MASTER'S THESIS

Analysis of changes in carotenoid biosynthesis in micro-tom tomatoes introduced with citrus *CCD1A* gene.

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Analysis of changes in carotenoid biosynthesis in micro-tom tomatoes introduced with citrus *CuCCD1A* gene.

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이 논문을 이학석사 학위논문으로 제출함

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정동식의 이학 석사학위 논문을 인준함

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Abstract

During the ripening process of citrus fruits, the orange color is caused by the accumulation of carotenoid pigments. Through differentially expressed gene(s) screening, the carotenoid cleavage dioxygenase (CCD) gene was isolated as a pigment-related gene with higher expression than the control group through gene analysis in the citrus eggplant mutant red fruit gene and in the citrus control group. CCD, which decomposes carotenoid metabolites, is an enzyme that catalyzes the reaction of bonding two oxygen atoms to a substrate by cleaving the double bond of C40 carotenoid. Four types have been reported in plants so far. CCD1 and CCD4 have broad substrate specificities ranging from phytoene to neoxanthin, CCD7 and CCD8 are involved in the synthesis of stringolactone from β-carotene, and 9-cis-epoxycarotenoid dioxygenases NCED2, NCED3, NCED5, NCED6, and NCED9 produce abscisic acid.

In this study, the expression changes of carotenoid biosynthesis genes were analyzed in transgenic Micro-Tom tomatoes isolated from citrus fruits introduced with CCD1A gene, total carotenoid content, flavonoid content, and phenolic compound content were analyzed, and antioxidant activity was analyzed to analyze CCDA1 The influence by genes was investigate.

1. Introduction

Citrus fruits are one of the plants growing worldwide, including Brazil, the United States, China, Mexico, Spain, India, and Asia. About 150 million tons of citrus fruits were cultivated in the 2000s, playing an important role in the diet (Liu, Heying et al. 2012).

Citrus fruits are composed of many compounds, such as vitamins A, C, E, carotenoids, and pectins, which have antioxidant properties. The reactive oxygen species is partially reduced as one electron e- is transferred to the oxygen molecule O2 to form a superoxide radical, and the superoxide radical thus formed can generate different reactive oxygen species, hydrogen peroxide and hydroxyl radical, respectively (Halliwell 1991). Free radicals accumulate in the body, causing cancer, (Liou and Storz 2010), inflammation and tissue damage (Mittal, Siddiqui et al. 2014), Aging (Stadtman 2004). According to the results of a recent study, it has been reported that antioxidant activity in citrus varieties depending on the degree of coloration of the fruit (Zacarías-García, Rey et al. 2021).

In the methylerythritol phosphate (MEP) pathway among carotenoid biosynthesis pathways, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) utilizes isoprenoids formed through the reaction of DA3P with pyruvate to form isopentenyl pyrophosphate (IPP) and allylic isomer dimethylallyl pyrophosphate (DMAPP) and mak- 1 -e other plastids as well as substrates for carotenoid biosynthesis (Hermanns, Zhou et al. 2020).

In the first step of carotenoid biosynthesis, phytoene synthase (PSY) produces

phytoene, a C40 carotene, and lycopene is produced through several desaturation and isomerization steps catalyzed by phytoene desaturase (PDS) and Z-carotene desaturase (ZDS) (Yuan, Zhang et al. 2015).

In All-trans-lycopene, it is divided into LCYE and LCYB. In LCYE, Lutein is formed through α-carotene and δ-carotene, and in LCYB, ɣ-carotene and β-carotene are produced. β-Cryptoxanthin and zeaxanthin are catalyzed by β-carotene hydroxylase (CHYB), zeaxanthin epoxidase (ZEP) acts on zeaxanthin and antheraxanthin to produce violaxanthin, and in the case of violaxanthin de-epoxydase (VDE), it acts in the reverse direction.(Yuan, Zhang et al. 2015, Hermanns, Zhou et al. 2020). Violaxanthin to Neoxanthin conversion occurs by Neoxanthin synthase (NXS), and Violaxanthin and Neoxanthin form abscisic acid (ABA) by 9-cis-epoxycarotenoid dioxygenase (Fraser, Enfissi et al. 2009).

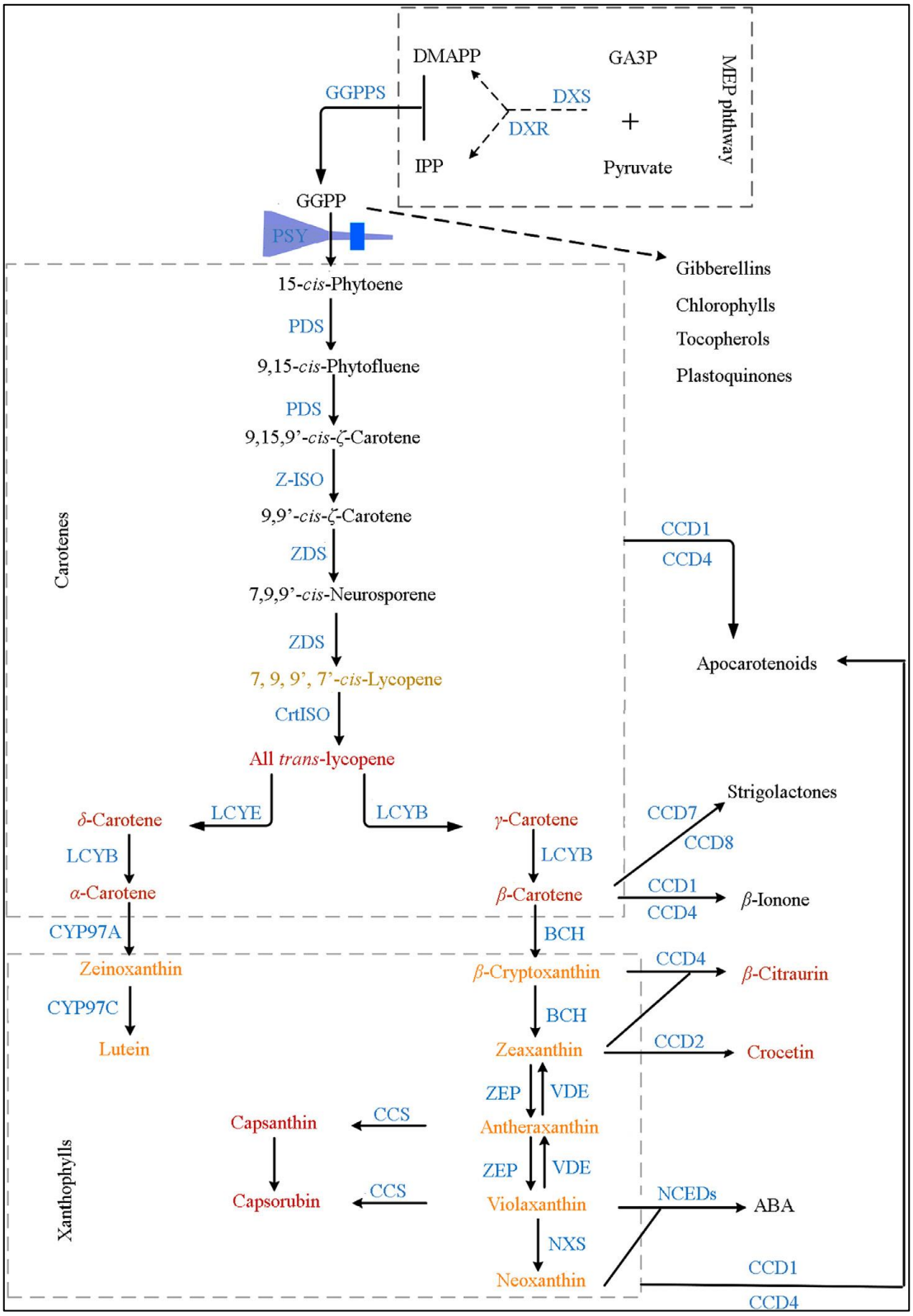

Fig 3. carotenoid biosynthesis pathway.

MEP, methylerythritol phosphate; GA3P, Glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate;

DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; DXS, 1-deoxy- *D* -xylulose 5-phosphate synthase; DXR, 1-deoxy- *D* -xylulose 5-phosphate reductoisomerase; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ - carotene isomerase; ZDS, ζ -carotene desaturase; CrtISO, carotenoid isomerase; LCYE, lycopene ε -cyclase; LCYB, lycopene β -cyclase; BCH, β -carotene hydroxylase; CYP97A, cytochrome P450 carotene β -hydroxylase; CYP97C, cytochrome P450 carotene ε -hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCD, carotenoid cleavage dioxygenase; NCED, 9- *cis* - epoxycarotenoid dioxygenase; ABA, abscisic acid.

CCD (carotenoid cleavage dioxygenase) family CCD1 and CCD4 have broad substrate specificities ranging from phytoene to neoxanthin, and in the case of CCD7 and CCD8, they are involved in the synthesis of stringolactone from β-carotene. 9-cis-epoxycarotenoid dioxygenase NCED2, NCED3, NCED5, NCED6, and NCED9 are involved in the production of the plant hormone Abscisic acid. As a result of isolating only the pigment-related genes with high expression in the control group through gene analysis of the red fruit gene from citrus eggplant mutation and the citrus control group, ZDS (Zeta-carotene desaturase), CCD1A (carotenoid cleavage dioxygenase), F3M (flavonoid 3) '-monooxygenase), MDDX (mevalonate diphosphate decarboxylase), and C4H2 (trans-cinnamate 4-monooxygenase) were selected. In this study, the expression changes of carotenoid biosynthesis genes in transgenic Micro-Tom tomatoes into which the CCD1A gene isolated from citrus was introduced. was analyzed, the total carotenoid content, flavonoid content, and phenolic compound content were analyzed, and the effect of CCDA1 gene was investigated by analyzing antioxidant activity.

2. Materials and Methods

2-1. plant materials

Plant selection genes in the pCAMBIA2300 vector are driven by a double-enhancer version of the CaMV35S promoter and terminated by the CaMV35S polyA signal. This vector contains minimal heterologous sequences for plant transformation and selection of transformants(Fig 2).

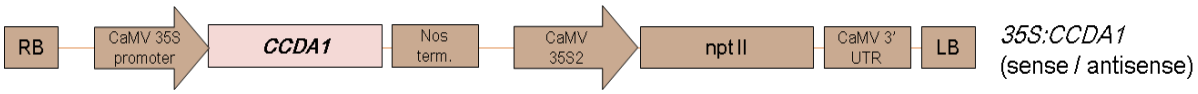

Fig 4. Transformation vector pCAMBIA 2300

CaMV35S : cauliflower mosaic virus(*CaMV*) *35S promoter*

npt II : Neomycin phosphotransferase

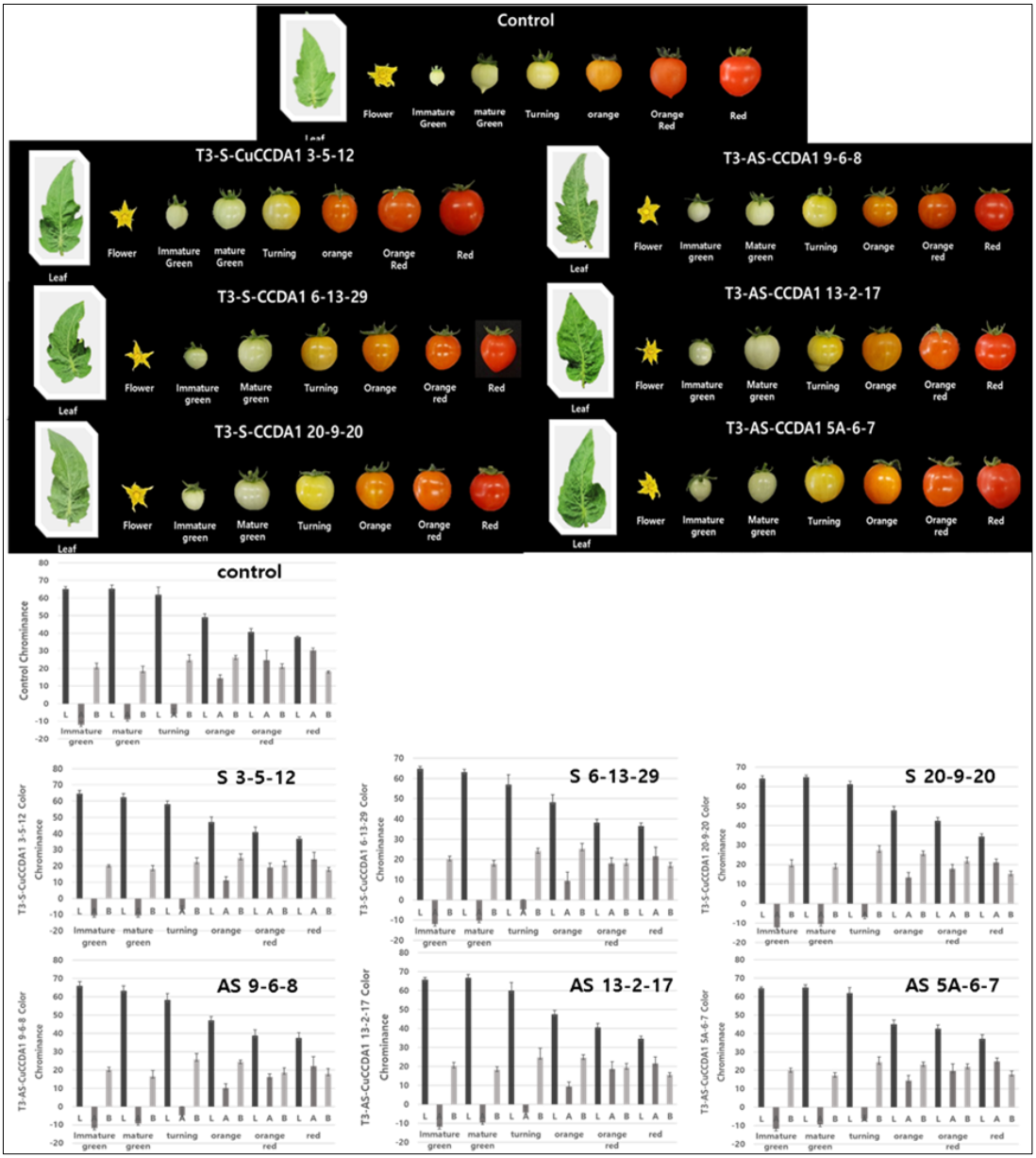

Fig 3. Sampling and color difference measurement by leaf, flower and fruit development.

For each line, flowers, leaves, and fruits were sampled in the order of immature green, mature green, turning, orange, orange-red, and red according to the development process.

The results of color difference by fruit development analyzed by Konica minolta's color difference machine are as follows (Fig. 3). Control-immature green showed the values of L(65.13±1.25), A(-12.08±1.58), and B(20.74±2.19), and mature green showed L(65.23±2.13), A(-9.03±1.85), It showed a value of B (18.59±2.67). In the

case of turning, the values of L(61.81±4.42), A(-5.90±2.9), and B(24.73±3.03) were shown, and Orange was L(49.08±1.91), A(14.41±1.86), B(26.18±1.26) was shown. Orange-red showed color differences of L(40.81±1.88), A(24.77±5.41), and B(20.90±1.68), and in case of red, L(37.96±0.48), A(30.34±1.16), B(18.31± 0.34) was shown. As a result of showing the average color difference values of three lines 3-5-12, 6-13-29, and 20-9-20 for the sense line, the sense line-Immmature green is L(64.52±0.29), A(-11.58± 0.93) and B(20.12±0.24), mature green showed L(63.52±1.23), A(-10.48±0.23), and B(18.89±0.56) color differences. Turning showed L(58.79±2.19), A(-6.09±1.16), B(24.67±2.45) color difference, and for Orange, L(47.71±0.58), A(11.32±1.98), B(25.31±0.29) seemed. For Orange-red, L(40.46±2.20), A(18.31±0.66), B(20.17±1.84), for Red, L(35.86±1.31), A(22.28±1.06), B(16.61±1.26) value was indicated. In the case of anti-sense, as a result of obtaining the average color difference values of 3 lines 9-6-8, 13-2-17, 5A-6-7, anti-sense-immature green is L(65.42±0.71), A(-11.89± 0.14) and B(20.11±0.12), and for mature green, L(64.97±1.77), A(-9.51±0.17), and B(24.96±0.69) values were shown. Turning showed color differences of L(59.99±1.91), A(-5.27±0.99), and B(24.96±0.69), and Orange showed L(46.56±1.34), A(11.34±2.74), B(24.00±0.77). For orange-red, L(40.6±1.96), A(18.14±1.79), B(19.98±1.72), for red, L(36.31±1.59),

A(22.83±1.82), B(17.05±1.28) value was indicated.

2-2. Genomic DNA extraction from seed.

DNA extraction from seeds was performed using the method of DiaStar™ Direct Multiplex/Fast PCR for plant (Solgent company). Lysis buffer is composed with a 60% PEG8000 and 20mM NaOH (pH 13.3). The seeds of the T2 generation are put into a new tube and the seeds are crushed so that the seeds are broken well. Then, 200㎕ of lysis buffer is added to the tubes and vortexed for about 5 seconds. Samples are precipitated by spin down and incubate at room temperature for 3 to 5 minutes, followed by centrifugation at 13000 rpm for 2 minutes. Transfer 100㎕ of the supernatant into a fresh tube.

2-3. Genomic DNA extraction from leaves.

