Further fine mapping of flavonoids biosynthesis in *capsicum* F6 lines

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Abstract

Flavonoid is a group of naturally occurring polyphenolic compounds. It can determine the pigment in fruits and repel the UV light. Also, the flavonoids intake is beneficial to human health. Meanwhile, *capsicum*, also known as pepper, is one of the top popular vegetables around the world with the vast amount of health-related compounds. This project is aimed to find the genetic regulation of flavonoids biosynthesis in capsicum. A QTL localized on chromosome 5 and a candidate gene, transcription factor MYB12-like gene, were proposed as part of the regulatory mechanism of flavonoids biosynthesis. The Recombinant Introgression Lines (RILs) developed from a cross between *C.annuum* 'Long Sweet' and *C.chinense* 'AC2212'. Metabolic analysis revealed the MYB12-12like gene strongly linked to flavonoids accumulation in ripened pepper fruits. The expression of *flavonol synthase* FLS and *flavanone-3'-hydroxylase* F3H have downregulated the production of dihydroflavonols and flavonols in mature green fruits. Besides, to confirm the function of the MYB12-like gene, transgenic tomato lines containing the MYB12-like gene with overexpressed 35S promotor were developed. However, there is no evidence to proof expression of the MYB12-like gene can increase flavonoids structural genes synthesis in transgenic tomato lines. In conclusion, the MYB12-like gene can activate the genes encoding enzymes involved in flavonoids biosynthesis in *capsicum*, but its function in tomato still need further study.

Key words: flavonoids biosynthesis, MYB12-like gene

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Introduction

Capsicum genus

Capsicum is a genus of Solanaceae family including 30 species [1]. The cultivated species are known as pepper which is the top popular vegetable around the world. Based on its morphology characters, the five species are identified including *Capsicum annuum L., Capsicum chinense Jacq., Capsicum frutescens L., Capsicum baccatum L., Capsicum pubescens Ruiz and Pav.* Most of them are autogamous and pollination is by pollinators or by wind. Among the five major species, the *Capsicum annuum*. is the largest group of varieties and grown worldwide especially in the United States and Mexico [1]. The fruit of Capsicum varies by mature stages. The color of mature Capsicum fruits ranges from yellow to red, while immature fruits are green. The shape of Capsicum fruits is also diverged, from long narrow to spherical.

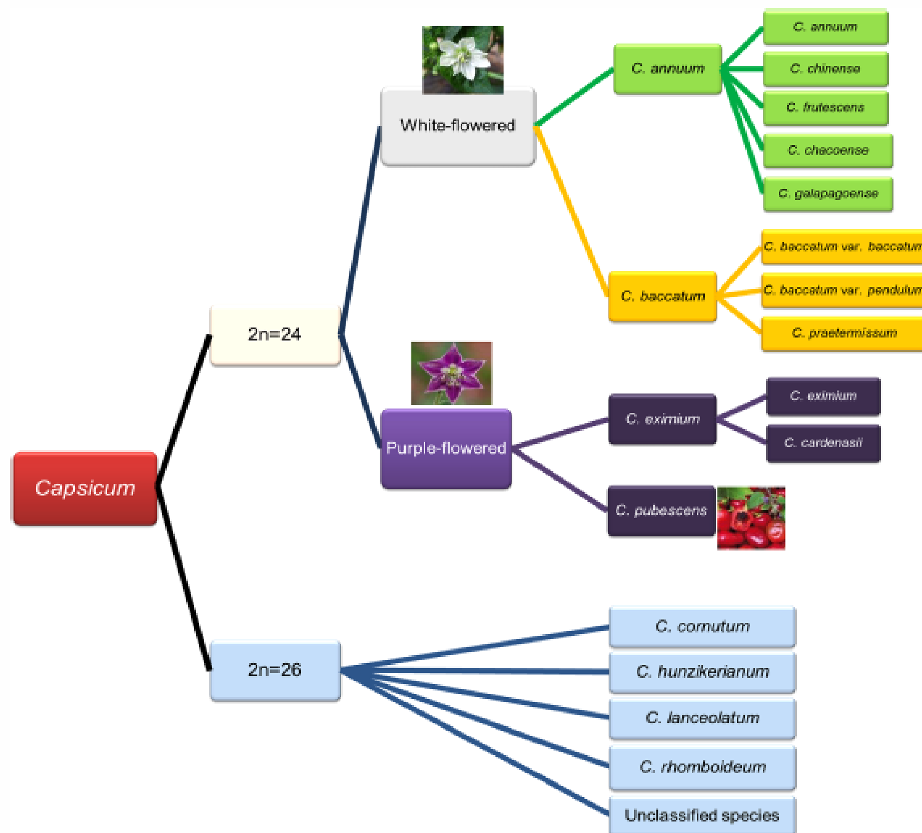


Figure 1 Capsicum family which contain two groups which are with 24 chromosomes and the other is 26 chromosomes [2].

Capsicum is diploid plants, but the number of chromosomes is not the same over all species (2n=24,26) [3]. A study showed that more semi-domesticated species are from 2n=24 and wild species are most likely from 2n=26 because of historical and botanical perspectives[2 3].
Due to species diversity, the use of pepper fruit is rich and colorful. Not only fresh but also dry pepper fruits as spice are widely used in the human diet. The sweet cultivated species, as known as sweet pepper or bell pepper, are wildly planted around the world as common dishes. While the pungent cultivated species which is known as paprika or chili peppers are more popular in Mexico, Indonesia, and China where local people like spicy tasty [3]. Since pepper fruits are abundant in health-related metabolites such as ascorbic acid (Vitamin C), tocopherols (Vitamin E) and flavonoids[2], pepper is also widely used in pharmaceutical industry.

Secondary metabolites

Secondary metabolites are wildly existed in the plant kingdom and used for pharmaceuticals, colors and food additives. However, secondary metabolites cannot be synthesized by the human, and 75% of the worldwide population relies on medicine based on plants materials five including pepper. Pepper as a top popular vegetable all over the world is a rich source of secondary metabolites including ascorbic acid, tocopherols, capsianoside, carotenoids and flavonoids related to human health. For example, ascorbic acid is an outstanding antioxidant which can reduce oxidative stress in the human body, therefore, reduce the possibility of oxidation related diseases[4]. In addition to the influence on people's health, flavonoids also have a function on plant defense system which can act as insecticidal phytochemicals and be used in insect pest management [6].

Flavonoids formation

Flavonoids are from secondary metabolites and universally distributed over plant kingdom. Flavonoids have various formations and are divided into six subgroups: flavanones, isoflavones, flavones, flavonols, flavan-3-ols, and anthocyanins (figure 2). Since flavonoids are the largest group of naturally occurring polyphenolic compounds[7], there are more than 6000 different flavonoids compounds exists now and this number is increasing with deep study[8].

Structurally, different flavonoids subgroups share the same skeleton (figure 2), which is a C6-C3-C6 structure [9]. This 15 carbon skeleton consists of two aromatic rings interconnected by heterocycle, which can be modified by methylation, methoxylation, oxidation, etc., which can result in flavonoids structural variations [2]. Based on the positions of modification, flavonoids can be classified into different classes, which are flavanones, isoflavones, flavones, flavonols, flavan-3-ols, and anthocyanins[10 11].

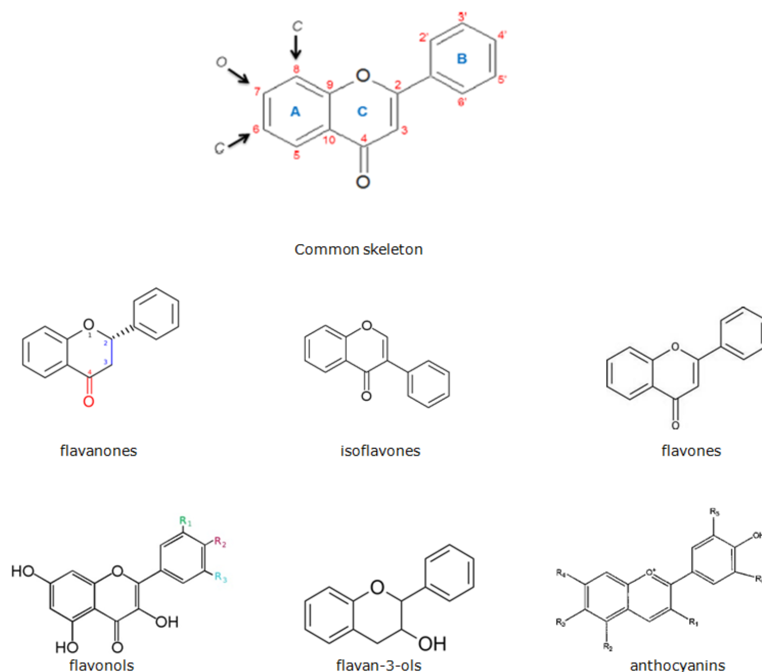


Figure 2 Flavones: are luteonin, apigenin and tangeritin; Flavonols: quercetin, kaempferol, myricetin, isorhamnetin, pachypodol and rhamnazin; Flavanones: hesteretin, naringenin and eriodictyol; Flavan-3-ols: catechins and epicatechins; Isoflavones: genistein, daidzein and glycitein; Anthocyanidins :cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin [12].

Flavonoids function

As a secondary metabolite, flavonoids are common and has different subgroups. Moreover, different flavonoids compounds have different biological functions not only at the plant but also for animals.

Flavonoids function in plants

Pigments in plants

The most well-known function of flavonoids is that it can help pigment color in flowers, fruits, and seeds[13]. Pigment study is one of the most interesting topics which have been studied for more than a century. Pigment coloring is a visual signal which can attract pollinators to have high fertilization rate. Compared to the background, the colorful flowers can be easily distinguished by pollinators[14].

The main factor of plant coloration is an accumulation of flavonoids. Over different flavonoids compounds, anthocyanins are an essential compound for pigment colouration[14] and synthesized through the multienzyme complex in plants. Anthocyanins are biosynthesized in ER and transported by glutathione transferase (GST) – like proteins into vacuole[14] where anthocyanins are accumulated.[8]. Nowadays, the enzyme coding genes have been isolated in Arabidopsis, tomato, etc. With further study, the expression of some structural genes is not stable during ripening stages and have the possibility to degrade over ripening[13].

UV protection

It is widely accepted that flavonoids can increase the protection again UV radiation in plants [15]. The studies of plant defense system are widespread in many model plants like Arabidopsis, Maize, and Petunia. All the study show that the UV radiation can induce flavonoids biosynthesis in plants[16]. Because of flavonoids structure, there is OH group which can inhibit the negative influence of free radicals by reducing free radical formation[10]. Over seven subgroups, flavonols play an uncharacterized role in the protection of UV radiation because it can function in numerous plants. Another flavonoids formation, flavones, also have a similar function in UV protection. In silks and maize, flavones are in high level when plants are exposed to UV-B radiation (280 -315 nm)[17]. Since FLS gene (figure 3) is the directly structural gene which is involved in flavonols biosynthesis, it can be regulated by UV-B radiation exposure intensity which can be affected by altitude[16].

Flavonoids function in human

Free radicals can be any molecules with single electron which is formed during ionization of oxygen, which can target cell membrane and cause DNA and protein damage[21]. The property changes of DNA and protein can lead to a devastating influence on cellular integrity as well as many diseases such as Malaria and HIV infection[19] [20]. To reduce the adverse effect of oxidants, antioxidants like flavonoids are an essential molecule for the human body to protect enzyme system. Recently, structure-activity studies show that flavonoids features are important to the cardio protective activity which can inhibit lipid peroxidation to maintain cellular homeostasis[22].

Moreover, it is shown that flavonoids can reduce the possibility of cancer by inhibition the core related enzyme [23] and reduce the oxidation lipoproteins. The previous study indicates that dietary intake of flavonoids can reduce the formation of oxidants. Because of its antioxidant function, flavonoids can be used in pharmacology as radical scavengers. Nowadays, flavonoids medical product can administer diseased tissue in the human body such as skin and throat[24].

Flavonoids biosynthesis pathway in plant

Flavonoids are a large group of polyphenolic compounds. They share the same skeleton with flavan nucleus which has two aromatic rings with six carbon atoms interconnected by a heterocycle [2] [25].

Flavonoids biosynthesis is through phenylpropanoids pathway. Phenylalanine is transferred into 4-coumaroyl-CoA which is an essential precursor for chalcone synthesis. However, it can also be transferred into P-coumaroyl quinic acid and return to phenylpropanoids biosynthesis cycle [26]. Chalcone is the primary flavonoids in this pathway, which is located on the central pathway [27]. There is a central pathway of flavonoids, and different groups of enzymes can participate to modified flavonoids skeleton[8]. In figure 3, isomerases, reductases and hydroxylases are directly participating flavonoids compounds biosynthesis and each of enzyme from the different group has different modification function [28].

As we are known, molecular regulation genes are including two classes: structural genes encoding the enzyme which can participate in metabolites biosynthesis and regulatory genes which can control structural gene, like transcription factor[29]. Thanks for the mutant study, the structural genes involved in flavonoids biosynthesis were isolated and confirmed the function in some model plants like Arabidopsis. In flavonoids biosynthesis pathway, each step is functioned by enzyme genes like *chalcone synthase* CHS and *chalcone isomerase* CHI. For regulatory genes, transcription factor CaMYB was isolated from chili pepper (*C.annuum L*.), and it can regulate FLS, CHI-2, CHS-2 and CHS-1 enzymatic activity[30 12].

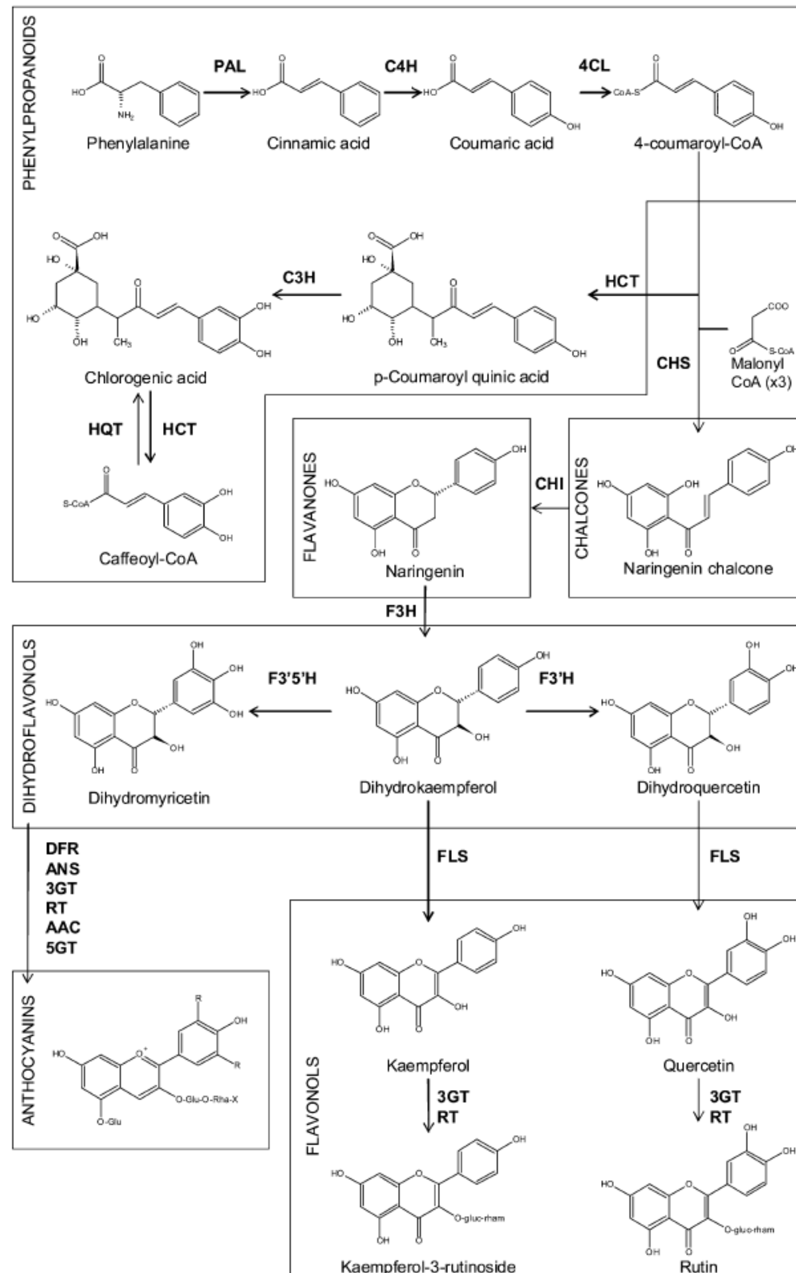


Figure 3 flavonoids biosynthetic pathway. Flavonoids biosynthesis in plants is through Phenylpropanoid [28]. HQT, hydroxycinnamoyl-CoA quinate transferase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3- hydroxylase; F3′H, flavonoid-3′-hydroxylase; F3′5′-H, flavonoid-3′5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3GT, flavonoid-3-O-glucosyltransferase; RT, flavonoid 3-O-glucoside-rhamnosyltransferase; AAC, anthocyanin acyltransferase; 5GT, flavonoid-5-glucosyltransferase.

Previous study in pepper

In Breeding for Quality group, we have previous projects about mapping flavonoids in pepper population. We analyzed a core collection of 31 diverse pepper accessions. Over these 31 accessions, we found *C.annuum* Long Sweet (No.12), which has significantly increased flavonoids level compared to other pepper accessions. However, another accession, AC2212 (No.24), it is with low flavonoids level but abundant in other health-related compound compared with most of other pepper accessions (figure 4). To elucidate the genetics underlying the high flavonoids trait, the high flavonoids phenotype accession, Long Sweet was crossed with AC2212 accession. Based on this cross, the segregated F2 population was developed and genotyped work co-operated with Ilan Paran lab, Israel. We analyzed F2 population and developed linkage map. Based on the linkage map we contracted, we found there are two QTL regions are related with high flavonoids phenotype (table 1). Q1 is located on chromosome 4 and linked with Quercetin, Capsianoside, and Luteolin. Another QTL is MYB12-like gene. Compared with Q1, MYB12-like (CA05g18430) QTL is a transcription factor that can regulate flavonoids biosynthesis. The total length of the MYB12-like gene is 1203 bp, and we have expressed the MYB12-like gene in the fruit of transgenic tomatoes with overexpressed 35S promotor. Meanwhile, some negative control tomato lines without overexpressed MYB12-like gene were selected as contrast.

