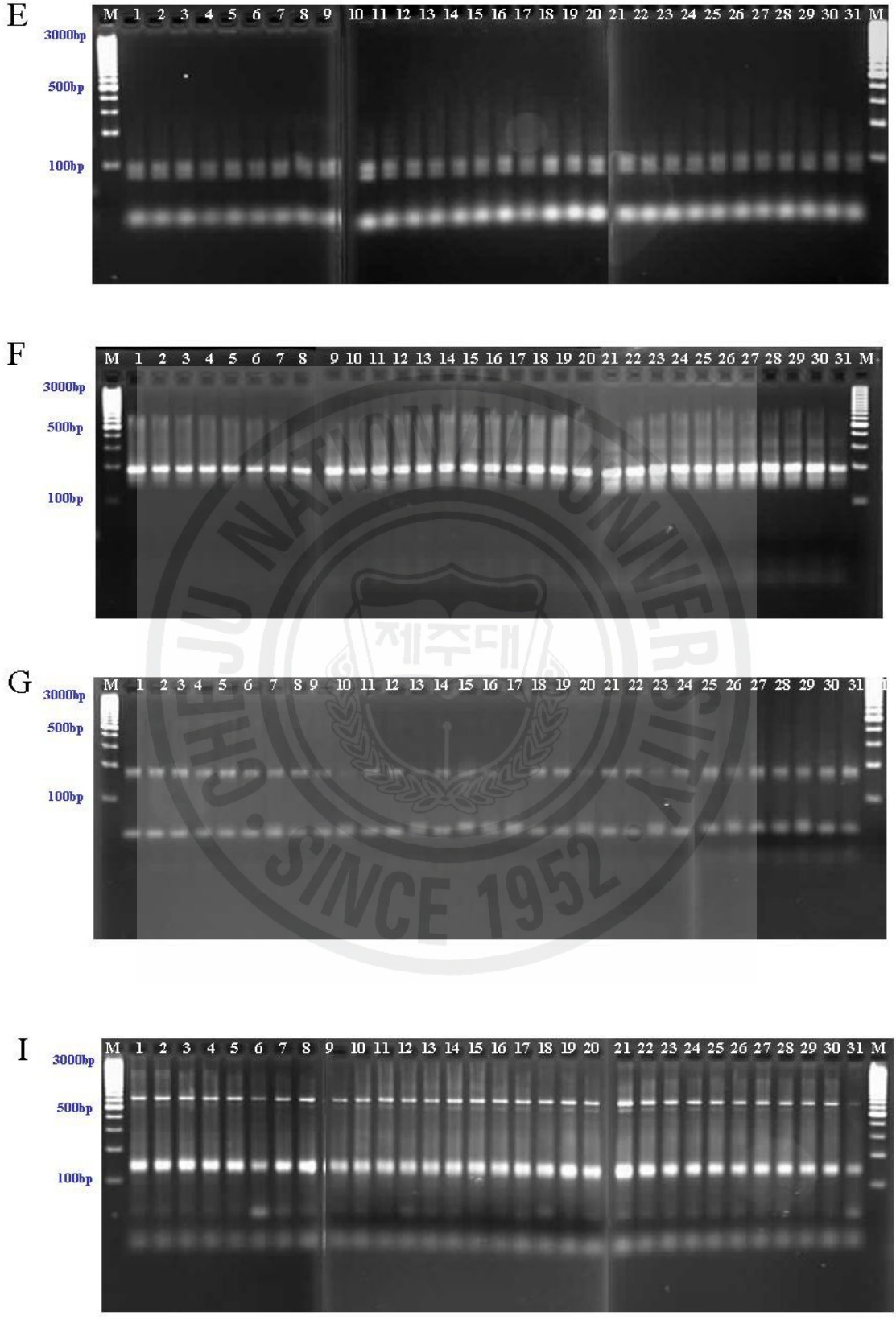
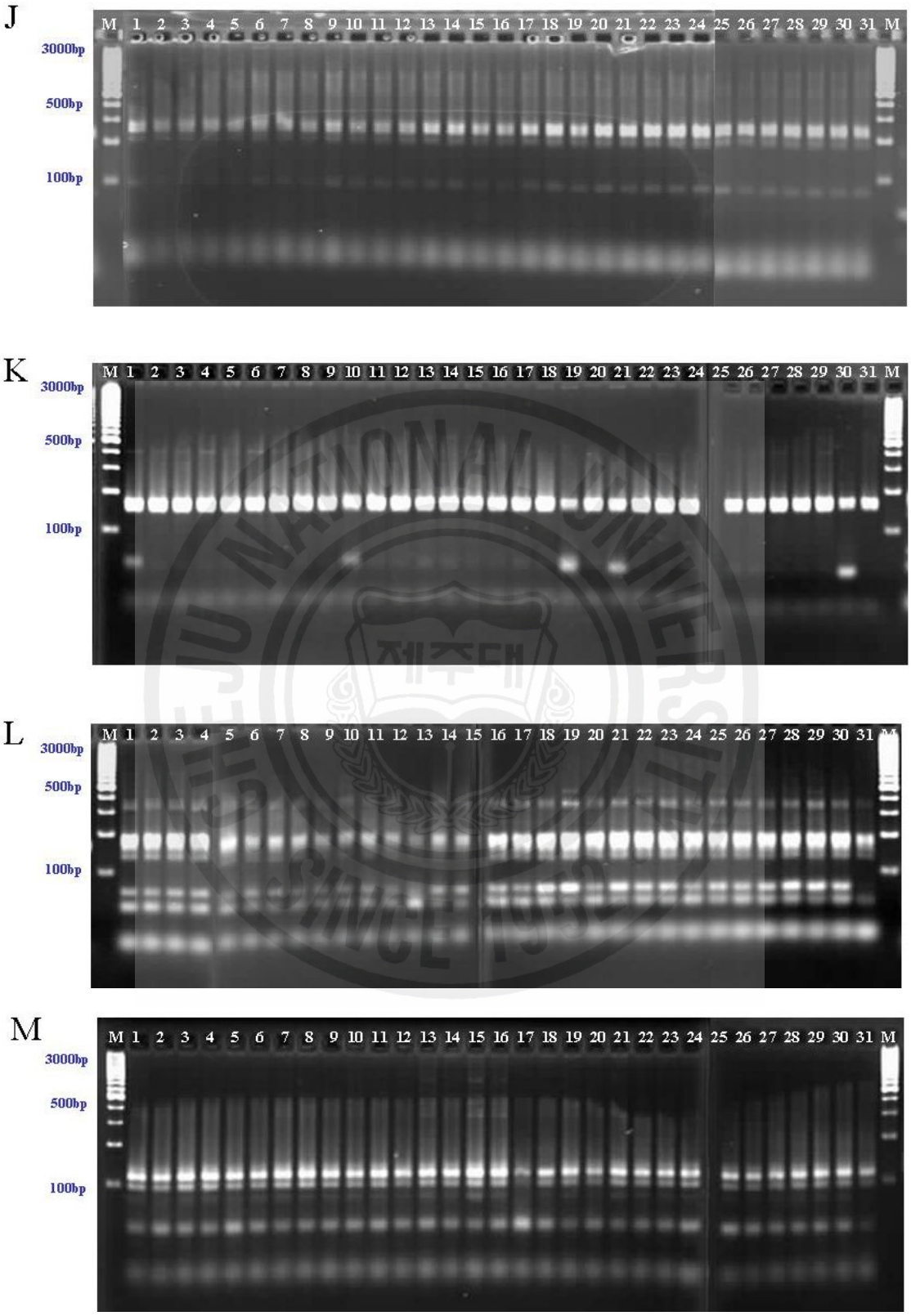


Fig. 5. (Continued)



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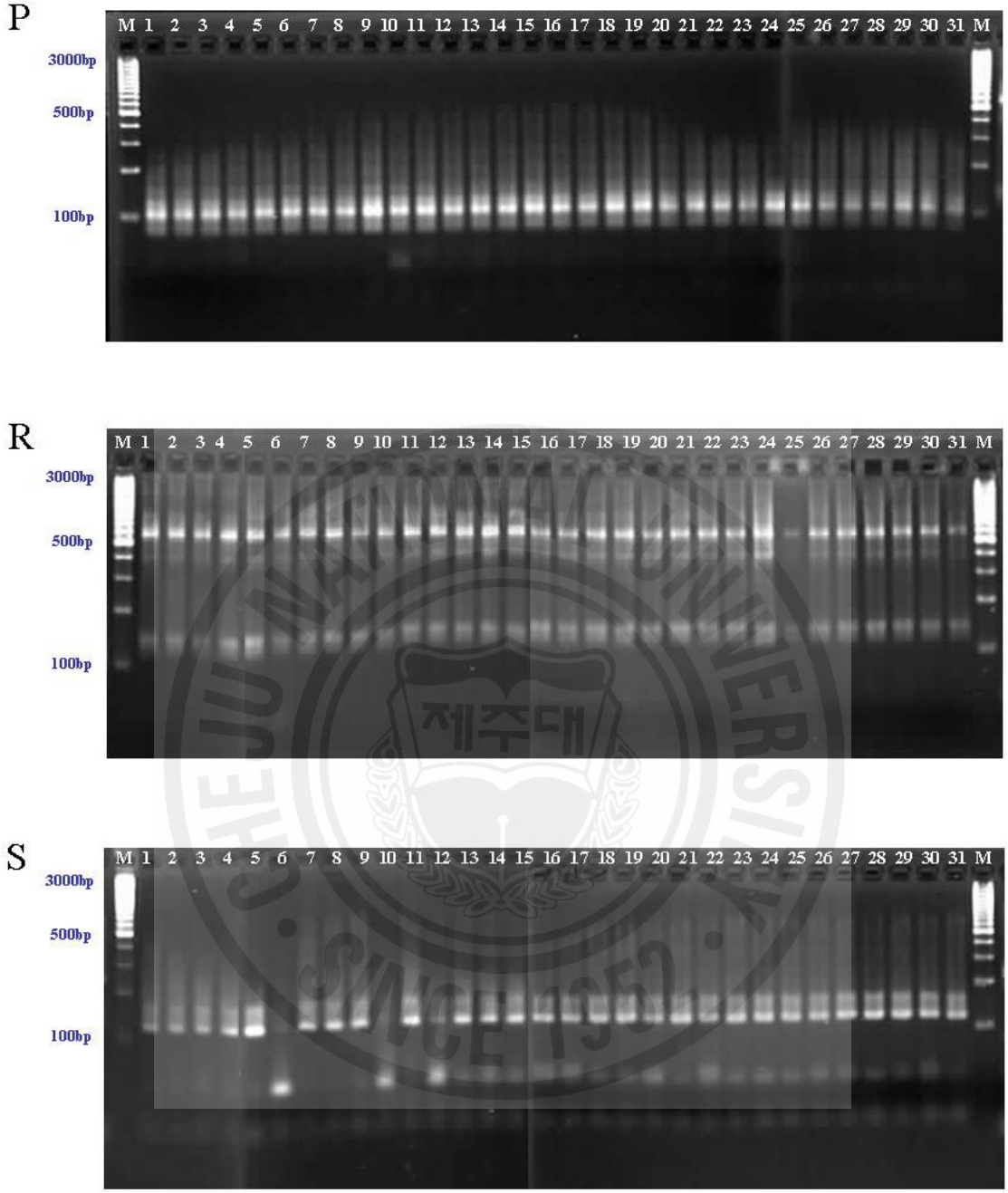


Fig. 5. (Continued)

Table 10. The number of countable bands, band size, and the number of polymorphic bands for SSR analysis.

Primer	No. of bands	Band size (base pairs)	No. of polymorphic bands	Primer	No. of bands	Band size (base pairs)	No. of polymorphic bands
A (UDK96-001)	5	50, 150, 180, 270, 300	0	K (UDK96-039)	2	50, 170	0
B (UDK96-015)	2	50, 120	0	L (UDK96-040)	5	50, 80, 150, 200, 350	0
C (UDK96-016)	3	200, 250, 300	0	M (UDK97-401)	3	50, 100, 120	0
D (UDK96-018)	2	50, 180	0	N (UDK97-402)	3	,80, 270, 500	0
E (UDK96-020)	2	80, 100	0	O (UDK97-403)	4	250, 400, 500, 1000	0
F (UDK96-023)	1	200	0	P (UDK97-408)	1	100	0
G (UDK96-026)	2	50, 180	0	R (UDK97-41)	3	150, 400, 600	0
I (UDK96-033)	2	150, 650	0	S (UDK97-414)	2	120, 180	0
J (UDK96-034)	4	80, 200, 240, 270	0				

Agrobacterium-mediated Transformation of Carotenoid Synthetic Genes in 'Hayward' Kiwifruit (*Actinidia deliciosa*)

I. Introduction

To develop new varieties of kiwifruit, breeding program has been mainly established through crossing as conventional method. In this fruit crop, it is difficult to carry out selection of excellent line developed by crossbreeding and study the valuable characters genetically because of different onset of blooming and longer juvenility. Moreover, this species also appeared to be dioecious and have a ploidy of considerable complexity. Cytogenetic analysis indicated that a base chromosome number for this genus is x = 29 (Zhang and Beuzenberg, 1983; McNeilage and Considine, 1989). One of the commercially cultivated kiwifruit is *A. deliciosa*, a hexaploid (6x) 174 chromosome number. On the other hand, there is economic potential *A. chinensis* in diploid to tetraploid (2x to 4x) which has 58 or 116 chromosome number (Huang et al., 2004). As a result, the developing of excellent line took a lot of time and effort. Therefore, biotechnologies need to selection at earlier stage, with molecular approach such as phylogenetic analysis, marker selection and genetic manipulation with cell fusion and transformation. Phylogenetic analysis for kiwifruit have been reported by Messina et al (1991), Testolin et al. (1997), Jung et al. (2003), and Kim et al. (2003). Marker assisted selection have also been developed by Harvey et al. (1997), Gill et al. (1998), Kim et al. (2004) Shirkot et al. (2001). In cell fusion, Xiao et al. (2004) reported interspecific somatic hybrid between *A. chinensis* and *A. kolomikta* and its cold tolerance. Transformation of

kiwifruit has been approached with functional substance as follows: basically to study reporter gene such as β-glucuronidase gene (GUS, Janssen and Gardner, 1993; Uemastu et al., 1991; Fraser et al., 1995), to reduce ethylene production (MacDiarmid, 1993; Whittaker, 1997), to improve drought and diseases resistance using *Agrobacterium rhizogenes rol* gene, β-1,3-endoglucanase, and rice homobox gene (*OSH1*) (Rugini et al., 1991; Nakamura et al., 1999; Kusaba et al., 1999), and to accumulate the bioactive compounds such as human epidermal growth factor and resveratrol-glucoside (Kobayashi et al.,1996, 2000).

The carotenoids are the most widespread group of pigments (yellowish to reddish) in nature, with over 600 characterized structures and an estimated yield of 100 million tons per year (Fraser and Bramley, 2004). All photosynthetic organisms such as plants, algae, and cyanobacteria or non-photosynthetic bacteria and fungi synthesize them. In plants, carotenoids are synthesized in the plastids (chloroplasts and chromoplasts) by themselves and they exist in all photosynthetic organisms such as roots, leaves, shoots, fruits, and flowers. They diverge markedly according to the oxygen excited, namely, xanthophylls containing oxygen but not carotene.

The animals, on the other hand, are incapable of synthesis carotenoids within their living body. So they must absorb by food with precursor in them that are converted provitamin A. The major component of carotenoids in crop plants such as vegetable, fruits, roots, and seeds are α-and β-carotene (3.6 mg/g and 10.8 mg/g in carrot), lycopene (2.9 mg/g in tomato), zeaxanthin (1.6mg/g in pepper), and lutein (10.7 mg/g in watercress) (Fraser and Bramley, 2004). The molecules of carotenoids have three major functions important to photosynthesis. First, they absorb solar energy in the blue-green region of the spectum and transfer it to the chlorophylls (the antenna functions or accessory light-harvesting role); second, they are important for the assembly pigment-protein complexes (a structural role); and third, they act as photoprotectors by preventing the formation of siglet oxygen by directly quenching

them and other harmful free radicals (Gracia-Asua et al., 1998).

Today, they have been widely applied as additives to poultry and fish feeds in industrial products or especially for disease prevention in human in addition to their original roles. They consisted of C_{40} and synthesized by isoprenoids pathway. Isoprenoids is commonly known as terpenes and terpenoids are large family of compounds with more than 20,000 members found in plants today. Their structures are diverse and range from relatively simple linear hydrocarbon chains to some of the most complex ring structures known (Chappell, 1995). They are generally lipophilic substances and also include hormones like geberellins and abscisic acid, the carotenoid pigments, sterols (e.g., cholesterol), latex (derived from rubber), and many of essential oil (e.g., menthol).

