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THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Biosciences, Fisheries and Economics, Department of Arctic and Marine Biology

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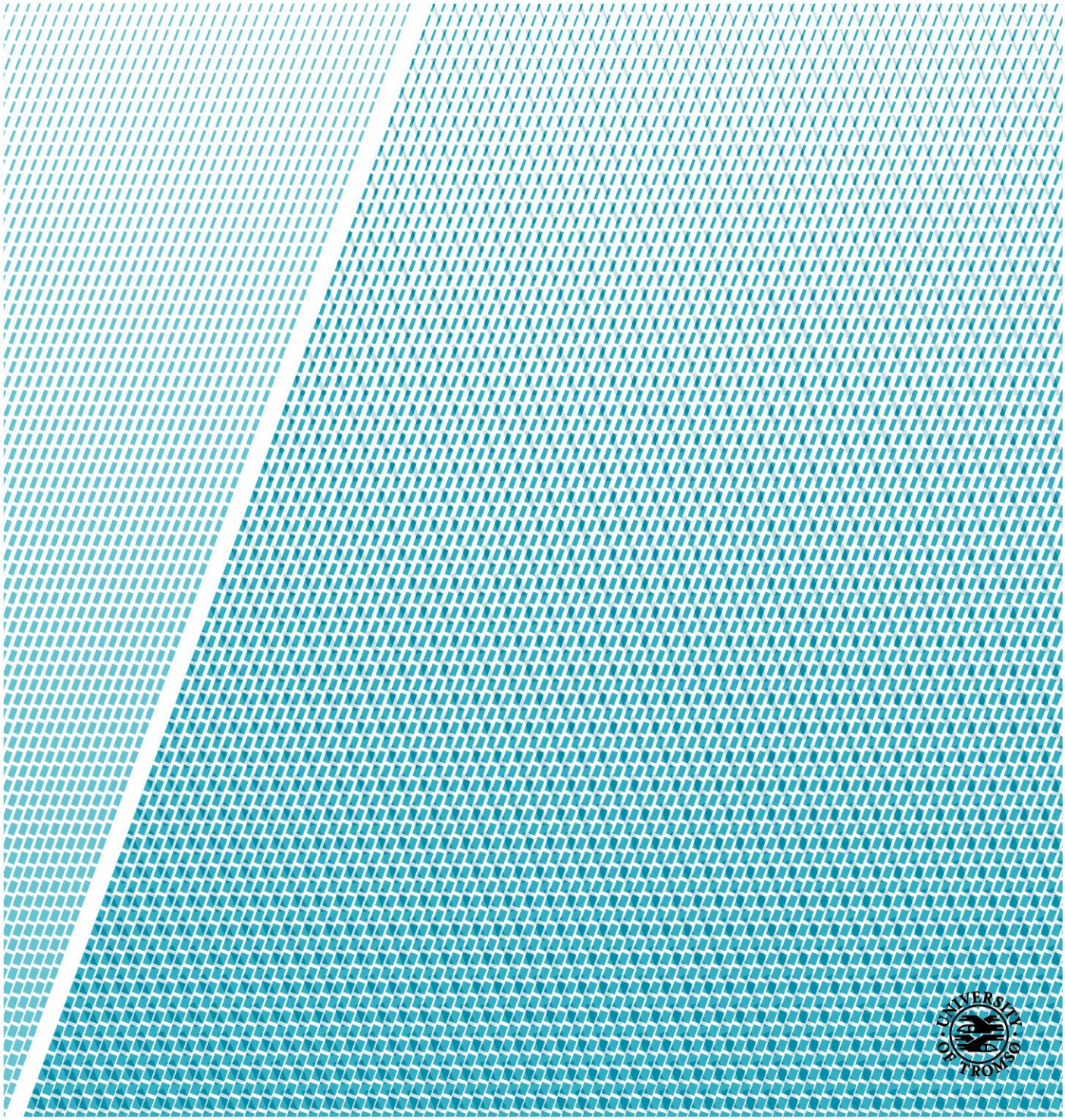
Florence Omowumi, Omole

Bio-3950 Master's Thesis in Biology … July, 2019



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Supervisors:

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Abstract

Flavonoids are a group of phenolic compounds produced in plants. They have many biological functions in plants and also beneficial bioactivities in human's health. Light quality is one of the major environmental factors regulating the accumulation of flavonoids in plants. Understanding plants accumulation of secondary metabolites in response to light qualities is important in order to optimize plants quality and cultivation conditions.

The biosynthesis of flavonoids is well understood and the structural genes of the pathway have been characterized from many plant species, including *Fragaria* spp. The *MYB-R2R3* transcription factors play significant roles in regulating the flavonoid biosynthesis genes. *FvMYB10* acts as a positive regulator of late anthocyanin biosynthesis gene in both leaves and third ripe stage berries, *FvMYB1* also acts as a repressor in the early fruit developmental stages. However, in leaf tissues, it interacts with *FvMYB10* to positively regulate flavonoid pathway genes.

In the present study, an experiment was carried out to study the effect of spectral light qualities (red, blue, far-red, white fluorescent light as control) on *F. vesca* clones from a different latitudinal origin; Italy, South Finland and North Norway, two separate clones from each origin. The results showed that red and blue LED lights had a prominent effect on the total anthocyanin accumulations and the related gene expression in all clones, although the effects vary between leaves and fruits. Also, *F. vesca* clones of different origins responded differently to the LED light treatments. The leaves and early stage fruits of the Italian clones responded most to blue light, whereas the leaves and the early stage fruits of the Finnish and North Norwegian clones responded most to red light. Interestingly, a shift in this pattern was detected at the end of fruit ripening, when the Italia clones responded more to red light, similarly to the Finnish clones, and the Norwegian clones responded more to blue light. Therefore, the results of this study give novel evidence on the role of the latitude related adaptations in the light perception affecting the flavonoid biosynthesis, and offer new tools for deeper understanding of this regulation.