DNA extraction from leaves was used by modifying the method of DNeasy plant mini kit (QIAGENE company). Place the leaves into a 1.5mL fresh tube with 1-2 stainless steel beads, and than added 400㎕ DNA extraction buffer, AP1 buffer(aka Lysis buffer) : 1mM EDTA (ethylene diamine tetraacetic acid, pH 8.0), 0.1% SDS solution (Sodium Dodecyl Sulfate, $C_{12}H_{25}SO_4Na$), 0.1M NaCl and 10mM Tris-HCl (pH 8.0) are required. After vortexing, incubate mixture for 10min at 65℃. Mix 2~3 times during incubation by inverting tube. Add 130㎕ of buffer AP2(aka Protein precipitation buffer) ; 3M potassium/5Macetate made by adding 60mL of 5M potassium acetate (98.14g in 200mL D.W.), 11.5mL of glacial acetic

acid, 28.5mL D.W. to the lysate, mix and incubate for 10min on ice. Centrifuge for 6min at 13000rpm and then, trans fer flow-through fraction to a fresh tube, without disturbing the pellet(About 300 μL of lysate is typically recovered). Add 1.5x volumes of Buffer AP3/E(aka binding buffer) ; 1M Guanidine Hydrochloride(4.78g in 50mL 100%EtOH), apply 700 μL of the mixture including any precipitate to the spin column sitting in a 2mL collection tube. Centrifuge for 1min at 8000rpm. Discard flow-through and reuse the collection tube. Add 500 μL Buffer AW ; 70% EtOH to the spin column and centrifuge 1min at 8000rpm, discard flow through and reuse the collection tube. Add 500 μL 100% EtOH to spin column and centrifuge for 2min at 13000rpm, discard flow through and centrifuge again at 13000rpm for 1min to dry. Carefully remove the spin column from the collection tube and transfer to 1.5mL tube and 50 μL of TE buffer directly onto the membrane. Incubate for 5min at room temperature and then centrifuge 6000rpm for 1min.

2-4. Total RNA extraction and DNase treatment

2-4-1 RNA extraction

The sample was stored at -80°C, and 0.2 g of powder was used by grinding the sample with a mortar. RNA추출은 Sabzevari, A. G., & RNA extraction was performed by Hosseini, R.(Sabzevari and Hosseini 2014) RNA extraction was modified from the method of Sabzevari, A. G. et al. Put 0.2g of sample powder into 2mL fresh tube, RNA Extraction Buffer; 100mM Tris-HCl pH 8.0, 10mM EDTA

(ethylene diamine tetraacetic acid, pH 8.0), 0.1M LiCl, 1% (w = v) SDS (Sodium Dodecyl Sulfate, C12H25SO4Na) 600μL was injected and vortexed. 600 μL of PCI (Phenole; Chlorofom; Isoamylalcohol/25:24:1) was added to the mixed solution and vortexed, followed by centrifugation at 14,620 rpm (20,000 g) for 10 minutes. The supernatant was transferred to a 1.5mL fresh tube, mixed with 500 μL of CI (Chloroform: Isoamylalcohol/24:1), vortexed, and centrifuged at 14,620rpm (20,000g) for 10 minutes. The supernatant was transferred to a 1.5mL fresh tube, and 270 μL of Ice-Cold 8M LiCl was added, inverted, and stored at -80℃ for 30 minutes, and then centrifuged at 14,620rpm (20,000g) for 30 minutes. After removing all of the centrifuged solution, 200 μL of DEPC, 0.1 volume of 3M sodium acetate (pH 5.2), and 2 volume of ice-cold absolute ethanol were mixed and stored at -80° C for 30 minutes after inverting. To collect RNA pellets, after centrifugation at 14,620 rpm (20,000 g) for 30 minutes, they were washed with ice-cold 70% ethanol, dried to evaporate ethanol, and dissolved in 30 μL of DEPC (Diethyl pyrocarbonate).

2-4-2. DNase treatment

For DNase treatment, Invitrogen TURBO DNA free™ Kit (Thermo Fisher company) was used. Dilute the sample to 10 μg nucleic acid/50 μL of total sample if possible. After Add 0.1volume 10X DNase™Buffer to the RNA, then mix gently that TURBO DNase™ Enzyme 1 μL to the RNA. Incubate samples at 37℃ for 30min and then, resuspend the DNase Inactivation Reagent by flicking or vortexing the tube before use. Add 0.2 volumes of resuspended DNase Inactivation reagent then mix well. Inactivate the sample for 5minutes at room temperature. Flick the tube 2~3times during the incubation period to redisperse the DNase

Inactivation Reagent. Centrifuge the samples, then carefully transfer the supernatant containing the RNA to a fresh tube, But do not disturb the pellet of DNase Inactivation Reagent.

2-5. PCR (Polymerase Chain Reaction) & Electrophoresis

2-5-1. Genotyping of T3 *S-CuCCDA1* / T3 *AS-CCDA1* lines from single seed

Genomic DNA was isolated from single seed of T2 *3-CuCCDA1* lines (3-5-12, 6-13-29 and 20-9-20) and carried out PCR to obtain T3 homo lines.

To perform PCR, 2 μL of genomic DNA from each lines, 0.5 μL of forward primer CCDA1 23582F(5'-GCAAACACTTGGTATGCTTG-3'), 0.5 μL of reverse primer t-NOSR(5'-AATTCCCGATCTAGTAACATAGA-3'), 5 μL of Prime Star(Dia star^{TM}Direct Mutiplex/Fast PCR, Solgent company), and 2 μL of deionized water (D.W.) were added in a 0.2 mL PCR tube.

The PCR conditions were as follows. Initial denaturation at 95℃ for 5 min, additional denaturation at 95℃ for 20 sec, annealing at 58-60℃ for 30 sec, extension at 72℃ for 30 sec, and final extension at 72℃ for 5 min. Repeat 35 cycles from denaturation to extension(Table 1). After the PCR reaction, 8 μL of PCR product and 2 μL of 6 × dye were mixed and electrophoresed at 100 V for 25 minutes on 1.2% agarose gel. The conditions of electrophoresis was electrophoresis on 1.2% agarose gel.

Genomic DNA was isolated from single seed of T3 *AS-CuCCDA1* lines (9-6-8, 13-2-17 and 5A-

6-7) and carried out PCR to obtain T3 homo lines and carried out PCR to obtain T3 homo lines, To perform the PCR, 2㎕ of genomic DNA from each lines, 0.5㎕ of forward primer CCDA1 2358R(5'-TCTTCCAGAACTTTGACGGC-3'), 0.5㎕ of reverse primer t-NOSR(5'-AATTCCCGATCTAGTAACATAGA-3'), 5㎕ of Prime Star, and 2㎕ of deionized water(D.W.) were added in a 0.2 mL PCR tube.

The PCR conditions were as follows. Initial denaturation at 95℃ for 5 min, additional denaturation at 95℃ for 20 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 30 sec, and final extension at 72℃ for 5 min. Repeat 35 cycles from denaturation to extension (Table 2). After the PCR reaction, 8㎕ of PCR product and 2㎕ of 6 × dye were mixed and electrophoresed at 100 V for 25 minutes.

Table 6. PCR condition of *T3-S-CuCCDA1* seed.

DNA	2㎕	95℃	5min	
CCDA1 2382F	0.5㎕	95℃	20sec	35cycles
tNOSR	0.5㎕	58~60℃	30sec	
Prime Star	5㎕	72℃	30sec	
D.W.	2㎕	95℃	5min	
	10㎕	8℃	∞	

Table 7. PCR condition of *T3-AS-CuCCDA1* seed.

DNA	2㎕	95℃	5min	
CCDA1 23+85R	0.5㎕	95℃	20sec	35cycles
tNOSR	0.5㎕	60℃	30sec	

Prime Star	5 μL	72℃	30sec	
D.W.	2 μL	72℃	5min	
	10 μL	8℃	∞	

2-5-2. Genotyping of T3-S-CuCCDA1 / T3-AS-CuCCDA1 lines from leaf

The seeds of T3 homo lines obtained from above genotyping PCR were sown on soil and cultivated in the green house. After 50~60 days from sowing, the leaf from each lines were sampled and isolated genomic DNA to reconfirm the genotype by PCR.

2 μL of genomic DNA from each lines, 0.5 μL of forward primer (CCDA1 23582F, 0.5 μL of reverse primer (t-NOSR), and 17 μL of deionized water(D.W.) were added in a 0.2 mL PCR tube of AccuPower® PCR PreMix(Top simple™ DryMix-nTaq, enzynomics company).

The T3-S-*CuCCDA1* PCR conditions were as follows. Initial denaturation at 95℃ for 5 min, additional denaturation at 95℃ for 20 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 30 sec, and final extension at 72℃ for 5 min. Repeat 35 cycles from denaturation to extension (Table 3). After the PCR reaction, 10 μL of PCR product electrophoresed at 100 V for 25 minutes.

To perform PCR in the case of T2-AS-*CCDA1*, 2 μL of genomic DNA from each lines, 0.5 μL of forward primer (CCDA1 2358R, 0.5 μL of reverse primer (t-NOSR), and 17 μL of deionized water (D.W.) were added in a 0.2 mL PCR tube of AccuPower® PCR PreMix (Table 4). After the PCR reaction, 8 μL of PCR product and 2 μL of 6 × dye are mixed and electrophoresed at 100 V for 25 minutes.

Table 8. PCR condition of *T3-S-CuCCDA1* leaf

DNA	2 μL	94℃	5min	
CCDA1 2382F	0.5 μL	94℃	20sec	35cycles
tNOSR	0.5 μL	60℃	30sec	
D.W.	17 μL	72℃	30sec	
		72℃	5min	
	20 μL	8℃	∞	

Table 9. PCR condition of T3-AS-CuCCDA1 leaf

DNA	2 μL	95℃	5min	
CCDA1 2358R	0.5 μL	95℃	20sec	35cycle
tNOSR	0.5 μL	60℃	30sec	
D.W.	17 μL	72℃	30sec	
		72℃	5min	
	20 μL	8℃	∞	

2-5-3. RT-PCR(Reverse Transcriptase Polymerase Chain Reaction)

After cDNA synthesis, microtomato CCD family genes were found in NCBI (National Center for Biotechnology Information) to perform RT-PCR. As a result, SLCCD1A, SLCCD1B, SLCCD4, SLCCD4-1, SLCCD (9'10'). SLCCD(9'10')-1 like and SLCCD('10')-2 like were searched, and primer information is in Table 5. PCR condition of CuCCD1A; Denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds were amplified in 28 cycles, and PCR conditions for SLCCD1A and SLCCD4; Denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds,

and elongation at 72°C for 30 seconds were amplified for 30 cycles. PCR condition of SLCCD1B; Denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 30 seconds were amplified in 30 cycles, SLCCD4-1, SLCCD7, CCD9' 10' like, CCD9'10'-1 like2, SLLCYB1, SLCHYB1, SLVDE PCR condition; Denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds were amplified for 30 cycles. Also, SLCCD8-2 PCR condition; Denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and elongation at 72°C for 30 seconds were amplified as 30 cycles. SLLCYB2 PCR condition; Denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds were amplified for 30 cycles. SLCHYB2 PCR condition; Denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 30 seconds were amplified as 30 cycles. SLZEP PCR condition; RT-PCR was performed with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, and elongation at 72°C for 30 seconds (Table. 6).

Table 10. RT-PCR Primer

RT-PCR primer	
Gene	Sequence(5' -> 3')
CuCCD1A F14	AAAAGCTGGAGAACGACGTC
CuCCD1A R196	GAGTCTCGTCAGGAACTGGA
SLCCD1A F909	GCTAGCTTTTACCTTTGATGC
SLCCD1A R1297	TTACCTTAGCAATGCTGTTC
SLCCD1B F911	CATACAGCTTTGACCCCACA
SLCCD1B R1136	GTGAAACCGTCACGCTGTTC
SLCCD4 F136	GAAAGACCACAAACAACCAC
SLCCD4 R260	GAGGGAAATGATGGTTCAAT
SLCCD4-1 F793	GTCTGGGGCATGACAGCTCA
SLCCD4-1 R1036	CGAGTATGAACTTGATCGGG
SLCCD7 F98	TCTTGCCACCGGCTAAACTG
SLCCD7 R200	GTTGGGGACGTGGTGATCGT
SLCCD8-2 F1165	ACCATCCTTGACAAGCTCCG
SLCCD8-2 R1400	CAAGGCCTCTTAGCACCACA
CCD9′10′F731	ATGAAGTAGGGTACGAGGTT
CCD9′10′R972	ATGGGCTGTGAATGATCGAG
CCD9′10′-1 like F848	AAAATACCAGATACGGGTGAACTT
CCD9′10′-1 like R1083	CCAAGCTCGTCTACTATAATATCT
CCD9′10′-1 like2 F544	CCTGGTTTTCTTCCCGCTAT
CCD9′10′-1 like2 R817	TAACAAGTTCGCTTGCTCCA
SLLCYB1 F297	ACAGCAAGTTTCTGAAGCAG
SLLCYB1 R519	CGATTTCAGCTGTTTCCGGT
SLLCYB2 F651	TGATGCGACGGGATTCTCTA
SLLCYB2 R919	ATCCAGGACGAGCTACAAGG
SLCHYB1 F81	ACCTACTTCGACAACCTCAC
SLCHYB1 R374	ACAGCCATAACAGCCATAGA
SLCHYB2 F477	TCCTTCAGGTAGGAATGGAG
SLCHYB2 R697	CAATCCAGCGCCGAAACAGA
SLZEP F121	AGGAGCTTGGAAAATGGGCA
SLZEP R402	CTGTATCTGTATTGGACCTC
SLVDE F435	GAAAGAGTGCAGGATAGAGC
SLVDE R730	TCAAACCGCGAGTGATGTAC

Table 6. RT-PCR condition

	RT-PCR condition						
	Denaturation	time	Anealing	time	Elongation	time	cycles
CuCCD1A	94℃	30	60℃	30	72℃	30	28
SLCCD1A SLCCD4	94℃	30	52℃	30	72℃	30	30
SLCCD1B	94℃	30	58℃	30	72℃	30	28
SLCCD4-1 SLCCD7 CCD(9'10') like CCD(9'10')-1like 2 SLLCYB1 SLCHYB1 SLVDE	94℃	30	55℃	30	72	30	30
SLCCD8-2	94℃	30	59℃	30	72℃	30	30
SLLCYB2	94℃	30	56℃	30	72℃	30	30
SLCHYB2	94℃	30	57℃	30	72℃	30	30
SLZEP	94℃	30	51℃	30	72℃	30	30

2-6. Anti-oxidant activity analysis

2-6-1. Total Carotenoid content

Mix 1 mL of ACN:MeOH:THF (50:45:5, by volume) with 5 mg of lyophilized powder, and shaken at 150 rpm for 2 hours. Centrifuge at 15000 rpm for 5 min at −5 ° C and supernatant was filtered through 0.45-mm. using a microplate reader (Thermo) at 450 nm wavelength read the plates. Carotenoid extraction was performed according to the method of DÓKA, Ottó.(Dóka, Ficzek et al. 2013).

2-6-2. Total Flavonoid content

Total flavonoid content was performed by modifying of Chang et al(Chang, Yang et al. 2002). To 20 μL of sample and standard, 10 μL of 50g/L $NaNO_2$ was added and stored at room temperature for 6 minutes. After add 15 μL of 100g/L $AlCl_3$ and incubate for 6min, add 75 μL of 1M NaOH. And then incubate in dark for

30 minutes, using a microplate reader (Thermo) at 510 nm wavelength read the plates. Quercetin was used as a standard at 0, 25, 50, 100, 200, 300 μg/mL to generate a calibration curve.

2-6-3. Total Phenolic content

Total phenolic content was performed by modifying the Folin-Denis analysis protocol by Rosa M et al(Lamuela-Raventós 2018). Mix 100μL of 10% folin-ciocalteu reagent with 20μL of sample and incubate for 5 mins. Add then 80μL of 10% sodium carbonate anhydrous saturated solution. After incubated in the dark at 25℃ for 60min, using a microplate reader (Thermo) at 750 nm wavelength read the plates. The total phenolic content was expressed as gallic acid equivalent(GAE) through a calibration curve 0, 25, 50, 100, 200, 400 μg/mL.

2-6-4. DPPH radical scavenging

DPPH radical scavenging activity assay was performed with a modification of the protocol of AOSHIMA et al. (Aoshima, Tsunoue et al. 2004). 160μL of 0.2 mM DPPH solution dissolved in methanol and sample 40μL were added and incubated in dark for 30 minutes. The plates were then read on a Microplate reader(Thermo) at 517nm wavelength. Used ascorbic acid as positive control.

2-7. HPLC(High Performance Liquid Chromatography)

For HPLC measurement, carotenoid was extracted according to the method of P

Gupta, and HPLC measurement was performed. Add 1.2 mL chloroform:dichloromethane (2:1, v/v) to the lyophilized plant sample and mix for 20 min at 1000 rpm at 4℃ using a thermal mixer. 0.4 mL of 1M sodium chloride solution was added, mixed by inversion, and centrifuged at 5000 g for 10 minutes to collect the organic phase. The remaining aqueous phase was re-extracted, and after mixing for 20 minutes at 1000 rpm at 4° C. using a thermal mixer, 0.2 mL of 1M sodium chloride solution was added and mixed by inversion.

After centrifugation at 5000 g for 10 minutes, the organic phase was collected and dried by centrifugal evaporation. It was also dissolved in 0.5 mL of methanol.

3. Result

3-1. Genotyping analysis of T3-S/AS-CuCCDA1 transformant lines using genomic DNA from single seed

To obtain S-*CuCCDA1* homo lines, I performed genotyping PCR using genomic DNA extracted from single seed (1~28 seeds) of each lines. The S-CuCCD1A의 경우 3-5-12, 6-13-29, 20-9-20 lines was only showed as homo lines because amplified the expected PCR product in all the tested PCR and the remained lines were not amplified the expected PCR product in the some of genotyping PCR from each lines(Fig. 2A). To obtain AS-CuCCDA1 homo lines, I performed genotyping PCR using genomic DNA extracted from single seed (1~28 seeds) of each lines. In the lines AS-CuCCDA transformant, the 9-6-8, 13-2-17, and 5A-6-7 line was only showed as homo lines because amplified the expected PCR product in all the tested PCR

and the remained lines were not amplified the expected PCR product in the some of genotyping PCR from each lines (Fig. 2B).