The pathways of carotenoid biosynthesis begin with the synthesis and the formation of phytone from geranylgeranyl pyrophosphate (GGPP) the 5-carbone compound isopentenyl pyrophosphate (IPP). Dimethylallyl pyrophosphate (DMAPP) is the initial, activated substrate for formation of long chain polyisoprenoid compounds such as GGPP, that C_{20} is formed by the sequential and linear addition of three molecules of IPP to one molecule of DMAPP. The geranylgeranyl pyrophosphate synthase (*GGPS*) catalyzes these reactions. The formation of the symmetrical phytoene (C_{40}) from two molecules of GGPP is the first reaction specific to the carotenoid biosynthesis pathway. The biosynthesis of phytoene from GGPP is a two-step reaction catalyzed by the enzyme phytone synthase (*PSY*). Phytoene undergoes a series of four desaturation reactions that result in the formation of first phytofluene and then, in turn, ζ-carotene, neurosporene, and lycopene. These desaturation reactions lengthen the conjugated series of carbon-carbon double bonds that constitute the chromophore in carotenoids pigments, and thereby transforming the colorless into the pink-colored lycopene. The four sequential desaturations undergone by phytoene are catalyzed by two related enzymes in plants such as the

phytoene desaturase (*PDS*), and the ζ-carotene desaturase (*ZDS*). The carotenoids in the photosynthetic apparatus of plants are bicyclic compounds, most commonly with two β or modified β rings. A single gene product, the lycopene β-cyclase (*LCYB*), catalyzes the formation of the bicyclic β-carotene from the linear, symmetrical lycopene in plants and cyanobacteria. Xanthophylls or oxygenated carotenoids comprise most of the carotenoid pigments in the thylakoid membranes of plants. Hydroxylation at the number three carbon of each ring of the hydrocarbons β-carotene and α-carotene will produce the well-known xanthophylls pigments zeaxanthin and lutein, respectively. Addition of carotenoids with a keto groups at the 4 positons of one or both rings of the yellow β-carotene will produce the reddish-orange to red pigments echinenon and canthaxanthin. The epocidase of zeaxanthin to form violaxanthin through antheraxanthin and de-epoxidation of violaxanthin to regenerate zeaxanthin comprise what is variously referred to as the xanthophylls, violaxanthin, or epoxide cycle. Carotenoids, and in particular the epoxy carotenoids violaxanthin and neoxanthin, have long been thought to be precursors for biosynthesis of the plant growth regulator ABA (Cunnigham and Gantt, 1998) (Fig. 6).

The Metabolic engineering of carotenoids in crops has been successfully obtained from transgenic canola (*Brassica napus*) and rice (*Oryza sativa*) etc. (Shewmaker et al., 1999; Ye et al., 2000) (Table 11). Increases in total carotenoid and carotene have been mainly achieved by manipulating such *PSY* and *PDS* due to phytoene biosynthesis and desaturation step.

The objectives of this study were to increase and characterize transformation of carotenoid synthesis genes, including *Cit-GGPS* (encodes geranylgeranyl pyrophosphate synthase), *Cit-PDS* (encodes phytoene desaturase*)*, *Cit-ZDS* (encodes ζ-carotene desaturase)*, Cit-CHX* (encodes β-carotene hydroxylase), and *Cit-PSY*

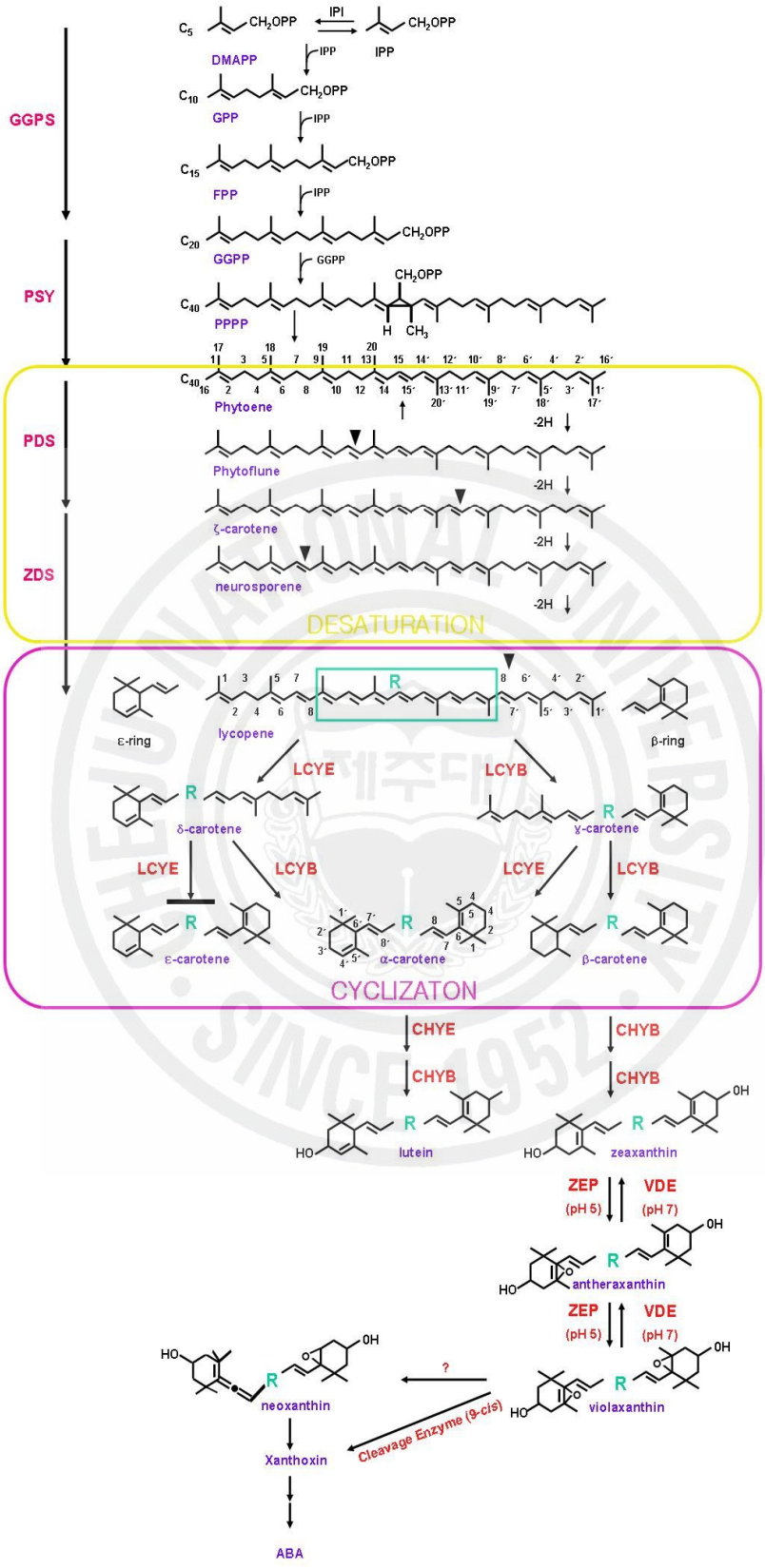


Fig. 6. Carotenoid biosynthesis pathway in plants (Cunnigham and Gantt, 1998)

Table 11. Engineering of carotenoid in different crops.

Crop name	Content	Gene	Reference
Tomato	400 folds increase in lycopene	down-regulation lycopene cylisation and up regulating phytoene synthase (*Psy*-1)	Fraser et al., 2001
Carrot	2 to 5 folds increase in beta-carotene	*Erwinia herbicola crt* gene	Ausich et al., 1991; Hauptmann et al., 1997
Potato	5 to 130 folds increase in zeaxanthin	Zeaxanthin epoxidasae	Römer et al., 2002
Canola	50 folds increase in carotenoids	*Erwinia uredovora* phytoene synthase (*crt*B)	Shewmaker et al., 1999
Rice	beta-carotene containing endosperm	Phytoene synthase and lycopene β-cyclase/ *E. uredovora* phytoene desaturase (*crt*I)	Ye et al., 2000

(encodes phytone synthase) gene and to determine whether these genes were functional in kiwifruit and could be used to increase the amount of carotenoids, especially β-carotene and lutein.

II. Materials and Methods

1. Plant materials and preparation of transformation explants

'Hayward' kiwifruit (*A. deliciosa*) was cultured in a 500 mL jar containing 100 mL of proliferation MS medium (Murashige and Skoog, 1962) supplemented with sucrose (3%, w/v), 1 mg/mL BA, 0.1 mg/mL IBA, and solidified with agar (0.8%, w/v) at pH 5.8 (adjusted with 1N NaOH before autoclaving at 121°C for 12 minutes). They were incubated at 25°C under a 16 hr photoperiod supplied by fluorescent lamps (45 μmol m^{-2} s^{-1}). When 1cm of internode appeared (usually after 2 months) MCS segments of stem (800 μm thick) were prepared by using a Vibratory Microtome (vibratome® series 1000 sectioning system, USA). Sliced segments were stored in half-strength MS medium without plant regulators until inoculations.

2. Transformation and regeneration

The transformation procedure using MCS method in kiwifruit is described in Figure 7.

2-1. Bacterial strains and vectors

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) harboring the binary vector pCABMIA1300 and *Agrobacterium tumefaciens* strain LBA4404 (Hoekema. et al. 1983) harboring the binary vector pBI121 were used for transformation experiments. Each plasmid contained the carotenoid synthesis genes such as *GGPS*, *PDS*, *ZDS*, *CHX*, and *PSY*. They were cloned from *Citrus unshiu.* Also, Hygromycin phosphotrasnferase II (*HPT*II) gene in pCABMIA1300 and neomycin phosphotrasnferase II (*NPT*II) gene in pBI121 were used as selectable marker (Figs. 8 and 9).

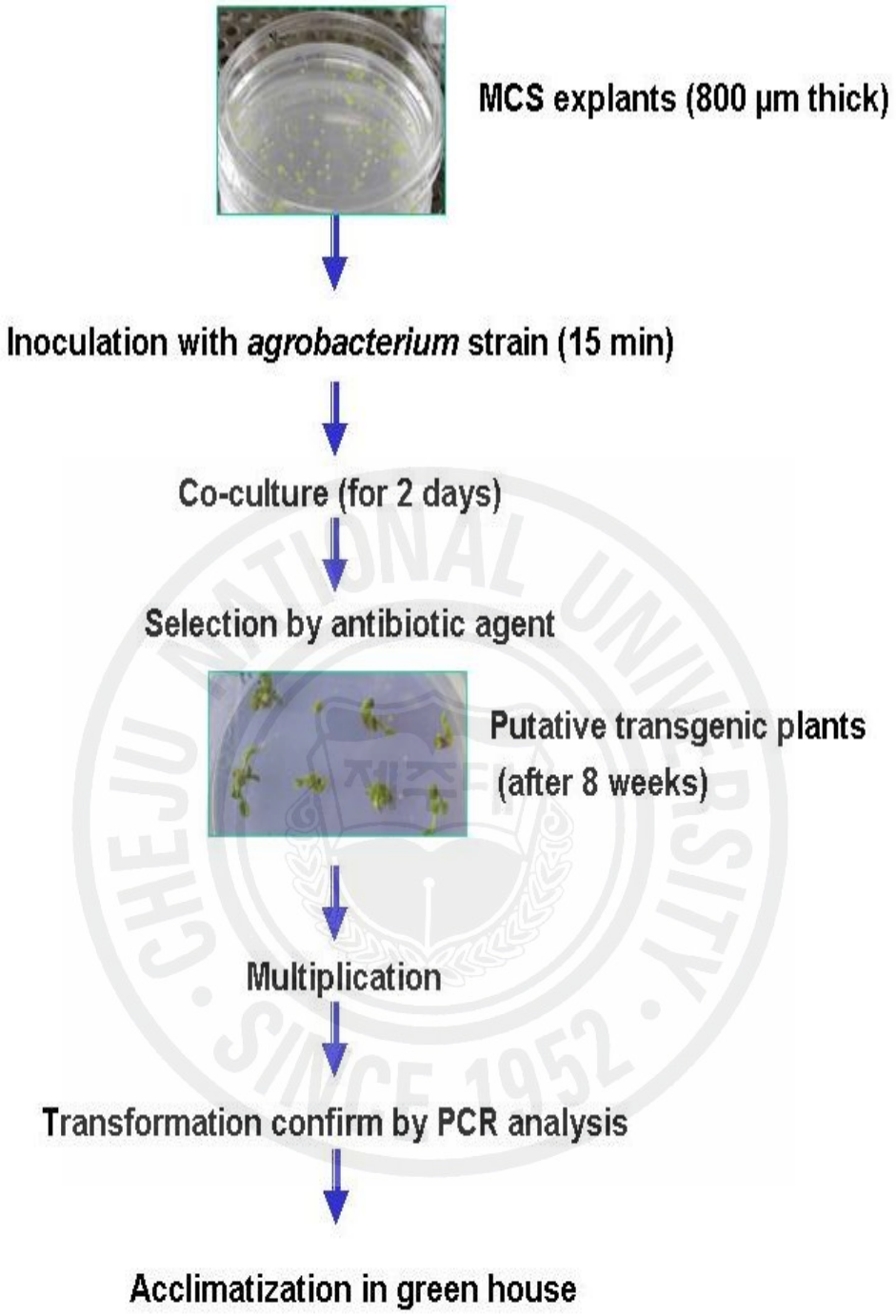


Fig. 7. Total transformation procedure using MCS method in 'Hayward' kiwifruit (*A. deliciosa*).

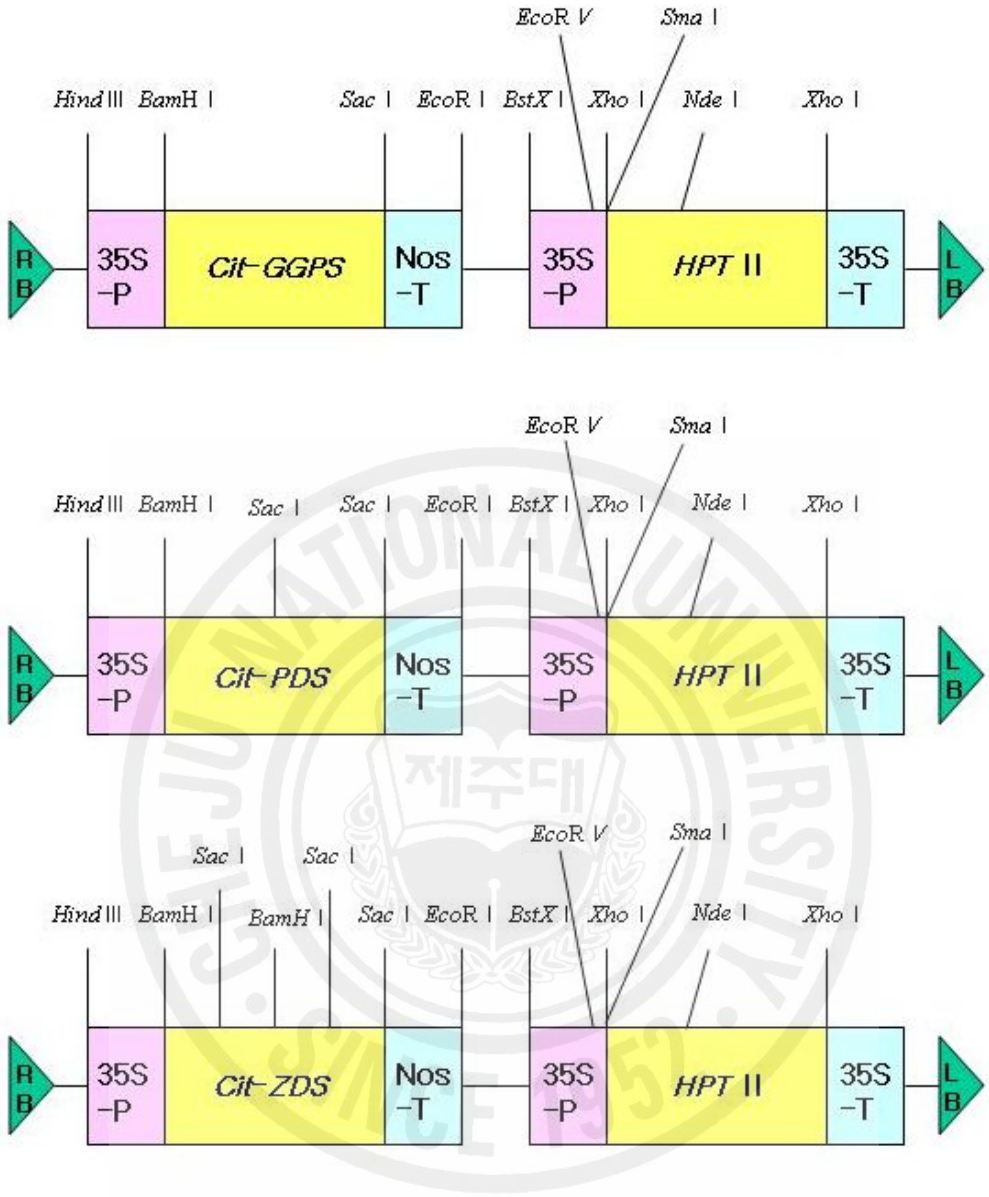


Fig. 8. Schematic representation of T-DNA regions of pCAMBIA1300 binary vector containing *GGPS*, *PDS*, *ZDS*, respectively. LB, left border; 35S-T, 35S terminator; *HPT*II, Hygromycin phosphotransferase II gene; 35S-P, 35S promoter; RB, right border.