Table 1 Two QTL regions related with high flavonoids phenotype

QTL name	QTL location	The metabolite
Q1	Chromosome #4	Quercetin, Capsianoside, Luteolin
MYB12-like	Chromosome #5	Quercetin, Luteolin, Naringenin

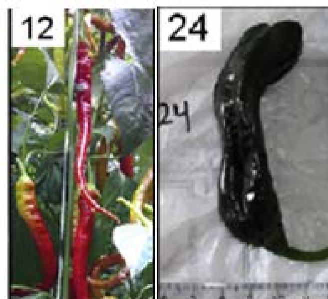


Figure 4 No.12 is Long Sweet accession and No.24 is AC2212. A genetic linkage map is constructed based on these two parental lines.

Research aim

In this project, we focused on the MYB12-like gene function and the expression level determination of structural genes involved in flavonoids biosynthesis:

1. Functional analysis of MYB12-like gene in transgenic tomato lines
2. Metabolic analysis of flavonoids in F6 pepper population
3. Functional analysis of MYB12-like gene in F6 pepper population

Material and Method

Plant material and sample preparation

Recently, the cDNA encoding MYB12-like gene has been cloned, and transgenic tomato with overexpressed 35S promotor has been developed. In contrast, tomato lines without MYB12-like gene are negative control samples in this project. In total, ten transgenic tomato samples and four negative control plants were developed in the I.Paran lab, Israel and shipped to Netherlands at -80.0 degrees. Meanwhile, seven families were selected which are 105, 107, 134, 165, 190, 208 and 47 in RILs F6 population based on marker assisted selection (MAS). Most regions are homozygous except certain QTL regions. Plants were cultivated in rock wool media under irrigated conditions and grown in a greenhouse in Wageningen (Netherlands). Fruits were harvested at mature green and mature red stage. In total, 16 mature red samples and 21 mature green samples from the F6 population were used to analyze the influence of the MYB12-like gene on flavonoids biosynthesis.

Extraction and Metabolic analysis:

After fruits had harvested, liquid nitrogen was applied to frozen them immediately and then were transported from Israel to Netherlands with -80-degree storage. Frozen samples were ground in liquid nitrogen by basic grinder into fine powders, which were stored in separate 50ml tubes at -80. After well ground, we weighted each powder of samples between 0.485 and 0.515 grams and stored them in 2ml Eppendorf at -80-degree. If the amount is larger than this interval, it would be difficult to balance some different samples during dilution. Biochemical analysis of flavonoids needs to dilute ground powder into 1.5mL of 99% methanol and 0.1% formic acid. After well vortex, the dilution was put in ultrasonic bath machine for 15 minutes, which can break cell walls and different compounds can dissolve well in liquid. We transferred supernatant into fresh tubes after centrifuging at 20,000 g for 15 min. 118 samples were divided into three plates, and on each plate, there is two quality control which is the mixture of same several types of samples. After extraction, the samples were applied Liquid chromatography–mass spectrometry(LC-MS).

RNA extraction and cDNA synthesis

RNA isolation in tomato lines

RNA extraction in tomato lines was used the same samples as those for metabolic analysis. The extraction was performed from two spoonfuls of frozen sample powers of transgenic tomato samples and negative control samples using Tripure, Chloroform isopropanol, and DNAse according to Tripure protocol (Appendix 1).

The RNA extraction in pepper was performed from 5mg of frozen ground samples using QuickGene RNA extraction kit according to manufacturer's instructions. Nanodrop spectrophotometer and QUIBIT measured the quantity of RNA. After quantity measurement, RNA samples were evaluated by electrophoresis using 2% TAE gel for quality check. cDNA was synthesized from 200ng RNA by TaqMan Reverse Transcription according to manufacturer's instructions. In total, ten ng cDNA was synthesized for each sample.

Structural gene selection and primer design

The structural genes related to flavonoids biosynthesis were selected for gene expression analysis from Solgenomics database *(https://solgenomics.net*). Also, the genomic information of the MYB12-like gene needs to be identified before performing expression analysis. Besides, the PCR primers of structural genes and the MYB12-like gene were designed by Clonemanager 8, and the Primer3Plus program(*http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi*) was employed for qPCR primers design. All primer information of structural genes and MYB12-like gene were described in Appendix 2, 3 and 4.

Gene expression analysis by qPCR

Real-time quantitative PCR was used to determine the expression level of specific genes. It is based on standard PCR principle which is based on cDNA amplification. It was executed as described in Protocol for Real-Time quantitative PCR by using Bio-Rad CFX96 Real-Time System. The qPCR is performed in a volume of 20 µL per well. Therefore, the reaction volume is 44 µL that means there are two replications of one sample (Appendix 1).

The qPCR result analysis is used by Bio-Rad iQ 5 program and Microsoft Excel. Bio-Rad iQ 5 was utilized for an open result and check the Ct value, but for analysis, data was copied on Excel and calculate relative gene expression. The expression level of reference gene which is a housekeeping gene was used to help calculate the relative gene expression (RGE). The calculation is based on the ΔCt calculation which is for data without wild type material. The formulas are as follow:

- $\Delta Ct = Ct_{(reference\ gene)} - Ct_{(gene\ of\ interest)}$
- $RGE = 2^{\Delta ct}$

Besides, LinRegPCR program was used to analyze PCR efficiency which should be in the range of 1.9 to 2.1.

Statistical analysis

All tables and figures in this project were based on mean ± S.E.M. The differences between genotypes and treatment were analyzed by one-way. If the P-value is smaller than 0.05 which is the level of significance, we believe there is a significant difference which can reject the null hypothesis.

For linkage analysis, LOG score is based on the P-value of one-way ANOVA. It is calculated by a formula: log^{10}(p-value). If LOG score is above 3, we believe there is a significant linkage between QTL and specific flavonoids compound. Meanwhile, the Genstat and Microsoft Excel were employed for the statistical analysis.

Result

Metabolic analysis of flavonoids phenotype

In RILs F6 lines, we have seven families including 105, 107, 134, 165, 190, 208 and 47, which are revealed from the F2 population. The metabolic analysis indicated the abundance of flavonoids, capsianosides and the glycosides of luteolin, quercetin, naringenin as well as naringenin chalcone were the major flavonoids in the F6 population. However, these metabolites varied from different F6 family referred to different genotype. The four QTLs (table 3) were employed to declare the presence of linkage ship with various F6 families.

There were no significant linkage relationships between QTLs and metabolites in family 105, 134 and 165 (table 2). The quercetin 3-O-rhamnoside and quercetin 3-O-rhamnoside-7-O-glucoside were predominant flavonol glycosides and highly linked with MYB12-like QTL in F6 family 47 208, 190 and 107. Besides, luteolin 6-C-hexoside, Luteolin 8-C-hexoside and Luteolin 6-C-hexoside-8-C-pentoside have significant linkage with MYB12-like QTL in family 190, 208 and 47. However, although the capsianosides and phenolic acids have been detected in F6 population, no significant linkage was shown in all F6 families.

Table 2 Result of metabolic analysis in each F6 lines. There are seven F6 lines which are 105, 107, 134, 165, 190, 208, 47.

Group	Family lines	105	107	134	165	190	208	47
Capsianoside	Capsianoside V							
Capsianoside	Capsianoside X-1							
Capsianoside	Capsianoside IX							
Capsianoside	Capsianoside IX							
Flavonoid	Quercetin rhamnoside-glucoside							Myb12
Flavonoid	Luteolin 6,8-di-C-hexoside					Myb12	Myb12	Myb12
Flavonoid	Naringenin-C-diglycoside I		Myb12					
Flavonoid	Quercetin 3-O-rhamnoside-7-O-glucoside							Myb12
Flavonoid	Luteolin 6-C-hexoside-8-C-pentoside					Myb12	Myb12	Myb12
Flavonoid	Apigenin 6,8-di-C-hexoside							
Flavonoid	Luteolin 6-C-pentoside-8-C-hexoside					Myb12	Myb12	Myb12
Flavonoid	Apigenin 6-C-pentoside-8-C-hexoside						Myb12	Myb12
Flavonoid	Luteolin 6-C-hexoside					Myb12	Myb12	Myb12
Flavonoid	Luteolin 8-C-hexoside					Myb12	Myb12	Myb12
Flavonoid	Naringenin-O-hexose 3		Myb12				Myb12	Myb12
Flavonoid	Naringenin-O-hexose 3		Myb12				Myb12	Myb12
Flavonoid	Phloretin-C-diglycoside						Myb12	Myb12
Flavonoid	Luteolin 7-O-(2-apiosyl)-glucoside							Myb12
Flavonoid	Luteolin-di-hexose					Myb12	Myb12	Myb12
Flavonoid	Quercetin 3-O-glucoside					Myb12		Myb12
Flavonoid	Kaempferol 3-O-rutinoside							
Flavonoid	Icariside E5.							
Flavonoid	Naringenin O-Pentose-diglucose		Myb12					Myb12
Flavonoid	Quercetin rhamnoside-glucoside							Myb12
Flavonoid	Quercetin 3-O-rhamnoside		Myb12			Myb12	Myb12	Myb12
Flavonoid	Luteolin (apiosyl-acetyl)-glucoside		Myb12					

Flavonoid	Luteolin-Methyl-O-di-hexose		Myb12					
Flavonoid	Kaempferol-synapoyl-hexose-pentose							
Flavonoid	Flavonoid glycosides		Myb12					Myb12
Flavonoid	Naringenin Chalcone							
Phenolic acid	Caffeic acid 3-glucoside							
Phenolic acid	3-Caffeoylquinic acid (Chlorogenic acid)							
Phenolic acid	Coumaric acid-hexose III							
Phenolic acid	Ferulic acid-hexose II							
Phenolic acid	Ferulic acid-hexose II_1							
Phenolic acid	trans-p-Sinapoyl beta-D-glucopyranoside							

Table 3 QTL information from the previous study. These four markers were selected by marker analysis with flavonoids phenotype. Q1, MYB12-like, J6 and EV1 are located different chromosomes and related with various flavonoids phenotype. In specific F6 lines, we can investigate QTL is related to specific replication lines.

QTL name	QTL location	The metabolite	Lines
Q1	Chromosome #4	Quercetin, Capsianoside, Luteolin	105, 107, 165
MYB12-like	Chromosome #5	Quercetin, Luteolin, Naringenin	47, 107, 190, 208
J6	Chromosome #9	Capsianoside	105, 134
EV1	Chromosome #10	Capsianoside	105, 190

It is shown in table 4 that the quercetin is linked with MYB12-like QTL in family 47 and 107. Also, Luteolin has significant linkage with MYB12-like QTL in 47, 190, 208, and Naringenin significantly linked with MYB12-like QTL in family 107,208 and 47. Besides, both Apigenin and Phloretin are associated with MYB12-like QTL in family 208 and 47.

Table 4 Metabolic result of F6 separate families.

The metabolite	Lines
Quercetin	47, 107
Luteolin	47, 190, 208
Naringenin	107, 208, 47
Apigenin	208, 47
Phloretin	208, 47

Functional analysis of transgenic tomato lines

Expression level of MYB12-like gene in tomato lines

Before starting the analysis, it is necessary to select a prepare reference gene. We have three housekeeping genes which are ß-Actin, L33 and L33-2 in our lab. After qPCR analysis of three housekeeping genes, L33 was selected as the reference gene in this section due to the smallest standard deviation.

For identify MYB12-like gene expression, two forward primers were designed, which is MYB12-like_FOR180 and MYB12-like_FOR 238. Primer sequences are described in Appendix 1.

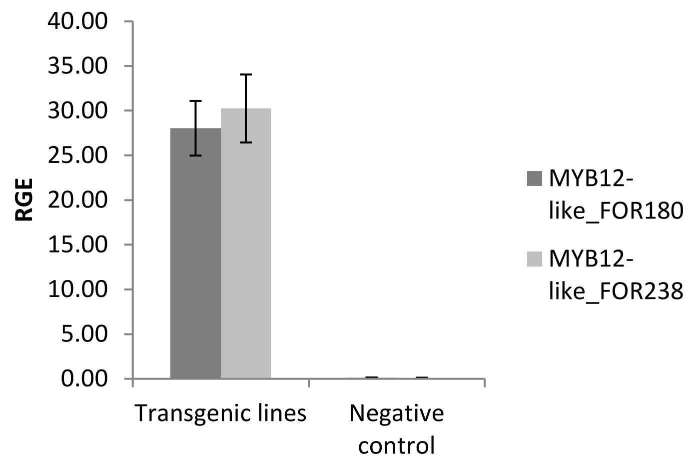


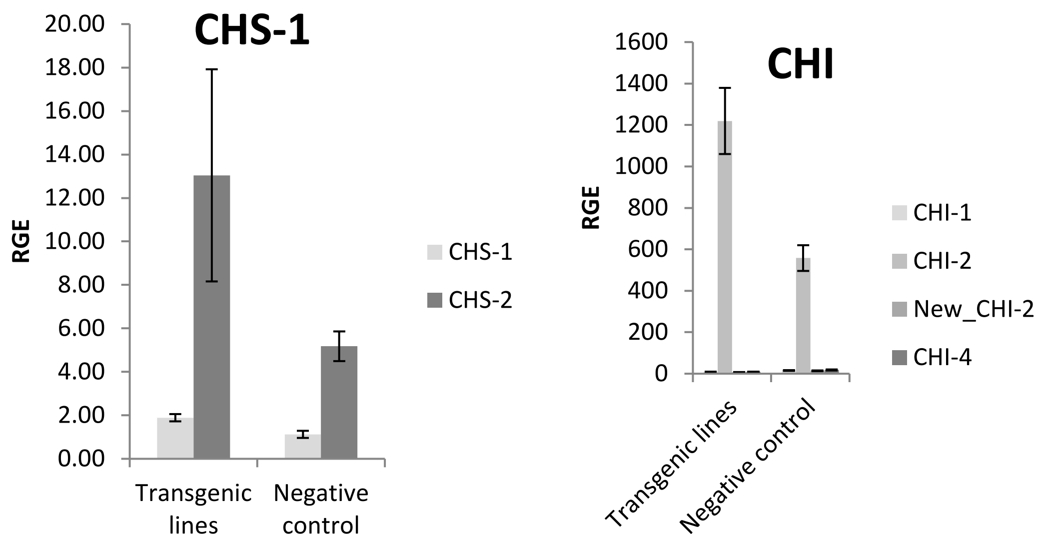
Figure 5 Expression level over tomato lines. ▇ is MYB12-like_FOR 180, ▇ is MYB12-like_FOR238. Standard error of MYB12_like_FOR180 is 0.041171 whereas MYB12

Based on the qPCR result, there are four samples which are NO.8, 14, 18 and -13 have obviously higher Ct value of MYB12-like gene compared with other samples. After Relative Gene Expression (RGE) calculation (Figure 5), mean of transgenic lines is 28.03 for MYB12_FOR180 while for MYB12-like_FOR238 is 30.25. In the negative control group, the average of MYB12-like_FOR180 is 0.11 compared with MYB12-like_FOR238 is 0.09.

Moreover, tomato samples are divided into transgenic group and negative control group based on the expression of the MYB12-like gene. The transgenic group is including No.1, 3, 5, 6, -7, 9, 11, 12, 16 which have higher expression level of the MYB12-like gene compared with sample No.8, 14 and 18 which are the negative control group.

Expression level of candidate structural genes involved in flavonoids biosynthetic pathway

To gain insight how MYB12-like gene regulated flavonoids biosynthetic pathway, the expression of structural genes including CHS, CHI, FLS, 3GT and F3H involved in flavonoids biosynthesis and phenylpropanoids biosynthesis was analyzed by real-time quantitative RT-PCR.



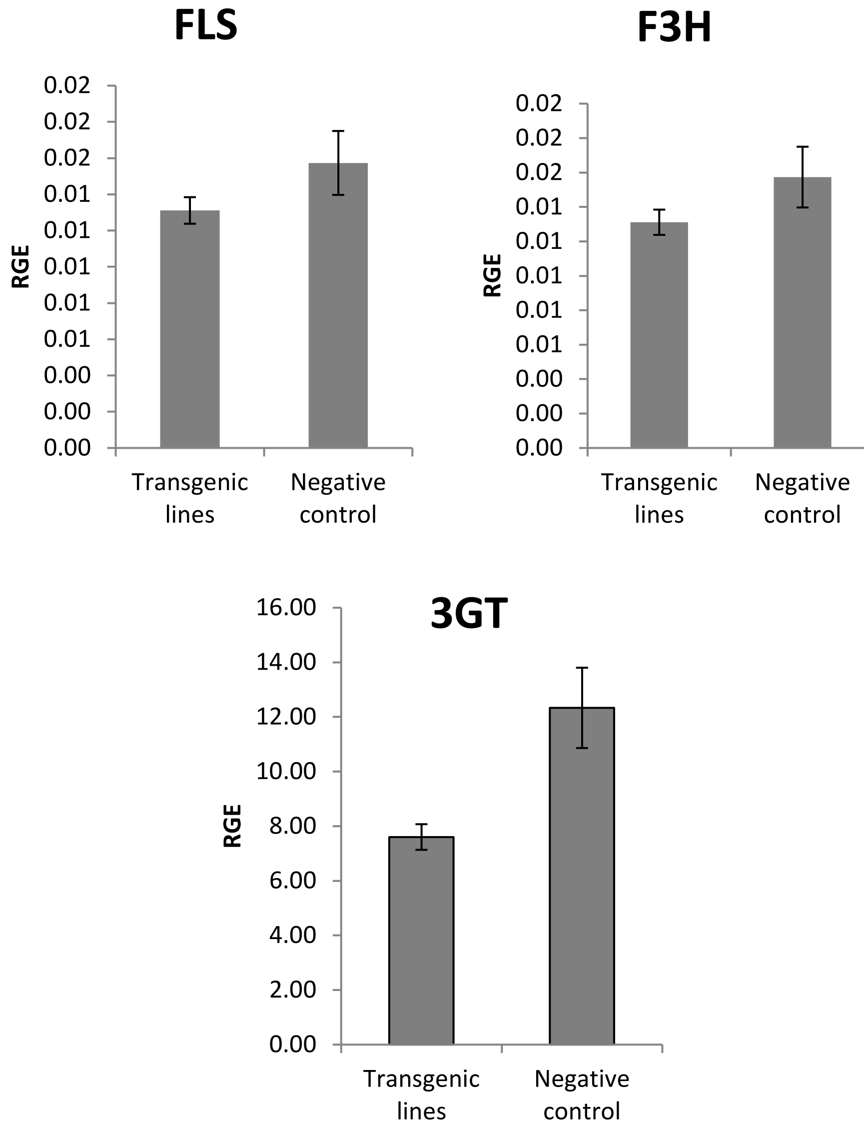


Figure 6 Mean of the expression level of structural genes involved in flavonoids biosynthesis. Error bars represent SEM. RGE was calculated based on LOG_2^{RGE} value of structural genes which are *Chalcone isomerase* CHI, *Chalcone synthase* CHS, *Flavonol synthase* FLS, *Flavanone 3-hydroxylase* F3H and *flavonoid-3-O-glucosyltransferase* 3GT.