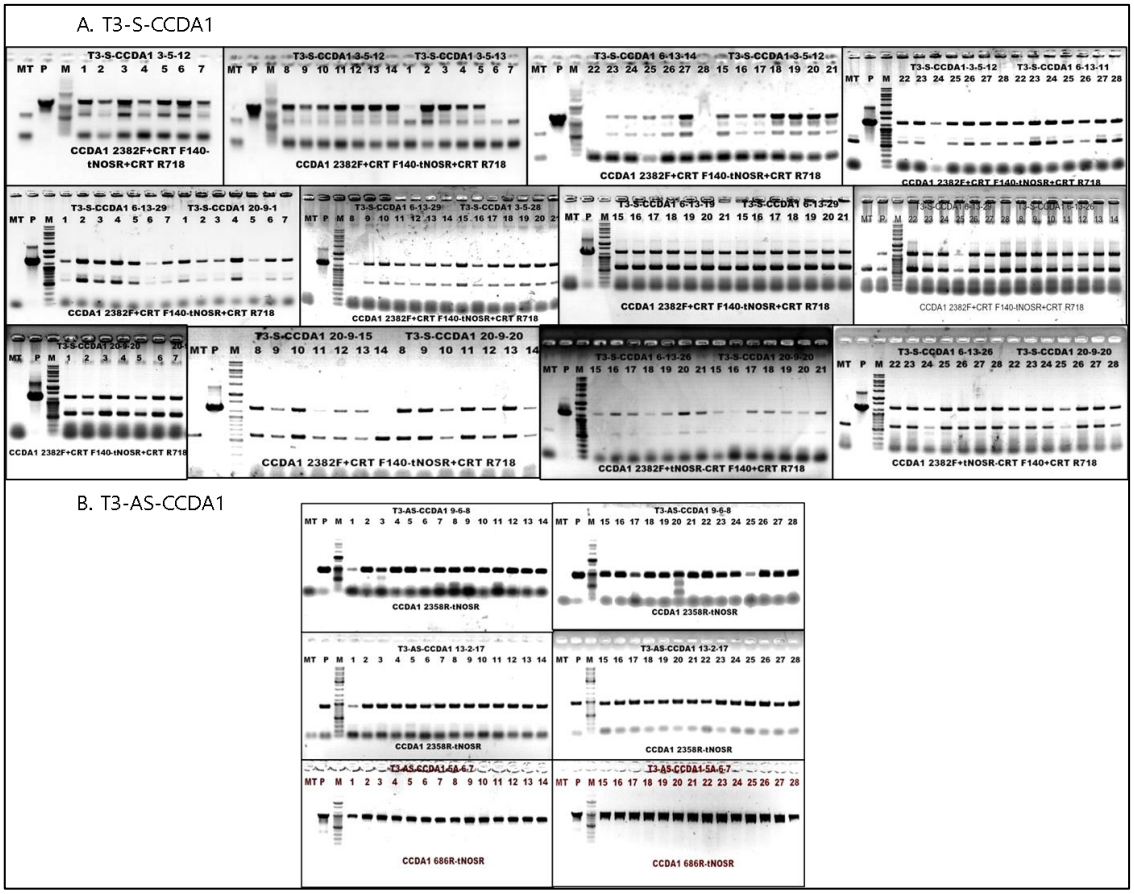

Fig 4. Genomic DNA extraction from Seed. A. Sense lines 3-5-12, 6-13-29 and 20-9-20, B. Anti-Sense lines 9-6-8, 13-2-17, 5A-6-7

3-2. Genotyping analysis of T3-S/AS-CuCCDA1 transformant lines using genomic DNA from single Leaf

As a result of genotyping of the T2-S/AS-CuCCDA1 transformant using genomic DNA from a single seed, three homo lines (3-5-12, 6-1329, 20-9-20) of the T2-S-CuCCDA1 transformant was obtained. and four homo lines

of the T2-AS-CuCCDA1 transformant (9-6-8, 13-2-17 and 5A-6-7).

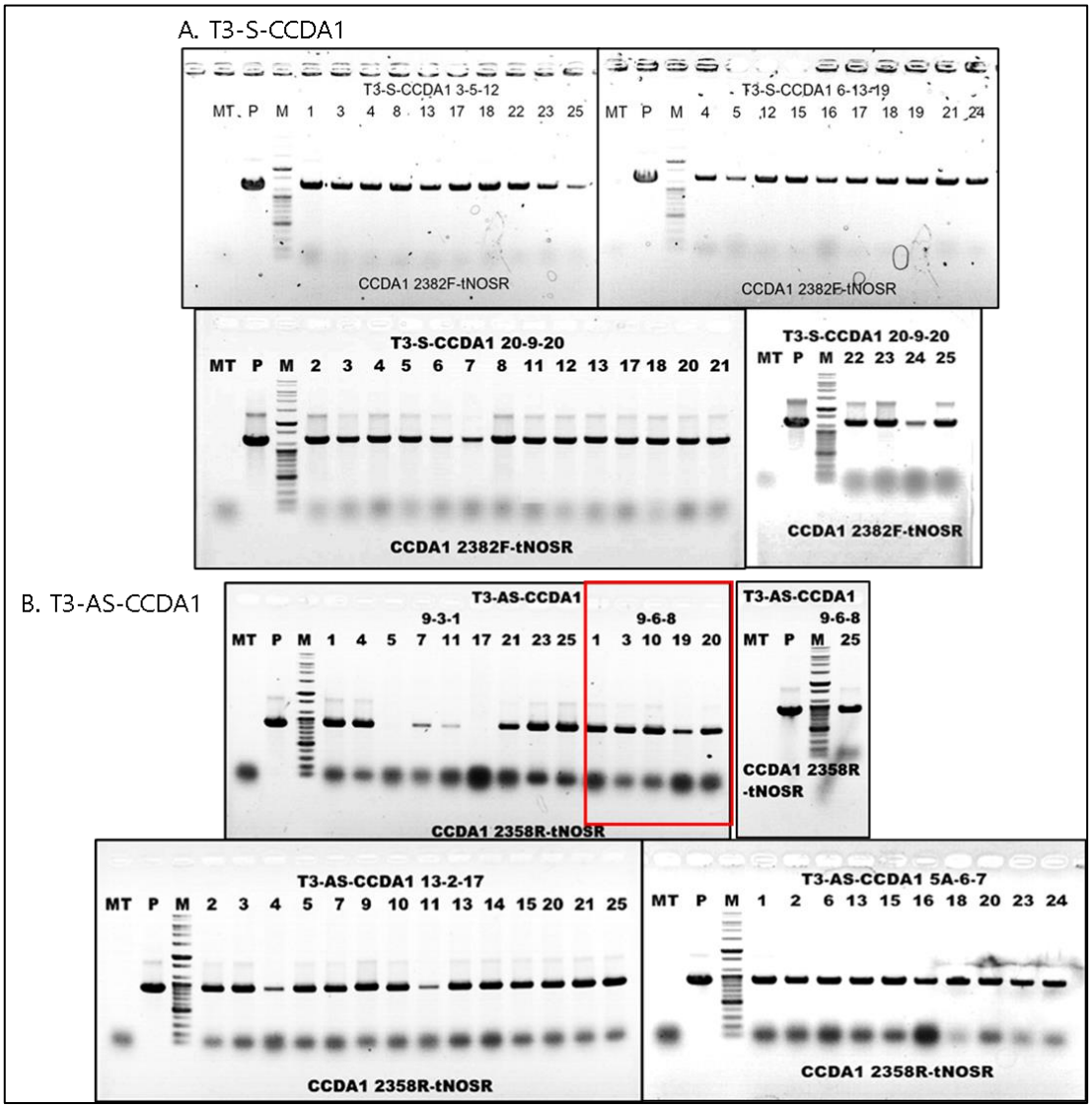

Fig 5. Genomic DNA extraction from Leaf. A. Sense lines 3-5-12, 6-13-29 and 20-9-20, B. Anti-Sense lines 9-6-8, 13-2-17, 5A-6-7

3-3. Total RNA extraction and DNase treatment

Total RNA was extracted from all samples, treated with DNase and electrophoresed (Fig. 4). In addition, the average values of nucleic acid, A260/280, and A260/230 of 3 lines of MT and 3 sense lines were obtained (Tables 7 and 8). Nucleic acid concentration of MT-Leaf was 296.9ng/μL, A260/280 showed a value of 1.98, A260/230 showed a value of 1.7, and MT-Flow's Nucleic acid concentration was 137.8ng/μL, A260/280 showed a value of 1.99, and A260/230 showed a value of 1.71. Nucleic acid concentration in Sense Leaf was 141.43±15.77 ng/μL, A260/280 was 1.97±0.03, A260/230 was 1.69±0.02, and Nucleic acid concentration in Sense Flower was 125.6±3.44 ng/μL and A260/280 was 1.95. A value of ±0.03 was shown, and A260/230 showed a value of 1.56±0.99. The nucleic acid concentration of anti-sense flower was 149.7±8.05 ng/μL, A260/280 1.95±0.05, and A260/230 1.65±0.1, and the nucleic acid concentration of anti-sense flower was 139.43±2.51, A260/ 280 showed a value of 1.95±0.01, and A260/230 showed a value of 1.71±0.03. Nucleic acid concentrations, A260/280 and A260/230 values for each fruit development were measured.

The concentration of MT-Immature green nucleic acid was 296.9 ng/μL, A260/280 showed a value of 1.93, and A260/230 showed a value of 1.82, and the nucleic acid concentration of mature green was 427.2 ng/μL, A260/280 showed a value

of 1.95 and A260/230 showed a value of 1.91. The nucleic acid concentration of Turning showed a value of 535.4 ng/μL, A260/280 showed a value of 1.99, A260/230 showed a value of 1.7, and the nucleic acid concentration of Orange showed a value of 225.9 ng/μL, A260/280 showed a value of 1.8 and A260/230 showed a value of 1.55. The concentration of nucleic acid in orange red was 275.4 ng/μL, A260/280 showed a value of 1.92, and A260/230 showed a value of 1.26, and the concentration of nucleic acid in red was 107.9 ng/μL, A260/ 280 showed 1.83 and A260/230 showed 1.62. Sense-Immature green Nucleic acid concentration was 104.1±34.66ng/μL, A260/280 showed a value of 1.9±0.14, A260/230 showed a value of 1.55±0.16. Nucleic acid concentration of mature green was 167.07±155.74ng/μL, 1.9±0.03 for A260/280, and 1.54±0.28 for A260/230. The nucleic acid concentration of Turning was 187.93±184.4ng/μL, A260/280 showed a value of 1.88±0.05, A260/230 showed a value of 1.49±0.28, and the nucleic acid concentration of Orange was 254.8±254.8± 283.71ng/μL, A260/280 showed a value of 1.72±0.07, and A260/230 showed a value of 1.50±0.27. The concentration of nucleic acid in orange-red was 219.37±244.79ng/μL, A260/280 showed a value of 1.78±0.12, A260/230 showed a value of 1.51±0.31, and the concentration of nucleic acid in red was 264.47. ±207.51 ng/μL, A260/280 showed

1.79±0.13, and A260/230 showed 1.62±0.24.

Anti sense-Immature green Nucleic acid concentration was 143.33±7.23ng/μL, A260/280 showed a value of 1.96±0.07, A260/230 showed a value of 1.71±0.08. The mature green Nucleic acid concentration was 138.23±4.51ng/μL, 1.92±0.02 for A260/280, and 1.61±0.09 for A260/230. The nucleic acid concentration of Turning showed a value of 133.4±36.57ng/μL, A260/280 showed a value of 1.9±0.06, A260/230 showed a value of 1.47±0.08, and the nucleic acid concentration of Orange was 122.93± 60.181ng/μL, A260/280 showed a value of 1.79±0.08, and A260/230 showed a value of 1.45±0.26. The concentration of nucleic acid in orange-red was 140.13±33.89ng/μL, A260/280 showed a value of 1.79±0.08, A260/230 showed a value of 1.51±0.10, and the concentration of nucleic acid in red was 101.00. ±63.34 ng/μL, A260/280 showed 1.78±0.11, and A260/230 showed 1.28±0.28.

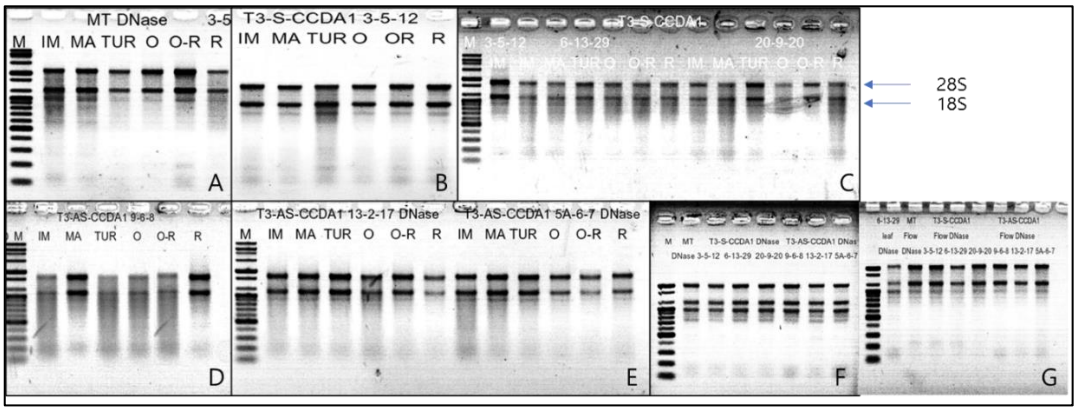

Fig 6. RNA extraction and DNase treatment.

(A) Electrophoresis of DNase-treated RNA by MT fruit development, (B) Electrophoresis of DNase-treated RNA by S 3-5-12 fruit development, (C) Electrophoresis of DNase-treated RNA by S 6-13-29 and 20-9-20 fruit development, (D) Electrophoresis of DNase-treated RNA by AS 9-6-8 fruit development, (E) Electrophoresis of DNase-treated RNA by AS 13-2-17, 5A-6-7 fruit development, (F) Electrophoresis of DNase-treated RNA by MT, Sense and Anti sense Leaf, (G) Electrophoresis of DNase-treated RNA by MT, Sense and Anti sense Flower.

Table 7. Nucleic acid concentration of MT and Sense.

		MT	S 3-5-12	S 6-13-29	S 20-9-20	AVER	STD
Leaf	Nucelic Acid	142.9	137.8	127.8	158.7	141.43	15.77
	260/280	1.98	2	1.95	1.97	1.97	0.03
	260/230	1.7	1.7	1.66	1.7	1.69	0.02
Flower	Nucelic Acid	137.8	126.9	128.2	121.7	125.60	3.44
	260/280	1.99	1.97	1.96	1.91	1.95	0.03
	260/230	1.71	1.62	1.61	1.46	1.56	0.09
Immature Green	Nucelic Acid	296.9	143.8	79.9	88.6	104.10	34.66
	260/280	1.93	2.01	1.98	1.76	1.92	0.14
	260/230	1.82	1.73	1.46	1.45	1.55	0.16
Mature Green	Nucelic Acid	427.2	355.9	86	86.3	176.07	155.74
	260/280	1.95	1.87	1.91	1.93	1.90	0.03
	260/230	1.91	1.86	1.44	1.32	1.54	0.28
Turning	Nucelic Acid	535.4	400.6	72.4	90.8	187.93	184.40
	260/280	1.66	1.93	1.86	1.84	1.88	0.05
	260/230	1.59	1.8	1.26	1.42	1.49	0.28
Orange	Nucelic Acid	225.9	582.4	90.4	91.6	254.80	283.71
	260/280	1.8	1.71	1.79	1.65	1.72	0.07
	260/230	1.55	1.81	1.32	1.36	1.50	0.27
Orange Red	Nucelic Acid	275.4	502	75.5	80.6	219.37	244.78
	260/280	1.92	1.89	1.78	1.66	1.78	0.12
	260/230	1.26	1.86	1.27	1.4	1.51	0.31
Red	Nucelic Acid	107.9	504	139.3	150.1	264.47	207.51
	260/280	1.83	1.91	1.66	1.8	1.79	0.13
	260/230	1.62	1.9	1.49	1.47	1.62	0.24

Table 8. Nucleic acid concentration of Anti-sense

		AS 9-6-8	AS 13-2-17	AS 5A-6-7	AVER	STD
Leaf	Nucelic Acid	150.7	141.2	157.2	149.70	8.05
	260/280	1.97	1.99	1.89	1.95	0.05
	260/230	1.71	1.71	1.54	1.65	0.10
Flower	Nucelic Acid	142.3	138.4	137.6	139.43	2.51
	260/280	1.96	1.95	1.94	1.95	0.01
	260/230	1.75	1.69	1.7	1.71	0.03
Immature Green	Nucelic Acid	149.3	145.4	135.3	143.33	7.23
	260/280	2	1.88	1.99	1.96	0.07
	260/230	1.78	1.63	1.72	1.71	0.08
Mature Green	Nucelic Acid	143.4	136.2	135.1	138.23	4.51
	260/280	1.94	1.91	1.91	1.92	0.02
	260/230	1.51	1.69	1.64	1.61	0.09
Turning	Nucelic Acid	147.9	91.8	160.5	133.40	36.57
	260/280	1.93	1.95	1.83	1.90	0.06
	260/230	1.54	1.49	1.38	1.47	0.08
Orange	Nucelic Acid	155.3	53.5	160	122.93	60.18
	260/280	1.83	1.84	1.69	1.79	0.08
	260/230	1.62	1.15	1.58	1.45	0.26
Orange Red	Nucelic Acid	170	103.3	147.1	140.13	33.89
	260/280	1.71	1.79	1.87	1.79	0.08
	260/230	1.51	1.41	1.6	1.51	0.10
Red	Nucelic Acid	36.4	103.6	163	101.00	63.34
	260/280	1.88	1.66	1.79	1.78	0.11
	260/230	1.03	1.22	1.58	1.28	0.28

3-4. RT-PCR(Reverse Transcriptase Polymerase Chain Reaction)

As a result of searching for CuCCD1A gene and SLCCD1A homology, 79% homology was found (fig. 4), and 3 μL of cDNA in 1.5 mL tube, 0.5 μL of CuCCD1A F14 0.5 μL-R196 0.5 μL, D.W. 16 μL mixing RT-PCR was performed. As a result of electrophoresis of 5 μL of the PCR product with 2% gel, in the case of MT, it was confirmed that the gene was not amplified as a control in which the CuCCD1A gene was not introduced. It was confirmed that the gene was amplified in plants into which was introduced (Fig. 5).

