MASTER'S THESIS

Changes in Flavonoid Ingredients in Micro-Tom Tomato Transgenic Plants Introduced with Citrus *CuCYP71A1* Gene

BATKHUU ENKHTUYA

Department of Biomaterials Science and Technology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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감귤 *cuCYP71A1*유전자가 도입된 Micro-Tom 토마토 형질전환 식물체에서의 플라보노이드 성분 변화

지도교수 김 인 중 (인)

바트후 엥흐토야

이 논문을 바이오소재공학 석사학위 논문으로 제출함

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석사학위 논문

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생명공학과

바트후 엥흐토야

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List of Abbreviations

CYP P450…… Cytochrome P450enzyme

CuCYP71A1..…….....Citrus Unshiu Cytochrome P71A1

gDNA..……...Genomic DNA

MT……..Micro-Tomato

WT…….Wild-type

AS……...Anti-Sense

S……..Sense

gPCR……..genotyping Polymerase Chain Reaction

MS medium…….....Murashige&Skoog medium

DW…….Distilled Water

RT-PCR…….....Reverse Transcription polymerase chain reactions

SL……...Solanum Lycopersium

CU……..Citrus unshiu

TFC…….....Total Flavonoid Content

TPC……Total Polyphenol Content

DPPH…….Free radical scavenging activity

HPLC: …….....High-performance liquid chromatopgraphy

F3’M…….....Flavonoid 3’ monooxygenase

Abstract

In this study, we researched the citrus gene *cuCYP71A1* developed from the red citrus mutant pigment is introduced into the Micro-Tom tomato seeds using pcambia2300vector. T2 generation seeds were sowed in the greenhouse, genomic DNA was extracted, and the introduction of the target gene was confirmed through genotyping PCR analysis. After confirming the gene introduction, tomato homozygous were prepared, sown in the greenhouse, and harvested by dividing leaves, flowers, and 6 fruit development stages. The color difference was taken for each stage of the fruit using a colorimeter, RNA was extracted, and cDNA synthesized. After cDNA synthesis, RT-PCR analysis confirmed the expression of the target gene. As a result, it was confirmed that the citrus gene *cuCYP71A1* introduced into tomatoes was expressed in all stages. After the introduction and expression of the target, the gene was confirmed as described above, the expression levels of the tomato-related genes *SLCYP71A1*, *SLCYP71A1-like*, *SLCYP85A3*, SL*CYP734A7*, and *SLCYP734A8* genes were confirmed through RT-PCR. The changed seed size of a transgenic tomato plant on the influence of citrus *cuCYP71A1* gene. At the time of infecting the citrus *cuCYP71A1* gene into to Micro-Tom tomato, the *SLCYP78A3* gene was suppressed and the decrease of the transgenic tomato seed size of the overexpressed sense line. Analysis of tomato total flavonoid content, total polyphenol content, and antioxidant activity (DPPH) was confirmed. As a result, it was confirmed that the total flavonoid content overexpression sense line was twice higher than the knock-out AS line and controlMT line in fruit. There was no difference between leaves and flowers. Total polyphenol content and antioxidant activity are confirmed was no difference in all stages and lines. The sense line in the total flavonoid content experiment was high in the fruit, the expression level of flavonoid-related genes in the tomato flavonoid pathway was confirmed through RT-PCR analysis. The result was confirmed that there was no difference in the amount of expression. As described above, the High-Performance Liquid

Chromatography experiment was confirmed by flavonoid analysis. The result was similar to the result of the total flavonoid content experiment, the sense line was twice higher than anti-sense, and control MT in the fruit. It was also confirmed that there was no difference between leaves and flowers.

I. Introduction

Citrus is the most important tree fruit crop in the world and is mainly consumed as a fresh fruit although it may be processed into canned- and juice-based products, with the current world production, far exceeding that of delicious tree fruits (apples, pears, peaches, plums). Citrus fruit contains rich bioactive compounds such as carotenoids, flavonoids, limonoids, nomilins, organic acids, soluble sugars, and volatile compounds. We performed differential expressed genes (DEG) screening between the red citrus mutant and the wild-type citrus. Several genes related to pigment biosynthesis were selected from genes with higher expression than in the wild type. As a result, flavonoid 3'monooxygenase(*CuF3M*), an up-regulated gene in the mutant, was isolated. After the citrus genome database have been updated, the *CuF3M* gene showed the highest homology with the *CuCYP71A1* gene involved in brassinosteroid biosynthesis. In this study, for functional analysis of the *CuCYP71A1* gene, overexpression transgenic Micro-tom tomato plants introduced with the *CuCYP71A1* gene were produced and the homozygous lines were selected. During the ripening of citrus, the *CYP71A1* mRNA and protein accumulate to relatively high levels. Tomato (Solanum Lycopersicum cv.Micro-Tom.) is one of the major vegetables which has achieved tremendous popularity over the last century. Tomatoes also accumulate semipolar metabolites, such as flavonoids, phenolic acids, and alkaloids, which are important health-promoting compounds. The main flavonoid classes are the flavones, flavonols, flavanones, flavanols, anthocyanidins, and isoflavones. More than 500 different forms of flavonoids are present in tomatoes, with the most major being the chalcone naringenin chalcone and various sugar conjugates of the flavonols quercetin and kaempferol, including rutin. In tomato fruits, the accumulation of flavonoids is restricted to the peel, with only traces found in the flesh, which comprises approximately 95% of the whole fruit. Links between primary and secondary metabolic pathways are further highlighted by the importance of pigments, flavonoids, and volatiles for tomato fruit quality. The advantage of

tomatoes is very fast growing. Increase of flavonoid compound in the fruits of tomato transformant overexpressing the *CuCYP71A1* gene. Tomato Ripening is also influenced by the balance of other hormones, including ABA, auxin, and brassinosteroid.

Overexpression of the *cuCYP71A1* gene affects seed development.

In our study, the role of *cuCYP71A1*gene in seed germination under stress conditions was investigated. Meanwhile decreased seed production was also observed in the cuCYP71A1 overexpressing transgenic lines, which triggered us to further investigate the underlying causes of this phenotype. In the 90day old plants, although no obvious difference was found between ControlMT and *cuCYP71A1* mutant, T3S CuCYP71A1 transgenic lines produced much shorter much than did the Control MT and knock out CYP71A1 mutant, T3AS CYP71A1 lines plants. (fig2) Fewer seeds and arrested embryo development were also observed in the opened siliques of T3S CYP71A1 transgenic plants. In addition, T3S CYP71A1 transgenic plants produced fewer seeds per silique, leading to decreased seed yield per plant, but decreased 100-seed-weight due to the decreased seed size. These results indicate that overexpression of *cuCYP71A*1 affected seed production in transgenic plants. Therefore we reported analysis and found no difference between control MT and knockdown T3AS CYP71A1 transgenic tomato plants. The result showed that three control MT, knockdown T3AS CYP71A1 and overexpression T3S CYP71A1 transgenic tomato plants seed size.

II. Materials and Methods

Plant material.

The T2 generation T2AS F3M (T2AS 1-8, 3-12, 7-5, 7-20, 1-7, 1-9, 1-12, 3-10, 3-12, 7-1) and T2S F3M (5-1, 1-11- 2-3, A4) line tomato seeds received by Dr. Eun Chang-ho were used, sowed in the glass greenhouse, and tested on seed, fruit, leaf, and flower.

The fruits, leaves, and flowers of transgenic tomatoes were sampled. Fruits were harvested at the following stages of development: IM: immature green, MA: mature green, TUR: turning. ORA: orange, O.R: orange-red, RED: red. All samples were using liquid nitrogen lyophilized and powdered

1. Genomic DNA isolation and confirmation of transgenic plant using PCR

To identify transgenic plants, genomic DNA that was the template for PCR was extracted. When extracting DNA from tomato young leaves, it was divided into two methods according to the extraction method.

50μl DNA extraction buffer was crushed with a pestle, 400μl DNA extraction buffer was put in and stored for 15 minutes at room temperature, Centrifugation was performed for 15 minutes at 13,000 rpm, 300μl was added to the upper layer of the new tube, Added 300μl 2-proposal was slowly drawn three times, Kept for 5 minutes in room temperature Centrifuge at 13000 rpm for 10 minutes. Remove the supplement. Add 1ml 70%ethanol (washing) Centrifuge at 13000rpm for 5minutes and remove the supplement. Dry and dissolve with 100μl TE buffer. For PCR confirmation of transgenic plants was performed using DryMIX-ntaq (Enzynomics) and AccuPCR premix (Bioneer,Korea). This PCR reaction included 2μl genomic DNA, 0.5μl forward and reverse primer, 17μl DW: the PCR conditions were 94°c for 5 min, 94°c for 30sec, followed by 30cycles of 58°c for 30sec, 72°c for 30sec, final extension of 5min at 72°c. The PCR products were analyzed on a 1.2% agarose gel 100v for25minDetected by band with ethidium bromide (EtBr).

2nd method:

Put the young leaves in a 1.5ml tube, put them in liquid nitrogen three times, crushed them with the pestle, add the AP1 buffer 400µl in voltex and store them at 65° for 10 minutes.

When storing to invert 3times, and the AP2 buffer was 130µl added and kept in ice for 10 minutes. Centrifuge at 13,000 rpm for six minutes and put 300µl in a new tube, mix it well with 450 µl AP3/E buffer, add 700µl in the upper solution, remove the upper layer at 8,000 rpm for one minute and add 500µl AW buffer for 8,000 minutes.

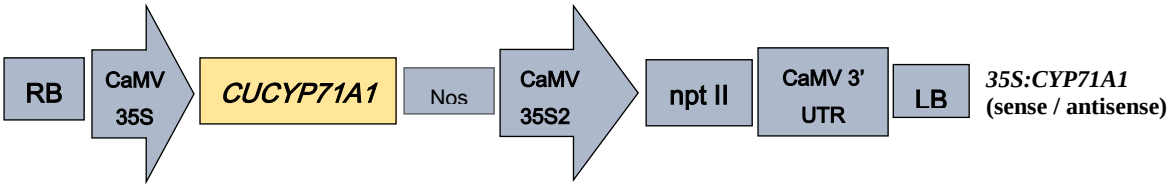
Transferred to a new 1.5ml tube and put 100µl TE buffer in room temperature for 5 minutes, Centrifuge was performed for 1 minute at 6,000 rpm, and the method up to 2 above was used.

For PCR confirmation of transgenic plants was performed using DryMIX-ntaq (Enzynomics) and AccuPCR premix (Bioneer,Korea). This PCR reaction included 2µl genomic DNA, 0.5µl forward and reverse primer, 17µl DW: the PCR conditions were 94°c for 5 min, 94°c for 30sec, followed by 30cycles of 58°c for 30sec, 72°c for 30sec, final extension of 5min at 72°c. The PCR products were analyzed on a 1.2% agarose gel 100v for25min.

2. When extracting DNA from tomato single seeds, following the extraction method, the following method was used The seed was crushed into a 1.5ml tube with a pestle, 100µl lysis buffer for seed was added to the voltex, spin down, Stored for 5 minutes in the actual operation, Centrifugation was performed for 5 minutes at 13,000 rpm, 50µl was put into the new tube; dilute it five times with TE buffer.

For PCR confirmation of transgenic plants was performed using DryMIX-ntaq (Enzynomics) and AccuPCR premix (Bioneer,Korea). This PCR reaction included 2µl genomic DNA, 0.5µl forward and reverse primer, 17µl DW: the PCR conditions were 94°c for 5 min, 94°c for 30sec, followed by 30cycles of 58°c for 30sec, 72°c for 30sec, final extension of 5min at 72°c. The PCR products were analyzed on a 1.2% agarose gel 100v for25min. Detected by band with ethidium bromide (EtBr).

Figure1. Construction of cuCYP71A1 over-expression and knock down vector with pCambia 2300 vector in plant.



Transformation into Micro-Tom tomato

LB left T-DNA border, CaMV 35S-pro, cauliflower mosaic virus 35S promoter, CYP71A1 gene, NOS-term, nopaline synthase terminator, CaMV S2 cauliflower mosaic virus 35S2 promoter, NPT- II neomycin phosphotransferase II,

2. Plant expression vector construction and production of transgenic tomato plants.

To express the Citrus Clementina *cuCYP71A1* gene in plants, the pCambia 2300 vector was chosen reconstructed for cloning. To perform this modification, the pCambia 2300 vector was digested with the BamHI enzyme at the site located between the CaMV 35S-promoter and NOS-terminator on T-DNA region and ligated into the target gene encoding *cuCYP71A1*.

The pCambia 2300-CYP71A1 construct was transformed into the Agrobacterium LB4404strain, and the Agrobacterium-mediated transformation method was used to produce transgenic tomato lines that were kanamycin-resistant and over-expressed and knockdown *cuCYP71A1* genes.

3. Identifying color difference from transgenic tomato homozygous different stage

Tomatoes (Lycopersicon esculentum cv. MT) were separated, according to the ripening stage, by a sensory panel into six groups, and color was measured on the tomato surface with a Minolta Chroma Meter CR-200 (Minolta Camera Co. Ltd., Osaka, Japan) tristimulus color analyzer, consisting of a head with an 8 mm diameter measuring area and a diffuse illumination/0° viewing, was used. The chroma meter was first calibrated with a white tile and checked for recalibration between measurements, although no adjustments were necessary. Readings are reported in the L*, a*, b* system. This study determined Immature green 100% green, mature green in color with lesser than 5% of immature green, turning lesser than 17% of immature green, orange between 26 and 50% and, red, orange-red between 56 and 90% of red, more than 92% red.

4. RNA isolation and cDNA synthesis

RNA extraction from selected homozygous transgenic Tomato fruit powder, Leaf, and Flower stored at –80℃ pestle with liquid nitrogen.

The extraction buffer is prepared and incubated in a water bath at +80℃ and inverted gently every 3min. This is carried out SDS is completely dissolved in the extraction buffer.

Samples are ground to a powder in liquid nitrogen using a prechilled pestle and mortar. About 0.1g of the still-frozen ground tissue is transferred into a 2-ml tube containing 900ml of hot extraction buffer, and the mixture is immediately shaken to suspend the powder evenly. Then all samples are vortexed for 45s prepare the sample powder 0.2g -and add RNA extraction buffer 600μl for each sample powder tube. To each tube 600μl water-saturated crystalline phenol (pH:4.30.2) and 400μl Chl:laa (24:1, v/v) are added, and then tubes are vortexed for 45s. The mixture is centrifuged at 14620rpm 4C for 10min. The aqueous phase is transferred into the new tube, and the phenol Chl:laa extraction is repeated. The aqueous phase is transferred into a new tube, and an equal volume of Chl:laa (24:1) is added, shaken, and

vortexed until the two phases form an emulsion; then, the tube is centrifuged as in step 3.

The aqueous phase is collected, and 1=3 volume ice-cold 8M LiCl is added. Tubes are inverted gently and placed at –80°C for 30min. Then tubes are allowed to thaw and centrifuged at 14620 for 30min. The supernatant is taken gently by a sampler and poured out. The pellet is dissolved in 200µl RNase-free water (DEPC), and then 0.1 volume 3M sodium acetate (pH 5.2) and 2volumes ice-cold absolute ethanol are added. Tubes are inverted gently and placed at –80C for 30min. The RNA pellet is collected by centrifugation at 14620 for 30min and then washed with 70% ice-cold ethanol, air dried, and dissolved in 20-30µl RNase-free water tubes are inverted gently and stored at –80°C.-Check-in Nucleic acid to Nano-drop.

-Check the RNA to Electrophoresis. 1.5% agarose gel (100v 25min)

5. cDNA synthesis and Gene expression analysis of transgenic plants using RT-PCR.

Total RNA extraction from (transgenic tomato) leaf, flower, and homozygous 6different stages and the concentration and purify of the RNA samples were determined by UV absorbance spectrophotometry (260nm/280nmration) and cDNA were performed as previously. The reverse transcription PCR reaction, which was performed with the 10µl pre-MIX ntaq, 0.5µl of forward and reverse primer, 3µl cDNA and DW to a 20µl final volume. The specific primer pairs used for amplification of *cuCYP71A1* gene included the forward primer 5'-CTT CTC AGT CGC TTG TAT GA -3', which was designed based on the *cuCYP71A1* cds and reverse primer 5'- ATG GCC TTG AAC TCC TGC-3', which belonged on the CDs too. Primers amplification of the reference gene (ACTIN) included tomato

The cDNA was synthesized from approximately 1µg RNA using a TOPscript RT Drymix (dT 18plus) tube. Template RNA Xµl (calculate to 1µg) RNAse free water (DEPC) up to volume 20µl. PCR condition is incubating at 42°C for 5minutes, Incubate at 60°C for 60minutes, Incubate at 94°C for 5minutes to inactivating the reaction. The cDNA used dilute 10more was then stored at -20°C.

Gene expression analysis of other related CYPgenes transgenic tomato plants using RT-PCR .

Total RNA extraction from (transgenic tomato) leaf, flower, and homozygous 6different stage and cDNA were performed as previously. The reverse transcription PCR reaction, which was performed with the 10µl pre-MIX ntaq, 0.5µl of forward and reverse primer, 3µl cDNA and DW up to a 20µl final volume. Primers amplification of the reference gene (ACTIN) included tomato.

The cDNA was synthesized from approximately 1µg RNA using a TOPscript RT Drymix (dT 18plus) tube. Template RNA Xµl (calculate to 1µg) RNAse free water (DEPC) up to volume 20µl. PCR condition is incubated at 42℃ for 5minutes, Incubate at 60℃ for 60minutes, Incubate at 94℃ for 5minutes to inactivating the reaction. The cDNA used dilute 10more was then stored at -20℃.

6. Analysis of Total Flavonoid Content (TFC)

The TFC of plant extracts was determined with a colorimetric aluminum chloride method as described by Herald et al. [32]. Briefly, 25 µL of each plant extract (5 mg/mL) and standard were added in a 96-well micro-plate and then mixed with 100 µL of distilled water and 10 µL of 50 g/L $NaNO_2$. After incubation for 5 min at room temperature, 15 µL of 100 g/L $AlCl_3$ was added to each assay well and then further incubated for 6 min at room temperature. After that, 50 µL of 1N NaOH and 50 µL of distilled water were added to each well, and absorbance was measured by a micro-plate reader at a wavelength of 510 nm. Catechin was used as a standard at 5–100 µg/mL to generate a calibration curve. All assays were performed in triplicate. The results were expressed as milligrams of catechin equivalent per gram of the dried plant extract.

7. Analysis of Total Polyphenol content:

The TPC of plant extracts was determined with a modified Folin–Ciocalteu method [32]. Twenty microliters of the plant samples (5 mg/mL), standard, and blank (DMSO) were added in a 96-well micro-plate and then mixed with a 10% (v/v) Folin–Ciocalteu reagent (100 µL). After incubation for 5 min at room temperature, a 7.5% (w/v) sodium carbonate solution (80 µL) was added to each assay well and was allowed to react for 60 min at 25 ◦C in the dark. Absorbance was measured by a micro-plate reader at the wavelength of 750 nm. Gallic acid was used as a standard at 12.5–400 µg/mL to generate a calibration curve (Figure S1A). All assays were performed in triplicate. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the dried plant extract.

8. DPPH Radical Scavenging and Ferric Reducing Anti-Oxidant Power (FRAP) Activity Assay

Free radical scavenging activity of the extracts was determined by a stable 2,2-diphenyl–1 picrylhydrazyl (DPPH) assay according to the method of Herald et al. [32]. Catechin was used as a standard control at 0.05–0.4 mg/mL. The results were expressed as the DPPH radical scavenging activity (%) of the dried plant extract. The FRAP assay was performed according to the manufacturer's directions in the FRAP assay kit (Abcam). Each assay was performed in triplicate. The results were expressed as µmol of the FRAP value per gram of the dried plant extract.

Methanol (MeOH) was purchased from Fisher Scientific (Seoul, Korea). Other chemicals are as follows: 2,2-diphenyl–1 picrylhydrazyl (DPPH), a Folin–Ciocalteu reagent, sodium carbonate, NaNO2, AlCl3, NaOH, catechin, gallic acid, and dimethyl sulfoxide (DMSO); these were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation

For the measurement of anti-elastase and anti-collagenase activity, 50 mg of lyophilized powder of each sample was mixed with 1 mL of 80% (v/v) MeOH, shaken for 72 h in the dark, and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a tube, and the residue was washed with 0.5 mL 80% (v/v) MeOH. Both supernatants were combined and filtered through a 0.45-μm syringe filter. The filtrate was removed by a centrifugal evaporator (CVE-3000, EYELA, Tokyo, Japan) and then dissolved in DMSO to a concentration of 100 mg/mL. These extracts were diluted to 10 mg/mL concentration with DMSO and stored at −20 °C until the use.

9 . HPLC analysis

Flavonoids are large family of polyphenolic compounds synthesized by plants and structurally derived from the parent substance flavone. Flavonoids present in fruits and leafy vegetables are thought to provide potential and versatile health benefits through radical scavenging and antioxidant activity.

HPLC analysis were performed with Quantitative analyses of four flavonoids **(**naringin, quercetin, myrcetin, luteolin) in the extracts were performed using a high-performance liquid chromatography system (SHIMADZU CTO-20A). Chromatographic separations were accomplished using a C18column (5 μm, 4.6 × 250 mm; GL Science, Tokyo, Japan). A pump was 0.1% formic acid and B pump was Acetonitrile (ACN). The flow rate was 1.0 mL/min and the column temperature was 35 °C. The injection volume was 20μL and the detection was performed at wavelengths of 254 and 270 nm. The chromatographic peaks of flavonoids were identified by comparing their UV–visible spectra and retention times to those of each standard compound, and quantified based on the calibration curves of standard compounds (Amaretti

et al., 2015; Kim and Lim, 2019)

Sample preparation : For analysis of flavonoids (caffeic acid**,** naringin, quercetin, luteolin, rutin, naringein, kaempferol, myrcetin) in the fruit development stage of transgenic tomato homozygous plant species (Leaf, flower, immature green, mature green, turning, orange, orange red, red) each 20g of fruits from transgenic species was extracted with 80% MeOH and evaporated in speedVac vacuum concentrator for 24hour. The residue was dissolved in 1ml of DMSO and filtered. The resulting solution was used for HPLC analysis

HPLC conditions:HPLC separation of flavonoids for quantitative analysis was performed using

Table1. Condition of HPLC for flavonoid.

Time	**Module**	**Action**	**Value**
0.01	**Pumps**	**B conc**	**1**
28	**Pumps**	**B conc**	**30**
33	**Pumps**	**B conc**	**30**
38	**Pumps**	**B conc**	**80**
43	**Pumps**	**B conc**	**30**
48	**Pumps**	**B conc**	**1**
60	**Pumps**	**B conc**	**1**
60	**Controller**	**Stop**	

A pump: 0.1% Formic acid

B pump: Acetonitrile (ACN)

Column: C18 column (250x4.6nm)

Figure2. Blast search of citrus CYP71A1 gene.

허지만 pCambia2300-S-F3M sequence : F3M이 아닌 것 같음.

