A THESIS

FOR THE DEGREE OF MASTER OF PHILOSOPHY

Transformation of *Carotenoid Isomerase* (*CRTISO*) Gene in Tobacco

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GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

December, 2006

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A thesis submitted in partial fulfillment of the requirement of
the degree of Master of Philosophy

December, 2006

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CONTENTS

요 약

카로티노이드는 자연계에 존재하는 isoprenoid계 물질로써 식물, 해조류, 미생물 등에서 합성되며 약 600여종 이상의 화합물이 있다고 알려져 있다. Carotenoid isomerase (CRTISO)는 식물의 카로티노이드 생합성 경로에서 노란색을 띄는 poly-cis-lycopene에서 빨간색을 띄는 all-trans-lycopene로 합성을 촉매하는 효소이다. 현재 CRTISO는 토마토 돌연변이체와 애기장대의 mutant 연구를 통하여 알려져 있다.

그래서 본 연구에서는 토마토의 과실에서 *CRTISO* 유전자를 T-벡터에 클로닝하였고, *CRTISO*가 카로티노이드 생합성 과정에서 어떠한 영향을 보이는지를 알아보기 위해 식물발현벡터에 이 유전자를 클로닝하여 담배에서 항시적 발현을 유도하였다. 식물발현벡터인 pBItCrtIso(+)는 *A. tumefaciens* LBA4404에 형질전환시켜 담배형질전환에 이용하였다. 담배형질전환은 기내에서 2일 동안 1㎎/ℓ의 benzyladenine (BA)과 0.01 ㎎/ℓ의 naphthylacetic acid (NAA)이 들어간 MS 배지에서 공동 배양하였고, 4주 동안 1 ㎎/ℓ의 BA, 0.01 ㎎/ℓ의 NAA, 100 ㎎/ℓ의 kanamycin, 500 ㎎/ℓ의 carbenicillin이 들어간 MS 배지에서 형질전환체를 선택 하였다. 선택된 형질전환체는 5주 동안 100 ㎎/ℓ의 kanamycin, 500 ㎎/ℓ의 carbenicillin이 들어간 MS 배지에서 뿌리유도를 시킨 후에 토양으로 순화 시켰다. 형질전환체로 추정되는 약 20개의 식물체를 얻었다.

담배형질전환체는 genomic DNA를 분리하여 PCR 분석법을 통해 유전자의 도입을 확인하였고, mRNA 발현을 보기 위하여 RT-PCR을 수행하였다. 형질전환 후 카로티노이드 생합성의 조성 변화와 양적인 변화를 보고자 추정되는 형질전환체 잎으로부터 HPLC 분석을 수행하였다. 후대검정을 위하여 T_1세대 형질전환체도 genomic DNA PCR과 HPLC를 통하여 분석되었다. T1세대 PCR 분석을 한 결과 500bp의 PCR product를 확인하였다.

이는 각각 1번 라인에서 18개체, 6번 라인에서 5개체의 식물에서 유전자 도입이 이루어졌음을 알 수 있었다. T_1세대의 분리비를 확인하기 위해 도입된 유전자의 kanamycin 저항성 성질을 이용하여 분리비를 조사하였다. 분리비는 카이제

곱 검정법에 의하여 1번 라인은 2.8:1, 6번 라인은 3:1임을 추정할 수 있었다. HPLC 방법을 이용하여 형질전환 된 T_1세대의 1번 라인에 카로티노이드 함량을 분석한 결과, lutein 함량은 대조군에 비하여 60% ~ 102%가, β-carotein 함량은 39% ~ 69% 가 증가되었음을 보여주었다. 이러한 결과를 살펴 볼 때, 식물에서 carotenoid isomerase (CRTISO)는 카로티노이드 생합성 경로에 관여하여 lutein과 β-carotene과 같은 xanthophyll의 양적인 변화를 유도한다고 생각된다.

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ABBRIVIATION

ABA : Abscisic acid

BHT : Buthylated hydroxytoluene

βLCY : β-cyclase

bp : Base pairs

BR : Breaker

CRTI : Bacterial phytoene desaturase

CRTISO : carotenoid isomerase

DDW : Distilled deionized water

DEPC : Diethylpyrocarbanate

EDTA : Etylenediamine tetraacetic acid

EtBr : Ethidium bromide

GGPP : Geranylgeranyl pyrophosphate

HPLC : High-performance liquid chromatography

hr : Hour(s)

IPP : Isopentenyl pyrophosphate

kb : Kilo base pair(s)

LB : Luria-Bertani medium

LCY-B : Lycopene β-cyclase

LCY-E : Lycopene ε-cyclase

MeOH : Methanol

PCR : Ploymerase chain reaction

PDS : Phytoene desaturase

PSY : Phytoene synthase

RT-PCR : Revers-transcriptase PCR

rpm : Revolution time

UV : Ultra violet

ZDS : ζ-carotene desaturase

I. INTRODUCTION

Plant carotenoids are 40-carbon isoprenoids with polyene chain that contain up to 15 conjugated double bonds. Because of their chemical properties carotenoids are essential components of all photosynthetis organisms.[1)] Carotenoids are the most widespread group of pigments in nature, with over 600 species characterised structurally.[2)] Carotenoids are responsible for the yellow, orange and red coloration of tissuese.[3)] These coloration pigments play important accessory roles in light harvest, protect against photooxidation, energy transfer to the chlorophylls and maintain structural integrity of the photosynthetic apparatus.[4)] Carotenoids furnish attractive colors to fruits, vegetables and ornamental flowers, and their composition in these crops has enormous economic value. β-carotene is the precursor of vitamin A and protect for degenerative disease in animals. Also, it is the major value in human nutrition, provide health benefits from their antioxidant activity in vivo. A number of carotenoids have industrial application, it use as colorants for human food and feed additives to enhance the pigmentation of fish and eggs, as cosmetics and as pharmaceutical products.[1)]

The pathway of carotenoid biosynthesis is shown Figure. 1. The biosynthesis of carotenoids occurs within the chloroplasts of plants and algae.[5)] The critical step in the formation of the first C_{40} acyclic hydrocarbon carotenoid, phytoene, is the tail-to-tail condensation of two molecules of the C_{20} intermediate geranylgeranyl pyrophosphate (GGPP). The biosynthesis of phytoene from geranylgeranyl pyrophosphate (GGPP) is produced by the enzyme phytoene synthase (PSY). This reaction yields the first C_{40} carotenoid, phytene, the bacbone of all plant carotenoids.[3)]

Phytoene undergoes a series of four desaturation reactions serve to lengthen the conjugated series of carbon-carbon double bonds that constitutes the chromophore in carotenoid pigment, and there by transform the coloress phytoene into the pink-colored lycopen. Four double bonds are introduced subsequently into phytoene by two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), each catalyzing two symmetric

dehydrogenation steps to yield ζ-carotene and lycopene, respectively.

In photosynthetic organisms, phytoene desaturase (PDS), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) catalyze the desaturation and isomerization reactions from phythoene to trans-lycopene. The enzyme phytoene desaturase (PDS) catalyze the conversions of phytoene to phytofluene to ζ-carotene, regulated steps in carotenoid and absisic acid (ABA) biosynthesis in many organisms. Phytoene desaturase (PDS) genes has been identified and isolated from soybean,[6] pepper,[7] and tomato[8,9]. ζ -carotene desaturase (ZDS) catalyses the conversions of ζ-carotene into lycopene via neurosporene. Encoding genes has been identified from peper,[10] Arabidopsis[11] and tomato.[8]

In plants, two desaturase, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), carry out these reactions, whereas in bacteria all four dehydrogenation reactions are carried out by a single gene product, named the bacterial phytoene desaturase (CRTI).[1]

The cyclization of lycopen in photosynthetic organisms marks an important branching point in the biosynthesis pathway of carotenoids. One branch leads to carotene and its derivative xanthophylls, whereas the other leads to α-carotene and lutein. Lycopene β -cyclase (LCY-B) catalyzes a two-step reaction that creates one β-ionone ring at each end of the lycopene molecule to produce β-carotene, whereas lycopen ε-cyclase (LCY-E) creates one εring to give δ-carotene.

Xanthophylls or oxygenated carotenoids coprise most of the carotenoid pigment in the thylakoid membranes of planta.[12] Several of the enzymes involved in carotenoid biosynthesis, carotenoids modified with oxygen containing groups, have also been and higher plants (Fig. 1).[13]

