Anthocyanin degradation in bell pepper fruit

Wageningen University

Plant Breeding Group

Narendra Kumar Kata (910806596050)

17-04-2018

Anthocyanin degradation in bell pepper fruit

Narendra Kumar Kata

910806596050

Supervisor: Ying Liu

Examiner: Arnaud Bovy; Yury Tikunov

Research Group: Plant Breeding

Wageningen University ● MSc Thesis ● 17-04-2017

Wageningen, Nederland

Abstract

In purple pepper genotypes the purple pigmentation is only observed in immature fruits; purple discolouration was noticed in mature fruits during ripening. The biosynthesis of anthocyanins was well characterized in many model plants and in *Solanaceae* crops. But very little information is available on anthocyanin degradation. The concentration of anthocyanins in mature fruits is by the balance between anthocyanin biosynthesis and degradation. The current project is conducted to study molecular mechanisms of anthocyanin degradation in pepper. In total, eight pepper genotypes were used to investigate the anthocyanin structure, concentration and accumulation pattern. In our study, the anthocyanins in purple genotypes were identified as delphinidin derivatives. The two forms of anthocyanins were delphinidin-3-coumaroylrutinoside-5-glucoside and delphinidin-3-caffeoylrutinoside-5-glucoside. From the results the concentration is correlated with fruit colour. Dark purple fruits had a high concentration and low levels were noticed in ripening fruits. The accumulation pattern of anthocyanins is first increased and then decreased during fruit development. Also, the environmental influence on colour pigmentation is investigated in purple-fruited genotypes with two temperature treatments 10ºC and 30ºC. No significant colour change in the fruits under low temperature and turn of fruit colour from purple to red was observed at high-temperature treatment.

Acknowledgement

Table of content

1. Introduction

Bell pepper (*Capsicum annum L.*) is one of the important vegetable consumed worldwide for its healthy reserves like vitamins, carotenoids and flavonoids (Ghasemnezhad et al., 2011). Bell peppers are available in various colours in the market. The variation in the colour is due to accumulation of different colour pigments. Some bell pepper species have immature purple fruits, which later turn to red colour. The purple colour is provided by the accumulation of anthocyanin (Borovsky et al., 2004). But, these immature purple fruits are not preferred by the consumers due to poor taste.

Anthocyanins are derivatives of flavonoid pathway which is one of the major secondary metabolites and protect plants from biotic and abiotic stresses, other than stress protection, they are also helpful in attracting pollinators and seed dispersers (Harborne & Williams, 2000; Hoballah et al., 2007). Plants under biotic and abiotic stresses synthesis more anthocyanins to scavenge Reactive Oxygen Species (ROS) produced under stress conditions (Ahmed et al., 2015). Anthocyanins are beneficial for humans as well, because of their antioxidant properties which could potentially prevent people from free radicals and harmful diseases like cancer, cardiovascular and even neurodegenerative problems (Materska & Perucka, 2005). Additional to health benefits, the concentration of anthocyanins determines the postharvest quality of several flowers, fruits, and vegetables (Tanaka & Ohmiya, 2008), which created a keen interest for researchers to study more in detail about the anthocyanins also for breeders to produce anthocyanin-rich crops for the market.

The accumulation of anthocyanins in pepper fruits is determined by two processes which include biosynthesis and degradation of anthocyanins. The process of biosynthesis has been well studied in many model plants like Maize, *Petunia*, Snapdragon, and *Arabidopsis*. But, only a few studies have been done in bell pepper to know how the metabolic pathways are controlled and what is the pattern of gene expression. Very less information is available on the degradation process and no proper mechanism was explained on degradation. Previous study Liu, Y. (2016) investigated on anthocyanin accumulation pattern and candidate gene expression in purple-fruited genotype cv. Tequilla. So far, delphinidin derivatives are the only forms of anthocyanins detected in the peppers (Arboretum, & Human, 2018; Lightbourn et al., 2008; Sadilova et al., 2006; Vega, 2013).

1.1 Anthocyanin biosynthesis

The transient accumulation of anthocyanin is due to anthocyanin biosynthesis, the rate of degradation and stability in the vacuoles (Fang et al., 2015). Additionally, the stage of crop development and crop production environment will have a significant influence on the concentration of anthocyanins. From the results of Liu, Y. (2016), anthocyanin accumulation pattern was first increased until stage five (big purple) and then decreased from stage six (purple to red) in purple-fruited genotype cv. Tequilla. In young green fruits, no purple pigmentation was observed. Dark purple pigmentation was noticed in immature purple fruits with high levels of anthocyanin concentration denoting dark purple colour in the

pericarp of the fruit is due to the accumulation of anthocyanins. In turning stage fruits the disappearance of purple colour and reduced anthocyanin concentrations can be by suppression of biosynthesis and induced degradation of anthocyanin in the purple fruits of cv. Teqilla genotype. Anthocyanin biosynthesis is a branch way of flavonoid pathway (Petroni & Tonelli, 2011). The structural and regulatory genes of anthocyanin biosynthesis have been very well characterized in many model plants like maize, *Arabidopsis*, and *Petunia* (Petroni & Tonelli, 2011). Three crucial transcription factors namely R2R3-MYB, bHLH and WD40 are found interesting for modulating the expression of structural genes for anthocyanin biosynthesis (Tanaka & Ohmiya, 2008). An MYB-bHLH-WD40 (MBW) transcriptional complex can be formed by combining R2R3-MYB and bHLH with WD40 proteins to activate the transcription of biosynthetic genes (Oa et al., 2011; Petroni & Tonelli, 2011). The schematic representation of anthocyanin biosynthesis and the enzymes involved is shown in (Figure 1). From the previous study, delphinidin-3-coumaroyl-rutinoside-5-glucoside and delphinidin-3-caffeoyl-rutinoside-5-glucoside are the only form of anthocyanins observed in the purple pepper fruits Liu, Y. (2016).
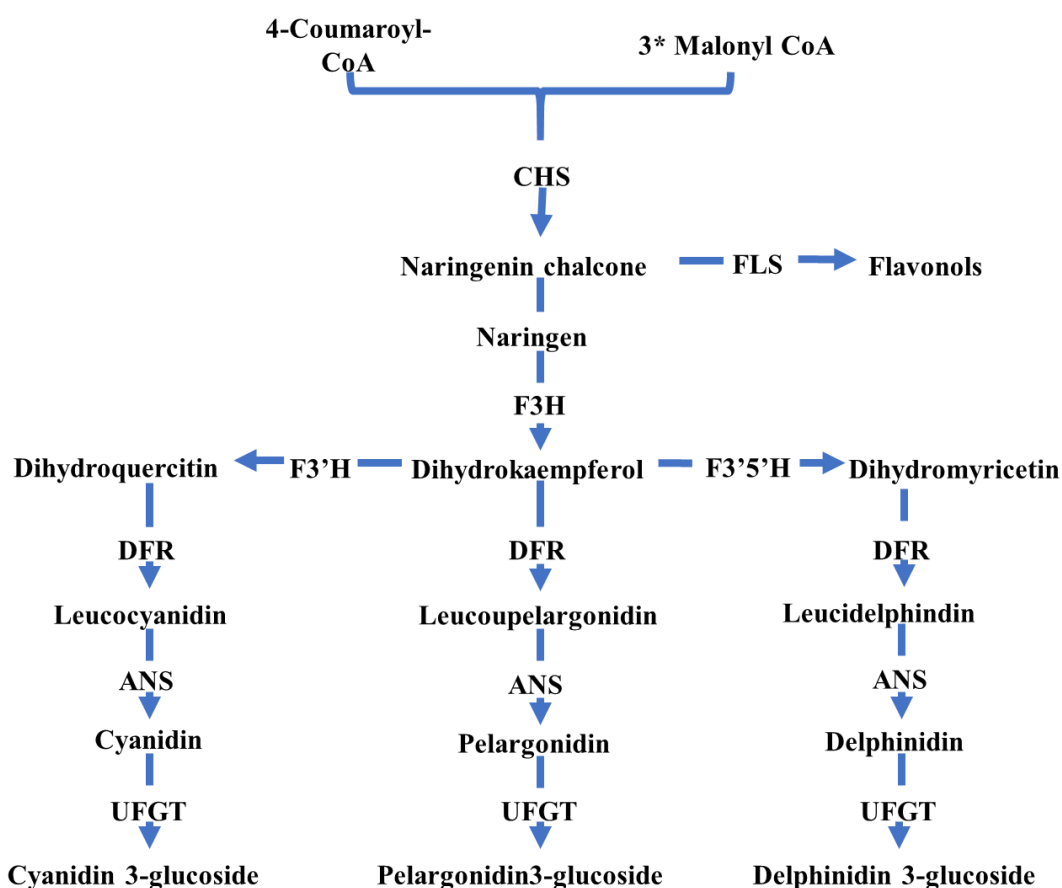

Figure 1: Anthocyanin biosynthetic pathway: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid 3-O-glucosyltransferase. Modified from (Liu et al., 2018; Petroni & Tonelli, 2011).

1.2 Anthocyanin degradation

In contrast to anthocyanin biosynthesis, degradation of anthocyanins is less understood. In plants, anthocyanin degradation occurs by regulation of specific genes and proteins in relation to growth and development of the plant (Oren-Shamir, 2009). In matured red fruits of purple-fruited genotype cv. Tequilla, the disappearance of purple colour might be due to continuous anthocyanin degradation process and termination of biosynthesis Liu, Y. (2016). The degradation process in plants is mainly contributed by three enzyme families namely, peroxidases (POD), polyphenol oxidases (PPO) and β-glucosidases (Oren-Shamir, 2009). From the previous study in purple pepper genotype, a candidate degradative gene *POD* (*CA02g17240)* expressed ubiquitously in the fruits, whereas, the expression of biosynthetic genes was shut down during the ripening stage. It indicates that disappearance of purple colour in mature ripening fruits might result from the termination of anthocyanin biosynthesis and continuous degradation process.

Several enzymatic and non-enzymatic factors influence the anthocyanin degradation, the enzymatic degradation is affected by oxidative enzymes peroxidases and polyphenol oxidases. The first active anthocyanin degradation in plants was reported in *Brunfelsia calycina* flowers. A class III peroxidase BcPrx01 was found responsible for the degradation of anthocyanins in *B. calycina* (Zipor et al., 2015). Three major enzyme families namely peroxidases or polyphenol oxidases and β-glucosidase proposed to be responsible for the degradation of anthocyanins in final ripening stages of blood orange and litchi fruits (Barbagallo et al., 2007; Zhang et al., 2001; 2005). Oren-Shamir (2009) proposed pre-assumed degradation mechanisms by three candidate enzyme families. The first mechanism is direct oxidation of anthocyanins by peroxidase enzyme. The second is a coupled oxidation process where β-glucosidase catalyses the anthocyanins and make a readily available form on anthocyanins by breaking the glycosidic bond, later oxidation of anthocyanin takes place by polyphenol oxidase or peroxidase enzyme (Fang et al., 2015; Oren-Shamir, 2009). Assuming in plants anthocyanin degradation takes place in cell vacuoles by oxidising enzymes mainly peroxidase that present in vacuoles than other enzymes located elsewhere. In the flower petals of *B. calycina* the increased total peroxidase activity was in correlation with anthocyanin degradation suggesting that peroxidases were responsible for degradation of anthocyanins (Vaknin et al., 2005). Non-enzymatic factors can also influence the colour and stability of anthocyanins by enhancing their vulnerability to enzymes that degrade anthocyanins. The colour and stability of anthocyanins are determined by the structure of anthocyanin. Increased hydroxylation of B-ring results more purple colour with unstable anthocyanins (Oodward et al., 2009). Glycosylation at C3 increases the stability of anthocyanins along with colour shift slightly towards red and acylation increases the stability also the colour shifts from red to blue (Lachman & Hamouz, 2005).