NCBI Blast : PREDICTED: Citrus clementina cytochrome P450 71A1 (LOC18036422), mRNA

caaagacttgtgttggtcactctctTACTATAAATACCCACCAGCagcacttgtaactacacaccataatcaacttgaaa
gtaacactccaatacactaaagctagatttacaacaATGGAGCTCATTCATCCCTCTTGGGTTTCCTACTTGACCGCTTG
GCTAGCAACCCTTGCCCTCGTCCTCCTCTCCGTTCGTCTCAGGCGCCGGAAACTCAACTTGCCGCCGGGTCCAAAACCCT
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CTTATGGACCATACTGGCGTCAGGCCCGTAAAATGTGTTTAATGGAATTGTTTAGTGCCAAACGCTCGATCATTCGAGTA
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CACCGCAGGAGTTCAAGGCCATGCTTGATGAATTGTTCCTGCTTAATGGGGTTTTAGATATCGGCGATTCGATTCCTTGG
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GCTGGATGAACATAACGCTAGAAGAAAAGGGGTCGAAAATTATGTGGCTAAAGATATGGTGGATGTGCTTTTGCAGCTTG
CTGATGATCCTTCTCTTGAAGTCAAGATTGAAAGACATGGTGTCAAAGCTTTTACACAGGACTTAATTGCTGGTGGGACT
GAGAGCTCAGCAGTAACAGTGGAATGGGCCATCTCTGAACTAATAAAAAAACCCGAAATCTTTGAGAAAGCAACAGAGGA
ACTAGATAGAGTAATTGGAAAAGAAAGGTGGGTTGAAGAAAAAGACATCGTTAATCTCCCATTTATTGATGCCATAGTTA
AAGAAACAATGCGTTTGCACCCTGTGGCTCCTTTCCTAGTGCCCCGAGCGGCTAGAGAAGATTGCAACGTAGCCGGCTAC
GACATCCCAAAAGGCACTCGAGTCCTTGTTAGTGTATGGGCGATTGGGAGAGACCCAAGTTTGTGGGAAAAGCCAAATGA
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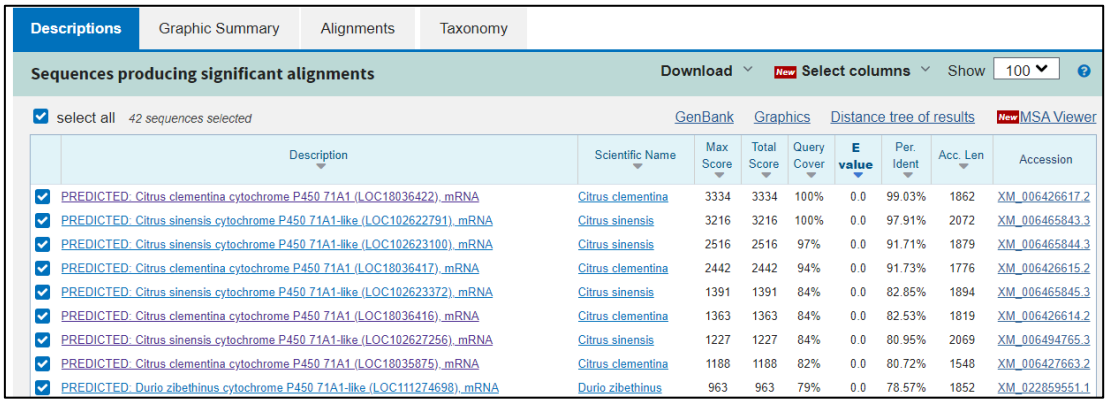
aaaaatcctcgtacaa



Descriptions | Graphic Summary | Alignments | Taxonomy

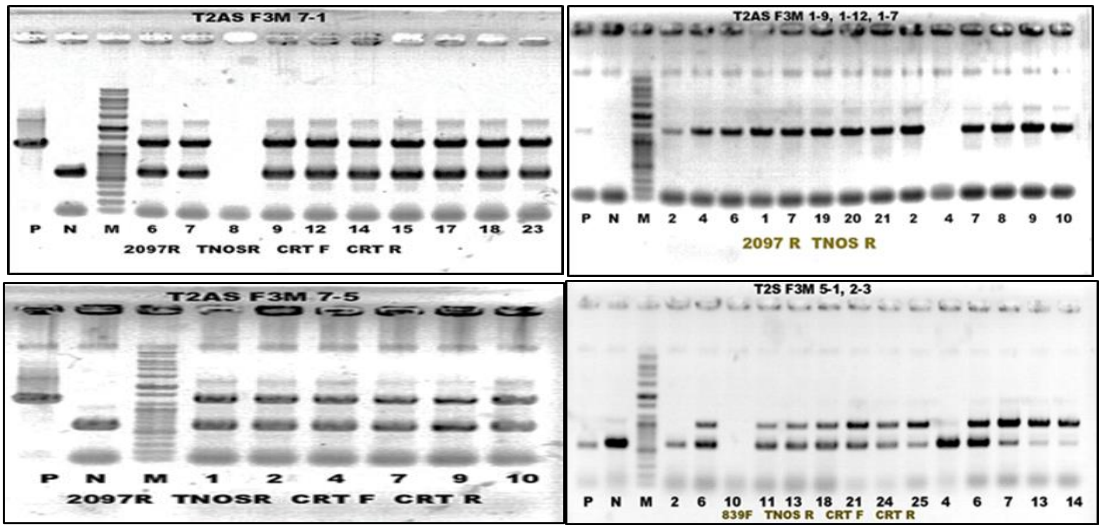
Sequences producing significant alignments — Download | New Select columns | Show 100

select all 42 sequences selected — GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
PREDICTED: Citrus clementina cytochrome P450 71A1 (LOC18036422), mRNA	Citrus clementina	3334	3334	100%	0.0	99.03%	1862	XM_006426617.2
PREDICTED: Citrus sinensis cytochrome P450 71A1-like (LOC102622791), mRNA	Citrus sinensis	3216	3216	100%	0.0	97.91%	2072	XM_006465843.3
PREDICTED: Citrus sinensis cytochrome P450 71A1-like (LOC102623100), mRNA	Citrus sinensis	2516	2516	97%	0.0	91.71%	1879	XM_006465844.3
PREDICTED: Citrus clementina cytochrome P450 71A1 (LOC18036417), mRNA	Citrus clementina	2442	2442	94%	0.0	91.73%	1776	XM_006426615.2
PREDICTED: Citrus sinensis cytochrome P450 71A1-like (LOC102623372), mRNA	Citrus sinensis	1391	1391	84%	0.0	82.85%	1894	XM_006465845.3
PREDICTED: Citrus clementina cytochrome P450 71A1 (LOC18036416), mRNA	Citrus clementina	1363	1363	84%	0.0	82.53%	1819	XM_006426614.2
PREDICTED: Citrus sinensis cytochrome P450 71A1-like (LOC102627256), mRNA	Citrus sinensis	1227	1227	84%	0.0	80.95%	2069	XM_006494765.3
PREDICTED: Citrus clementina cytochrome P450 71A1 (LOC18035875), mRNA	Citrus clementina	1188	1188	82%	0.0	80.72%	1548	XM_006427663.2
PREDICTED: Durio zibethinus cytochrome P450 71A1-like (LOC111274698), mRNA	Durio zibethinus	963	963	79%	0.0	78.57%	1852	XM_022859551.1

III. Results and Discussion

Figure3.genomicPCR analysis : gDNA extraction from young leaf



We check the gDNA to PCR and electrophoresis 1.2% agarose gel to 100wt for 25minutes. Detected by a band with ethidium bromide (EtBr).

Figure4a. genomicPCR analysis; Genomic DNA extraction from transgenic tomato single seed. (Anti-Sense)

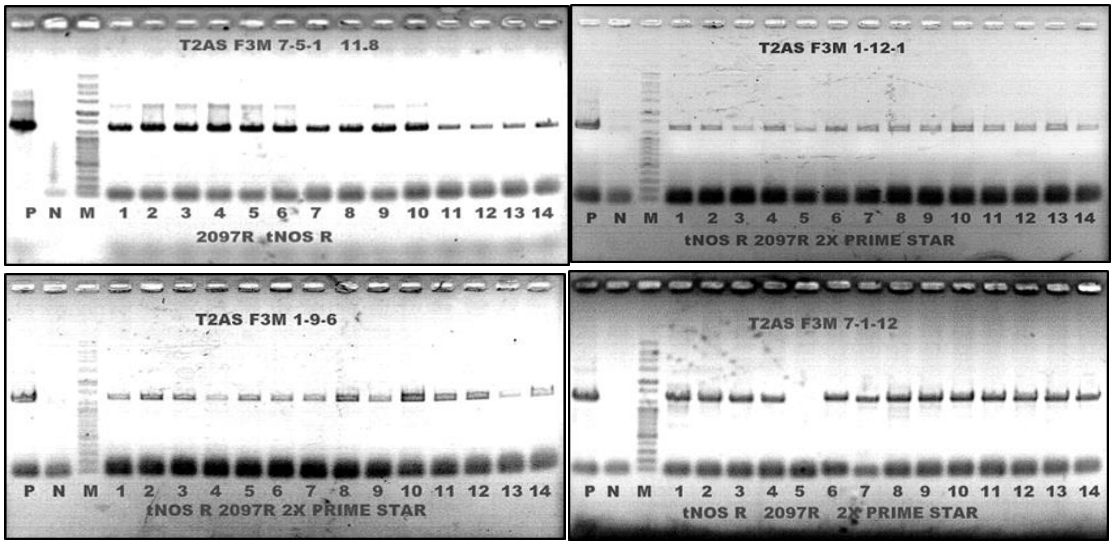


Figure4. genomicPCR analysis Genomic DNA extraction from a transgenic tomato single

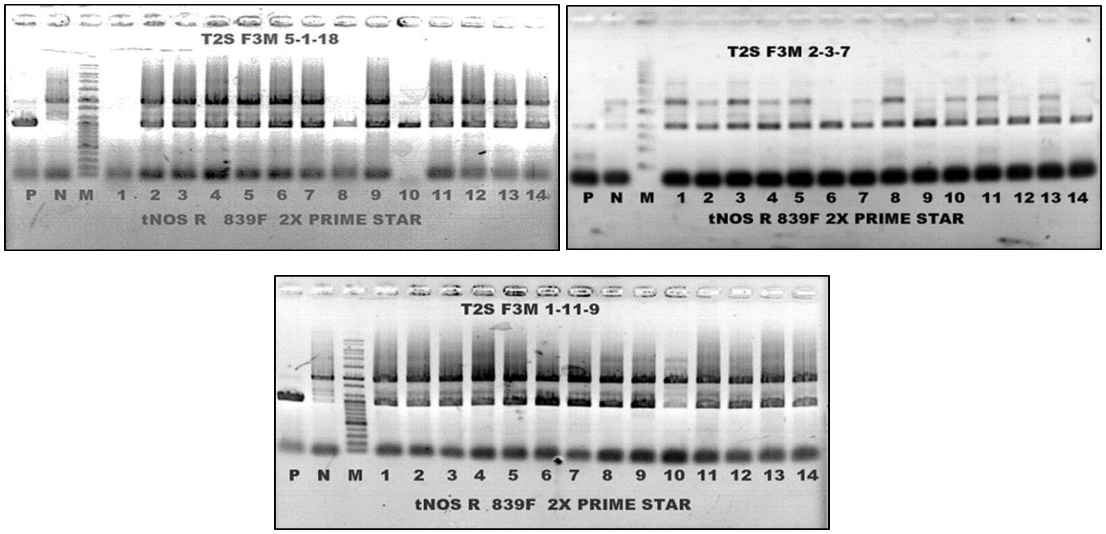
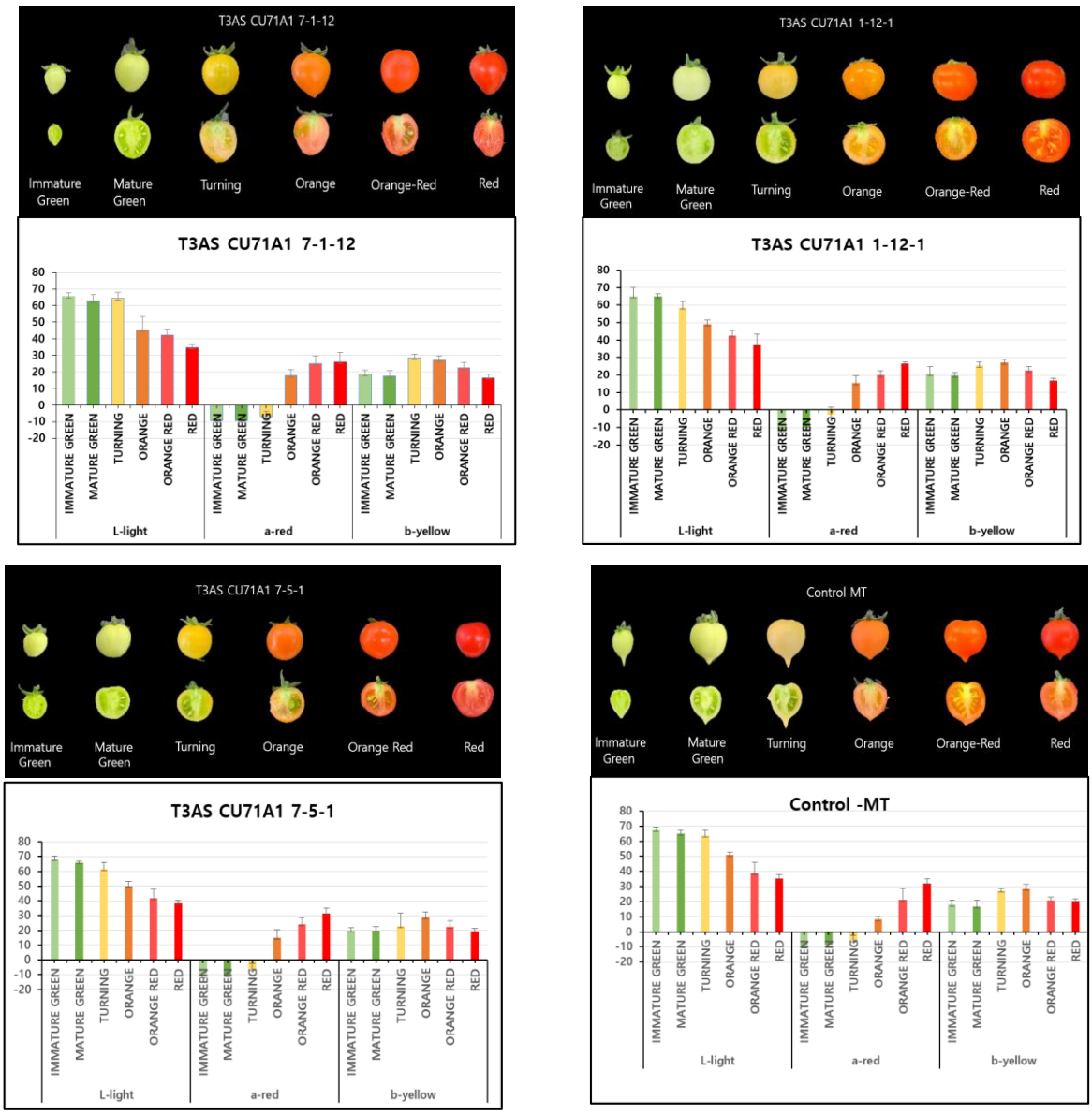
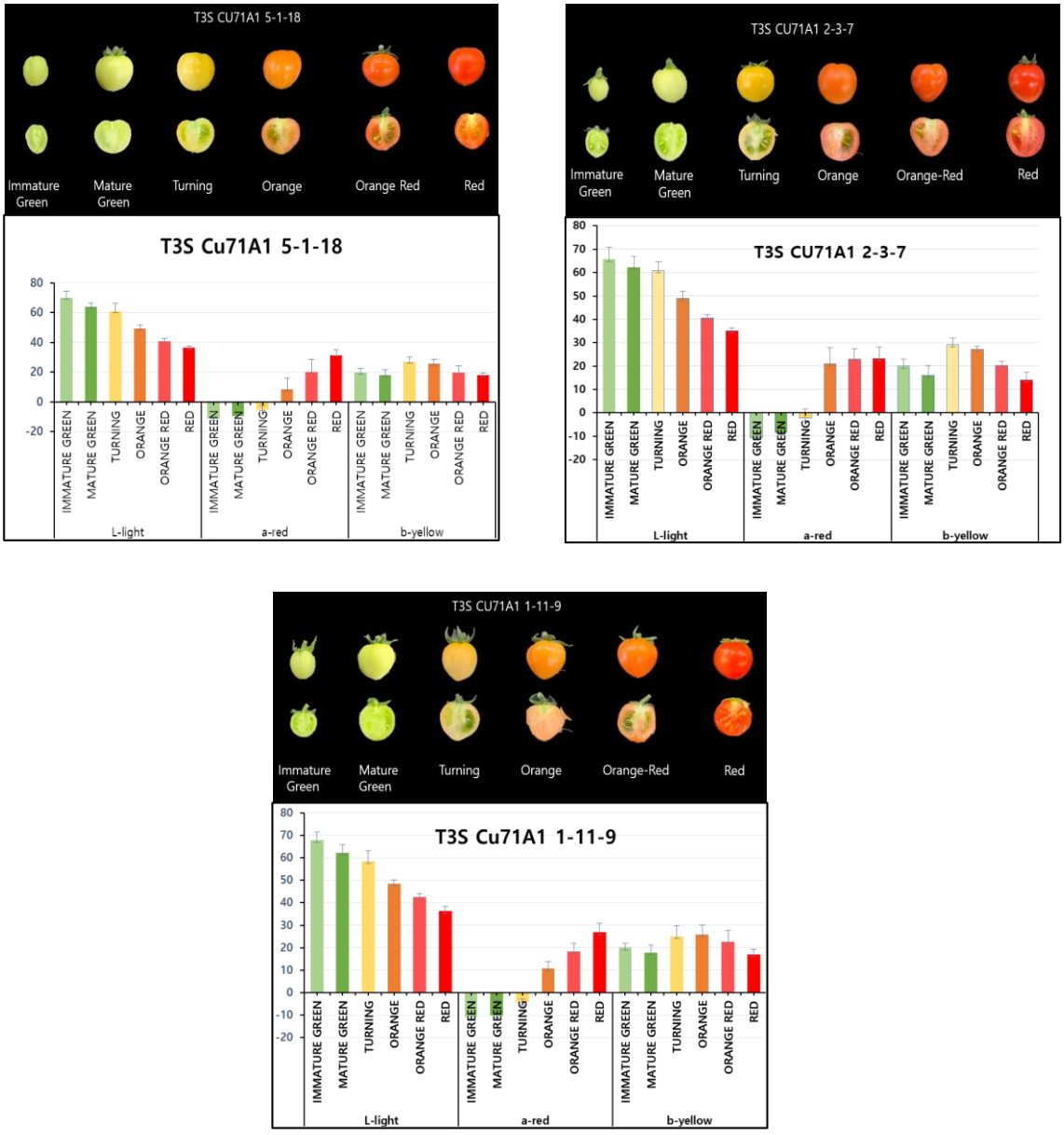


Figure5a. Identifying color difference from tomato homozygous different stage (AS,MT)



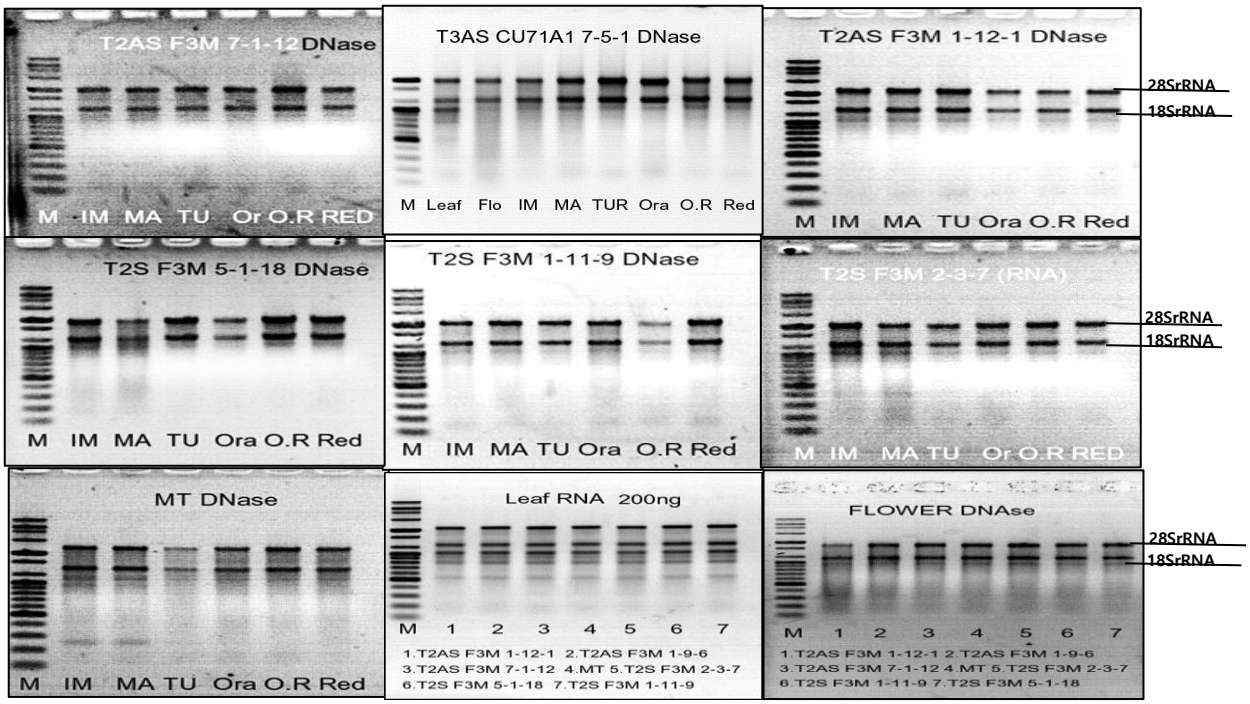
L phase is light, a phase is red, b phase is yellow indicated in tomato color developing stages.

Figure6. Identifying color difference from tomato homozygous different stage (Sense)



L phase is light, a phase is red, b phase is yellow indicated in tomato color developing stages.