The FLS and F3H showed deficient expression levels compared with other structural genes (figure 6), suggesting MYB12-like not with a big influence on the expression of FLS and F3H in tomato. From figure 6, the expression level of CHS-1 and CHI-2 with highest expression level over other homologs and it suggested they are the most informative candidate genes in the transgenic tomato lines. Although expression level of CHS and CHI genes related to flavonoids biosynthesis is higher in transgenic tomato group compared with negative control group, there is no significant difference between two groups. This section was extended with other candidate genes from the upstream phenylpropanoid pathway, i.e., PAL, 4CL, C4H, HQT, and 3GT.

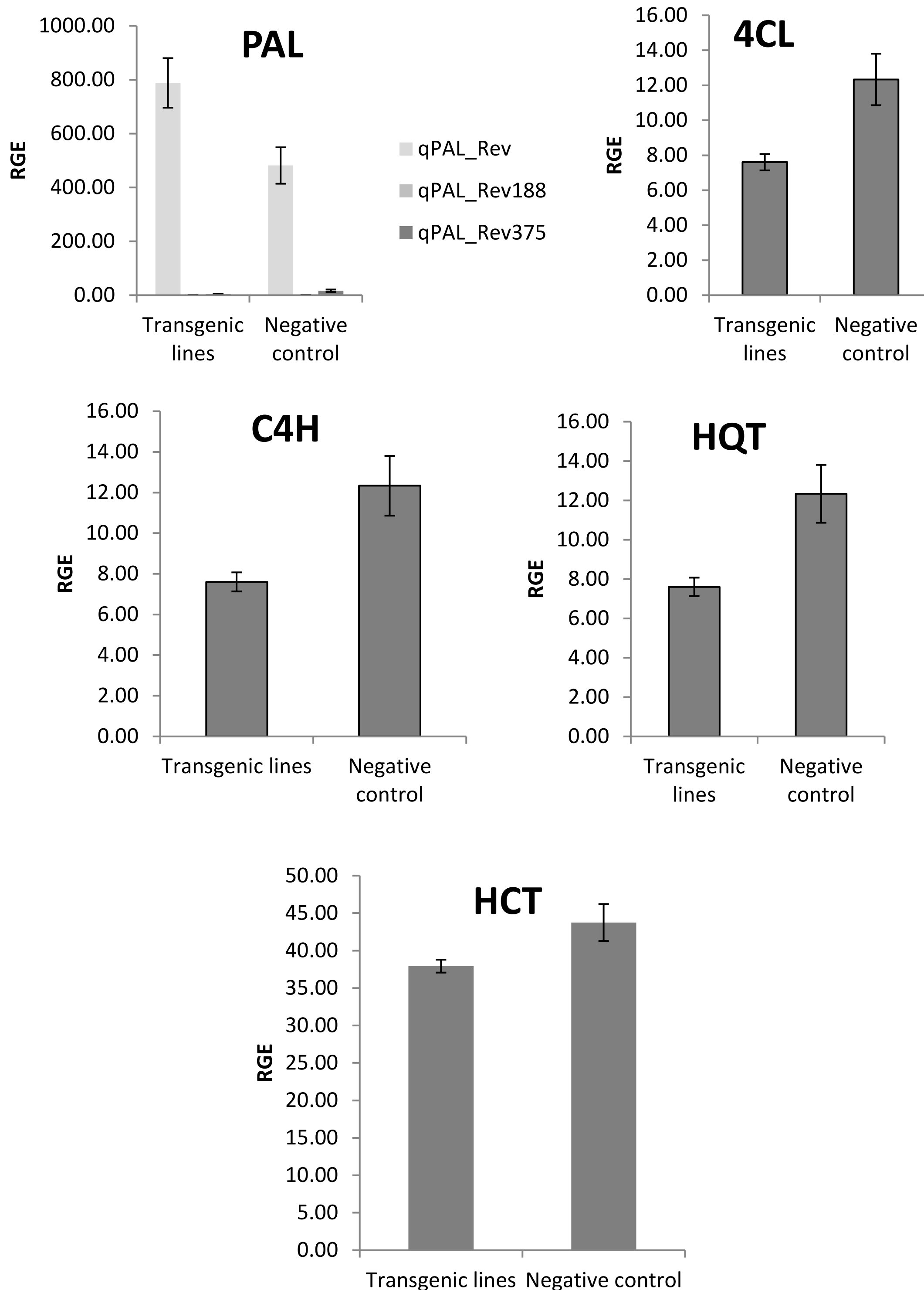


Figure 7 Mean of the expression level of structural genes involved in Phenylpropanoids biosynthesis. Error bars represent SEM. RGE was calculated based on the LOG_2^{RGE} value of structural genes which are *Phenylalanine ammonia lyase* PAL, *4-coumarate: CoA ligase* 4CL, *Cinnamate 4-hydroxylase* C4H, *hydroxycinnamoyl-CoA quinate transferase* HQT and *Hydroxycinnamoyltransferase* HCT.

The expression analysis was shown in figure 7. The PAL with primer qPAL_Rev is the most active PAL gene over three homologs. Moreover, only the PAL gene showed higher expression level in transgenic tomato lines. For the case of other structural genes, the expression levels in transgenic lines were lower

than in negative control lines. However, although the expression of PAL indicated a higher expression in transgenic lines, there is no significant difference between two groups (P-value of T-test is 0.3402).

Sequencing of MYB12-like gene

Sequencing technology was carried out to confirm the cDNA of the MYB12-like gene was successfully cloned in tomato samples. Six cDNA fragments which are amplified by PCR were used for sequencing (figure 8). Two pairs of primers were designed for the ideal fragments by PCR and primer sequences were described in Appendix 2.

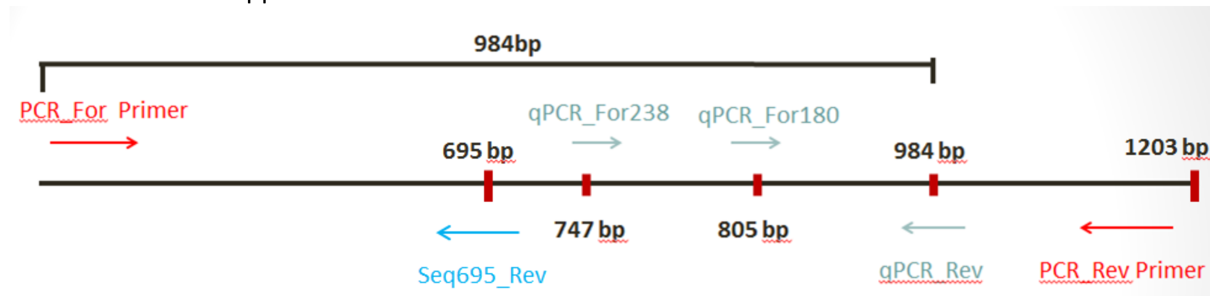


Figure 8 Graph of primers. The total length of the MYB12-like gene is 1203bp. Based on three forward primers and three reverse primers, six DNA fragments are used to analyze.

Compared the consensus with parent lines (No.12 and No. 24), two amino acids changes were determined (figure 9). Mutant of position 441 is that Leucine which is from hydrophobic side group turned into Arginine from positive electrically charged side chain group. Glutamine is from uncharged side chain group and changed into Arginine at position 632 (table 5). However, these two amino acid changes cannot consequence protein function loss (by *provean.jcvi.org/index.php)*.

Table 5 Amino acid changes between consensus and parental lines. The result of protein function check by Provean (*provean.jcvi.org/index.php*). "Neutral" means the function will not be modified because of amino acid mutant. L and R; Q and R are the amino acid difference between parent lines and consensus. Position 161 and 212 means in which amino acid position they changed.

position	Theoretical	Amino acid group	Consensus	Amino acid group
441	Leucine	Hydrophobic side chains	Arginine	Positive electrically charged side chains
632	Glutamine	Uncharged side chains	Arginine	Positive electrically charged side chains

```
MYB12-like-1      1 mkapccekvglkrgkwtieedellvkyiqangegswrslpknagllrcgkscrlrwtnylrpnlkrgkft
MYB12-like-2      1 mkapccekvglkrgkwtieedellvkyiqangegswrslpknagllrcgkscrlrwtnylrpnlkrgkft
consensus MY     1 mkapccekvglkrgkwtieedellvkyiqangegswrslpknagllrcgkscrlrwtnylrpnlkrgkft

MYB12-like-1    211 aeedetivklhstlgnrwsliaqdlpgrtdneiknywnsnlrrrlysfrnfkeliktttnvpkiavdgds
MYB12-like-2    211 aeedetivklhstlgnrwsliaqdlpgrtdneiknywnsnlrrrlysfrnfkeliktttnvpkiavdgds
consensus MY    211 aeedetivklhstlgnrwsliaqdlpgrtdneiknywnsnlrrrlysfrnfkeliktttnvpkiavdgds

MYB12-like-1    421 deslrkqdtstmskegsiesllpednivvdrgstrknrslcvgsnegatwsegdstesllpenctvdigs
MYB12-like-2    421 deslrkqdtstmskegsiesrlpednivvdrgstrknrslcvgsnegatwsegdstesllpenctvdigs
consensus MY    421 deslrkqdtstmskegsiesrlpednivvdrgstrknrslcvgsnegatwsegdstesllpenctvdigs

MYB12-like-1    631 mqkpysscprgkddvpwyrggstesllleniei nfaceensigsllmencsspggrddtswseeggsfks
MYB12-like-2    631 mrkpysscprgkddvpwyrggstesllleniei nfaceensigsllmencsspggrddtswseeggsfks
consensus MY    631 mrkpysscprgkddvpwyrggstesllleniei nfaceensigsllmencsspggrddtswseeggsfks

MYB12-like-1    841 lllenietnfscpkgtcnatwseggsveffslencvvdlgsnrenkpvwsdkggsieyllltenytvdigf
MYB12-like-2    841 lllenietnfscpkgtcnatwseggsveffslencvvdlgsnrenkpvwsdkggsieyllltenytvdigf
consensus MY    841 lllenietnfscpkgtcnatwseggsveffslencvvdlgsnrenkpvwsdkggsieyllltenytvdigf

MYB12-like-1   1051 nretnssscprgnaeamwyeegssiessstesyivdlgktsqtssilinv*
MYB12-like-2   1051 nretnssscprgnaeamwyeegssiessstesyivdlgktsqtssilinv*
consensus MY   1051 nretnssscprgnaeamwyeegssiessstesyivdlgktsqtssilinv*
```

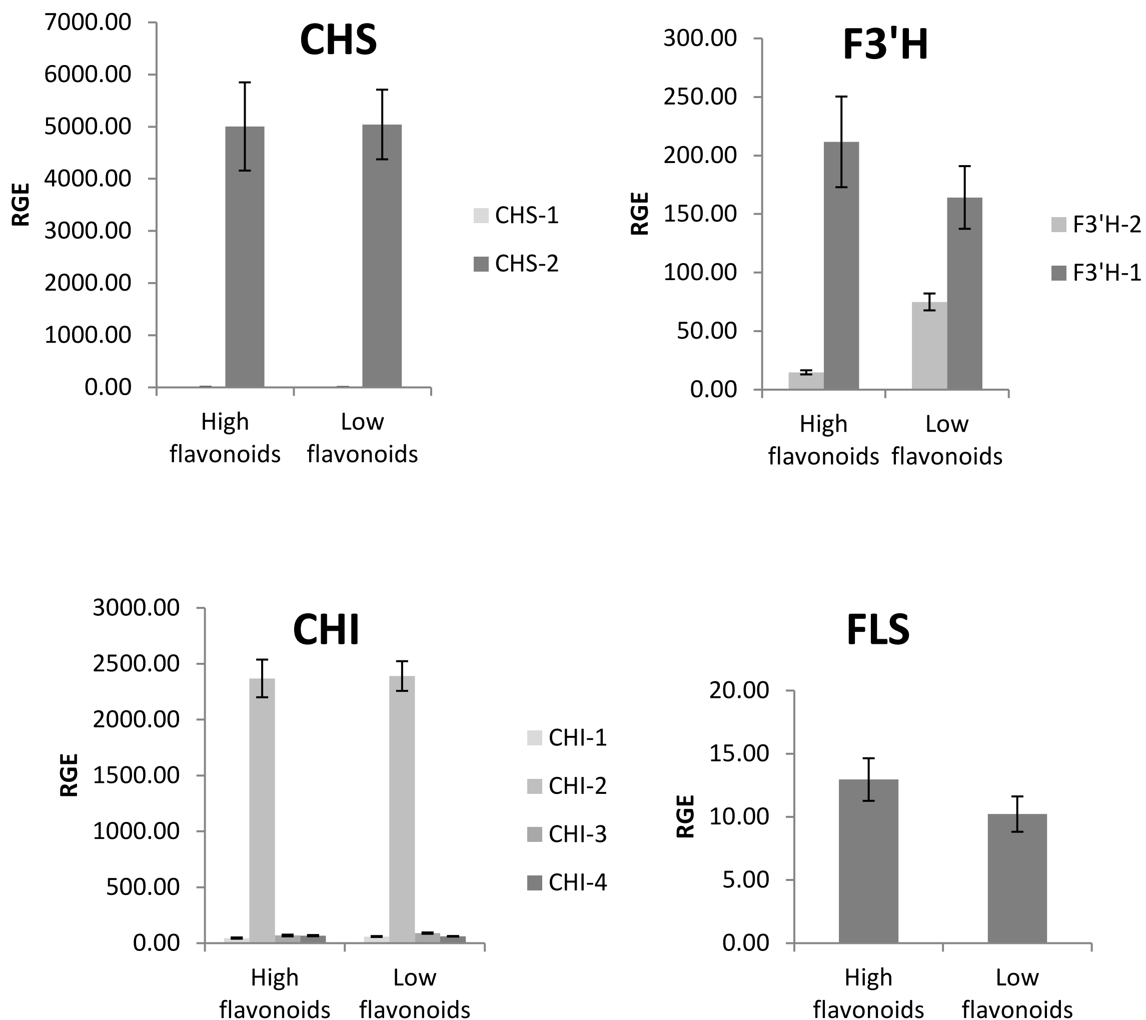
Figure 9 Alignment with MYB12-like gene amino acid over two parent lines (No.12 and No.24) and consensus. There are two amino acid changes.

Functional analysis of pepper F6 population

Function of MYB12-like gene in pepper F6 lines

In this section, Ubiquitin gene was selected as reference gene due to the smallest standard deviation. 20 pepper F6 samples have been selected based on flavonoids variation, and they are separated into the high-flavonoids group with MYB12-like gene and low-flavonoids group without the MYB12-like gene. However, the Ct value of the MYB12-like gene in pepper is quite high, and all Ct values are above 35 cycles. Normally, in the qPCR result, if the Ct value is above 35.0, it means there is almost nothing expressed.

Expression level of candidate genes in pepper F6 lines



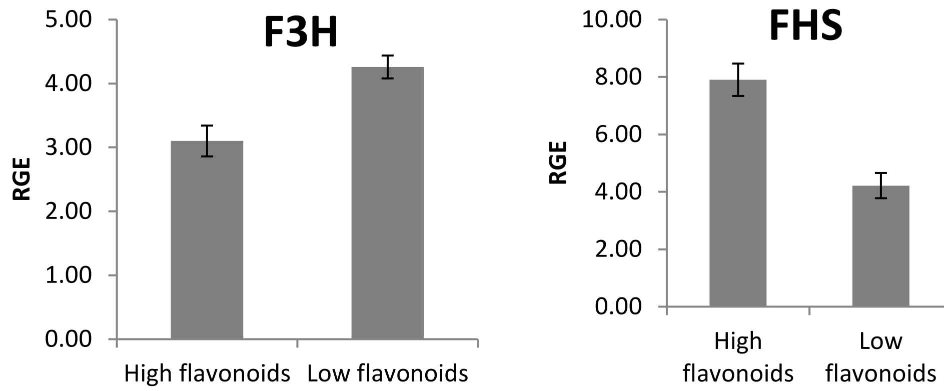


Figure 10 Mean expression level of structural genes involved in flavonoids biosynthesis. Error bars represent SEM. RGE was calculated based on the LOG_2^{RGE} value of structural genes which are *Chalcone isomerase* CHI, *Chalcone synthase* CHS, *Flavonol synthase* FLS, *Flavanone 3-hydroxylase* F3H, *Flavonoid 3`-hydroxylase* F3′H, *Flavonol synthase* FLS and *Flavonoid hydroxylases* FHS.

Two CHS homologs were tested, and the CHS-2 showed higher expression level over both high and low-flavonoids groups, suggesting the CHS-2 is the most functional CHS candidate (figure 10). Also, CHI-1 and F3'H-1 showed higher expression level over other homologs, and MYB12-like can lead to a positive influence on them. However, the statistically significant difference was not determined among all structural genes.