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Table of Contents

List of Equations

List of Tables

List of Figures

Abbreviations

LED: Light-emitting diode

RT-qPCR: Real-time polymerase chain reaction

dNTP: Deoxynucleoside triphosphate

ACs: Anthocyanins

CTAB: Cetyl Trimethyl Ammonium Bromide

IAA: Isoamyl Alcohol

RNA: Ribonucleic acid

cDNA: Complementary Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

PVP: Polyvinylpyrrolidone

TAE: Tris-acetate-EDTA

DTT: Dithiothreitol

MW: Molecular Weight

DF: Dilution Factor

Keywords

Flavonoid pathway, Strawberry, Gene expression, Total anthocyanin, Light-emitting diode, Light spectrum, Latitude

1 Introduction

1.1 Woodland strawberry (*Fragaria vesca* L.)

The strawberries of genus *Fragaria* are perennial herbaceous plants that belong to the Rosaceae plant family containing 23 species at varying ploidy levels, including 13 diploids, four tetraploids, one hexaploid, and four octoploids (Folta & Davis, 2006). Woodland strawberry (*Fragaria vesca L.)* is a diploid species with chromosome number ($2n = 2x = 14$). It is a wild relative of the cultivated garden strawberry (*Fragaria* × *ananassa*) (Egan et al., 2018), and one of the most wildly distributed natural species. It is largely appreciated because of its natural appearance, great taste, aroma, and high composition of essential nutrients and bioactive compounds. Berries are majorly consumed either fresh or in processed products such as juices, jams, yoghurts and as flavours in cakes and pies. Both berries and leaves of woodland strawberry have traditionally been used for medicinal purposes (Dias et al., 2016; Mudnic et al., 2009). Its small genome size (240 Mb) makes it a favourable model species for genetics studies. *F. vesca* grows naturally in the wild mostly in the Northern hemisphere and can also be cultivated in the garden. In Norway, its distribution is from the south to 70°30′N latitude at Tana in the county of Finnmark and reaches an altitude of 1220 m above sea level at Hardangervidda (Heide & Sønsteby, 2007).

F. vesca can be propagated asexually by runners (stolon) or sexually by the seeds. It has a rootstock stem with short internode and leaves that are arranged spirally in a way that every sixth leaf is above the first. Individual leaf comprises three leaflets attached to the tip of an extended petiole emerging above the crown. The leaflets are spherical to oblong in shape with saw-toothed edges and a thick cuticle layer. The apical bud, together with the uppermost part of the rootstock is called crown (Heide et al., 2013). From the crown, the adventitious root is formed in the underground part, which extends deeply into the soil to form several lateral roots to take nutrient and water into the plant and on the aerial, auxiliary crown, leaves, runners and inflorescence are formed (Taylor, 2002). At the vegetative state, the apical meristem of the crown produces new internodes and one trifoliate leaf with an extended petiole in each node; one axillary bud is also formed in every node. The auxiliary bud of the crown may remain inert or produce branched crown or stolon, depending on the growing conditions. The stolon consists of two elongated internodes, which are terminated by a daughter plant on the second node. After

the daughter plant has been formed and is in good growing conditions, the second auxiliary bud on the runner plant will form secondary or elongate runner (Hytönen, 2009). The first node usually remains dormant or develops another small stolon. A runner plant can only become independent without support from its mother plant after it develops several lateral roots in the presence of good soil moisture. Runner plants are mainly used for the cultivation of strawberries commercially.

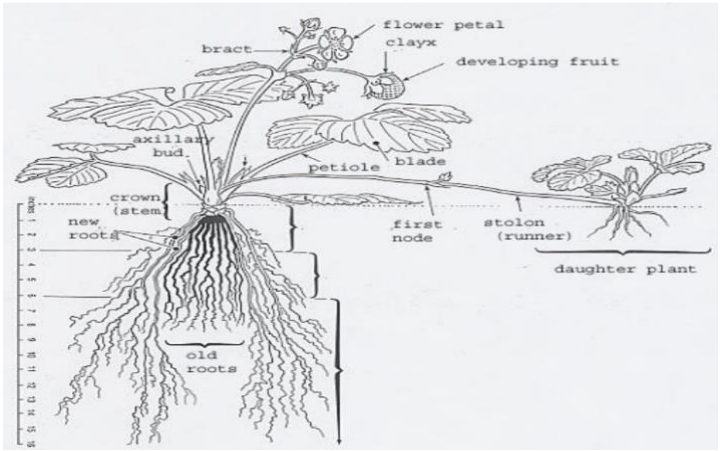


Figure 1: Anatomy of a strawberry plant (Poling, 2012)

Inflorescence develops at the apex of the crown. The induction started by the elongation of the apical growing point, which also includes the broadening and flattening of the apex. Flower

initiation in *F. vesca* is controlled by low temperature between the narrow range of 10 to 15 °C. (Brown & Wareing, 1965; Koskela, 2016). Inflorescence initiation is immediately followed by the development of secondary flowers, which can be seen on the axis of the inflorescence. The branch structures of the strawberry inflorescence are characterized as "dichasial cymes" where each truss show incredible cultivar of structures (Taylor et al., 2008). The main flora peduncle terminates with a primary flower; the primary terminal flower is the largest and produces the largest fruit. Secondary flowers develop terminally on each of the two to four branches formed underneath the primary flower. Afterwards, two tertiary flowers are developed from each secondary branch, and also quaternary flowers from each tertiary branch, which will after pollination further develop to berries (Heide et al., 2013).

The fruit size is determined by the size of the flower and the number of floral parts, especially carpel. However, external factors also play a role. In the diploid *F. vesca.*, the flower usually contains ten sepals, five petals, 20 anthers, and numerous carpels situated on a fleshy receptacle (Heide et al., 2013). The fruit of strawberry is neither a berry nor a true fruit, as the actual fruit consists of numerous dry achenes (or seeds) that dot the surface of the enlarged receptacle, a fleshy modified shoot tip. Strawberry is regarded to be non-climacteric because unlike other Rosaceae family crops, such as apple and peach, the flesh does not ripen in response to ethylene (Shulaev et al., 2011).

Early fruit development of *F. vesca* from inflorescence to fertilization and to green fruit is divided into five stages, which involve complex developmental, morphological, physiological, and hormonal changes. In stage one, the flowers are opened. This is the pre-fertilization stage. Stage 2 is the post-anthesis when fertilization has just taken place and signs of senescence begin to show. This includes loss of all petals, the colour of styles turning pink, and enlargement of the ovary. Inside the seed of stage 2 is a globular stage embryo. Stage 3 is characterized by red and dry styles, complete loss of anthers, and a heart stage embryo inside each seed. At stage 4, embryos adopt morphology of torpedo or walking stick. The embryo's two cotyledons stay upright at stage 5 and fill the whole seed. The cotyledons turn from transparent to white, indicating the maturation of the embryo. Stage 5, therefore, marks the maturation of the embryos and achenes. Size-wise, achenes gradually expand from stage 1 to stage 3 but stay comparatively constant from stage 3 to stage 5. In contrast, the receptacle stays comparatively

constant in size between stages 1 and 2 but gradually expands from stages 2 to 5, revealing more and more receptacle tissues between achenes (Kang et al., 2013).