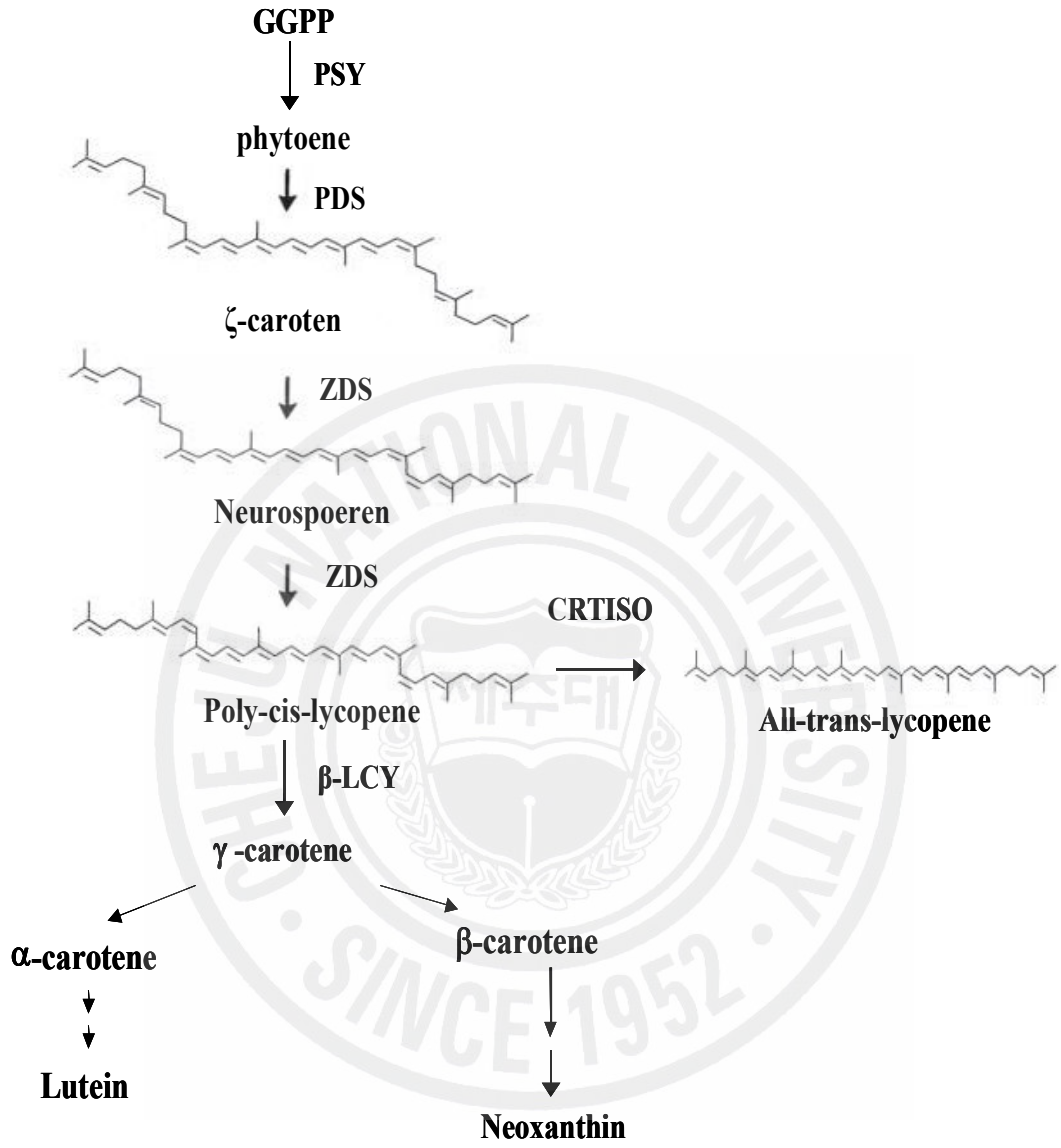

Fig. 1. The carotenoid biosynthesis pathway in plants (revised by Park et al).

PDS ; Phytoene desaturase, PSY ; Phytoene synthase,

ZDS ; ζ-carotene desaturase, CRTISO ; carotenoid isomerase, βLCY ; β-cyclase

In higher plants, the isoprenoid chains of carotenoids contain numerous double bonds that allow the formation of a variety of cis or trans geometric isomers. The cis-trans configurations are critical features of carotenoids, because they determine the spectral quality and resulting photochemical properties and colors of these pigments.[4)]

Carotenoid isomerization in plant has been mystery since the tangerin (t) mutant of tomato, with its orange-tinted flowers and characteristic tangerine-orange fruit, was described by Zechmeister et al. (1941) and Tomes et al. (1953).[15,16)] The fruit of tangerine accumulate a poly-cis-isomer of the carotenoid lycopene, named prolycopene, whereas the wild-type red tomato varieties produce mainly trans-isomers of lycopene and other carotenoids.[15)] Like tomato, most plants appear to produce mainly trans forms of lycopene and other carotenoids.[17)]

The tomato (*Solanum lycopersicum*) mutant tangerine is well-studied example of cis-carotene. 7,9,7',9'-tetra-cis-lycopene (prolycopene) replaces the all-trans-lycopene in the orange-colored fruit of this mutant. In tangerine locus, CrtIso gene have cloned, and reported that mutations in this gene abolish in tangerine a specific cis-trans-isomerrization of carotenoids, carrying the accumulation of prolycopene and other cis-isomers of its upstream precursors. By functional expression of CrtIso cDNA in E. coli CRTISO is a carotene isomerase involved in the biosynthesis of all trans-lycopene.[14)]

Isaacson et al. identified carotenoid isomerase (CRTISO) from tomato and Park et al. identified carotenoid isomerase (CRTISO) from Arabidopsis, respectively. The tomato *CRTISO* gene identified at tomato mutant locus via map-based cloning using introgression line of a wild tomato species, *Lycopersicon pennellii*, in the cultivated tomato *L. esculentum.*[14)] Park et. al. isolated the *CRTISO* mutant (ccr2) as a carotenoid and chloroplast regulation mutant in Arabidopsis.[18)] The mutants lacking CRTISO accumulate prolycopene and ζ-carotene and very little trans-lycopene, and the CRTISO enzymes are shown to be capable of converting poly-cis-lycopene to all-trans-lycopene when expressed in *Escherichia coli*. Interestingly, although the plant CRTISO lacks desaturase activity, the sequences were found to be homologous with those of the

bacterial phytoene desaturase (CRTI) and are not related to the plant desaturases PDS and ZDS.[4)]

In this study, *carotenoid isomerase* (*CRTISO*) gene from tomato was cloned by PCR using the primer designed based on the sequence of tomato (Genbank, AF416727). *CRTISO* gene from tomato was overexpressed in tobacco by *Agrobacterium* mediated transformation, and its carotenoid composition were investigated. Putative tobacco transformants confirmation by genomic DNA PCR, and RT-PCR analysis. Also, carotenoids extracted from putative tobacco transformants leaves and composition of carotenoids were analyzed by HPLC. This study aimed to show the effect of consititutive expression of CRTISO in tobacco and quantity regulation in the carotenoid biosynthesis pathway.

II. MATERIALS AND METHODS

Plant materials

The tobacco (*Nicotina tobacum* cv. NC) was used in this study. The seeds were sterilized by immersion in YUHANROX solution (commercial bleach containing 4% sodium hypochloride) for 10 min and washed three times with sterile distilled water. The sterilized seeds were blot-dried on sterile filter paper for 20 min. The seed were placed on petri dishes containing hormone-free Murashige and Skoog (MS) medium[19] supplemented with 30 g/ℓ of sucrose and solidified with 8 g/ℓ of plant agar. Each culture plate was incubated at 25℃ under a 16/8 hr (day/night) photoperiod.

Isolation of *CRTISO* gene

Tomato carotenoid isomerase cDNA was isolated by RT-PCR (Genbank, AF416727). PCR product was cloned into T-vector (Promega). CrtIso-L primer and CrtIso-R primer designed for cloning (Table. 2) and performed RT-PCR from immature fruit of tomato. *CRTISO* gene were confirmed by sequencing and NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST).[20]

Bacterial strain and *Agrobacterium* strain

The bacterial strain, *E. coli* XL-1 blue was used as a primary host for transformation and amplification of plasmid. pGEM-T easy (Promega) was used in T-vector cloning and pBI121 (CLONTECH) was used for the construction of plant expression vector (Table. 1). The detailed procedures for construction of each recombined plasmid are described in results. The *Agrobacterium tumefaciens* strain LBA4404 caring the binary vector pBI121 was used. *Agrobacterium* was cultured on YEP madium[21] supplemented with 25 ㎎/ℓ of kanamycin sulfate (Sigma-Aldrich) using standard procedures.[22]

Table 1. Plasmids used in this study

Plasmid	Characteristics	Reference
pGEM-T easy	Apr	Promega
pBI121	Kmr CaMV35S(P)-gus-nos(T)	Clontech
pBICI	Kmr CaMV35S(P)-CI-nos(T)	In this study

***Agrobacterium* mediated transformation**

Agrobacterium-mediated transformation of tobacco was conducted by the method of Horsch et al.[23] Leaves were cut into around 0.5-1cm discs. The explants were introduced with *A. tumefaciens* suspensions for 10 min. Then transferred to co-culture medium in MS[19] medium supplimented with 1 ㎎/ℓ of benzyladenine (BA) and 0.01 ㎎/ℓ of naphthalneacetic acid (NAA). Co-culture medium were incubated at dark for 2 days. After 2 days of co-culture, explants were transferred to selective regeneration medium consisting of MS medium with 1 ㎎/ℓ of BA, 0.01 ㎎/ℓ of NAA, 100 ㎎/ℓ of kanamycin, 500 ㎎/ℓ of carbenicillin. After 4 weeks, the developing shoots were transferred to MS medium supplimented with 100 ㎎/ℓ of kanamycin, 500 ㎎/ℓ of carbenicillin for rooting. The rooted plants (T_0) were transferred to soil and grown in a growth chamber.

Extraction of genomic DNA

DNA was isolated from the young leaves of putative transgenic plants according to Gawel and Jarret (1991).[24] Approximately, 500 ㎎ of frozen young leaves were ground fine powder with liquid nitrogen using motor and pestle. Fine powder heated at 65℃ for 30min contained with 1ml of extraction buffer, 2% of Mercaptoethanol and 10 ㎕ of protenase K in 1.5 ㎖ of micro centrifuge tube. After heating, sample tube quite was chilled on ice for 5 min and was added 500㎕ of chloroform. Sample tubes were centrifuged at 12000 χg for 10 min and taken supernatant. Then was precipitaated incubated at -20℃ for 3 hr over with same volume of isopropanol, and 1/10 volume of 3M sodium acetate (pH 4.8). Collected pellet were washed 500 ㎕ of 70% ethanol and dried at 37℃ for 30 min. Dried pellet were dissolved at 800 ㎕ of sterile distilled water.