1.3 Temperature effect

Environmental factors such as high irradiation, UV/blue light and temperature can influence the metabolism of anthocyanin (Jiang et al., 2016). Temperature is one of the major factors determining the

anthocyanin metabolism. It is well demonstrated that anthocyanin concentration is temperature and light dependent in the leaves of *Arabidopsis thaliana*. High temperature resulted in the loss of red colour in the leaves of overexpressed *PAP 1*(Production of anthocyanin pigments 1) transcription factor. The disappearance of colour at high temperature is due to down-regulation of biosynthesis and degradation of existing pigments (Rowan et al., 2009). The down-regulation of biosynthesis have been noticed within 24 hours and the degradation of existing pigments have been seen over a period of 6 days with the disappearance of red pigmentation and decrease in the concentration of anthocyanins by a combination of both anthocyanin degradation and the inhibition of transcription (Rowan et al., 2009). Other than leaves, the effect of temperature on the anthocyanin concentration is also seen in fruits of red pear and apple. An increased temperature 35ºC resulted in preharvest degradation of anthocyanins in pears and apples (Huysamer. M, 1998). The effects on anthocyanin composition and the gene expression levels in response to high temperature were studied in *Vitis vinifera L. cv.* Sauvignon. The fruits from the high-temperature treatment (35˚C) showed a reduced level of total anthocyanin content compared to control (25˚C) (Mori et al., 2007). The reduction of anthocyanin content is due to enzymatic degradation of anthocyanin. Under high temperature in grapes induced activity of genes encoding peroxidases and oxidoreduction enzymes was observed by oxidative stress, thus how the degradation of anthocyanin occur in the skin of grape berries (Mori et al., 2007). In *Vitis vinifera L. cv.* Sangiovese the effect on anthocyanin in relation to two temperature treatments, low (20 and 29˚C) and high (22 and 36˚C) temperatures, were studied. In high-temperature treatment, the anthocyanin biosynthetic processes were suppressed both at the transcriptional and enzymatic levels, with increased activity of peroxidase enzyme VviPrx31 (Movahed et al., 2016). This suppression is due to the combined impact of reduced biosynthesis and increased anthocyanin degradation (Movahed et al., 2016). To check the effect of VviPrx31 on anthocyanin accumulation in other species, the *VviPrx31* was overexpressed under stress condition in Petunia petals (*Petunia hybrida E.Vilm.*). Results showed VviPrx31 was involved in the anthocyanin degradation. At high temperatures peroxidase activity is stimulated and thus degradation on anthocyanins is seen in ripening grape berries (Movahed et al., 2016).

1.4 RNA isolation

Extraction of quality RNA from fruit tissues rich in secondary metabolites is complicated especially from pepper fruits compared to other Solanaceae family crops. The quality of RNA is essential for gene expression studies, no proper RNA isolation technology is available for pepper fruit tissues. From the past experiences from our group no standard protocol is available for extraction of quality RNA. So, we compared two RNA isolation techniques, CTAB miniprep method and GenElute Mammalian Total RNA miniprep kit for total RNA isolation with few modifications in the protocol. The differences in the quality of total RNA was analysed by measuring the RNA with Nanodrop spectrophotometer and gel electrophoresis.

2. Research Aim

The aim of this study is to investigate metabolic profile and mechanism of anthocyanin degradation in pepper fruits to provide insight into the process of anthocyanin metabolism during fruit ripening. And to examine proper protocol for RNA isolation in pepper fruits.

3. Research questions and hypothesis

1. What is accumulation pattern of anthocyanins in pepper fruits different genotypes during fruit ripening?

In fruits, colour is due to the accumulation of a different group of pigments such as anthocyanins, carotenoids etc. The purple-fruited genotypes are mainly the due to the presence of anthocyanins whereas, the fruit colour in purple-fruited genotype changes during the fruit development from green to purple and then to red/orange/yellow/brown colour. From the previous study, it is confirmed that in cv. Tequilla, the anthocyanin concentration first increased and then decreased during fruit ripening Liu, Y. (2016). The intensity of fruit colour is associated with concentrations of anthocyanins in purple pepper cv. Tequilla.

Hypothesis 1: The anthocyanin accumulation pattern might first increase and then decrease during fruit development of purple fruited genotypes and no anthocyanin accumulation may see in green fruited genotypes.

2. What is the effect of temperature on anthocyanin accumulation?

Besides developmental stages, environmental factors can also influence the anthocyanin content, in which temperature is one of the principal factors. At low temperature, the anthocyanin content increases by induction of anthocyanin biosynthesis (Qiu et al., 2016). Whereas at high temperature, the anthocyanin content decreased by induction of peroxidase activity in grapevine (Movahed et al., 2016).

Hypothesis 2: The anthocyanin concentration will be high in the fruits at low-temperature 10ºC compared to fruits in high-temperature 30ºC.

3. What is the best method for RNA isolation from pepper fruit tissues?

Extraction of quality RNA from fruit tissues rich in secondary metabolites is complicated especially from pepper fruits compared to other Solanaceae family crops. The quality of RNA is essential for gene expression studies, no proper RNA isolation technology is available for pepper fruit tissues. We compare two RNA techniques CTAB miniprep and GenElute Mammalian Total RNA miniprep kit for total RNA isolation with few modifications in the protocol. Assuming good quality of RNA will be harvested from mammalian kit compared to the CTAB method because the isolation procedure for CTAB is very lengthy also several rounds of washings are involved this might result in degradation of total RNA at the end.

Hypothesis 3: The quality of RNA from the mammalian kit will be better compared to RNA from CTAB method.

4. Can a *β-Actin* gene be the good reference gene for different peppers? What is the expression pattern of β-Actin in bell peppers with different isolation methods?

Hypothesis 4: The expression level of reference gene (*β-Actin*) will be same in all samples and Ct values might be within once cycle difference.

4. Material and methods
4.1 Plant materials

Eight pepper genotypes were used in this study. Fruits from respective genotypes were harvested from the plants growing in the greenhouse (Unifarm 10.10) with standard cultivation procedures under natural light conditions. The genotypes vary from each other in colour and size, from green, purple, black, yellow, orange, and red and small round, long slender medium and big round. Fruits with different developmental stages were harvested from respective genotype and individual plant. Fruit stage was determined based on size, colour, and firmness. Fruits from four plants of each genotype were harvested and peel was separated from the flesh, collected in 25ml flat base polystyrene tubes. The list of pepper genotypes, number of plants, developmental stages and type of sample used for metabolomics analysis (Appendix 1). In total 163 fruit samples, frozen with liquid nitrogen were ground into fine powder with the help of IKA A11 basic. The powder samples are labelled and stored at -80ºC for further analysis.

4.2 Methods
4.2.1 Metabolomic analysis (Extraction and analysis of Anthocyanins)

The 163 powder samples were pooled into 90 samples by combing samples from plant one and three as one replicate and two and four as another replicate. Frozen ground samples were separately weighed 0.3 grams (0.15 grams from each plant) for each sample by using analytical balance and taken into 2ml Eppendorf tube. In total 96 samples were prepared for anthocyanin extraction of which six was standard samples from an old tequilla purple fruit. For extraction of anthocyanins a composition of 99% methanol and 1% formic acid was prepared with 300 ml of 100% methanol and 3 ml of formic acid mixed in a 500ml glass bottle. Then 0.7 ml of chemical solution with 99% methanol and 1% formic acid added to 0.3 grams of powder sample in 2 ml Eppendorf tube. Same was done for all the 96 samples and then tubes were shaken for five minutes for the complete dissolving of powder sample in solution. Later these tubes were kept in the ultrasonicate bath for ten minutes. Then centrifuge samples at 13330 rfx for 10 minutes, then extracts were transferred to AcroPrep filter plate (Figure 2) 1ml, by using BIO-RAD vacuum control where the samples were filtered with 0.2 μm PTFE membrane filter. The filtered sample was collected at the bottom plate. The extracts were further analysed by using liquid chromatography-mass spectrometry (LC-MS) as per the protocol and anthocyanins were observed by the absorption spectrum at a wavelength of 520 nm and molecular weight of observed anthocyanins (Wahyuni & Yury, 2013).
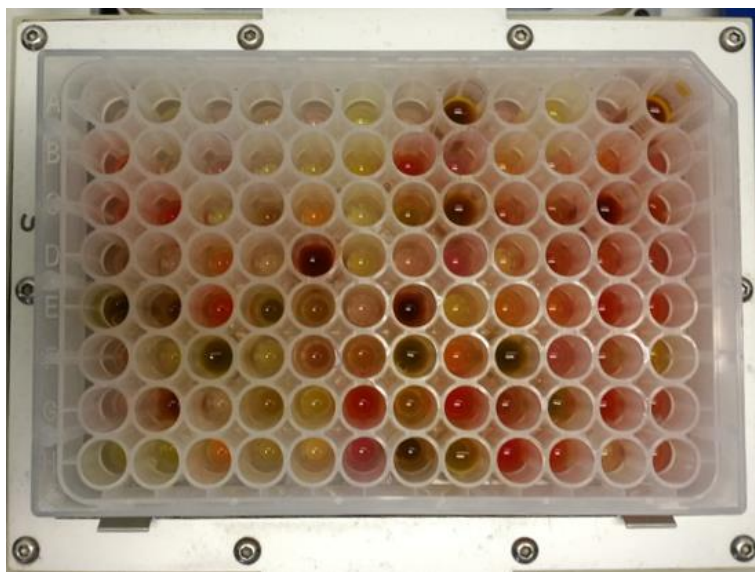
Figure 2: AcroPrep filter plate with 1ml filtrate extract sample mix.

4.2.2 Temperature experiment

To study the effect of temperature on anthocyanin concentration we planned an experimental set up with four pepper genotypes, cv. Tequilla, CGN22179, CGN23272 and cv. Black pearl with two temperature treatments 10°C and 30°C and relative humidity (RH) as 100%. To create a standard environment, white rectangular ribbed containers 400 x 267 mm were used with a filter paper inside to hold the moisture and aluminium foil container 150 x 70 mm to place the fruits and box is closed with a perforated plastic lid. Containers were labelled, later two fruits from respective genotypes of individual plants with different developmental stages were harvested from the greenhouse (Unifarm 10.10) and distributed into respective containers. Before storing the filter, papers were moisture to maintain the desired RH nearly 100%. The boxes were stored one in 10°C and 30°C from each plant of each genotype to compare the differences between two treatments regarding colour change and anthocyanin concentration. Images were taken on every alternative day to record visual differences in colour change. Damaged and fungus infected fruits were replaced with fresh fruits and moisture level of filter paper was checked regularly. The same process was continued for at least 10-14 days; storage time depends on the developmental stages and genotypes. After the storage peel was separated from flesh then frozen in liquid nitrogen and stored at -80°C for later use.