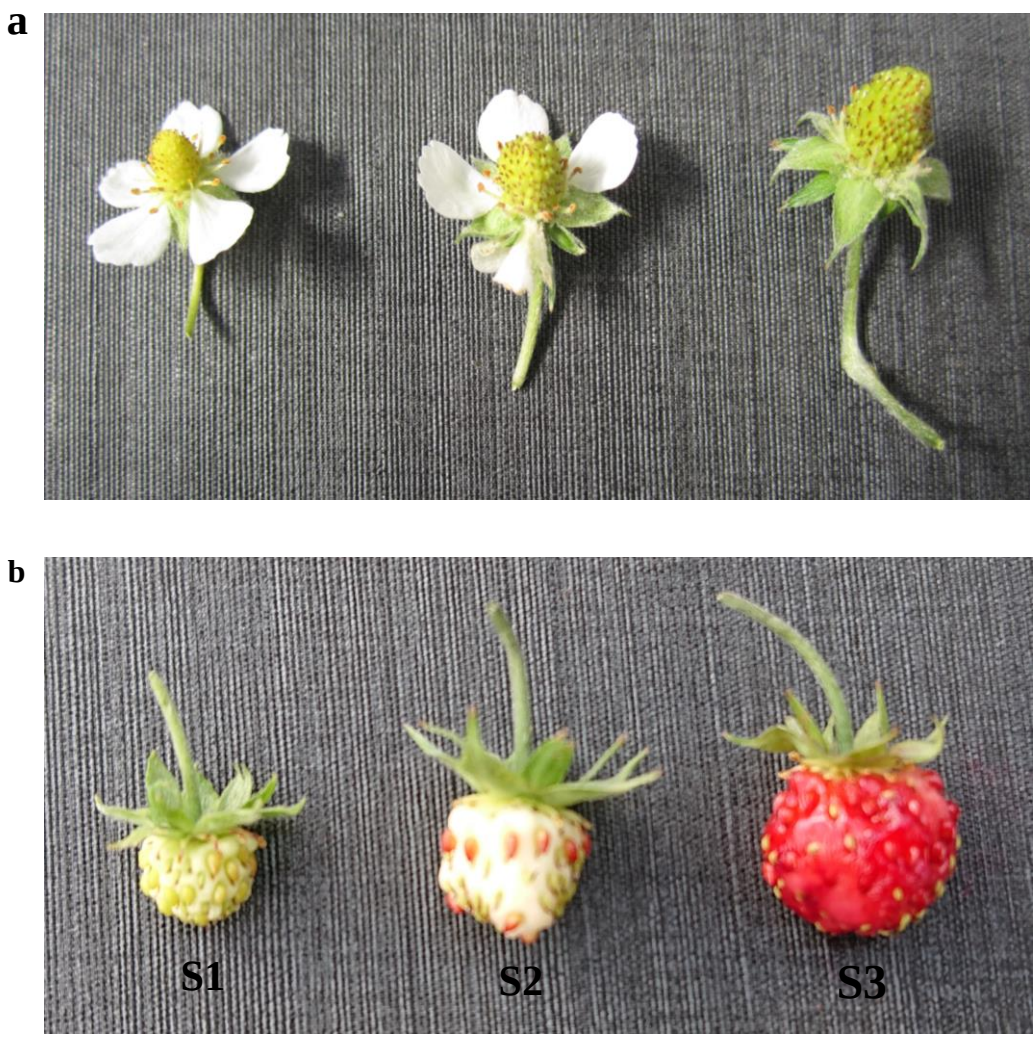


Figure 2: Developmental stages of *F. vesca* berries a) Different flowering stages to early unripe berries, b) three stages of berry development; stage 1 (S1), stage 2 (S2) and stage 3 (S3).

Vegetative growth, as well as floral initiation and the formation of stolon, are controlled by environmental factors such as light, temperature, photoperiod and also nutrition. There are two main classes of flowering and fruiting pattern in strawberry, the seasonal flowering (SF) and the everbearing flowering (EB). The SF produces flowers and fruits once in the spring and EB produces more or less continuous flowering and fruiting throughout the growing season.

Octoploid cultivated strawberry, *Fragaria x ananassa*, and diploid woodland strawberry, *F. vesca* both exhibit the two growing pattern, and independently of ploidy level, (SF) genotypes are mostly short-day plants, while (EB) genotypes are mostly long-day plants (Heide et al., 2013).

1.2 Nutritional and antioxidant properties of wild woodland strawberries (*Fragaria vesca* L.*)*

Woodland strawberry fruits are excellent sources of essential nutrients, for instance; carbohydrates (e.g., sucrose), soluble dietary fibers, and polyunsaturated fatty acids, mainly linoleic and linolenic acids, as well as other elements such as citric and succinic acids, and vitamins B9 and E (mostly γ-tocopherol) (Dias et al., 2016). They are also found to produce a wide range of phytochemicals of which phenolic compounds predominate. These compounds are synthesized in plants for their physiological development as well as for defence mechanisms against environmental stress; for instance, protection against UV-B, pathogens and pests attack. However, they also have been shown to possess several health beneficial activities in the human diet, including potent antioxidant, anticancer, anti-atherosclerotic and anti-neurodegenerative effects, (Giampieri et al., 2012; Seeram, 2015), which makes them interesting research target for several studies.

Woodland strawberry has mostly been known for its aroma and strongly flavoured berries with a higher composition of phenolic compounds than commercial varieties (Doumett et al., 2011; Urrutia et al., 2017), even though it has lower productivity. The major class of phenolic compounds is represented by the flavonoids (mainly anthocyanins, with flavonols and flavanols providing a minor contribution), followed by hydrolyzable tannins (ellagitannins and gallotannins) and phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), with condensed tannins (proanthocyanidins) being the minor constituents (Giampieri et al., 2012). High-performance liquid chromatography (HPLC) coupled with ion spray mass spectrometry in the tandem mode with both negative and positive ionization was used for investigating a variety of polyphenolic compounds in *F. vesca* berries, and about 60 phenolic compounds belonging to the compound classes of phenolic acids, ellagitannins, ellagic acid derivatives,

flavonols, monomeric and oligomeric flavanols, dihydrochalcones and anthocyanins were reported (Del Bubba et al., 2012).

1.3 Anthocyanins

Anthocyanins (ACs) are quantitatively the most important and best known polyphenolic compounds in strawberries (Giampieri et al., 2012). These compounds are accumulated only at the ripe phase of most fruits. ACs are important pigments in flowers, fruits and leaves, contributing to the quality of fruits and serving as visual signals for pollinators and seed dispersers (Koes et al., 2005). Additionally, they are recognized as compounds that have potential health benefits, as they have shown to exhibit antioxidant, anticarcinogenic, antimicrobial, and antiviral properties (Zhang et al., 2018). ACs are water-soluble pigment compounds belonging to the flavonoid class, their pigments are predominantly present in glycosylated forms of their aglycon, which contribute to their increased stability and aqueous solubility in vacuoles. They are structurally composed of an anthocyanidin aglycone bound to one or more sugar moieties (Jaakola, 2013).