Functional analysis of pepper F6 mature green samples

Expression level of MYB12-like gene

Compared with the expression of the MYB12-like gene in mature red fruits, it was much higher, and the mean Ct value is 31.01 for MYB12-like_128 and 31.00 for MYB12-like_208. Form figure 11, it is evident that mean RGE value of low flavonoids group is lower than high flavonoids group. However, the difference between two groups is not significant based on statistical analysis (p-value is 0.3578 which is larger than 0.05).

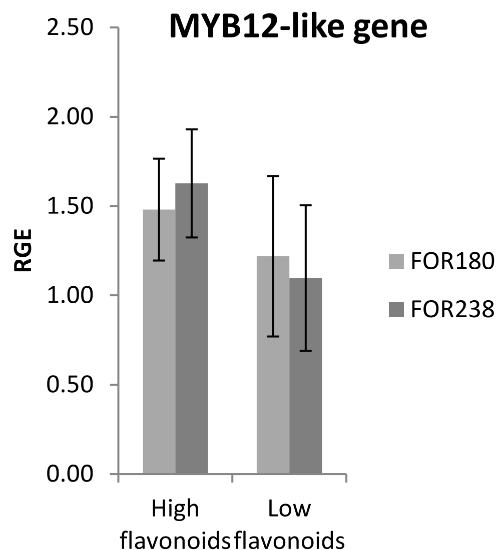


Figure 11 Relative gene expression of the MYB12-like gene in pepper F6 lines. Error bars are based on SEM. ■ is MYB12-like_FOR 180, ■ is MYB12-like_FOR238.

Expression level of structural genes in mature green fruits

In this section, six structural flavonoids pathway genes were tested in mature green pepper, which is CHS, CHI, FLS, F3H, F3'H and FHS-2 and primer sequences are described in Appendix 3.

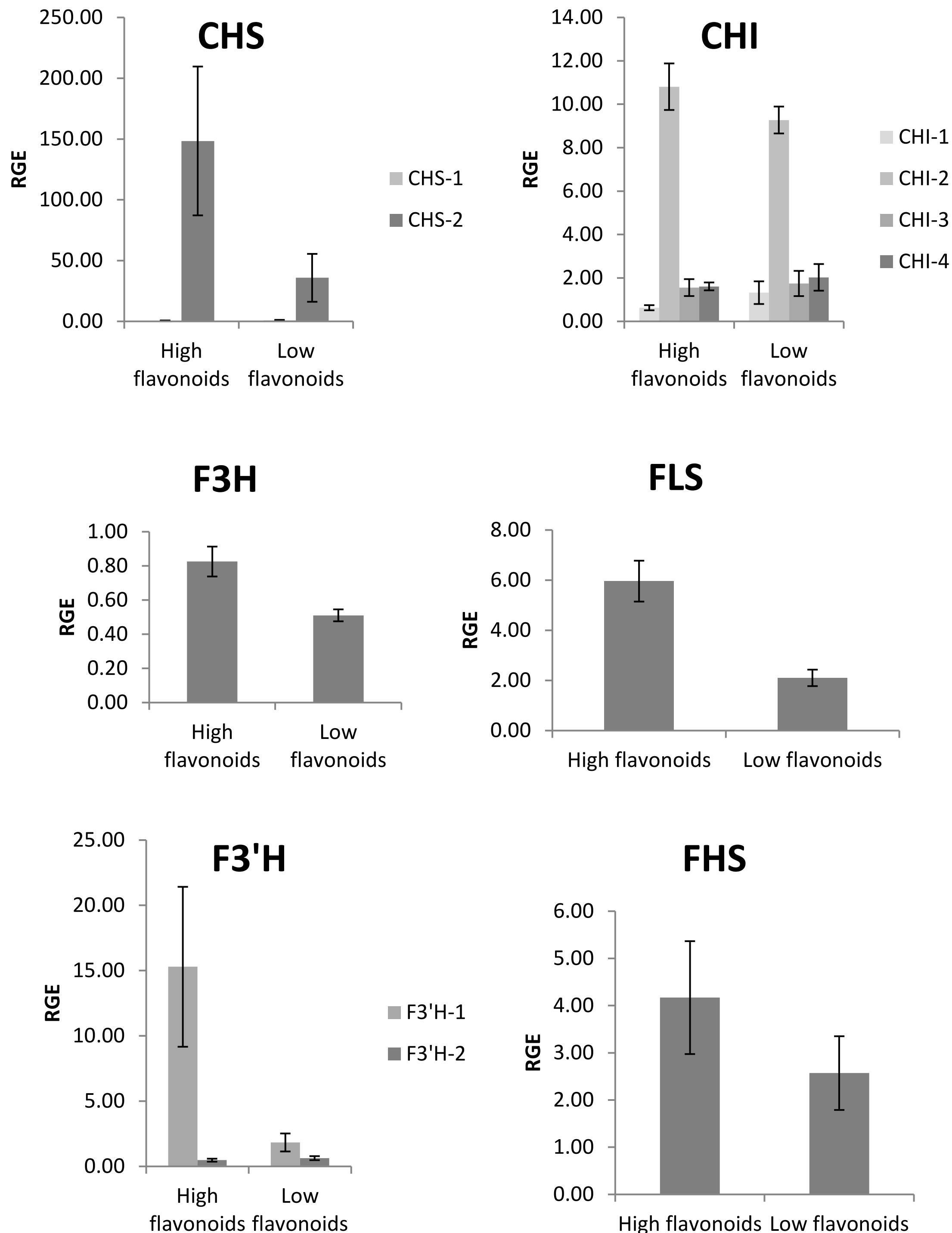


Figure 12 Mean expression level of structural genes involved in flavonoids biosynthesis. Error bars represent SEM. RGE was calculated based on the LOG_2^{RGE} value of structural genes which are *Chalcone isomerase* CHI, *Chalcone synthase* CHS, *Flavonol synthase* FLS, *Flavanone 3-hydroxylase* F3H, *Flavonoid 3`-hydroxylase* F3'H, *Flavonol synthase* FLS and *Flavonoid hydroxylases* FHS.

The qPCR result revealed that the CHS-2, CHI-2, F3'H-1 were easily influenced by MYB12-like gene over all homologs due to the highest increased expression level. Also, the CHS-2 expression higher in the high-flavonoids group compared with the group without the MYB12-like gene. However, there is no

significant difference between two groups by use of statistical analysis ($P>0.05$). It is interesting to note that the significant difference between high- and low- flavonoids groups was observed in the expression of F3'H-1 and FLS ($P<0.05$).

Question mark

We can investigate from figure 13, No.12-1 and No.24-2 have the similar color which is bright red. Besides, No.12-2 and No.24-1 have similar dark green color. However, both of two replicated should have the same color. From the previous study, mature fruit of No.12 is red color whereas No.24 is dark green color.

Figure 13 Photo of four parent sample powders. From left to right, the samples are No.12-1, No.12-2, No.24-1, and No.24-2.

Discussion

We have analyzed the metabolic and molecular aspect associated with flavonoids biosynthesis. The flavonoids variation was investigated in RILs F6 population and related to the presence of the MYB12-like gene. In tomato, MYB12 which is also known as SIMYB12 can regulate naringenin chalcone production [28,32]. In *Capsicum,* CaMYB12 was isolated from pepper fruit and the expression level correlated with the structural gene involved in flavonoids biosynthesis expression [12]. In pepper population, the metabolic analysis revealed a large difference between the F6 population in flavonoids accumulation. Based on metabolite profile, the MYB12-like gene has significant linkage with luteolin in family 190, 208, 47. Besides, naringenin variation can be observed in family 208 and 47. This metabolic information can be used for next generation selection with specific flavonoids compounds.
Transgenic tomato with overexpressed MYB12-like gene was as a tool to investigate genetic regulation of flavonoids biosynthesis. However, fruit-specific overexpression of the *Capsicum* MYB12-like gene in transgenic tomato plants cannot lead to induction of flavonoids and phenylpropanoids pathway. It suggests that there is no direct link between flavonoids level and activation of the MYB12-like gene in transgenic tomato lines. In contrast, MYB12 gene can downstream regulate flavonoids structural genes such as *chalcone synthase* CHS [33] thus increase naringenin chalcone accumulation. It is mainly because transgenic tomato in this project is T1 population. In T1 population, it is possible that the transgene did not fully function and cannot lead to flavonoids variation. Also, the frameshifting in the MYB12-like gene is also a possibility. Frameshifting mutation is an error when transcription and translation allow DNA information to be communicated into making proteins. The introns were not completely removed, but some exons were removed during RNA processing into mRNA. This mutant can result in the protein function change, or function loss.
Since MYB12-like gene was found in *Capsicum*, pepper RILs F6 population was analyzed to demonstrate the role of MYB12-like in flavonoids biosynthesis. Based on metabolite profile, we investigated mature red fruits with MYB12-like gene accumulated high levels of flavonoids. To get a deep insight into the molecular regulation of this pathway, the expression of several structural genes and the MYB12-like gene related to flavonoid biosynthetic pathway was analyzed by real-time quantitative RT-PCR. Indeed, the MYB12-like gene expressed at pretty low level and Ct value of all samples are above 35.0 cycles. Due to the low expression level of the MYB12-like gene, we conclude that it does not play a major role in the regulation of the flavonoids pathway in pepper mature red fruits.
However, flavonoids variation was investigated, and another factor that may contribute to the high flavonoids levels is the expression of structural genes. With the analysis of mature red fruits, the expression of structural genes was not significantly different between two phenotype groups.
In tomato, it was demonstrated that accumulation of naringenin chalcone was dependent on MYB12 gene expression but stopped accumulating upon fruit ripening and the ripening-dependent induction of MYB12 as well its downstream target flavonoids genes, such as CHS was severely reduced [28,32,33]. The previous study shows that MYB12-like gene has higher expression level at the early green stage and the expression is reducing with ripening (Appendix 9a). Based on the qPCR result, the Ct value of mature green samples was relatively higher than in mature red samples, and the expression of the MYB12-like gene was with a higher level in the high-flavonoids group. However, there was no significant difference investigated by use of statistical analysis, which is not in agreement with the observation of transgenic tomato lines with Arabidopsis MYB12 gene [35]. Besides, of all tested structural genes, there was a significant difference in the expression of *flavanone-3'-hydroxylase* F3'H and *flavonol synthase* FLS between high-flavonoids and low-flavonoids phenotype groups. For other structural genes, although there was no significant difference based on statistical analysis, we observed high expression level of *chalcone synthase-2* CHS-2 in the high-flavonoids group. It was in agreement with an observation from pink tomato mutants [28] and pepper F2 population [33]. In pink mutant tomato, expression of MYB12 gene can increase *chalcone synthase* CHS expression, suggesting an accumulation of the CHS product naringenin chalcone. In pepper F2 population, accumulation of naringenin chalcone in ripen fruits was following the upregulation of the CHS-2 gene, and whose

encoded enzyme is responsible for the production of naringenin chalcone from 4-coumaroyl CoA and malonyl CoA [33].

Question mark of sample label

Since the colour of two replicates is different, we believe that one of parent No.12 was switched to a replicate of No.24 based on the colour of samples powder (figure 13). Parent No.24 is dark green color while No.12 is the red colour. It is shown that NO.24-1 was switched to No.12-2. This mistake can be made during labeling, but it is also possible the wrong information was used for population selection. There is a possibility that the metabolic data of NO.12-2 was used for No.24-2 selection. If it is true, all data need to be reanalyzed again.

Conclusion

Metabolic analysis result can be used for next generation selection. Q1 and J6 QTL can be used to select high Capsianoside phenotype, while MYB12-like can be utilized for high flavonols phenotype selection. In F6 family 107,190,208 and 47, they have a different linkage between MYB12-like gene and flavonoids compounds. Since MYB12-like gene can increase flavonols biosynthesis, Quercetin as one important flavonol can be selected in family 47 and 107.

As a transcription factor, the MYB12-like gene cannot activate other structural genes related to flavonoids biosynthesis in tomato lines. Besides, we did not observe flavonoids variation in transgenic tomato lines. A further study should be proposed in the next transgenic generation. In pepper F6 population, although expression level of the MYB12-like gene is shallow (Ct value is above 35.0) in pepper mature red fruits, no significant difference was investigated over all tested structural genes. The expression of the MY1B2-like gene was with a higher level in mature green fruits which agreed with the previous study that the MYB12 expression level is reducing during fruits ripening. There was a significant difference between high-flavonoids and low-flavonoids phenotype groups over *flavanone 3-hydroxylase* F3H and *flavonol synthase* FLS genes. F3H is a key enzyme in the flavonoid biosynthetic pathway, providing a branching point for the biosynthesis of different flavonoids and can converts flavanones to dihydroflavonols. F3'H can convert dihydrokaempferol into dihydroquercetin which is a substrate of FLS for flavonols biosynthesis. Meanwhile, the MYB12-like gene can increase CHS expression, suggesting CHS, F3H, and FLS catalyze successive steps in the biosynthetic pathway leading to the production of flavonols. Although the first generation of transgenic tomato lines with overexpressed MY1B2-like gene cannot provide positive information of regulatory mechanism of flavonoids biosynthesis, the different expression of MYB12-like was investigated. Generally, this project provides valuable information for further study. For further study, early stage green pepper samples and next generation transgenic tomato lines would be analyzed for a deep insight of MYB12-like gene function in flavonoids biosynthesis.

Recommendation

This research cannot provide sufficient knowledge about the function of the MYB12-like gene in flavonoids biosynthesis. It is a transcription factor which can increase flavonoids biosynthesis and only investigated two of structural genes expression level increased which are *flavanone 3-hydroxylase* F3H and FLS in mature green pepper F6 samples. Based on what we achieved, here are some recommendations:

1. Although MYB12-like gene cannot activate other structural genes expression, it is possible that MYB12-like gene did not fully function since the transgenic tomato lines are T0 population. The expression level experiment can be applied to the next generation of transgenic tomato to investigate whether the MYB12-like gene has an influence on flavonoids biosynthesis in developed population.
2. Since parent No.12-2 was switched to No.24-1, the wrong metabolic data may be used for samples selection. First, it is necessary to check whether this mistake was only about the wrong label or not. If the wrong information was used, new selection and metabolic analysis need to be done.
3. The candidate genes involved in this research is based on the previous study. However, some of the genes did not have significantly different expression levels. Besides, there are more potential candidate genes which can affect flavonoids biosynthesis. Therefore, new candidate genes need to be identified in the further study.
4. Because some samples in negative or low-flavonoids phenotype group have relatively high expression level, it is possible that the environmental conditions are different. For this reason, more strict environmental regulation will be required in the following experiments.
5. Since MYB12-like gene expression is reducing during ripening, early green stage pepper samples need to be analyzed in the further research. In this project, we only analyzed mature green samples which are not truly green samples. However, we investigated MYB12-like gene can affect three structural genes. We believe the analysis of early green fruits can provide more information about MYB12-like gene function.

Appendix

Appendix1 Protocol of qPCR

Isolating RNA from plant material:

Material (RNase free):
- Safety glasses and gloves.
- Tripure (Roche, #1166757001 50 ml, #11667165001 200 ml)
- Yellow and blue tips
- Spoon
- 2 ml Eppendorf tubes
- Chloroform (pure, no isoamyl alcohol added!)
- Isopropanol
- EtOH 75%
- RNase free water

Method:
1. Put on the safety glasses, gloves and work in fume hood!
2. Collect plant material tubes from -80°C into liquid nitrogen container.
3. Add 1.5 ml TriPure in 2 ml eppendorf tube.
4. Add a spoonful of plant material powder into eppendorf and vortex.
   (Make yourself sure no liquid nitrogen is added to the phenol, it will literally explode in a closed eppendorf)
5. Centrifuge 10 min micro centrifuge max speed at 4°C temperature
6. Transfer supernatant into a fresh tube (2 ml)
7. Add 300 µl chloroform and shake vigorously by hand for 15 seconds.
8. Incubate at room temperature for 3 minutes
9. Centrifuge 15 min micro centrifuge 14,000 g at 4ºC
10. Transfer the supernatant to a fresh tube (2.0 ml)
11. Add 750 µl isopropanol and mix by hand
12. Incubate at room temperature for 10 minutes
13. Centrifuge 10 min micro centrifuge max speed at 4ºC
14. Remove supernatant and wash the pellet with 900 µl 75% EtOH
15. Centrifuge 5 min micro centrifuge max speed at 4ºC
16. Remove supernatant and semi dry the pellet briefly in draught of fume hood
17. Dissolve pellet in 80 µl RNase free MQ. RNA pellet will float on surface of the water. 18. Incubate 5 minutes at 50ºC to dissolve the RNA completely
19. Keep on ice from this moment on 4ºC
20. Store the RNA at -80°C or continue to the DNase I reaction.

DNAse I treatment.

Material:
- Deoxyribonuclease I kit (Invitrogen #18068-015)

Method:
RNA solution 80 µl
10* DNAse buffer 9 µl
DNAse I enzyme 1 µl
1. Incubate 15 min at room temp
2. Add EDTA (25mM) 10 µl
3. Incubate 10 min at 65 °C to stop the reaction.

Purify RNA.

Material:
RNeasy mini kit (Qiagen #74104(50), #74106 (250))

Method:
According to the added protocol booklet: RNeasy Mini Handbook following the protocol for RNA Clean up. Elute in 2* 30uL RNase free water warmed at 50°C

Concentration measurement of RNA by Nanodrop.

Material:
- Nanodrop device in lab 1.058
- MQ
- White tips, pipette and gloves

Method:
1. Log in on computer.
2. Open program ND-1000 3.7.1 → Nucleic Acid
3. Clean machine with a tissue and water
4. Add 1.5 µl MQ and press OK. Spectrometer is initializing
5. Select sample type RNA-40 (ε = 40 in Lambert – Beer law)
6. Press **Blank** in upper button row
7. Wipe the MQ droplet, Fill in Sample ID **Blank**.
8. Add 1.5 µl MQ and press **Measure**
9. If blank gives a value of about 0 ng/µl, the blank is good. If not go back to third point
10. Give a RNA name in Sample ID field
11. Add 1 – 1.5 µl of your RNA sample and press **Measure**
12. Wipe away the RNA droplet
13. After measuring your RNA close the program
14. Press shortcut to Nucleic Acid
15. Select Nucleic Acid file (YYYY MM DD) with right mouse button →
16. Open with: Microsoft Office Excel
17. Select the header and your samples. Copy data.
18. Open new Excel sheet paste data.
19. Save data on your M drive
20. Close sheets and log off the computer

If the ratios of 260/280 and 260/230 are above 1.8, the purity and quality of RNA is good for RT-PCR. If the quality is not good enough, such as the value of 260/230 is quite low, purify that RNA sample with the kit again.