4.2.3 RNA extraction by the CTAB miniprep method

The isolation procedure was followed as per the protocol of CTAB miniprep for RNA with some modifications with same materials used for metabolomic analysis. Firstly 10 µl of 2-Mercaptoethanol is added to 900 µl of CTAB buffer (1 reaction) and 1ml of solution mix was taken into 2ml Eppendorf tube. Approximately 150 mg of powder sample was added to the tube and mixed thoroughly until the powder sample is completely dissolved in liquid. Then 500 µl of chloroform was taken into Eppendorf

tube shake well and vortex for two minutes at 14,000 X g. Transfer the upper phase of the solution to a new tube and again add (1chloroform:2upper phase solution) ratio of chloroform shake it and spin again. Transfer the upper phase to a new tube and add (1:1) ratio of isopropanol for precipitation of DNA and RNA and then spin the tubes for 15 minutes at room temperature at 3600 rpm. Discard the solution from the tube without disturbing the pellet at the bottom of the tube. Wash the pellet with 1ml of 70% ethanol and spin for 5 minutes at 14,000 X g. Discard the solution and dry the pellet at 37°C for 3-5 minutes. Dissolve the pellet with 200 µl of MilliQ and add 70 µl of 8M lithium chloride solution and precipitate it for 20 minutes at -20°C. Spin the tubes for 30 minutes at 4°C at 3600 rpm. The supernatant contains genomic DNA. Transfer or discard the supernatant and wash the RNA pellet briefly with 1ml of 70% ethanol and spin for 5 minutes at 14,000 X g. Discard the supernatant and dry the pellet and then dissolve the pellet in 80 µl of DNase buffer plus MilliQ (90 percent of MilliQ in 9 percent of buffer), add 20 µl of DNase enzyme and incubate at room temperature for 15 minutes. RNA clean-up procedure was done according to the protocol of QIAGEN RNeasy mini kit. To continue with clean-up procedure, the sample volume should be of 100 µl if not adjust the volume to 100 µl by adding RNase-free water and add 350 µl of RLT buffer (Lysis buffer) and mix well. Add 250 µl of (96-100%) ethanol and mix well only by pipetting. Transfer the (700 µl) solution into an RNeasy mini spin column with 2ml collection tube (pink) and centrifuge for 15 seconds at ≥ 8000 x g speed. Discard the flow through and add 500 µl of RPE buffer (Washing buffer) and centrifuge for 15 seconds at ≥ 8000 x g speed. Discard the flow through and repeat the step by spinning for 2 minutes at same speed. Make sure that no liquid remains in the RNeasy spin column if necessary spin again for 1 minute. Transfer the RNeasy spin column to the 1.5ml collection tube and add 30-50 µl of RNase-free water directly on the spin column membrane and spin it for 1 minute at ≥ 8000 x g speed. The concentration and quality of eluted RNA were measured by using Nanodrop Spectrophotometer. The quality of RNA is confirmed by gel electrophoresis with 1.5% of TAE buffer and ethidium bromide as fluorescent tag. Gel preparation: To make sure that working in a cleaned RNase free condition whole procedure was worked in a clean environment. Autoclaved TAE buffer was used, gel plate, trays and combs were soaked overnight in the soap solution and rinsed with MilliQ water to get rid of RNases that can degrade RNA. Working sample preparation: 200 ng/µl RNA samples were prepared with a total volume of 10 µl includes 8 µl of RNA plus MilliQ and 2 µl of loading dye and run on the gel with 1kb marker ladder. The samples with good quality RNA were labelled and stored in -80°C for further studies.

4.2.4 RNA extraction with GenElute Mammalian Total RNA miniprep kit (SIGMA-ALDRICH)

Firstly, 10 µl of 2-mercaptoethanol was added to 1ml of lysis solution. Transfer 500 µl of solution mix to 2ml Eppendorf tube and add up to 40 mg of powder sample and mix by vortex until the powder sample is completely dissolved. Transfer the 500 µl of lysate to filtration column (blue) with 2ml collection tube and spin for 2 minutes at 14,000 X g. Discard the filtration column and add 500 µl of

70% ethanol into the collection tube and mix thoroughly by pipetting. Transfer up to 700 μl of lysate mixture to binding column (Red) with a 2ml collection tube and spin for 15 seconds at 14,000 X g. Discard the flow through and repeat with the remaining lysate mixture. On-Column DNase I digestion was prepared by mixing 10 μl of DNase I with 70 μl of DNase Digest buffer for each reaction mix only by using pippet. Add 80 μl of reaction mix directly on to the binding column and incubate at room temperature for 15 minutes. Wash the column with 250 μl of wash solution 1 and spin for 15 seconds at 14,000 X g speed. Transfer the binding column to the new 2ml collection tube and wash the column with 250 μl wash solution 1 and spin for 15 seconds at 14,000 X g. Discard the flow through and wash the binding column with 500 μl of wash solution 2 and spin for 15 seconds at 14,000 X g. Repeat the step now spin for 2 minutes at 14,000 X g make sure that the membrane is free from ethanol repeat the spin for 1 minute if necessary. Transfer the binding column to the 1.5ml new collection tube and add 50 μl of the eluting solution to harvest total RNA from membrane and spin for 1 minute. The concentration and quality of eluted RNA were measured by Nanodrop Spectrophotometer. The quality of RNA is confirmed by gel electrophoresis with 1.5% of TAE buffer and ethidium bromide as fluorescent tag. Later the good quality RNA samples were frozen in liquid nitrogen and stored at -80°C for further studies.

4.2.5 cDNA synthesis

To perform the qRT-PCR, RNA should be converted into complementary DNA (cDNA). We used Taqman® Reverse transcription reagents kit. First, thaw the reagents on ice and prepare the master mix according to the guidelines given in kit (Table 1). Add the RNA sample, seal and incubate the reactions in a thermal cycler as following 25°C for 10 minutes (Annealing of primer), 48°C for 30 minutes (Extension) and 95°C for 5 minutes (Deactivating the RT enzyme). The final obtained reaction is 20 ng cDNA/μL with a volume of 50 μl and stores the samples at -20°C for further studies.

Table 1: Reverse transcription reaction mixture

Materials	Quantity (μl)
10* Taqman RT buffer	5
Mg cl2	11
dNTP mix	10
Oligo dT	2.5
RNase inhibitor	1
MultiScribe rev. Transcriptase	1.25
RNA (1μg)	X
MilliQ	19.25-X
Total volume	50

4.2.6 Real-time PCR

The amplification of cDNA by Real-time PCR was performed according to the protocol of qRT-PCR. Touch Deep Well TM thermal cycler (BIO-RAD Touch deep well TM Real-time PCR system) is used in our laboratory. The RT-PCR mix was prepared as per the guidelines of protocol shown in (Table 2). For each sample two technical replicates were prepared and water is used for non-template control (NTC). *β-actin* is used as a reference gene and the delta Ct values of reference gene were calculated in Microsoft Excel 2016.

Table 2: Preparation of RT-PCR mixture for Duplo.

Materials	Volume (µl)
2*iQ SYBR GREEN super mix	22
MilliQ	11
Forward primer (3µM)	4.4
Reverse primer (3µM)	4.4
cDNA (10ng/µl)	2.2
Total volume	44

5. Result

5.1 Anthocyanin concentration and accumulation pattern.

The results of our study identified two anthocyanins. Delphinidin-3-coumaroylrutinoside-5-glucoside (D-CmRut-G) was identified at a retention time of 18.07 minutes with a mass of m/z [M-H] 917.2363. Delphinidin-3-caffeoylrutinoside-5-glucoside (D-CfRut-G) was identified at a retention time of 15.67 with a mass equal to m/z [M-H] 933.2306. From the results of LC-MS the concentration and accumulation pattern of anthocyanins is also known. In purple capsicum genotypes cv.Tequilla, CGN22179 and CGN23273 the accumulation pattern of D-CmRut-G and D-CfRut-G was first increased and then decreased during the fruit development (Figure 3A, B and C). The concentration of D-CmRut-G was highly measured in dark purple fruits; the amount is increased from stage two to stage five. No significant difference was observed in quantity of D-CmRut-G in cv. tequilla between stage two to five and in stage six and seven the concentration was reduced (Figure 3A, B and C). Whereas the amount of D-CfRut-G was significantly increased from stage 4 and reached a maximum at stage five and later decreased in stage six and seven. No change in the concentration of D-CfRut-G in CGN22179 at stage four and five. However, in purple pepper fruit of CGN16951 (Figure 3D), the accumulation pattern of D-CmRut-G was gradually decreased from the first stage to the sixth stage but differences were observed only from stage three to six and the concentration of D-CmRut-G was similar in stage one and two. The concentration of D-CfRut-G was very low in all stages and similar to the concentrations of green fruit genotypes. In cv. Black pearl the concentration of D-CmRut-G was decreased from stage one to three and very low concentration of D-CfRut-G was observed in all three stages (Figure 3E). The accumulation pattern of D-CmRut-G in CGN23223 was increased in stage two and decreased from stage three to five. No significant changes in the quantity of D-CfRut-G were observed and the concentration was very low and it remained constant in all the stages (Figure 3F). No significant level of D-CmRut-G and D-CfRut-G was observed in control green-fruited genotypes Yuni12 and Yuni24 (Figure 3G and 3H).