There are about 17 anthocyanidins found in nature, whereas only six of them, namely pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt), and malvidin (Mv), are frequently present in plants. Of these, cyanidin is most common and can be found in over 82% of examined fruits and berries (Jaakola, 2013). The major anthocyanin in strawberries is Pelargonidin 3-glucoside, and the presence of cyanidin-3-glucoside seems also constant in all varieties but in smaller proportions (da Silva et al., 2007). Furthermore, although glucose appears to be the most common substitute sugar in strawberry anthocyanins, also rutinose, arabinose and rhamnose conjugates have been discovered in some strawberry cultivars (Giampieri et al., 2012).

1.4 Flavonoid biosynthetic pathway

Genes involved in flavonoid biosynthesis have been analyzed in *F. vesca* (Yuchao Zhang et al., 2015) and many other plant species, such as *Arabidopsis thaliana* (K. Saito et al., 2013) and

Vaccinium myrtillus (Jaakola, 2002). The pathway involves several genes that act in two stages, namely, early and late biosynthetic stages. Early biosynthetic stages start in the phenylpropanoid pathway with the catalysis of phenylalanine to yield cinnamic acid and coumaroyl-CoA followed by the synthesis of chalcone. These reactions are catalyzed by a group of enzymes, which are as follow: phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate: CoA ligase (*4CL*), and chalcone synthase (*CHS*). Chalcone synthase (*CHS*) acts in the first step of the flavonoid biosynthetic pathway, it catalyzes the condensation of one molecule of 4-coumaroyl-coenzyme A (*CoA*) and three molecules of malonyl-CoA, to form naringenin chalcone. In the second step, chalcone isomerase (*CHI*) catalyzes the isomerization of chalcone into flavanone. Afterwards, flavone synthase (*FNS*) introduces a double bond between the C2 and C3 positions of flavanone, converting flavanone into flavone. Flavanone 3-hydroxylase (*F3H*), also referred to as flavanone 3β-hydroxylase (*FHT*), catalyzes the 3-hydroxylation of flavanone which results in dihydroflavonol. Flavonoid 3`-hydroxylase (*F3`H*) and flavonoid 3`, 5`-hydroxylase (*F3`5`H*) catalyze the hydroxylation of the B ring of flavonoids at the 3` and the 3` 5`-position, respectively. Flavonol synthase (*FLS*) catalyzes the desaturation of dihydroflavonol into flavonol (Hossain et al., 2018; K. Saito et al., 2013).

In the late biosynthetic step, dihydroflavonol 4-reductase (*DFR*) compete with *FLS* for the same substrate. It catalyzes the reduction of dihydroflavonol into leucoanthocyanidin. Anthocyanidin synthase (*ANS*), also termed leucoanthocyanidin dioxygenase (*LDOX*), catalyzes the conversion of leucoanthocyanidin into anthocyanidin (Deng et al., 2018). Anthocyanidins are finally glycosylated to anthocyanins via the enzyme uridine diphosphate (*UDP*)-glucose:flavonoid-O-glycosyl-transferase (*UFGT*). The flavonoid biosynthetic enzymes are localized in the cytosol. After biosynthesis, flavonoids are transported to vacuoles or cell walls (Jaakola, 2013).

The transcription of structural genes encoding the enzymes that directly participate in the formation of flavonoids are controlled by the regulatory *MYB-bHLH-WD40* "*MBW*" protein complex, which is formed by highly conserved transcription factors (TFs) *R2R3-MYB* interacting or not with MYC-like basic helix-loop-helix (*bHLH*) proteins and/or with *WD40*-repeat