For SLCCD1A, 3 μL of cDNA, 0.5 μL of SLCCD1A F909-R1297 0.5 μL, D.W. After

RT-PCR was performed by mixing 16 μL, 5 μL of the PCR product was loaded on a 2% gel and electrophorese. In the case of MT, since only CCD1A of tomato itself was present, it was confirmed that its own gene was amplified properly, and in the case of 3 sense lines and 3 anti-sense lines, electrophoresis was performed and similar patterns were shown (Fig. 6). .

Fig 7. Homology of *CuCCD1A* and *SLCCD1A*

Homology was searched through NCBI. The upper part marked in red is the sequence position for CuCCD1A F14-R196, and the lower part marked in red is the sequence position for SLCCD1A F909-R1297.

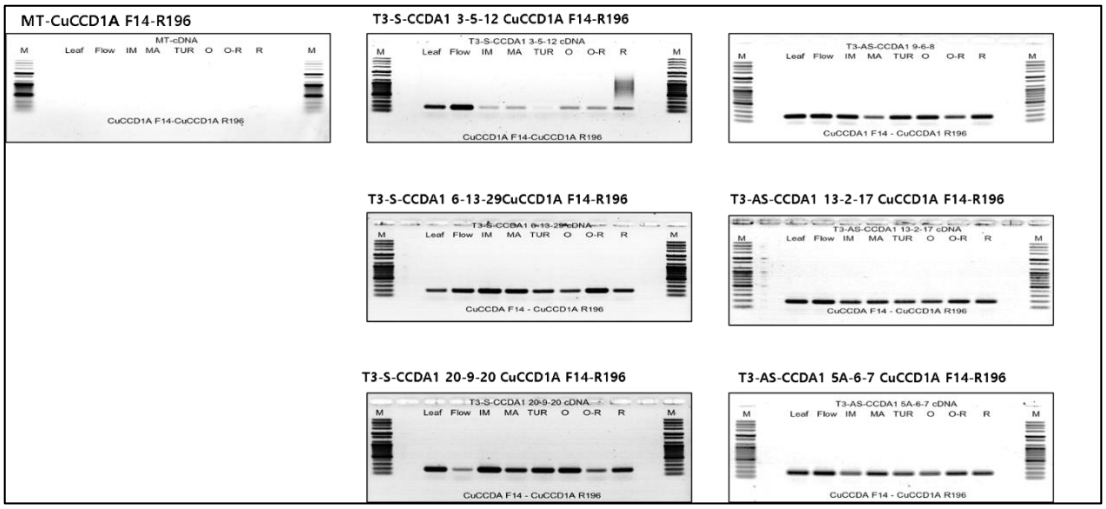

Fig 8. Electrophoresis of *CuCCD1A*

Gene expression analysis of CuCCD1A F14-R196. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

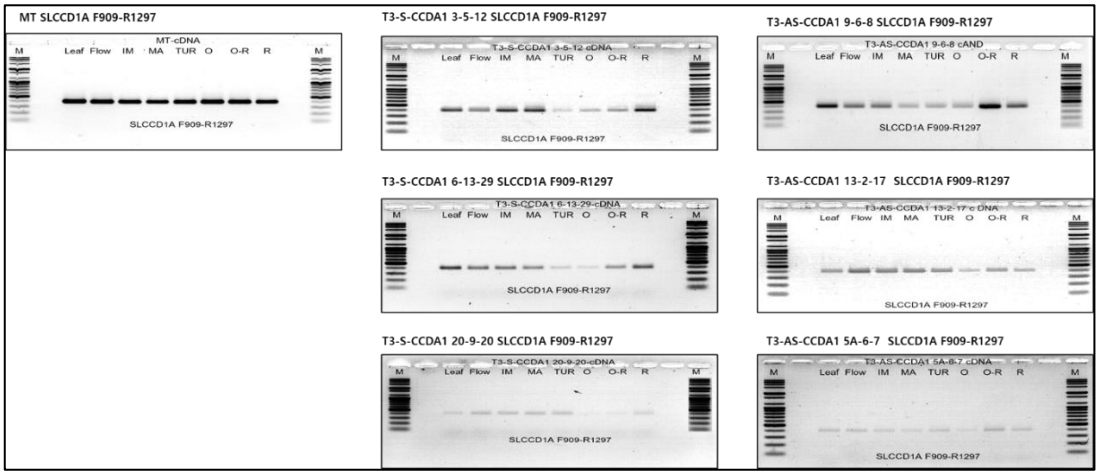

Fig 9. Electrophoresis of *SLCCD1A.*

Gene expression analysis of SLCCD1A F14-R196. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

As a result of confirming the homology of CuCCD1A and SLCCD1B, 77% of homology was shown (Fig. 7). 3 μL of cDNA, 0.5 μL of SLCCD1B F911-0.5 μL of SLCCD1B R1297, D.W. 16 μL was mixed and RT-PCR was performed. In addition,

5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 8).

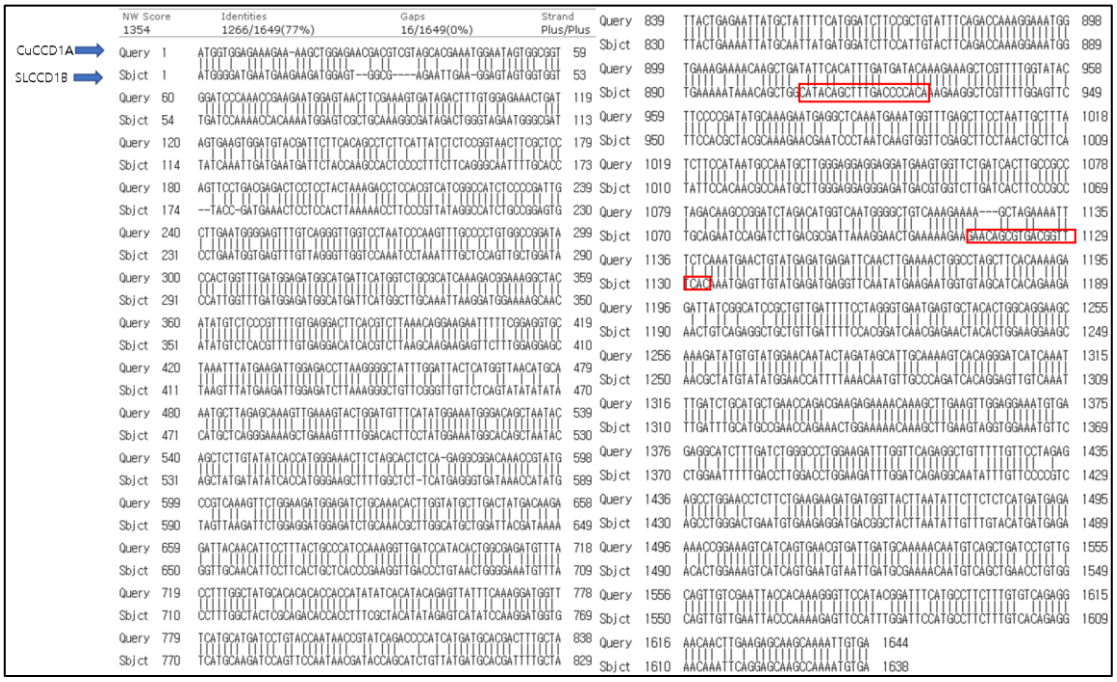

Fig 10. Homology of *CuCCD1A* and *SLCCD1B*

Homology was searched through NCBI. The lower part marked in red is the sequence position for SLCCD1B F911-R1136.

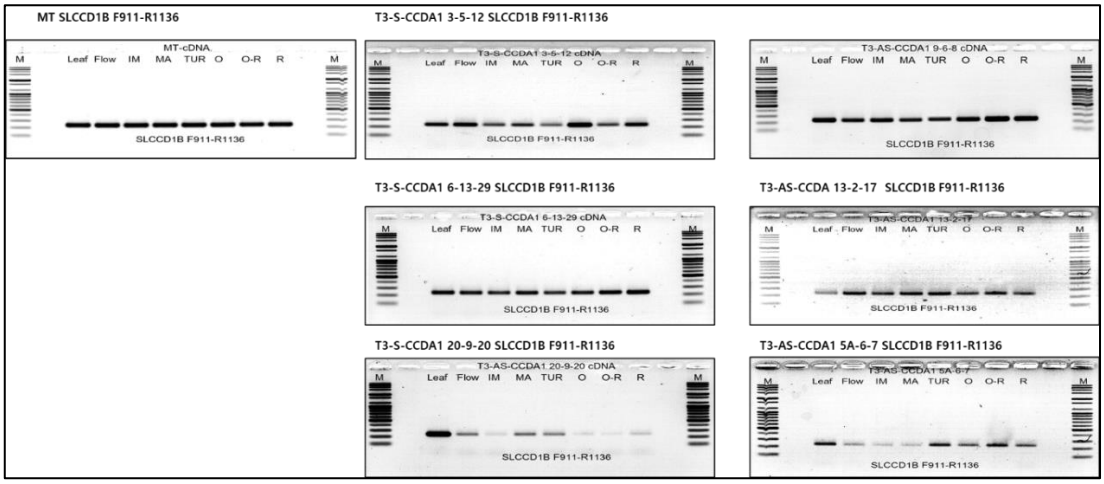

Fig 11. Electrophoresis of *SLCCD1B.*

Gene expression analysis of SLCCD1B F911-R1136. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

As a result of checking the homology of CuCCD1A, SLCCD4, and SLCCD4-1, the homology was about 50%, and 3 μL of cDNA, 0.5 μL of SLCCD4 F136, 0.5 μL of SLCCD4 R260, D.W. After RT-PCR by mixing 16 μL, 5 μL of PCR product was loaded on 2% gel and electrophoresis was performed (Fig. 10A). Homology of LCCD4 and 4-1 was 82% (Fig. 9B). cDNA 3 μL, SLCCD4-1 F793 0.5 μL, SLCCD4-1 R1036 0.5 μL, D.W. After RT-PCR was performed by mixing 16 μL, 5 μL of the PCR product was loaded on 2% gel and electrophoresed (Fig. 10B).

In the case of SLCCD4 and SLCCD4-1, it was confirmed that they were amplified in flowers and leaves. This shows that CCD4 is a gene acting on flowers and leaves. In the experiment of HAI, Nguyen Thi Lam, *Lilium brownii var. Colchesteri's* flower color changed from white to yellow during the flowering period. (Hai, Masuda et al. 2012).

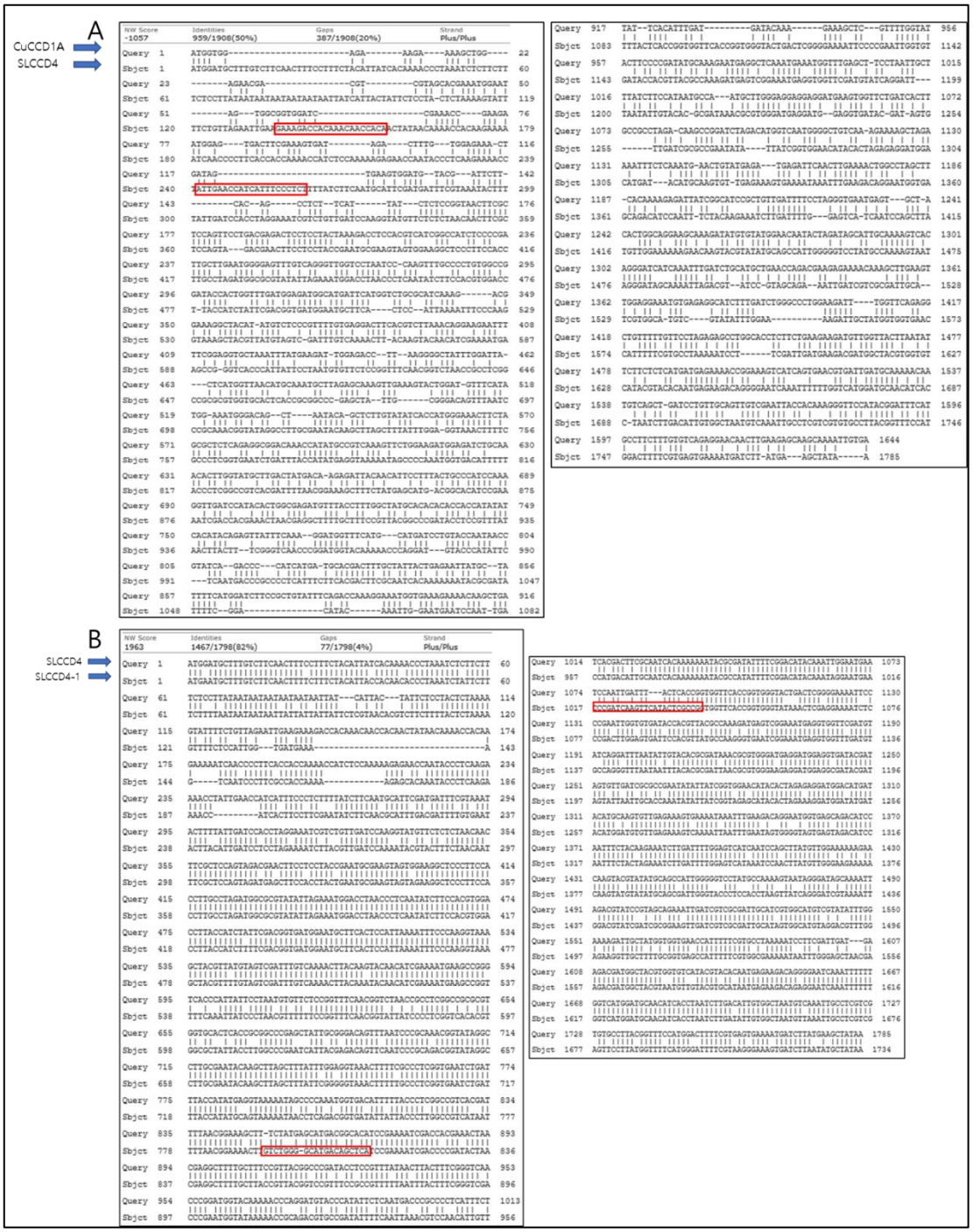

Fig 12. Homology of *CuCCD1A*, *SLCCD4*, *SLCCD4-1*

Homology was searched through NCBI. (A)The lower part marked in red is the sequence position for SLCCD4 F136-R260, and the lower part marked in red is the sequence position for SLCCD1A F793-R1036.

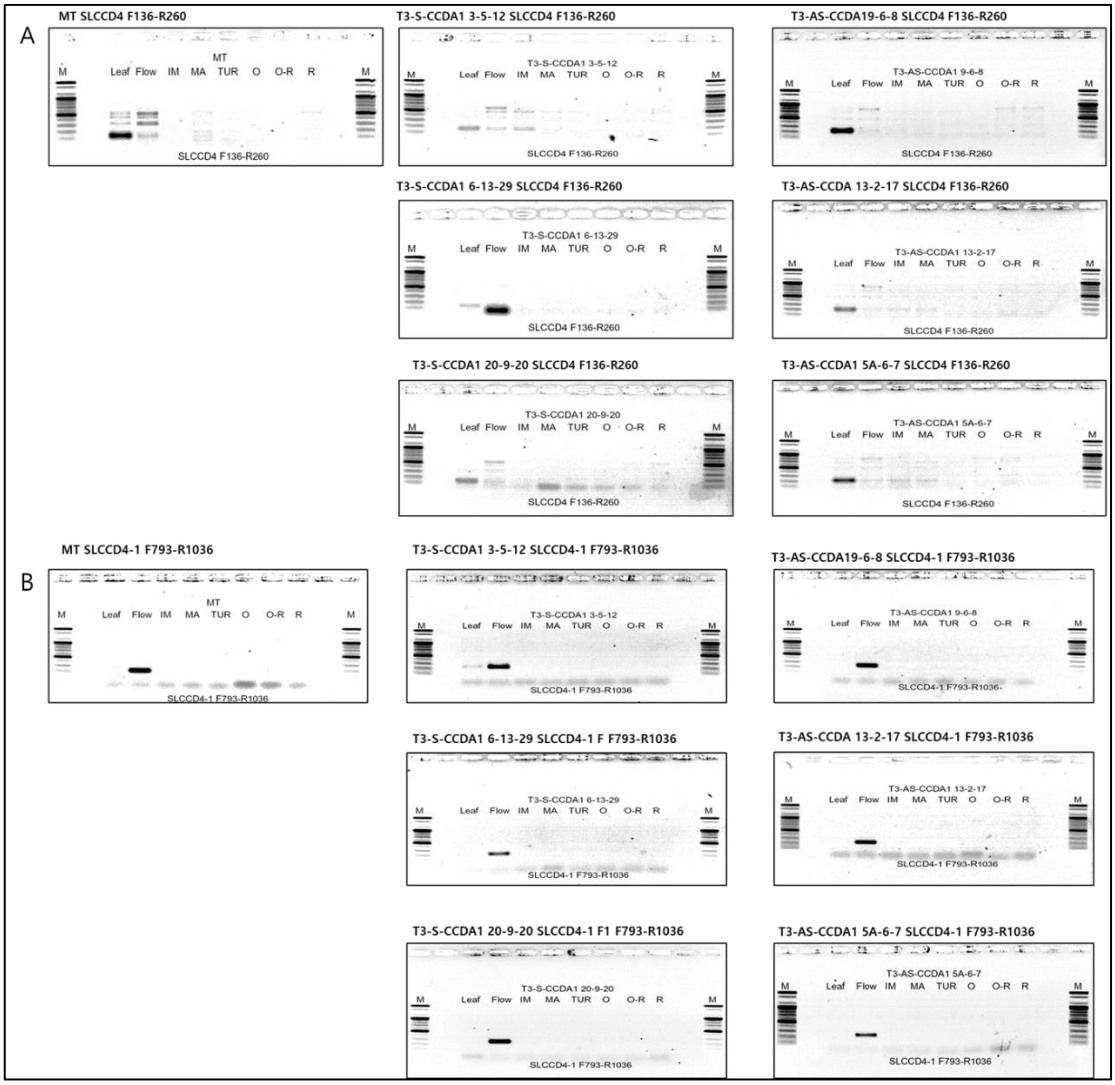

Fig 13. Electrophoresis of *SLCCD4* and *SLCCD4-1.*

(A) Gene expression analysis of SLCCD4 F136-R260. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (B) Gene expression analysis of SLCCD4 F793-R1036. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

As a result of searching for the homology of CuCCD1A, SLCCD7, and SLCCD8-2, the homology of CuCCD1A and SLCCD7 was 49% (Fig. 11A). In a 1.5 mL tube, 3 μL of cDNA, 0.5 μL of SLCCD7 F98, 0.5 μL of SLCCD7 R200, DW After RT-PCR was performed by mixing 16 μL, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 12A).