The results of the metabolic analysis showed anthocyanins highly accumulated in dark purple colour pericarp samples it's interesting to study the relation between pericarp colour and anthocyanin concentration. A correlation study was done between RGB of the fruit and total anthocyanin content (TAC) a strong correlation was seen in purple-fruited genotype cv. Tequilla, cv. Black pearl and CGN22179 with R^2 of 0.83 (Figure 4), 0.86 (Figure 5) and 0.69 (Figure 6). Very poor correlation was seen in CGN16951the R^2 was 0.24 (Appendix 2) and no correlation was observed in CGN23223 with an R^2 of 0.0 (Appendix 3). A moderate correlation was present in Yuni12 with an R^2 of 0.56 (Appendix 4) and very poor correlation was seen in Yuni24 with R^2 of 0.21 (Appendix 5).
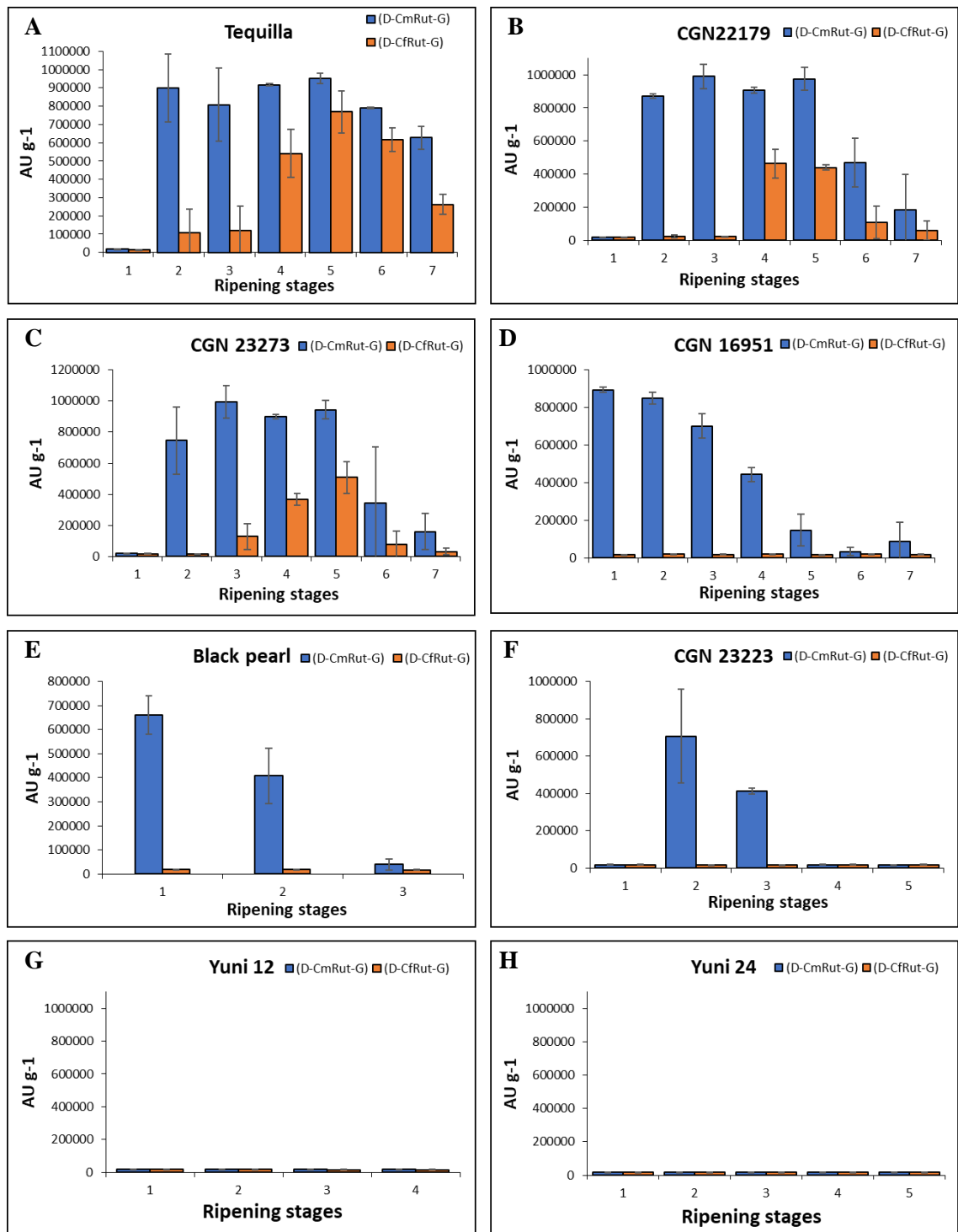
Figure 3: Accumulation pattern of anthocyanins in peppers:
On X-axis ripening fruit developmental and absorbance unit per gram on Y-axis. The developmental stages of individual genotypes are as follow, cv. Tequilla, CGN22179, CGN232731-Small green, 2- Green to purple, 3- Small purple, 4-Medium purple, 5- Big purple, 6- Purple to red, 7- Red. CGN16951 1- Small purple, 2- Medium purple, 3- Big purple, 4- Purple to white, 5- Purple to yellow, 6- Orange 7- Red. Cv. Black pearl 1-Small black, 2- Big black, 3- Red. CGN23223 1- Small green, 2- Small black, 3- Big green, 4- Green, 5- Brown. Yuni12 1- Young green, 2- Green, 3- Turning, 4- Red. Yuni24 1- Small green, 2- Medium green, 3- Big green, 4- Matured green, 5- Brown.

13

Figure 4: Correlation of fruit colour and total anthocyanin content (TAC) in cv. Tequilla: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1-Small green, 2- Green to purple, 3- Small purple, 4-Medium purple, 5- Big purple, 6- Purple to red, 7- Red.
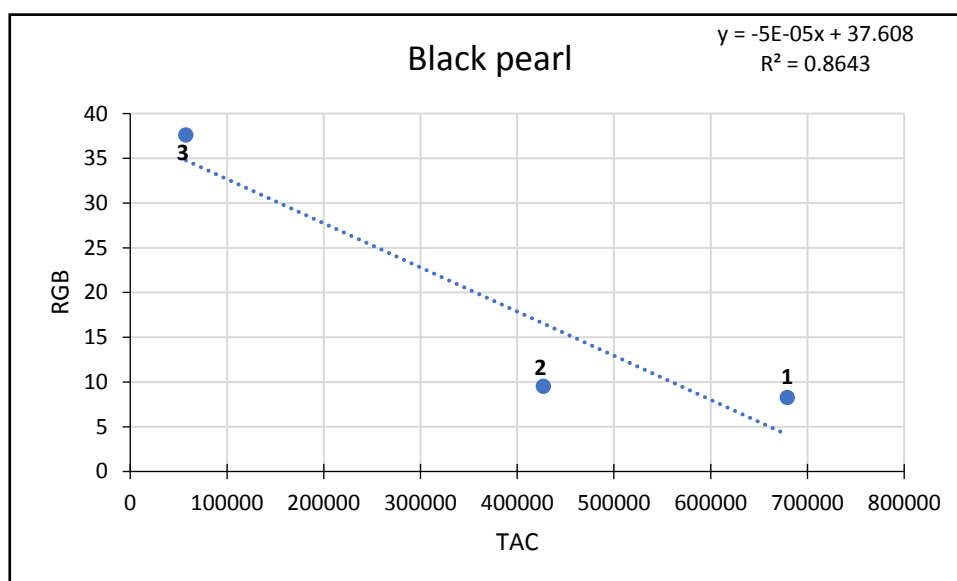
Figure5: Correlation of fruit colour and Total anthocyanin content (TAC) in cv. Black pearl: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1-Small black, 2- Big black, 3- Red.
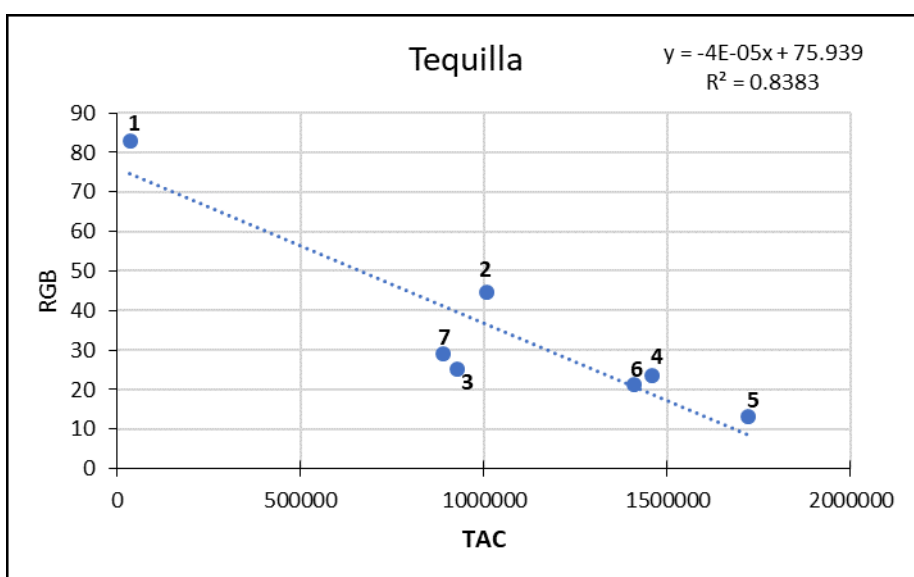
Figure6: Correlation of fruit colour and Total anthocyanin content (TAC) in CGN22179: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1-Small green, 2- Green to purple, 3- Small purple, 4-Medium purple, 5- Big purple, 6- Purple to red, 7- Red.
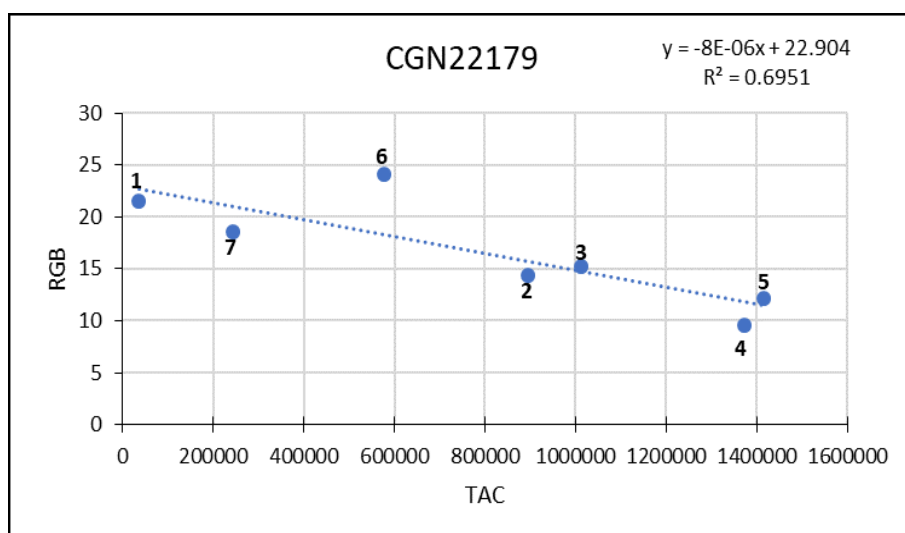
5.2 Temperature effect on anthocyanin concentrations.

This experiment was designed to study the effect of temperature on anthocyanin degradation. Two temperature treatments were established with three pepper genotypes described in materials and methods, their physical changes in the colour of the fruit were compared between two treatments with respective genotypes at different developmental stages.

In cv. Tequilla stage five (Big purple) fruits at 10˚C after five days the fruits remain freshed with no colour difference (Figure 7A), but from 30˚C after five days the fruit colour was changed, the fruit colour completely turned to red from purple (Figure 7B). Whereas in another capsicum genotype CGN22179 showed the same result with stage four (medium purple) fruits. No difference in the colour of fruits was observed in case of 10˚C treatment even after twenty days (Figure 7C), but a complete turnover of fruit colour from purple to red was observed from 30˚C treatment after two weeks (Figure 7D). In lateral stage (Red) the fruits remained same even after twenty days, no changes were observed from 10˚C treatment. Whereas in 30˚C fruit cracks, fungus growth and colour changed to dark red (Appendix 6). Same results were observed in fruits of CGN23273 and in black pearl, no differences in fruit colour were seen at 10˚C even after two weeks but, at 30˚C the fruits entirely turned to red from purple and few fruits showed the rotting and fungus growth symptoms.