RNA gel.

Material:
- Gel container
- Tray
- Comb
- 1*TAE sterile (1l)
- Demi water sterile (1l)
- Agarose
- Ethidium Bromide 10 mg/ml

Method:
1. Put a soap solution (Rosal) overnight in gel container, tray and comb.
2. Autoclave 1 l of TAE and 1 l of Demiwater
3. Next day, rinse the gel container, tray and comb with sterile demiwater.
4. Make a 1.5% Agarose gel in 1*TAE and autoclave.
5. Poor the gel in tray
6. Add 1*TAE in container
7. Calculate the µl for 200 ng of RNA and add MQ to 10 µl
8. Add 3 µl of RNA loadingbuffer
9. Run the gel. After run the 2 rRNA bands (1.1 kB and 700bp band) and mRNA smear should be visible. A quality and quantity check can be made.

Reverse transcription reaction

Material:
- Taqman Reverse Transcription Reagent kit (Life Technologies, Applied Biosystems #N8080234)
- Oligo d(T)16 (50µM) (Life Technologies, Applied Biosystems #8080128)

Method:
Calculate the amount of RNA solution needed for 1 µg RNA. The system is optimized for
1 µg RNA. Double the amounts when 2 µg of RNA has to be transcribed. (minimum concentration is 52 ng/µl)

5.00 µl 10* Taqman RT buffer

11.00 µl $MgCl_2$

10.00 µl dNTP mix

2.50 µl oligo dT

1.00 µl RNase inhibitor

1.25 µl MultiScribe rev. transcriptase

X   µl RNA (1µg)

19.25-X µl MQ

50.00   µl total volume

Reverse transcription program:
- 25 °C   10 min (annealing of primer)
- 48 °C   30 min (extension)
- 95 °C   5 min (deactivate the RTenzyme)

The concentration is 20 ng cDNA/µl

Designing qPCR primers with Primer design Program

Copy the naked sequence of your gene of interest into a WORD file and save as a TXT file.

Open the txt file and copy the sequence.

Open Program Primer3 Plus

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

Paste the sequence into the sequence field. Change at Tab General Settings 'select special settings here' in qPCR. Now primer will be designed according the suitable parameters.

Find primers by pressing 'Pick Primers'

In the program also Alternate primers are given:

- The best option is given #1. When you would like to see the other options, scroll down
- A preference is to have primers designed at the 3'side of the gene. Less chance of breakage of the RNA strand.
- In the Excel sheet the amount of base pairs are counted by the function   =LEN(cell primer).

qPCR experiment

Notes:

1. Plan your experiment and pipetting scheme with care. Use the 96 wells scheme!
2. Give thoughts about suitable reference genes and No Template Controls
3. iQ SYBR GREEN super mix 2* is stored at -20 °C. Once it's thawed, it is stored at 4°C. It will be stable for at least 6 months.
4. The primers are designed by the software "Beacon Designer" program. This program is designed for RT-PCR primers.
5. The primer and enzyme work is strictly separated from the cDNA work. Therefore the primer/ enzyme work is performed in the fume hood with special DNA free pipettes and matching tips.
The cDNA is added on your lab spot with your own pipettes and tips!
6. Always prepare a mixture of "No Template Control (NTC)" by adding MQ water instead of cDNA template and a mixture of "RNA template control" by adding RNA instead of cDNA template.

Material:

- iQ SYBR GREEN super mix 2* (BioRad 25*5 ml tubes #172-5006 CUST)
- Optical quality 96-well thin wall PCR plates (BioRad 25 plates skirted #223-9441)
- Micro seal B Optical quality sealing tape (BioRad 100 pieces # MSB-1001)
- Primers Biolegio, dissolve in MQ with DNA free pipettes and tips
  - Scale: 40 nmols
  - Method: Economy
  - No purification
  - Quality check: Normal no extra
  - Chemistry DNA
  - Dissolved: No
- Rainin Tips for DNA free work (Mettler Rainin)
  - 0.5-20 µl (#17005860)
  - 10-200 µl (#17005859)
  - 100-1000 µl (#17007081)
- DNA free Rainin pipettes (Drawer nearby qPCR machine)

Method:
Prepare RT-PCR mixture. The PCR is performed in a volume of 20 µl per well. Therefore the reaction volume is 44 µl (duplo) or 65µl (triplo)! Work clean avoid cross contamination.

Duplo	Triplo	
22.0 µl	32.50 µl	2*iQ SYBR GREEN super mix
11.0 µl	16.25 µl	MQ
4.4 µl	6.50 µl	Forward primer (3 µM)
4.4 µl	6.50 µl	Reverse primer (3 µM)
Add per sample:		
2.2 µl	3.25 µl	cDNA (10 ng/µl)
44.0 µl	65.00µl	total volume

3. Fill the 96 wells plate according to your scheme with 20µl reaction mixture per well (duplo or triplo). For every well use a fresh tip! When dividing the mixture, cover the other wells you are not working with!

4. Carefully seal the RT-PCR plate with special film (Micro seal, adhesive sealer). When taking out the film, only hold its edge which is not transparent. Do not touch the transparent part of the film! After sealing the film PCR plate, use a small plastic board (can be found in the top drawer near RT-PCR machine) to make certain every well is sealed properly.

5. Calibrate the plates including the centrifuging trays. Centrifuge the RT-PCR plate in plate centrifuge for 2 minutes at 2500 rpm.

6. Run RT-PCR machine.
- Turn on the RT-PCR machine and then the computer to establish the communication between the device and the computer.
- The PCR machine and optical mode must be pre-warmed at least 10 min in advance. b. Put your RT-PCR plate in the machine.
- When using the machine for the first time:
  - Find the qPCR program file under C:\ Users\ \Public\Public Documents\Bio-Rad\ CFX\ Plant Breeding
  - Here you find the PCR program needed
  - Press ‘OK’ and go to Tab ‘Plate’
  - Here you select ‘Create New’ and fill in the 96 well scheme according your design.
  - In ‘Sample Type’ Unknowns or NTC (No Template Control) can be selected.
  - Select all sample wells and at ‘Load’ check the ‘SYBR’ box. Now you gave your samples a sample type.
  - Press ‘OK’ Save your File and press”Next>>’
  - Be sure that you select the proper qPCR machine.
  - Press ‘Open Lid’. Place your plate in the machine and press ‘Close lid’
  - When this is done press ‘Start Run’
  - Save your data on the N drive or on a USB stick for analyzing your data later on.
  - An overview will appear of the progress of the run.
  - One RT-PCR run will take 100 minutes

Analysis of the data

Analysis of the Ct values and PCR products

- If the Bio-Rad iQ 5 program is not available on your computer you can install this by pressing START (Left Down your screen) →WUR→!Available Software and select CFXManager 3.1
- Activate the program.
- Open ‘File’ open ‘Data File’ and find your saved data file

- The Plots will appear in the program including a common threshold cycle (green line). The Ct’s appearin the panel right under.
- A specific Threshold cycle is needed for your samples with one primer pair combination. Therefore select the wells done with one primer pair combination. You will see the Threshold cycle and Ct values will change a bit. This makes the values specific for the selected primer pair.
- Select the samples then copy the result table and paste this in a new worksheet called “raw data” in the Excel file you already made for the plate designed at the start of your experiment for further data analysis.

- Press Melt Curve/Peak. Two plots appear. One with the melting curve and the second is the first derivative of the first plot. If there is only one peak for each sample, that means there is a unique PCR product and the primers you designed are unique for the gene you selected.

Analysis of PCR Efficiency

- In a perfect PCR reaction the PCR product will double in each PCR cycle. The real efficiency should be around 2 otherwise the next formulas and calculations are not valid. The real efficiency can be analyzed in the by pressing "Export"→ Export All data sheets TEXT: (*.txt). Save data on your drive and select Quantification Amplification Results_SYBR.txt
- Copy these data into an Excel file and delete the first empty column.
- Start the program LinRegPCR.
- Fill in the empty fields (In a full plate Column A to CS; Row 1 to 41) and select Bio_rad iCycler. The plots appear again. Select Fit Options → Fit all. The efficiency per well appears in the program.
- By pressing "Sample" the individual efficiency appears. This can be adjusted with upper- and lower limit.
- When the analysis is finished, press File → save to excel. Fill in the field column: A and row 1 → OK. The efficiencies and R^2 will appear in the excel sheet. The values can be paSted in the raw data sheet of the qPCR data and analyzed.

Calculation Relative Gene Expression

- For a good reference gene it is necessary that the Ct values are the same over the different cDNA's. IE The reference gene is expressed in the same level over the different tissues. The Ct's should not differ more than one cycle.
- The raw data are copied in a worksheet 'Raw Data" Fill in the names in the column Identifier to name every well.
- Copy the columns "Well Identifier, Ct mean and Ct Std. Dev." from the raw data to another Excel sheet, and name it "RGE" (relative gene expression). Do not work on the raw data directly.
- The calculation starts now. It depends on the data obtained how to continue.
    - Data without wild type material → δCt calculation
    - Data with wild type material → δδCt calculation.

δCt calculation:
This is how to calculate the Relative Gene Expression compared to a reference gene.

- The δCt is calculated as follows: $\delta Ct = Ct_{(gene\ of\ interest)} - Ct_{(reference)}$
- The RGE is calculated as: RGE= POWER(2;-δCt)*factor
    - A factor (f.i.100.000) is needed to enlarge the figures needed for the Log calculation
- The Log 2 of the RGE is calculated as: LOG = LOG(RGE;2)

δδCt calculation:
This is how to calculate the Gene Expression Transformant (Tr) compared to wild type(Wt) material.

- The $\delta Ct_{(Tr)}$ is calculated as follows: $\delta Ct = Ct_{(gene\ of\ interest,\ Tr)} - Ct_{(reference,\ Tr)}$
- The $\delta Ct_{(Wt)}$ is calculated as follows: $\delta Ct = Ct_{(gene\ of\ interest,\ Wt)} - Ct_{(reference,\ Wt)}$
- $\delta\delta Ct = \delta CT_{(Tr)} - \delta Ct_{(Wt)}$
    - A factor is needed to enlarge the figures needed for the Log calculation
- The Log 2 of the RGE is calculated as: LOG = LOG(RGE;2)

Appendix 2 Oligo nucleotides of phenylpropanoid and flavonoid genes used for SYBR Green RT-PCR analysis in transgenic tomato lines.

Gene	SubjectId	Primer	pairs
		Forward	Reverse
PAL	Solyc09g007910	ATTGGGAAATGGCTGCTGATT	TCAACATTTGCAATGGATGCA
C4H	Solyc11g028170	CCAATGGCAACGACTTCAGA	TCTGCACCAAACGTCCAATG
4CL	Solyc03g117870	TGTGAGGAACGCAGAGATG	CAATATCGCCAGTGTGTAACC
HQT	Solyc07g005760	GTGTTTTGTTTGTTGAGGCTGA	TGATGAAGTGGATGGATGAGAG
C3H	Solyc10g078240	TCTCAGTTTACTTCGGTTCAC	TTCAATACTTGTTTCGCTAA
HCT	Solyc03g117600	AGGTGAAAAACTCAACGATGGT	ACACTAGGCGTGTGGAAATTAG
CHS1	Solyc09g091510	GTTCCGTGGACCCAGTGAAT	AAAAGGGCTTGGCCTACCA
CHS2	Solyc05g053550	GGCCGGCGATTCTAGATCA	TTTCGGGCTTTAGGCTCAGTT

CHI-1	Solyc05g010320	GAAGCAGTGCTCGATTCCATAAT	GTTTTTCACAAACCAACAGTTCTGAT
CHI-like	Solyc05g052240	GCGATAGAAGGTAAGGA	AGCCAAAGAAGAAATAGTTGT
F3H	Solyc02g083860	CACACCGATCCAGGAACCAT	GCCCACCAACTTGGTCTTGTA
F3'H	Solyc03g115220	GCACCACGAATGCACTTGC	CGTTAGTACCGTCGGCGAAT
FLS	Solyc11g013110	GAGCATGAAGTTGGGCCAAT	TGGTGGGTTGGCCTCATTAA
3GT	Solyc10g083440	CGAACGACGAAACACTGTTGA	TGCAGCATAGATGGCATTGG

Appendix 3 Oligo nucleotides of phenylpropanoid and flavonoid genes used for SYBR Green RT-PCR analysis in pepper F6 lines.

Gene	SubjectId	Primer	pairs
		Forward	Reverse
CHS-1	CA03G02050	GGGCGCTAGAGTTCTTGTTG	AATGTAAGCCCAACCTCACG
CHS-2	CA05G17060	CATTGGGGATTTCTGATTGG	GGCCTTTCTCATTTCATCCA
CHI-1	CA11G02280	AGCTCCAAGGAGTTGAACGA	TTGGCTCGAACCATCTATCC
CHI-2	CA00G42910	CAGGCAAAACTTTGGGATGT	TGCACTCCAACTGAATCTGC
CHI-3	CA02G08970	TTCAAGGCTCCCAGGATATG	TGAAGGCCCTGCAGAGTAGT
CHI-4	CA06G26920	GTCCCTGGTCAGTGCGTAAT	TCACATCAGCATCATCAGCA
F3H	CA02G21550	GCCTTAACCAAGGCATGTGT	AACCGTGATCCAAGTTTTGC
FS-2	CA06G24290	AGCTAAGGAGACGACGGTGA	AAATCAAACCACCCACCAAA
FLS	CA04G08330	CTTGTCACTTGGGCTTGGAT	TAAACACTTGGAGGCCTTGG
F3'H-1	CA03G29350	CATTGCTCGTGATCCAAATG	AACGAATGGACCAAAGTTGC
F3'H-2	CA04G13910	GCAATTTGAACCTGCTTGGT	TGCCCATGTCATGTCTGAGT

Appendix 4 Oligo nucleotides of reference genes used for SYBR Green RT-PCR analysis in pepper F6 lines.

Reference gene	Primer sequence
ß-Actin 1167F	CCTCAGCACATTCCAGCAG
ß-Actin 1358R	CCACCAAACTTCTCCATCCC
GAPDH-517F	ATGACTACAGTTCACTCCATAACC
GAPDH-654R	AACCTTTCCAACAGCCTTAGC
18Srib-182F	CCTTCGGGATCGGAGTAATG
18Srib-283R	TTCGCAGTTGTTCGTCTTTC
Ub-345F	GCACAAGCACAAGAAGGTTAAGC
Ub-449R	CCACACTCAGCATTAGGACACTC

Appendix 5 Sequence of MYB12-like gene

Consensus sequence of MYB12-like gene:

ATGAAGGCACCTTGTTGTGAAAAAGTTGGGCTAAAGAGAGGAAAATGGACAATTGAAGAAGATGAATTATTGGTGAAGTAT
ATTCAAGCTAATGGTGAAGGTTCATGGAGGTCTCTTCCCAAAAATGCTGGTTTATTAAGATGTGGAAAGAGTTGCAGATTAA
GATGGACAAATTATTTGAGACCAAATTTGAAGAGAGGCAAATTTACTGCAGAAGAAGATGAAACTATTGTCAAATTGCATAG
CACCTTGGGAAATAGGTGGTCTTTGATAGCCCAGGATTTACCCGGCCGAACAGATAATGAAATAAAGAACTATTGGAACTC
CAATTTACGTCGGAGACTTTACTCCTTCAGGAACTTCAAAGAACTTATTAAGACCACTACAAATGTACCAAAAATCGCGGTG

GATGGCGATTCTGATGAATCCTTGAGAAAACAAGATACAAGTACAATGTCGAAAGAGGGTTCAATTGAATCTCGTTTGCCAG
AAGATAATATTGTTGTTGATAGAGGTTCCACTCGAAAAAACAGGTCATTATGTGTTGGAAGCAATGAAGGGGCTACGTGGT
CTGAAGGGGATTCAACTGAATCTCTTTTACCGGAAAATTGTACAGTTGATATAGGTTCCATGCGGAAACCCTACTCCTCGTG
TCCTAGAGGCAAGGACGATGTTCCTTGGTATAGAGGGGGTTCAACTGAATCTCTTTTGCTGGAAAATATAGAAATAAACTTC
GCGTGTGAAGAGAATTCGATTGGGTCCCTTTTGATGGAAAATTGTTCTTCTCCTGGAGGCAGGGATGATACGTCGTGGTCT
GAAGAAGGGGGTTCATTTAAATCTCTTTTGTTGGAAAATATAGAAACCAACTTTTCATGTCCCAAAGGCACATGCAATGCTA
CGTGGTCTGAAGGGGGTTCAGTTGAATTTTTTTCGTTGGAAAATTGTGTTGTTGATCTAGGTTCTAATCGAGAAAACAAACC
CGTGTGGTCTGACAAAGGGGGTTCAATTGAATACCTGTTGACGGAAAATTATACTGTTGATATAGGTTTTAATCGAGAAACC
AATTCCTCATCATGTCCCAGAGGCAACGCCGAAGCTATGTGGTATGAAGAAGGGAGTTCAATTGAATCCTCATCGACAGAA
AGTTATATCGTTGATCTAGGAAAAACAAGCCAAACTTCTAGTATATTGATAAATGTGTAG

Theoretical sequence of MYB12-like gene:

ATGAAGGCACCTTGTTGTGAAAAAGTTGGGCTAAAGAGAGGAAAATGGACAATTGAAGAAGATGAATTATTGGTGAAGTAT
ATTCAAGCTAATGGTGAAGGTTCATGGAGGTCTCTTCCCAAAAATGCTGGTTTATTAAGATGTGGAAAGAGTTGCAGATTAA
GATGGACAAATTATTTGAGACCAAATTTGAAGAGAGGCAAATTTACTGCAGAAGAAGATGAAACTATTGTCAAATTGCATAG
CACCTTGGGAAATAGGTGGTCTTTGATAGCCCAGGATTTACCCGGCCGAACAGATAATGAAATAAAGAACTATTGGAACTC
CAATTTACGTCGGAGACTTTACTCCTTCAGGAACTTCAAAGAACTTATTAAGACCACTACAAATGTACCAAAAATCGCGGTT
GATGGCGATTCTGATGAATCCTTGAGAAAACAAGATACAAGTACAATGTCGAAAGAGGGTTCAATTGAATCTCTTTTGCCAG
AAGATAATATTGTTGTTGATAGAGGTTCCACTCGAAAAAACAGGTCATTATGTGTTGGAAGCAATGAAGGGGCTACGTGGT
CTGAAGGGGATTCAACTGAATCTCTTTTACCGGAAAATTGTACAGTTGATATAGGTTCCATGCGGAAACCCTACTCCTCGTG
TCCTAGAGGCAAGGACGATGTTCCTTGGTATAGAGAGGGTTCAACTGAATCTCTTTTGCTGGAAAATATAGAAATAAACTTC
GCGTGTGAAGAGAATTCGATTGGGTCCCTTTTGATGGAAAATTGTTCTTCTCCTGGAGGCAGGGATGATACGTCGTGGTTT
GAAGAAGGGGGTTCATTTAAATCTCTTTTGTTGGAAAATATAGAAACCAACTTTTCATGTCCCAAAGGCACATGCAATGCTA
CGTGGTCTGAAGGGGGTTCAGTTGAATTTTTTTCGTTGGAAAATTGTGTTGTTGATCTAGGTTCTAATCGAGAAAACAAACC
CGTGTGGTCTGACAAAGGGGGTTCAATTGAATACCTGTTGACGGAAAATTATACTGTTGATATAGGTTTTAATCGAGAAACC
AATTCCTCATCATGTCCCAGAGGCAACGCCAAAGCTATGTGGTATGAAGAAGGGAGTTCAATTGAATCCTCATCGACAGAA
AGTTATATCGTTGATCTAGGTAAAACAAGCCAAACTTCTAGTATATTGATAAATGTGTAG

Parent No.12 sequence of MYB12-like gene:

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ATTCAAGCTAATGGTGAAGGTTCATGGAGGTCTCTTCCCAAAAATGCTGGTTTATTAAGATGTGGAAAGAGTTGCAGATTAA
GATGGACAAATTATTTGAGACCAAATTTGAAGAGAGGCAAATTTACTGCAGAAGAAGATGAAACTATTGTCAAATTGCATAG
CACCTTGGGAAATAGGTGGTCTTTGATAGCCCAGGATTTACCCGGCCGAACAGATAACGAAATAAAGAACTATTGGAACTC
CAATTTACGTCGGAGACTTTACTCCTTCAGGAACTTCAAAGAACTTATTAAGACCACTACAAATGTACCAAAAATCGCGGTT
GATGGCGATTCTGATGAATCCTTGAGAAAACAAGATACAAGTACAATGTCGAAAGAGGGTTCAATTGAATCTCTTTTGCCAG
AAGATAATATTGTTGTTGATAGAGGTTCCACTCGAAAAAACAGGTCATTATGTGTTGGAAGCAATGAAGGGGCTACGTGGT
CTGAAGGGGATTCAACTGAATCTCTTTTACCGGAAAATTGTACAGTTGATATAGGTTCCATGCAGAAACCCTACTCCTCGTG
TCCTAGAGGCAAGGACGATGTTCCTTGGTATAGAGGGGGTTCAACTGAATCTCTTTTGCTGGAAAATATAGAAATAAACTTC
GCGTGTGAAGAGAATTCGATTGGGTCCCTTTTGATGGAAAATTGTTCTTCTCCTGGAGGCAGGGATGATACGTCGTGGTCT
GAAGAAGGGGGTTCATTTAAATCTCTTTTGTTGGAAAATATAGAAACCAACTTTTCATGTCCCAAAGGCACATGCAATGCTA
CGTGGTCTGAAGGGGGTTCAGTTGAATTTTTTTCGTTGGAAAATTGTGTTGTTGATCTAGGTTCTAATCGAGAAAACAAACC
CGTGTGGTCTGACAAAGGGGGTTCAATTGAATACCTGTTGACGGAAAATTATACTGTTGATATAGGTTTTAATCGAGAAACC
AATTCCTCATCATGTCCCAGAGGCAACGCCGAAGCTATGTGGTATGAAGAAGGGAGTTCAATTGAATCCTCATCGACAGAA
AGTTATATCGTTGATCTAGGTAAAACAAGCCAAACTTCTAGTATATTGATAAATGTGTAG

Parent No.24 sequence of MYB12-like gene:

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ATTCAAGCTAATGGTGAAGGTTCATGGAGGTCTCTTCCCAAAAATGCTGGTTTATTAAGATGTGGAAAGAGTTGCAGATTAA
GATGGACAAATTATTTGAGACCAAATTTGAAGAGAGGCAAATTTACTGCAGAAGAAGATGAAACTATTGTCAAATTGCATAG
CACCTTGGGAAATAGGTGGTCTTTGATAGCCCAGGATTTACCCGGCCGAACAGATAATGAAATAAAGAACTATTGGAACTC
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GATGGCGATTCTGATGAATCCTTGAGAAAACAAGATACAAGTACAATGTCGAAAGAGGGTTCAATTGAATCTCGTTTGCCAG
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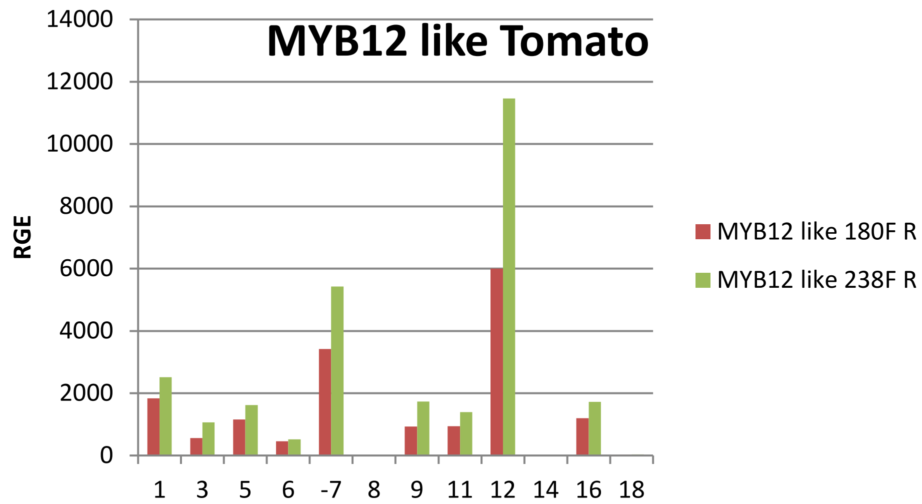
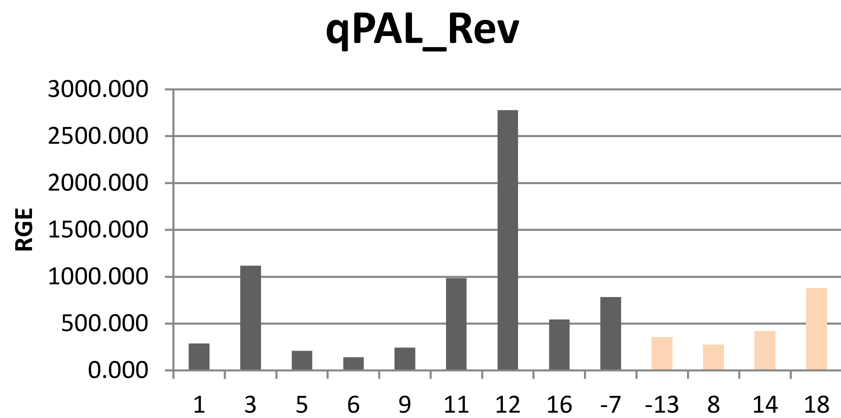
TCCTAGAGGCAAGGACGATGTTCCTTGGTATAGAGGGGGTTCAACTGAATCTCTTTTGCTGGAAAATATAGAAATAAACTTC
GCGTGTGAAGAGAATTCGATTGGGTCCCTTTTGATGGAAAATTGTTCTTCTCCTGGAGGCAGGGATGATACGTCGTGGTCT
GAAGAAGGGGGTTCATTTAAATCTCTTTTGTTGGAAAATATAGAAACCAACTTTTCATGTCCCAAAGGCACATGCAATGCTA
CGTGGTCTGAAGGGGGTTCAGTTGAATTTTTTTCGTTGGAAAATTGTGTTGTTGATCTAGGTTCTAATCGAGAAAACAAACC
CGTGTGGTCTGACAAAGGGGGTTCAATTGAATACCTGTTGACGGAAAATTATACTGTTGATATAGGTTTTAATCGAGAAACC
AATTCCTCATCATGTCCCAGAGGCAACGCCGAAGCTATGTGGTATGAAGAAGGGAGTTCAATTGAATCCTCATCGACAGAA
AGTTATATCGTTGATCTAGGTAAAACAAGCCAAACTTCTAGTATATTGATAAATGTGTAG

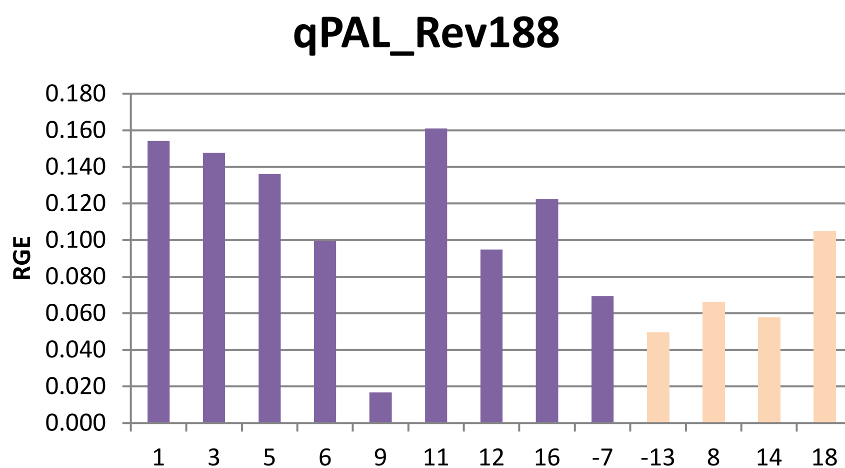
Appendix 6 Relative expression of phenylpropanoid and flavonoid candidate genes in transgenic tomato lines.

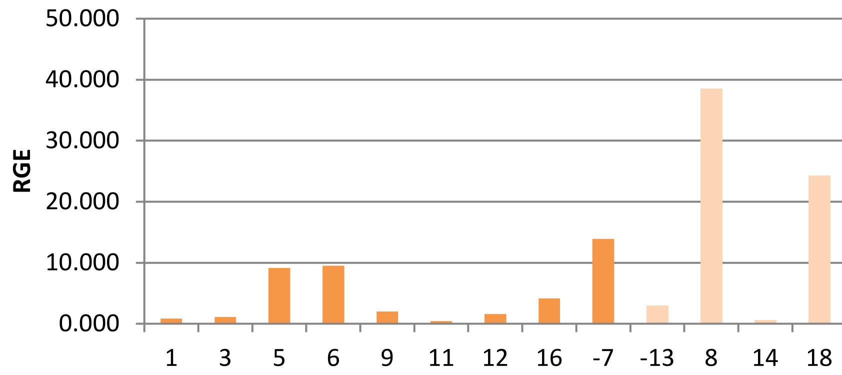
A. MYB12-like gene



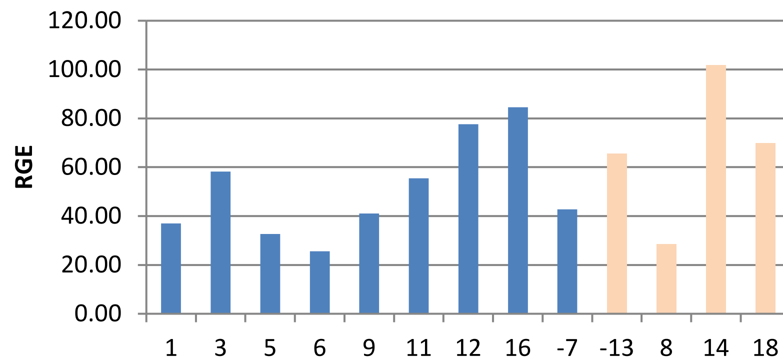
B. Phenylalanine ammonia lyase (PAL) genes