RNase A reaction was performed at 37℃ for 30 min. Then, centrifuged with same volume of phenol/chloroform (1:1), same volume of chloroform. After than, genomic DNA was precipitated with same volume of isopropanol and 1/10 volume of 3M sodium acetate (pH 4.8) at -20℃ overnight. Repeated washing step was used 70% ethanol, and dissolved pellet at 50 ㎕ of sterile distilled water.

Confirmation of transgenic plants by genomic DNA PCR

Genomic DNA was amplified by PCR using *CRTISO* specific primers to represent 500 bp. PCR amplification of a 500 bp *CRTISO* fragment was performed using primers: the forward primer (5'- TGG AGA GCT ATG GCA GTA GTG A -3') and the reverse primer (5'- GAA CTG GCC AAA AAG GTA GAT G -3'). These primers designed on the basis of the conserved regions of the previously reported *CRTISO* (Accession No. AF416727). To amplify the presence of a full sequence of *CRTISO* gene, the 35S forward primer, 5'- CAC TAT CCT TCG CAA GAC -3' and Nos-T reverse primer, 5'- GCA ACA GGA TTC AAT CTT AAG -3' were designed from regions between 35S promoter and Nos terminator. This primers represent in Table. 2.

Plant genomic DNA (1 ㎕ approximately 200 ng) was mixed with a pair of primers, 10 pmoles each, 5 ㎕ of 10× PCR buffer, 5 ㎕ of 2.5 mM dNTP mixture, and 0.5 ㎕ of Taq DNA polymerase (Takara Taq 0.25 units), and adjusted with double stilled water to a final volume of 50 ㎕. The PCR was carried out in a thermal cycler (MJ Resarch, USA) under the following conditions: 30 cycles of 94℃ denaturing for 30 sec, 55℃ annealing for 30 sec, and 72℃ extension for 30 sec and another 10 min at 72℃ final extension. Amplfied PCR products were electrophoresed on 1.2 % agarose gel and detected by ethidium bromide (EtBr) staining, photographed under ultraviolet light.

Table 2. Primer sequences of primers designed for PCR amplification used in this study

Primer	nucleotide sequences	characteristics
Cariso-L	5' ATGTGTACCTTGAGTTTTATGTATCC3'	Gene cloning
Cariso-R	5'TCATGCTAGTGTCCTTAACC3'	Gene cloning
CI-up(sn245)	5'TGGAGAGCTATGGCAGTAGTGA3'	PCR analysis
CI-dn(EN753)	5'GAACTGGCCAAAAAGGTAGATG3'	PCR analysis
35S FG	5'CACTATCCTTCGCAAGAC3'	PCR analysis
Nos-T FG	5'GCAACAGGATTCAATCTTAAG3'	PCR analysis

Extraction of total RNA and cDNA synthesis

Extraction of total RNA was isolated using TRIzol reagent and the method of Chomczynski and Sacchi.[25] 1g of tobacco tissue was ground to a find powder with liquid nitrogen in a motor and pestle, resuspended with 15 ㎖ of TRIzol reagent and incubated for 5 min at room temperature. The suspension was centrifuged at 4℃ for 10 min at 12000 ㄨg and decanted supernatant into new screw-cap tube. Then, extracted with 3 ml of chloroform to destroy residual protein. RNA was precipitated with isopropanol and 0.8 M sodium citrate/1.2 M NaCl, 1/2 volume of the aqueous phase each. After incubated at -20℃ for 3 hr, centrifuged at 4℃ for 15min. The pellet were collected by centrifuging at 4℃ for 15min at 12000 ㄨg, washed with 75% of ethanol, and dried at room temperature. The pellet dissolved in 150 ㎕ of DEPC-treated water. If the RNA is not to be used immediately, the RNA solution placed at -70℃.

A 3 ㎍ of total RNA was denaturated with Oligo dT_{18} primer at 70℃ for 5 min. After chilled on ice, heat at 37℃ for 5 min with buffer mixture (containing 4 ㎕ of 5 xRT buffer, 2 ㎕ of 0.1 M DTT and 4 ㎕ of 2.5 mM dNTP). Then, reacted at 42℃ for 60 min with a 1 ㎕ of reverse transcriptase (M-MuLV, MBI).

Transgene segregation of T_1 progeny of transgenic plants

Seeds were surface sterilized in the detergent for 10 min and 4% sodium hypochloride for 10 min. After rinsed 3 times in steril water, seeds were dried and placed on MS medium containing 100 ㎎/ℓ of kanamycin in petri dish. This treatment was carried out in triplicate (30 seeds per dish) and was repeated four times in each transformant lines. After 4 weeks, kanamycin-resistant and kanamycin-sensitive seedling were counted and data were analysed by the Chi-square(χ^2) test at $p = 0.05$.

HPLC analysis

Extraction of carotenoid from leaves of transgenic tobacco were based on Rodriguez-Amaya.[26] Approximately 1g of leaf was ground using mortar and pestle with liquid nitrogen. The find powder was extracted with 50 ml of extraction solvent (Methanol: Ethyl acetate=1:1 containing 0.1 % BHT). The mixture was stirred incubation for 1 hour and filtered through a fitted glass funnel. The residue was extracted two times with same extraction solvent indicated above. The liquid chromatograph apparatus is consisted of a Thermo Separation Products (Table 3). Solvent run at a flow rate of 1 ㎖/min, and 20 ㎕ of crude carotenoids sample was injected.

Table 3. Operation condition of HPLC for carotenoides analysis

Instrument:	P4000 gradient pump, UV6000LP dedector, SCM1000 vacuum degasser, As1000 auto sampler with column oven
column:	Waters PAH C_{18} 5μm 4.6x250mm Column
Mobile phase #A:	ACN : MeOH : Ethyl acetate(80:15:5, v/v)
Mobile phase #B:	ACN : MeOH : Ethyl acetate(60:25:15, v/v)
Gradient:	1% to 99% #A and 99% to 1% #B mobile phase with a linear for 0 to 60 min
Flow rate:	1㎖/min
Wavelength:	450nm
Other condition	- Sample inject : 20㎕ - Equilibration time : 3.0min - Run time : 60.0min

Ⅲ. RESULT AND DISCUSSION

Cloning of *CRTISO* gene

To isolate *CRTISO* gene from tomato, RT-PCR was carried out using specific primer of *CRTISO* gene designed from the nucleotide sequence from Genbank (AF416727) (Fig 2.). Amplified 1.8 Kb fragment was ligated to pGEM-T easy vector (Promega) yielding plasmid pGEM-T-CI. Oligonucleotide primers are shown in Table 2 and cloning scheme in Figure 4. The nucleotide sequence of *CRTISO* gene from immature tomato are shown in Figure 3. The nucleotide sequence has a homology of 98 % with *Lycopersicon esculetum* (Fig 3) from BLAST search.[20)]

The cDNA of CRTISO contains an open reading frame of 600 codons that encodes a polypeptide with a calculated molecular mass of 66 KDa. Most carotenoids in plants occur in the all-trans-configuration. The carotenoids intermediates through the four desaturation recations from phytoene to lycopene have been considered to be in the all-trans-configuration.[7,17)] It is a well-studied fact that the central C15-15' double bond in phytoene is cis-configured.[27)]

Construction of plant expression vector

The plasmid, pBICrtIso(+), was constructed by cloning the tomato carotenoid isomerase cDNA (Genbank Accession number, AF416727) in the orientation into the binary vector, pBI121 which contains a cauliflower mosaic virus (CaMV) 35S promoter and neomycin phosphotransferase (NPT II) gene for antibiotic selection (Fig. 4, Fig. 5). This vector was introduced into *A. turmefaciens* strain LBA4404 (CLONTECH) by freeze-thaw method.

Fig. 2. Amplification of CRTISO cDNA by RT-PCR.

Total RNA extracted from immature tomato fruits and used as a template. RT was done with M-MuULV reverse transcriptase and oligo dT_{18} primer. PCR was amplified with Taq DNA polymerase and synthetic oligomer.