As a result of searching for homology of CuCCD1A and SLCCD8-2, the homology was 50% (Fig. 11B), and 3 μL of cDNA, 0.5 μL of SLCCD8-2 F1165, 0.5 μL of SLCCD8-2 R1400, D.W. After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 12B).

It was confirmed that amplification was not performed in SLCCD7 and SLCCD8-2, and in the study of jUNWEI Liu, CCD7 and 8 genes appear to affect the root and stem growth of plants (Liu, Novero et al. 2013).

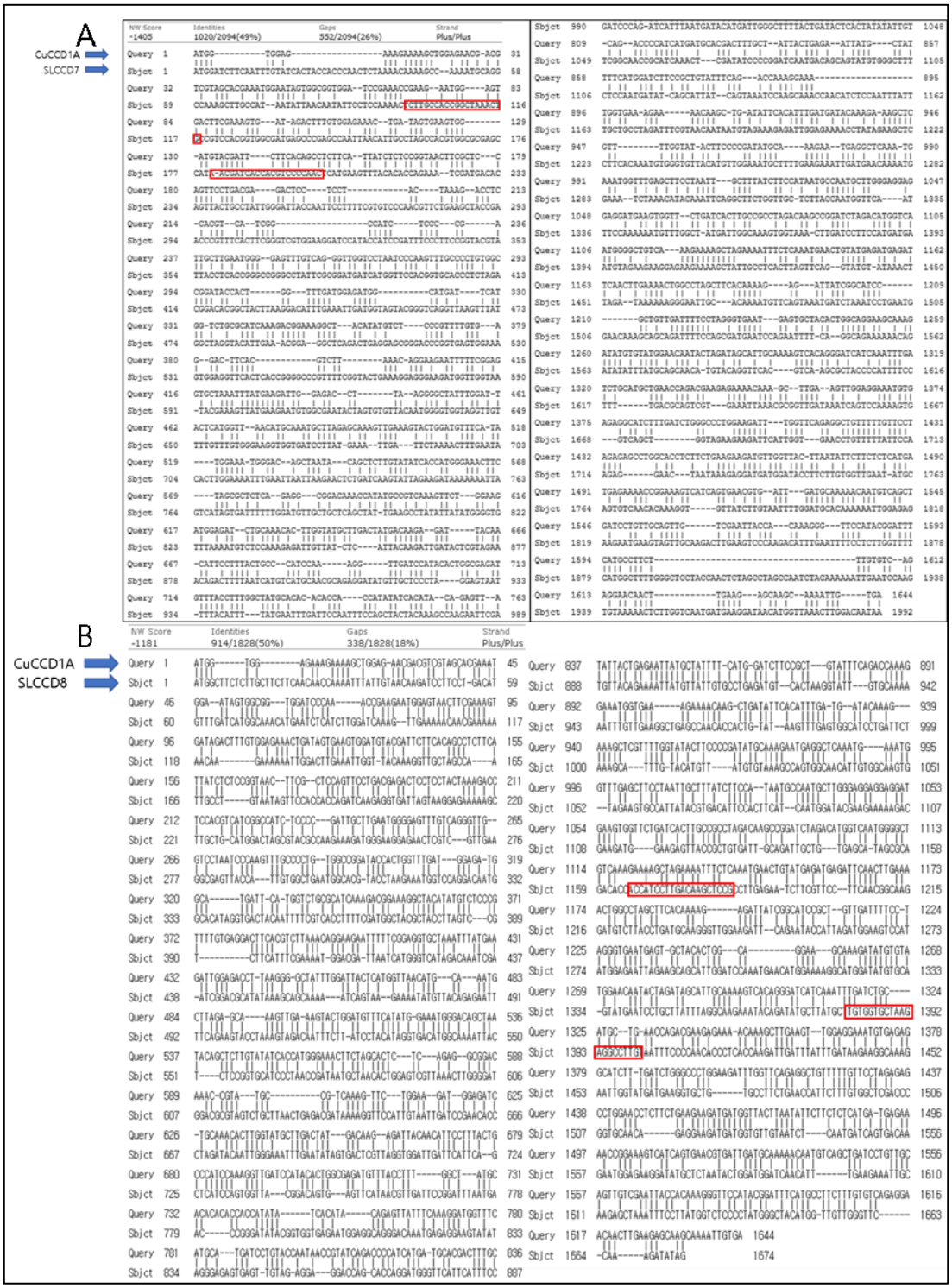

Fig 14. Homology of *CuCCD1A*, *SLCCD7*, *SLCCD8*

Homology was searched through NCBI. (A)The lower part marked in red is the sequence position for SLCCD7 F98-R200, and the lower part marked in red is the sequence position for SLCCD8-2 F1165-R1400.

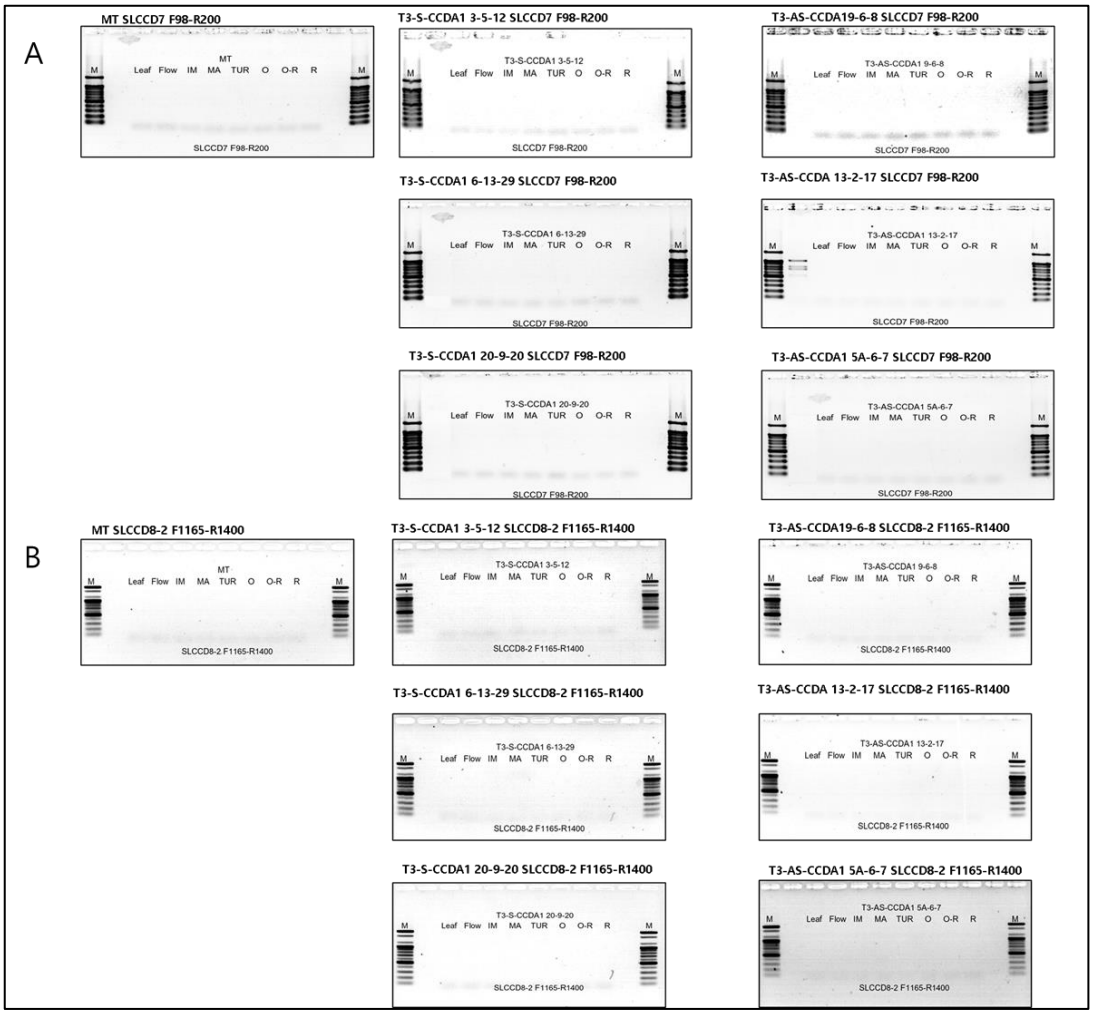

Fig 15. Electrophoresis of *SLCCD7* and *SLCCD8-2*.

(A) Gene expression analysis of SLCCD7 F98-R200. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (B) Gene expression analysis of SLCCD8-2 F1165-R1400. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

Homology of CuCCD1A, SLCCD(9'10') Like, SLCCD(9'10')-1 Like, SLCCD(9'10')-1 Like2 was searched. CuCCD1A and SLCCD (9'10') Like confirmed 49% homology (Fig. 13A). In a 1.5mL tube, 3 μL of cDNA, 0.5 μL of SLCCD(9'10') Like F731, 0.5L of SLCCD(9'10') Like R972, D.W. After mixing 16L and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 14A). SLCCD(9'10')-1 Like Homology showed 51% Homology(Fig 13B), 3 μL of cDNA in 1.5mL tube, 0.5 μL of SLCCD(9'10')-1 Like F913, SLCCD(9'10') -1 Like R1083 0.5 μL, DW After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed(Fig 14B). SLCCD(9'10')-1 Like2 Homology showed 52% Homology(Fig. 13C), 3 μL of cDNA in 1.5mL tube, SLCCD(9'10')-1 Like2 F544 0.5 μL, SLCCD(9'10') -1 Like2 R817 0.5 μL, DW After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed(Fig. 14C).

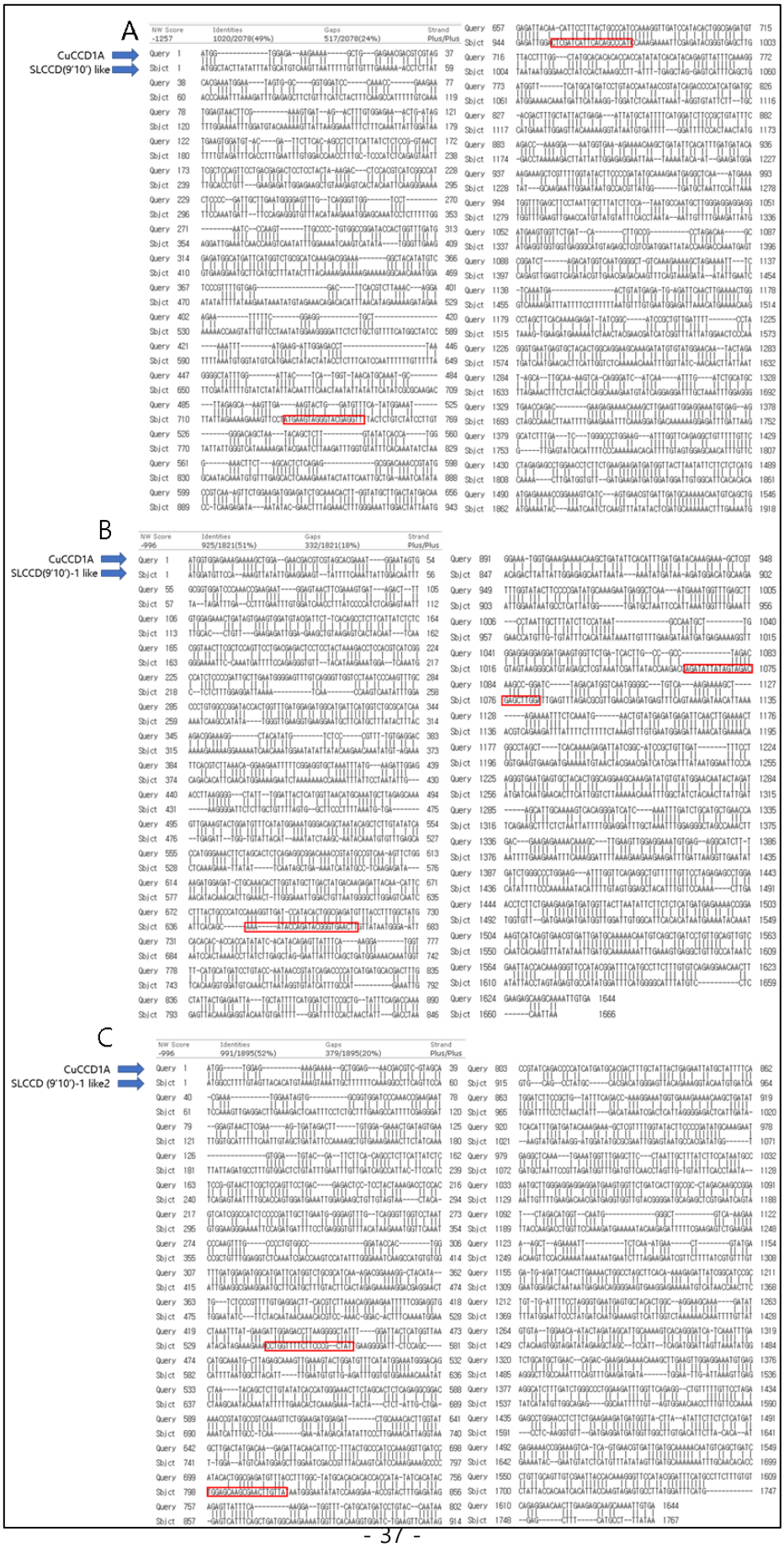

Fig 16. Homology of CuCCD1A, SLCCD(9’ 10’)like, SLCCD(9’ 10’)-1 like, SLCCD(9’ 10’)-1 like2

Homology was searched through NCBI. (A)The lower part marked in red is the sequence position for SLCCD(9′10′) like F731-R972, (B) The lower part marked in red is the sequence position for SLCCD(9′10′)-1 like F913-R1083, (C) The lower part marked in red is the sequence position for SLCCD(9′10′)-1 like2 F544-R817.

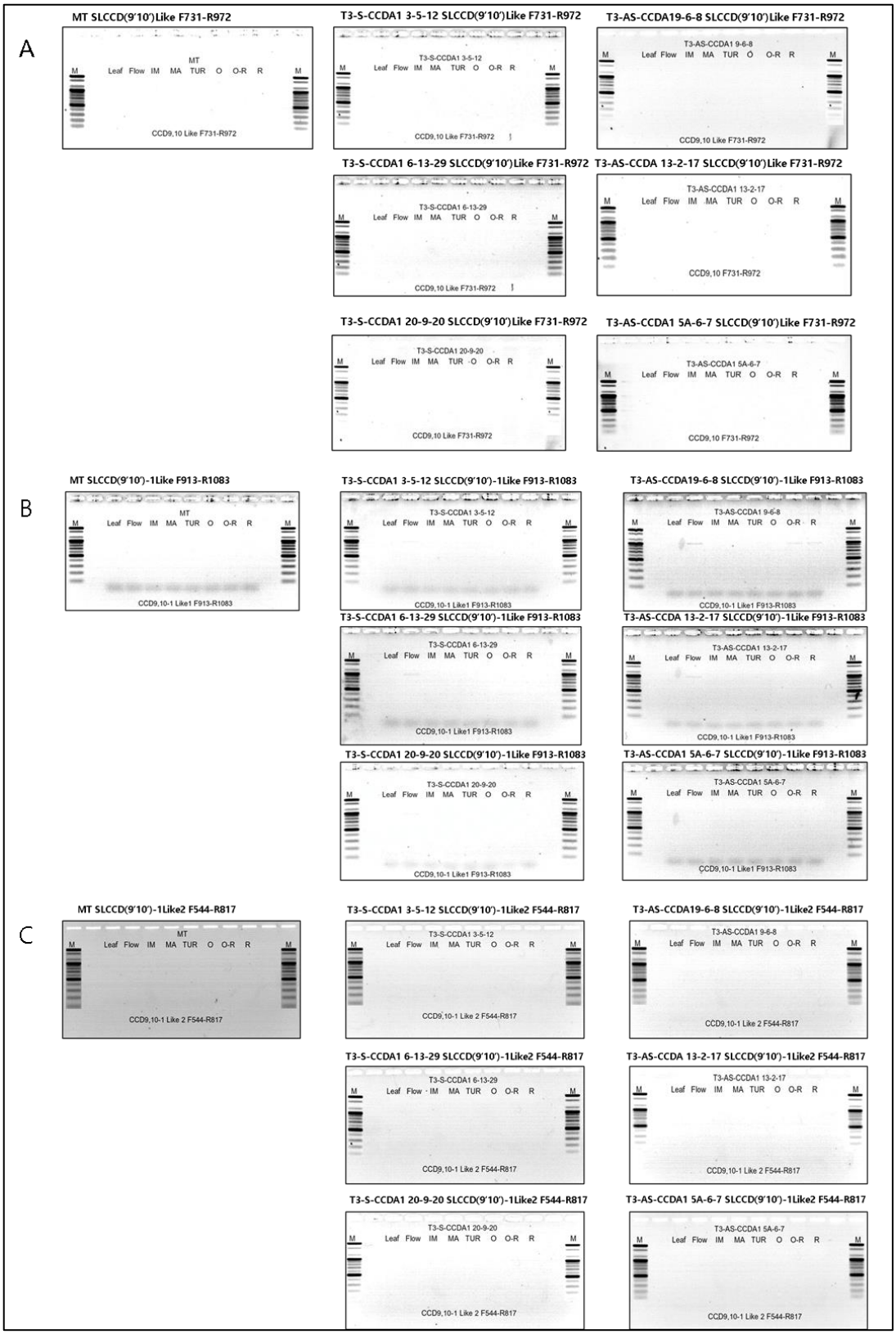

Fig 17. Electrophoresis of *SLCCD*(9′10′) Like, *SLCCD*(9′10′)-1 Like and , *SLCCD*(9′10′)-1 Like2.