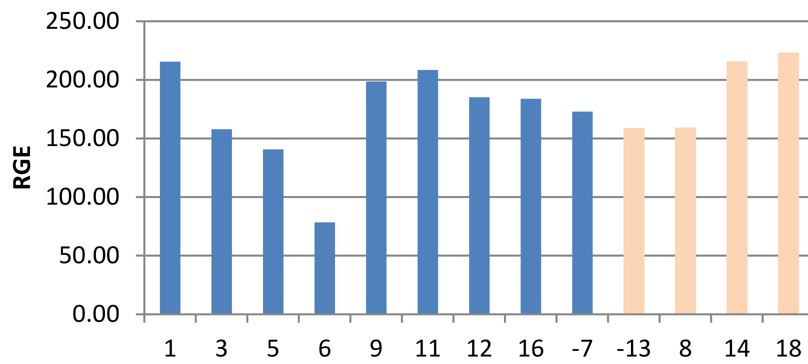




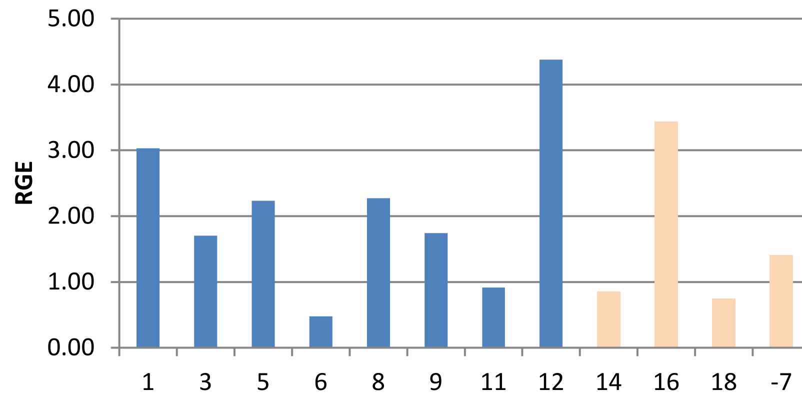
C. Cinnamate 4-hydroxylase (C4H) gene

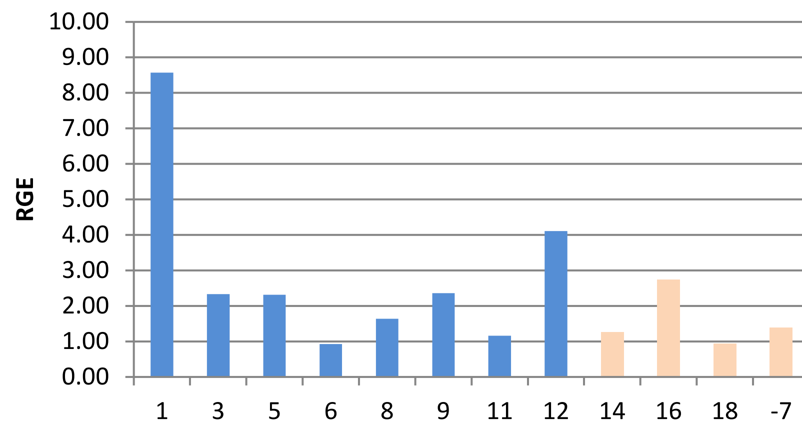


D. 4-coumarate: CoA ligase (4CL) gene

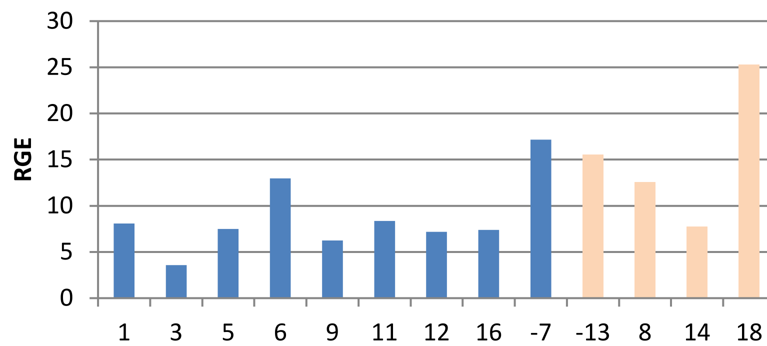


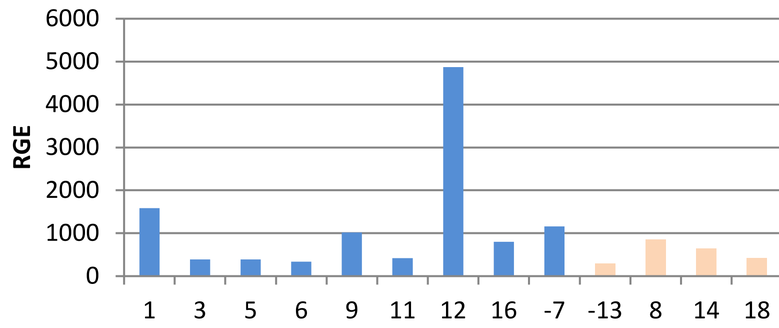
E. Chalcone synthase (CHS) genes



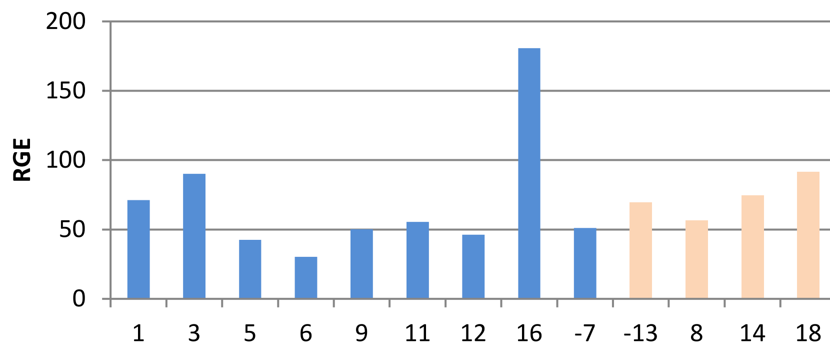


F. Chalcone isomerase (CHI) genes

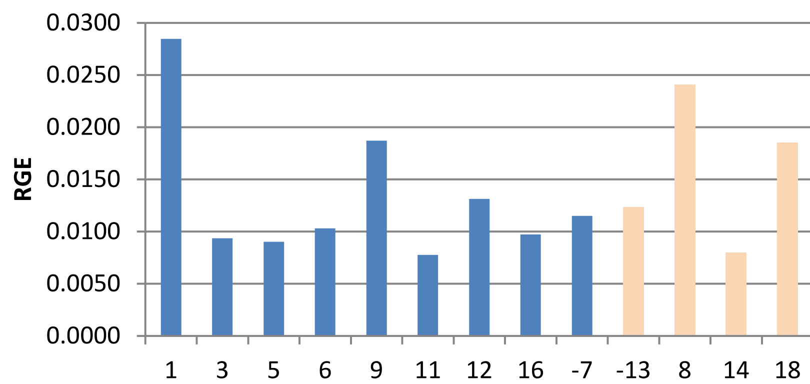




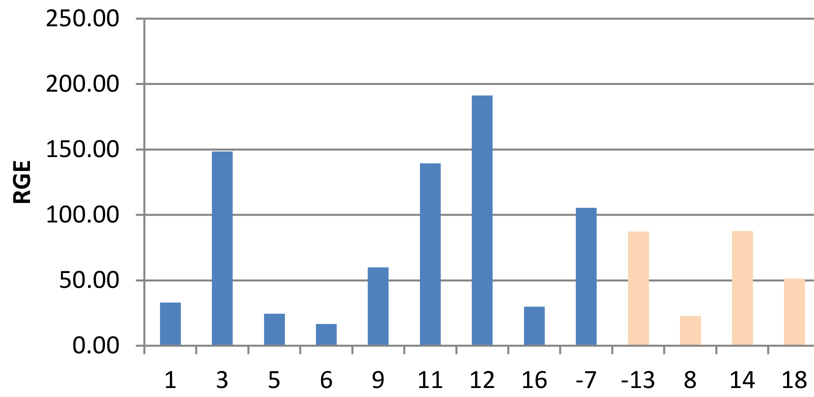
G. Flavanone 3-hydroxylase(F3H) gene



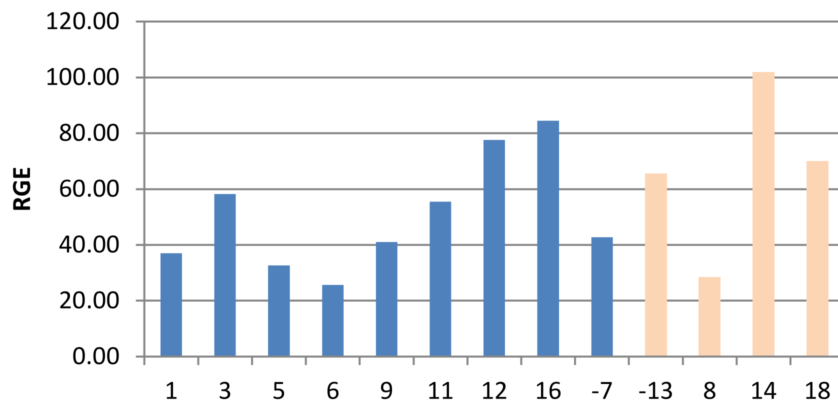
H. Flavonoid 3 hydroxylase (F3′H) gene



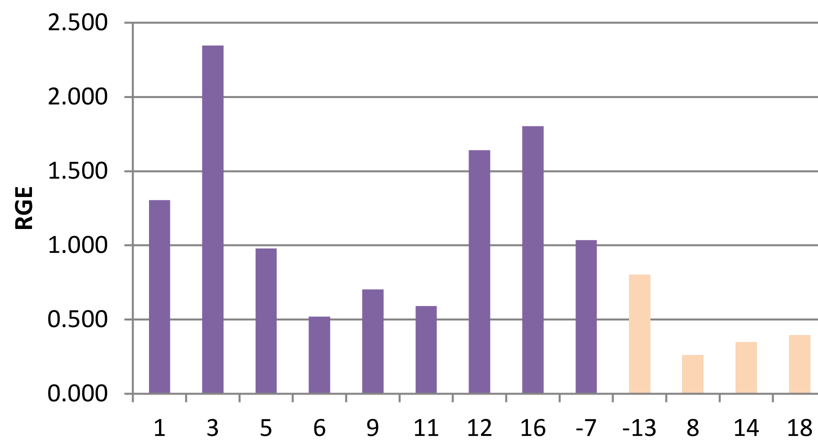
I. Hydroxycinnamoyltransferase (HCT) gene



J. P-coumarate 4-hydroxylase (C4H) gene

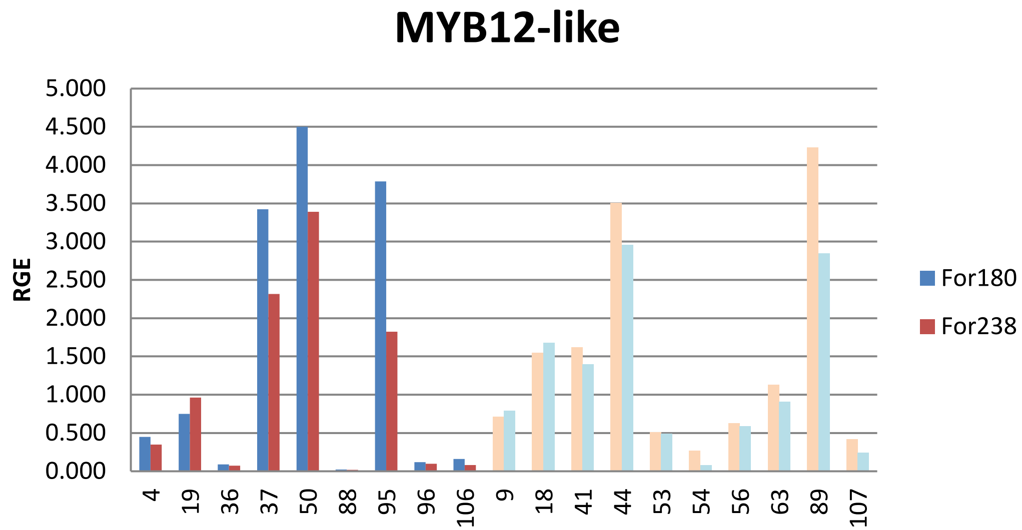


K. Flavonol synthase (FLS) gene

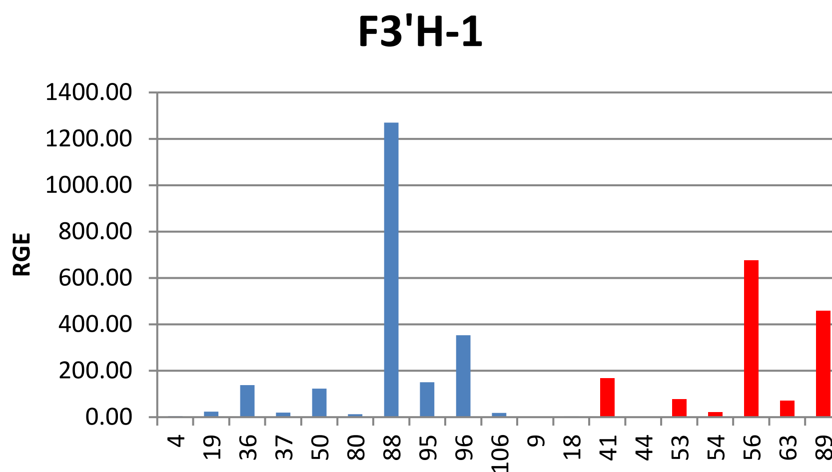


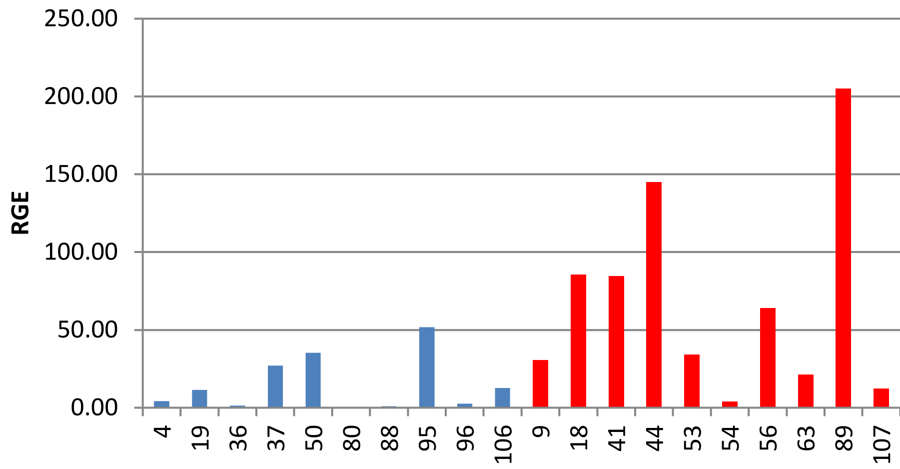
Appendix 7 Relative expression of flavonoid candidate genes in pepper F6 lines