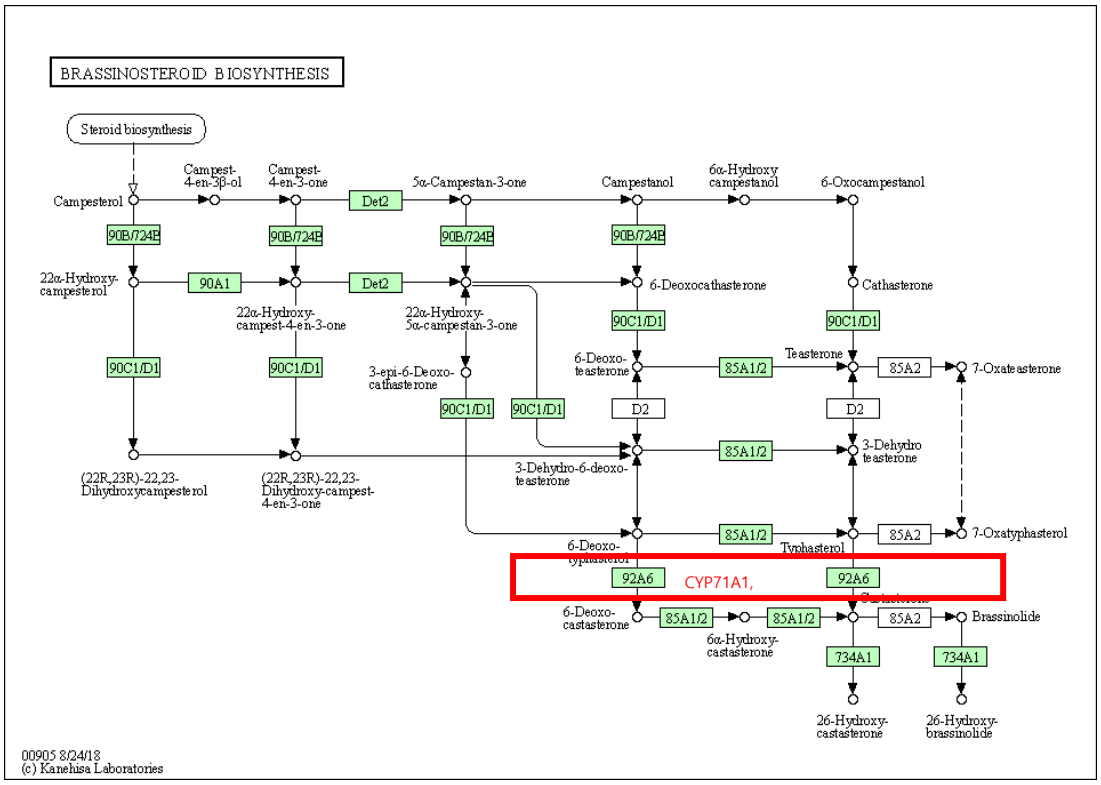
Figure8. RNA extraction from transgenic tomato homozygous line leaf, flower, 6different stage



Flo- flower, IM- immature green, MA-mature green, TUR-turning, Ora-orange, O.R-orange red Red-red

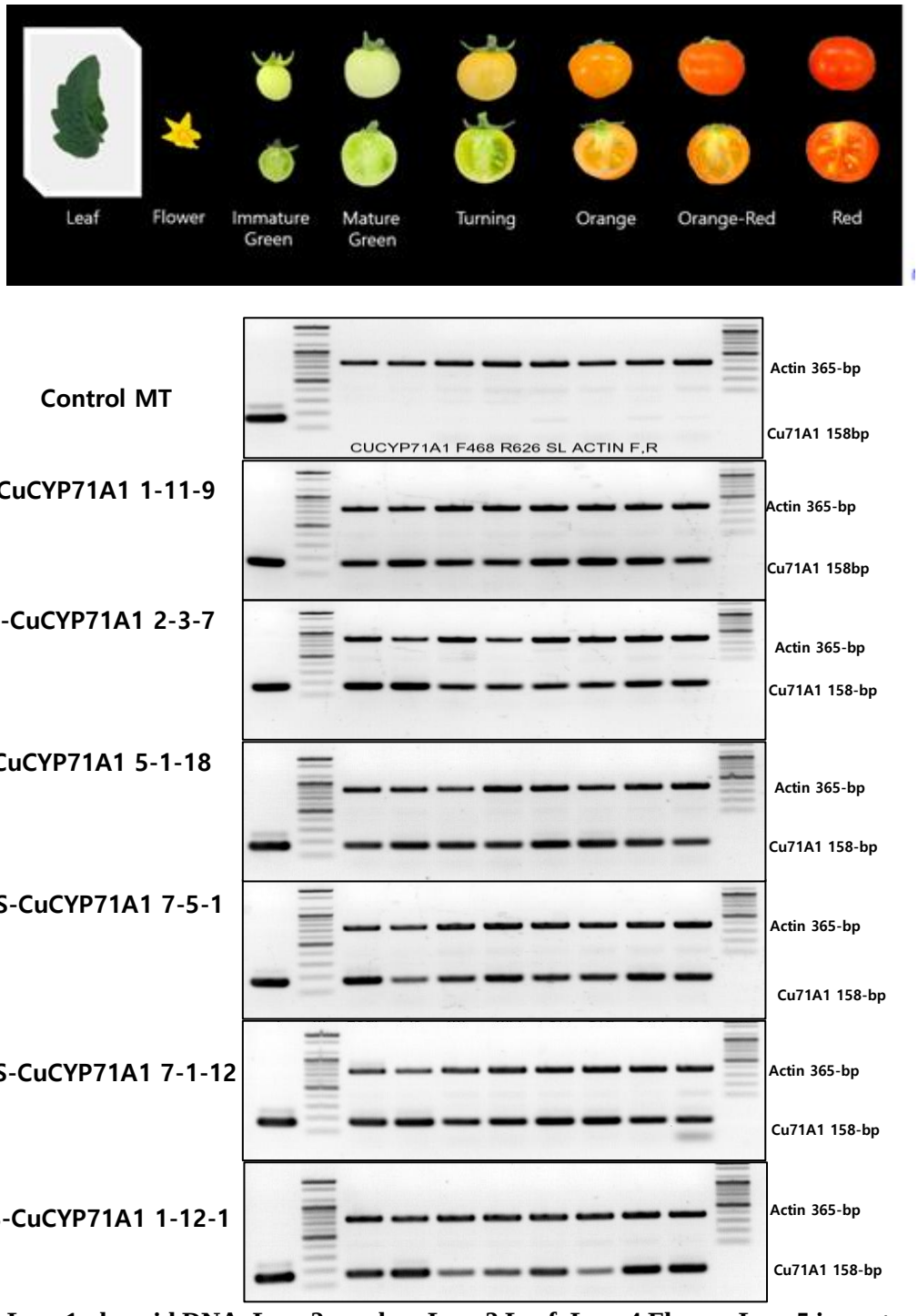
Figure8. KEGG biosynthesis pathway (Citrus clementina)

Brassinosteroid biosynthesis – Citrus clementina (Orange)



KEGG Brassinosteroid pathway of citrus clementina. We researched CYP71A1 gene of this pathway.

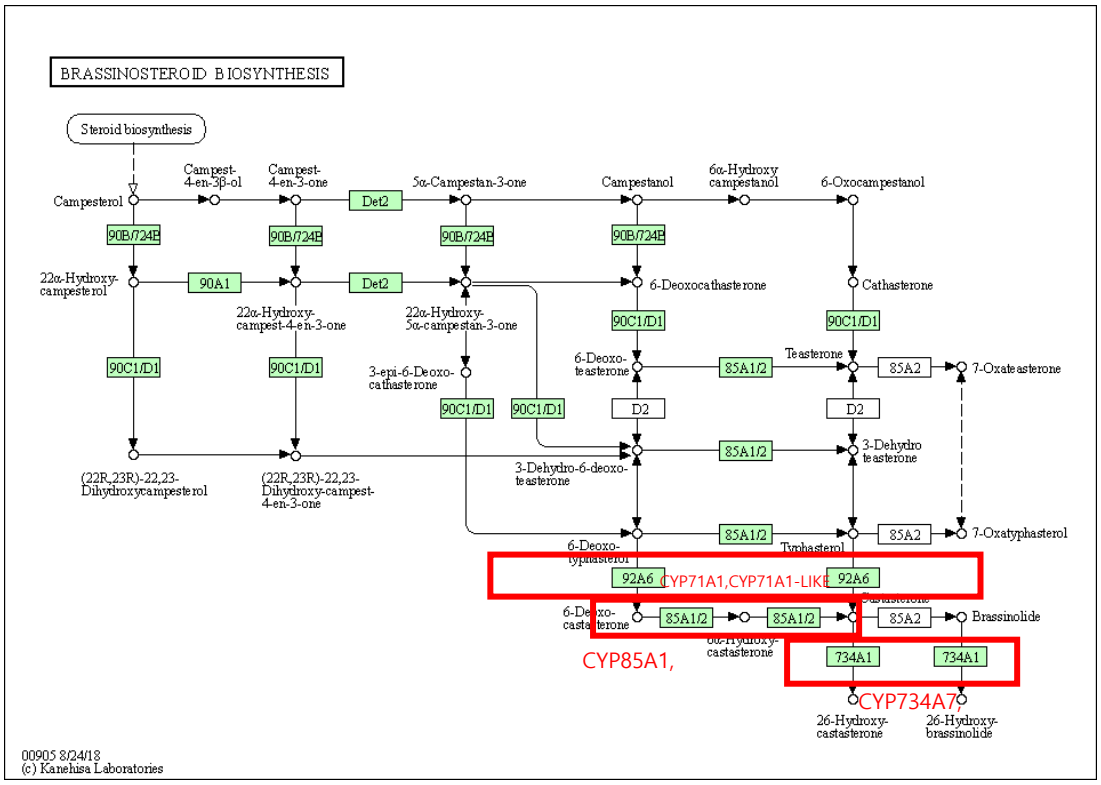
Figure9. GENE expression of CUCYP71A1 gene using transgenic tomato plant**.**



Lane1 plasmid DNA, Lane2 marker, Lane3 Leaf, Lane4 Flower, Lane5 immature green, Lane6 mature green, lane7 turning, Lane8 Orange , Lane9 Orange red, Lane10 Red-

Figure10. KEGG biosynthesis pathway (Solanium lycopersicum) Tomato

Brassinosteroid biosynthesis – (Solanium lycopersicum) Tomato



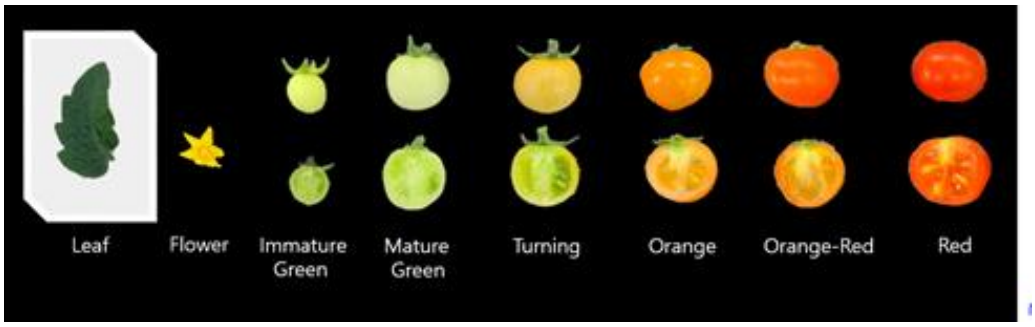
All gene's was confirmed by sequencing and NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST).

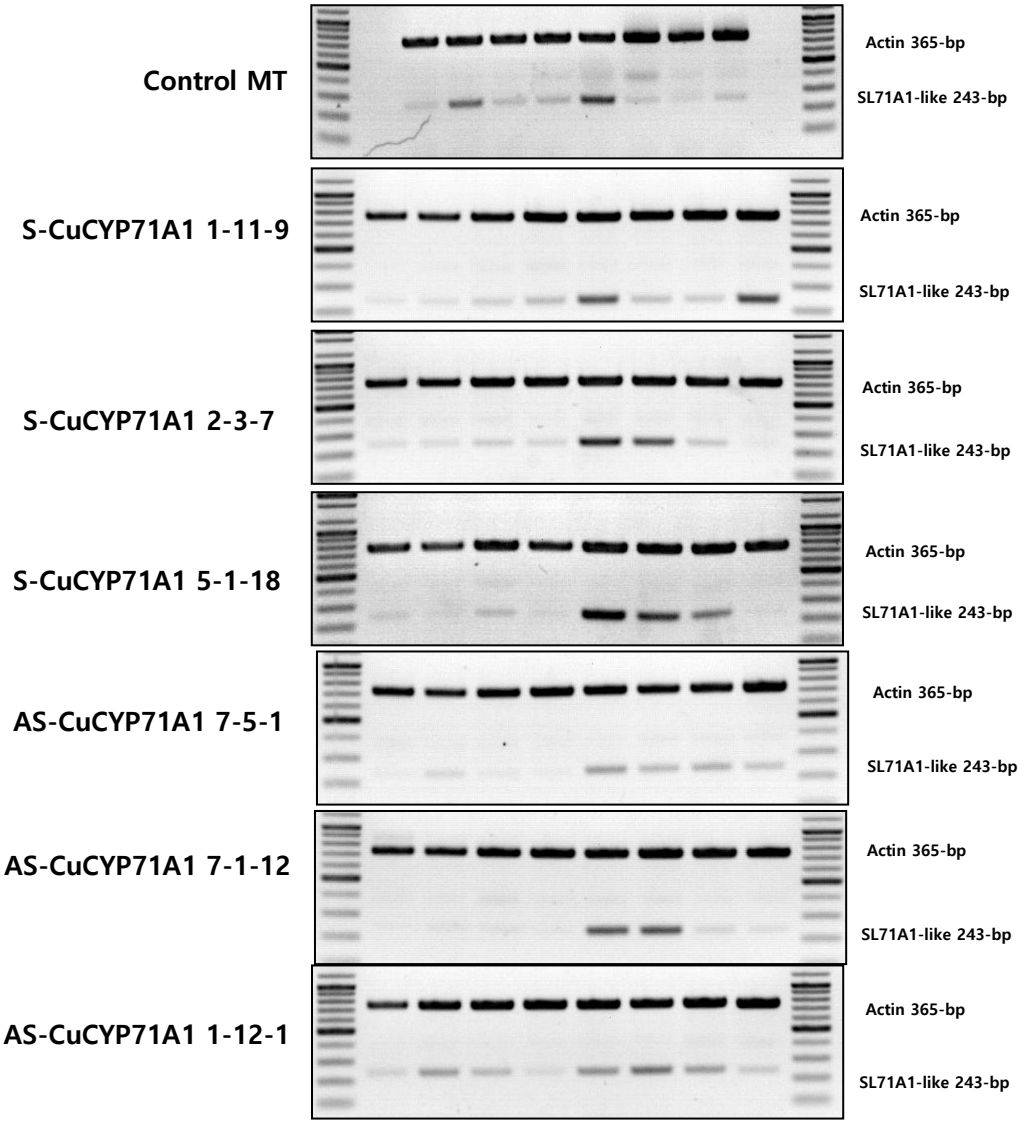
Table2. RT-PCR primer used in this study related of CYP450.

Primer name	Primer sequence	Product size
Cu CYP71A1 F468	CTT CTC AGT CGC TTG TAT GA	158bp
Cu CYP71A1 R626	ATG GCC TTG AAC TCC TGC	
SL CYP71A1-1 F1318	ATG TGC CCT GGA TAC AGC TT	234bp
SL CYP71A1-1 R1552	CAG GGC ATC AAG TCA AAC AA	
SL CYP71A1-2 F14	CCT GGA CTG CAA CTG CAG TG	222bp
SL CYP71A1-2 F236	CCAACT ACG ACG GGA AAA GA	
SL CYP85A1 F345	TGC AGC TGT CAA TGG TTC AG	218bp
SL CYP85A1 R563	GAG GTA GAT TCA ATA CCA GC	
SL CYP85A3 F301	GGC TAC CCA CAG TCC ATG TT	266bp
SL CYP85A3 R566	GAG CTT GTT GCA AAC CCA CC	
SL CYP734A7 F1099	CTG ACG TGG ACC ACC ATT TT	187bp
SL CYP734A7 R1285	GTC TAA TTG CTG CCA CTG CT	
SL CYP734A8 F949	TTG GGC CTT ATG ATC CAA GC	286bp
SL CYP734A8 R1234	ACA CTT TGA TCA CCT CGT CG	
SL ACTIN F	CTG CGG GTC TCC ATG AGA CT	368bp
SL ACTIN R	CGGGAA ACA GAC AGG ACA CT	

All gene's was confirmed by sequencing and NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST).

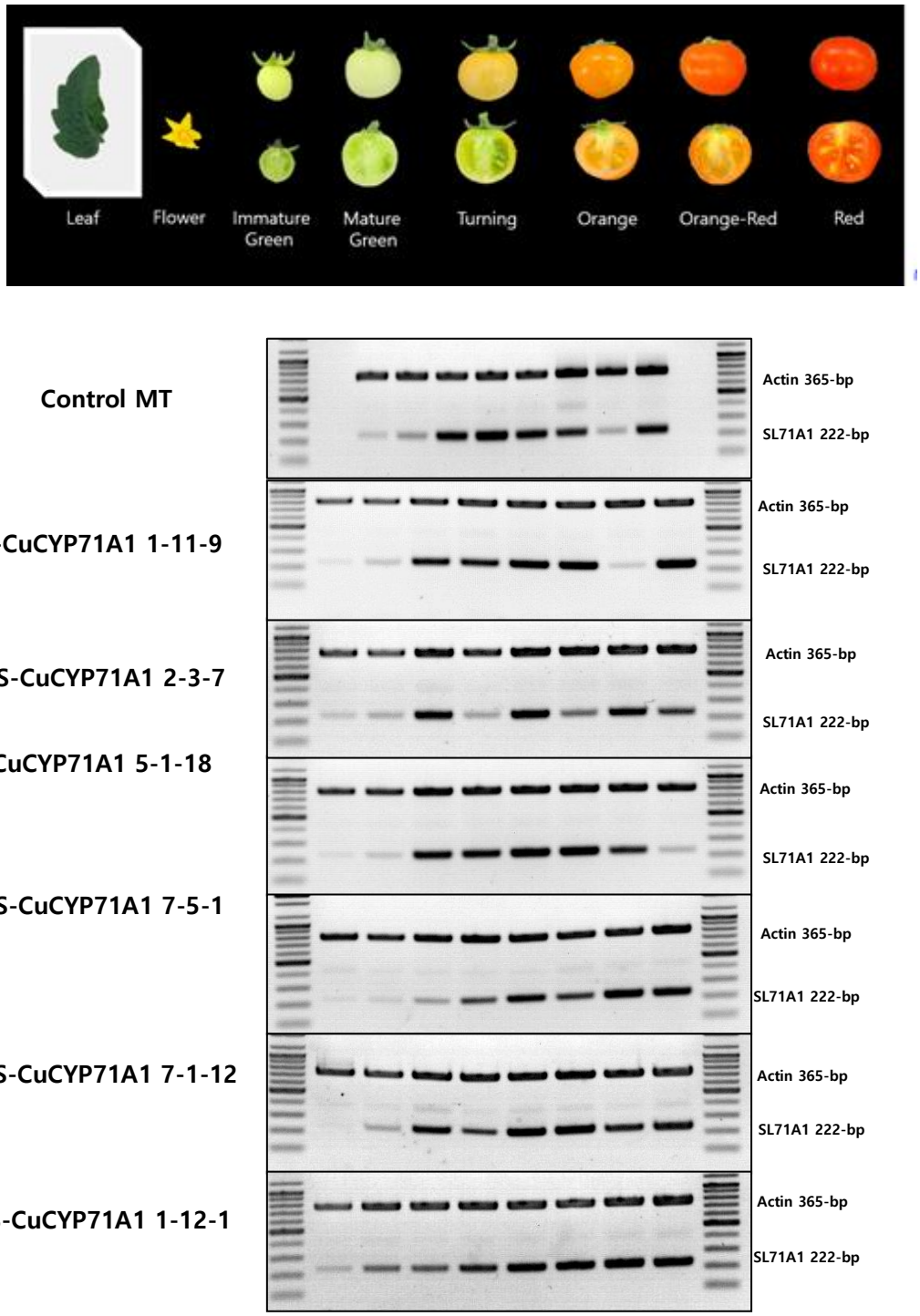
Figure11. GENE expression of SLCYP71A1-like gene using transgenic tomato plant.





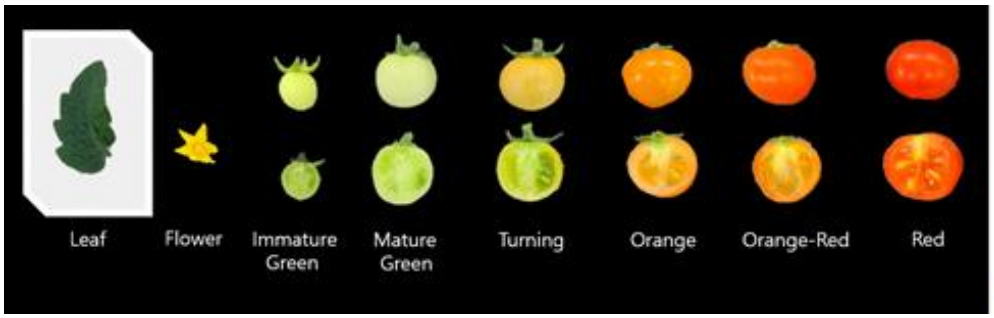
Lane1 marker, Lane2 Leaf, Lane3 Flower, Lane4 immature green, Lane5 mature green, lane6 turning, Lane7 Orange , Lane8 Orange red, Lane9 Red-

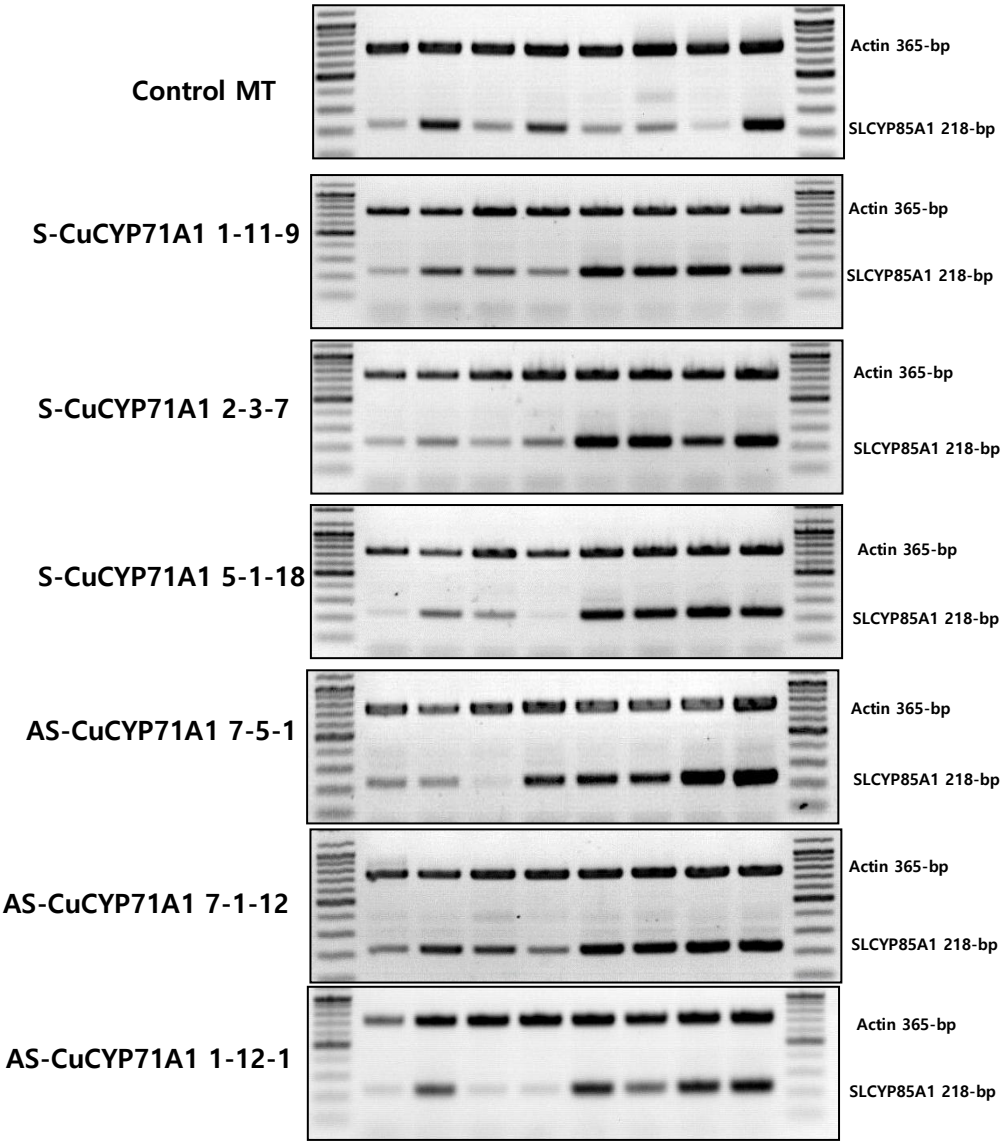
Figure12. GENE expression of SLCYP71A1 gene using transgenic tomato plant.