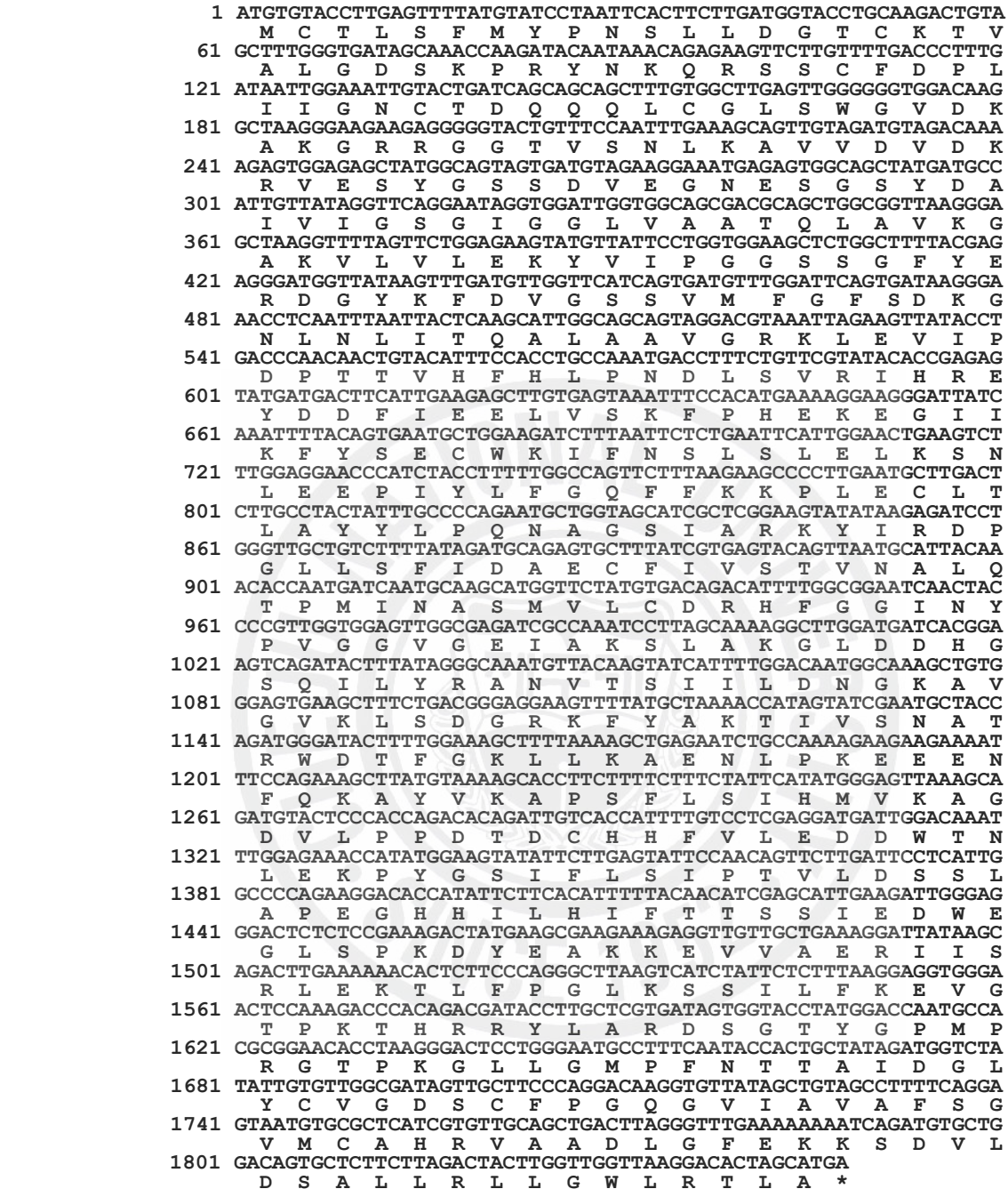

Fig. 3. Nucleotide sequence of *CRTISO* gene and its deduced amino acid sequence from immature tomato. Nucleotides are numbered at the right of the figure in the 5' to 3' direction, beginning with the first nucleotide of the cDNA insert. The deduced amino acid residues are indicated below the nucleotide triplets.

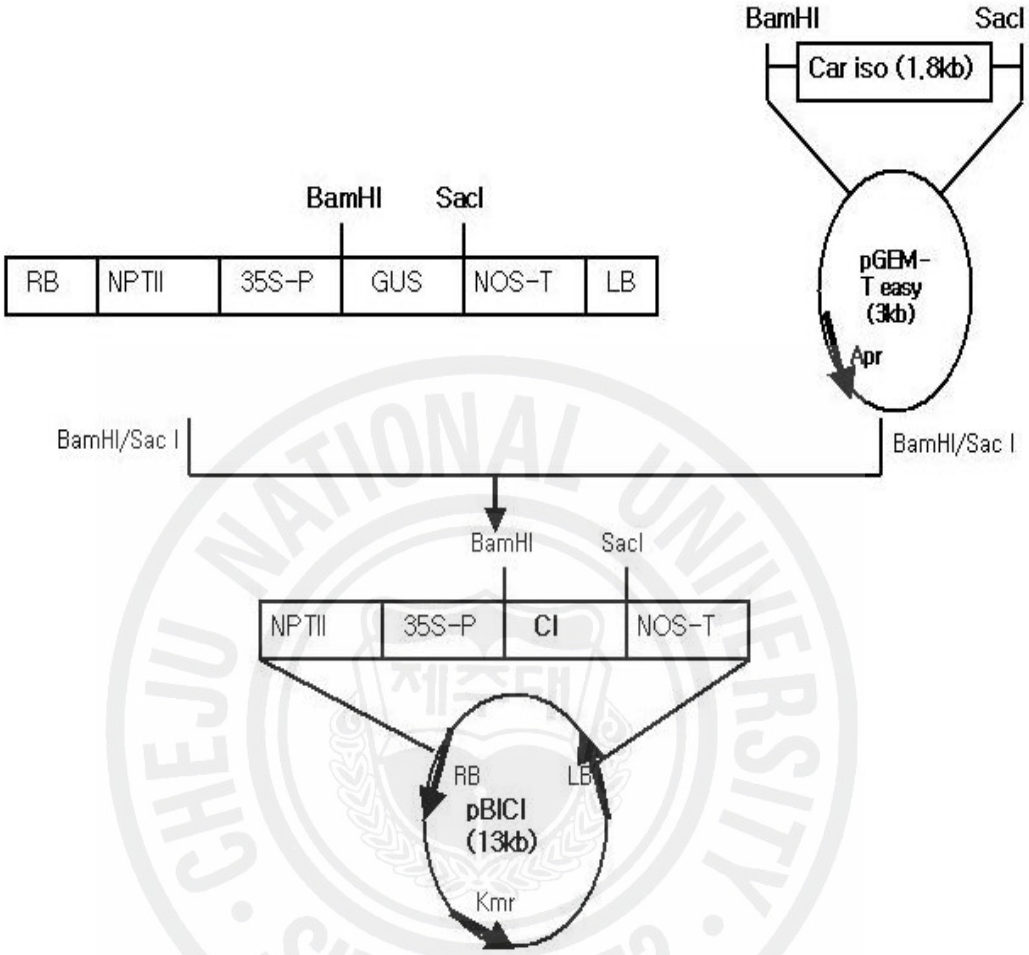

Fig. 4. Cloning strategy of carotenoid isomerase gene from tomato and construction of plant expression vectors. pBICI vector contains CaMV 35S promoter for strong induction and kanamycin resistance for selection of transformants. Insertion of any target gene is available at the same site of BamHI and SacI around *CRTISO*.

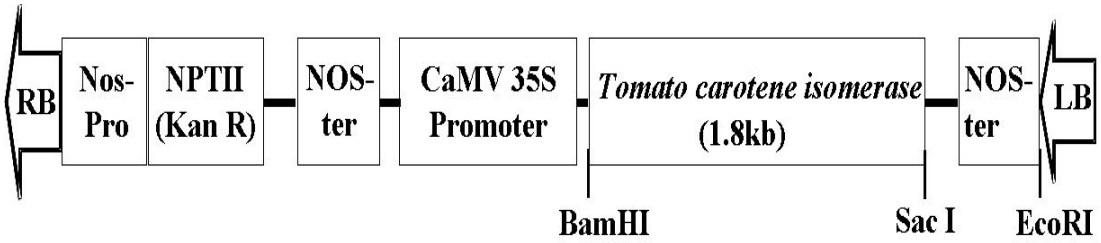

Fig. 5. Construction of plant expression vector, pBItCrtIso(+) plasmid.

It has CaMV 35S promoter and nos terminator as regulatory sequence. It constitutively express the tomato carotene isomerase genes in transformed plants.

Transformants selection

Plant expression vector, pBICI was transformed in *A. tumefaciens* by freeze-thaw method.[23] Tobacco (*N. tobaccum cv.* nc) segments were co-cultured on co-culture medium consisting of MS medium supplimented with 1 ㎎/ℓ of benzyladenine (BA) and 0.01 ㎎/ℓ of naphthaleneacetic acid (NAA).

About twenty of kanamycin-resistance callus formation was observed on surface of forty segments 4 weeks after culture on regeneration medium. It consist of MS medium with 1 ㎎/ℓ of BA, 0.01 ㎎/ℓ of NAA, 100 ㎎/ℓ of kanamycin, 500 ㎎/ℓ of carbenicillin. Adventitious shoots were developed from callus after 5 weeks (Fig. 6 -A, B).

Rooted putative transformants were acclimatized and grown in plastic greenhouse after 12 weeks. Twenty putative transformants, called T_0 progeny transformants, were produced by transforming *N. tobaccum* with pBICI using *Agrobacterium*-madiated transformation of tobacco leaf after 30 weeks (Fig. 6-C, D). These plants were transplanted in the environmentally controlled greenhouse and flowered after 7 months (Fig. 6-D) and self-pollinated seeds were produced after 3 weeks since flowering.

The transgenic plants exhibited nomal growth in morphology and seed yield. Three of these lines were used of futher studies. Seeding of T_1 generation seeds were sown on soil. Growth rate of T_1 generation were similar to T_0 generation.