(A) Gene expression analysis of SLCCD(9′10′) Like F731-R972. Left is MT, middle is 3-5-

12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (B) Gene expression analysis of SLCCD(9′10′)-1 Like F913-R1083. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (C) Gene expression analysis of SLCCD(9′10′)-1 Like2 F544-R817. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

As a result of confirming the homology of SLLCYB1 and SLLCYB2, 85% of homology was shown (Fig. 15A). 3 μL of cDNA, 0.5 μL of SLLCYB1 F297, 0.5 μL of SLLCYB1 R519, D.W. 16 μL / cDNA 3 μL, SLLCYB1 F651 0.5 μL, SLLCYB2 R919 0.5 μL, D.W. After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on 2% gel and electrophoresed (Fig. 16A,B). As a result of confirming the homology of SLCHYB1 and SLCHYB2, the homology was 75% (Fig. 15B). In a 1.5 mL tube, 3 μL of cDNA, 0.5 μL of SLCHYB1 F81, 0.5 μL of SLLCYB1 R374, D.W. 16 μL / cDNA 3 μL, SLCHYB2 F477 0.5 μL, SLLCYB2 R697 0.5 μL, D.W. After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 16C,D). SLZEP primer was synthesized as follows (Fig. 15C). 3 μL of cDNA, 0.5 μL of SLLCYB1 F297, 0.5 μL of SLLCYB1 R519, D.W. After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on a 2% gel and electrophoresed (Fig. 16E).

SLVDE primer was synthesized as follows (Fig. 15D). 3 μL of cDNA, 0.5 μL of SLLCYB1 F297, 0.5 μL of SLLCYB1 R519, D.W. After mixing 16 μL and performing RT-PCR, 5 μL of the PCR product was loaded on 2% gel and electrophoresed (Fig. 16F).

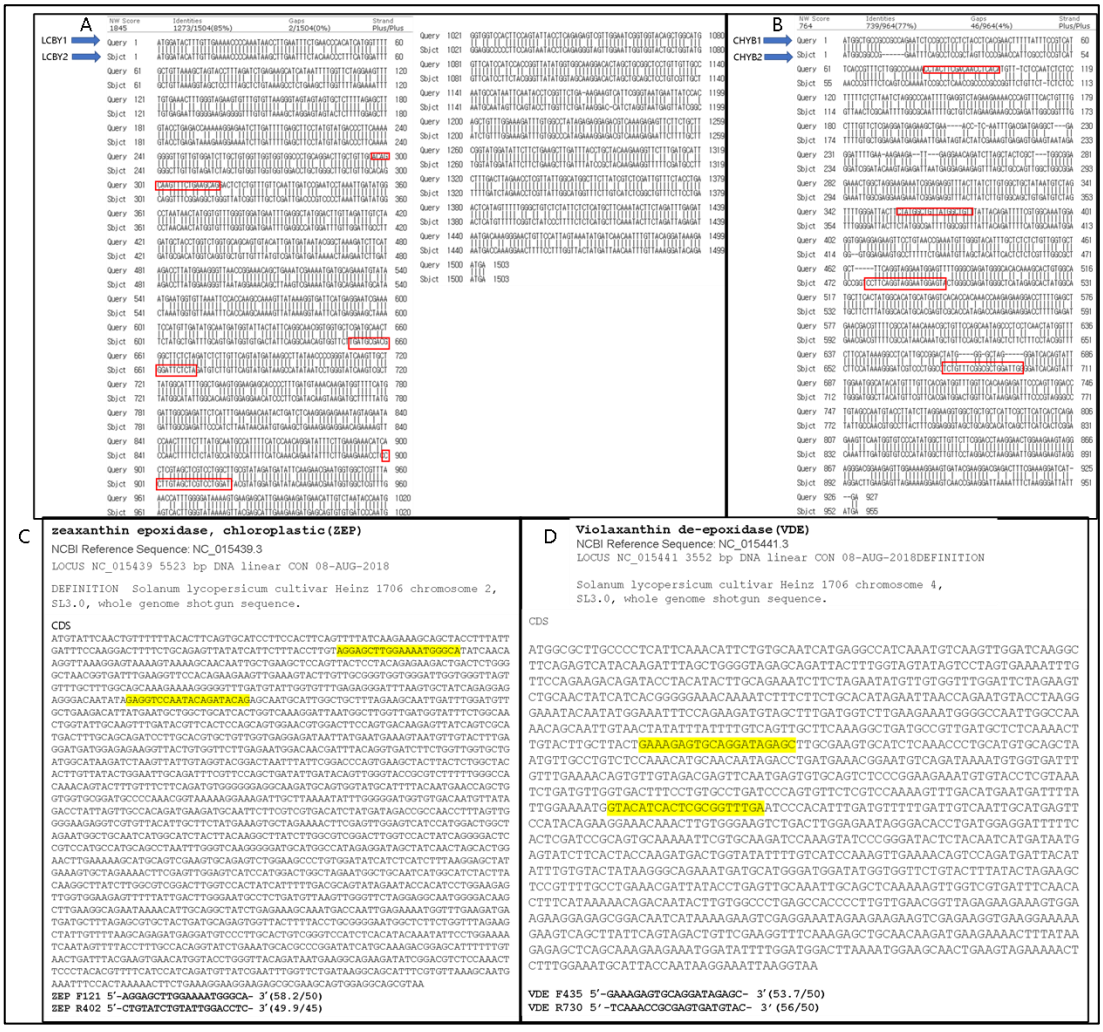

Fig 18. Gene expression analysis from LCYB1 to VDE in carotenoid biosynthesis.

Homology was searched through NCBI. (A) Homology was searched through NCBI. The upper part marked in red is the sequence position for SLLCYB1 F297-R519, and the lower part marked in red is the sequence position for SLLCYB2 F651-R919, (B) Homology was searched through NCBI. The

upper part marked in red is the sequence position for SLCHYB1 F81-R374, and the lower part marked in red is the sequence position for SLCHYB2 F477-R697, (C)The yellow part marked is the sequence position for SLZEP F121-R402, (D)The yellow part marked is the sequence position for SLVDE F435-R730.

Fig 19. Electrophoresis results from SLLCYB1 to SLVDE.

(A) Gene expression analysis of SLLCYB1 F297-R519. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (B) Gene expression analysis of SLLCYB2 F651-R519. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (C) Gene expression analysis of SLCHYB1 F81-R374. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (D) Gene expression analysis of SLCHYB2 F477-R697. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (E) Gene expression analysis of SLZEP

F121-R402. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line. (F) Gene expression analysis of SLVDE F435-R730. Left is MT, middle is 3-5-12, 6-13-29, 20-9-20 3 sense lines, right is 9-6-8, 13-2-17, 5A-6-7 3 Anti -sense line.

3-5. Anti oxidant activity analysis

3-5-1. Total carotenoid content

The results of measuring the total carotenoid content by flower, leaf and fruit development are as follows (Fig. 5). MT leaf showed values of 4.18±0.17 and flowers, 1.4±0.07, Sense leaves 3.18±0.2, flowers 1.26±0.06, Anti sense, leaves 3.52±0.22, flowers 1.04±0.08 value of (Fig. 5a).

MT-Immature green showed a value of 0.19±0.009, and mature green showed a value of 0.07±0.004. Turning showed a value of 0.1±0.006, and Orange showed a value of 0.11±0.004. Orange red showed a value of 0.22±0.11, and red showed a value of 0.22±0.29. Sense immature green showed a value of 0.13±0.008, and mature green showed a value of 0.07±0.005. Turning showed a value of 0.11± 0.006, and Orange showed a value of 0.09±0.006. Orange red showed a value of 0.17±0.01, and Red showed a value of 0.26±0.017. Anti sense immature green showed a value of 0.14±0.009, and mature green showed a value of 0.07±0.004. Turning showed a value of 0.07±0.005, and orange showed a value of 0.11± 0.007. Orange red showed a value of 0.2±0.014, and red showed a value of 0.24 ±0.018 (fig 5b).

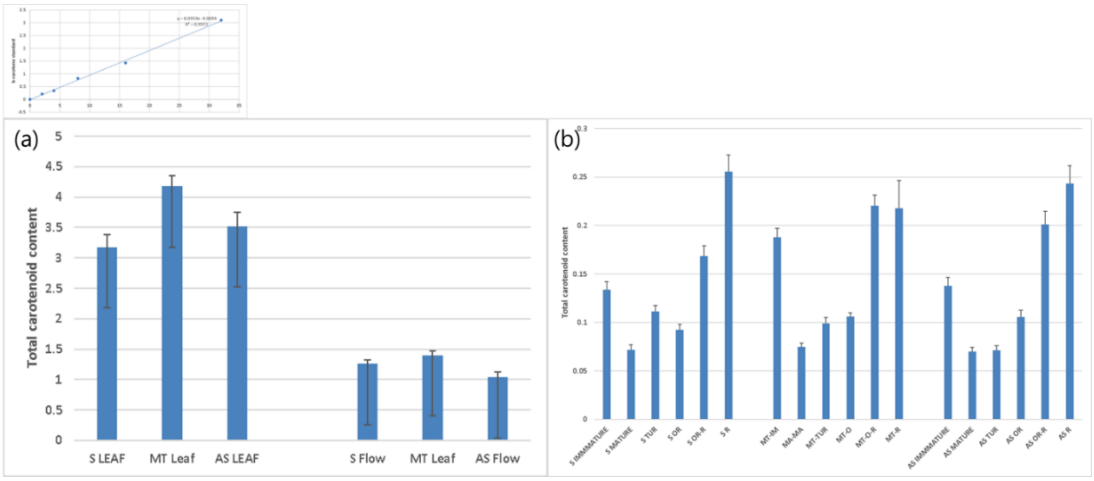

Fig 20. Total carotenoid content of control MT, Sense line, Anti sense line.

(a) Total carotenoid content in flowers and leaves, (b) Total carotenoid content by fruit development in sense, anti-sense, and control

3-5-2 Total flavonoid content.

The flavonoid content of three control and sense lines and the flavonoid content value of three anti sense lines were confirmed (Fig. 6). As a result of obtaining the average value of the total flavonoid content of the three Sense lines and the total flavonoid content of the three Anti Sense lines, Control leaves showed values of 23.15±0.73 and flowers of 41.36±0.55, while leaves of sense showed values of 22.31 ±1.08 and flowers of 44.5±14.56. In the case of anti sense, the leaf showed a value of 20.72±2.73 and the flower showed a value of 44.97±4.49.

As a result of checking the total flavonoid content in the fruit, control immature green showed a value of 17.40±1.21, and mature green showed a value of 13.57± 0.73. In addition, Turning showed a value of 11.01±0.73, and Orange showed a content of 14.21±1.00. Orange red showed a value of 12.61±1.00, and red showed

a value of 9.73±1.54. Sense Immature green showed a value of 13.43±1.09, and mature green showed a value of 11.79±1.33. Turning showed a value of 8.51±1.14 and Orange showed a value of 9.79±0.92. Orange Red showed a value of 10.1± 0.84, and Red showed a value of 9.52±1.13. Anti Sense Immature green showed a value of 9.68±1.78, and mature green showed a value of 6.71±2.69. Turning showed a value of 8.47±2.6, and Orange showed a value of 10.28±1.04. Orange red showed a value of 9.53±2, and red showed a value of 8.7±1.28.

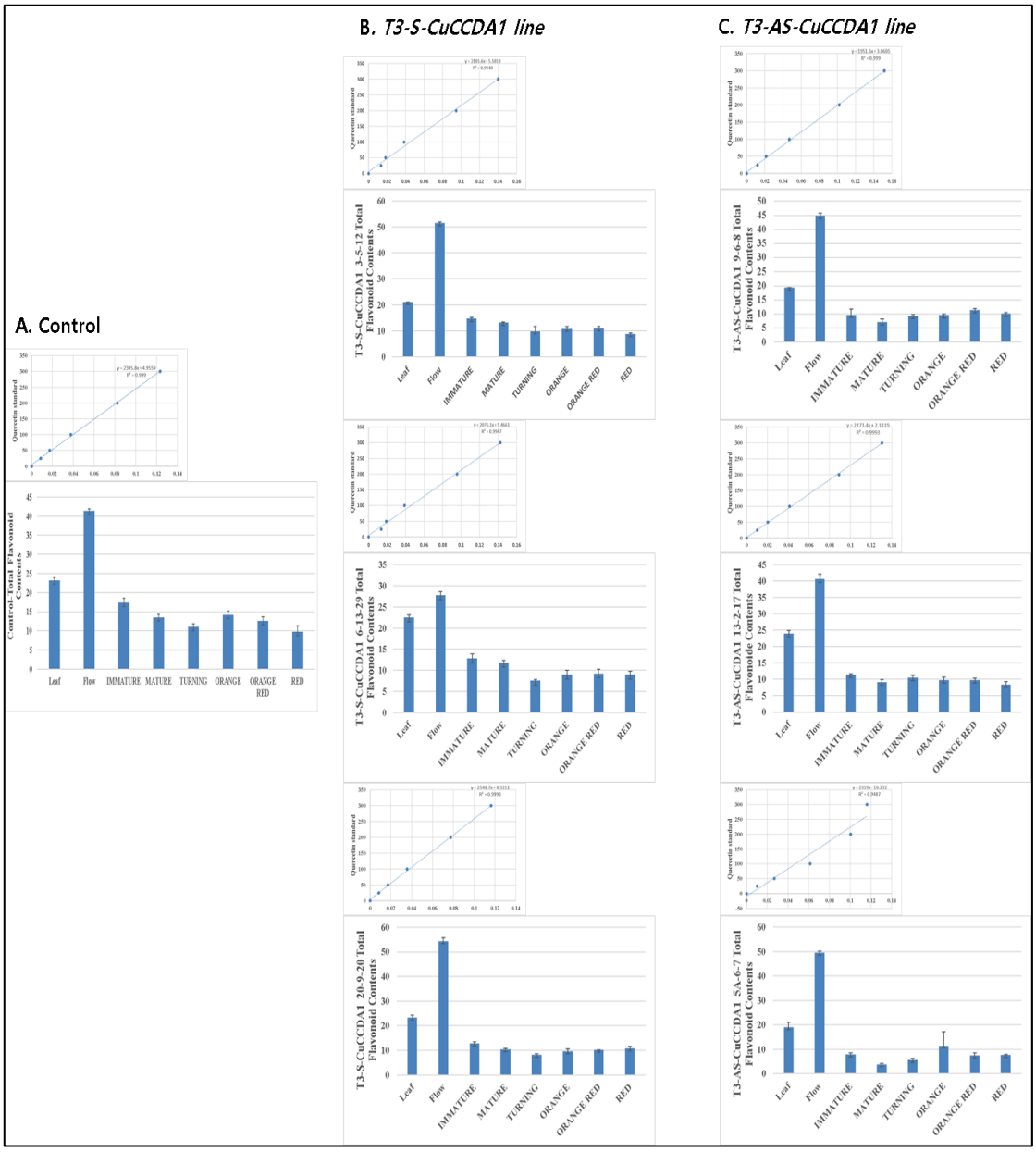

Fig 21. Total flavonoid content of control MT, Sense line, Anti sense line.

(A) Total flavonoid content of MT, (B) Total flavonoid content of Sense line, (C) Totlal flavonoid content of Anti-Sense

3-5-3 Total phenolic content

The total polyphenol content of the three control and sense lines and the total

polyphenol content of the three anti sense lines were checked (Fig. 7). As a result of obtaining the average of the total polyphenol content of the three sense lines and the total polyphenol content of the three anti sense lines, the control leaf showed a value of 9.32±0.04, and the flower showed a value of 15.43±0.008. The value of Sense leaves was 7.67±0.63 and that of flowers was 15.00±2.95. Anti Sense leaves showed a value of 9.16±0.91, and flowers showed a value of 19.76±1.47.

As a result of measuring the total polyphenol content in the fruit, control immature green showed a value of 6.71±0.002, and mature green showed a value of 5.13± 0.003. Also, Turning showed a value of 5.94±0.0006, and Orange showed a value of 7.07±0.004. Orange Red showed a value of 8.05±0.002, and Red showed a value of 6.92±0.004. Sense immature green showed a value of 6.83±0.93, and mature green showed a value of 6.40±0.41. Turning showed a value of 5.99±0.57, and orange showed a value of 7.63±0.35. In the case of orange red, a value of 11.21±1.54 was shown, and in the case of red, a value of 11.74±1.75 was shown.