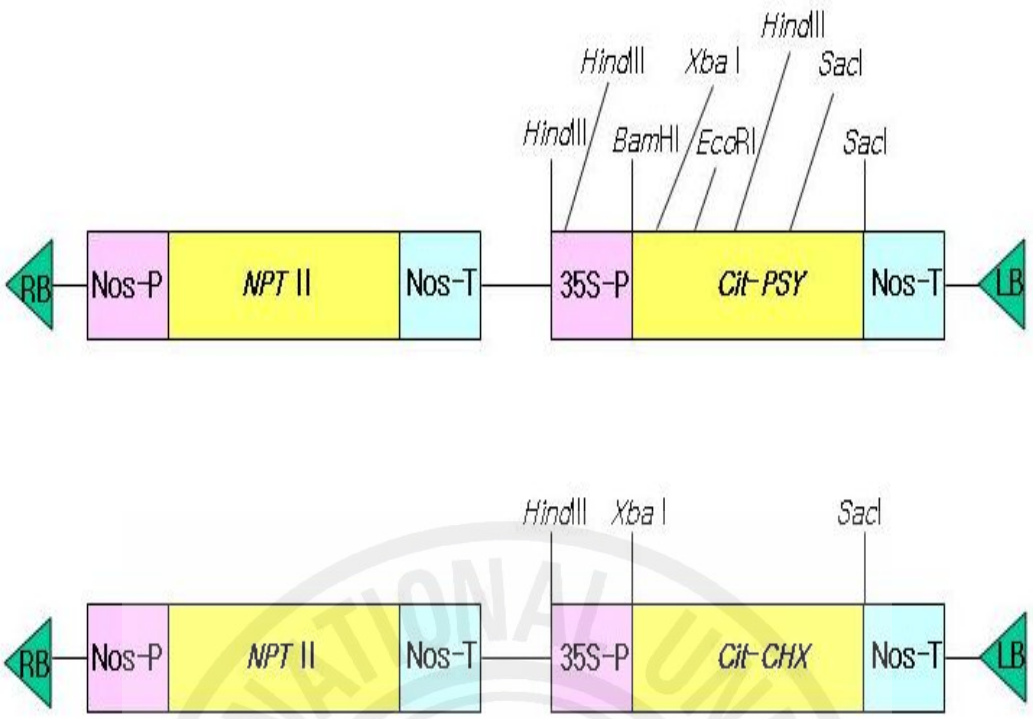


Fig. 9. Schematic representation of T-DNA regions of pBI121 binary vector containing *PSY* and *CHX*, respectively. LB, left border; Nos-T, Nos terminator; *NPT*II, Neomycin phosphotransferase II gene; 35S-P, 35S promoter; RB, right border.

2-2. Selection of suitable antibiotic

MCS segments were analyzed with hygromycin and kanamycin for concentration of appropriate antibiotic. They were cultured on half-strength MS liquid medium supplemented with sucrose (3%, w/v), 0.001 mg/mL 2,4-D, 0.1 mg/mL zeatin, pH 5.8 and either 0 to 50 μg/mL of hygromycin or 0 to 150 μg/mL of kanamycin. The segments were incubated at 25 ± 2°C under a 16 hr photoperiod supplied by fluorescent lamps (45 μmol m^{-2} s^{-1}) for 4 weeks. And they were examined for their resistance to antibiotic agent, including survival rate, fresh plant weight, and the number of shoots produced per explant.

2-3. Inoculation with *Agrobacterium*

Agrobacterium was incubated at 28°C, 180 rpm in 50 mL LB liquid medium (0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.0) for overnight to the $OD_{600} \geq 1.5$. They were collected by centrifugation at 4,000 rpm for 10 min. Pellet was re-suspended in 10 mL of re-suspension medium (half-strength MS liquid medium, containing sucrose (3%, w/v), 0.001 mg/mL 2,4-D, 0.1 mg/mL zeatin, and 20 μM acetosyringone) with shaking incubator at 28°C for 1 hr. The explants were inoculated by briefly shaking for 15 min then they transferred to sterilized filter paper to dry for 10 min. They were placed on half-strengh MS medium supplemented with sucrose (3%, w/v), 0.001 mg/mL 2,4-D and 0.1 mg/mL zeatin. The pH was adjusted to 5.8, and plant agar (0.8%, w/v, Duchefa, The Netherlands) was included as the gelling agent. Two reagents were also added to the medium after filter sterilization. The one was zeatin, while the other acetosyringone, and inoculated explants were incubated at 25°C with a dark condition for 2 days.

2-4. Selection of explants

Transgenic plants were selected in 1/2-strength MS medium supplemented with sucrose (3%, w/v), 0.001 mg/mL 2,4-D and 0.1 mg/mL zeatin, 500 μg/mL cefotaxime sodium, 5 μg/mL hygromycin in *GGPS*, *PDS*, and *ZDS*, or 25 μg/mL kanamycin in *CHX* and *PSY* after co-cultivation.

3. Molecular confirmation for transformation

3-1. The total DNA extraction and purification

The genomic DNA was isolated from untreated green leaves as control and transgenic plants according to Dellaporta et al. (1983) with some modifications.

3-2. PCR analysis

Putative transgenic kiwifruits were analyzed for the presence of each gene insertion. DNA (15 ng) was used as template in a reaction mixture (total volume 25 μL) that contained 3 μL of 10x reaction buffer, 500 μM of each dNTP, 0.25 μM of primer, and 1.5 units *Taq* polymerase (Takara, Japan). The amplification of gene was performed in a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, USA) with initial denaturation for 2 min at 94°C, then 30 cylces of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by 10 min of final extension at 72°C. The PCR products were confirmed by 1% agarose gel in 1x TBE buffer (Tris-Borate/ EDTA, pH 8.3).

Table 12. List of primer sequences from PCR and RT- PCR analysis.

Section	Primer		Sequence (5'→3')
PCR	35S	sense	CTGACGTAAGGGATGACGCA
	GGPS	antisense	AAGCATCTTCTACTCCATCA
	PDS	antisense	TCATAGAGTGCTCCTTCCAC
	ZDS	antisense	TGCGCGAATCCCATGTAACG
	*HPT*II	sense	ATG TCC TGCGGG TAAATAGC
		antisense	GCC TCCAGAAGAAGATGTTG
	*CHX**	sense	GCACCCAGATCGAGACTTTC
		antisense	ACGAACATGTAGGCCATCCC
	*PSY**	sense	CGGACTGCTGTGTTTAATTC
		antisense	TGCAGCATCAAGCATATCAA
	*NPT*II	sense	GAGGCTATTCGGCTATGACT
		antisense	AATCTCGTGATGGCAGGTTG
RT-PCR	*RBCS*1	sense	CATGTATGGGTGTACCGATTCG
		antisense	GTTGAAGATCCGATGAACAAGC
	*GGPS*160	sense	TGCCATGGAAGAGTTCCCTCAGTT
		antisense	AATCGCAGCTGCAGCAAGATTAGC
	*PDS*163	sense	TCCGAGATAGTGAACCGATGG
		antisense	CGAAATGACGAAGACAAGTAAGC
	*ZDS*269	sense	CCAGAAGACTACTACAGAGAAGG
		antisense	TATGAGCCAGCAAGGAAGAAG

*These primers also used in RT-PCR analysis

3-3. RNA isolation and purification

The total RNA was extracted from young leaves of non-transgenic and transgenic kiwifruit using the RNeasy Plant Mini Kit (Quagen, USA) with the following some modifications as follows. Approximately 100 mg of *in vitro*-grown tissues in liquid N_2 were homogenized using a mortar and pestle. The procedure of RNA isolation was performed according to the manufactures's instruction. Dnase I (Quagen, USA) digestion, phenol : chloroform : isomyalcohol (25:24:1), and Chloroform : iso-myalcohol for clean up RNA were treated after RNA isolation.

3-4. Reverse transcription (RT)-PCR analysis

The transgenic kiwifruits were analyzed for the presence of each gene's transcripts using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) which was performed according to the manufactures's instruction.

The cDNA (15 ng) was used as template in a reaction mixture (total volume 30 μL) that contained 3 μL of 10x reaction buffer, 500 μM of each dNTP, 0.25 μM of primer, and 1.5 units *Taq* polymerase (Takara, Japan). The amplification of gene was performed in a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, USA) with initial denaturation for 5 min at 96°C, then 30 cylces of 15 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C, followed by 5 min of final extension at 72°C. The RT-PCR products were confirmed by 1.2 % agarose gel in 1x TBE buffer (Tris-Borate/ EDTA, pH 8.3).

3-5. Southern blot analysis

Genomic DNA was isolated from *GGPS* transgenic plants using the DNeasy Plant Maxi Kit (Qiagen, USA), and then quantified using spectrophotometer (260/280).

About 30 μg of each DNA per lane was digested with *EcoR*I restriction enzymes (Promega, USA) and separated by electrophoresis through a 1% (w/v) agarose gel. The DNA was transferred to a Nytran® Supercharge Nylon Membrane (Schleicher & Schuell, Inc) by capillary transfer. The membrane was washed in 2x SSC (20x SSC is 3 M NaCl, 0.3 M sodium citrate) and fixed by oven at 80°C for 2 hr. The Southern blots were hybridized with a DNA fragment of reporter gene (*HTP*II) that was labeled with [α-32P]-dCTP probes using the Rediprime II random prime labeling system (GE Healthcare, Corp). The membranes were prehybridized at 62°C hybridization buffer for 4 hr and then hybridization was accomplished by overnight shaking at 62°C. The hybridized membranes were washed with 2x SSC (with 0.1% SDS) and then with 1x SSC (with 0.1% SDS) for 30 min at 62°C while shaking. The membrane was finally washed with 0.5x SSC for 20 min at room temperature. The autoradiographic image of the membrane is obtained by Phosphor Imager.

4. Carotenoid analysis by HPLC

4-1. Carotenoid extraction

Two lines of non-transgenic plants were used as control. About 1g of leaves two month old *in vitro* grown transgenic plants were ground in liquid nitrogen using a mortar and pestle. It was extracted with 30 mL of 80% acetone (with 1% 2,6-Di-tert-butyl-4-methylphenol; BHT). After the extracts were centrifuged in 10,000 rpm for 10 min, aliquots of the extracts were transferred with equal volume of diethyl ether. This procedure was performed for more than 3 times until pigment was removed. The extracts were transferred in a separatory funnel and added with 30mL of distilled water and equal volume of diethyl ether. The lower aqueous phase was discarded and the upper layers were collected. These layers were dried in evaporator at 35°C. The concentrate was dissolved in a 10 mL ethyl ether and an equal volume of 10%

potassium hydroxide in methanol to remove residual chlorophyll. They were carefully incubated in a shaker for 1 hr in dark condition. The reacted products were then transferred in a separatory funnel and added with saturated ammonium chloride and then washed 7 times with water to remove residual chlorophyll and salts. The carotenoid phase was collected and dried with sodium sulfate and evaporator. The residue was diluted in 5 mL 1:1 (with 0.1% BHT) = ethyl acetate: methanol and filtered with 0.50 μm PTFE Filter. The total extraction procedure of carotenids is described in Figure 10.

4-2. HPLC analysis

The HPLC operational condition and gradient condition of mobile phase for carotenoid identification are presented in Table 13. All samples were extracted and injected in triplicates for HPLC analysis. For quantification, standard of lutein (Fuluka, Japan) and β-carotene (Calbiochem, USA) were used to prepare calibration curves with correlation coefficient values of greater than 0.999 (Fig. 20).

4-3. Statistical analysis

Data were analyzed using SAS (2002) Version 6.12, and significant differences among means were tested using Duncan's multiple range tests at $P \leq 0.05$.

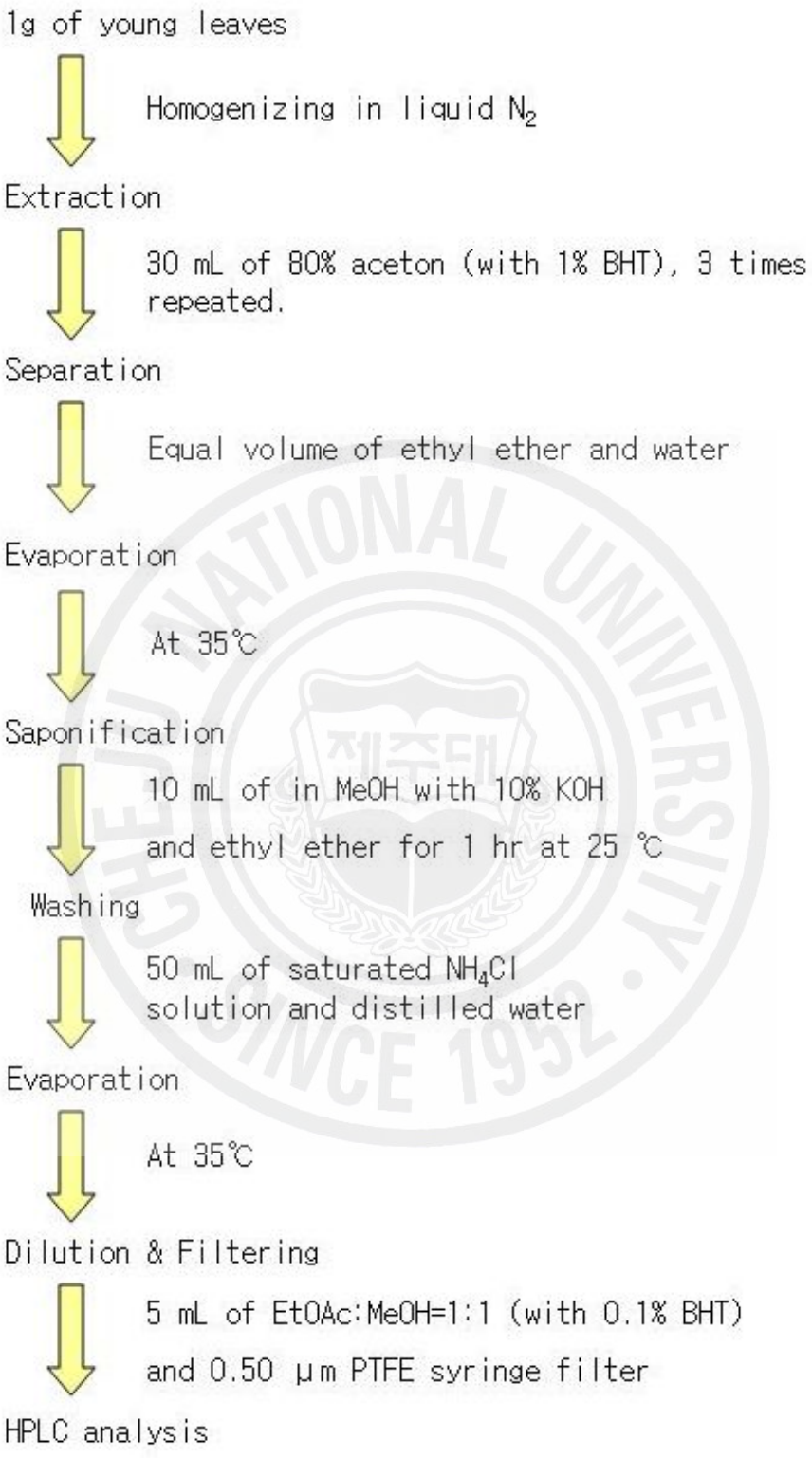


Fig. 10. Schematic diagram for extracting carotenoids from leaves of 'Haywrad' kiwifruit (*A. deliciosa*).

Table 13. Operational conditions of HPLC for carotenoid analysis.

Instrument	Waters 2996 photodiode array detector and Waters 2695 separations module.			
Column	μBondaPak C18 125 Å, 10 μm column (3.9 x 150mm, Waters Corp.)			
Column temp.	30°C			
Gradient	Time	MeOH:H_20=75:25	EtOAc (100%)	Curve
	0	70	30	1
	5	70	30	6
	19	0	100	6
	20	0	100	6
	21	70	30	6
	30	70	30	6
Flow rate	1 mL/min			
Wavelength	430 nm			
Sample inject	20 μL			

Ⅲ. Results and Discussion

1. Establishment of selection condition with antibiotics

Hygromycin phosphotransferase II (*HPT*II) gene and neomycin phosphotransferase II (*NPT*II) gene were used as selectable marker in this study. MCS segments were very susceptible to antibiotic agent because they had surface-to-volume ratio greater than the conventional tissues such as leaves and petiole in absorptive property. In order to identify the most appropriate concentration of antibiotic agent, explants were tested with 0 to 50 μg/mL of hygromycin and 0 to 150 μg/mL kanamycin in half-strength MS medium containing sucrose (3%, w/v), 0.001 mg/mL 2,4-D, 0.1 mg/mL zeatin for 4 weeks (Figs. 11 and 12). Callus only formed with below 2.5 μg/mL hygromycin, where 75% of survival rate was indicated. Although kanamycin had more survival than hygromycin, regeneration was not obtained in all concentrations (Tables 14 and 15). This result was similar to the previous report that hygromyin has more sensitive than kanamycin in *Populus* (Lee et al., 2001). Based on the results of this study, antibiotic agent determined with 5 μg/mL hygromycin and 25 μg/mL kanamycin for early step selection of putative transgenic plants.