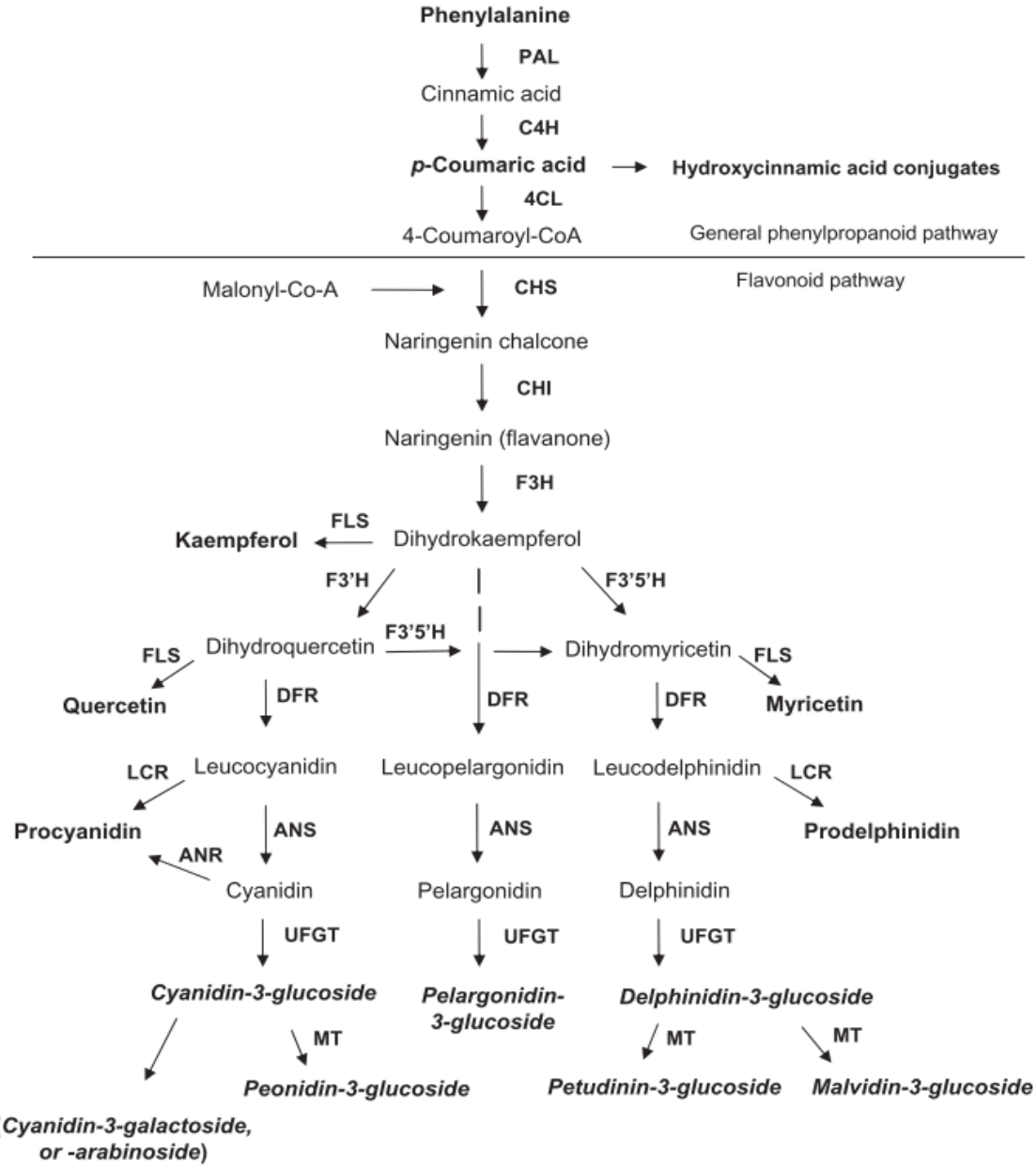


Figure 3: A schematic of the major pathway of flavonoid biosynthesis. Enzyme abbreviations: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumaroyl:*CoA* ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone 3-hydroxylase; *F3'H*, flavonoid 3′ hydroxylase; *F3'5'H*, flavonoid 3′5′ hydroxylase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol 4-reductase; *LCR*, leucoanthocyanidin reductase; *ANR*, anthocyanidin reductase; *ANS*, anthocyanidin synthase; *UFGT*, *UDP* glucose-flavonoid-O-glycosyl-transferase; *RT*, rhamnosyl transferase; *MT*, methyltransferase (Jaakola & Hohtola, 2010).

proteins. They are involved in the regulation of the pathways of diverse secondary metabolites, signal transduction, developmental changes, and disease resistance. MBW complex is unique to plants but varies among species, for instance in Arabidopsis, the early biosynthetic genes can be regulated by an independent *R2R3-MYB* co-activator, while late biosynthetic genes are known to be regulated by ternary MBW complex. In grape and apple, *MYB-bHLH* complex (lacking *WD40*) regulates anthocyanin biosynthesis. In strawberry, *FaMYB10* is reported as the main regulator of anthocyanin biosynthesis. Most of the MYBs involved in the control of flavonoid biosynthesis are positive regulators that enhance the expression of the structural flavonoid pathway genes. However, repressors have also been characterized, such as *FaMYB1* and *FcMYB1* in strawberry and *AtMYB3*, *AtMYB4* and *AtMYBL2* in Arabidopsis. The combined interactions of the regulatory genes activate or inhibit the expression of individual or set of structural genes (some of which might have pleiotropic effects) that act in a coordinated way to regulate the flux of different branches of the pathway, which eventually determines the flavonoid composition in the tissue. The pathway is also modulated by environmental factors, particularly light and temperature (Hossain et al., 2018; Zoratti et al., 2014).

1.5 Environmental effects on the accumulation of flavonoids

The biosynthesis of secondary metabolites in *Fragaria vesca* is influenced by the plant's genotype and environmental factors such as light, temperature, soil water, nutrient, fertility and salinity (Bian et al., 2015). External factors may trigger modifications in the composition of phenolic compounds in plant tissue, but the plant's genetic background is the primary determinant (Carbone et al., 2009). Among these factors, light has been reported in strawberry and other plants to play a significant role. Light is one of the most important environmental factors as plants depend on it for their sole photosynthetic energy. Many physiological responses in plants, including flavonoid biosynthesis, are controlled by the composition of visible light spectrum from solar radiation. The biologically active radiation consists of the spectrum from approximately 300 to 800nm including UV-light (below 400 nm). The visible light spectrum ranges between 400 and 710nm and is subdivided in blue (400– 495 nm), green (495–570 nm), yellow (570–590 nm), and red (590–710 nm) wavelengths. There is far-red light (710–750 nm) at the extreme end of the visible light spectrum, followed by infrared radiation (Zoratti, Karppinen, et al., 2014).

Plants, through their photoreceptors, which perceive light signals, react to changes in light intensity, photoperiod, direction and wavelength (Folta & Childers, 2008). These include photoreceptors absorbing red/far-red light (*PHYA*, *PHYB*, *PHYC*, *PHYD*, *PHYE*) as well as cryptochromes (*CRY1*, *CRY2*, *CRY3*), and phototropins (*PHOT1*, *PHOT2*) sensing UV- A/blue light, and UV-B photoreceptor *UVRESISTENCE LOCUS8* (*UVR8*), which was identified recently (Figure 4). When light is absorbed, these photoreceptors activate different signal transduction cascades to control light-dependent responses and related gene expression in plants (Zoratti et al., 2014). Protected cultivation system such as the use of greenhouses, tunnels and growth chambers has become popular across the globe as they contribute to sustainable agriculture. Improvement of these production systems has led to the development of different innovations, one of which is LEDs illumination. In the past decades, conventional lighting system such as high-pressure sodium lamps (HPSL), metal halide lamps, incandescent lamps, and fluorescent lamps, which are characterized by broad spectral power distribution, with limited control over the emissions of UV or infrared radiation, are used in protected cultivation facilities to supplement solar light. These lighting systems have been reported to improve light conditions and optimize plants quality and biomass production in greenhouses. However, certain disadvantages exist when these light sources are used. For instance; only 30% of the consumed electricity by HPSL is converted to light with 70% of the electricity lost as heat (Bian et al., 2015).

Unlike conventional light sources, light-emitting diodes (LEDs) can provide narrowly-centred spectrum with the properties of small size, lightweight, longevity, low energy consumption and heat output. The spectral output of an LED lighting system can be matched to plant photoreceptors and optimized to provide maximum production without wasting energy on nonproductive (Morrow, 2008; Rini et al., 2018)