15

5.3 Analysis of candidate RNA isolation method from the peel of pepper fruits.

This section identifies the candidate RNA isolation method from fruit tissues of pepper by comparing the three extraction procedures such as CTAB miniprep method and GenElute Mammalian Total RNA miniprep kit (SIGMA-ALDRICH). The results of the gel indicate it is possible to extract RNA by using the CTAB miniprep method, but the quality of the RNA is not up to the desired level for gene expression studies. Because for many samples the intensity of bands is not uniform and few samples of RNA was degraded (Figure 8). Compared to CTAB gel results the RNA from GenElute Mammalian Total RNA miniprep kit the quality of RNA is good with uniform intensity in bands for many samples and no RNA degradation was observed for Tequilla samples (Figure 9). Compared with cv. Tequilla, the quality of total RNA regarding band intensity was not uniform in case of CGN22179 and Yuni 24 (Figure 10).

Figure5: Temperature effect on anthocyanin degradation: **A.** Cv.Tequilla Big purple from 10ºC treatment. **B. Cv.**Tequilla Big purple from 30ºC treatment. **C.** CGN22179 Medium purple from 10ºC treatment. **D.** CGN22179 Medium purple from 30ºC treatment.
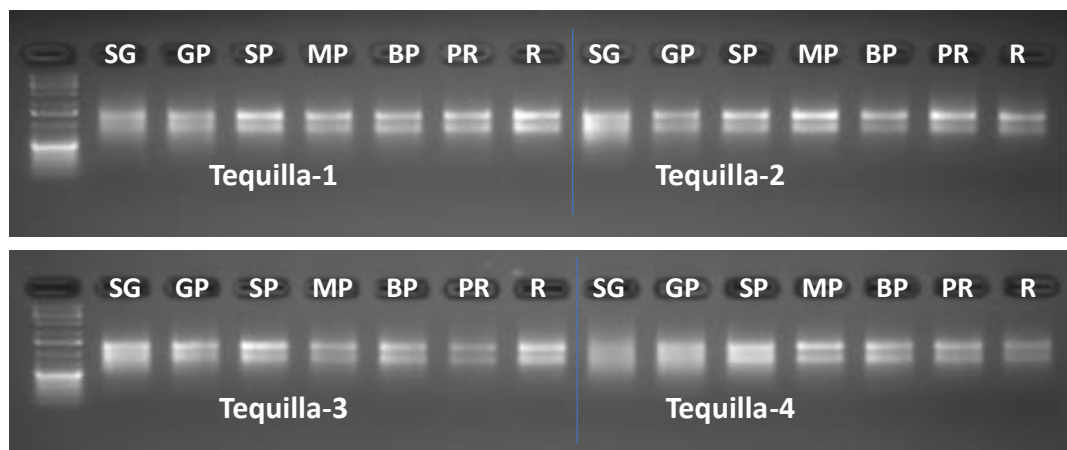
Figure 6: RNA gel for tequilla samples extracted by CTAB method: Left side 1Kb DNA ladder, RNA samples of different developmental stages. The sample codes represent as follow SG-Small green, GP-Green to purple, SP-Small purple, MP-Medium purple, BP-Big purple, PR- Purple to red, R- Red.

Figure 7: RNA gel for tequilla samples extracted by mammalian kit method: Left side 1Kb DNA ladder, RNA samples of different developmental stages. The sample codes represent as follow SG-Small green, GP-Green to purple, SP-Small purple, MP-Medium purple, BP-Big purple, PR- Purple to red, R- Red.

Figure 8: RNA gel of CGN22179 and Yuni24 samples extracted by mammalian kit method: Left side 1Kb DNA ladder, RNA samples of different developmental stages. The sample codes represent as follow SG-Small green, GP-Green to purple, SP-Small purple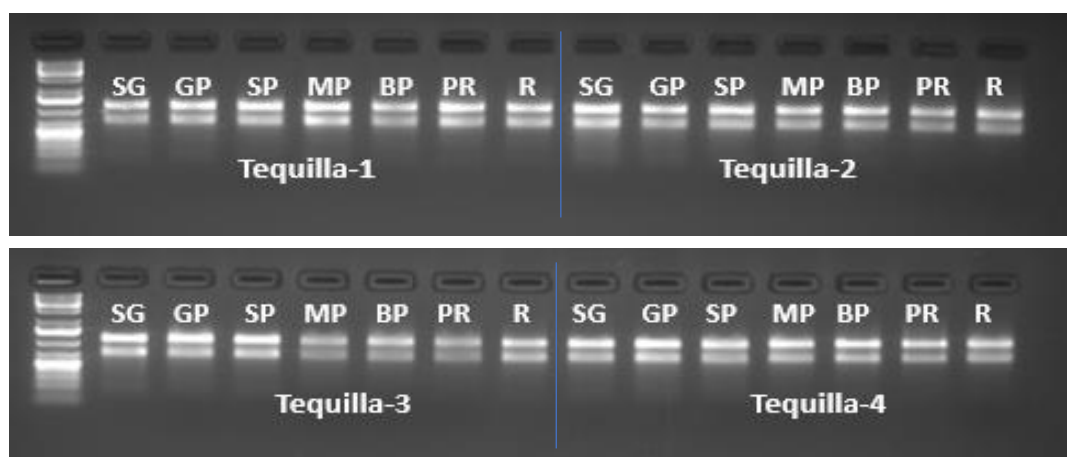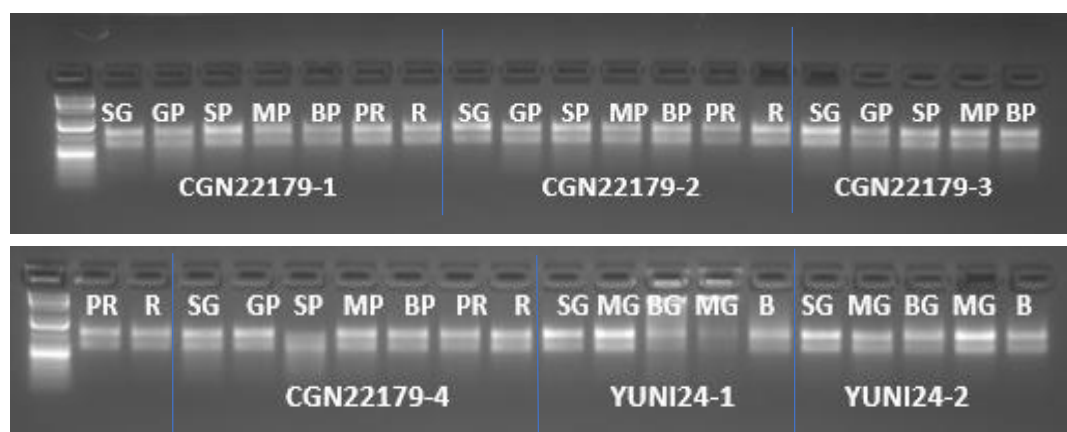, MP-Medium purple, BP-Big purple, PR- Purple to red, R- Red and SG- Small green, MG-Medium green, BG-Big green, MG-Matured green and B-Brown.

5.4 Validation of reference gene

In our study *β-actin* is used as a reference gene and the Ct values determined the expression of *β-actin* in three pepper genotypes and comparison between Ct values of cv. Tequilla from CTAB method and Mammalian kit. Most of the samples showed a stable expression but the Ct values were not within one cycle difference in three genotypes (Ct = 21.96 ± 0.67). However, the delta Ct values were not as we expected they differ in each genotype (Table 3). A good reference gene should be within one cycle difference. Therefore, the *β-actin* cannot be used as a housekeeping gene for gene expression studies.

Table 3: Delta Ct values of a reference gene in three genotypes.

Genotype	Extraction method	Del.ct values
Tequilla	CTAB	2.74
Tequilla	Mammalian kit	3.49
CGN22179	Mammalian kit	1.95
Yuni24	Mammalian kit	1.99

6. Discussion

The accumulation of anthocyanins in pepper fruits is mainly by two processes, namely anthocyanin biosynthesis and their degradation during the fruit ripening stages. The structure and metabolic profile of anthocyanins in peppers are studied. In this section, the concentration and accumulation pattern of anthocyanins in different pepper genotypes, the effect of temperature on anthocyanin degradation, the RNA extraction methods and the test for a reference gene were discussed.

6.1 The accumulation pattern of anthocyanins in different pepper genotypes.

In peppers so far only two forms anthocyanins, delphinidin derivatives, were observed which are delphinidin-3-caffeoyl-rutinoside-5-glucoside and delphinidin-3-coumaroyl-rutinoside-5-glucoside (Arboretum, & Human, 2018; Lightbourn et al., 2008; Sadilova et al., 2006; Vega, 2013). The anthocyanins were observed only in purple peel tissues, indicating that anthocyanins are responsible for purple colour and only accumulated in the pericarp of the fruit.

The accumulation pattern is first increased and then decreased in cv. Tequilla, CGN22179 and CGN23273 genotypes. Anthocyanin concentration is increased in early immature fruits and then decreased in matured ripening fruits of cv. Tequilla, CGN22179, CGN23273 and CGN16591 genotypes. But in case of control green fruit genotypes Yuni 12 and Yuni 24 no significant levels of anthocyanins were observed, the results were in agreement with our **Hypothesis 1: The anthocyanin accumulation pattern might first increase and then decrease in the matured ripe fruits of purple fruited genotypes and no anthocyanin accumulation may see in green fruited genotypes.** The concentration and stability of anthocyanins can be influenced by developmental stages. From the previous study Liu, Y. (2016), the results of anthocyanin accumulation were similar with (Borovsky et al., 2004), indicating that reduced level concentrations during ripening are by anthocyanin degradation. Liu, Y. (2016), mentioned the expression of candidate biosynthetic genes were stopped before turning stage and candidate degradative gene *POD CA02g17240* encodes with peroxidase to degrade anthocyanin. From our results the concentration of anthocyanins was increased in early fruit stages and reduced during ripening stages. The hypothesized mechanism is as follows. The high level of anthocyanin concentration in early stages might be due to induced biosynthetic processes whereas, the decreased concentration of anthocyanins in later stages might be induced degradation and inhibition of biosynthesis leads to discolouration of fruit. The concentration of anthocyanins is associated with fruit colour as the concentration was increased with the increased intensity of the purple colour and decreased with the colour change from purple to red.

In our study, we tried to correlate the anthocyanin concentration with RGB (average of red, green and blue) in order to find a non-destructive method to quantify anthocyanin concentration. The correlation results were not clear to use the non-destructive to correlate colour with anthocyanin content because the correlation was only seen in three genotypes. Also, a moderate correlation was observed in control

green-fruit genotype Yuni12 which cannot be true as the anthocyanin concentration levels was very low from metabolomics data.