Lane1 marker, Lane2 Leaf, Lane3 Flower, Lane4 immature green, Lane5 mature green, lane6 turning, Lane7 Orange , Lane8 Orange red, Lane9 Red-

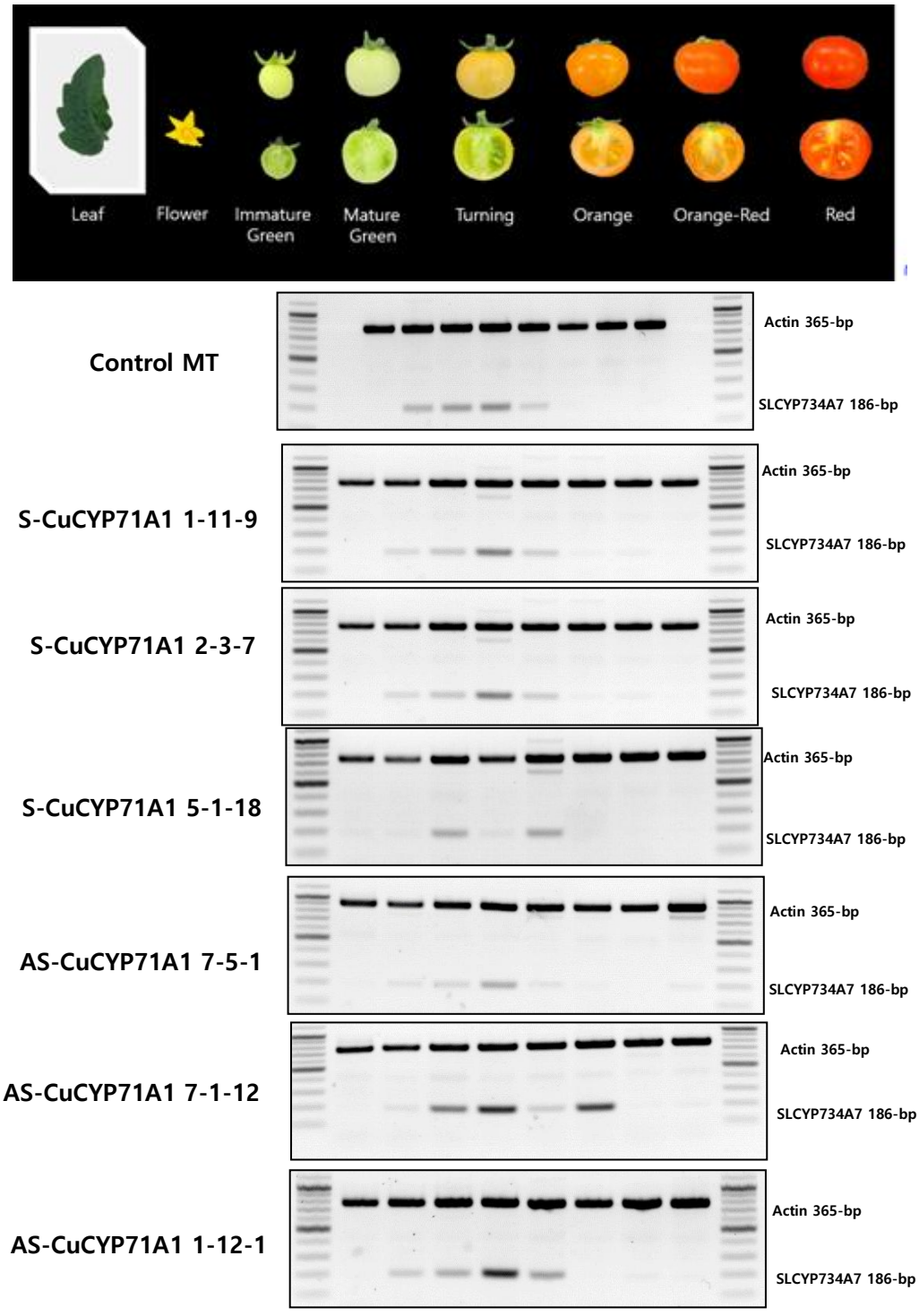
Figure13. GENE expression of SLCYP85A1 gene using transgenic tomato plant.





Lane1 marker, Lane2 Leaf, Lane3 Flower, Lane4 immature green, Lane5 mature green, lane6 turning, Lane7 Orange , Lane8 Orange red, Lane9 Red-

Figure14. GENE expression of SLCYP734A7 gene using transgenic tomato plant.

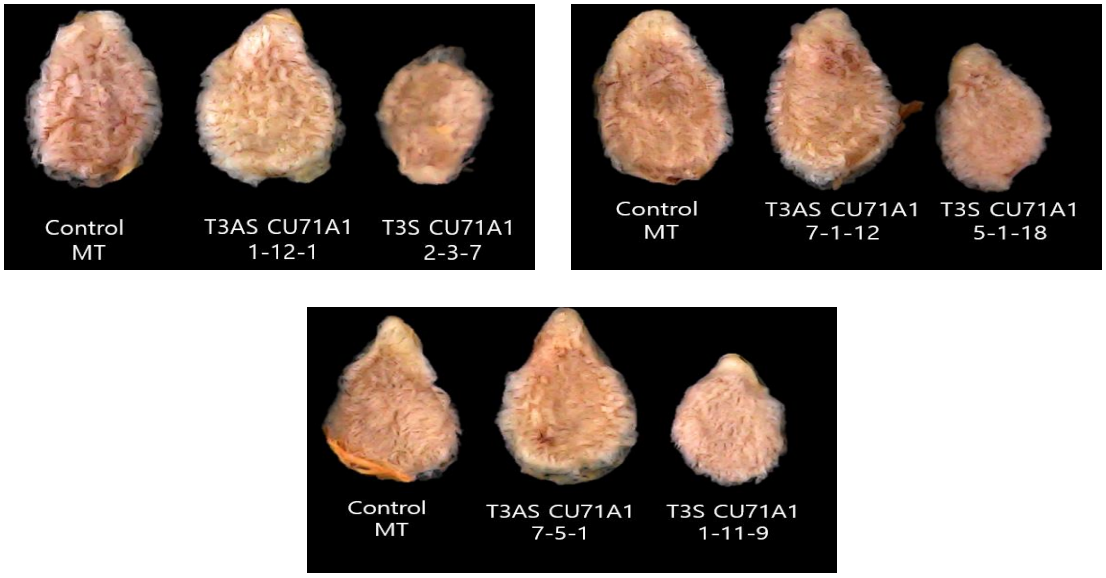


Lane1 marker, Lane2 Leaf, Lane3 Flower, Lane4 immature green, Lane5 mature green, lane6 turning, Lane7 Orange , Lane8 Orange red, Lane9 Red-

9 Seed development of transgenic tomato plants.

Seed area measurement for transgenic tomato 100dry seeds from each genotype plants were spread on the single white paper sheet and scanned as images. Seed areas were measured and calculate with grain scanner.

Figure15. Overexpression of *CuCYP71A1* gene affects seed production



1.90day old plants of wild-type Control MT 2.Knockdown of *CuCYP71A1* gene T3AS CuCYP71A1 7-1-12 line transgenic tomato plant 3.Overexpression of CuCYP71A1 gene T3S CuCYP71A1 5-1-18 line transgenic tomato plant.

(The picture was captured using a digital microscope)

Table3a Transgenic tomato plants seed size of using gram scanner program

Seed size results of grain scanner.

Line	area_mm2	perimeter_mm (둘레)	majellipse_mm (횡경)	minellipse_mm (종경)
Mic-Tom	6.548350069	12.13594234	3.590083862	2.33945531
AS-7-5-1	6.527060382	12.10151878	3.538327481	2.36935374
AS-7-1-12	6.436248585	12.15445604	3.627785472	2.28852066
AS-1-12-1	6.737272162	12.28696486	3.638701216	2.388073649
S-1-11-9	2.35223	7.227859649	2.093569649	1.448401754
S-2-3-7	2.436585579	7.444071579	2.211908211	1.415286884
S-5-1-18	2.530238788	7.588442424	2.252127576	1.445843182

Table3b. Transgenic tomato seed size results of area and perimeter.

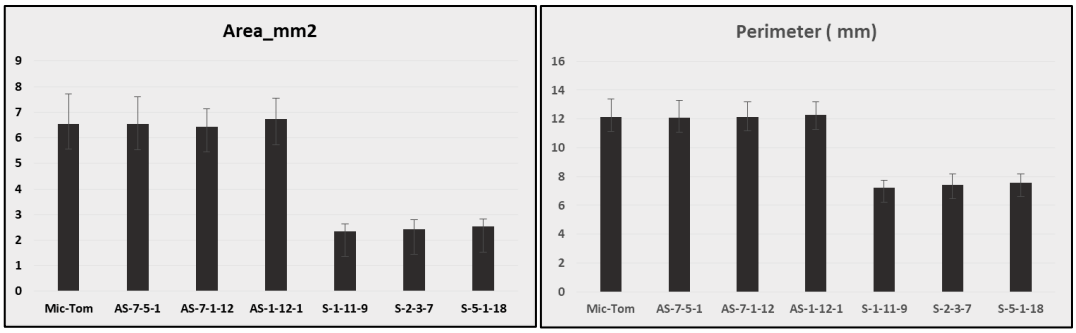


Table3c. Transgenic tomato Seed size results of majellipse and minellipse. (height and breadth).

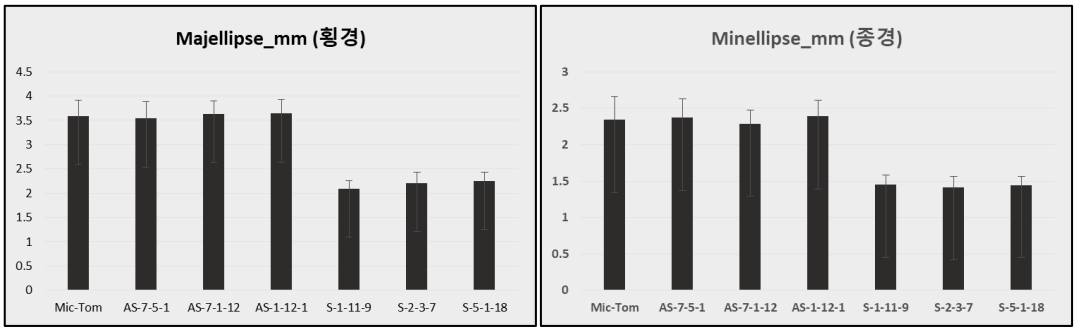
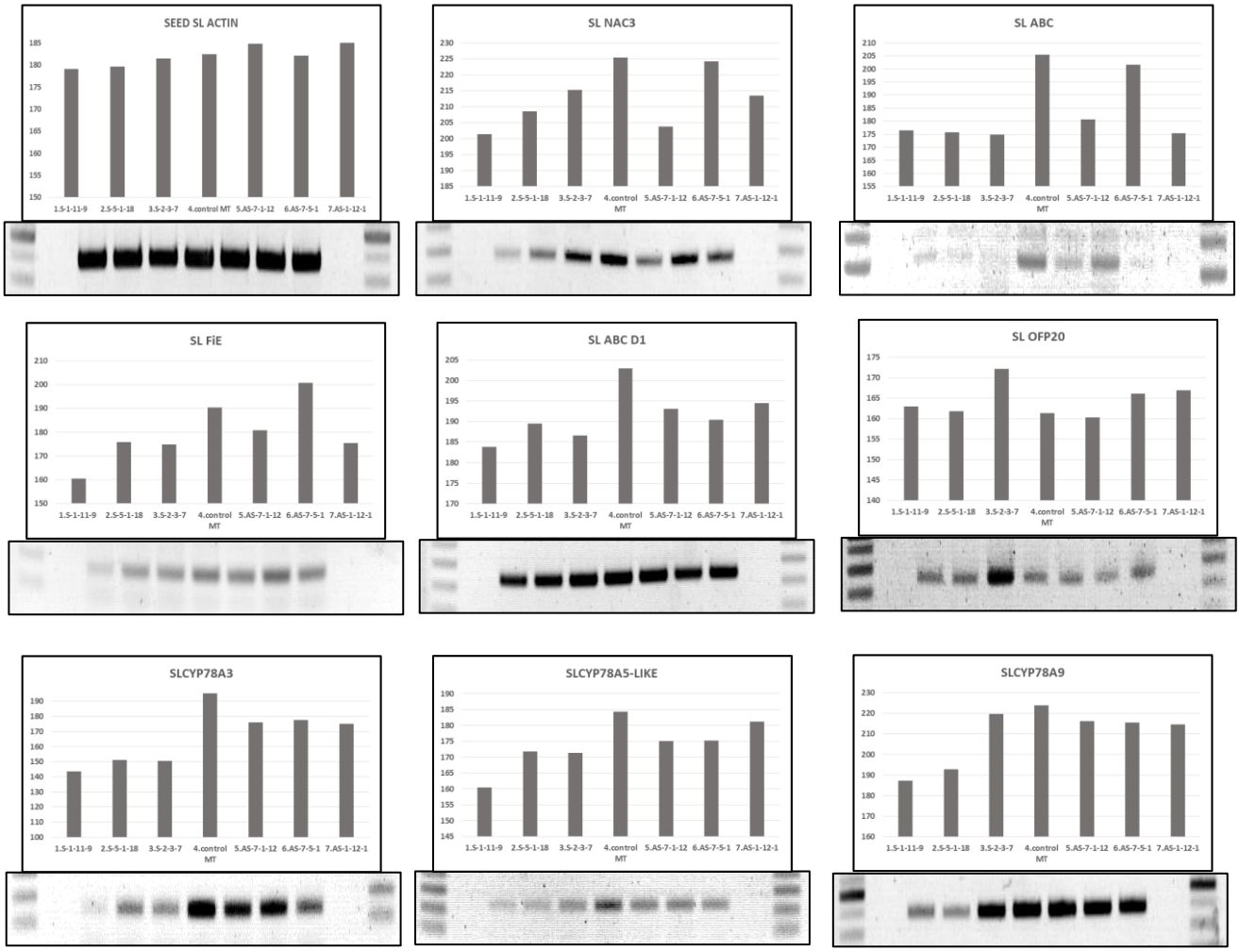


Table4. RT-PCR primer used in this study related of seed gene expression

Primer name	Primer sequence	Product size
SL CYP78A7 F1200	ACT TTT ATC GTG GGC CCG TT	132bp
SL CYP78A7 R1331	GAC GGG TTA TCC CAC ACG TT	
SL CYP78A5-like F136	AAA GCT CGC GTC TCC ATT CC	212bp
SL CYP78A5-like R347	GCA AAA GCC GAG CTA CCA AG	
SL CYP78A3 F1239	AAC GGT GGA TGG GTA TCA CG	204bp
SL CYP78A3 R1442	AGT GTT TTA CCG GGG CAA GT	
SL CYP78A9 F448	GTG GTC CAG CTT GGG GTA AA	380bp
SL CYP78A9 R827	CAG GAC GCG CAA CAA AAG AT	
SL FIE F958	GAA GTA CAA ACA AGC CCG CC	153bp
SL FIE R1110	TGT TGC TAC CAC ATC CCA GC	
SL ABC F194	GGC TCA TAA GGA GCA TGG CA	325bp
SL ABC R518	CCC ATG CCT AAG CCA TTT GC	
SL NAC3 F10	ACC GAT TCA TCA ACC GGC TC	272bp
SL NAC3 R284	GAA GTT GCC GCC CTA TTT GG	
SL ABC D F4243	GGG AAG ACG AAC CAA ATGAA	203bp
SL ABC D R4446	CGG GAA CTA GGC GCT ATA CA	
SL OFP20 F241	AAC GTC CAC ATC ACT GAG CC	243bp
SL OFP20 R483	ACA GTC GCA AGA CGG TGA AA	

All gene's was confirmed by sequencing and NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST).

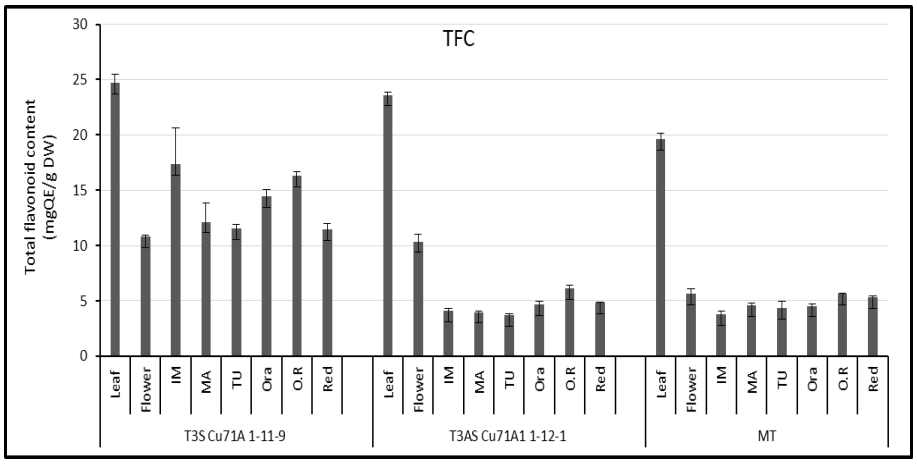
Figure16. RT-PCR analysis of seeds

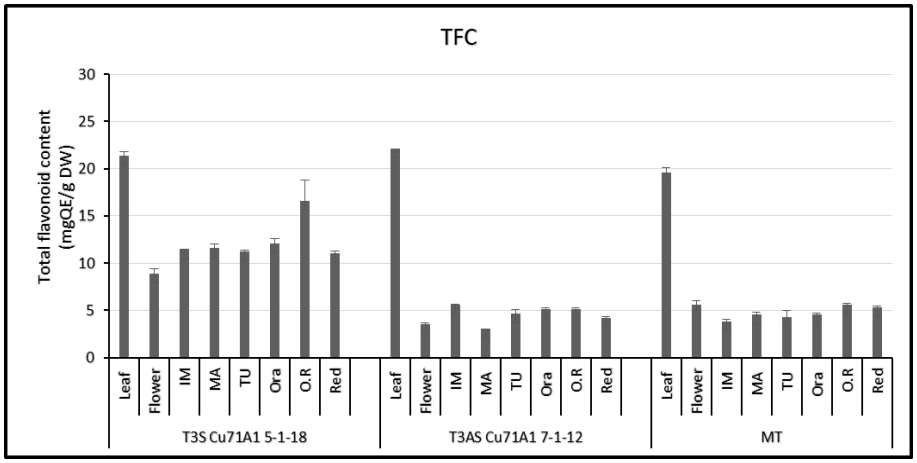


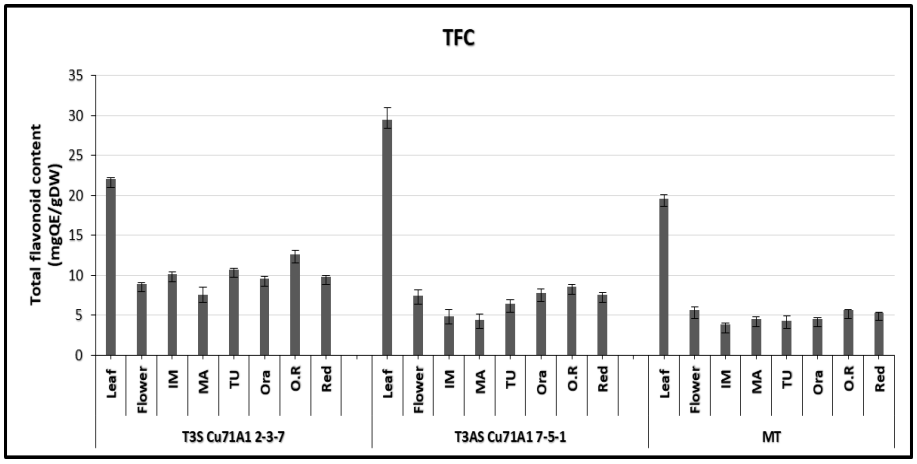
The RT-PCR analysis of seed *SLNAC3*, *SLABC*, *SLCYP78A3*, *SLCYP78A5-like*, *SLCYP78A9* gene expression result is an overexpressed sense line is lower than controlMT and knockdown AS line. As a result is at the time of infecting the citrus *cuCYP71A1* gene into to Micro-Tom tomato, the *SLCYP78A3* gene was suppressed and the decrease of the transgenic tomato seed size of the overexpressed sense line.