A. MYB12-like gene

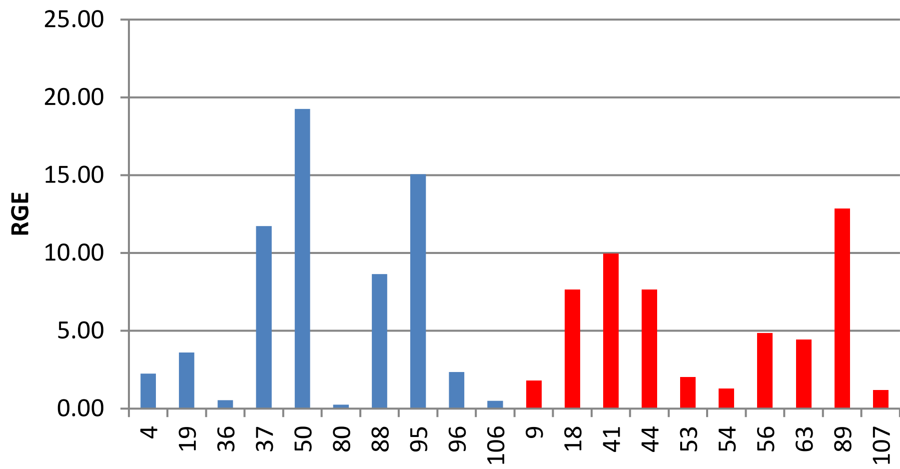


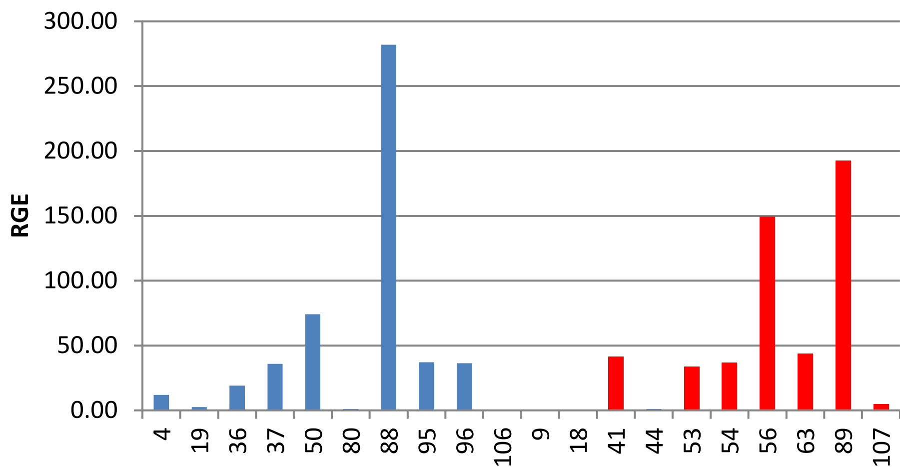
B. Flavonoid 3 hydroxylase (F3′H) gene



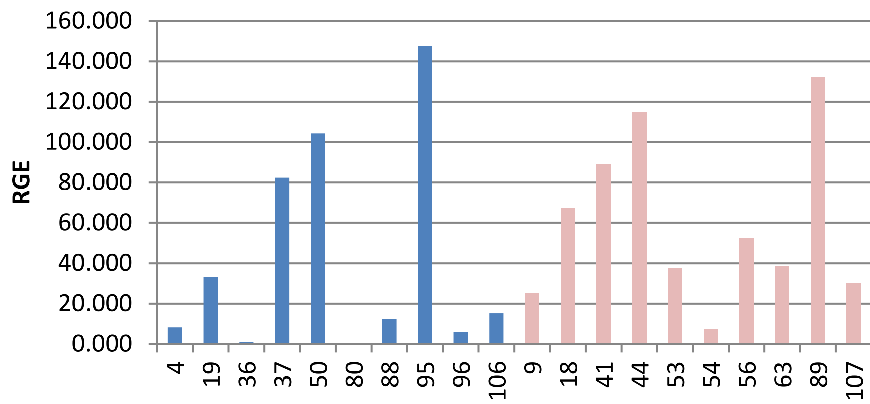


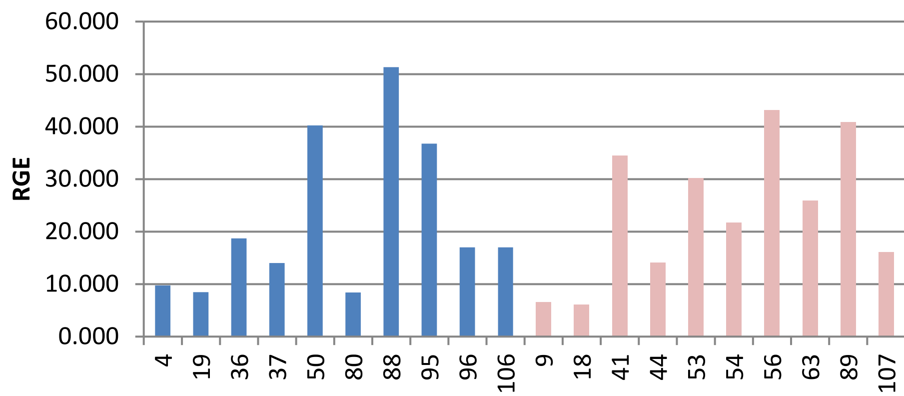
C. Chalcone synthase (CHS) genes

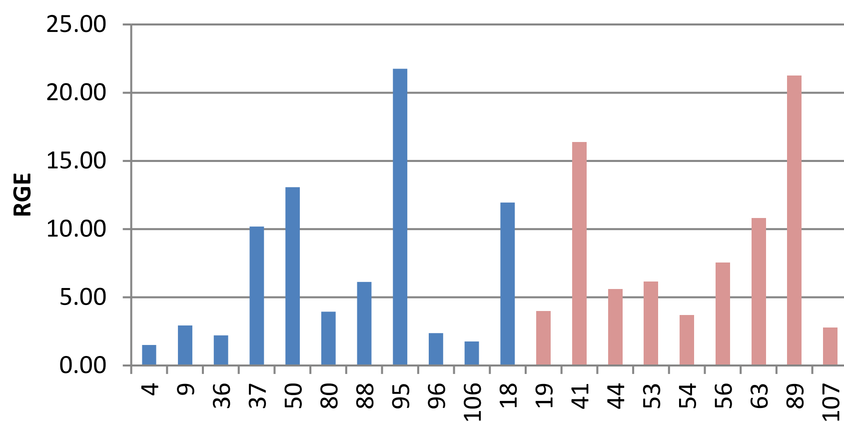


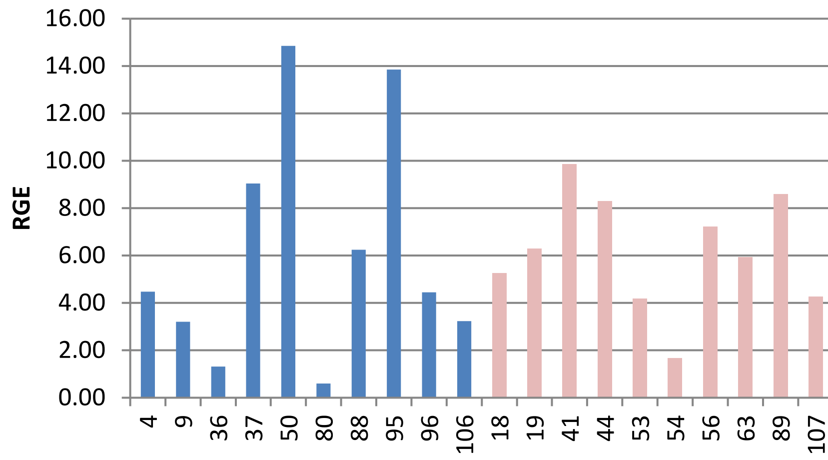


D. Chalcone isomerase (CHI) genes

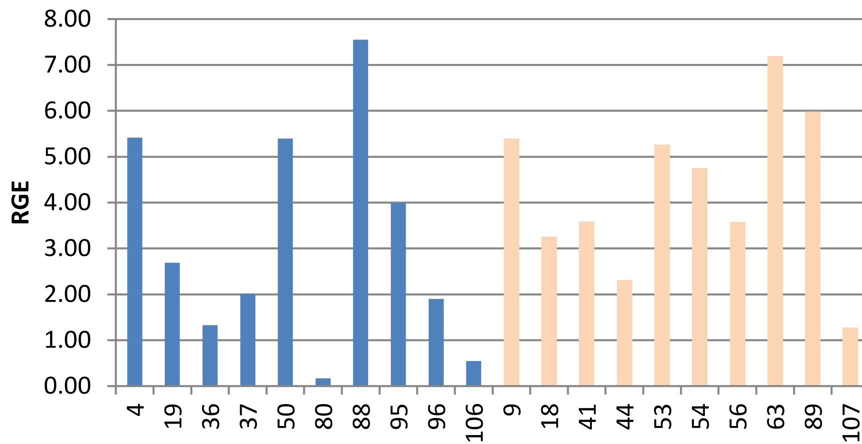




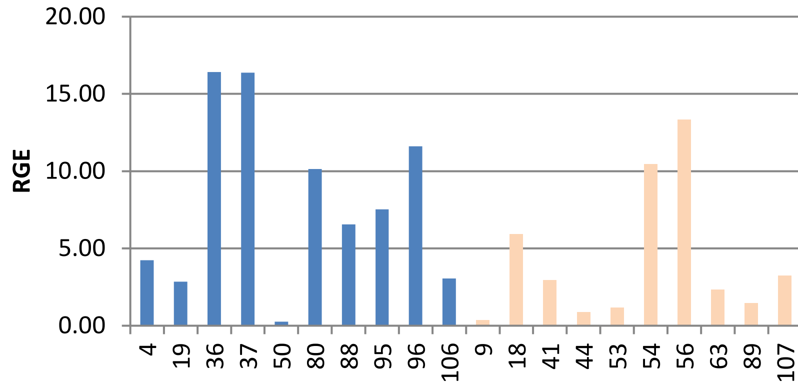




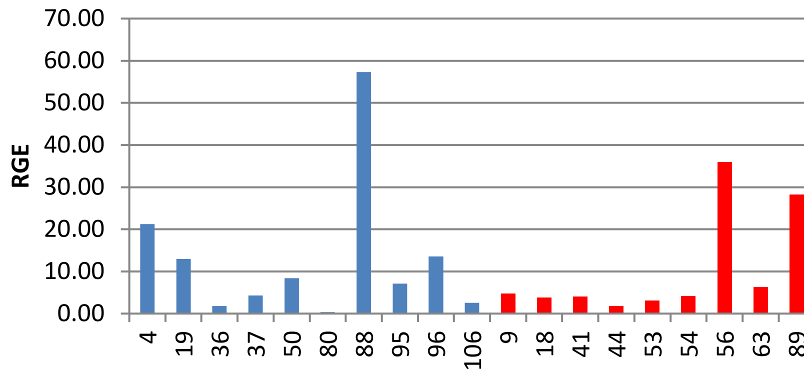
E. Flavanone 3-hydroxylase (F3H) gene



F. Flavone synthase-2 (FHS-2) gene

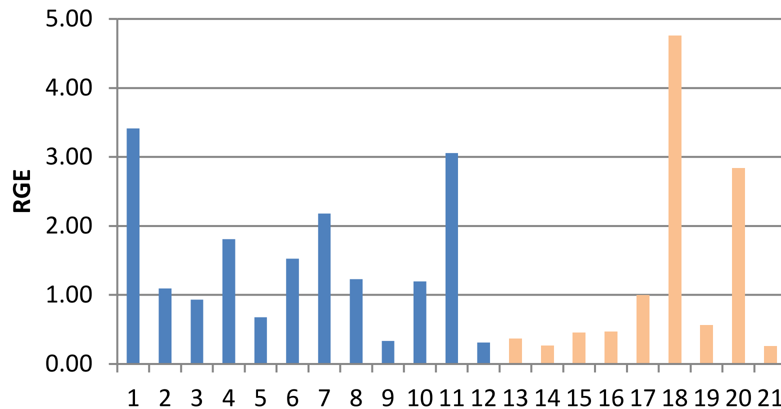


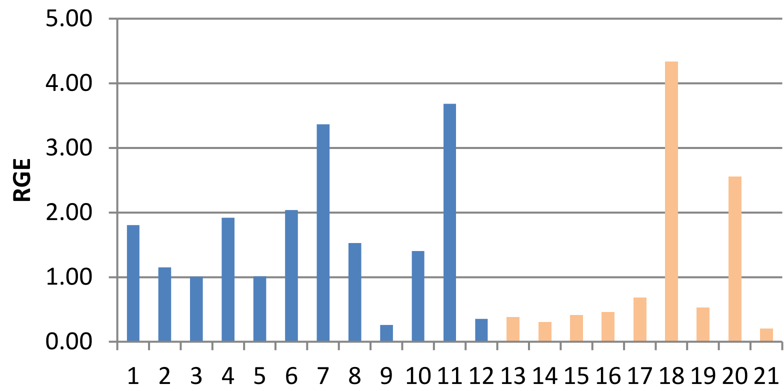
G. Flavonol synthase (FLS) gene



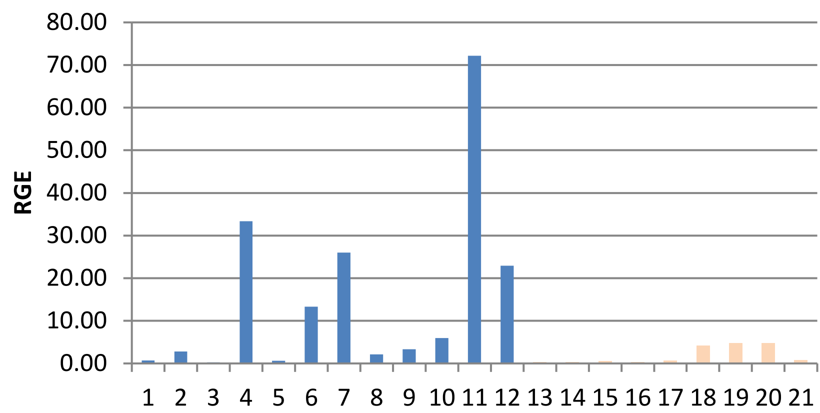
Appendix 8 Relative expression of flavonoid candidate genes in pepper F6 mature green lines

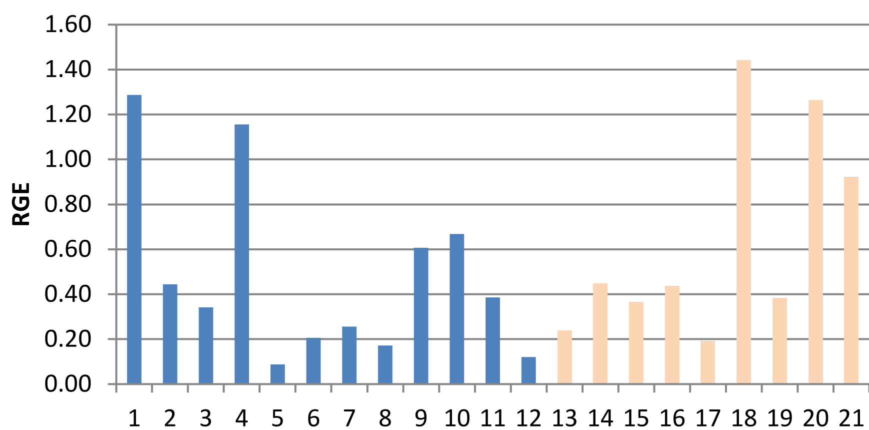
A. MYB12-like gene



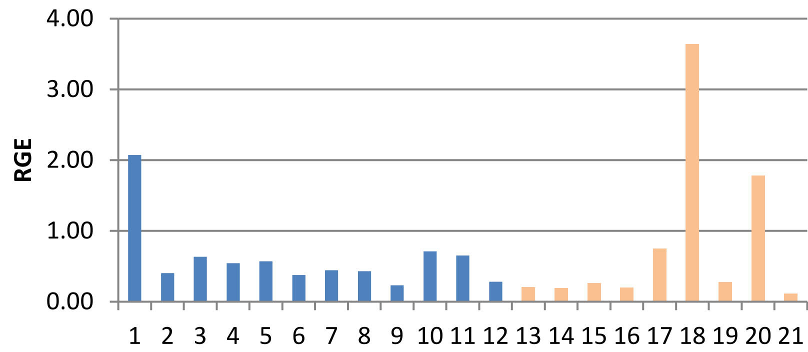


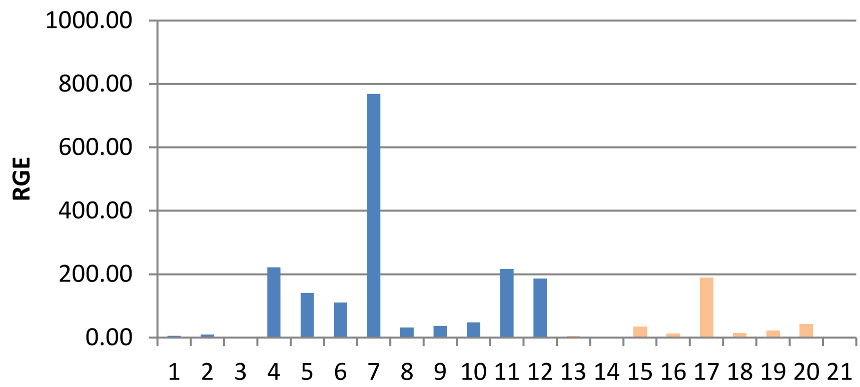
B. Flavonoid 3 hydroxylase (F3′H) genes



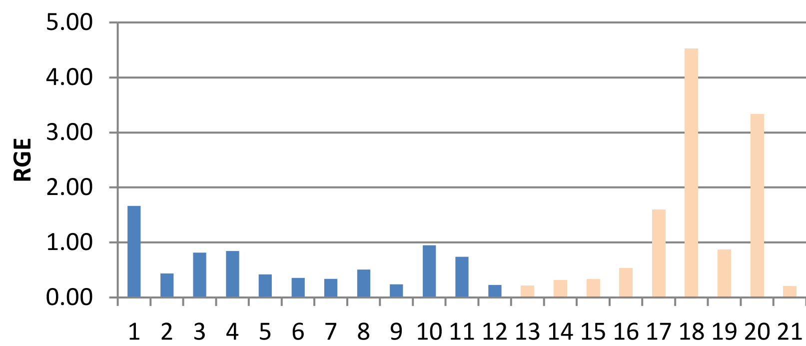


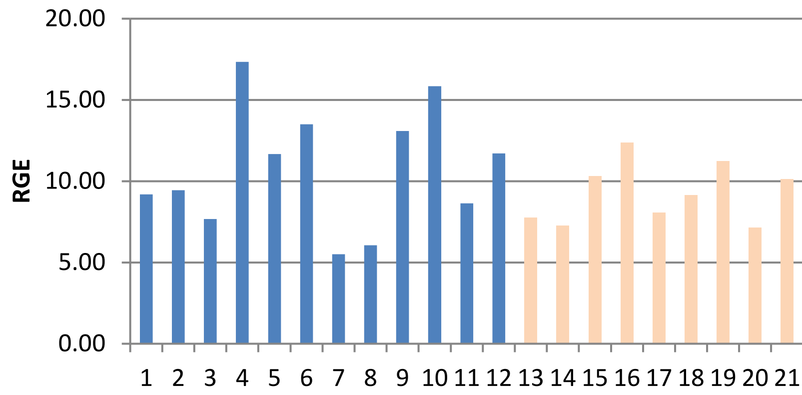
C. Chalcone synthase (CHS) genes

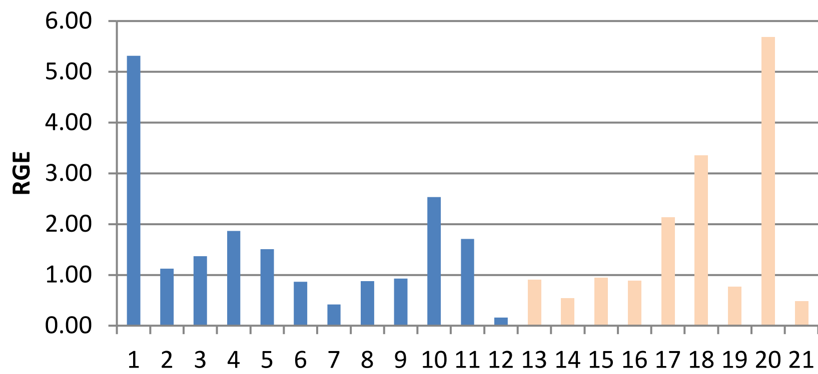


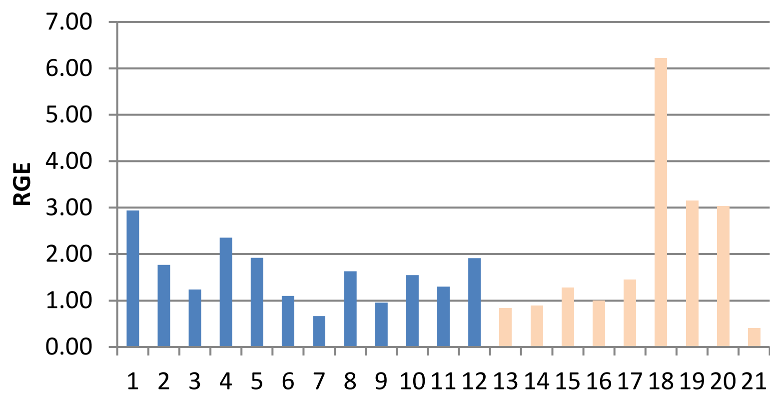


D. Chalcone isomerase (CHI) genes

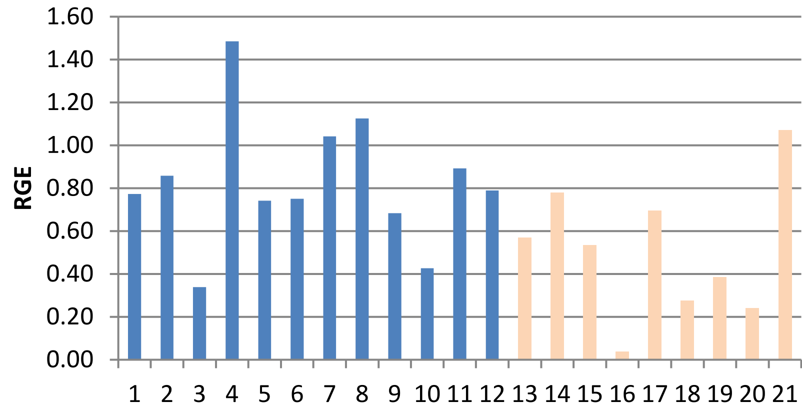




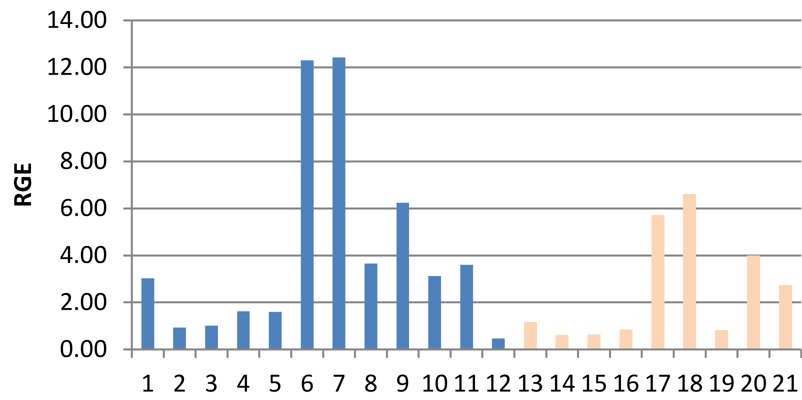




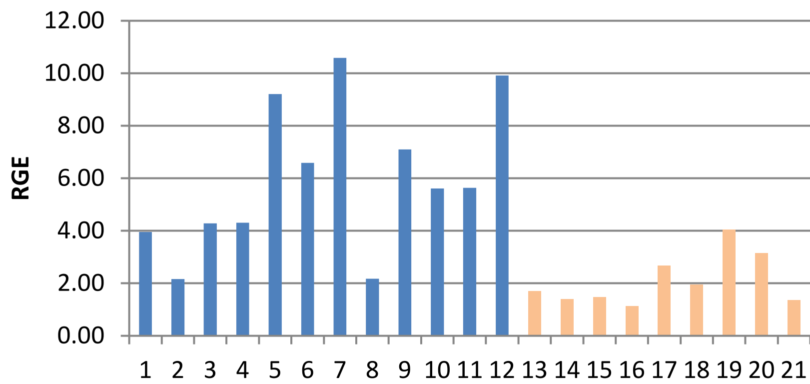
E. Flavanone 3-hydroxylase (F3H) gene



F. Flavone synthase-2 (FHS-2) gene

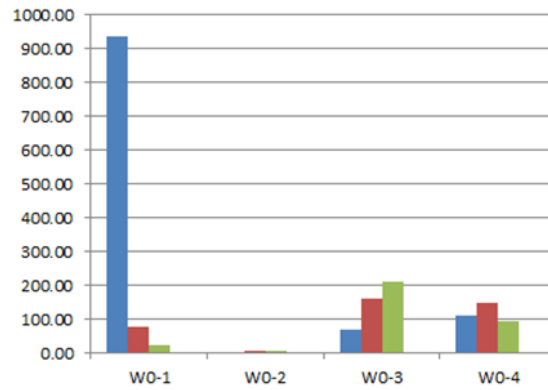


G. Flavonol synthase (FLS) gene

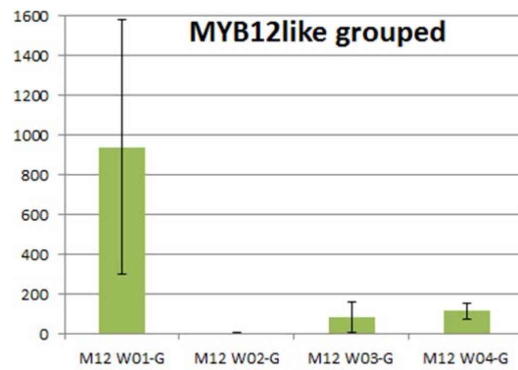


Appendix 9 Previous data.

a. MYB12-like expression level is degraded with fruit ripening. W0-1 is No12 while W0-3 is No.24.



b. MYB12-like expression level in green fruit is higher in parent line W01 (No.12) than W03 (No.24).



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