2. *Agrobacterium*-mediated transformation with carotenoid synthetic genes

The MCS segments transformed with carotenoid synthetic genes, including *GGPS, PDS*, *ZDS, CHX,* and *PSY* gene, also contained reporter gene such as *HPT*II or *NPT*II. The surface of the MCS segments formed calli after culturing on the selection medium for 2 weeks, while shoots were regenerated after 4 weeks. They were later transferred to multiplication medium (MS medium supplemented with sucrose (3%, w/v), 0.001 mg/ 2,4-D, 0.1 mg/mL zeatin, and 500 μg/mL cefotaxime sodium) when

Fig. 11. Hygromycin test of MCS segments.

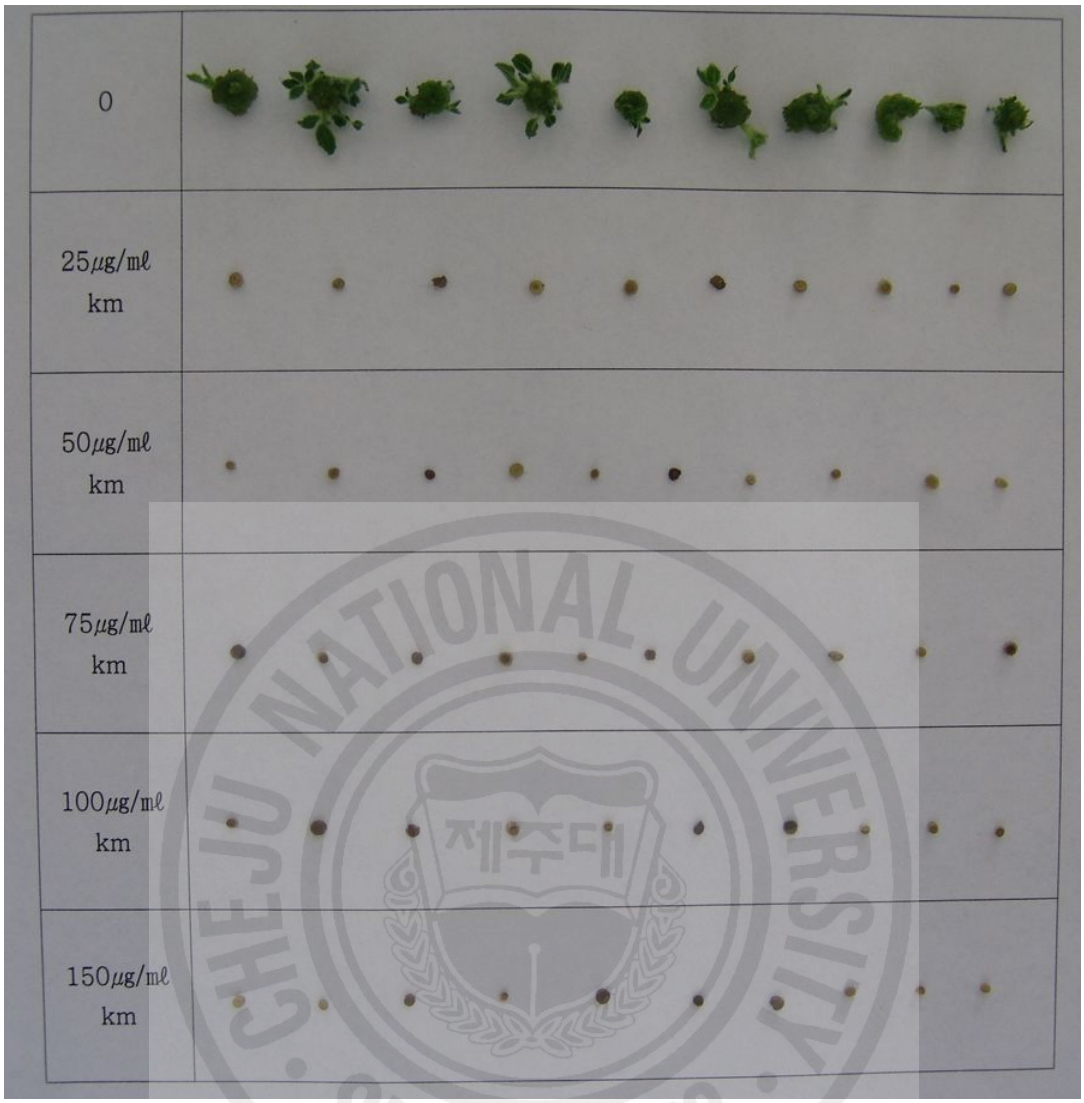


Fig. 12. Kanamycin test of MCS segments.

Table 14. Hygromycin resistance test for MCS segments.

Concentration (μg/mL)	Survival (%)	Callus formation (%)	Shoot formation (%)	No. of shoot/ explant
0	100	100	100	7.0
2.5	75	100	0	0
5	0	0	0	0
10	0	0	0	0
20	0	0	0	0
50	0	0	0	0

Table 15. Kanamycin resistance test for MCS segments.

Concentration (μg/mL)	Survival (%)	Callus formation (%)	Shoot formation (%)	No. of shoot per explant
0	100	10	100	4.1
25	100	0	0	0
50	80	0	0	0
75	80	0	0	0
100	70	0	0	0
150	100	0	0	0

regenerated shoots had 3 to 4 of leaves after 10 weeks (Figs. 13 and 14). The regeneraion efficiency of transformed shoots with each gene was evaluated by survival rate and the number of regenerated shoots. Also, the efficiency of the transformation was confirmed by PCR analysis for putative transgenic kiwifruit.

The survival rate which means production of callus or shoot for total treated explants in the selection medium, were indicated as 13.3% in *GGPS*, 4.3% in *PDS*, 16.4% in *ZDS*, 35.3% in *CHX*, and 68.4% in *PSY*. The shoot regeneration efficiencies were similar to survival rate except for 14.1% in *CHX* and 37.9% in *PSY* lines (Table 16). This result was similar to previously reported findings for transgenic kiwifruit (Uematsu et al., 1991; Janssen and Gardner, 1993; Wang et al., 2007). Uematsu et al. (1991) and Janseen and Gardner (1993) indicated various regeneration frequency using different binary vectors. Wang et al. (2007) reported that various kinds of *Agrobacterium* strain show appreciable effect on percentage calli formation from explants in kiwifruit. Therefore, regeneration efficiency could be determined by *Agrobacterium* strains or binary vectors.

3. Transgene expression analysis

The transformation efficiency was confirmed by PCR analysis, which indicated 8% in *GGPS*, 2.9% in *PDS*, and 12.3% in *ZDS,* 9.4% in *CHX*, and 21.1% in *PSY* transgenic plants in the target specific gene and 12% in *GGPS*, 2.9% in *PDS*, and 13.7% in *ZDS,* 14.1% in *CHX*, 24.2% in *PSY* transgenic plants in the reporter gene for the total number of explants (Fig. 15 and 16). The PCR positive for regenerated shoot was 66.7% in *GGPS*, 100% in *PDS*, 90% in *ZDS*, 66.7% in *CHX*, and 87.5% in *PSY* in target specific gene, while reporter gene was indicated to be 100% except for 95.8% in *PSY* transgenic plants (Table 16). The transformation efficiencies were obtained ranged from 75% to 83% of PCR positive for the tested plants that transformed with *NPT*II using PCR analysis in Duncan grapefruit (*Citrus paradise*

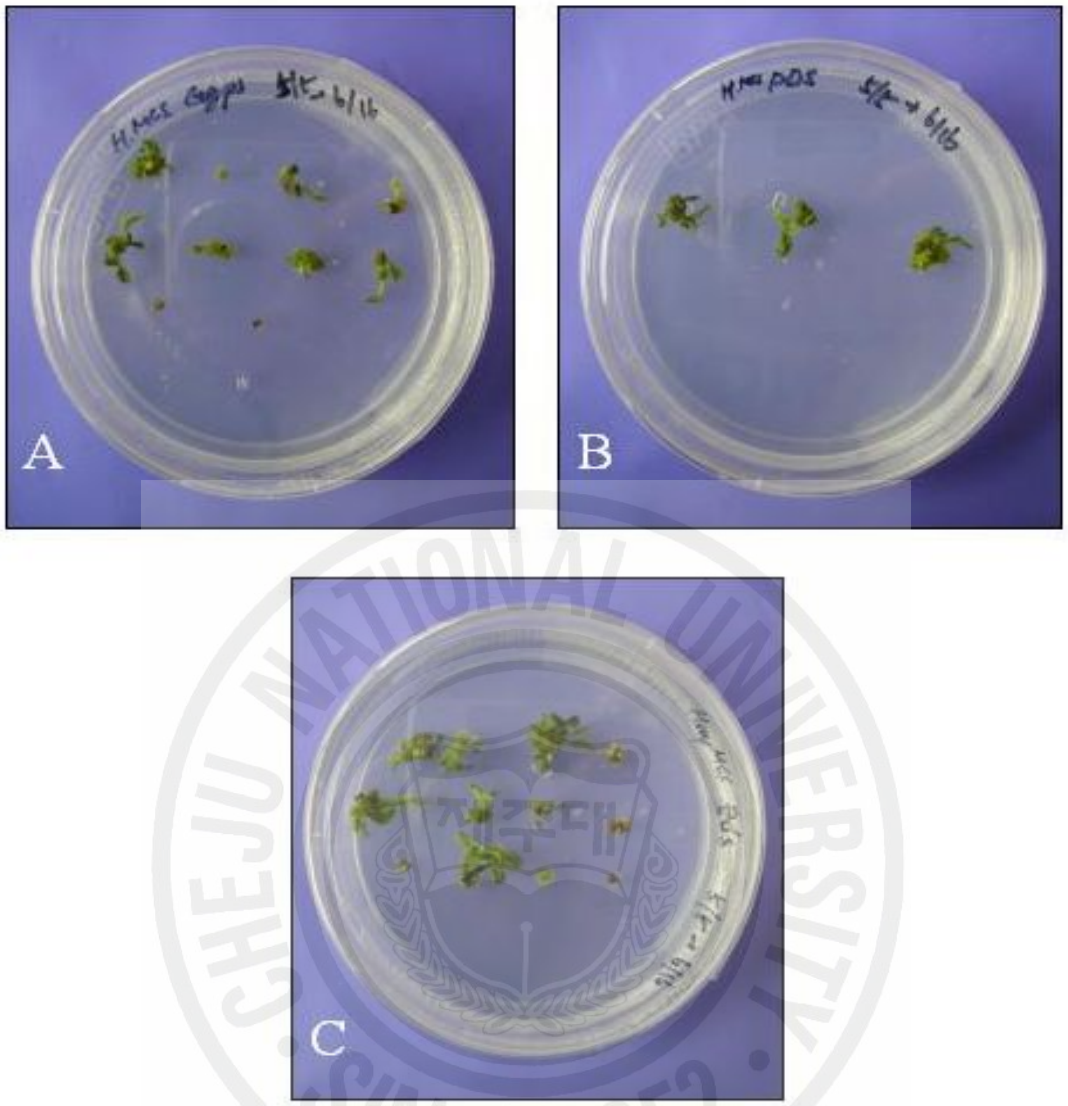


Fig. 13. Selection of transgenic plants by antibiotic resistance gene on selection medium with hygromycin B. Putative transgenic shoot was regenerated in MS medium with 0.1 mg/mL zeatin and 500 μg/mL cefotaxime sodium. A, *GGPS*; B, *PDS*; C, *ZDS* transgenic plants.

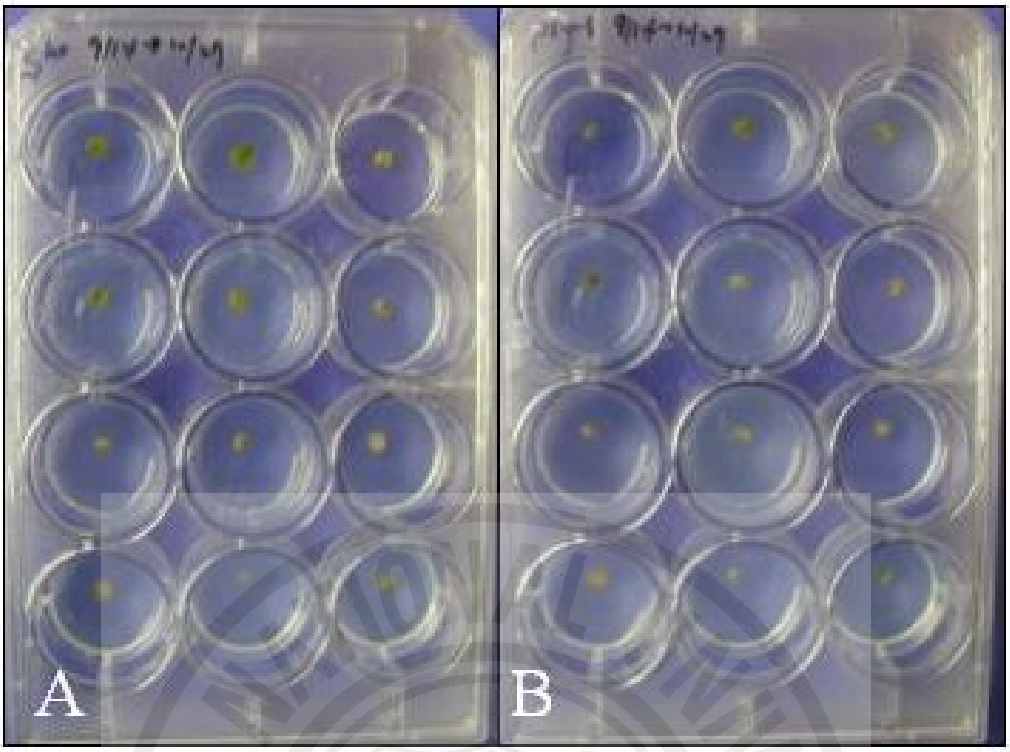


Fig. 14. Selection of transgenic plants by antibiotic resistance gene on selection medium with kanamycin A. Putative transgenic shoot was regenerated in MS medium with 0.1 mg/mL zeatin and 500 μg/mL cefotaxime sodium. A, *CHX*; B, *PSY* transgenic plants.

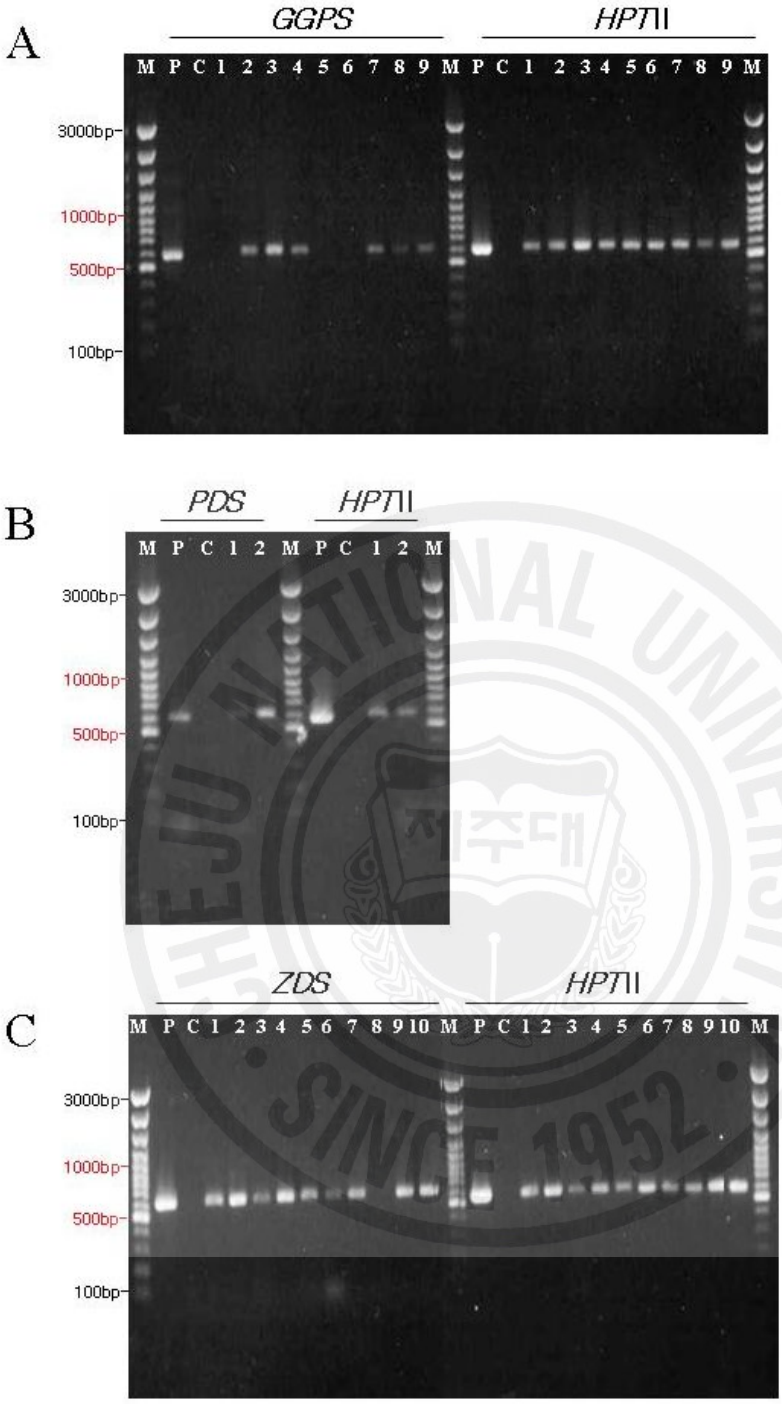


Fig. 15. PCR analysis of *GGPS*, *PDS*, and *ZDS* in transgenic plants. Lanes: M, DNA size marker (GeneRuler^{TM} 100bp Ladder Plus); P, positive control; C, untransformed control plant; Number, putative plants of each gene. A, *GGPS*; B, *PDS*; C, *ZDS* transgenic plants.