1.T3S-CU71A1-1-11-9line 2. T3S-CU71A1-5-1-18line 3. T3S-CU71A1-2-3-7line 4.controlMT 5.T3AS CU71A1 7-1-12 6. T3AS CU71A1 7-5-1 7. T3AS CU71A1 1-12-1

Table4 Compared Analysis of Total flavonoid content (TFC)

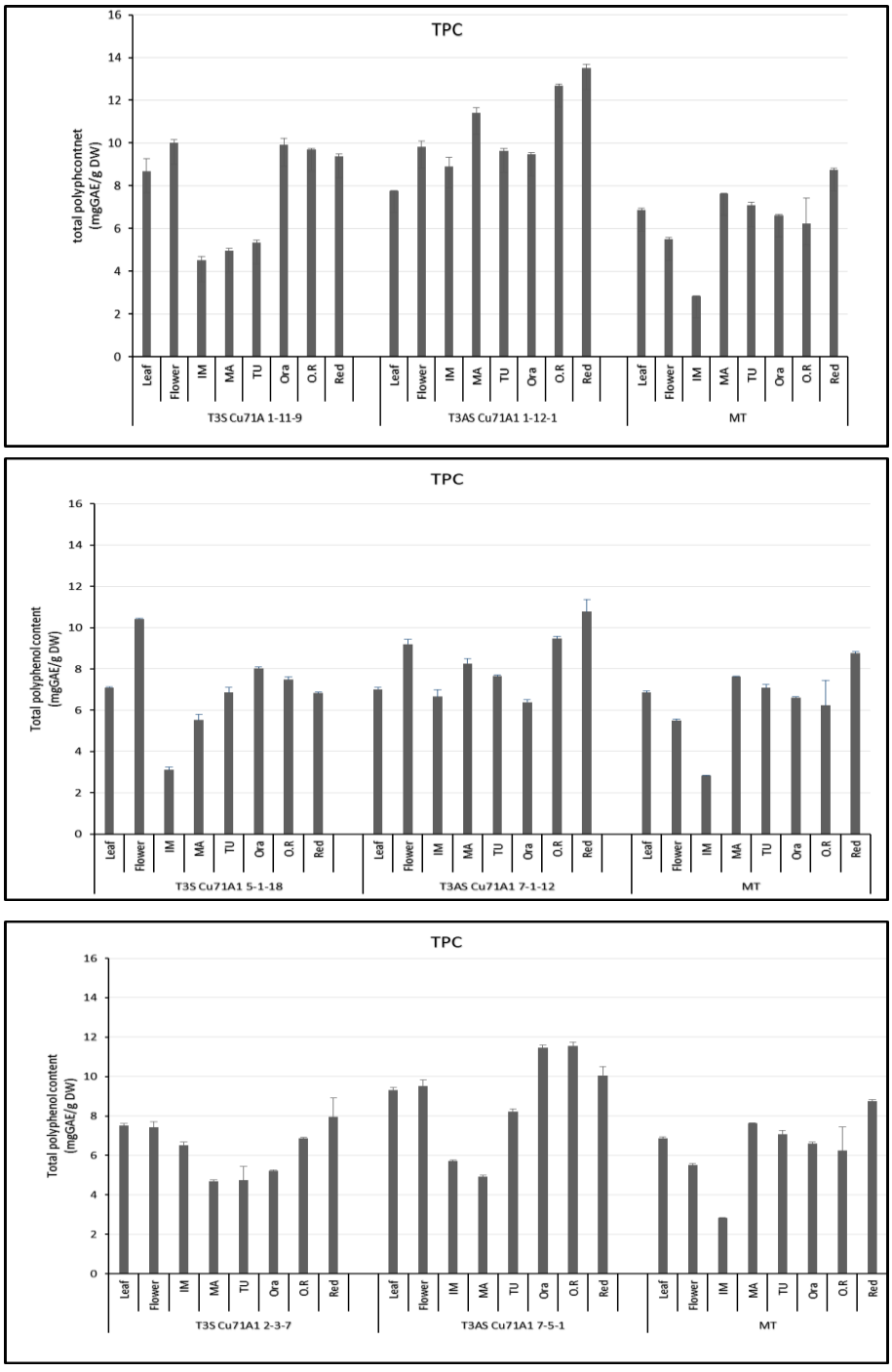






The TFC result is all of the lines Leaf and overexpression S line flower, immature green, mature green, turning, orange, orange red, red stages are higher than control MT and AS flower, immature green, mature green, turning, orange, orange red, red stages. So we researching gene expression of KEGG flavonoid biosynthesis pathway tomato related genes. (fig21)

Table5 Compared Analysis of Total polyphenol content (TPC)



The TPC result is shown sense lines between 5.9-12.1mg/ml, control MT is between 2.5-9.2mg/ml, Anti sense lines between 6.2-11.9mg/ml. All stages flower, orange, orange red, red stages higher than other stages.

Table6 Compared Analysis of Free radical scavenging activity (DPPH)

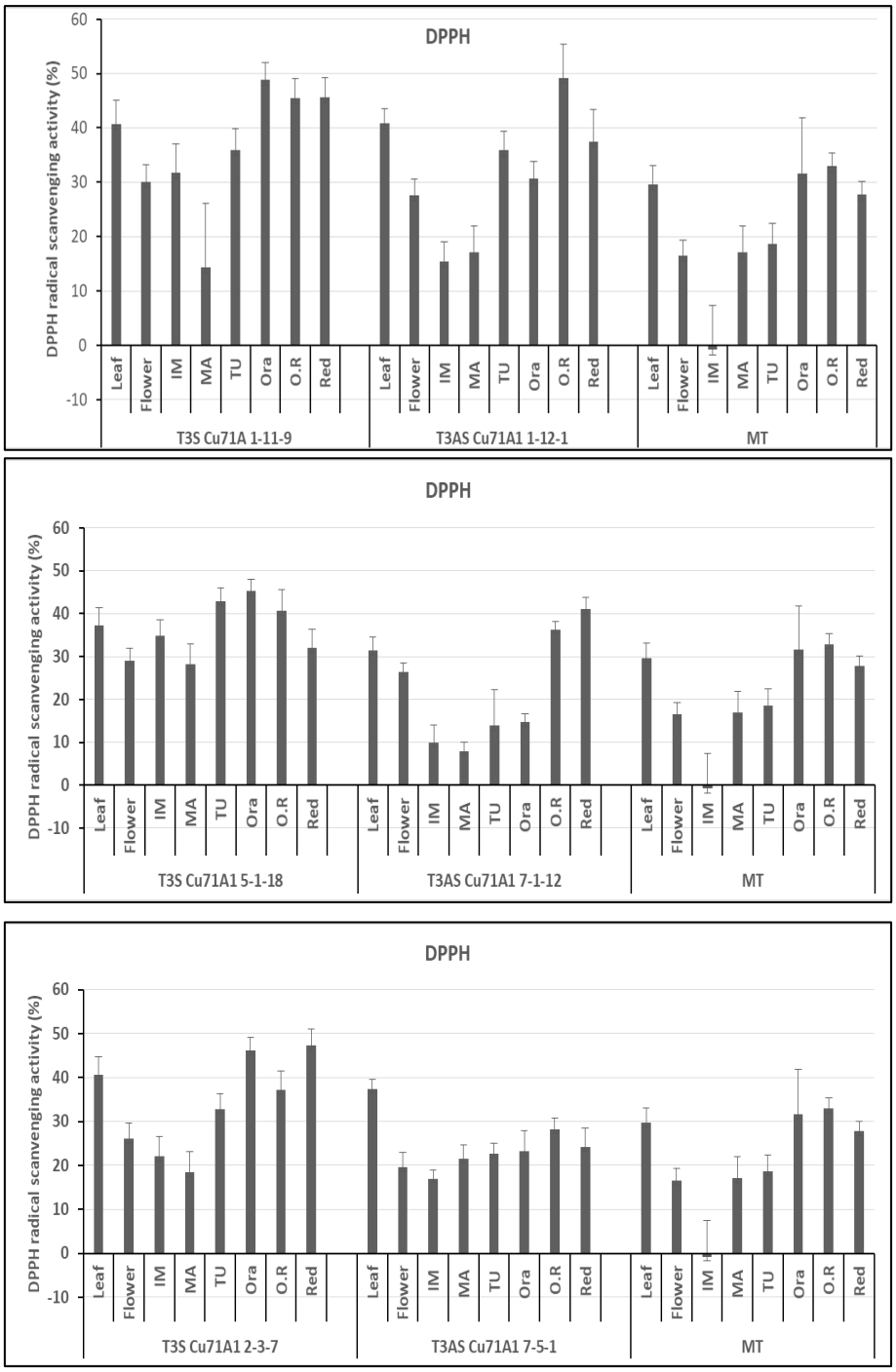
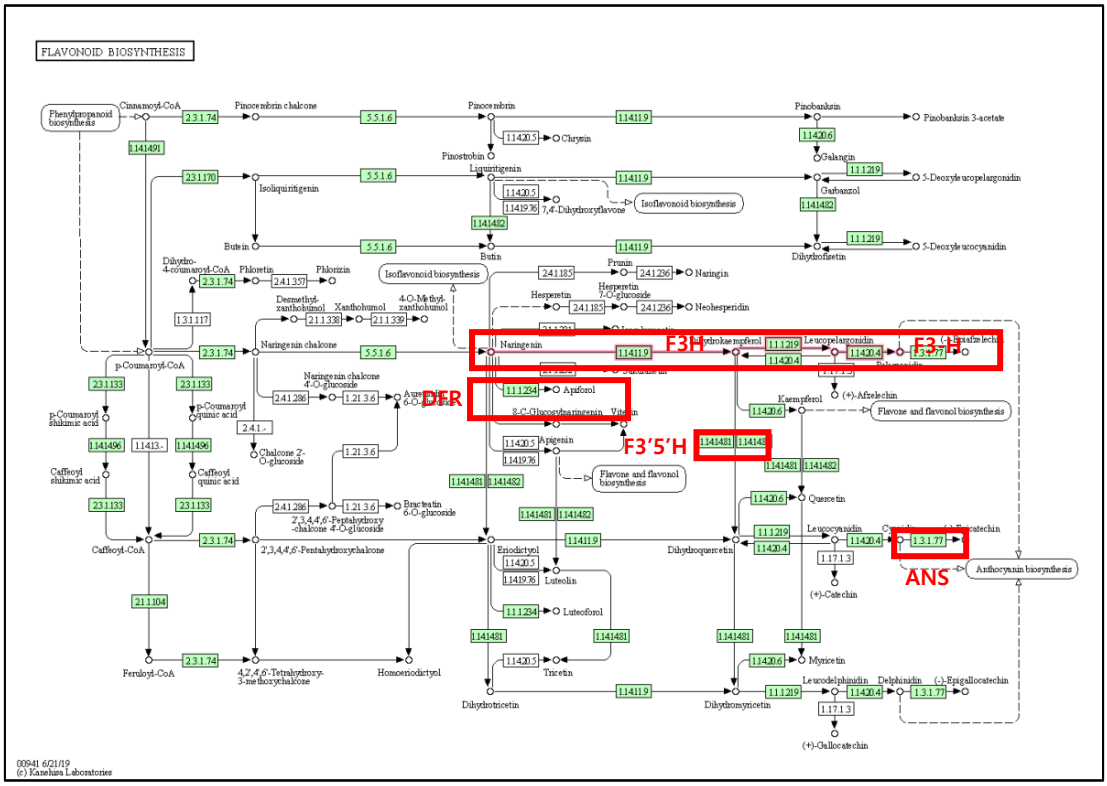


Table7 RT-PCR primer used in this study related of Flavonoid.

Primer name	Primer sequence	Product size
SL F3H F870	ATT CCA GAA TCC AGC ACC AG	189bp
SL F3H R1059	CTC CAA CTT GGC CTC TTC AG	
SL F3-H F683	GGC CTG ATT TGG CTC TGG GA	279bp
SL F3-H R962	GGC CCA ACT TCA TGC TCT GA	
SL DFR F75	AGC TGG ATT TAT CGG CTC TT	247bp
SL DFR R322	CTG GAT CCT TGG ACT CGA AA	
SL ANS F209	AAT GCC ACC AGG AGT TGA AG	196bp
SL ANS R405	TGC TAG CTT GCT TCC ATA GC	
SL FLS F233	AGG CTA GTA AAG AGT GGG GT	246bp
SL FLS R479	ATA GCA GGA GGA GGC CAA AT	
SL F3’5’H F1277	AGC CAA AGA TTG AAC CTC GT	217bp
SL F3’5’H R1494	AGC TTC TAG AGG GAC AGC TT	

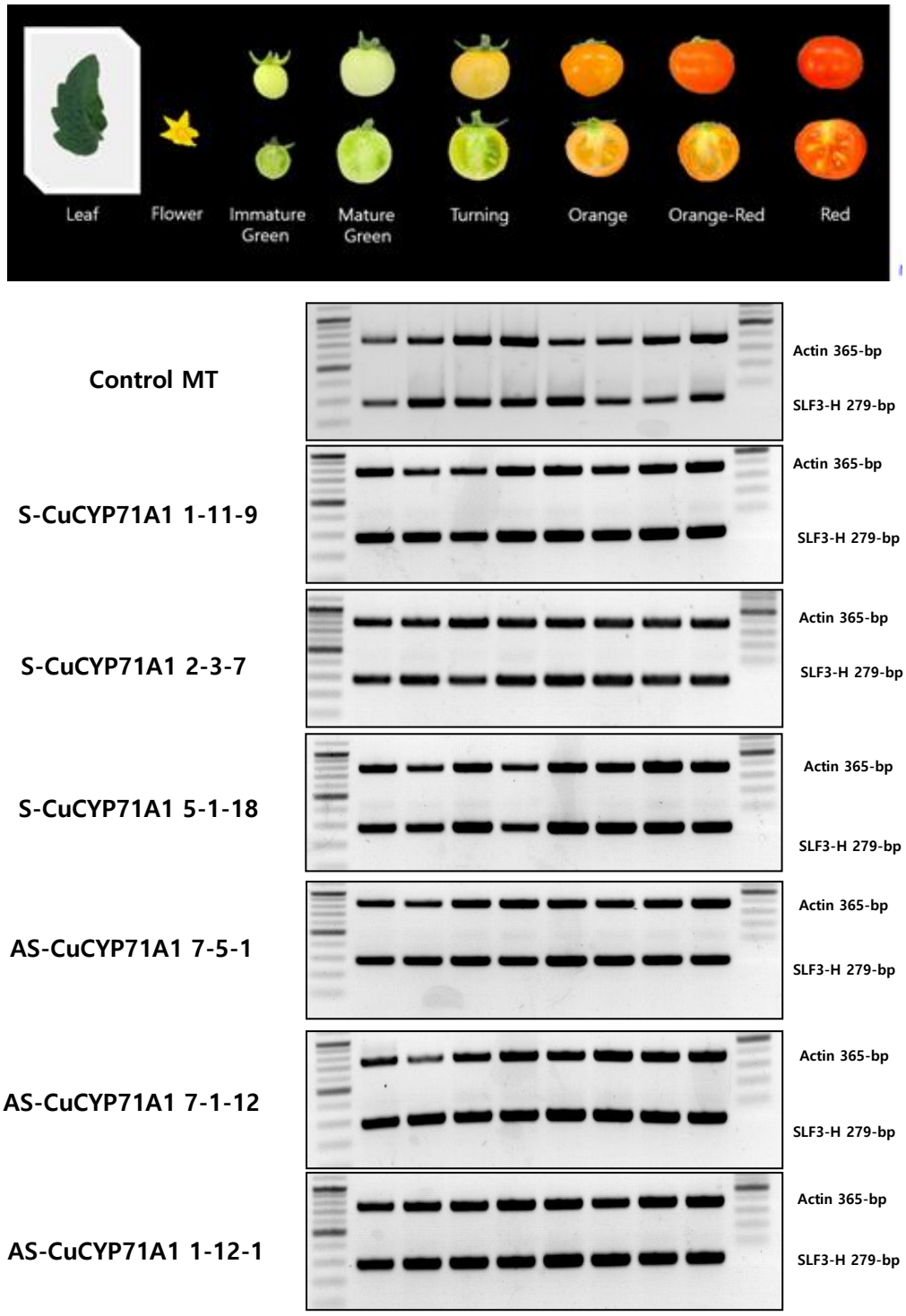
All gene’s was confirmed by sequencing and NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST).

Figure17. Flavonoid biosynthesis: Solanum lycopersicum (Tomato)



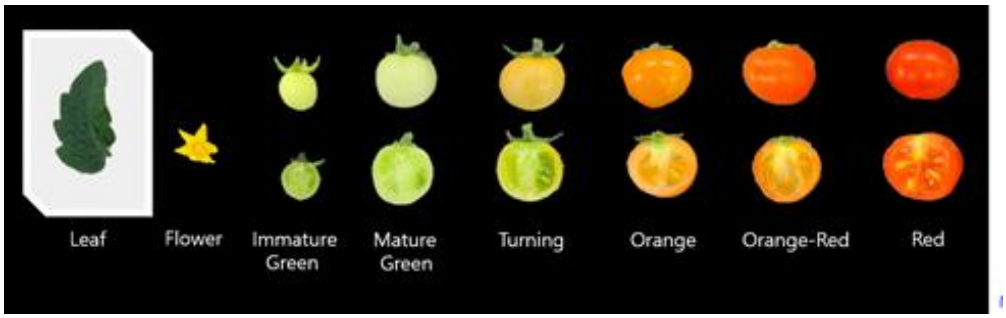
KEGG flavonoid biosynthesis of transgenic tomato.

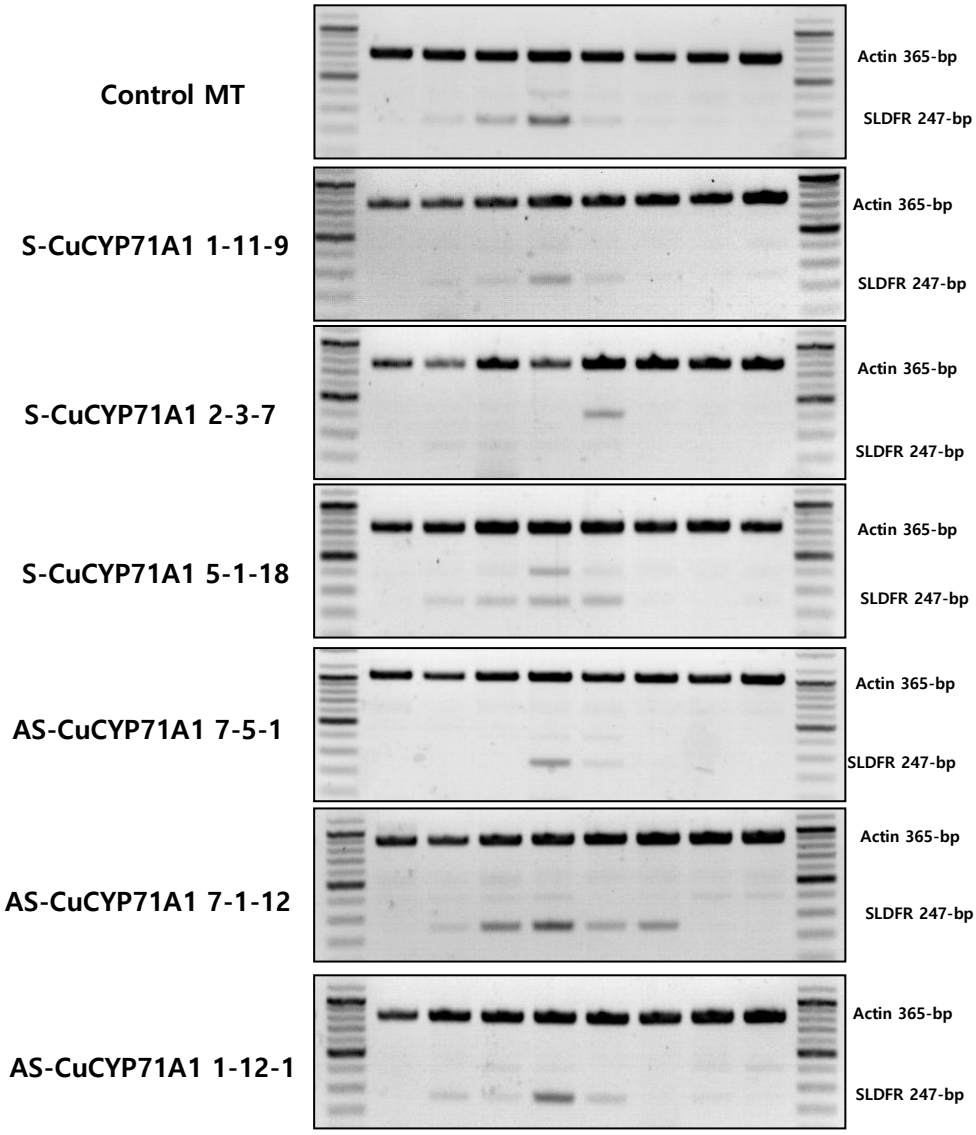
Figure18. GENE expression of SLF3-H gene using transgenic tomato plant.



Lane1 plasmid DNA, Lane2 marker, Lane3 Leaf, Lane4 Flower, Lane5 immature green, Lane6 mature green, lane7 turning, Lane8 Orange , Lane9 Orange red, Lane10 Red-

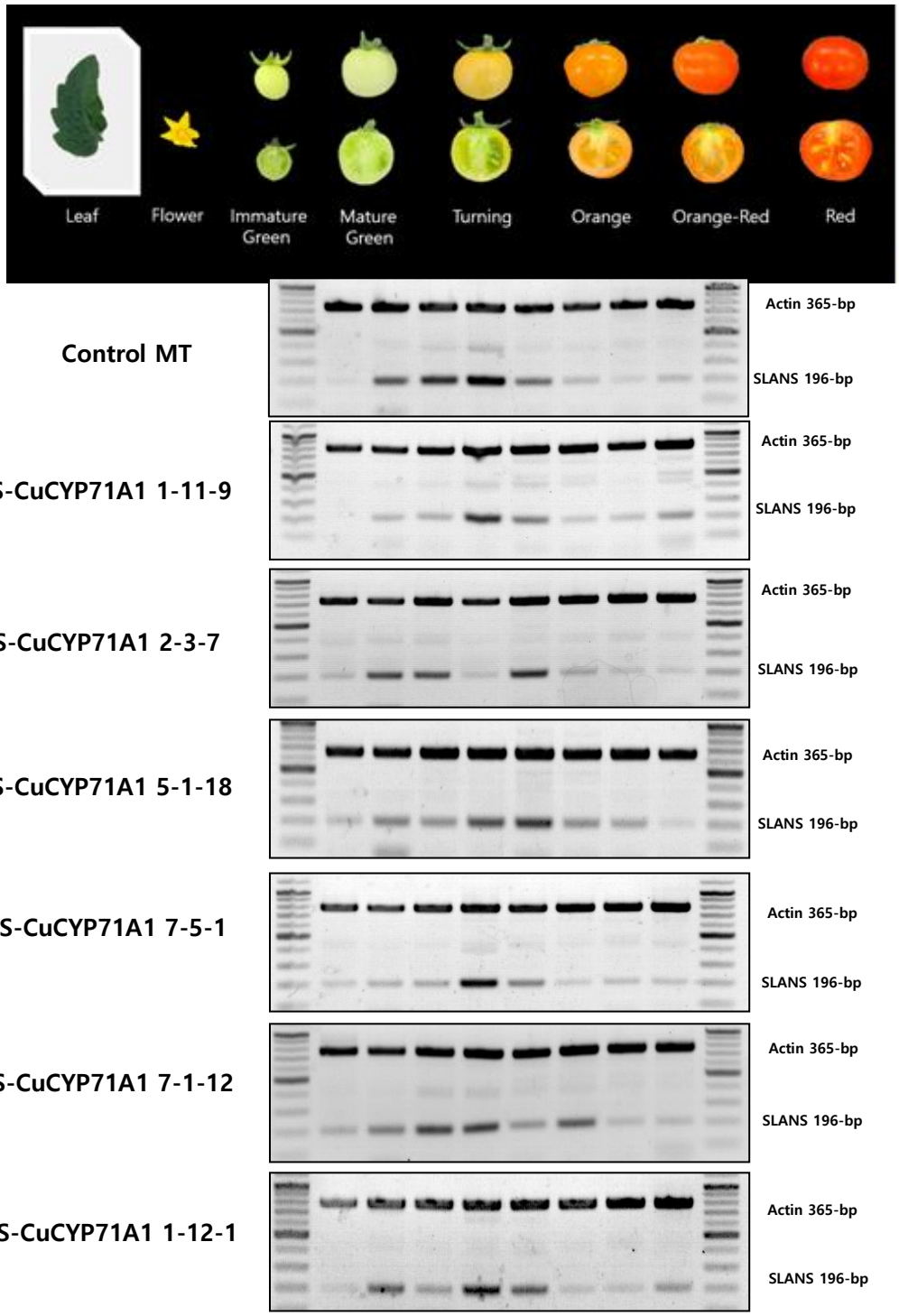
Figure19. GENE expression of SLDFR gene using transgenic tomato plant.