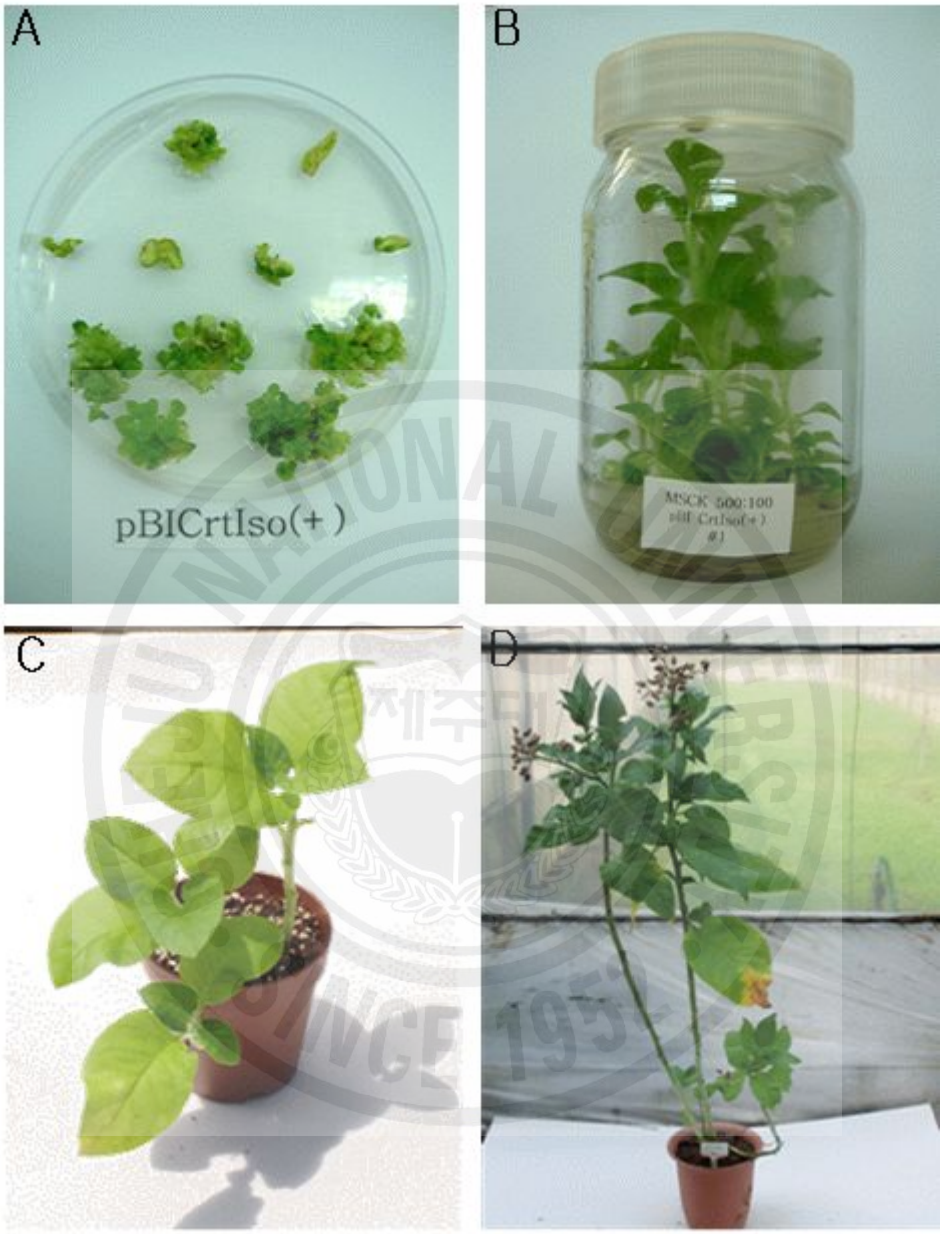

Fig. 6. Selection of putative transgenic plants

A; Formation of pBICrtIso(+) callus and shoots at selection medium. B; Putative transformants in rooting medium, aged 9 weeks. C; Acclimatized transformants in soil, aged 12 weeks. D; Acclimatized transformant in soil, aged 30 weeks.

Confirmation of putative transformants

The presence of the *CRTISO* gene in putative transformants were confirmed by genomic DNA PCR analysis. PCR analysis revealed the expected DNA fragment bands of 500 bp (Fig. 7-A) using specific primer (tCrt Iso up, dn). Also PCR analysis revealed the expected DNA fragment bands of 1.8 Kb using 35S-FG and Nos-Term primer (Fig. 7-B) with CrtIso as primers, an expected 500 bp band was found in transgenic plants and in the positive control, but not in wild-type plants (Fig. 7A). A 1.8Kb band was amplified using 35S-FG and Nos-T as primers in transgenic plants line and in the positive control, but not in the wild-type plants (Fig. 7B). These result showed that the *CRTISO* gene cloned from immature tomato could be expressed in the transgenic tobacco.

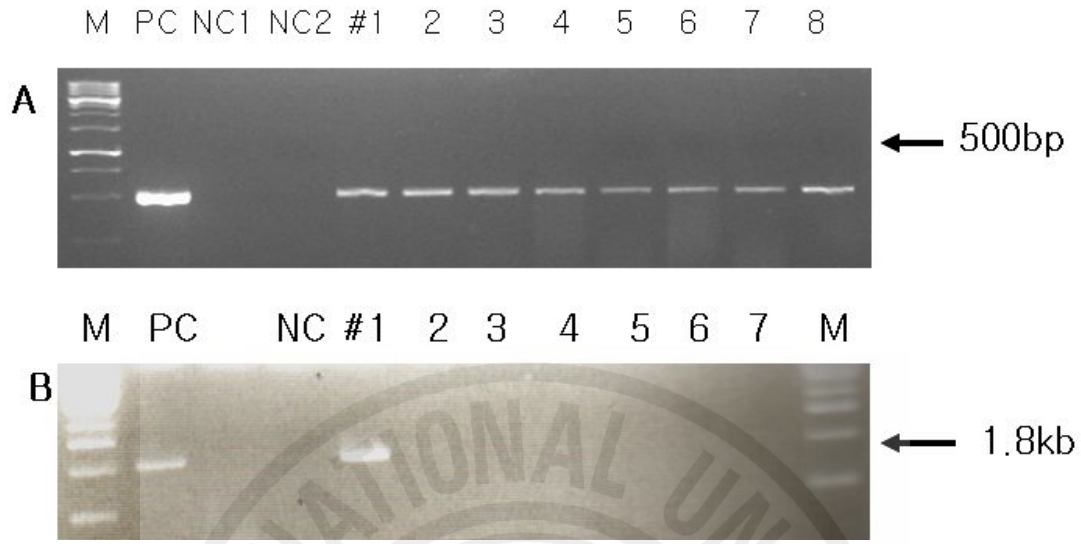

Fig. 7. Confirmation of putative transformants (T_0) by PCR analysis.

A ; specific primer (tCrt Iso up, tCrt Iso dn) , B ; 35S FG and Nos-Term primer,

M ; 1Kb DNA ladder marker, #1~9 ; Putative transformants,

Reverse transcription-Polymerase chain reaction (RT-PCR)

CRTISO gene in the putative transformants tobacco revealed by reverse transcription PCR. The total RNA of transformants were isolated using TRIzol method.[25] cDNA synthesis were conducted by M-MuLV reverse transcriptase protocols (MBI). Three of putative transformants (line 1, line 6, line12) represented 500 bp by RT-PCR analysis (Fig. 8). Plants transformed with pBICI were analysed for the presence of CRTISO transcripts. Fig. 8 shows transcripts of 500 bp. This results indicated *CRTISO* gene was expressed in mRNA level.

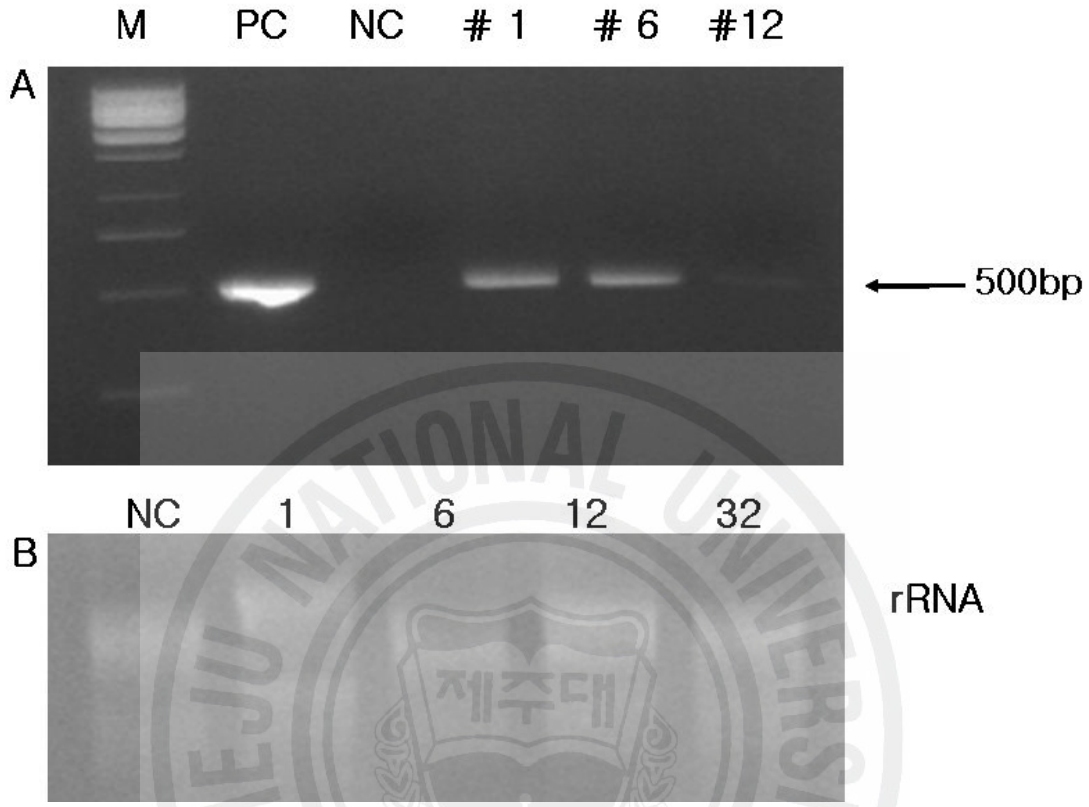

Fig. 8. Confirmation of T_0 transgenic tobacco plants by RT-PCR.