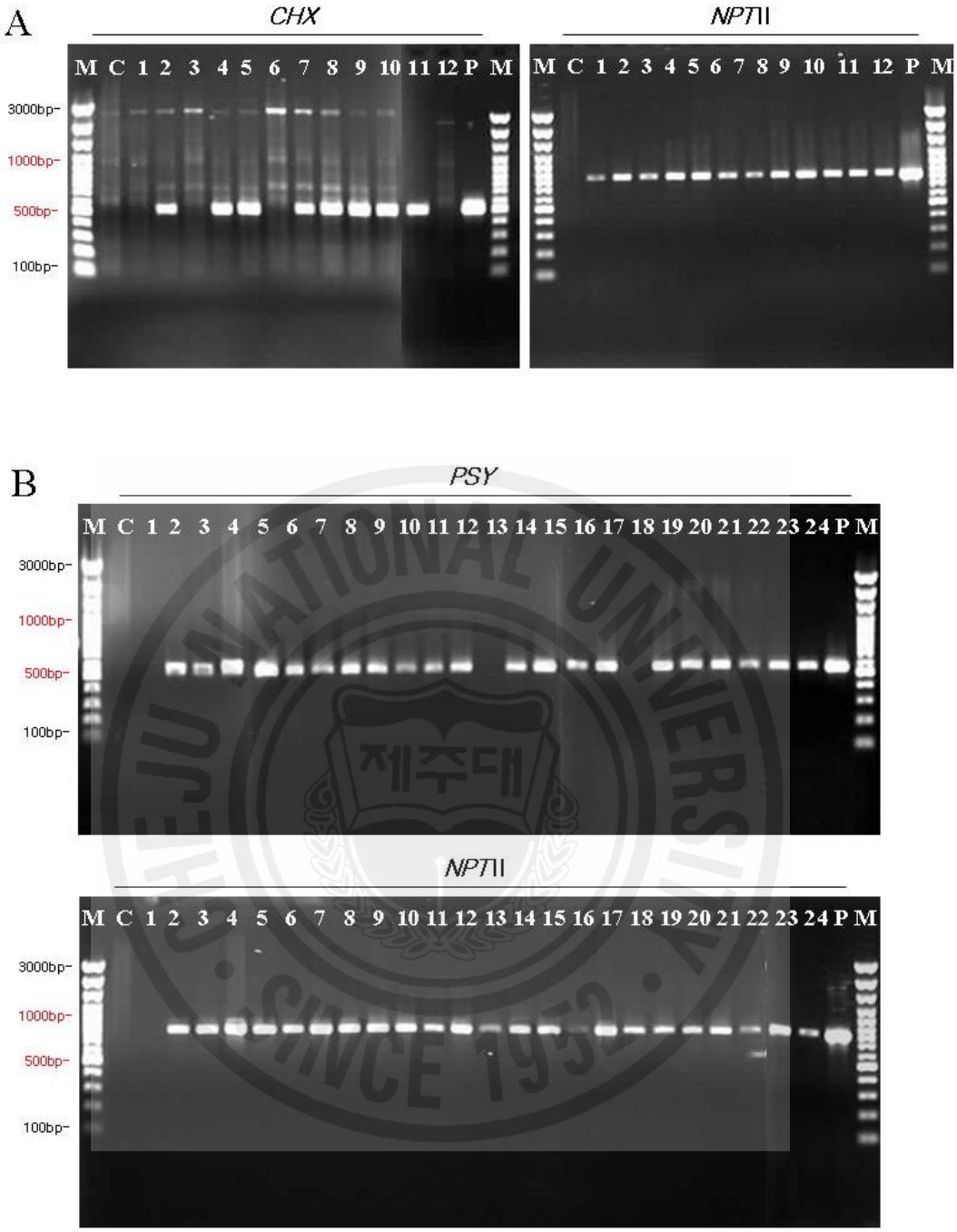


Fig. 16. PCR analysis of *CHX* and *PSY* in transgenic plants. Lanes: M, DNA size marker (GeneRuler^{TM} 100bp Ladder Plus); C, untransformed control plant; P, positive control; Number, putative plants of each gene. A, *CHX*; B, *PSY* transgenic plants.

Table 16. Regeneration and transformation efficiency for each gene in this study.

Gene	Segments treated	Survival rate (%)	No. of Regenerated shoots	No. of PCR confirmed	Transformation efficiency (%)[Z]	
					Target gene	Reporter gene
GGPS	75	13.3	10	9	8.0 (6/9)[y]	12.0 (9/9)
PDS	70	4.3	3	2	2.9 (2/2)	2.9 (2/2)
ZDS	73	16.4	12	10	12.3 (9/10)	13.7 (10/10)
CHX	85	35.3	12	12	9.41 (8/12)	14.1 (12/12)
PSY	95	68.4	36	24	22.1 (21/24)	24.2 (23/24)

[Z]The percentage of transformation efficiency was calculated as the total number of PCR postive shoots per total number of explants evaluated x 100.

[y]Parenthesis indicates PCR positive shoots per total number of explants.

Macf.) (Costa et. al, 2002). The results of this study contributed that non-transgenic plants so called 'escape' was remarkably able to decrease because putative transgenic plants were strictly selected by early step selection of in this study. Therefore, transformation by MCS method suggests that selection of putative transgenic plants is very easy due to lower survival rate of non- transgenics with antibiotic agent used in this study.

The RT-PCR results showed that the transcripts of each inserted genes with the expected size were 160 bps for *GGPS*, 163 bps for *PDS*, 269 bps for *ZDS*, 508 bps for *CHX*, and 540 bps for *PSY* (Fig 17).

Using Southern blots analysis, the *GGPS* transgenic plants were carried out with single copy or multiple copies of *HPT*II targeted-DNA (Fig. 18).

The confirmed transgenic plants through PCR analysis were briefly dipped in 0.4 mg/mL IBA for rooting and then planted in artificial soil (1:1 of perlite and vermiculite) in the greenhouse (Fig 19).

4. Analysis of carotenoid contents by HPLC

The main carotenoid in leaves known is lutein (about 45%), β-carotene (usually 25-30%), violaxanthin (15%), and neoxanthin (15%) (Britton, 1991). HPLC analysis was used to confirm the accumulation of carotenoid such as β-carotene and lutein in transgenic plants that contain five genes. HPLC analysis indicated that β-carotene were not different in *GGPS*, *PDS*, and *ZDS* transgenic plants. *GGPS* clone 8 increased by 1.2 folds with 0.25 mg/g in lutein content than non-transgenic plants. *CHX* transgenic plants were increased by 1.6 to 2.0 folds with 0.33 mg/g to 0.39 mg/g in lutein content and by 1.7 to 2.0 folds with 4.3 mg/g to 5.6 mg/g in β-carotene content than the non-transgenic plants. *PSY* transgenic plants also increased by 1.6 to 1.9 folds with 0.3 mg/g to 0.34 mg/g in lutein content and by 1.35 to 1.7-fold with 3.4 mg/g to 4.3 mg/g in beta-carotene content than the non-transgenic plants (Figs.

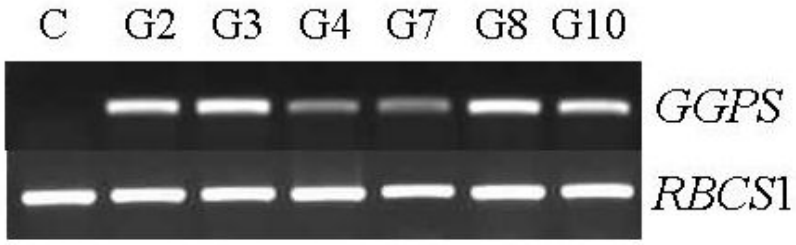


Fig. 17-1. RT-PCR analysis for the transgenic plants overexpressing *GGPS*. *RBCS*1 transcripts were amplified as PCR control. C, control plant; G2 to G10, transgenic plants

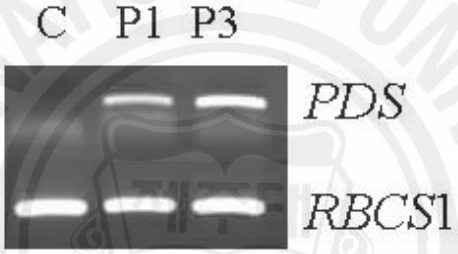


Fig. 17-2. RT-PCR analysis for the transgenic plants overexpressing *PDS*. *RBCS*1 transcripts were amplified as PCR control. C, control plant; P1 and P3, transgenic plants.

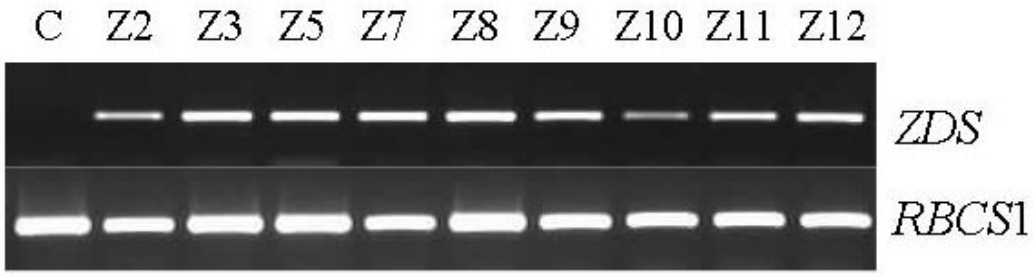


Fig. 17-3. RT-PCR analysis for the transgenic plants overexpressing *ZDS*. *RBCS*1 transcripts were amplified as PCR control. C, control plant; Z2 to Z12, transgenic plants.

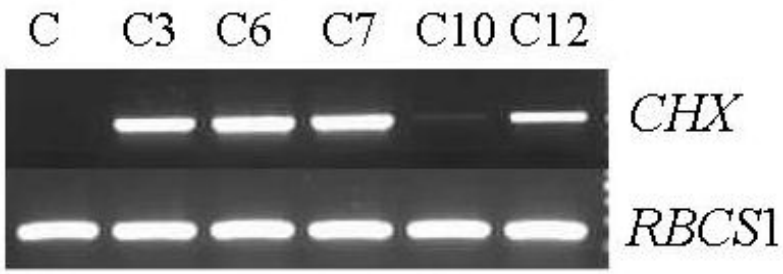


Fig. 17-4. RT-PCR analysis for the transgenic plants overexpressing *CHX*. *RBCS*1 transcripts were amplified as PCR control. C, control plant; C3 to C12, transgenic plants.

Fig. 17-5. RT-PCR analysis for the transgenic plants overexpressing *PSY*. *RBCS*1 transcripts were amplified as PCR control. C, control plant; PS2 to PS8, transgenic plants.

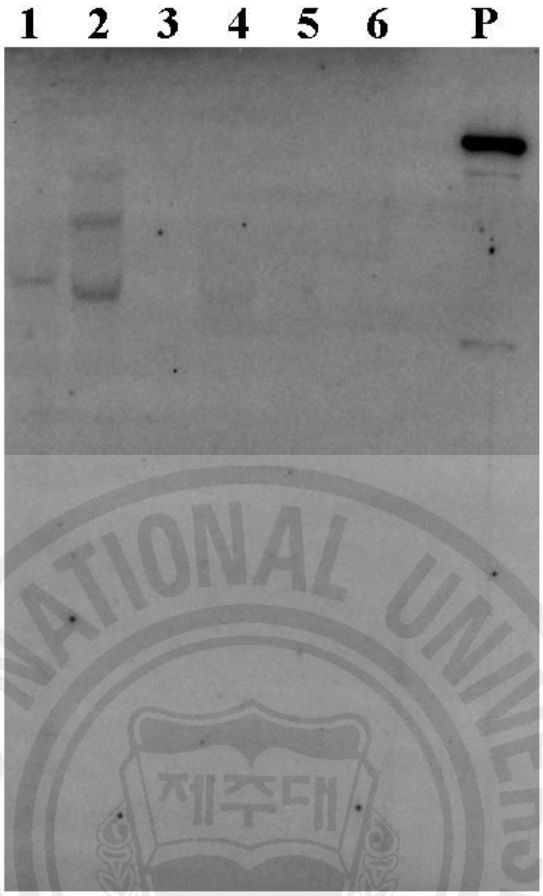


Fig. 18. Southern blot analysis of the *HPT*II gene in *GGPS*-transgenic plants. Lane 1-6, *GGPS* transgenic plants; lane P, positive control. Genomic DNA was loaded after digestion with *Eco*RI and hybridized with a *HPT*II probe.

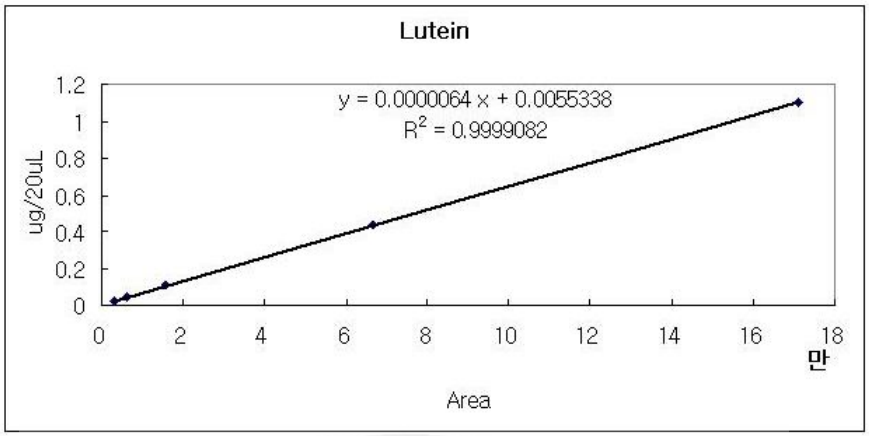
Fig. 19. Two-year-old transgenic plants in green house.

21 to 23 and Table 17). Therefore, *CHX* and *PSY* genes were indicated to be more effective than other genes.

Increases in total carotenoid have been mainly reported by manipulating *PSY* and *PDS* according to phytoene biosynthesis and desaturation step (Shewmaker et al., 1999; Ye et al., 2000; Fraser et al., 2001). Transgenic *chy*B plants (encodes β-carotene hydroxylase) distinctly increased with violaxanthin amount by two folds than wild type in *Arabidopsis* (Davison et al., 2002). This genes, however, did not affect the lutein and β-carotene contents. The *non dormant*-1 mutant (*nd*-1), with deficiency at the gene coding for ζ-carotene desaturase in sunflower (*Helianthus annuus* L.) showed an albino and viviparous phenotype and the absence of xanthophylls such as lutein, β-carotene, violaxanthin, and cis-neoxanthin in carotenoid analysis (Conti et al., 2004). This result suggested that those *ZDS* genes are also concerned in the accumulation of carotenoid content. In contrast, we achieved plants with high content of carotenoid from *CHX* and *PSY* transformants than other genes.

In conclusion, we suggest that *CHX* and *PSY* genes are functional and potentially useful to increase carotenoid production than *GGPS*, *PDS*, *ZDS* genes.