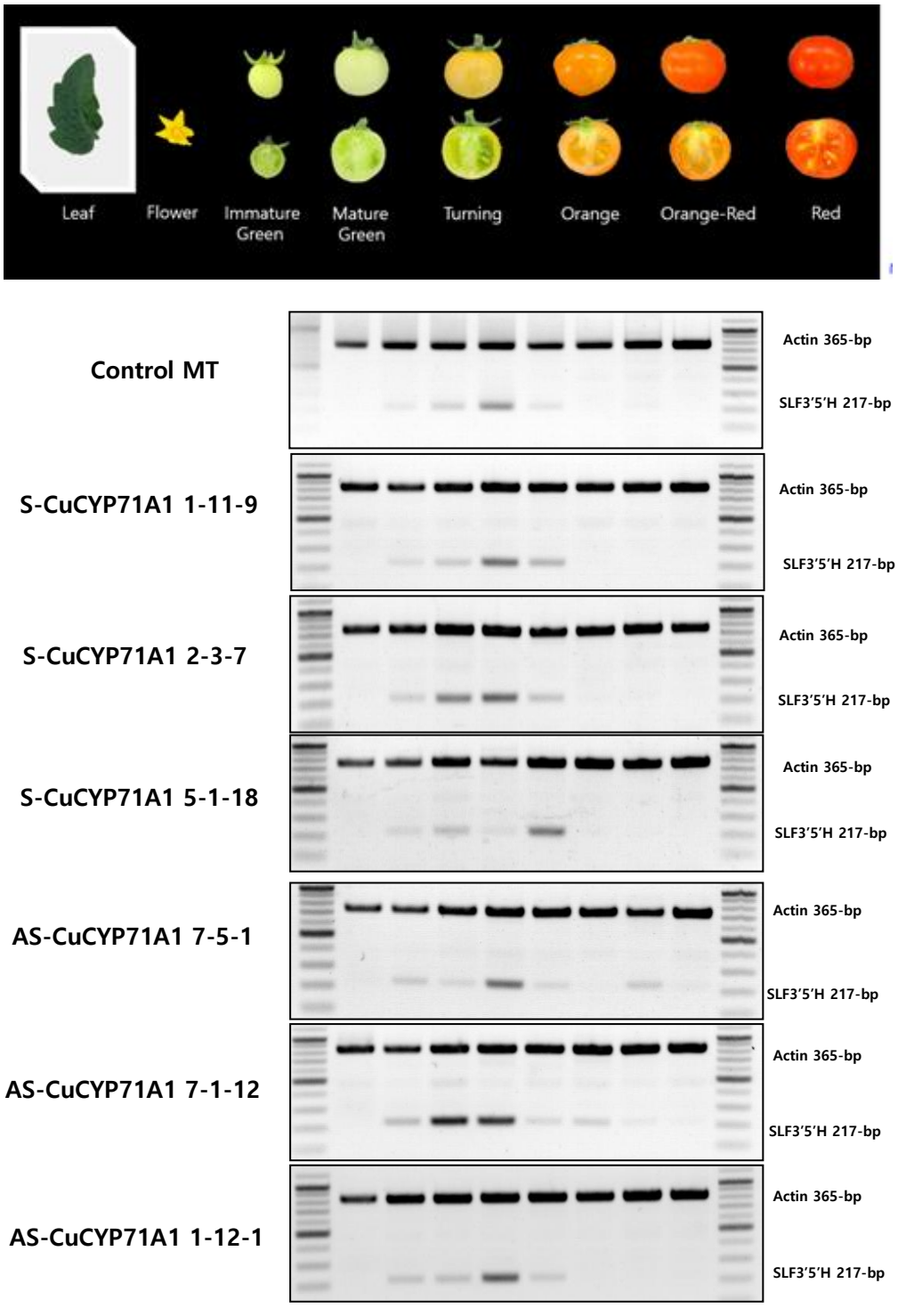
Lane1 plasmid DNA, Lane2 marker, Lane3 Leaf, Lane4 Flower, Lane5 immature green, Lane6 mature green, lane7 turning, Lane8 Orange , Lane9 Orange red, Lane10 Red-

Figure20. GENE expression of SLANS gene using transgenic tomato plant.



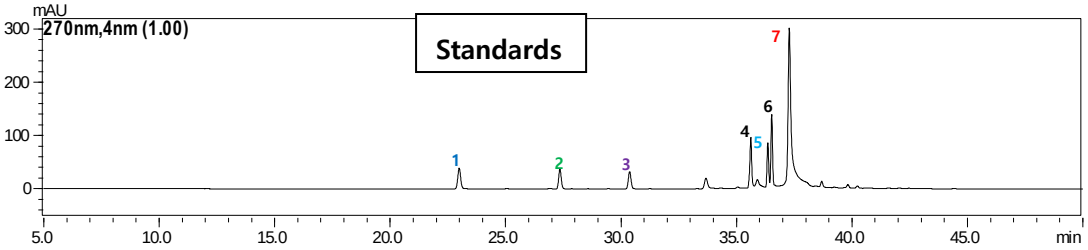
Lane1 plasmid DNA, Lane2 marker, Lane3 Leaf, Lane4 Flower, Lane5 immature green, Lane6 mature green, lane7 turning, Lane8 Orange , Lane9 Orange red, Lane10 Red-

Figure21. GENE expression of SLF3’5’H gene using transgenic tomato plant**.**



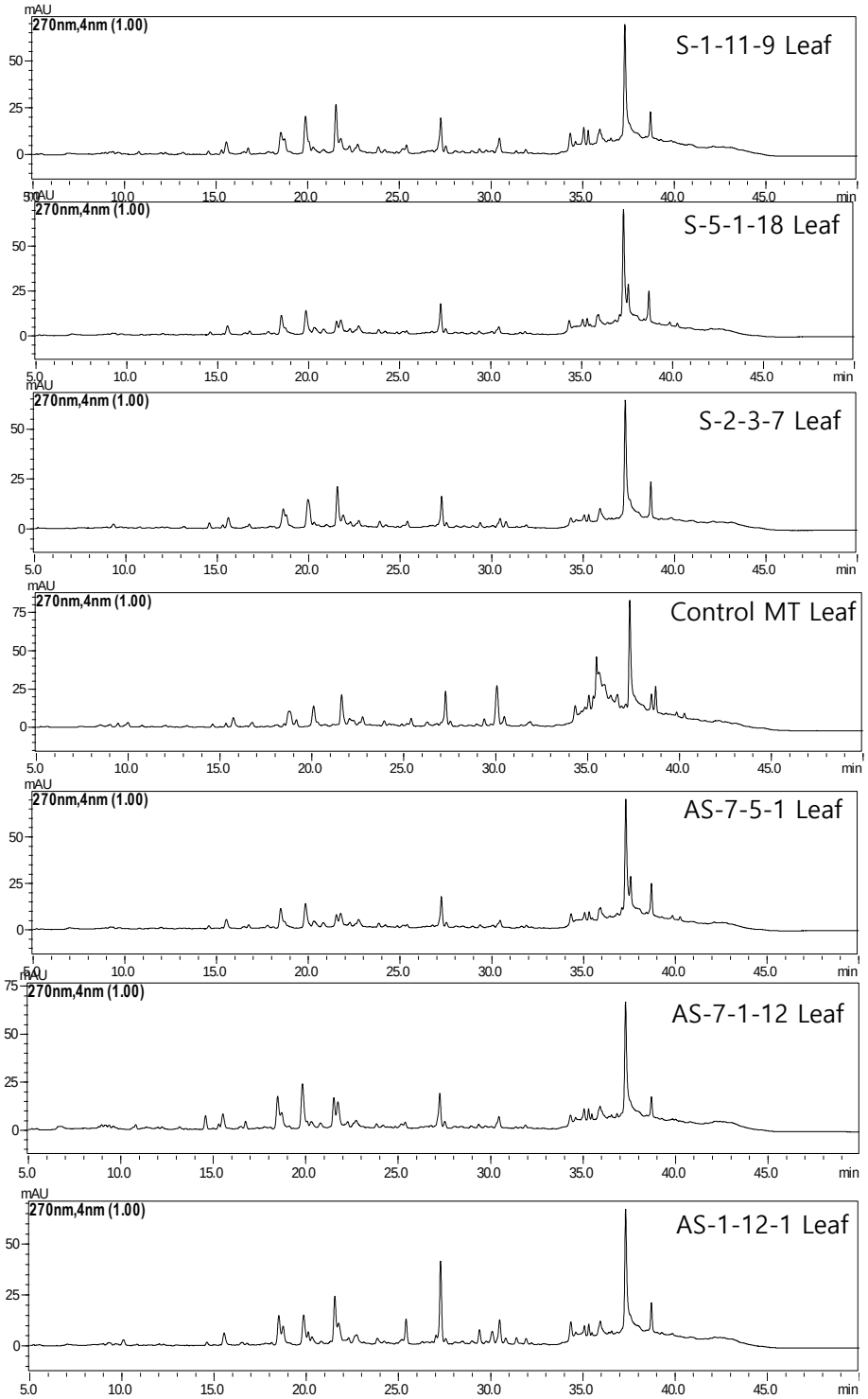
Lane1 plasmid DNA, Lane2 marker, Lane3 Leaf, Lane4 Flower, Lane5 immature green, Lane6 mature green, lane7 turning, Lane8 Orange , Lane9 Orange red, Lane10 Red-

Figure22. Standards of flavonoids



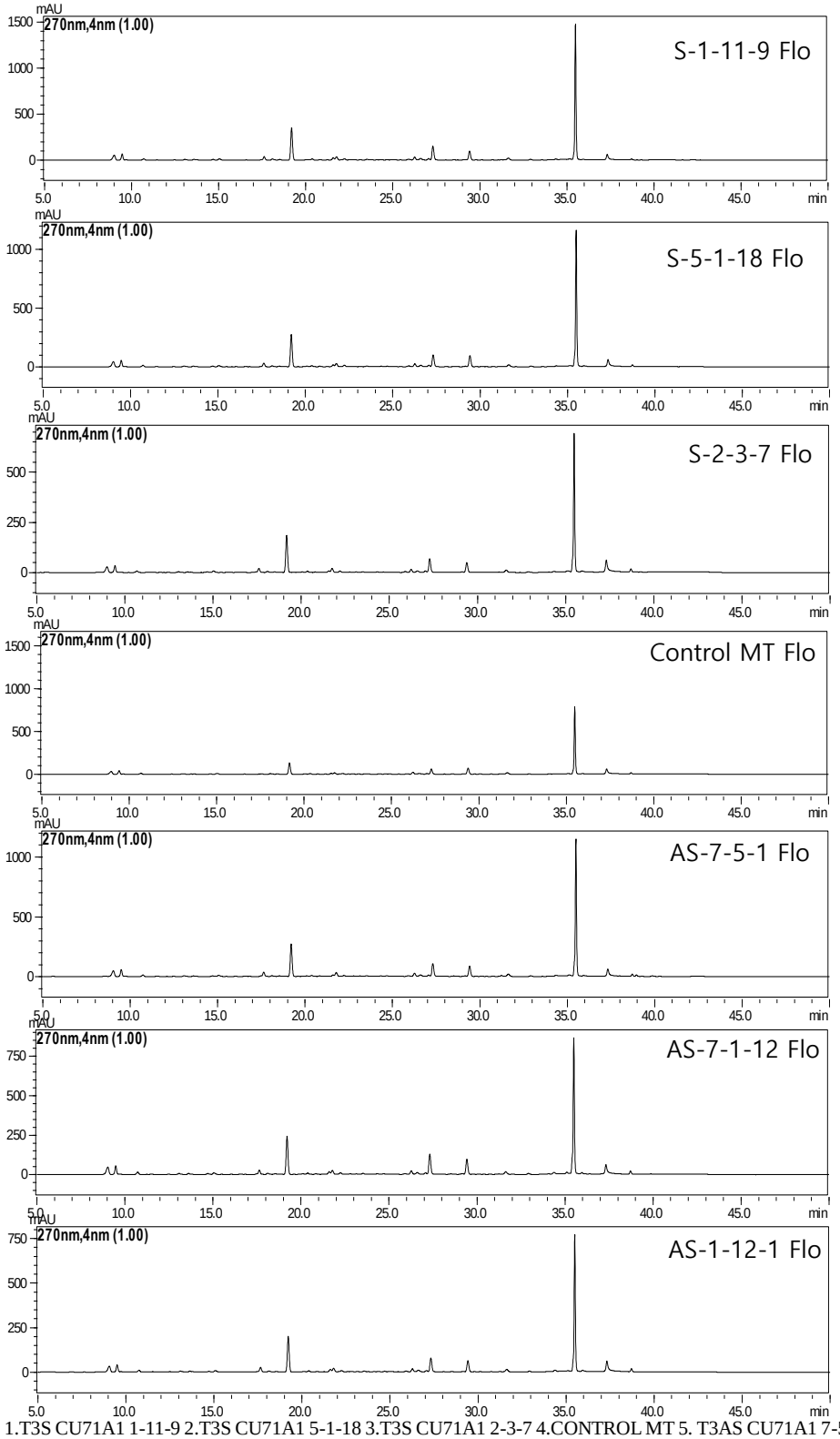
no	Standard name	Retention time	Area	Height	Start time	Stop time
1	Caffeic acid	22.981	338839	39203	22.709	22.709
2	Naringenin	27.237	437379	55851	27.051	27.691
3	Naringin	30.262	413573	49812	30.048	30.773
4	Quercetin	35.592	640205	145679	35.381	35.723
5	Rutin	36.332	780769	126731	36.224	36.459
6	Kaempferol	36.518	780769	139847	36.437	36.843
7	Myrcetin	37.250	2190866	158408	36.843	38.549

Figure23. HPLC analysis of transgenic tomato leaf.



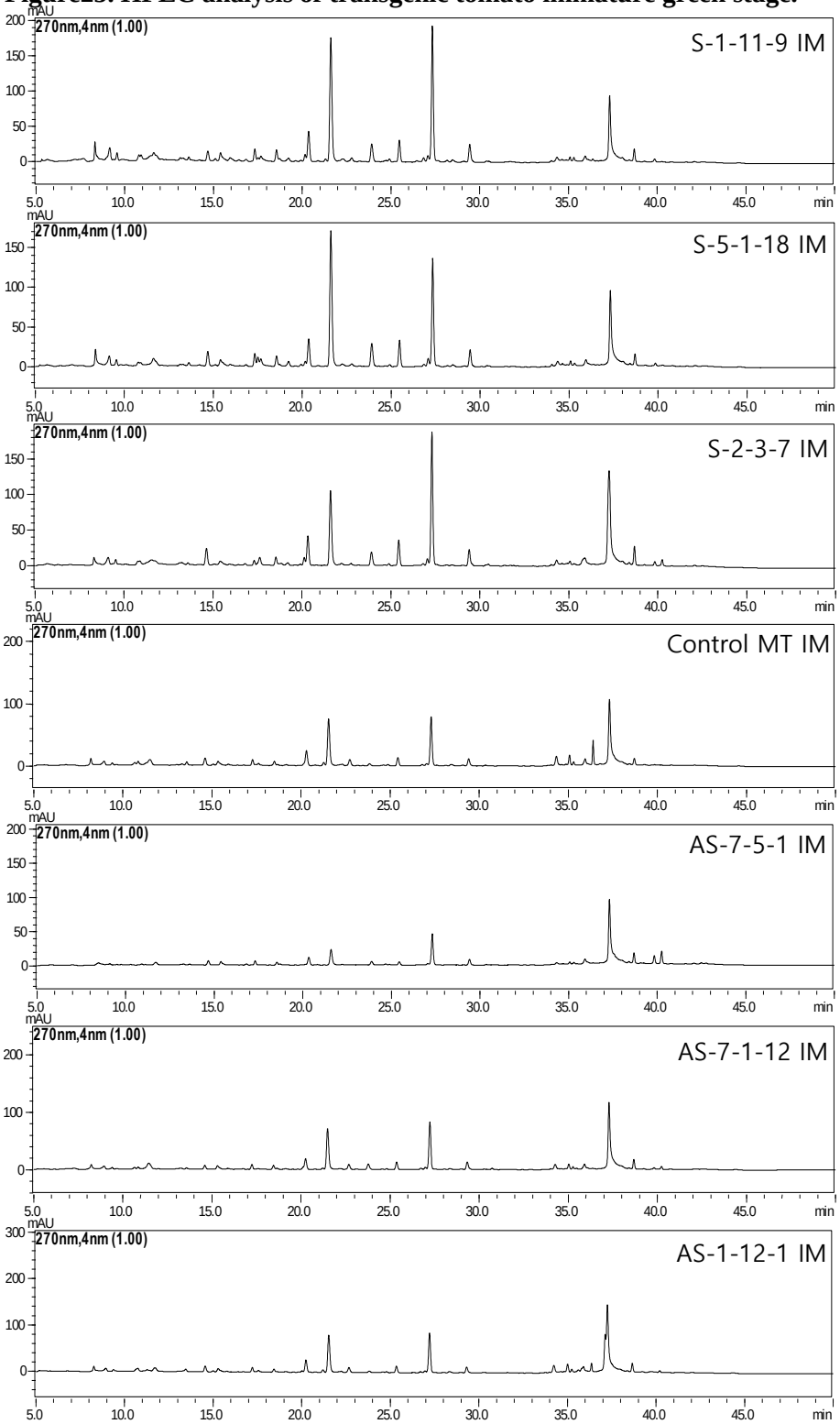
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure24. HPLC analysis of transgenic tomato flower.



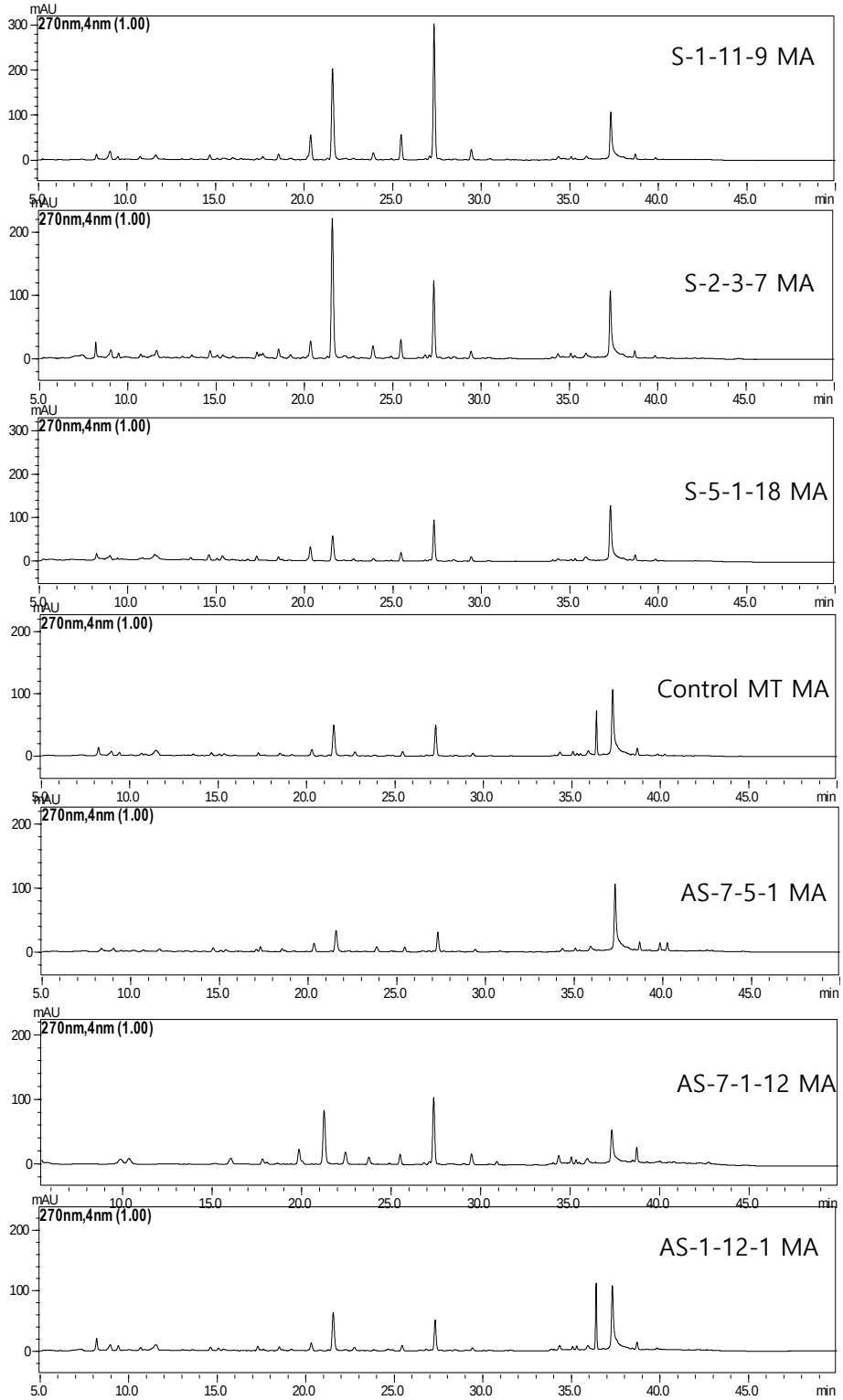
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure25. HPLC analysis of transgenic tomato immature green stage.