The relation between coumaroyl and caffeoyl

Delphinidin-3-coumaroyl-rutinoside-5-glucoside and delphinidin-3-caffeoyl-rutinoside-5-glucoside are the major forms of anthocyanins observed in purple peppers. In purple peppers delphinidin-3-coumaroyl-rutinoside-5-glucoside acylated by *p*-coumaroyl acid represents about 90-95 % of total anthocyanin content followed by delphinidin-3-caffeoyl-rutinoside-5-glucoside (Sadilova et al., 2006). Hydroxycinnamic acids are non-flavonoid phenolics which are abundant in plants characterized by C6-C3 structures, helps the plants in defence strategy. The cinnamic acid derivatives are present in several fruits and vegetables, *p*-coumaric acid and caffeic acid are most commonly present in *Solanaceae* crops associate with antioxidant activity. *P*-coumaric acid is synthesized from the cinnamic acid by 4-cinnamic acid hydroxylase (C4H) enzyme. Later the *p*-coumaric acid is converted to caffeic acid by hydroxylation, in plants, the conversion hydroxylation is described to be catalysed by two possible pathways either by copper containing polyphenol oxidases or by P450 dependent hydroxylases (Strack & Lea, 2001). The conversion of the *p*-coumaric acid to caffeic acid is the first step of biosynthesis of flavonoids which is as follow:

p-coumaric acid + O_2 + AH_2 $\longrightarrow$ Caffeic acid + H_2O + A. In which AH_2 denotes for electron donors like NADH, ascorbic acid or L-dopa and the reaction can also be catalysed by several catechol oxidases (Meyer, 2009). Compared to *p*-coumaric acid, caffeic acid is superior in antioxidant activity this can be due to the presence of the *o*-dihydroxyl group in the phenyl ring (Andreasen et al., 2001; Meyer et al., 1998).

6.2 Temperature effect on anthocyanin content

The results of temperature experiment with two treatments 10°C and 30°C showed no significant change in fruit colour of stage 5 (Big purple) purple pepper genotype cv.Tequilla from 10°C (Figure 7A). whereas, a clear disappearance of purple colour was noticed in the stage 5 fruits of purple pepper genotype cv.Tequilla from 30°C treatment (Figure 7B) also similar results were observed in stage 4 fruits of CGN22179 (Figure 7C and 7D) was in agreement with our **Hypothesis 2: The anthocyanin concentration will be high in the fruits at low-temperature 10°C compared to fruits in high-temperature 30°C.** The disappearance of fruit colour in 30°C temperature might be hypothesized as either reduced biosynthesis or increased anthocyanin degradation or by a combination of both. Anthocyanin metabolism can also be influenced by environmental factors such as high irradiation, UV/Blue light and temperature (Jiang et al., 2016). Several studies have shown that temperature affected the anthocyanin biosynthesis. The low temperature induced the anthocyanin biosynthesis in tomato crop in hypocotyls, leaves and fruits (Qiu et al., 2016). In *Vitis vinifera L. cv. Sauvignon* fruits from high temperature (35˚C) showed a reduced level of total anthocyanin content compared with control (25˚C)

(Mori et al., 2007). In another grape cultivar *Vitis vinifera L. cv.* Sangiovese fruits at high temperature (36˚C) anthocyanin biosynthesis processes were suppressed at both transcriptional and enzymatic levels, with increased activity of peroxidase enzyme VviPrx31 (Movahed et al., 2016). This suppression is due to the combined impact of reduced biosynthesis and increased anthocyanin degradation (Movahed et al., 2016), which indicates VviPrx31 is responsible for anthocyanin degradation at high temperature. The increased peroxidase activity is the indicator of anthocyanin degradation. From our results anthocyanin accumulation was reduced during fruit development and reduced level of anthocyanins was observed in ripening fruits. The observations from temperature experiment showed at high temperature 30ºC the fruit colour was turned from purple to red. The disappearance of purple colour in fruits is hypothesized might be because of increased degradation of anthocyanins by peroxidase enzyme. At high temperature the peroxidase activity will be increased and degrade the anthocyanins (Movahed et al., 2016; Niu et al., 2017).

6.3 RNA extraction by different methods.

In this section, the only comparison between CTAB and GenElute Mammalian Total RNA miniprep kit was discussed.

The quality of RNA extracted by CTAB is not uniform among the samples compared to Mammalian kit and it is very laborious and lengthy process and several difficulties were encountered during the extraction of RNA by CTAB method and chances of RNA degradation was higher in CTAM method. Because of lengthy process and several rounds of pellet dilution and washings. The final harvested RNA still consists of genomic DNA in several samples even after DNase-1 treatment (Appendix 7). RNA degradation is also observed in many samples with the CTAB method. Whereas, mammalian kit extracted good quality of RNA compared to CTAB but still genomic DNA contamination was seen on the gel even after DNase-1 treatment (Appendix 8). Later increased lysis (500 µl) combination with on-column DNase treatment yielded good quality RNA with uniform band intensity and no contamination of genomic DNA was observed on RNA gel (Figure 9).

6.4 Expression of a candidate gene in different genotypes.

In this section only, the expression of a reference gene (*β-actin*) in three pepper genotypes is described in our group *β-actin* is used as candidate reference gene for gene expressions studies in peppers. To be a good reference gene, the reference gene should be expressed at the same level over different samples with Ct values less than one cycle difference. In our study, the expression of reference gene is not uniform in fruit tissues of three pepper genotypes and the Ct values differ in all three genotypes with more than one cycle difference. The results were not in agreement with our **Hypothesis 4: The expression of reference will be same in all samples and Ct values might be within once cycle difference.** One possible explanation for difference of Ct values might be the difference in the concentration of RNA.

7. Conclusion

In peppers anthocyanins accumulation is due to anthocyanin biosynthesis and degradation. The accumulation is seen only in the pericarp of the fruits obtained purple pigmentation. In young green fruits of purple genotypes, the purple pigmentation is not observed this can be due to the delay of anthocyanin biosynthesis. Later the purple colour was changed to red in matured ripening fruits. The purple discolouration was due to suppression of biosynthesis and induction of degradation. In our study two forms of anthocyanins were detected delphinidin-3-coumaroylrutinoside-5-glucoside and delphinidin-3-caffeoylrutinoside-5-glucoside. The accumulation pattern of anthocyanins was first increase and then decrease in purple fruit cv. Tequilla, CGN2217 and CGN23273 genotypes. The concentration of anthocyanins is associated with the intensity of fruit colour. The darker the fruit colour the higher concentration of anthocyanins was detected. Over fruit ripening stages of cv. Tequilla, darker fruits were observed in big purple stage and concentration of anthocyanins was detected higher in the big purple stage.

The colour shift of purple fruits of cv. Tequilla and CGN22179 from purple to red at 30ºC treatment, the possible explanation for degradation of anthocyanins at high-temperature can be due to increased peroxidase activity. The expression of reference gene is not same among all the samples and the Ct values were not within one cycle difference so *β-actin* cannot be a good reference gene. Therefore, a further research should be done for selecting a good reference gene with Ct difference less than one cycle.

8. Further prospective

8.1 Research on anthocyanin degradation

In our study, only anthocyanin concentration was measured in different genotypes and the gene expression study was not done. It is interesting to study the expression of candidate degrative genes in different genotypes. Also, metabolic analysis of temperature samples should be measured to know more information on the mechanism of anthocyanin degradation and enzymes involved in degradation that might result in a new candidate gene for further degradation studies.

8.2 Research on the antioxidant superiority

So far delphinidin-3-coumaroylrutinoside-5-glucoside and delphinidin-3-caffeoylrutinoside-5-glucoside are the only form of anthocyanins observed in peppers. Among the two anthocyanins caffeic acid is superior in antioxidant activity even though the percent of caffeic acid is very less in total anthocyanin content compared with *p*-coumaric acid. Antioxidants are the important characteristics of purple peppers so, it is interesting to study to increase the caffeic acid percentage by biotransformation of *p*-coumaric acid to caffeic acid.

8.2 Selection of RNA extraction kit

In our lab there was only once standard RNA kit Quick Gene RNA cell culture kit (FujiFilm Life Science) was available before the group relay on this kit due unknown reasons the kit was not available now. In our study I tried two different RNA extraction methods as a CTAB method, GenElute Mammalian Total RNA miniprep kit (SIGMA-ALDRICH). The total RNA quality from the mammalian kit was good with desired and similar concentration and uniformity band intensity. I would recommend a mammalian kit with on-column DNase can straight away start for RNA extraction.

9. Reference

Agricultural, B., Plant, N., Arboretum, U. S. N., & Human, B. (2018). Effects of Anthocyanin and Carotenoid Combinations on Foliage and Immature Fruit Color of Capsicum annuum L ., *99*(April), 105–111. https://doi.org/10.1093/jhered/esm108

Ahmed, N. U., Park, J., Jung, H., Hur, Y., & Nou, I. (2015). Anthocyanin biosynthesis for cold and freezing stress tolerance and desirable color in Brassica rapa, 383–394. https://doi.org/10.1007/s10142-014-0427-7

Andreasen, M. F., Landbo, A.-K., Christensen, L. P., Hansen, Å., & Meyer, A. S. (2001). Antioxidant Effects of Phenolic Rye (*Secale cereale* L.) Extracts, Monomeric Hydroxycinnamates, and Ferulic Acid Dehydrodimers on Human Low-Density Lipoproteins. *Journal of Agricultural and Food Chemistry*, *49*(8), 4090–4096. https://doi.org/10.1021/jf0101758

Barbagallo, R. N., Palmeri, R., Fabiano, S., Rapisarda, P., & Spagna, G. (2007). Characteristic of $^{\text{N}}$ᴸ - glucosidase from Sicilian blood oranges in relation to anthocyanin degradation, *41*, 570–575. https://doi.org/10.1016/j.enzmictec.2007.05.006

Borovsky, Y., Oren-Shamir, M., Ovadia, R., De Jong, W., & Paran, I. (2004). The A locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to Anthocyanin2 of Petunia. *Theoretical and Applied Genetics*, *109*(1), 23–29. https://doi.org/10.1007/s00122-004-1625-9

Dixon, R. A., Liu, C., & Jun, J. H. (2013). Metabolic engineering of anthocyanins and condensed tannins in plants. *Current Opinion in Biotechnology*, *24*(2), 329–335. https://doi.org/10.1016/j.copbio.2012.07.004

Fang, F., Zhang, X., Luo, H., Zhou, J., Gong, Y., Li, W., … Pang, X. (2015). An Intracellular Laccase is Responsible for the Epicatechin Mediated Anthocyanin Degradation in Litchi Fruit Pericarp. *Plant Physiology*, *169*(December), 2391–2408. https://doi.org/10.1104/pp.15.00359

Ghasemnezhad, M., Sherafati, M., & Ali Payvast, G. (2011). Variation in phenolic compounds, ascorbic acid and antioxidant activity of five coloured bell pepper (Capsicum annum) fruits at two different harvest times. *Journal of Functional Foods*, *3*, 44–49. https://doi.org/10.1016/j.jff.2011.02.002

Harborne, J. B., & Williams, C. A. (2000). Advances in ⁻avonoid research since 1992, *55*.