A



B

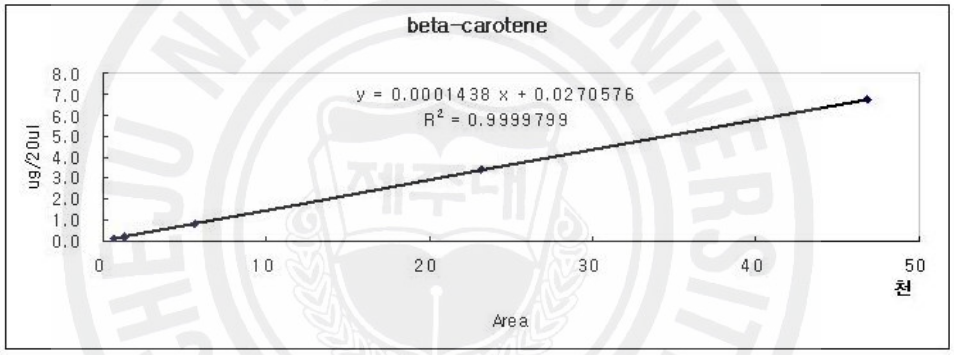


Fig. 20. Calibration curve of lutein (A) and β-carotene (B) for gradual dilution standard by HPLC analysis.

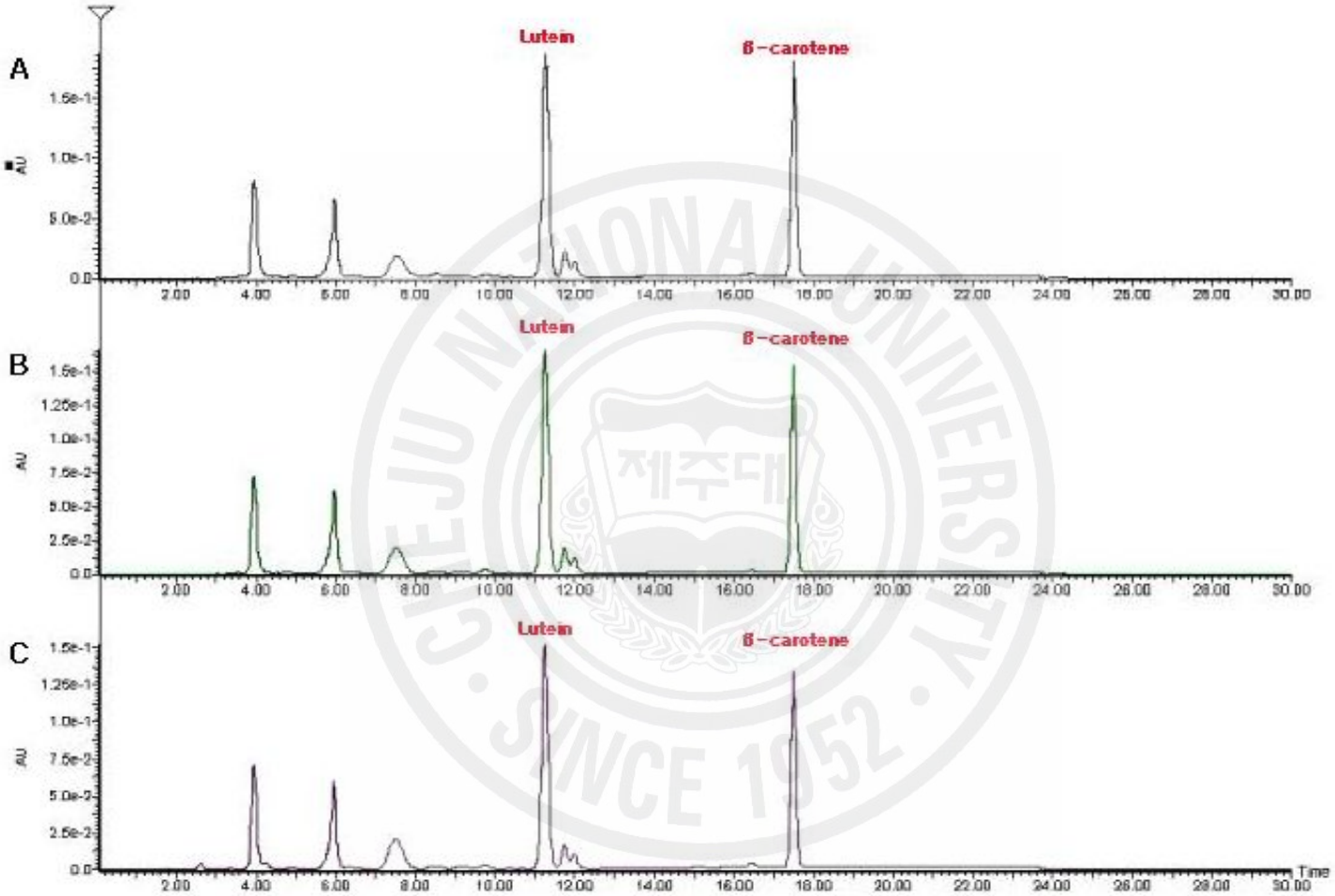


Fig. 21. HPLC profiles of carotenoids pigments from non-transgenic and transgenic plants.
A, Control plant 1; B, G2-1 transgenic plant; C, G3-2 transgenic plant.

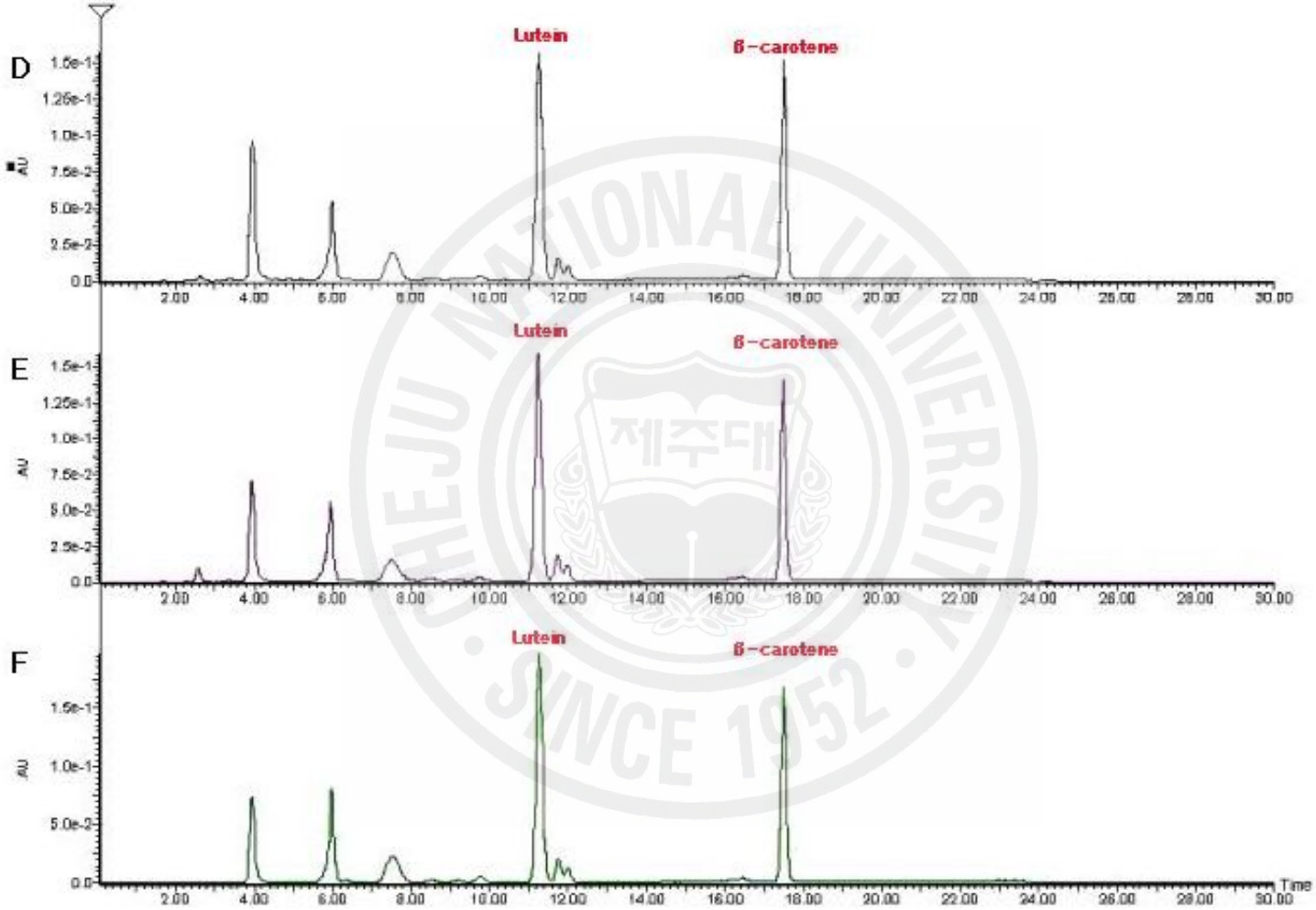


Fig. 21. (Contiuned). D, G4-1 transgenic plant; E, G7-3 transgenic plant; F, G8-2 transgenic plant.

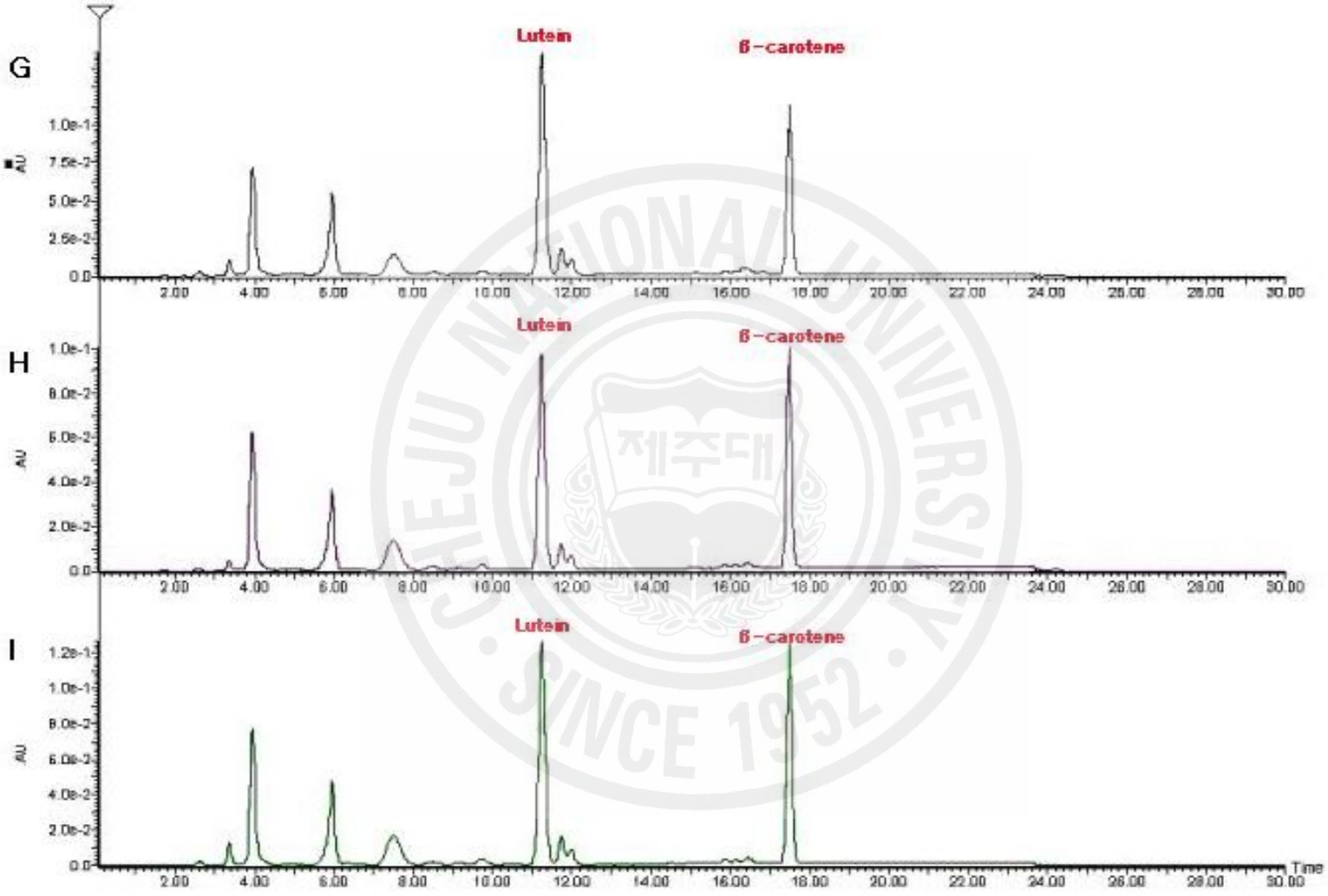


Fig. 21. (Contiuned). G, G10-2 transgenic plant; H, Z2-3 transgenic plant; I, Z3-1 transgenic plant.

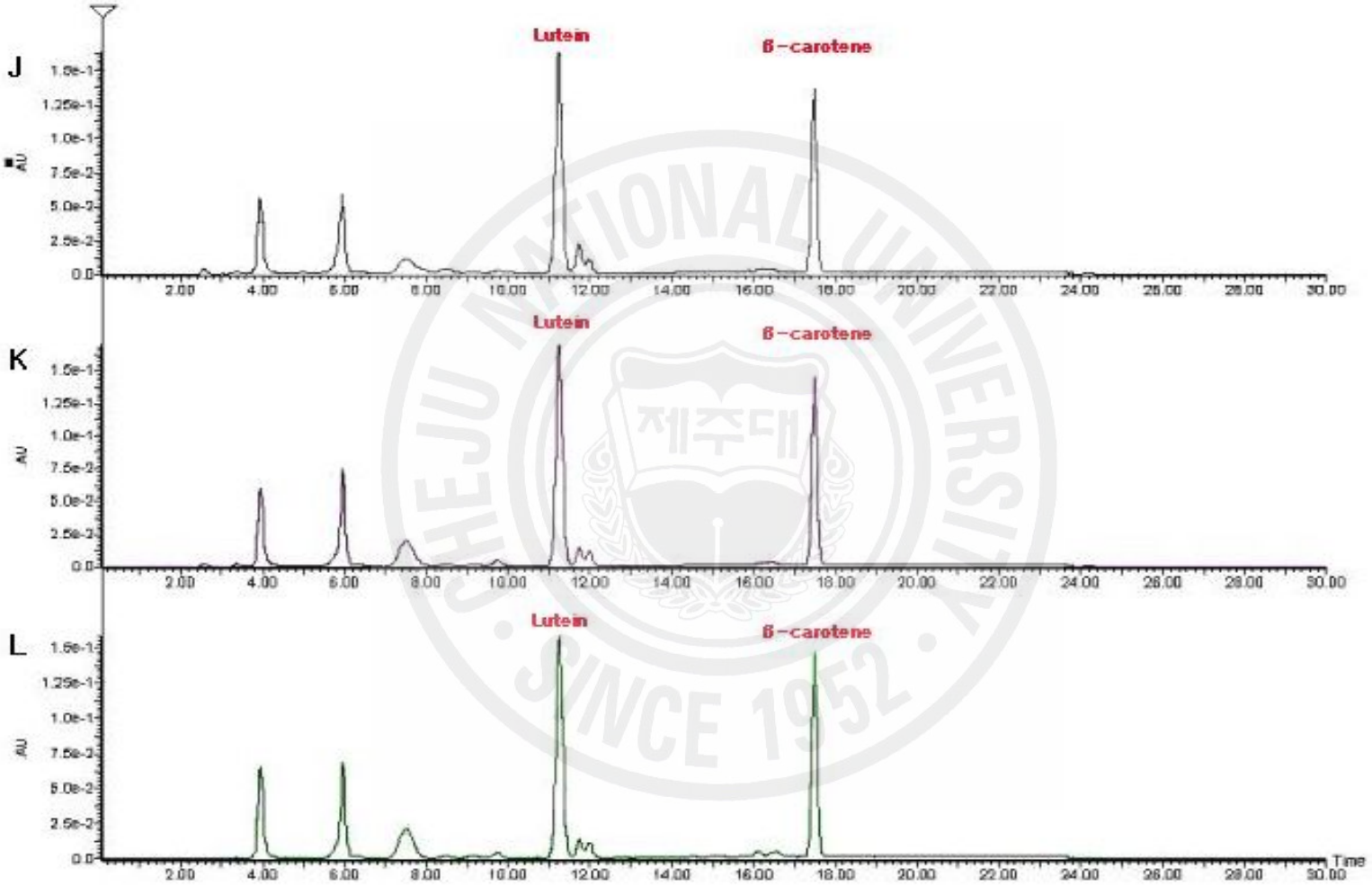


Fig. 21. (Contiuned). J, Z5-3 transgenic plant; K, Z6-2 transgenic plant; L, Z7-2 transgenic plant.