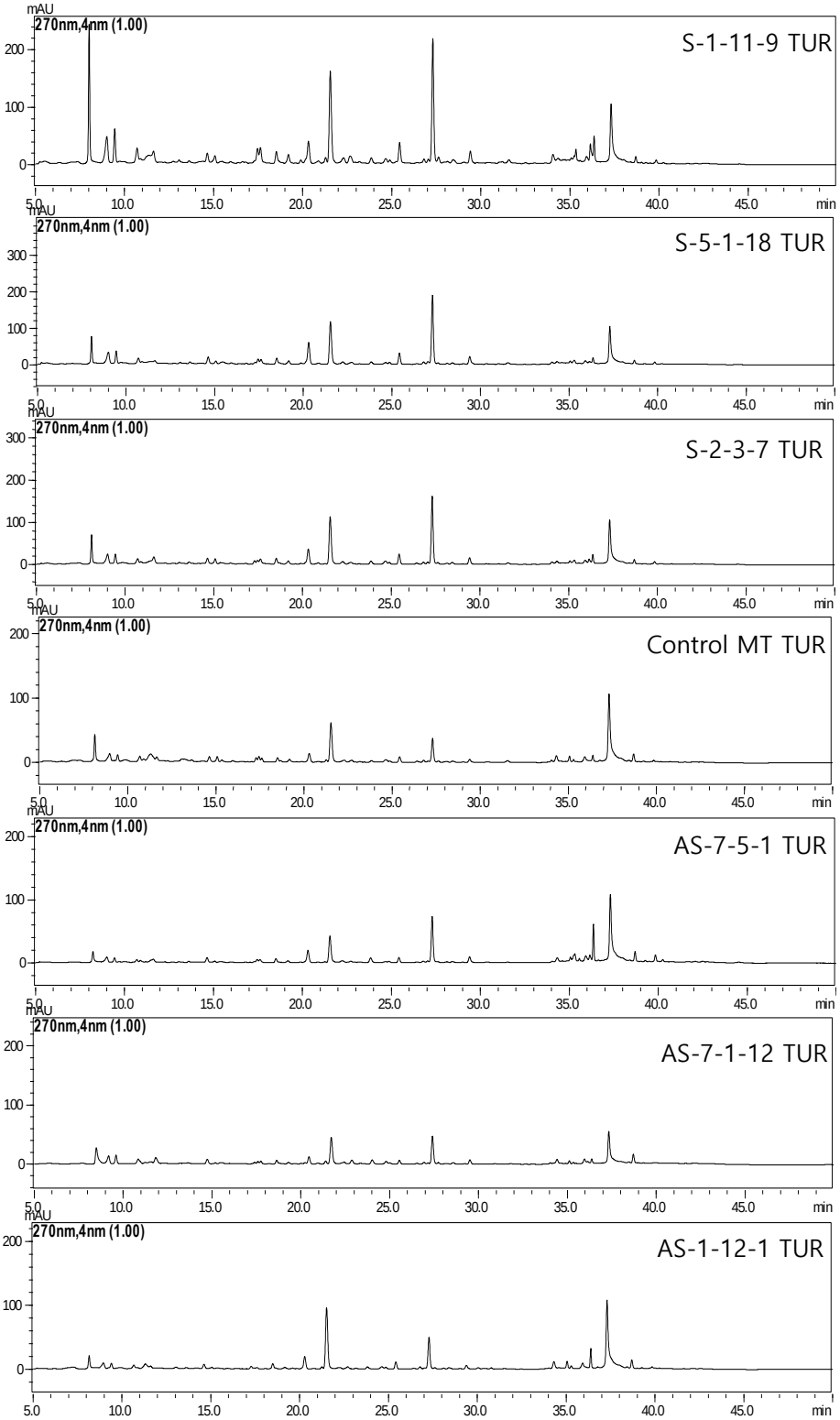
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure26. HPLC analysis of transgenic tomato mature green stage.



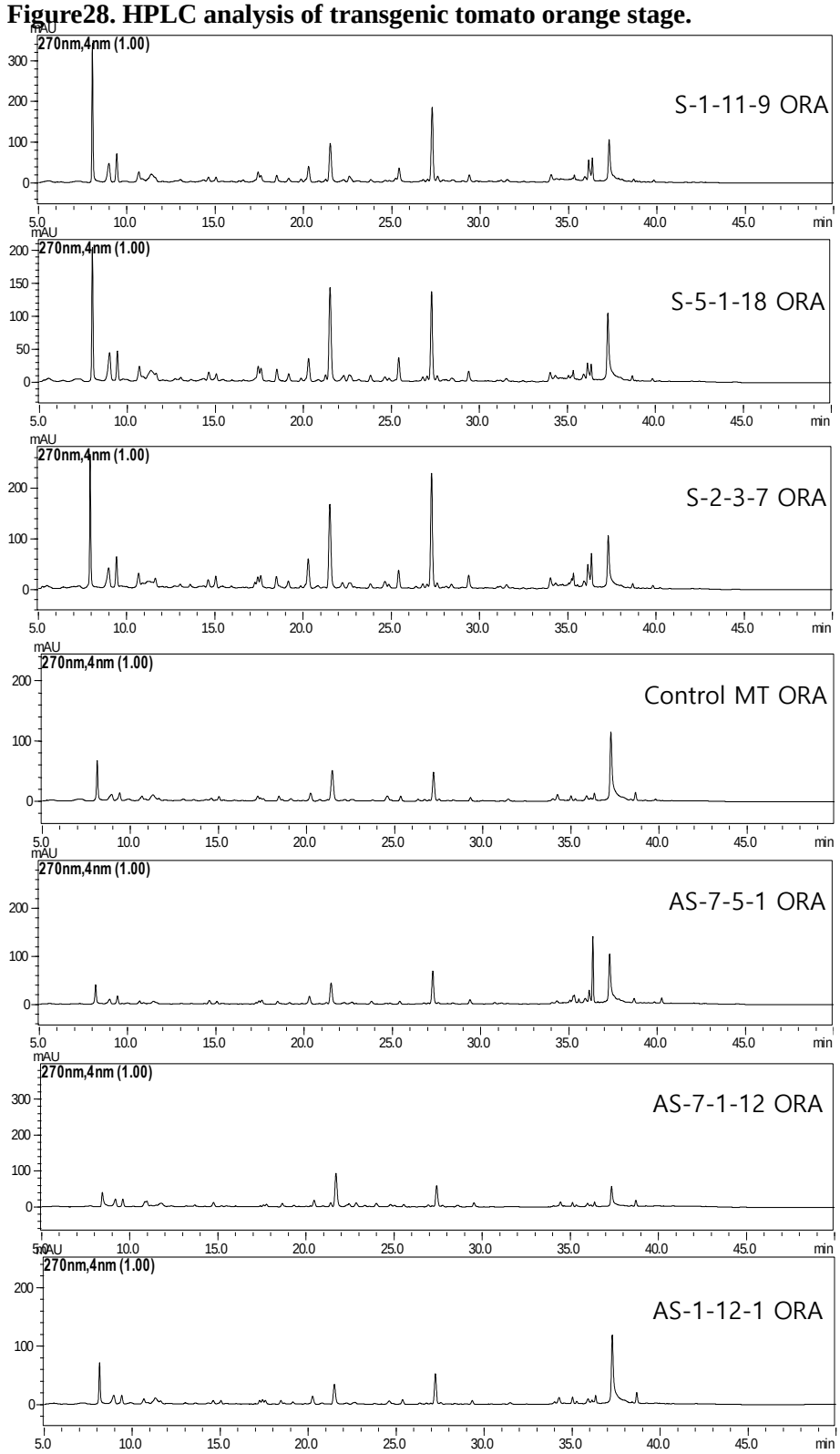
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure27. HPLC analysis of transgenic tomato turning stage.



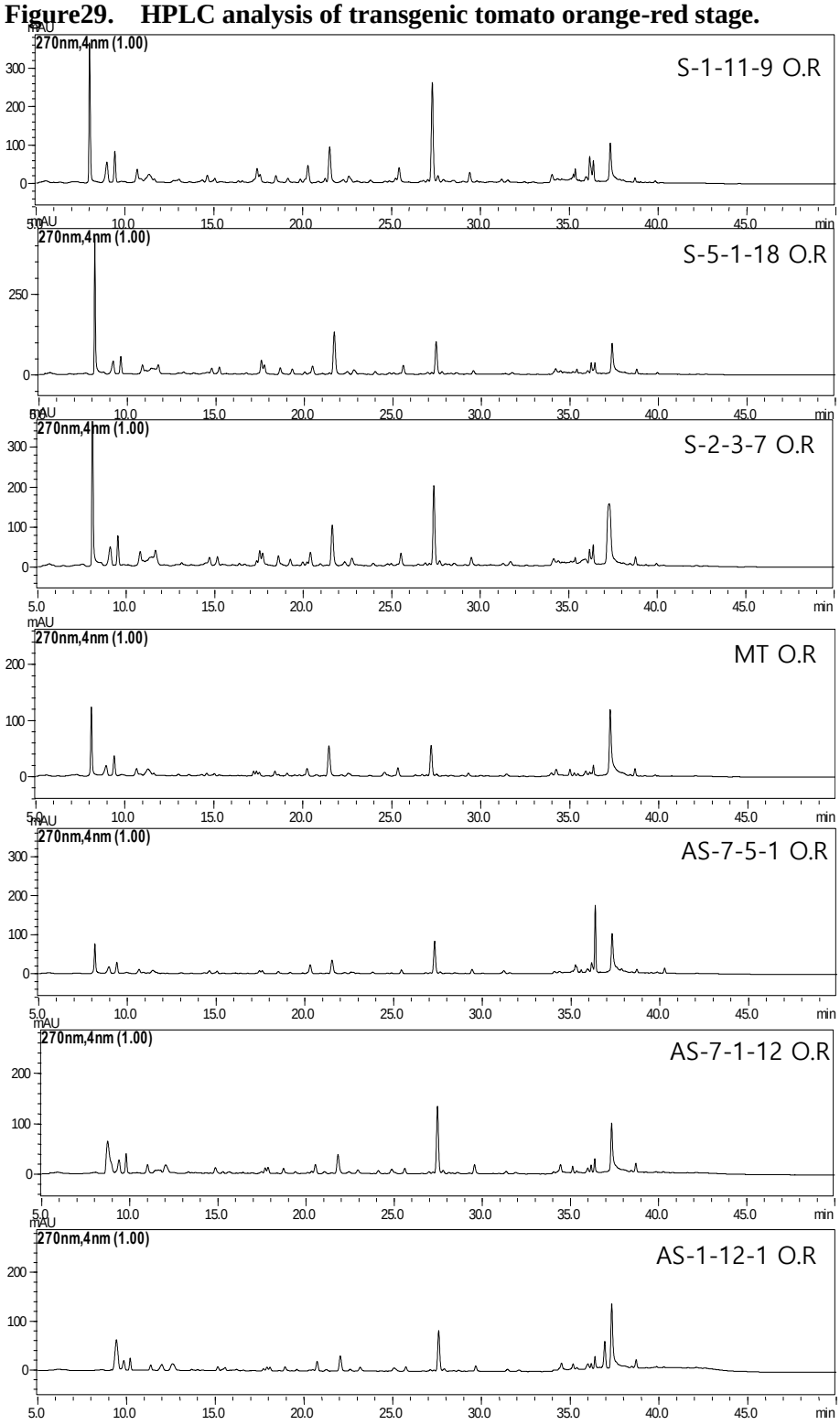
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure28. HPLC analysis of transgenic tomato orange stage.



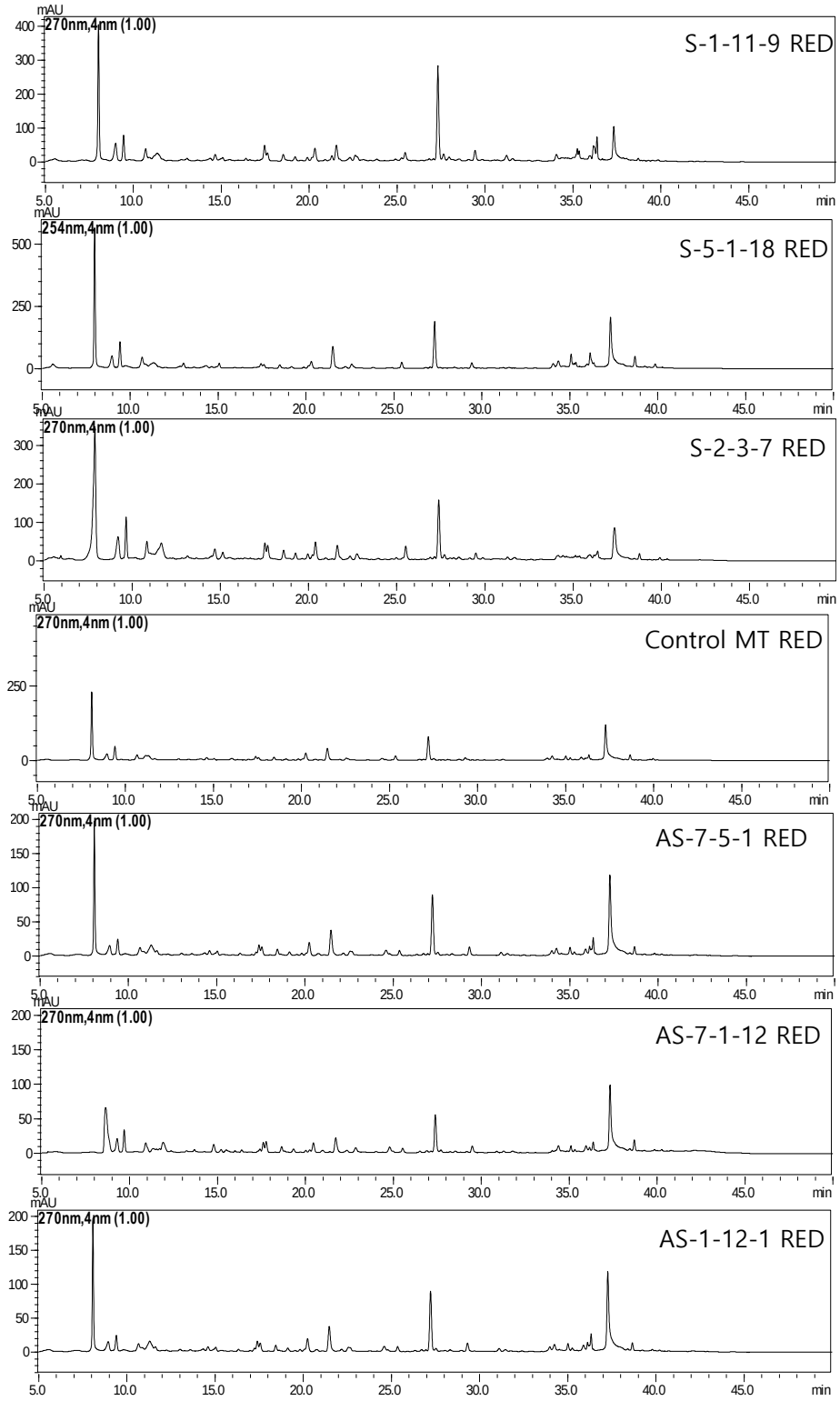
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure29. HPLC analysis of transgenic tomato orange-red stage.



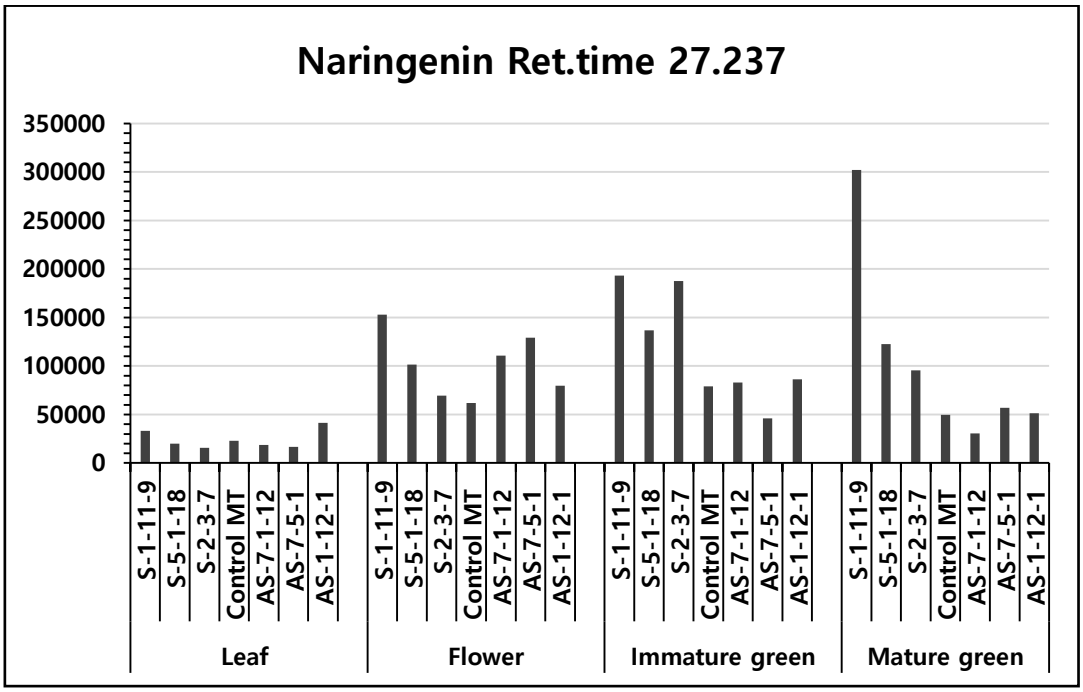
1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-12-1

Figure30. HPLC analysis of transgenic tomato red stage.



1.T3S CU71A1 1-11-9 2.T3S CU71A1 5-1-18 3.T3S CU71A1 2-3-7 4.CONTROL MT 5. T3AS CU71A1 7-5-1 6.T3AS CU71A1 7-1-12 7.T3AS CU71A1 1-1

Table8 HPLC Result of Naringenin.



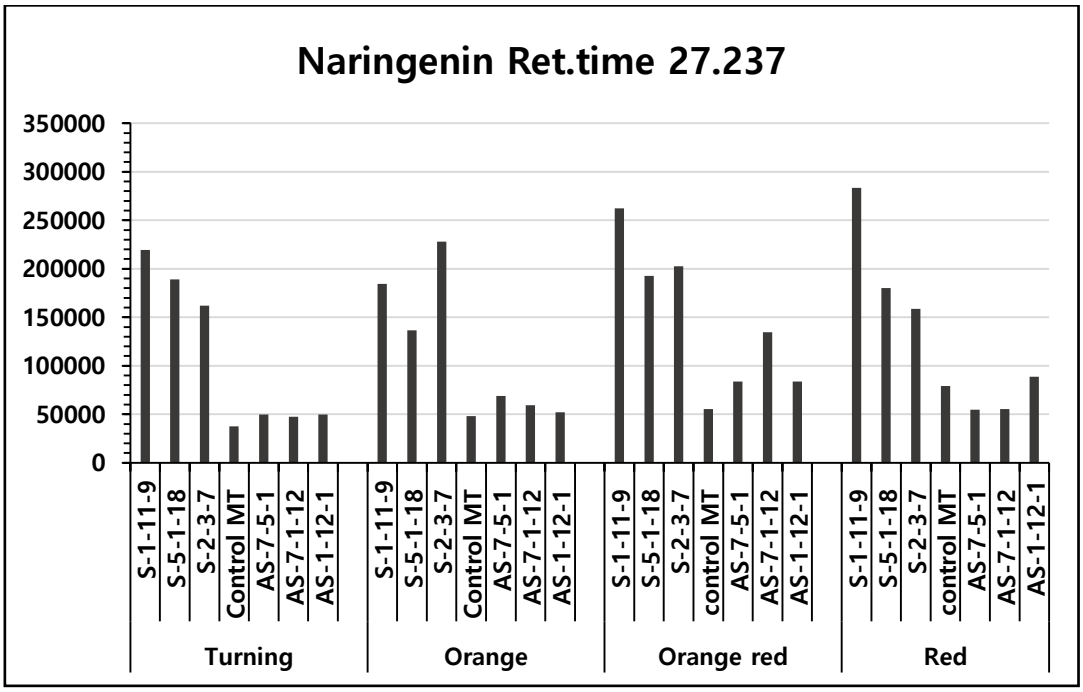
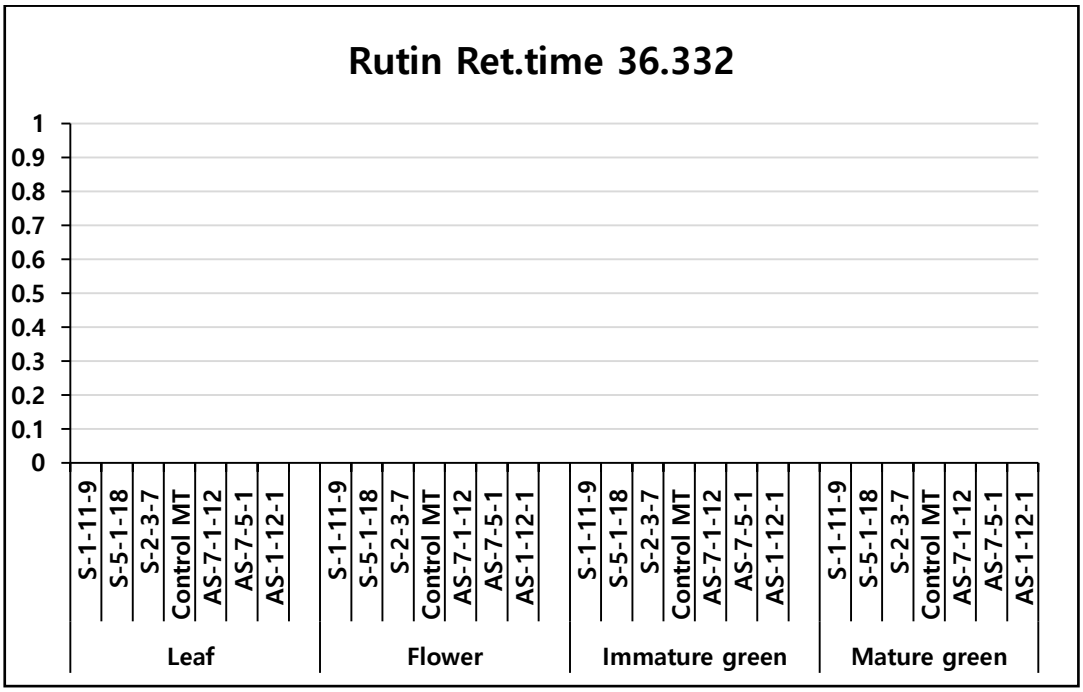