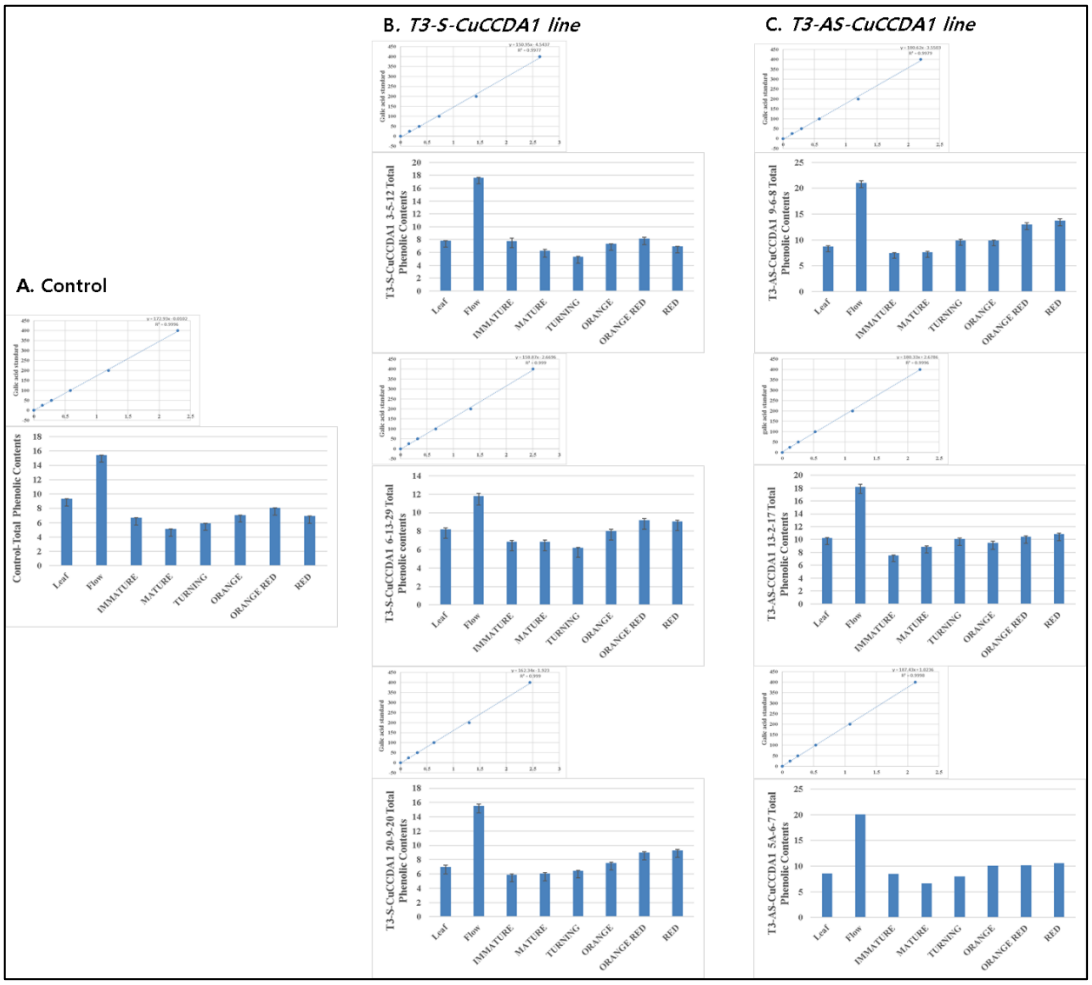

Fig 22. Total Phenolic content of control MT, Sense line, Anti sense line.

(A) Total Phenolic content of MT, (B) Total Phenolic content of Sense line, (C) Total Phenolic content of Anti-Sense

3-5-4. DPPH radical scavenging activity

The DPPH radical scavenging ability of three control and sense lines and the DPPH radical scavenging ability of three anti sense lines were confirmed (Fig. 8). As a result of obtaining the average value of the DPPH radical scavenging activity of the dog sense lines and the DPPH radical scavenging activity of the three anti sense lines, the control leaves showed a scavenging activity of 35.9±0.33, and the flowers showed a scavenging activity of 70.75±0.16. Sense leaves exhibited a scavenging activity of 31.75±7.32, and flowers exhibited a scavenging activity of 67.77±7.68. Anti sense leaves exhibited a scavenging activity of 26.81±3.43 and flowers of 75.56 ±2.25.

As a result of measuring DPPH radical scavenging activity in fruits, control immature green showed a scavenging activity of 25.29±0.17 and mature green showed a scavenging activity of 13.19±0.08. Turning showed a scavenging ability of 19.84±0.2 and 23.76±0.31 for orange, 25.59±0.18 for orange and 24.01±1.45 for red. Sense immature green showed a scavenging ability of 23.16±10.87 and mature green showed a scavenging ability of 19.97±7.28. Turning showed a scavenging ability of 21±4.75, orange showed a value of 29.06±6.50, orange red showed a scavenging ability of 32.3±6.79 and red showed a scavenging ability of 30.91±5.08. Anti-sense immature green showed a scavenging ability of 21.36±3.77, and mature green showed a scavenging ability of 21.66±3.67. Turning showed a scavenging ability of 27.44±3.75, orange showed a scavenging ability of 24.54±3.14, orange red showed a scavenging ability of 32.02±3.35, and red showed a scavenging ability of 38.44±3.57.

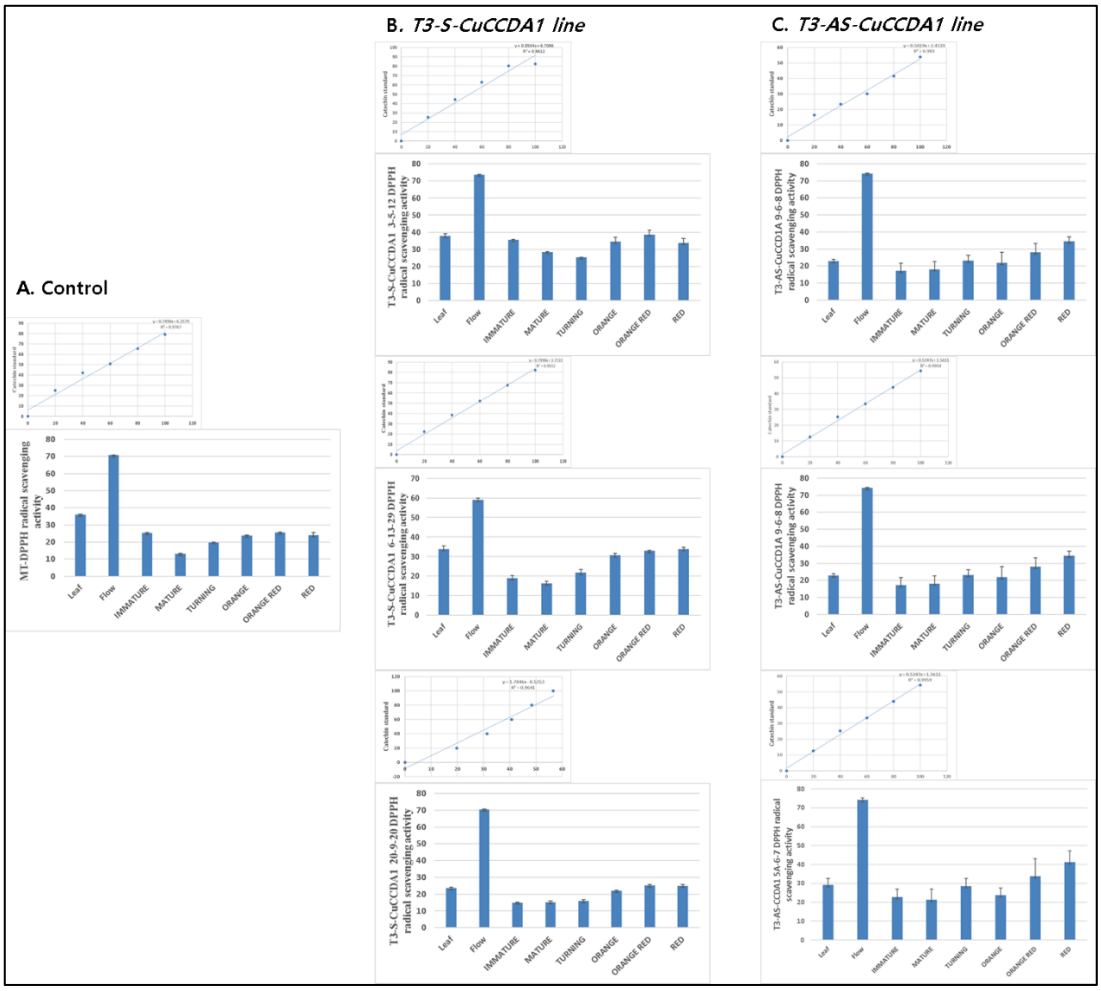

Fig 23. DPPH radical scavenging activity of control MT, Sense line, Anti sense line.

(A) DPPH radical scavenging activity of MT, (B) DPPH radical scavenging activity of Sense line, (C) DPPH radical scavenging activity of Anti-Sense

3-5. HPLC(High Performance Liquid Chromatography)

As a result of HPLC on leaves in the MT, Sense, and Anti-sense lines (Fig. 22), the peaks were high at 3.5 min and 15 min in the case of MT. S3-5-12 had a high peak at about 12.5 minutes, and in the case of S 6-13-29, the peak was high at 14 minutes. In addition, in S 20-9-20, the peaks were high at 10.5 and 13.5 minutes, and in the case of AS 9-6-8, high peaks were shown at about 14 minutes. AS 13-2-17 showed a high peak at 14 min, and AS 5A-6-7 showed a high peak between about 10 min, 10.5 min, and 13 min to 13.5 min.

Table 9. HPLC condition.

Time	A	B
	MeOH:Water (95:5)	MTBE
0.01	80	20
2.00	70	30
13.00	50	50
18.00	45	55
23.00	0	100
28.00	80	20

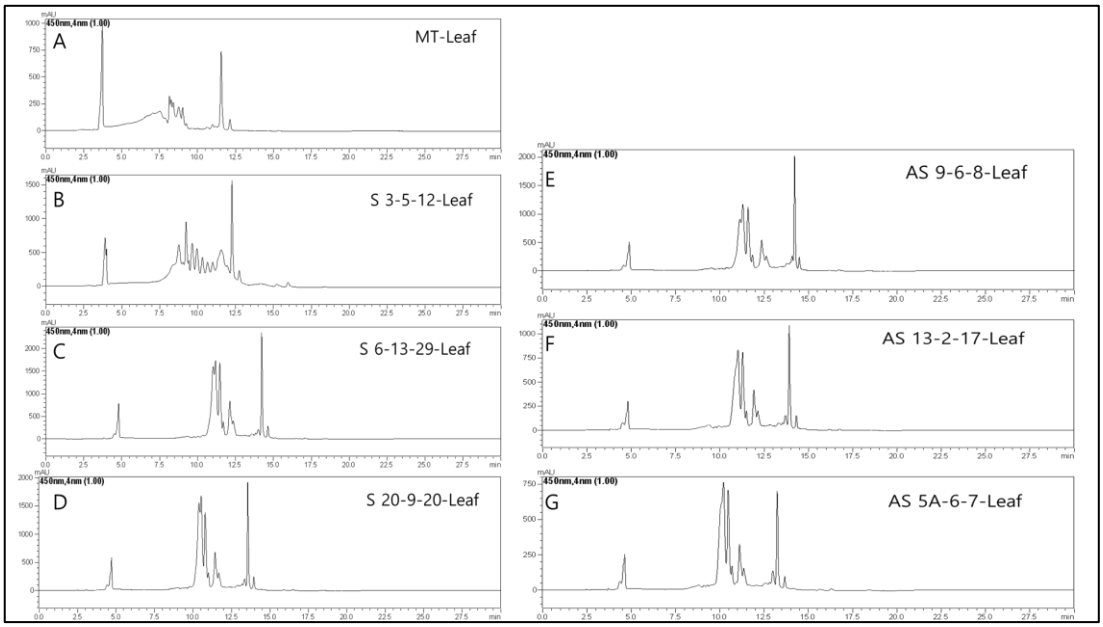

Fig 24. HPLC on leaves among MT, Sense, and Anti sense lines.

(A)MT Leaf, (B)S 3-5-12 Leaf, (C)S 6-13-29 Leaf, (D)S 20-9-20 Leaf, (E)AS 9-6-8 Leaf, (F)AS 13-2-17 Leaf, (G)AS 5A-6-7 Leaf

As a result of HPLC for flowers in the MT, Sense, and Anti-sense lines (Fig. 23), the peaks were high at 3 min and 12.5 min in the case of MT. S3-5-12 had a high peak at about 12.5 min, and in the case of S 6-13-29, the peak was high at 9 min. In addition, in S 20-9-20, the peaks were high at 15 min and min, and in the case of AS 9-6-8, a high peak was shown at about 12.5 min. AS 13-2-17 showed a high peak at 13.5 min, and AS 5A-6-7 showed a high peak at about 3.5 min.

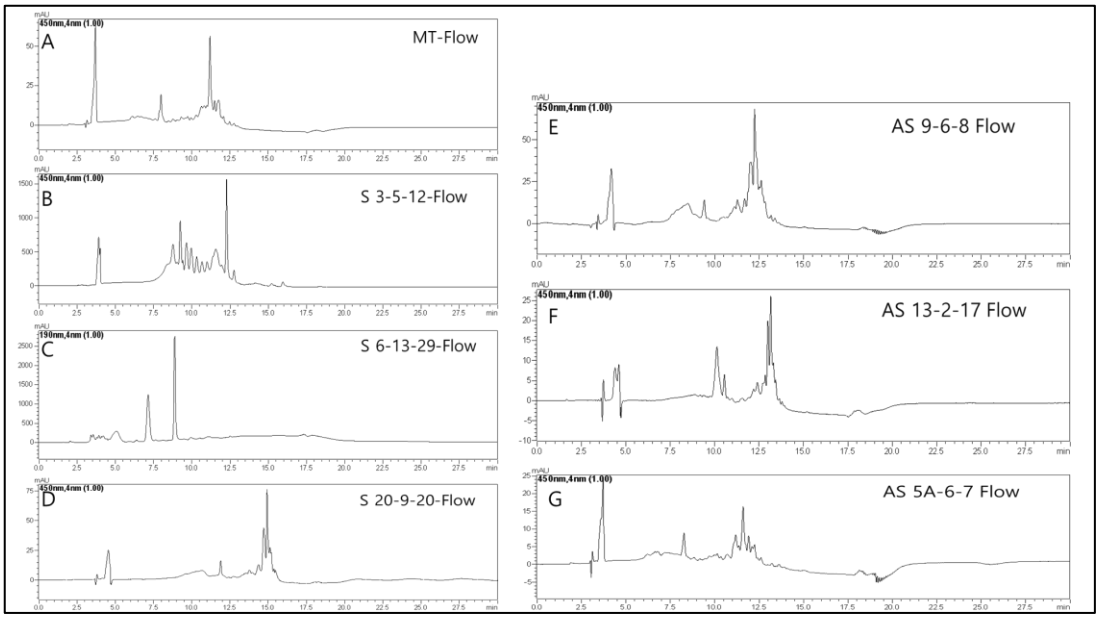

Fig 25. HPLC on Flower among MT, Sense, and Anti sense lines.

(A)MT Flower, (B)S 3-5-12 Flower, (C)S 6-13-29 Flower, (D)S 20-9-20 Flower, (E)AS 9-6-8 Flower, (F)AS 13-2-17 Flower, (G)AS 5A-6-7 Flower

As a result of HPLC for immature green in MT, Sense, and Anti-sense lines (Fig. 24), MT peaks were high at 11 and 11.5 minutes. S3-5-12 had a high peak at about 13 to 13.5 minutes, and in the case of S 6-13-29, the peak was high at 13 to 14 minutes. In addition, in S 20-9-20, the peak was high at 12.5 min and min, and in the case of AS 9-6-8, a high peak was shown at about 14.5 min. AS 13-2-17 showed a high peak at 12.5 min, and AS 5A-6-7 showed a high peak at about 11 min.

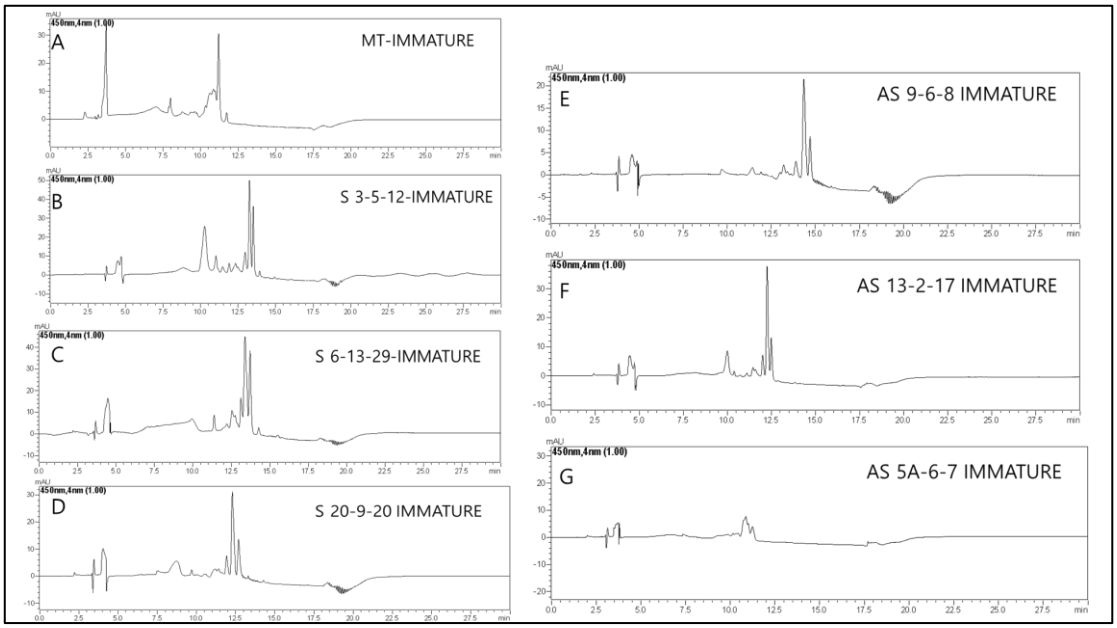

Fig 26. HPLC on Immature green among MT, Sense, and Anti sense lines.