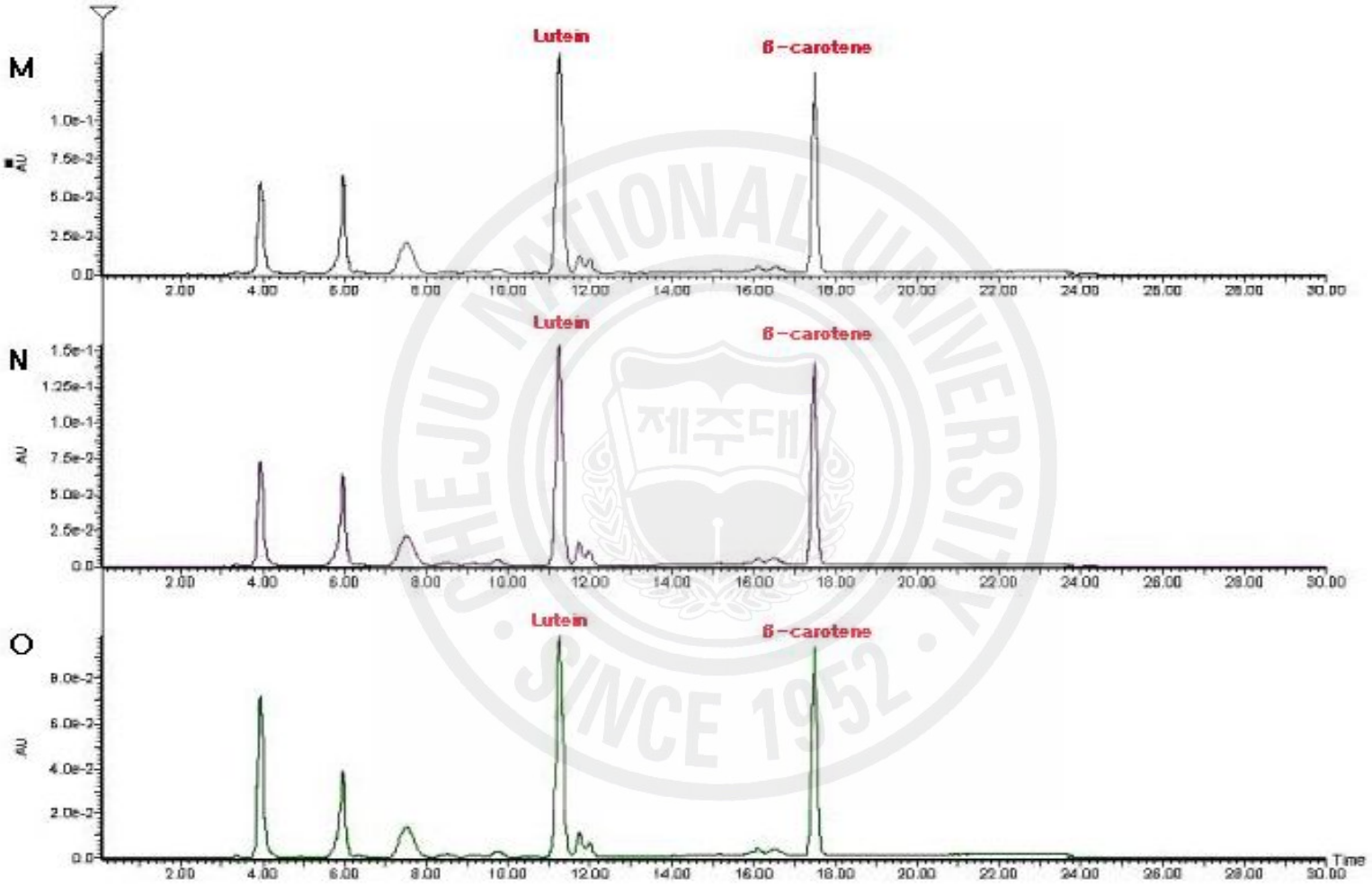


Fig. 21. (Contiuned). M, Z8-2 transgenic plant; N, Z9-1 transgenic plant; O, Z11-2 transgenic plant.

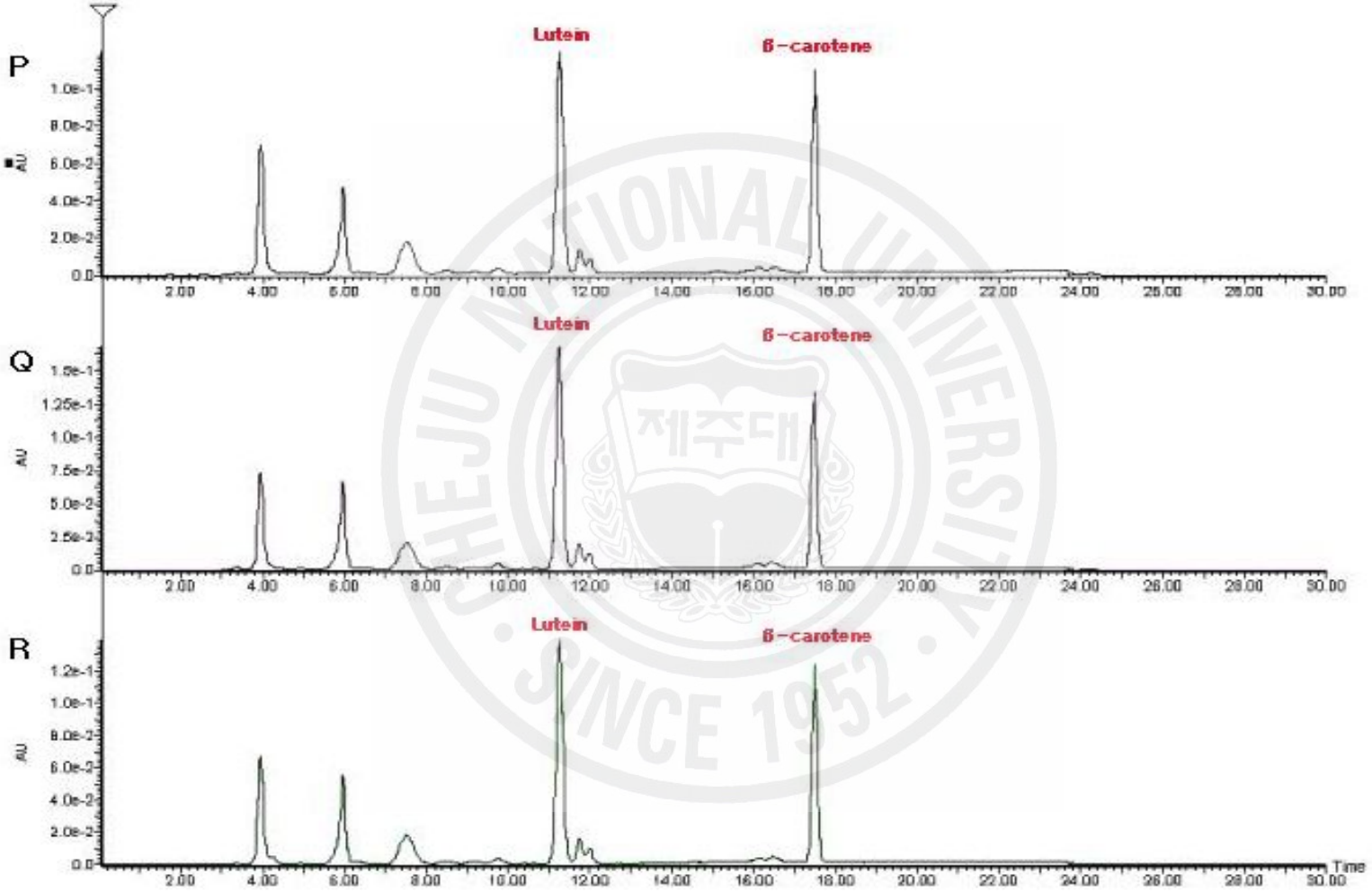


Fig. 21. (Contiuned). P, Z12-3 transgenic plant; Q, P2-3 transgenic plant; R, P3-8 transgenic plant.

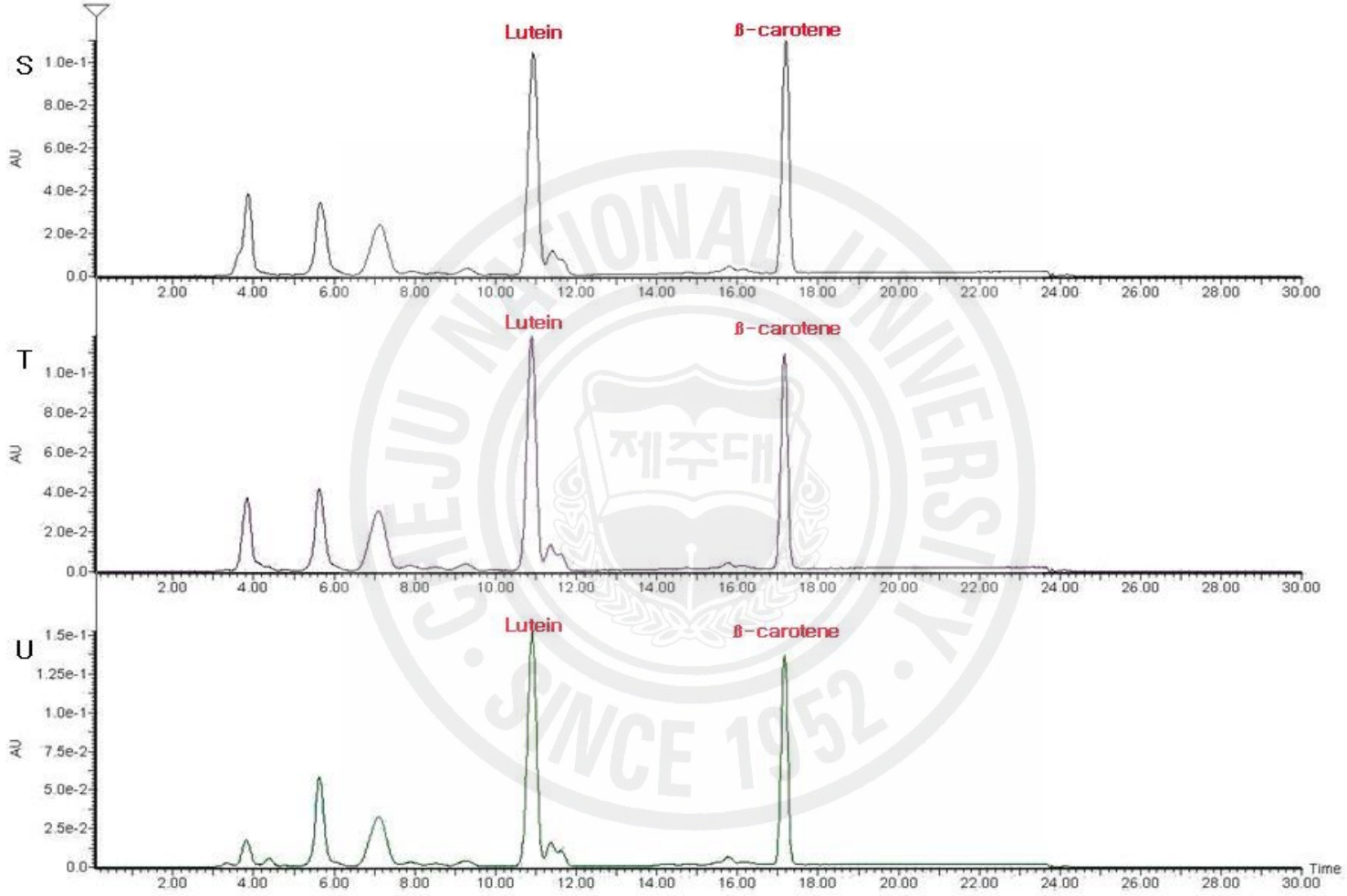


Fig. 21. (Contiuned). S, C3-1 transgenic plant; T, C6-1 transgenic plant; U, C6-1 transgenic plant.

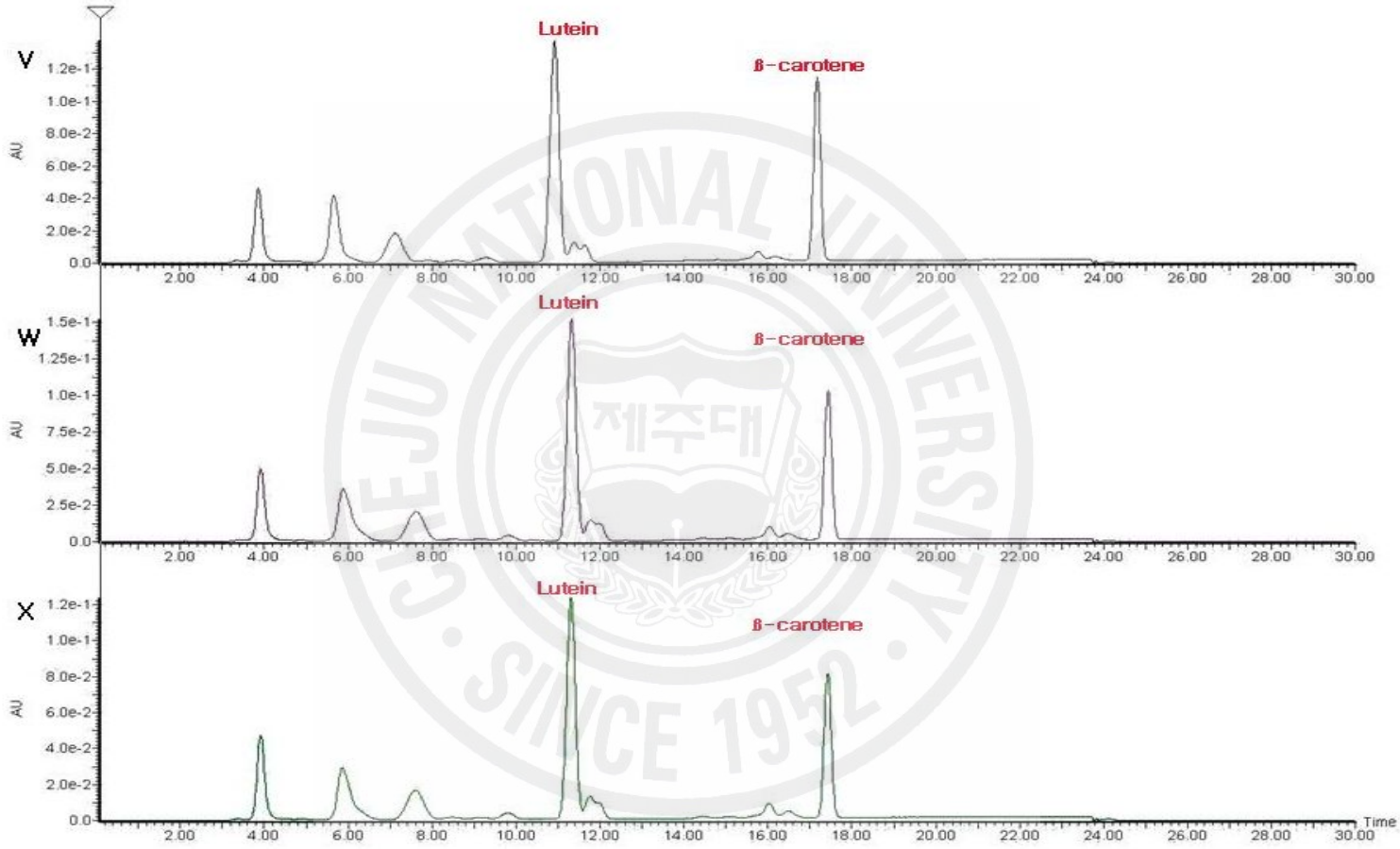


Fig. 21. (Contiuned). V, PS2-1 transgenic plant; W, PS3-2 transgenic plant; X, PS4-1 transgenic plant.

Table 17. Carotenoid contents among non-transgenic and transgenic plants.

Line	Lutein[z] (μg/g)	β-carotene (μg/g)
Cont 1	193.33 ± 11.260 def	2523.00 ± 111.27 cd
Cont 2	196.33 ± 13.132 def	2499.00 ± 42.36 cd
G2-1	198.33 ± 6.386 de	2472.67 ± 157.43 d
G3-2	177.33 ± 6.888 ef	2320.67 ± 214.76 d
G4-1	153.33 ± 12.441 ef	2006.67 ± 85.76 d
G7-3	182.00 ± 14.468 def	2220.33 ± 111.14 d
G8-2	235.33 ± 10.171 d	2607.33 ± 137.14 cd
G10-2	163.67 ± 7.219 ef	2191.33 ± 278.16 d
Z2-3	154.33 ± 25.783 ef	2402.67 ± 532.01 d
Z3-1	143.67 ± 23.786 f	2108.67 ± 346.44 d
Z5-3	169.00 ± 10.970 ef	2266.33 ± 144.17 d
Z6-2	165.00 ± 15.535 ef	2269.67 ± 147.47 d
Z7-2	184.33 ± 3.844 def	2630.00 ± 254.64 cd
Z8-2	165.67 ± 15.762 ef	2288.67 ± 251.56 d
Z9-3	181.67 ± 13.860 def	2556.33 ± 269.33 cd
Z11-2	140.33 ± 19.055 f	2086.67 ± 391.52 d
Z12-3	153.00 ± 17.156 ef	2286.67 ± 414.68 d
P2-5	184.00 ± 22.113 def	2538.00 ± 403.50 cd
P3-8	150.00 ± 3.215 ef	2159.33 ± 176.15 d
C3-1	333.33±10.477 bc	5057.00 ± 178.06 a
C6-1	320.67 ± 14.836 bc	4311.33 ± 282.32 a
C7-1	389.67 ± 22.040 a	5058.67 ± 462.51 a
PS2-1	345.33 ± 20.301 abc	4212.00 ± 386.62 ab
PS3-2	3640.00 ± 27.875 ab	4320.00 ± 366.51 a
PS4-1	304.00 ± 21.939 c	3409.33 ± 185.87 bc

[z]Means separation within each column by Duncan's multiple range test at $P \leq 0.05$.