A ; Confirmation by RT-PCR analysis, B ; separated RNA,

M ; Marker, PC ; Positive control(plasmid), NC ; Negative control,

#1,6,12,32 ; CrtIso lines 1, 6, 12, 32

Analysis of carotenoid composition by HPLC

Chromatogram of carotenoids in non-transgenic tobacco and transgenic tobacco were shown in Fig. 9. A wavelength of 450 nm chosen to maximize absorbance in the 300 ~ 600 nm region of visible spectrum, which is an optimal area for studying carotenoids. Carotenoids were identified by characteristics absorption spectra, distinctive reaction times, and some cases, comparison with standards. The peaks were identified as lutein and β -carotene. Lutein and β-carotene peak confirmed at 4 ~ 5 min, 29 ~ 31 min, respectively.

Profiles of transformants were steep slop, whereas profiles of control were gentle slop formation, it means carotenoids composition of putative transformants were increased than wild-type. Chlorophyll and carotenoids were extracted from leaves of transformed lines and untransformed control plants. The amount of total pigments was increased in transformed ones than the controls (Fig. 9). Determination of pigment distribution by HPLC demonstrated that lutein, β-carotene, chlorophyll a, and chlorophyll b were increased to the wild-type. Futhermore, β-carotene was higher in transformants lines than in untransformed controls. Peak area of line 1, lutein composition were increased about 459 %, and β-carotene composition were increased about 225 % (Fig. 10). This results indicated that expression of CRTISO effected the valance of carotenoid composition, such as xanthophyll, carotenoid isomerase essential for carotenoid complex in plant.[27)]

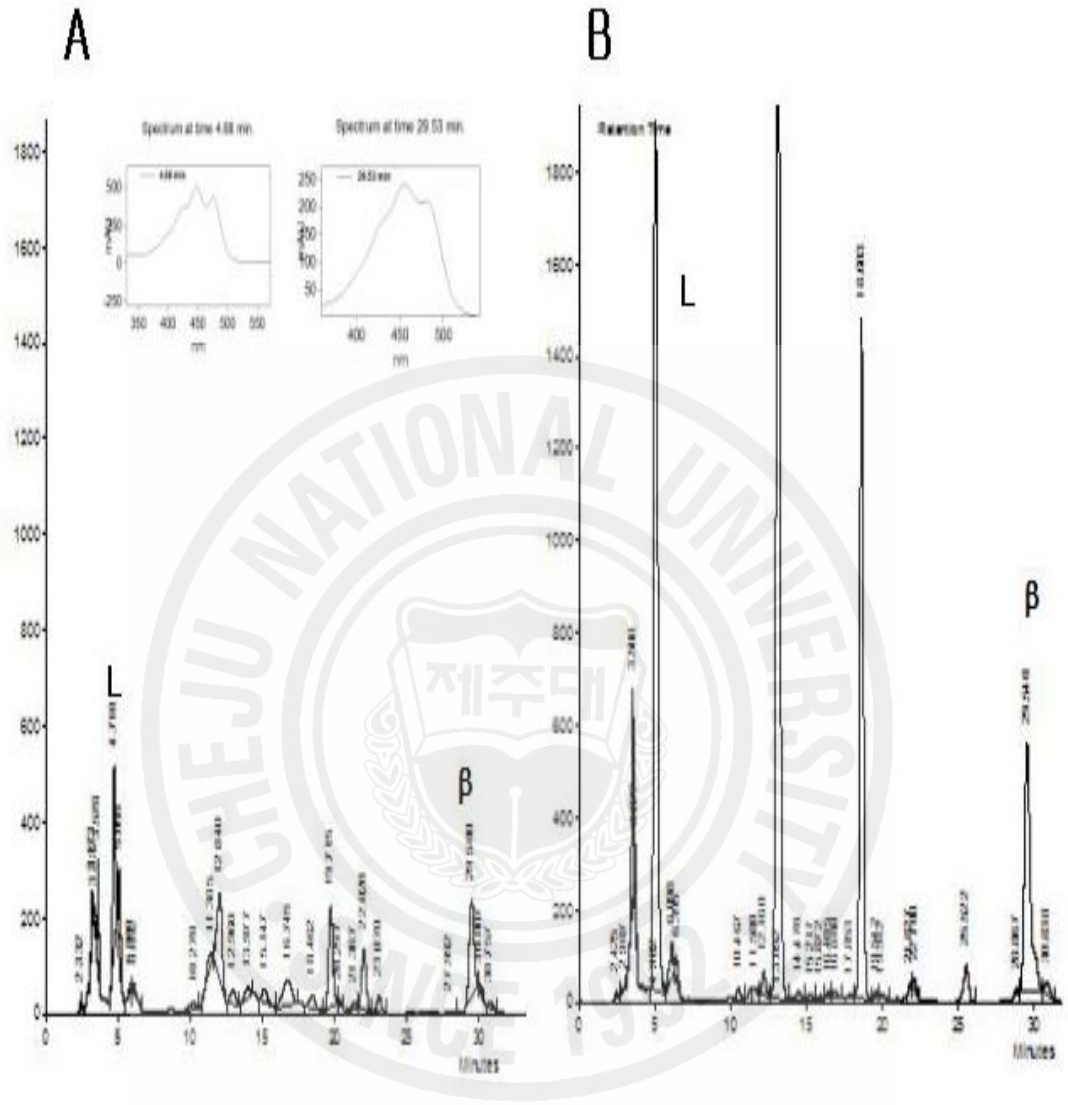

Fig. 9. Profiles of crude carotenoids from transgenic tobacco leaves by HPLC.

A; Profile of control plant, B; Putative transgenic tobacco line 1 (T_0) transformant

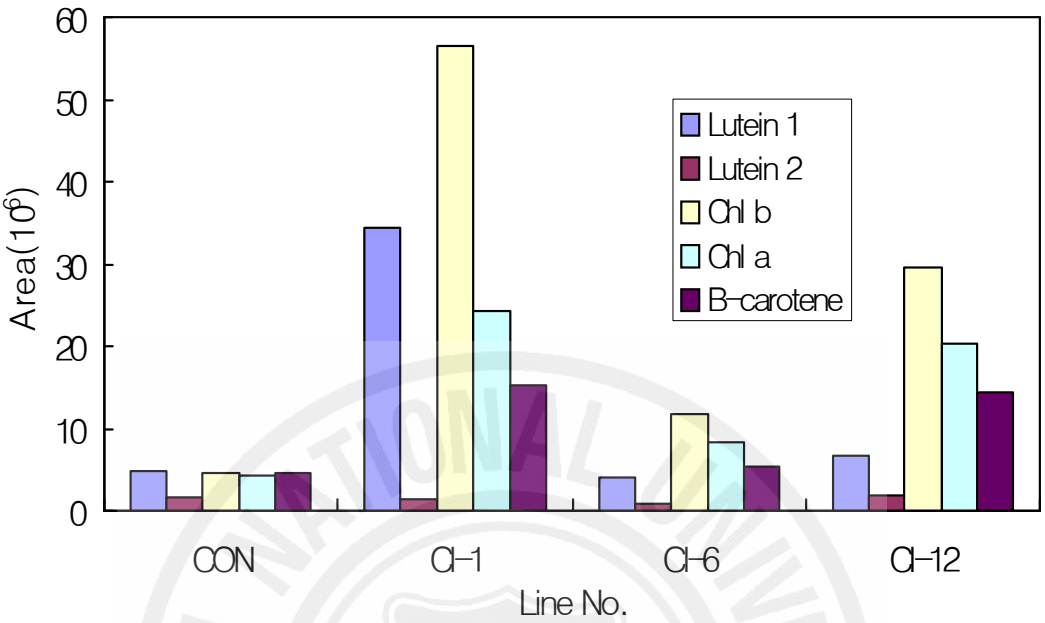

Fig. 10. Carotenoid composition of T_0 transgenic generation

Transgene segregation in T_1 progeny of transgenic plants

Transformed tobacco seeds (T_1) were collected at adult transformants (T_0). Seeds of T_1 generation germinated at MS medium containing 100 ㎕/㎖ of kanamycin, because pBICI vector could be identified for the kanamycin-resistance at neomycin phosphotransferase (*npt* II) (Fig. 11). The reporter gene, *Npt* II, encode enzymes with specificities not normally found in plant tissues.[28] This treatment was carried out in triplicate (30 seeds per dish) and was repeated four times in each transformant lines. While the kanamycin-sensitive seedlings became albinos in a couple of days of germination, the resistant ones developed normally. After 4 weeks, kanamycin-resistance plants developing true leaves were counted and data analysed by the Chi-square (χ^2) test at p = 0.05 (Table. 4). Line 1 of T_1 transformants showed 2.8:1 segregation ratios for the kanamycin resistance (χ^2 = 0.40, p-value = 0.82) and line of T_1 transformants showed 3:1 segregation ratios for the kanamycin resistance (χ^2 = 2.53, p-value = 0.30). Among the progenic of self-pollinated T_1 transgenic plant lines, 1 and 6 lines showed 3:1 segregation ratio. From this results, the transgene was inherited according to Mendelian law.