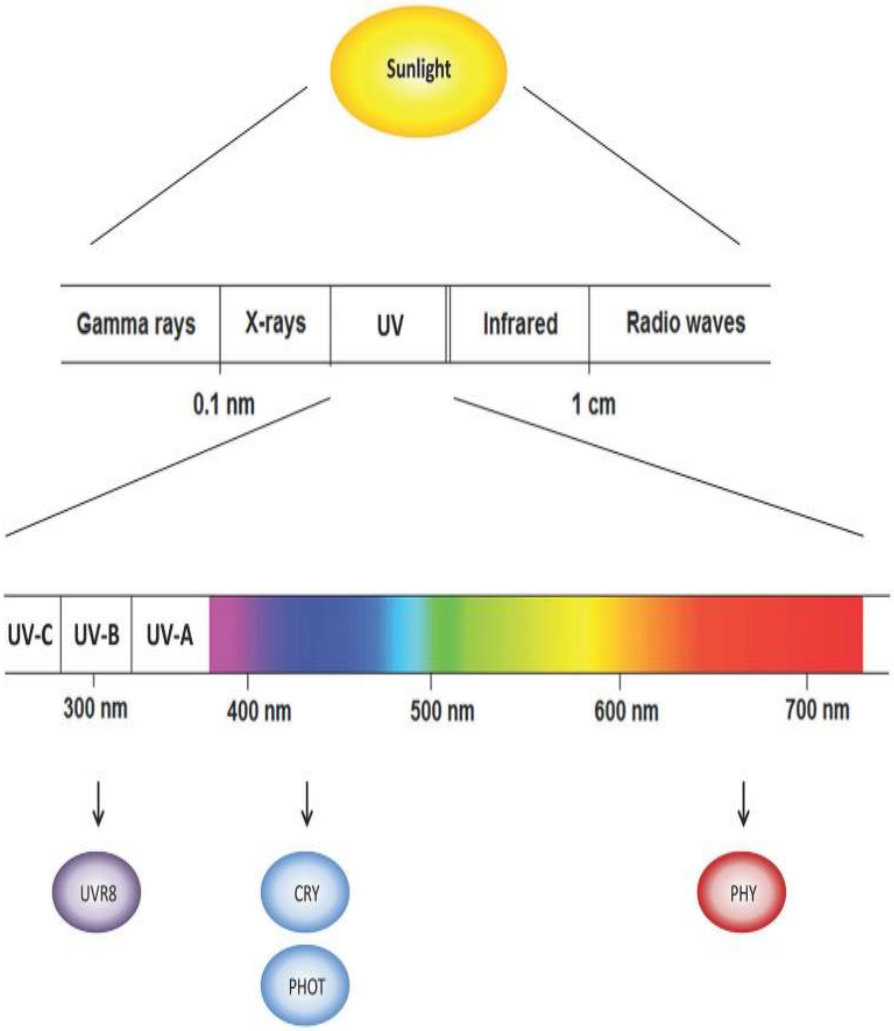


Figure 4: The spectrum of solar radiation reaching from gamma rays to radio waves with a closer view on visible wavelengths and plant photoreceptors absorbing specific wavelength regions. Cry, cryptochromes; Phy, phytochromes; Phot, phototropins; UV, ultraviolet; UVR8, UV-B photoreceptor (Zoratti, Karppinen, et al., 2014)

Red light is similarly essential for the growth of plant photosynthetic apparatus and can boost the accumulation of starch in several plant species by inhibiting the translocation of photosynthates from the leaves, while blue light is important in chlorophyll formation,

chloroplast development, stomatal opening, enzyme synthesis, activation of the circadian rhythm of photosynthesis and photomorphogenesis (Schuerger et al., 1997). Among artificial lighting systems, LEDs have the highest photosynthetically active radiation potential (80–100), which is used for the formation of various metabolic pathways, such as those involved in the synthesis of phenolic compounds (Schuerger et al., 1997). LED lamps have been used to successfully grow strawberry and other fruits and vegetables in greenhouses and growth chambers with the production of higher yields, qualities and improved defence mechanism against stress and pathogen attacks (Bantis et al., 2018; Rini et al., 2018; Zhang et al., 2018).

1.6 Latitude and altitude effects

Latitude appears to affect the accumulation of flavonoids in plants. Plants that are genetically similar may have developed different strategy, which assists them to adapt well to a distinct environment, one of which is the additional accumulation of secondary metabolites, which protect the plant from damages from an increased UV light radiation. The exposure of plants to high amounts of UV radiation can cause damage to their macromolecules, such as DNA. Altitude also affects the contents of secondary metabolites in higher plants. Alpine sites are exposed more to higher radiation, (especially UV-B) than lower habitats. Higher solar radiation at higher altitudes has often been indicated as having an effect on the secondary metabolite profile (Jaakola & Hohtola, 2010).

2 Aim of the study

The aim of the work is to investigate the anthocyanin accumulation and changes in expression of the flavonoid biosynthesis pathway genes of *Fragaria vesca* L. under different spectra of LED light supplement treatments. The study was also focused on investigating latitude specific variations, in order to explain recent findings that show that flavonoid biosynthesis genes expression in plants varies among different origins. There have been several studies on Octoploid cultivated strawberry (*Fragaria x ananassa)* that investigated the expression of flavonoid biosynthesis pathway genes under different spectra of LED light, and there is evidence that genes respond differently to a different wavelength. However, only a few studies have been carried out on diploid woodland strawberry (*Fragaria vesca* L.), also detailed studies that focus on comparing woodland strawberry clones from different latitudes are still missing.

The specific aims of the study were:

1. To study the effect of different wavelengths of supplemental LED light on the expression of flavonoid biosynthesis pathway genes.
2. To study variations in gene expression of plants originating from different latitudes, under the different wavelength of the LED light.
3. To analyze the variation in anthocyanin accumulation of ripe berries under different LED light treatment using a spectrophotometer. Metabolic profiling using HPLC/MS will be performed at later stages of this project.

The genomes of the woodland strawberry clones used in the experiment have been sequenced at the University of Helsinki, Finland (Research group of Dr Timo Hytönen).

3 Materials and Methods

3.1 Plant materials and experimental design

The research was carried out at the Climate laboratory in Holt, Tromsø a joint facility between UiT The Arctic University of Norway, and The Norwegian Institute of Bioeconomy Research (NIBIO), Tromsø. Wild strawberry clones (*Fragaria vesca* L.) from three different latitudes were used in the experiment (clones were gotten from the research laboratory at the University of Helsinki, Finland). These clones according to their latitudes were; Italia 1 (Tenno, Ville del Monte, Tennosee 45°93' N 10°81' E, Altitude 590m), Italia 4 ((Sp129) da Salorno, Pochi, Alto Adige, Italy 46°23' N 11°23' E, Altitude 535m), Finland 50 (Raasepori, Karjaa 60°10' N 23°67' E), Finland 53 (Lohja 60°20' N 23°80' E), Norway 2 (Alta1, Leirbukta 69° 93' N 23°09' E), Norway 13 (Indre Nordnes (N1) 69°53' N 20°38' E). Runners from these clones were planted in plastic pots using a mixture of Agra-perlite and peat. After they are fully rooted, they were moved to the greenhouse and were grown under 12 hours' daylight at 18 °C until they started producing the first stage of berries. After flowering, small paintbrushes were used to facilitate self-fertilization in order to guarantee viable pollination. Plants were watered once in a day, with fertilized water 5 times in a week.

The experiment was initially carried out in spring of 2018 in enclosed dark chambers with single-wavelength light from Heliospectra RX30 lamps (Figure 5). The monochromatic light quality treatment was too intense, thereby most of the plants started to senesce even before their berries are fully grown to the ripe stage, especially under far-red light treatment (Figure 6). Therefore, the experiment was repeated in Autumn 2018 as an enhanced light treatment allowing a small amount of ambient backup light in all treatments.