Hoballah, M. E., Gu, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., … Kuhlemeier, C. (2007). Single Gene – Mediated Shift in Pollinator Attraction in Petunia, *19*(March), 779–790. https://doi.org/10.1105/tpc.106.048694

Jiang, M., Ren, L., Lian, H., Liu, Y., & Chen, H. (2016). Plant Science Novel insight into the mechanism underlying light-controlled anthocyanin accumulation in eggplant (Solanum melongena L .). *Plant Science*, *249*, 46–58. https://doi.org/10.1016/j.plantsci.2016.04.001

Lachman, J., & Hamouz, K. (2005). Red and purple coloured potatoes as a significant antioxidant source in human nutrition – a review, *2005*(1), 477–482.

Lightbourn, G. J., Griesbach, R. J., Novotny, J. A., Clevidence, B. A., Rao, D. D., & Stommel, J. R. (2008). Effects of Anthocyanin and Carotenoid Combinations on Foliage and Immature Fruit Color of Capsicum annuum L. *Journal of Heredity*, *99*(2), 105–111. https://doi.org/10.1093/jhered/esm108

Liu, Y. (2016). Anthocyanin regulation in bell pepper fruit Anthocyanin regulation in bell pepper fruit, (890427523050), 1–3.

Materska, M., & Perucka, I. (2005). Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (Capsicum annuum L.). *Journal of Agricultural and Food Chemistry*,

53(5), 1750–1756. https://doi.org/10.1021/jf035331k

Meyer, A. S. (2009). Grape skins (Vitis vinifera L .) catalyze the in vitro enzymatic hydroxylation of p-coumaric acid to caffeic acid enzymatic hydroxylation of p -coumaric acid to caffeic acid, (June 2014). https://doi.org/10.1007/s10529-009-0103-5

Meyer, A. S., Donovan, J. L., Pearson, D. A., Waterhouse, A. L., & Frankel, E. N. (1998). Fruit Hydroxycinnamic Acids Inhibit Human Low-Density Lipoprotein Oxidation in Vitro. *Journal of Agricultural and Food Chemistry*, *46*(5), 1783–1787. https://doi.org/10.1021/jf9708960

Mori, K., Goto-Yamamoto, N., Kitayama, M., & Hashizume, K. (2007). Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany*, *58*(8), 1935–1945. https://doi.org/10.1093/jxb/erm055

Movahed, N., Pastore, C., Cellini, A., Allegro, G., Valentini, G., Zenoni, S., … Filippetti, I. (2016). The grapevine VviPrx31 peroxidase as a candidate gene involved in anthocyanin degradation in ripening berries under high temperature. *Journal of Plant Research*, *129*(3), 513–526. https://doi.org/10.1007/s10265-016-0786-3

Niu, J., Zhang, G., Zhang, W., Goltsev, V., Sun, S., & Wang, J. (2017). Anthocyanin concentration depends on the counterbalance between its synthesis and degradation in plum fruit at high temperature. *Scientific Reports*, (January), 1–16. https://doi.org/10.1038/s41598-017-07896-0

Oa, W., Gou, J.-Y., Felippes, F. F., Liu, C.-J., Weigel, D., & Wang, J.-W. (n.d.). Negative Regulation of Anthocyanin Biosynthesis in Arabidopsis by a miR156-Targeted SPL Transcription Factor. https://doi.org/10.1105/tpc.111.084525

Oodward, G. A. R. Y. W., Roon, P. A. U. L. K., Assidy, A. E. C., & Ay, C. O. K. (2009). Anthocyanin Stability and Recovery : Implications for the Analysis of Clinical and Experimental Samples, 5271–5278. https://doi.org/10.1021/jf900602b

Oren-Shamir, M. (2009). Does anthocyanin degradation play a significant role in determining pigment concentration in plants? *Plant Science*. https://doi.org/10.1016/j.plantsci.2009.06.015

Petroni, K., & Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science*, *181*, 219–229. https://doi.org/10.1016/j.plantsci.2011.05.009

Qiu, Z., Wang, X., Gao, J., Guo, Y., & Huang, Z. (2016). The Tomato Hoffman ' s Anthocyaninless Gene Encodes a bHLH Transcription Factor Involved in Anthocyanin Biosynthesis That Is Developmentally Regulated and Induced by Low Temperatures, 1–22. https://doi.org/10.1371/journal.pone.0151067

Rowan, D. D., Cao, M., Lin-Wang, K., Cooney, J. M., Jensen, D. J., Austin, P. T., … Allan, A. C. (2009). Environmental regulation of leaf colour in red 35S:PAP1 Arabidopsis thaliana. *New Phytologist*, *182*(1), 102–115. https://doi.org/10.1111/j.1469-8137.2008.02737.x

Sadilova, E., Stintzing, F. C., & Carle, R. (2006). Anthocyanins , Colour and Antioxidant Properties of Eggplant (Solanum melongena L .) and Violet Pepper (Capsicum annuum L .) Peel Extracts.

Strack, D., & Lea, P. J. (2001). Enzymes Involved in Hydroxycinnamate Metabolism, *25*(3), 70–81.

Tanaka, Y., & Ohmiya, A. (2008). Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. https://doi.org/10.1016/j.copbio.2008.02.015

Vaknin, H., Bar-Akiva, A., Ovadia, R., Nissim-Levi, A., Forer, I., Weiss, D., & Oren-Shamir, M. (2005). Active anthocyanin degradation in Brunfelsia calycina (yesterday–today–tomorrow) flowers. *Planta*, *222*(1), 19–26. https://doi.org/10.1007/s00425-005-1509-5

Vega, O. M. D. E. L. A. (2013). Anthocyanin accumulation and expression analysis of biosynthesis-related genes during chili pepper fruit development, *57*(1), 49–55. https://doi.org/10.1007/s10535-012-0265-1

Wahyuni, Y., & Yury, A. B. (2013). Metabolomics and molecular marker analysis to explore pepper (Capsicum sp .) biodiversity, 130–144. https://doi.org/10.1007/s11306-012-0432-6

Zhang, Z., Pang, X., Ji, Z., & Jiang, Y. (2001). Role of anthocyanin degradation in litchi pericarp browning, *75*, 217–221.

Zhang, Z., Pang, X., Xuewu, D., Ji, Z., & Jiang, Y. (2005). Food Chemistry Role of peroxidase in anthocyanin degradation in litchi fruit pericarp, *90*, 47–52. https://doi.org/10.1016/j.foodchem.2004.03.023

Zipor, G., Duarte, P., Carqueijeiro, I., Shahar, L., Ovadia, R., Teper-Bamnolker, P., … Oren-Shamir, M. (2015). In planta anthocyanin degradation by a vacuolar class III peroxidase in Brunfelsia calycina flowers. *New Phytologist*, *205*(2), 653–665. https://doi.org/10.1111/nph.13038

10. Appendix

Appendix 1: List of samples used for metabolomics analysis.

Genotype	Developmental stages	Replications	No.of fruits harvested from each plant for each replicate	Sample type
Tequila	Small green	4	4	Peel
	Green purple	4	3	Peel
	Small purple	4	4	Peel
	Medium purple	4	2	Peel
	Big purple	4	1	Peel
	Purple to Red	4	2	Peel
	Red	4	1	Peel
CGN16951	Small purple	4	5	Peel + Flesh
	Medium purple	4	4	Peel + Flesh
	Big purple	4	4	Peel + Flesh
	Purple to white	4	4	Peel + Flesh
	Purple to yellow	4	4	Peel + Flesh
	Orange	4	4	Peel + Flesh
	Red	4	4	Peel + Flesh
CGN22179	Small green	4	3	Peel + Flesh
	Green purple	4	2	Peel
	Small purple	4	2	Peel
	Medium purple	4	2	Peel
	Big purple	4	1	Peel
	Purple-red	4	1	Peel
	Red	4	1	Peel
CGN23223	Small green	4	3	Peel + Flesh
	Small black	4	3	Peel
	Black green	4	2	Peel
	Green	4	2	Peel
	Brown	4	2	Peel
CGN23273	Small green	3	3	Peel + Flesh
	Green purple	3	3	Peel
	Small purple	3	3	Peel
	Middle purple	3	2	Peel
	Big purple	3	1	Peel
	Purple-red	3	1	Peel

	Red	3	1	Peel
Black pearl	Small black	4	5	Peel + Flesh
	Big black	4	5	Peel + Flesh
	Red	4	5	Peel + Flesh
Yuni-12	Young green	4	1	Peel
	Green	4	1	Peel
	Turning	4	1	Peel
	Red	4	1	Peel
Yuni-24	Small green	2	5	Peel + Flesh
	Medium green	2	5	Peel
	Big green	2	3	Peel
	Matured green	2	3	Peel
	Brown	2	3	Peel

Appendix 2: Correlation between RGB and TAC in CGN16951
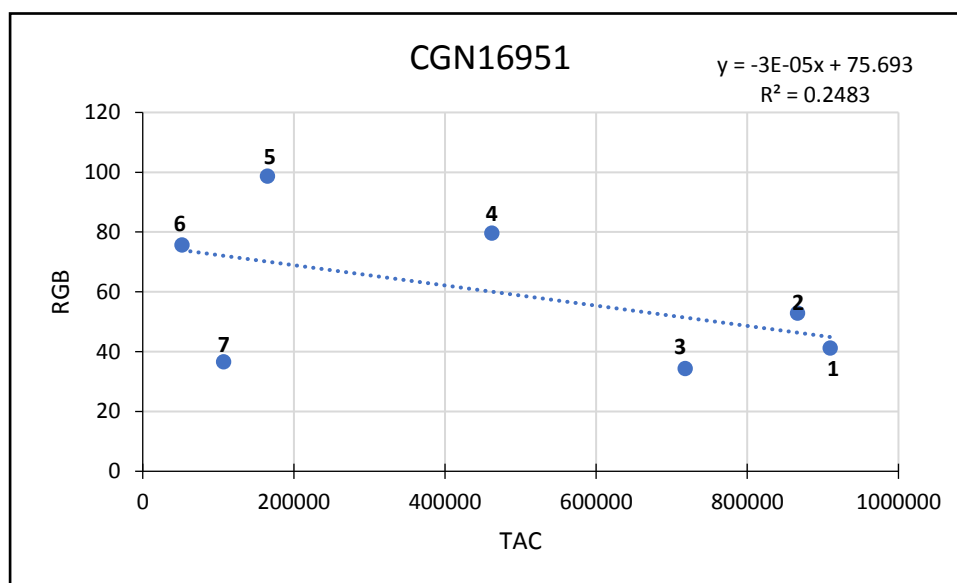
Figure1: Correlation of fruit colour and total anthocyanin content (TAC) in CGN16951: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1- Small purple, 2- Medium purple, 3- Big purple, 4- Purple to white, 5- Purple to yellow, 6- Orange 7- Red.