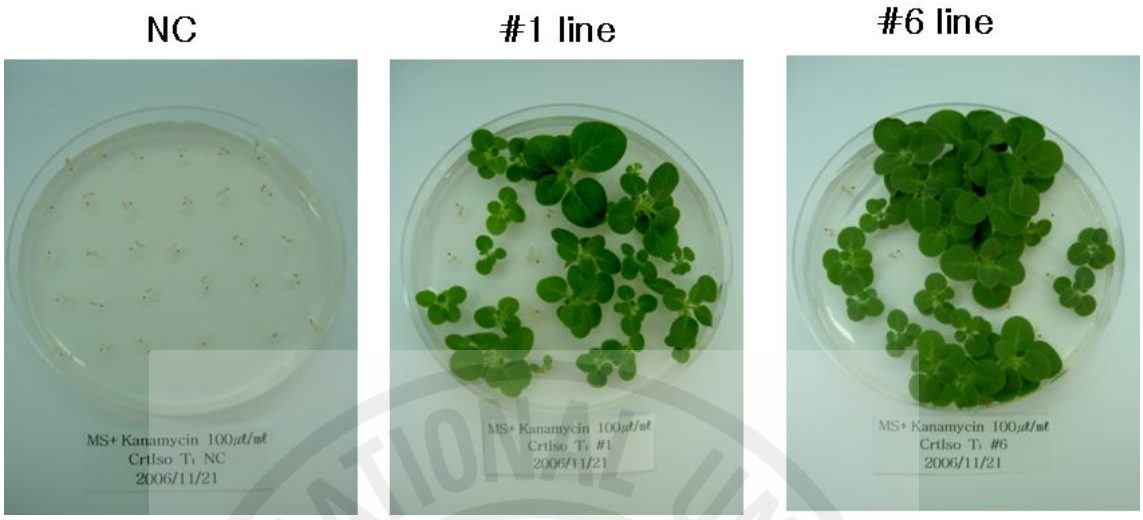

Fig. 11. Phenotype of T_1 seedling derived from wild-type tobacco controls and transformants with kanamycin.

NC ; Control, #1,6 ; CI transformants lines (line 1, line 6).

T_1 generation seeds were germinated on MS medium containing 100 ㎍/㎖ of kanamycin.

Table 4. Segregation of the resistance to kanamycin in the T_1 generations

Lines	No. of seeds		Ratio	Expected		
	Kmr^R	Kmr^S	(R:S)	ratio	χ^2-value	p-value
# 1	22.0±0.4	8.0±0.4	2.8:1	3 : 1	0.40	0.82
# 6	22.5±1.5	7.5±1.5	3.0:1	3 : 1	2.53	0.30

Each value represents mean ± S.E. of four replicates. χ^2-value indicated significant fit to the expected ratio. Kmr^R; Kanamycin resistance progeny, Kmr^S; Kanamycin susceptible progeny.

Confimation of T_1 progeny transgenic plants

Seeds of T_1 generation were collected self-pollinated of T_0 generation transformants. After 16 weeks, 0.5g of leaf gathered from transformants, respectively. Genomic DNA were shown in Figure 12. Genomic DNA (T_1) were separated at 1% of agarose gel at 100V for 1hr (Fig.12). Genomic DNA were used template for genomic DNA PCR, and PCR amplification performed using specific primer (tCrtIso up/dn). Ampificated of PCR product were separated at 1% of agarose gel containing EtBr (Fig. 13) and confirmed at 500 bp. *CRTISO* gene revealed at T_1 generation transformants (line 1, line 6). This means that *CRTISO* gene was stably inherited to T_1 generation.

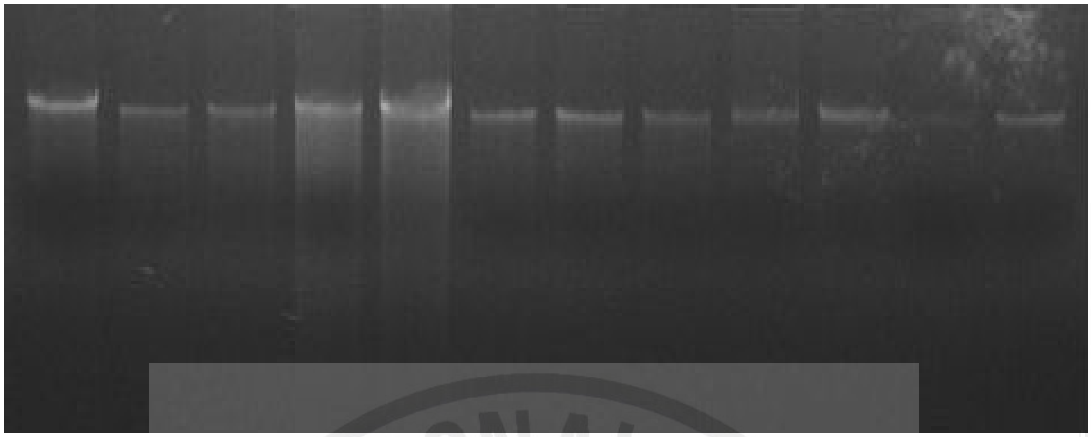

Fig. 12. Extraction of genomicDNA T_1 generation transformants by CTAB method.

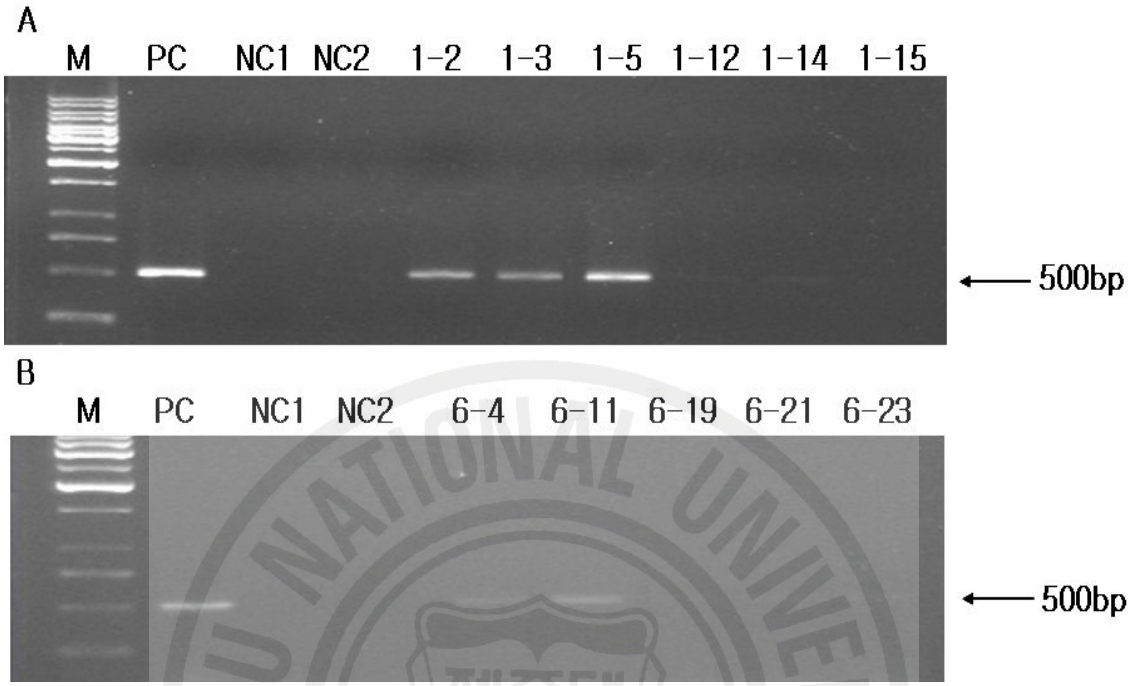

Fig. 13. PCR analysis of T_1 progeny transformants using specific primer (tCrtIso up/dn).

A ; line 1, B ; line 6, M ; Marker, PC ; Positive control, NC ; Negative control.

T_1 progeny transformants analysis by HPLC

The carotenoid composition of lutein and β-carotene were showed in T_1 progeny plants (Figure 14). Lutein composition increased about 60% ~ 102% than control. β-carotene composition increased about 39% ~ 69% than control. Expecially, line 1-14 of carotenoids were highly increased. It implies that expression of carotenoid isomerase are related with quantitative variation. The overexpression of CRTISO showed related to increment of lutein and β-carotene.

Isaacson et al., (2004) et al. discovered that CRTISO was unable to isomerize the cis-isomers of ζ-carotene in the in vitro assay, while it was actively converting tri-cis-lycopene (prolycopene).[29] During fruit ripening, all the ζ-carotene detected in the wild-type tomato, whereas, ζ-carotene detected in large amounts in fruits of a mutant that is impaired in ζ-carotene desaturation. ZDS is capable of converting 9',9-di-cis-ζ-carotene to produce prolycopene via 7,9,9'-tri-cis-neurosporene.[30] Therefore, Isaacson (2004) reported rhat CRTISO acts in conjunction with or immediately after ZDS during the desaturation of ζ -carotene to all-trans-lycopene.[29] In addition, several tomato lines of evidence designated showed CRTISO as a genuine carotenoid isomerase, which served the function of carotenoid biosynthesis in oxygenic photosynthetic organisms. From the pattern of expression of CRTISO, Isaacson et al., showed that its mRNA level of CRTISO in wild-type fruit in different stages of fruit development increased 10-fold during the breaker stage of fruit ripening, whereas no mRNA of CRTISO could be detected in fruit of *tangerine*^{3183} during fruit ripening.[14] Our results indicated that overexpression of CRTISO in tobacco leaf increased the β-carotene content than wild-type. Therefore, it was considred carotenoid isomerase essential for the production of β-carotene and xanthophylls in plants.