For the new experiment, three chambers covered with photo reflective material were set up for the treatments. The chambers were supplied with three wavelengths of varying LED light conditions (Heliospectra-RX30); Blue (450nm), Red (660nm), or Far-Red (735nm), using the maximum intensity of each wavelength (Figure 8). The LED lights treatments were supplemented with ambient light (Osram Compact Fluorescent Lamp 20W) from the top. A fourth chamber equipped with only white fluorescent light bulb was also set up as control (Figure 7). Strawberry plants at first stage of berries were then separated in two clones for each

latitude into the chambers, thereby having 12 pots per treatment. The plants continue to grow under 24 hours of light treatments and at 18°C.

Figure 5: Experimental set up in dark rooms with single-wavelength light from Heliospectra lamps.

Figure 6: The morphological changes in strawberry plants under experimental set up in dark rooms with single-wavelength light from Heliospectra lamps.

Figure 7: The experimental layout of the present study (Chambers with enhanced light exposure covered with photo reflective material).

3.2 Sample collection

To determine the expression of genes and metabolite accumulation at different light treatments, samples were collected at different time points and fruit developmental stages. For gene expression analysis, leaves were collected at 0 hours before the treatment, and at 2 days, 4 days, 7 days and 11 days after treatment. Fruits were collected at the first green stage, second white stage and third ripened stage of the fruit development (Figure 9). Leaf samples were collected for metabolite analysis at the 0 hours before light treatment and at the end of the experiment. Also, fully ripened coloured berries among different treatments were collected. Leaf and fruit samples were collected in aluminium foil and falcon tubes respectively and immediately flash-frozen in liquid nitrogen. Samples were stored in -80°C until RNA isolation.

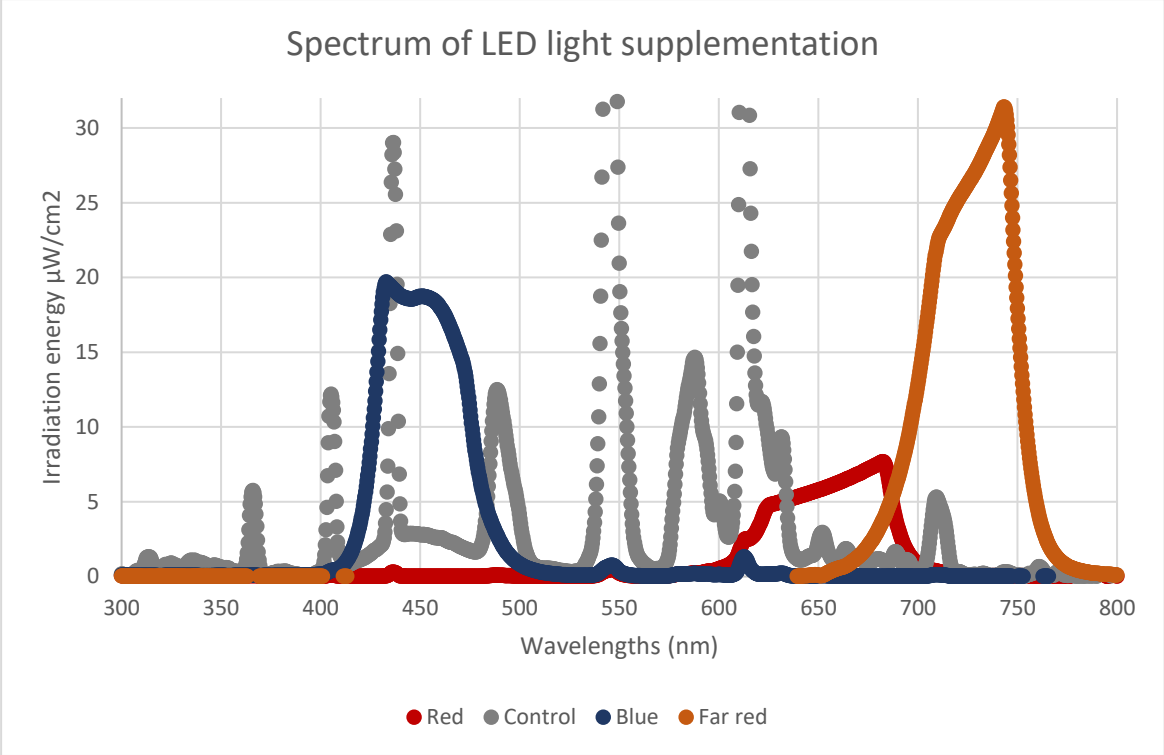


Figure 8: Spectral irradiance measurement of different light treatments in strawberry using Jaz spectrometer (Ocean Optics Inc).

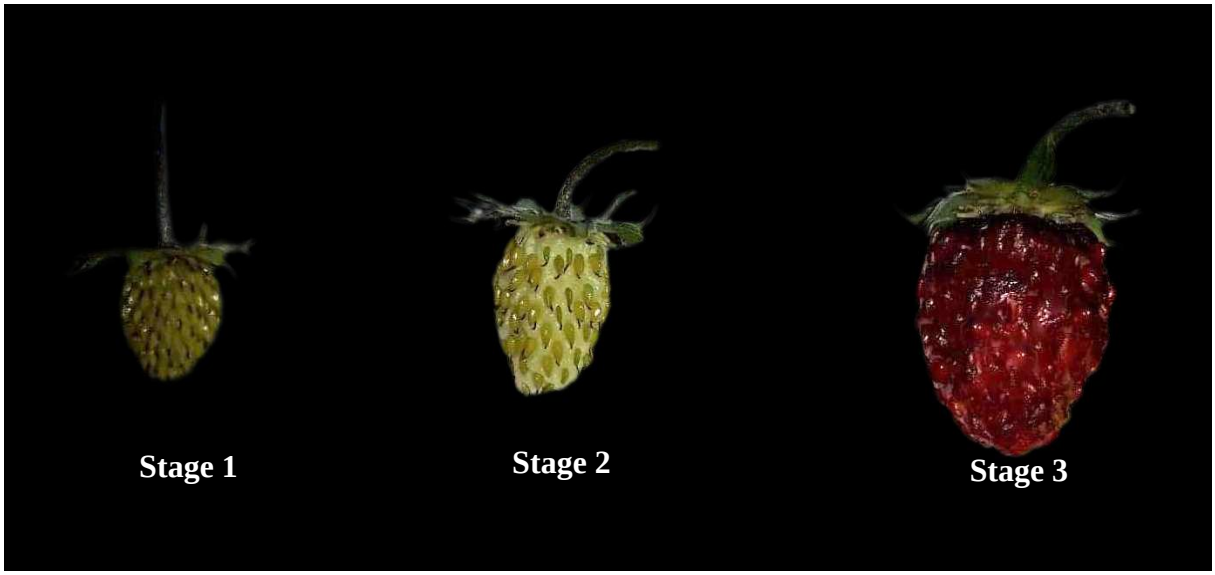


Figure 9: Three stages of berries collected for the analyses.