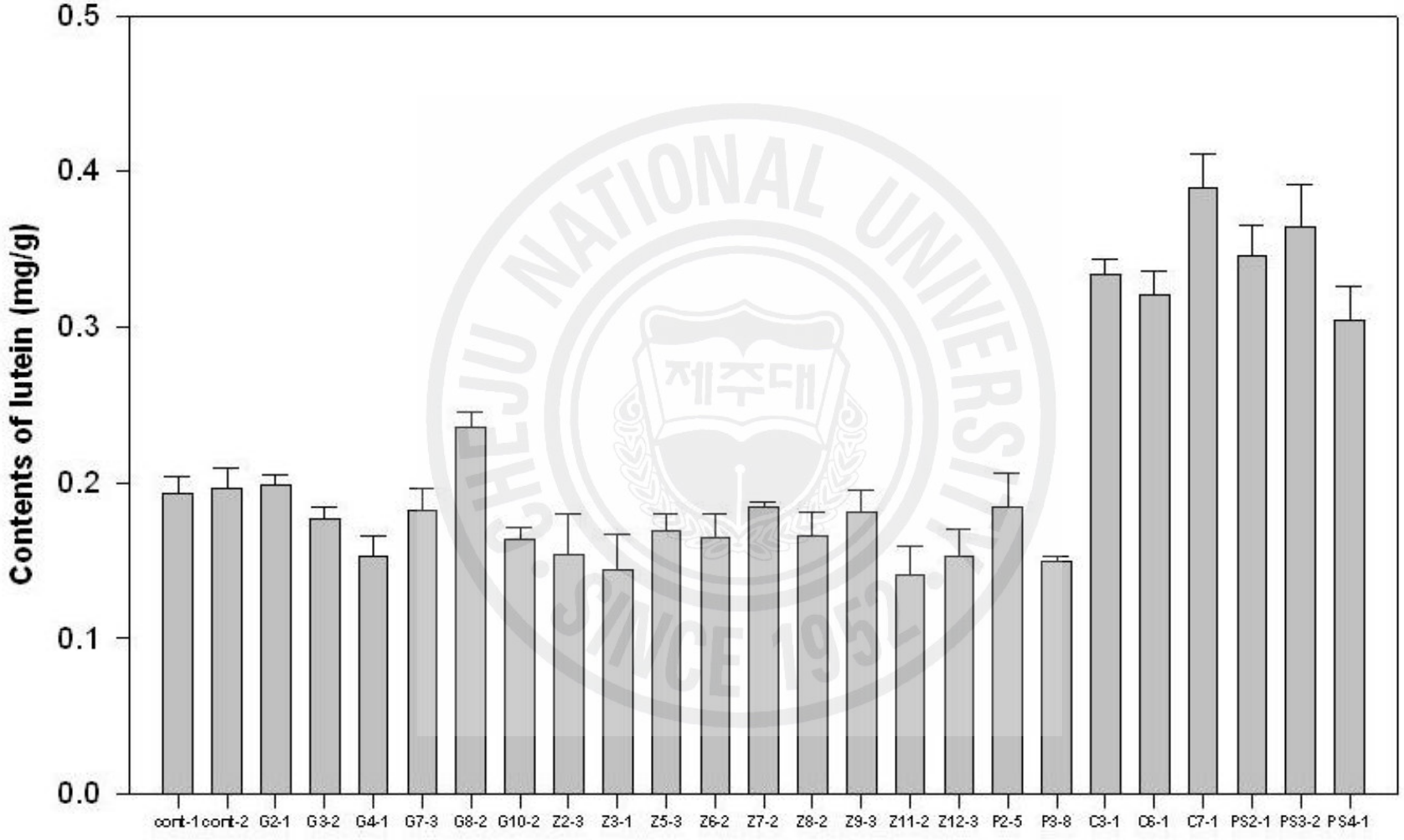


Fig. 22. Lutein content among non-transgenic and transgenic plants. Bars represent means ± SE.

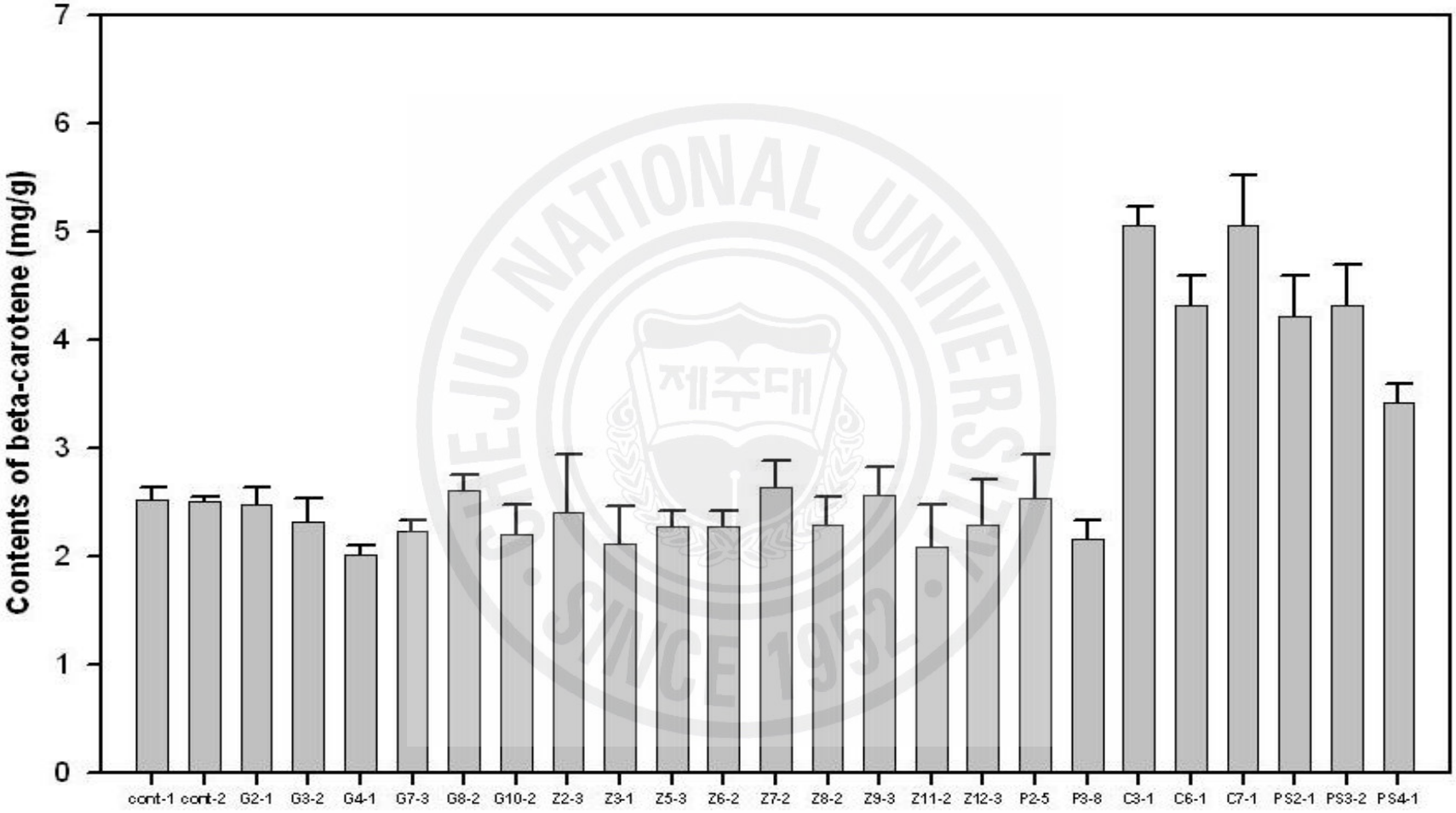


Fig. 23. β-carotene content among non-transgenic and transgenic plants. Bars represent means ± SE.

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ABSTRACT IN KOREAN
(국문 초록)

본 연구는 참다래에서의 미세절단 방법 (micro-cross sections, MCS)을 이용한 급속증식 기술개발과 카로티노이드의 함량을 증가시키기 위하여 수행되었다.

미세절단 시스템을 이용한 효율적인 급속증식 시스템 확립을 위하여 적절한 무기염, 식물 생장조절제, 절편체의 크기, 절편체의 종류를 검토하였다. 무기염은 1/2 MS 배지가 MS 배지보다 절편체의 생존율이 높게 나타났으며, 절편체의 크기는 800 μm 크기가 가장 적절한 것으로 나타났다. 또한, 식물생장조절제는 2,4-D와 zeatin이 thidizuron이 캘러스 형성과 신초 형성에 효과적으로 나타났다. 하지만 thidiazuron이 비정상적이 신초를 많이 생성하였기 때문에 zeatin을 신초 형성에 이용하였다. 이를 토대로 1/2 MS 배지에 2,4-D와 zeatin를 조합하여 잎, 엽병, 줄기 절편을 치상한 결과, 2,4-D 0.001 mg/mL와 zeatin 0.1 mg/mL가 첨가된 줄기조직에서 가장 높은 신초 증식 효율을 보여주었다. 캘러스 형성율과 신초 형성율은 각각 100%와 94%였으며, 절편체당 신초 수는 2.61개를 나타내었다. 또한, 이 방법을 통해 재분화된 식물체의 체세포 변이 유무를 확인하기 위하여 MCS로부터 재분화된 식물 29개와 기내에서 배양된 모본을 RAPD와 SSR를 이용한 유전자 분석을 수행하였다. 먼저, RAPD분석에서는, 24개의 프라이머 중 19개에서 500 ~ 3500 bps의 범위를 지닌 63개의 뚜렷한 밴드를 나타냈다. SSR 분석에서는 19개의 SSR primer 중 17개에서 50 bps ~ 1500 bps의 범위을 지닌 46개의 뚜렷한 밴드가 관찰되었다. RAPD와 SSR 분석결과 재분화된 식물체와 모본 사이에 polymophic한 밴드는 관찰되지 않았다. 따라서, RAPD와 SSR 분석 결과는 미세절단 방법을 통해 재분화된 식물체들이 유전적으로 안정됨을 보여주었다. 결과적으로, 이들 결과들은 줄기 조직을 이용한 미세절단 배양법이 키위 증식에 있어 유용한 방법임을 나타내었다.

카로티노이드 함량이 높은 참다래를 개발하기 위하여 카로티노이드 합성과 관련된 유전자인 geranylgeranyl diphosphate synthase (*GGPS*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), phytone synthase (*PSY*), and β-carotene hydroxylase (*CHX*) 등 5가지 유전자를 이용하였다. hygromycin phosphotransferase II (*hpt*II) 유전자와 neomycin phosphotransferase II (*npt*II) 유전자를 선발마커로 사용하였다.

형질전환은 1/2 MS배지에 2,4-D 0.001 mg/mL와 zeatin 0.1mg/mL, hygromycin 2.5 μg/mL 또는 kanamycin 25 μg/mL 그리고 500 μg/mL cefotaxime을 이용하였다.

형질전환된 식물체에서의 생존율은 *GGPS*에서 13.3%, *PDS*에서 4.3%, *ZDS*에서 16.4%, *CHX*에서 35.3%, 그리고 *PSY*에서 68.4%이었다. 신초 재분화율은 *GGPS*에서 13.3%, *PDS*에서 4.3%, 그리고 *ZDS*에서 16.4%, *CHX*에서 14.1%, 그리고 *PSY*에서는 37.9%로 나타났다. PCR 분석에 의하면 전체 처리된 식물체에 대한 목표유전자의 형질전환율은 *GGPS*에서 8%, *PDS*에서 2.9%, *ZDS*에서 12.3%, *CHX*에서 9.4%, *PSY*에서 22.1%였다. 리포터 유전자의 형질전환율은 *GGPS*에서 12%, *PDS*에서 2.9%, *ZDS*에서 13.7%, *CHX*에서 14.1%, *PSY*에서 24.2%였다. 재분화된 식물에 대하여 PCR을 실시한 결과에 목표유전자에 대하여 *GGPS*에서 66.7%, *PDS* 100%, *ZDS* 90%, *CHX*에서 66.7% 그리고 *PSY*에서 87.5%를 나타냈으며, 리포터 유전자에 대해서는 *PSY*에서의 95.8%를 제외한 모든 형질전환체에서 100%를 나타내었다.

RT-PCR 결과 *GGPS*에서 160 bps, *PDS*에서 163 bps, *ZDS*에서 269 bps, *CHX* 508 bps, 그리고 *PSY* 540 bps를 나타내어 유전자가 발현됨을 확인하였다.

*GGPS*가 삽입된 형질전환체의 서던 분석결과 1 copy 또는 multi copies로 유전자가 삽입된 것으로 확인되었다.

HPLC 를 이용한 카로티노이드 분석 결과 *GGPS*, *PDS*, *ZDS* 형질전환체 사이에는 베타카로틴에 있어서 함량 차이를 나타내지는 않았지만, 루테인에서는 *GGPS* 클론 8 이 25 mg/g 으로 대조구에 비해 함량이 1.2 배 증가 하였다. 하지만, *CHX*

형질전환체에서는 대조구에 비교하여 루테인이 0.33 mg/g ~ 0.39 mg/g 으로 1.6 배 ~ 2 배, 베타카로틴이 4.3 mg/g ~ 5.6 mg/g 으로 약 1.7 배 ~ 2.0 배 증가하였다. 또한, *PSY* 형질전환체에서도 대조구에 비교하여 루테인이 0.3 mg/g ~ 0.34 mg/g 으로 약 1.6 ~ 1.9 배 증가하였고, 3.4 mg/g ~ 4.3 mg/g 으로 약 1.35 ~ 1.7 배 증가하였다. 따라서 카로티노이드 합성에 관여하는 5 개의 유전자 중에서 참다래의 카로티노이드 함량을 높이기 위해서는 *CHX* 와 *PSY* 가 *GGPS*, *PDS* 및 *ZDS* 보다 유리할 것으로 판단된다.

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(감사의 글)

실험을 마치고 귀가할 때 느끼는 밤공기의 상쾌함과 그 하늘을 수놓는 수많은 별들, 그리고 따뜻한 커피 한 잔은 지난 4년간의 학위과정 동안 저에게 피로회복제와 같은 존재였습니다. 그리고 저에게는 한 걸음 더 앞으로 나아갈 수 있게 디딤돌이 되어주신 고마운 분들이 있습니다. 이 지면을 빌어 그 분들께 감사의 말씀을 드리고자 합니다.

저에게 아낌없는 지도와 사랑으로 부족한 저를 이 자리까지 이끌어주셨으며 지금은 미국으로 교환교수로 가 계시는 송관정 교수님께 마음 깊이 감사 드립니다. 그리고 논문이 완성되기까지 세심한 조언을 아끼지 않으신 문두길 교수님, 소인섭 교수님께 마음 깊이 감사 드립니다. 지금까지 저에게 많은 가르침과 관심을 가져주시는 박용봉 교수님, 강훈 교수님, 한상헌 교수님께도 감사 드립니다. 바쁘신 와중에도 많은 조언과 격려 그리고 연구에 대한 열정을 보여주신 명지대학교의 김호방 박사님께도 마음 깊이 감사 드립니다.

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본 연구를 수행하는데 있어 부족한 저에게 항상 끊임없는 조언과 격려를 아끼지 않으신 난지농업연구소의 김성철 박사님께 마음 깊이 감사 드립니다. 모르는 것 있을 때마다 많이 귀찮게 해드린 장기창 박사님 감사 드립니다. 항상 저를 격려해주시는 문두영 연구관님, 성기철 연구관님, 김천환 연구사님, 송은영 연구사님, 노나영 연구사님, 이진수 연구사님, 실험실의 전능재, 오병돈 후배님에게도 감사 드립니다. 그리고 난지원예작물과 여러 선생님과 연구원 분들께도 감사 드립니다.

연구하는데 있어 많은 배려를 해주신 난지농업연구소의 서효덕 전 소장님, 난지원예작물과의 강경희 과장님, 만날 때마다 저를 걱정해주시는 난지환경과의 전승종 과장님께 감사 드립니다.

언제나 전화를 걸 때마다 반갑게 대해 주시는 생물종다양성 연구소의 정용환 박사님 그리고 많은 조언을 해주신 축산센터의 한상현 박사님께도 감사 드립니다.

짧은 기간이었지만 실험을 하는 동안 많은 배려를 해주신 명지대학교 생명과학정보학부의 최상봉 교수님 및 연구원 분들께 감사 드립니다. 많은 도움을 준 안효민 후배님에게도 감사합니다.

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