Table9 HPLC Result of Rutin



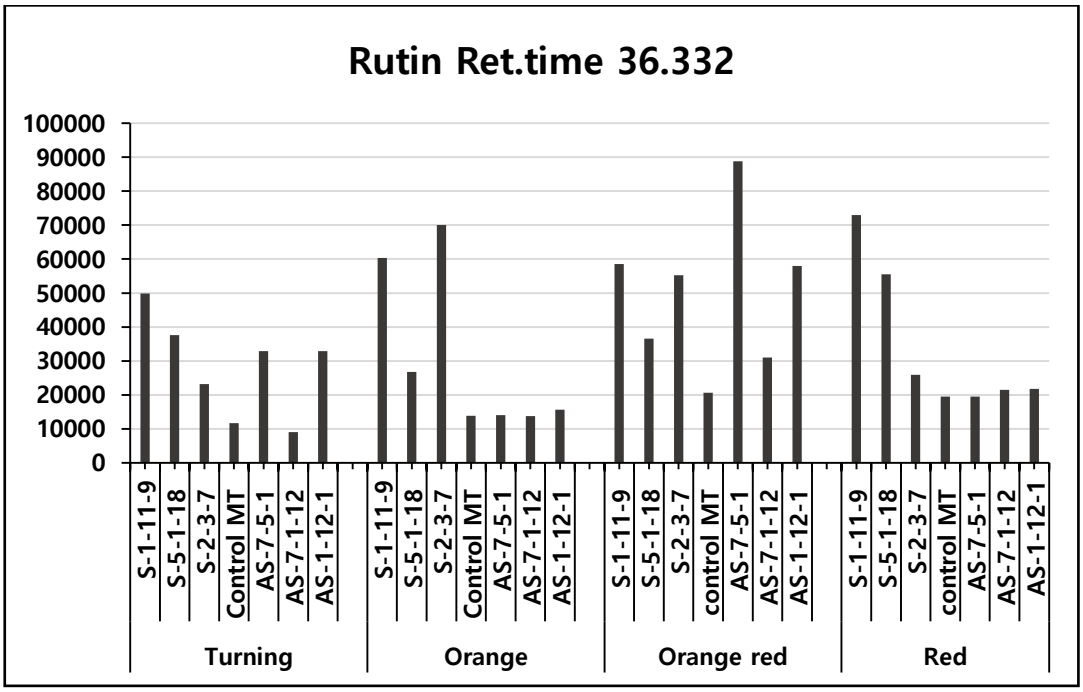
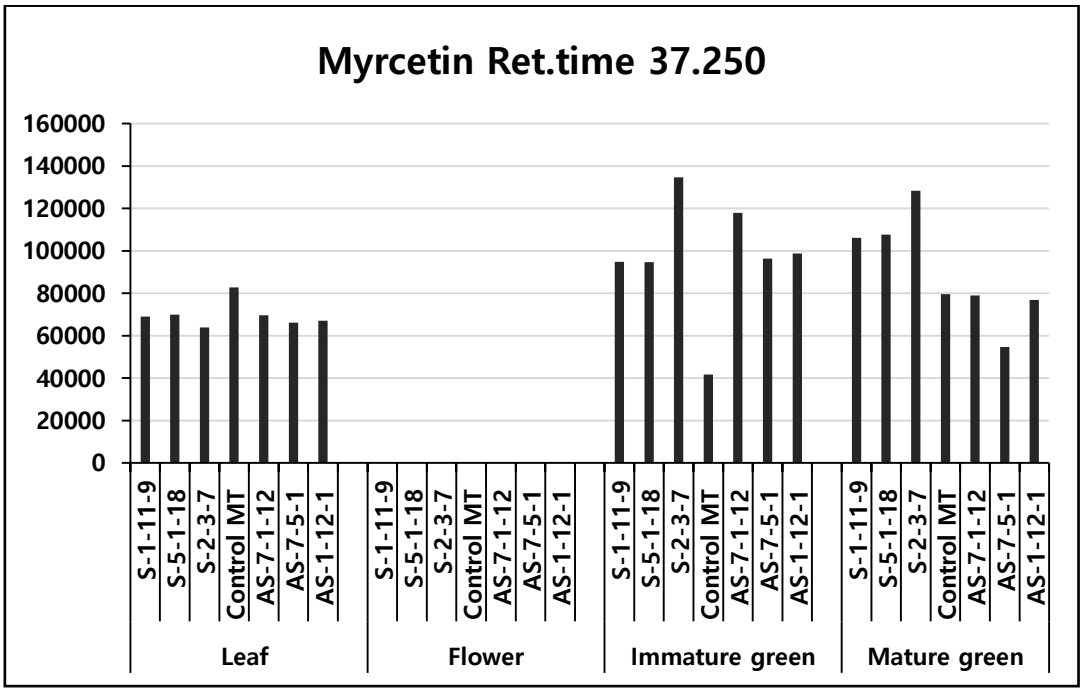


Table10 HPLC Result of Myrceitn.



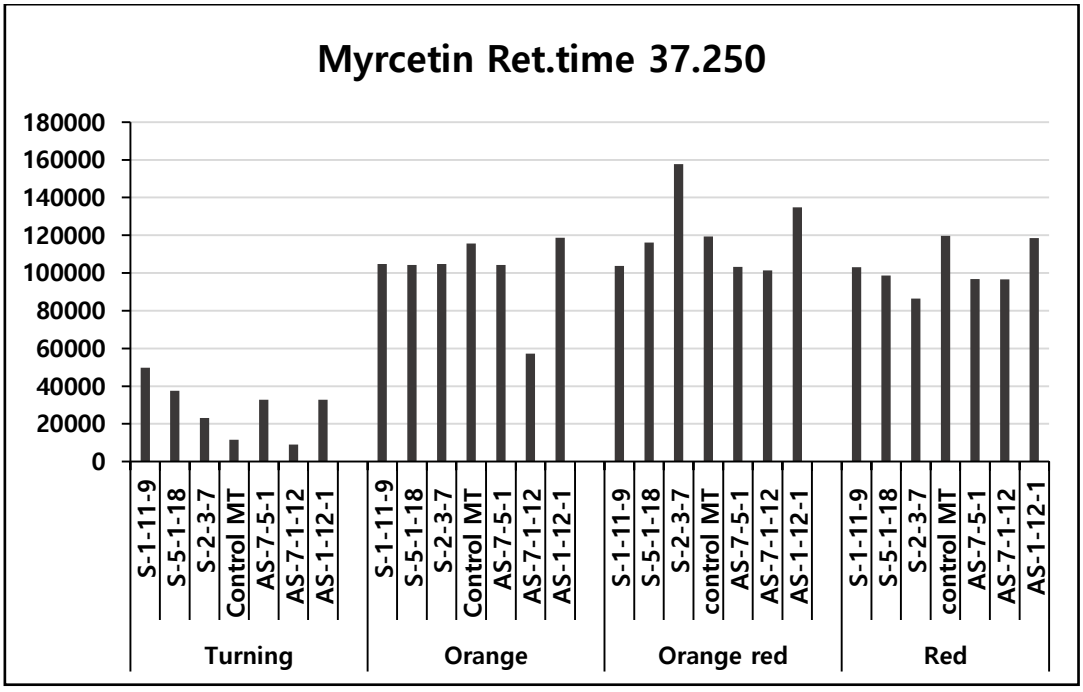
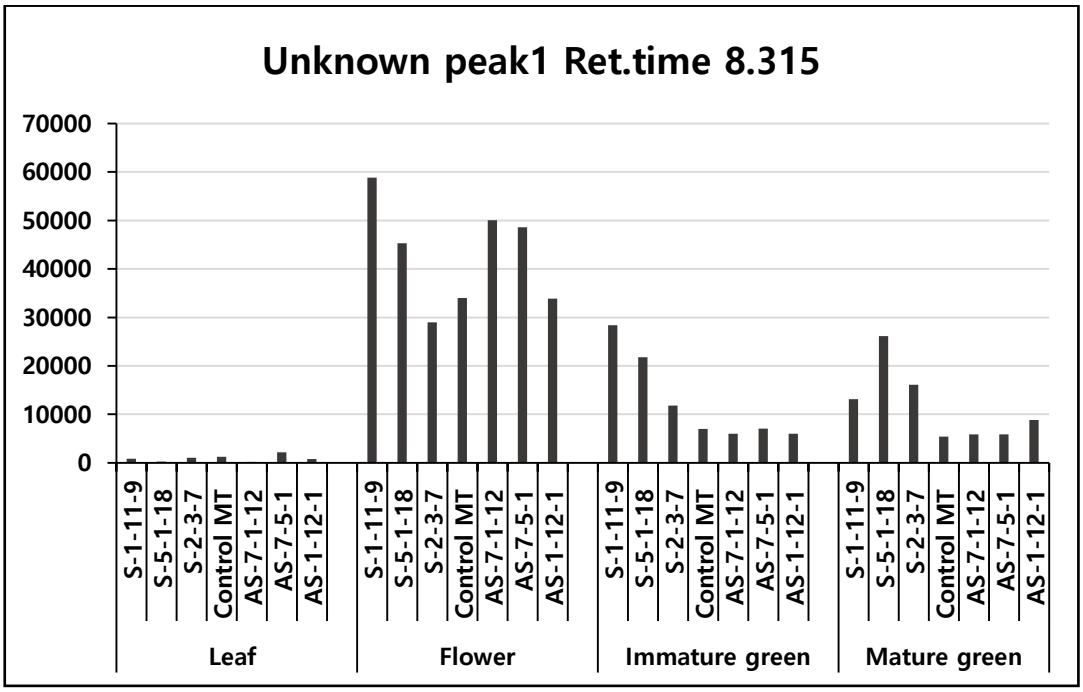


Table11 HPLC Result of unknown peak1.



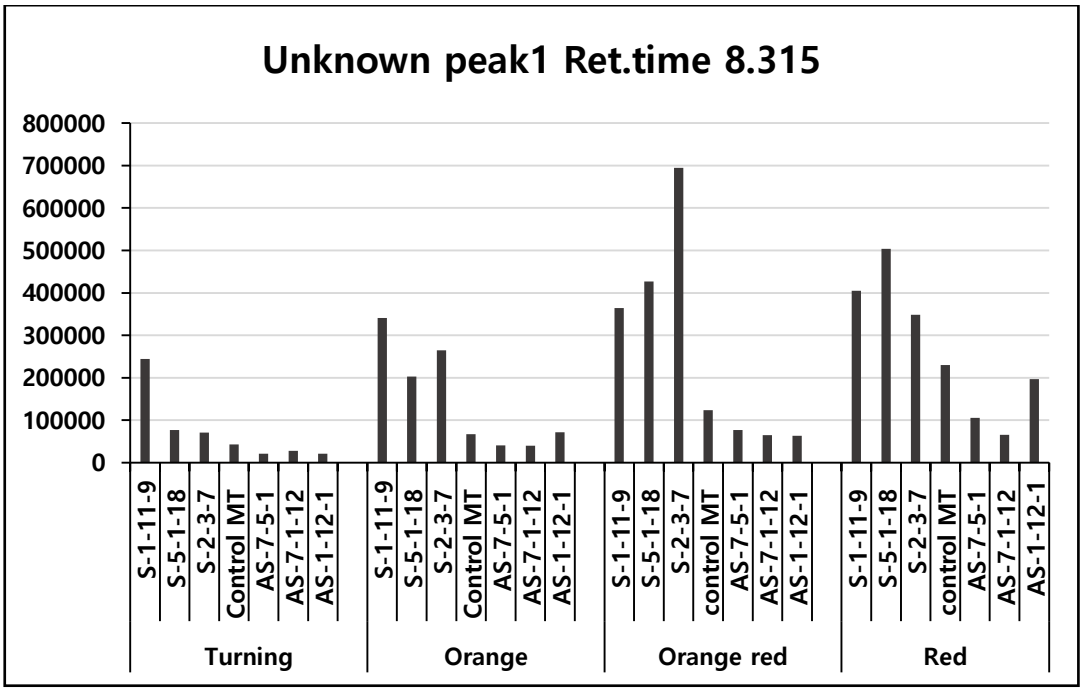
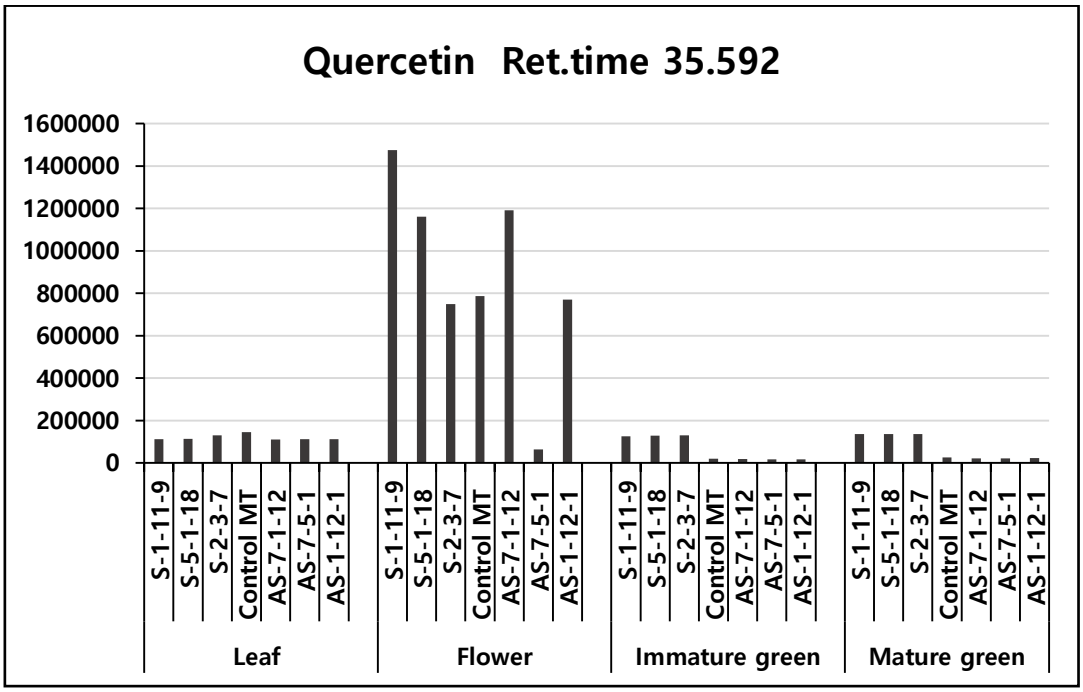


Table12 HPLC Result of quercetin.



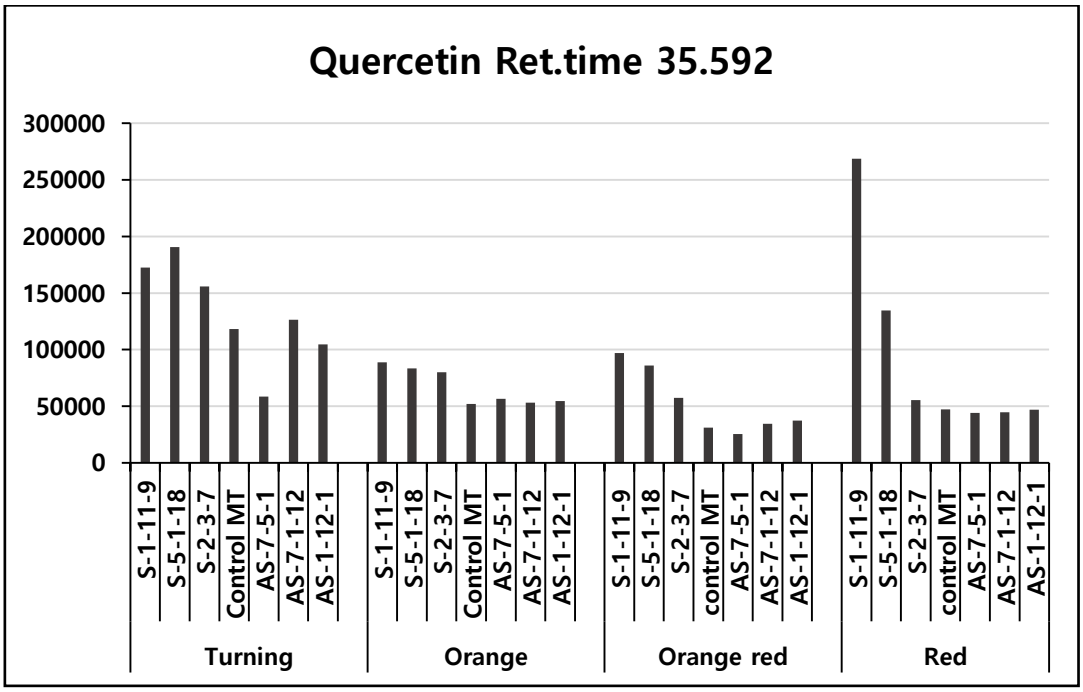
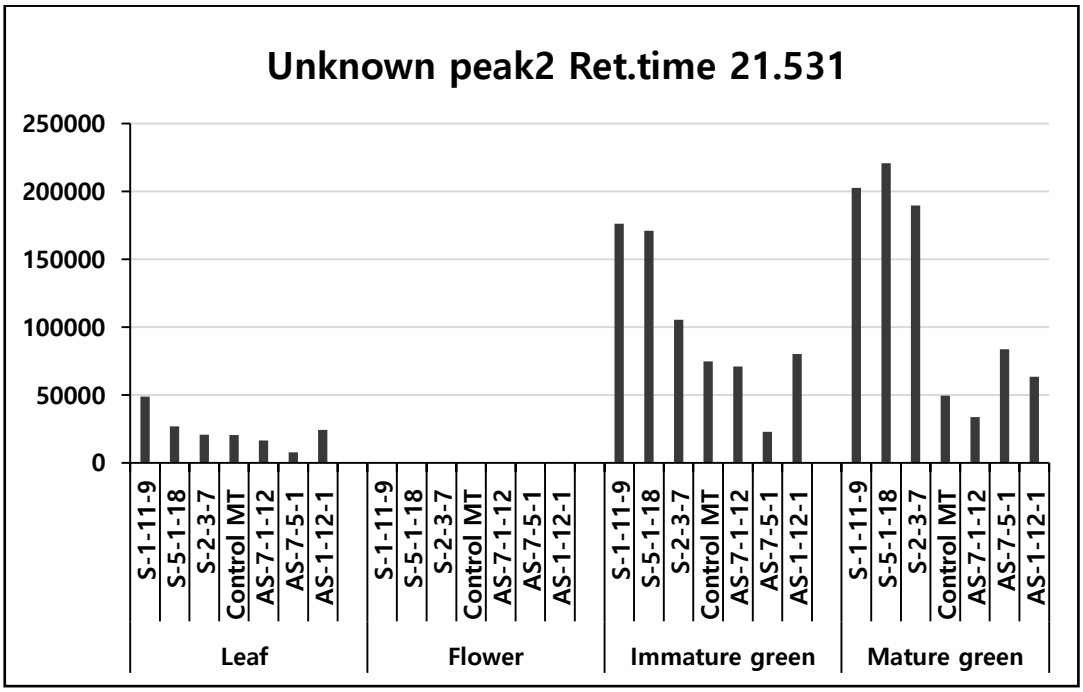
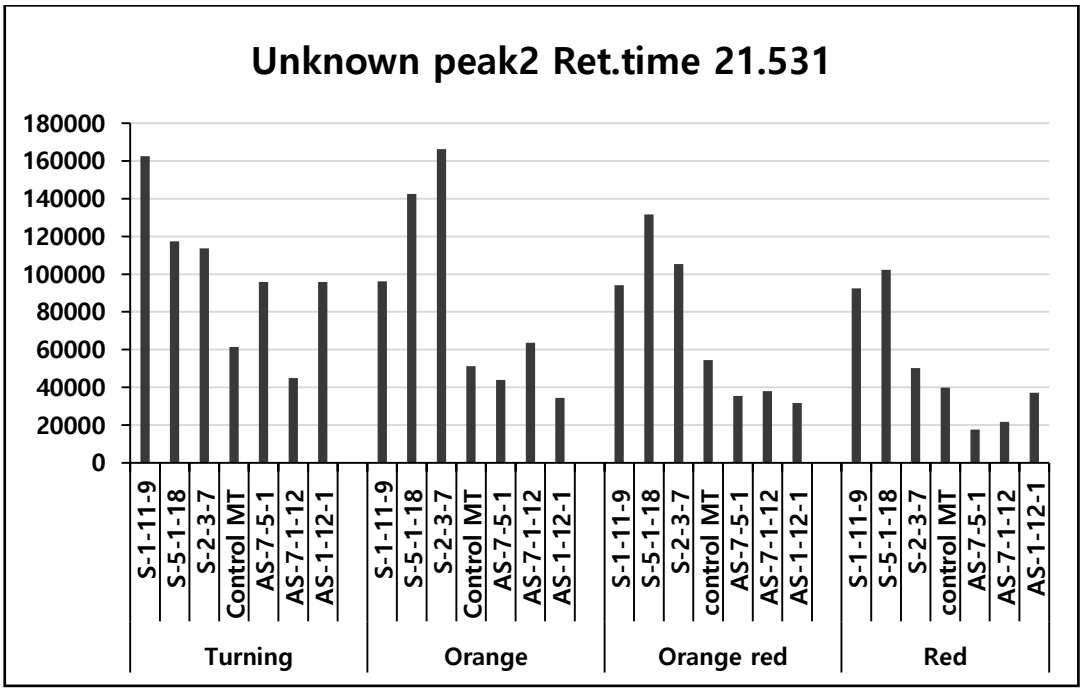


Table13 HPLC Result of unknown peak2.





IV.Conclusion

In this study We performed differential expressed genes (DEG) screening between the red citrus mutant and the wild-type citrus. Several genes related to pigment biosynthesis were selected from genes with higher expression than in the wild type. As a result, flavonoid 3'monooxygenase(*CuF3M*), an up-regulated gene in the mutant, was isolated. After the citrus genome database have been updated, the *CuF3M* gene showed the highest homology with the *CuCYP71A1* gene involved in brassinosteroid biosynthesisThe citrus *cuCYP71A1* gene is expressed in all of the transgenic tomato homozygous line flower, leaf, and fruit 6different stages. (fig11) The *CYP71A1* gene is more involved in the ripening process of fruit, and it can see that the expression of this gene has increased from the immature green stage to the red stage. The other CYP gene expressions are no difference between sense line, anti-sense line, and control MT(fig16,17,18,19,20). In the TFC experiment result is overexpressed sense line twice higher than antisense and control MT in fruit stages. There was no difference between leaves and flowers. Total polyphenol content and antioxidant activity are confirmed was no difference in all stages and lines. The sense line in the total flavonoid content experiment was high in the fruit, the expression level of flavonoid-related genes in the tomato flavonoid pathway was confirmed through RT-PCR analysis. The result was confirmed that there was no difference in the amount of gene expression. As described above, the High-Performance Liquid Chromatography experiment was confirmed by flavonoid analysis. The result was similar to the result of the total flavonoid content (TFC) experiment, the sense line was twice higher than anti-sense, and control MT in the fruit. It was also confirmed that there was no difference between leaves and flowers. The HPLC experiments we using for standards caffeic acid, naringenin, naringin, quercetin, myrcetin, rutin, and kaempferol. (fig22) Leaf and flowers are no different from all of the stage and line. Overexpressed sense line is twice higher than antisense and control MT on Naringenin, Rutin, Quercetin.(table8,9,12) No difference on

myrcetin. (table10) As a result effect of *cuCYP71A1* gene in transgenic tomato plant decreased seed size, increased tomato fruit flavonoid content. Citrus *cuCYP71A1*gene influenced micro-tom tomato seed size. When the citrus *cuCYP71A1* gene into the micro-tomato dry seeds, the size of the seed becomes smaller in the overexpression sense line, and the size of the seed not changed in the knock-out antisense line. In the selected T3 homo line *(cuCYP71a1),* the seed size of *CuCYP71A1*was smaller than that of the control plant. The number of seeds formed per fruit was higher in *CuCYP71A1*han in the control. In addition, the *CuCYP71A1*had smaller flowers, shorter root length, and lower germination rate than those of the WT. Previously reported genes related to seed size and development, were selected from the Micro-Tom tomato genome database and expression of the genes in *CuCYP71A1* plants was analyzed by the RT-PCR technique. It showed that some genes have different expressions compared to the WT. The results of this study suggest the possibility that the *CuCYP71A1* gene may play some important roles in seed size and development. (fig2, table3a3b3c)

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