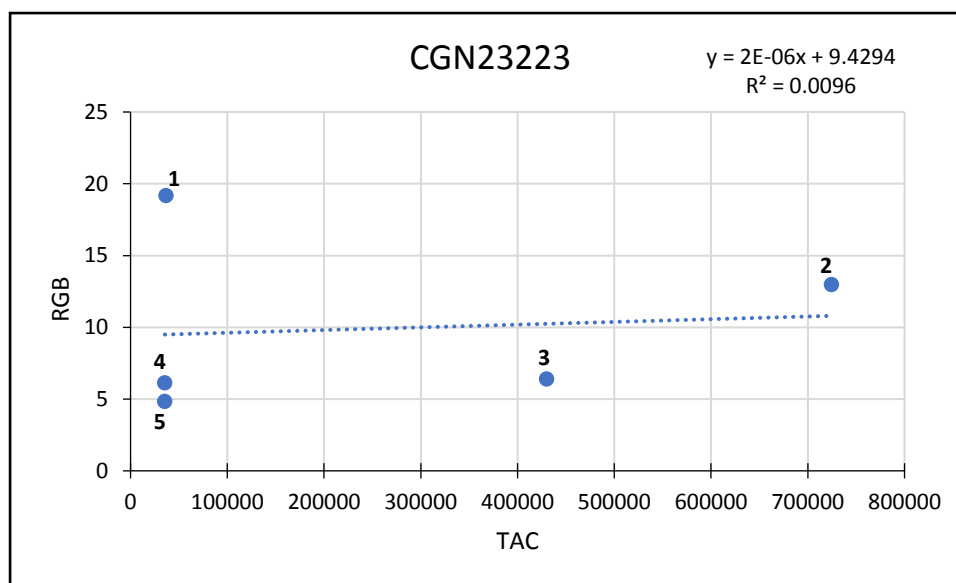## Appendix 3: Correlation between RGB and TAC in CGN23223

Figure2: Correlation of fruit colour and total anthocyanin content (TAC) in CGN23223: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1- Small green, 2- Small black, 3- Big green, 4- Green, 5- Brown.
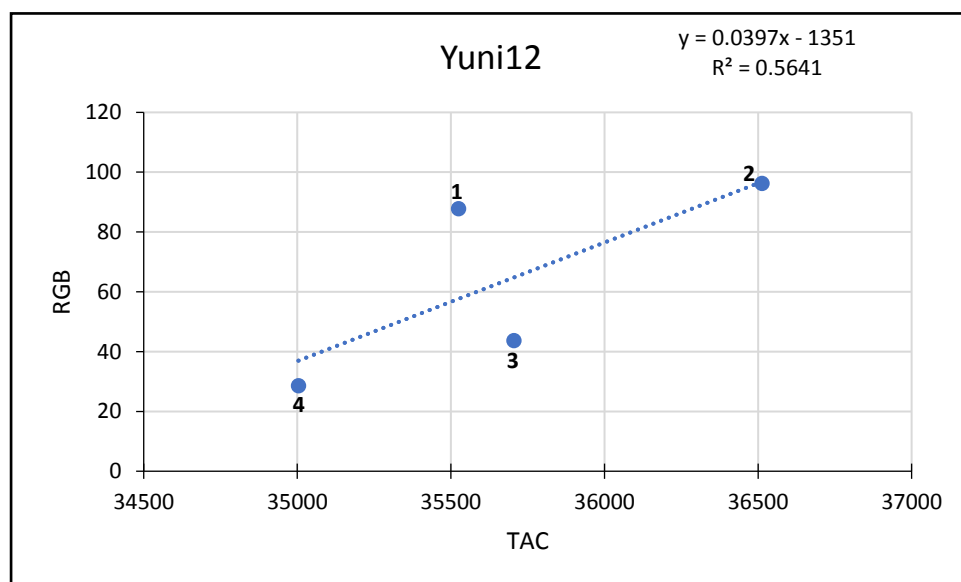
Appendix 4: Correlation between RGB and TAC in Yuni12

Figure3: Correlation of fruit colour and total anthocyanin content (TAC) in Yuni12: On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1- Young green, 2- Green, 3- Turning, 4- Red.

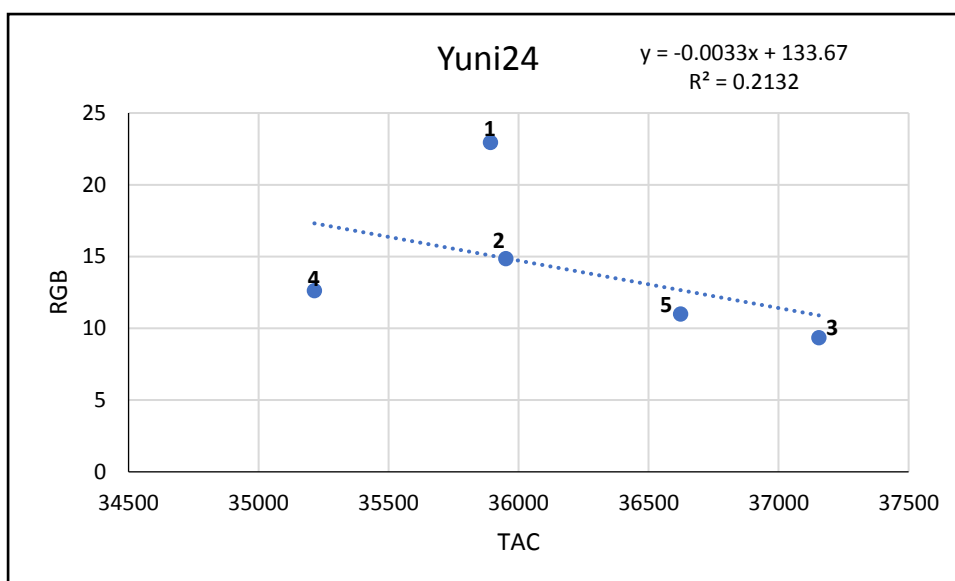**Appendix 5: Correlation between RGB and TAC of Yuni24**

Figure4: Correlation of fruit colour and total anthocyanin content (TAC) in Yuni24:
On Y-axis average RGB values of fruit colour and X-axis indicates the TAC absorbance unit per gram. The numbers on the trend line denotes ripening stages of fruit as follow, 1- Small green, 2- Medium green, 3- Big green, 4- Matured green, 5- Brown.

Appendix 6: Matured red fruits of CGN22179 from 10 ºC and 30ºC treatment.

Figure59: A. From 30ºC on 1st day of storage B. Same fruits after two weeks of storage, C. From 10ºC on 1st day of storage D. Same fruit after two weeks of storage.

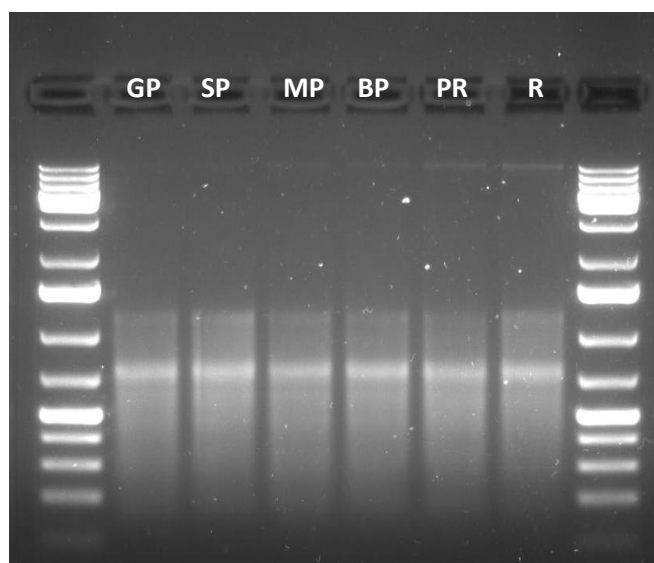**Appendix 7: RNA gel of tequilla samples with DNase-1 treatment after CTAB method**

Figure6: RNA gel of tequilla smaple from CTAB miniprep method: Left side 1Kb DNA ladder, RNA samples of different developmental stages. The sample codes represent as follow, GP-Green to purple, SP-Small purple, MP-Medium purple, BP-Big purple, PR- Purple to red, R- Red.

Appendix8: RNA gel of Tequilla samples with DNase-1 treatment after GenElute Mammalian RNA Kit method
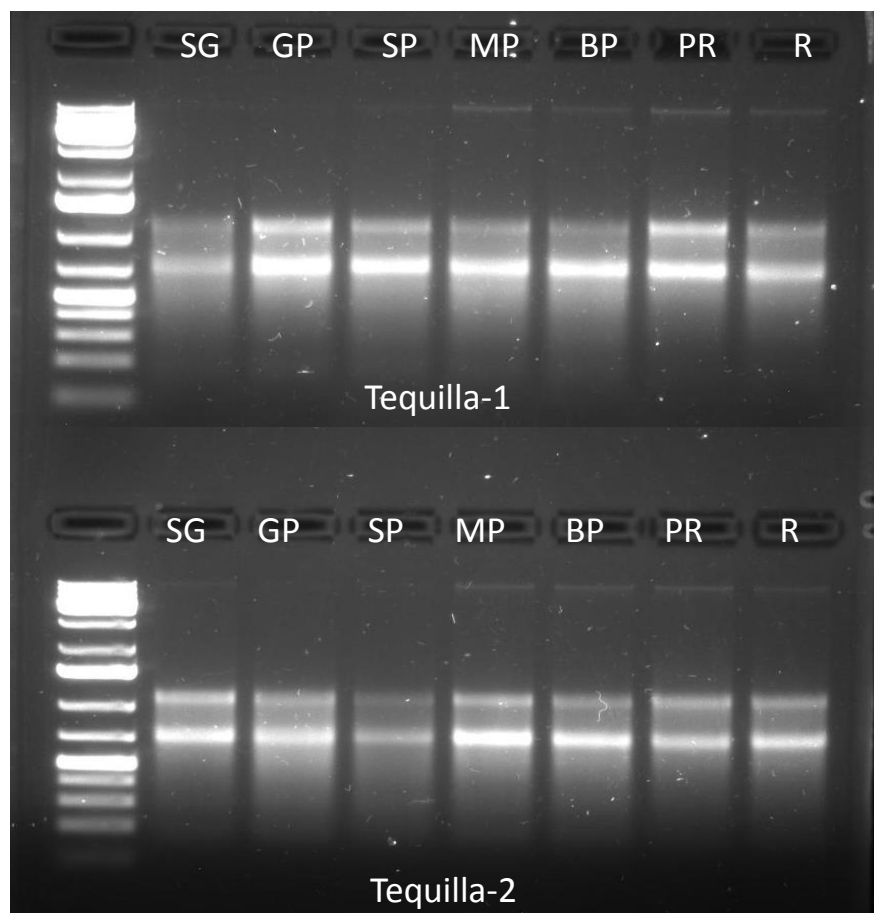

Figure7: RNA gel of tequilla smaple from GenElute Mammalian RNA Kit: Left side 1Kb DNA ladder, RNA samples of different developmental stages. The sample codes represent as follow SG-Small green, GP-Green to purple, SP-Small purple, MP-Medium purple, BP-Big purple, PR- Purple to red, R- Red.