(A)MT Immature green, (B)S 3-5-12 Immature green, (C)S 6-13-29 Immature green, (D)S 20-9-20 Immature green, (E)AS 9-6-8 Immature green, (F)AS 13-2-17 Immature green, (G)AS 5A-6-7 Immature green

As a result of HPLC for mature green in MT, Sense, and Anti-sense lines (Fig. 25), the peak at 11.5 min for MT was high. S3-5-12 had a high peak at about 3.5 min, and in the case of S 6-13-29, the peak was high at 3.5 min and 11~11.5 min. In addition, in S 20-9-20, the peaks were high at 3.5 and 12 minutes, and in the case of AS 9-6-8, high peaks were shown at about 12 to 12.5 minutes.. AS 13-2-17 showed high peaks at 7 and 9 min, and AS 5A-6-7 showed low peaks.

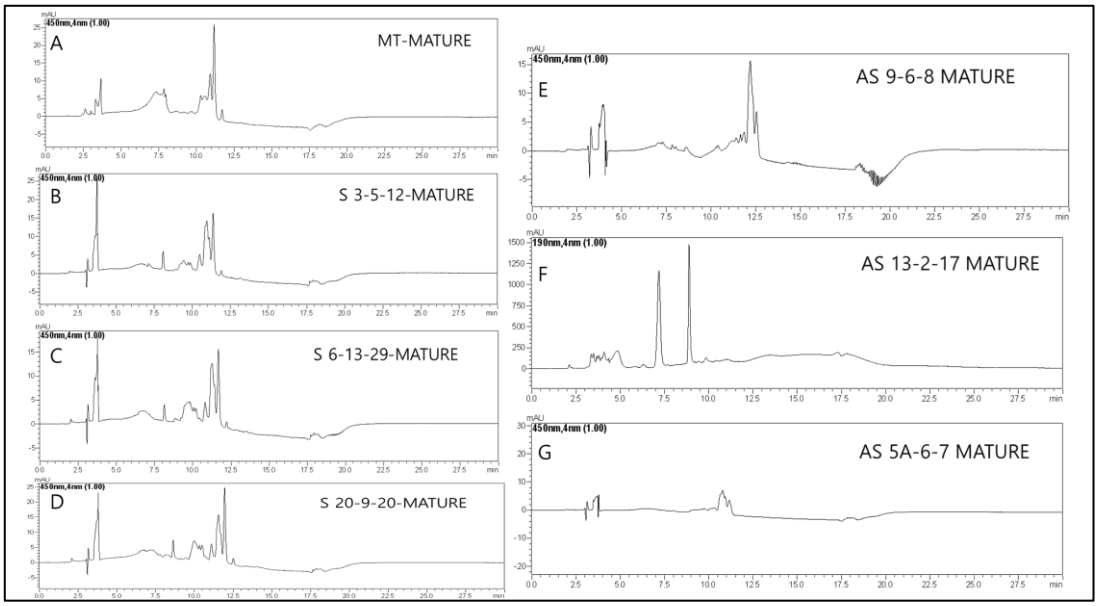

Fig 27. HPLC on Mature green among MT, Sense, and Anti sense lines.

(A)MT mature green, (B)S 3-5-12 mature green, (C)S 6-13-29 mature green, (D)S 20-9-20 mature green, (E)AS 9-6-8 mature green, (F)AS 13-2-17 mature green, (G)AS 5A-6-7 mature green

As a result of HPLC for turning in MT, Sense, and Anti-sense lines (Fig. 26), in the case of MT, the peak was high at 11.5 to 12. S3-5-12 had a high peak at about 11.5 to 12.5 minutes, and in the case of S6-13-29, the peak was high at 13 minutes. In addition, in S 20-9-20, the peak was high at 12.5~13 min and min, and in the case of AS 9-6-8, a high peak was shown at about 13 min. AS 13-2-17 had a high peak at 12.5-13 min, and AS 5A-6-7 had a low peak.

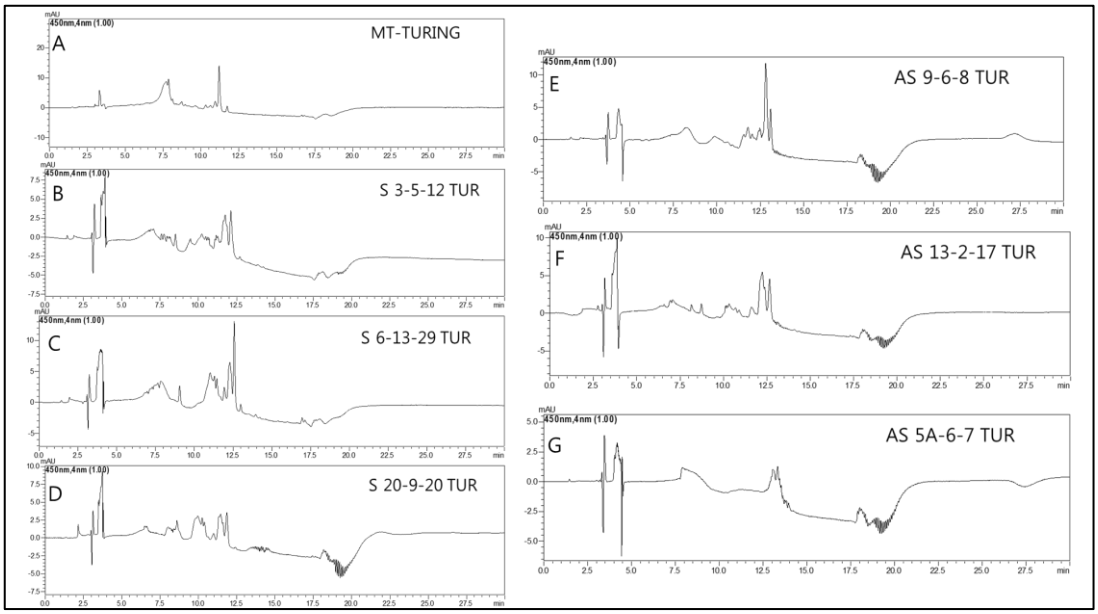

Fig 28. HPLC on Turning among MT, Sense, and Anti sense lines.

(A)MT Turning, (B)S 3-5-12 Turning, (C)S 6-13-29 Turning, (D)S 20-9-20 Turning, (E)AS 9-6-8 Turning, (F)AS 13-2-17 Turning, (G)AS 5A-6-7 Turning

MT, Sense, Anti-sense라인 에서의 Orange에대한 HPLC 결과로(Fig. 27), MT의 경우 3.5분과 The peak was high between 11 and 11.5 minutes. S3-5-12 had a high peak at about 13 minutes, and in the case of S 6-13-29, the peak was high at 10-12 minutes. In addition, in S 20-9-20, the peaks were high at 9 and 13 minutes, and in the case of AS 9-6-8, high peaks were shown at about 12.5 minutes. AS 13-2-17 showed a high peak at 11-12 min, and AS 5A-6-7 showed a high peak at about 17 min.

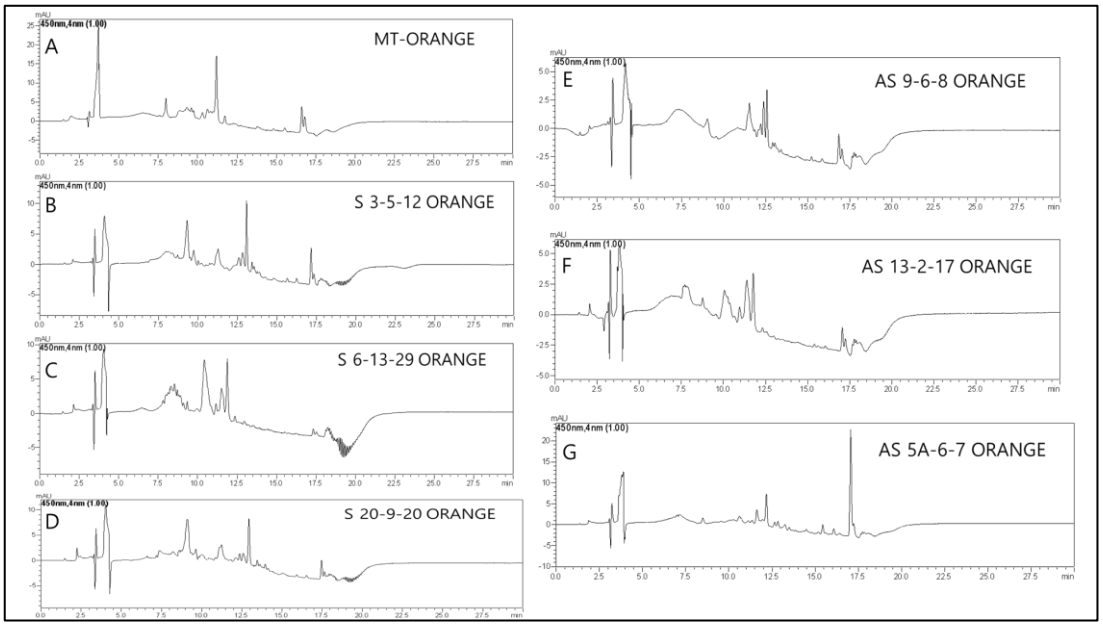

Fig 29. HPLC on Orange among MT, Sense, and Anti sense lines.

(A)MT Orange, (B)S 3-5-12 Orange, (C)S 6-13-29 Orange, (D)S 20-9-20 Orange, (E)AS 9-6-8 Orange, (F)AS 13-2-17 Orange, (G)AS 5A-6-7 Orange

As a result of HPLC for Orange-red in MT, Sense, and Anti-sense lines (Fig. 28), the peak at 16.5 min for MT was high. S3-5-12 showed a low peak, and in the case of S 6-13-29, the peak was high at 17.5 min. In addition, in S 20-9-20, the peak was high at 17.5 min and min, and in the case of AS 9-6-8, the peak was high at about 17 min. AS 13-2-17 showed a high peak at 17.5 min, and AS 5A-6-7 showed a high peak at about 17.5 min.

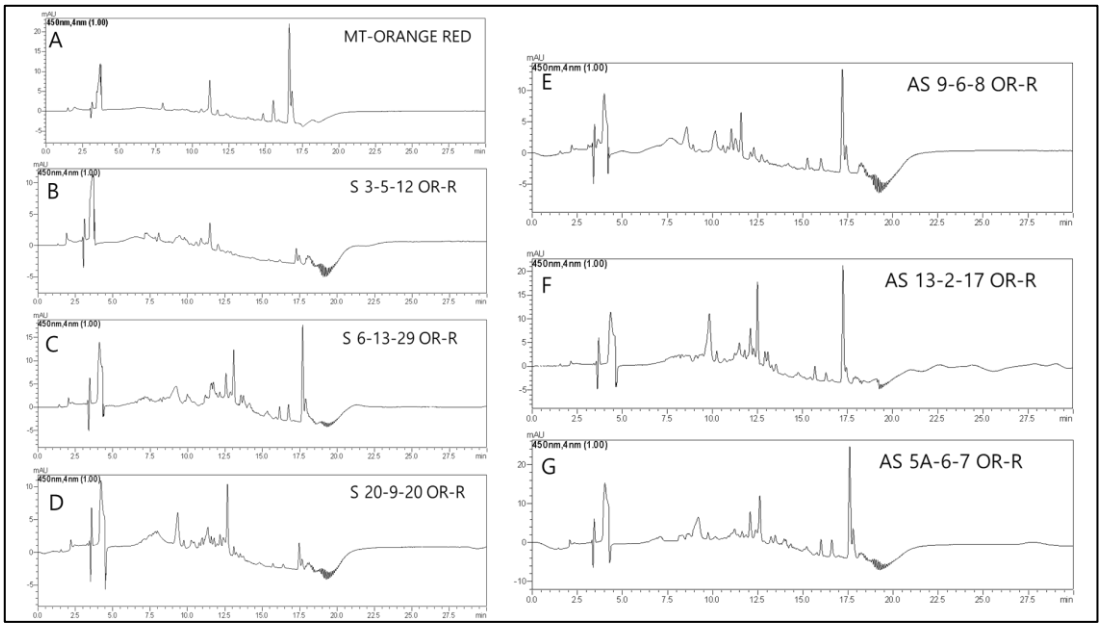

Fig 30. HPLC on Orange-Red among MT, Sense, and Anti sense lines.

(A)MT Orange-red, (B)S 3-5-12 Orange-red, (C)S 6-13-29 Orange-red, (D)S 20-9-20 Orange-red, (E)AS 9-6-8 Orange-red, (F)AS 13-2-17 Orange-red, (G)AS 5A-6-7 Orange-red

As a result of HPLC for Red in the MT, Sense, and Anti-sense lines (Fig. 29), the peaks were high at 3 min and 16.5 min for MT. S3-5-12 had a high peak at about 17 minutes, and in the case of S 6-13-29, the peak was high at 17 minutes. In addition, in S 20-9-20, the peak was high at 17.5 min and min, and in the case of AS 9-6-8, a high peak was shown at about 17.5 min. AS 13-2-17 showed a high peak at 17.5 min, and AS 5A-6-7 showed a high peak at about 17.5 min.

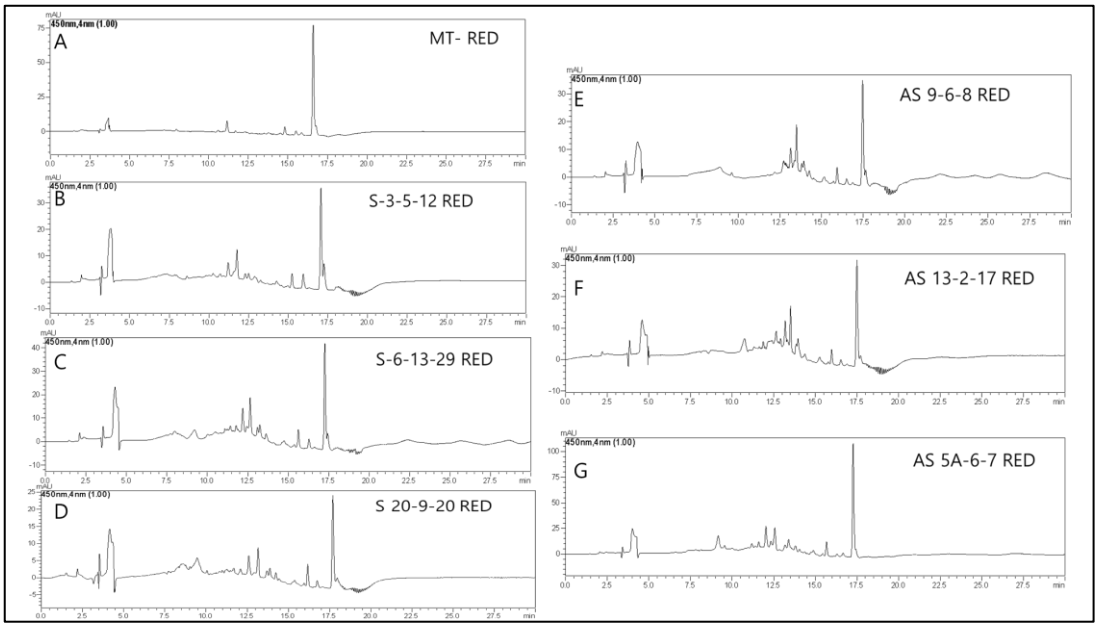

Fig 31. HPLC on Red among MT, Sense, and Anti sense lines.

(A)MT Red, (B)S 3-5-12 Red, (C)S 6-13-29 Red, (D)S 20-9-20 Red, (E)AS 9-6-8 Red, (F)AS 13-2-17 Red, (G)AS 5A-6-7 Red

4. Summary and Conclusions

Homo line was found from transgenic tomatoes introduced with CuCCD1A carotenoid gene from citrus fruits, and gene expression analysis, activity analysis, and HPLC analysis were performed. As a result, the transformed microtomato was compared with the general control group in the gene expression analysis, but in the experimental group and the control group did not feel any difference.

Also, in the activity assays Total Carotenoid Content, Total Phenolic Content and Total Flavonoid Content, and DPPH radical scavenging, no significant difference was found between the control group and the experimental group.

Therefore, it is necessary to additionally conduct experiments on gene expression analysis and activity analysis, and it is also necessary to perform additional analysis of changes in the composition of substances by performing HPLC measurement several times.

In the case of gene activity analysis, when the genes of the overexpressed Sense line and the suppressed Anti Sense line function normally, the difference in DNA band and the difference between the overexpressed Sense line and the suppressed Anti sense line in the case of activity analysis should be shown. Through this experiment, the change in carotenoid biosynthesis can be observed when the CCD1A gene isolated from citrus is transformed into tomato, and it has great academic and commercial value through activity analysis and material analysis.

감사의 글

대학원 석사 과정으로 있는 동안 저 뿐 만이 아닌 실험실 소속 대학원생들과 학부생들을 지도해 주신 지도교수님께 감사의 인사를 드리고 싶습니다. 또한 실험적인 부분이나 궁금한 점이 있을 때 저희에게 가르쳐 주신 실험실 박사님께도 감사의 인사를 드립니다.

또한 대학원과 학교에 발전을 위해 힘을 써 주시는 여러 조교선생님들에게도 감사의 인사를 드리는 바입니다.

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