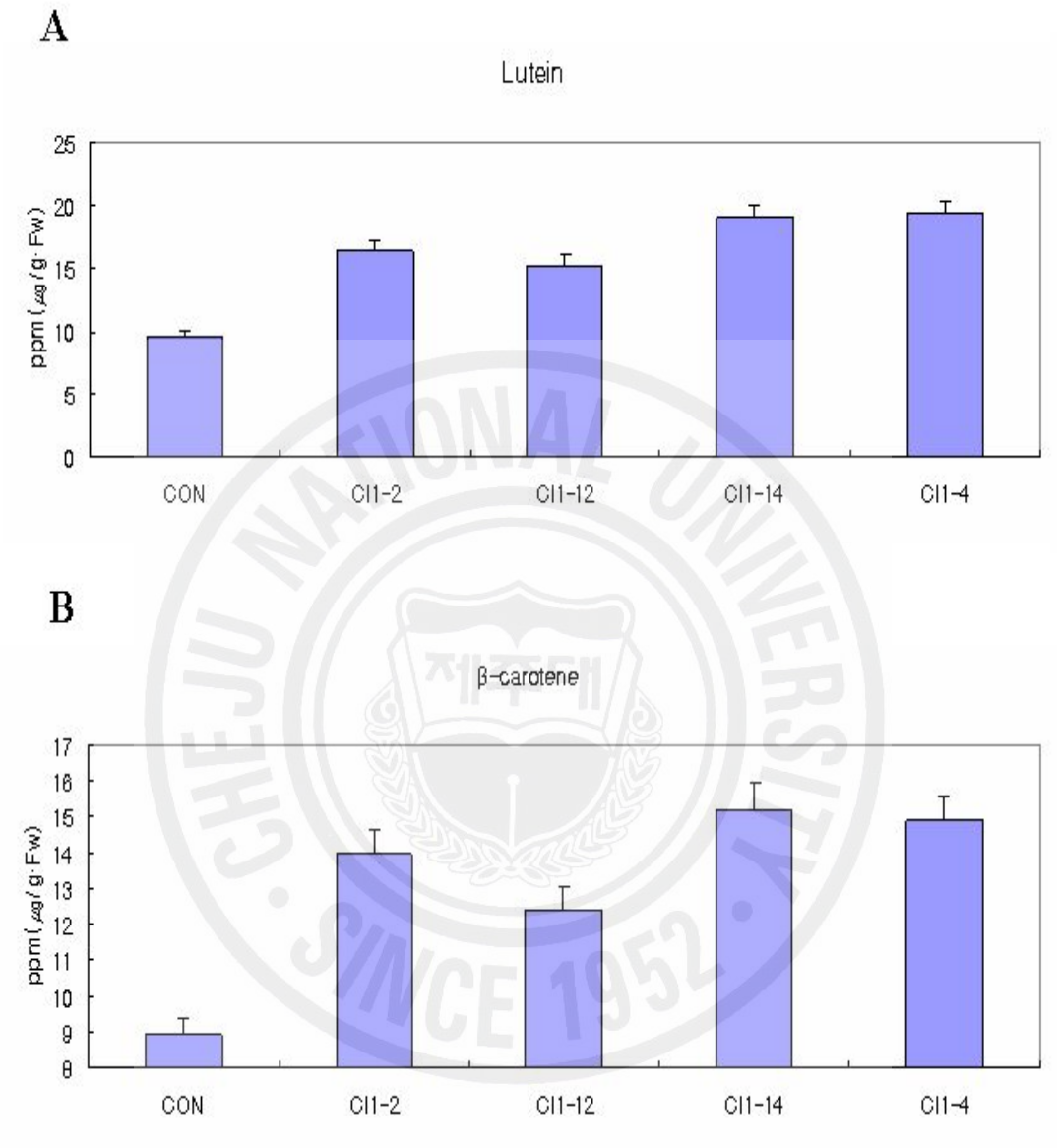

Fig. 14. Carotenoid content of CI T_1 generation.

A; Lutein composition (g/g FW), B; β-carotein composition (g/g FW)

ABSTRACT

Carotenoids are the most widespread group of isoprenoid-derived compounds in nature, with over 600 species characterised structurally. Carotenoids are synthesized by plants, algae, bacteria, and fungi. The CRTISO enzymes catalyzes the formation of the red pigment trans-lycopene from the yellow pigment poly-cis-lycopene. Isaacson et al. and Park et al. present the identification of carotenoid isomerase (CRTISO) from tomato mutant (*tangerine*) and Arabidopsis mutant (*ccr2*).

In this study, we report the isolation of the *CRTISO* gene from immature fruit of tomato. It was shown by sequence similarities with carotenoid isomerase from tomato fruit. *carotenoid isomerase* gene was inserted into the binary vector pBI121 for *Agrobacterium*-mediated transformation. In the carotenoid biosynthetic pathway, the effects of carotenoid isomerase were studied in tobacco by *Agrobacterium*-mediated transformation.

The plant expression vector, pBItCrtiso(+), was introduced into *A. tumefaciens* LBA4404. Transformed tobaccos were pre-cultivated for 2 days, co-cultivated for 2 days and selected on Murashige & Skoog (MS) medium supplemented with 100 mg/ℓ of kanamycin, 500 mg/ℓ of carbenicillin for 5 weeks. Finally, twenty putative transgenic plants were obtained. The integration of the *carotenoid isomerase* gene into the tobacco genome was confirmed by genomic DNA polymerase chain reaction (PCR). T_0-generation putative transgenic plants gave the expected PCR product, 500 bp amplified by specific primers. Also, putative transformants were confirmed by RT-PCR. In addition, the quantatative changes of carotenoid composition were analyzed by HPLC.

T_0 investigated the progeny test, T_1- progeny transformants were confirmed by genomic DNA PCR and HPLC analysis. line 1 progeny transformants were confirmed 18 transformants and line 6 progeny transformants were confirmed 5 transformants by genomic DNA PCR analysis. T_1 transformants seeds were germinated at MS medium supplemented 100 ㎍/㎖ of kanamycin for the segregation pattern. Carotenoid composition of T_1- progeny transformants were confirmed by HPLC analysis. Lutein composition of line 1 progeny

transformants were increased about 60% ~ 102% rather then composition of control. β-carotene composition of line 1 progeny transformants were increased about 39% ~ 69% rather then composition of control. These mean that CRTISO is involved in the valance of carotenoids, such as xantophyll in plants.

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ACKNOWLEDGEMENT

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이 연구를 수행 할 수 있도록 도와주신 Bio-Agr. 분들께도 감사드립니다. 이세영 교수님을 비롯한 전경용 팀장님, 홍권춘, 오순열 선생님, 현정, 난희, (김)현정, 소영, 효민, 그리고 순복언니. 이 전공을 선택하는데 있어 많은 도움을 주셨고, 연구하는 동안 보여주신 많은 가르침과 조언, 흔들리지 않도록 해 주신 격려 등은 다음 진로설계에 있어서 많은 도움이 되리라 생각 됩니다.

실험실 식구들-진우, 정용, 능재오빠, 윤정, 하나, 정순, 정아-에게도 감사에 뜻을 전합니다. 맏언니면서도 자주 챙겨주지 못해서 미안했는데, 항상 반갑게 맞아주고 학문 등에 의견을 교류 할 수 있어서 고마웠습니다. 새로운 학과에 적응할 수 있도록 이끌어 주신 부경환, 이도승, 고형석 선생님, 상협오빠, 유왕이 등 대학원 동기들을 비롯한 선·후배님들, 진규, 승희 등 생명공학부 선·후배님들, 주화 선생님을 비롯한 학과 조교 선생님들께도 감사에 뜻을 전합니다.

희노애락을 함께 나누어준 중앙운영위 식구들-홍준, 승남, 승호, 재휘, 영철, 슬기오빠, 경원언니 그리고 기현오빠-에게도 감사드립니다. 특히 힘들었던 시기마다 함께 고민 해 주고 용기를 북돋아 주셔서 고맙습니다. 학교라는 한 울타리 안에서 서로에 학문을 독려하고 격려했던 교생식구들-문종, 광수 선생님, 효정언니, 선희, 미경이, 재연이, 그리고 교사에 길을 가고 있는 은경이-에게도 감사드립니다. 저에 멘토 같은 원준오빠를 비롯한 지희, 세미, 윤선언니, 지연이, 은미, 혜숙이, 석호 등 식품공학과 선·후배, 동기들에게도 감사에 뜻을 전합니다.

자주 만나지 못했어도 항상 격려하고 응원해준 친구들-미리, 의정, 은아, 유리, 현숙, 우정, 효범, 현빈, 승윤 등-과 은숙언니, 형준오빠를 비롯한 선배님들, 후배들-유미, 시우, 한맘, 으로 등-에게도 감사에 뜻을 전합니다. 이 외에도 제주영화제 식구들, 소영언니, 유택 선생님 등과 함께 꿈꾸던 사람들, 봉사활동으로 인연을 맺은 나에 제자 요한에게 감사드립니다.

마지막으로 뒷바라지 하시느라 고생하시는 부모님과 하숙생 같은 생활을 하느라 집안일도 제대로 챙기지 못한 맏언니를 이해 해 주는 동생들, 일본에 계시는 할머니와 고모님들, 멀리서나마 묵묵히 응원 해 주시는 외삼촌 식구들과 24시간이 모자란 막내고모, 도서관에 놀러가기 좋아하는 아이들을 둔 사촌언니 식구들에게도 감사에 뜻을 전합니다. 지켜 봐 주셔서 고맙습니다, 사랑합니다.

저를 아끼고 격려 해 주시는 주위에 모든 분들께 감사드립니다. 항상 응원해 주셔서 고맙습니다. 초심에 자세로 일신우일신하여 사상누각이 되지 않도록 노력하겠습니다.

힘찬 새해를 맞이하는 어느 날.