3.3 Total RNA isolation

For the optimal extraction of total RNA, different kits were tested initially; RNeasy Plant mini kit (QIAGEN, Germany), Spectrum Plant Total RNA Kit (Sigma Life Science, USA), and E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, USA). Optimum yield and quality of total RNA was achieved using the combination of modified CTAB (Cetyl trimethylammonium bromide) extraction methods (Chen et al., 2012), and Omega Bio-Tek Total RNA kit I washing protocols. The amount of 150mg to 180mg of ground strawberry leaves and berries were used for the extraction. The samples were ground in liquid nitrogen using RNase free ceramic mortar and pestle, then weighted directly into 2mL Eppendorf tubes. Then, 1mL of pre-heated extraction buffer was added on the samples and heated on 65° C for 10 minutes; samples were vigorously vortexed at intervals within these periods. Amount of 800µL of chloroform: IAA was added directly at the top of the samples and inverted few times for mixture, and subsequently centrifuged at 15,000 x g for 15 minutes at 6° C. Supernatants were transferred into new tubes. Then, 800µL of chloroform: IAA was again added to the supernatants, mixed and centrifuged at 15,000 x g for 15 minutes at 6° C. Supernatant was transferred into new tubes and one-quarter volume of 10M LiCl (Lithium chloride) of the sample's volume was added to the samples, gently mixed and precipitated overnight on ice in the -4° C cold room.

The following day, the samples were centrifuged at 15,000 x g for 25 minutes at 6° C to form pellets, supernatants were removed and pellets formed were washed by adding 600µL 70% ethanol, then centrifuged at 15,000 x g for 7 minutes at 6° C. The ethanol was decanted and tubes were pressed against paper towel to remove excess ethanol. Samples were later treated with DNase and incubated in room temperature for 15 minutes. The DNase treatment for each sample was prepared with 40µL H_2O, 5µL DNase buffer, and 5µL DNase (Sigma-Aldrich.com, USA). Afterwards, 150uL of RNase free water, 200µL of TRK buffer and 200µL of absolute ethanol were added into the tubes and the whole volume were pipetted into Omega Total RNA Kit 1 columns. The samples were further centrifuged at 15,000 x g for 1 minute at 6° C and liquid in the collection tubes were discarded. Thereafter, 500µL of Washing Buffer II was added to the columns and centrifuged at 15,000 x g for 1 minute at 6° C, liquids were discarded and the washing step was repeated again. Columns were then transferred to another collection tubes and centrifuged for at 15,000 x g for 2 minutes at 6° C to dry the columns of excess Washing Buffer. The tubes were discarded and columns were transferred to clean 1.5mL Eppendorf

tubes. Lastly, 33µL of 70° C RNase free water was added directly on the centre of the membrane in the columns and incubated in room temperature for 1 minute, tubes were centrifuged at 15,000 x g for 1 minute at 6° C and the step was repeated again with the elute. The quality of RNA was checked using NanoDrop spectrophotometer, and samples were stored at -80° C until use.

RNA isolation buffer used contained 2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8), 25mM EDTA, 2.0 M NaCl, 0.5g/l Spermidin (mixed and autoclaved), 2% β-merkaptoethanol was added in autoclaved buffer just before use (Chen et al., 2012).

3.4 RNA integrity/quality and quantity check

During the testing of different extraction protocols for optimum isolation, the integrity of total RNA was subsequently observed under agarose gel electrophoresis (Bio-Rad). 1% (0.6g) of agarose was melted in 60mL of TAE buffer by heating in a microwave for 2 minutes. Amount of 1.5ug of RNA (reconstituted with nuclease-free water to 6°C) of RNA was mixed with 1µL of gel loading dye and loaded onto the gel. 1kb gene ruler (Thermo Scientific) produces on the first and last well of the gel. The electrophoresis chamber was filled with 1X TAE buffer and gel was run at 50V for 30 minutes. The gel image was acquired using a gel documentation system (UVP BioImaging Systems, Upland, CA, USA) and RNA band sizes were determined by comparing with a commercial size marker.

The quality and quantity of isolated RNA were additionally examined using spectrophotometer (NanoDrop 2000c Spectrophotometer, Thermo Scientific). The assessment of purity and concentration of RNA samples is needed for further utilization in reverse transcription, contaminated RNA samples can affect downstream applications. Samples were measured at the ratio of 260 and 280nm absorbance, which examines protein contaminations. Pure RNA was determined with a ratio of approximately 2.1; lower ratio signifies protein contaminations. Samples were also examined for the presence of organic contaminants for example Phenol, chaotropic salt and other aromatic compounds. Measurements at the ratio 260 and 230nm absorbance should be approximately 2.0, a ratio below 1.8 indicates these contaminants.

3.5 cDNA synthesis

cDNA is synthesized from single-stranded RNA prior qPCR amplification in order for accurate quantification to analyze gene expression. The transcription is catalyzed by an enzyme called Reverse Transcriptase with oligo (dT) as a primer which binds to the poly-A tail of RNA, and the first strand is made by adding deoxyribonucleotides to the 3` end. SuperScript IV First-Strand cDNA transcriptase (Thermo Fisher Scientific) was used in the experiment to generate first-strand cDNA for two-step RT-qPCR applications. RNA dilution of 1:10 was made before cDNA synthesis for accurate amplification. For the reaction, 0.5µL each of 50µM anchored Oligo d(T) primers and 10mM dNTP mix were first measured in a PCR tube, 2.5µg of template RNA was pipetted into the tube and nuclease-free water was then added to reach the volume of the final reaction of 7µL. The reactions were mixed briefly, heated at 65° C for 5 minutes, and then incubated on ice for 1 minute.

A master mixture of 3µL per sample was made for Reverse Transcriptase reactions, the reactions include; 2µL 5 x SSIV Buffer, 100mM DTT and superscript IV Reverse Transcriptase. The 5 x SSIV Buffer was vortexed and briefly centrifuged before use. The reactions were mixed briefly and added to annealed RNA samples. The combined reaction mixture was incubated at the following conditions:

Steps	Temperature	Time
Incubation	52° C	30 minutes
Enzyme inactivation	80° C	10 minutes
Hold/store	4° C	30 minutes

Reverse Transcriptase reactions were stored in -20° C until use.

3.6 Quantitative Real-Time PCR (qPCR) analysis

qRT-PCR was carried out using 48 well plates on the MiniOpticon real-time PCR system (Bio-Rad, USA). All PCR samples were composed of 15µL reactions mixture which contained 7.5µL SsoFast EverGreen supermix reaction cocktail (Bio-Rad, USA), 1.5µL of each forward and reverses primer (5µM) (Sigma, USA), 1ul of 1:10 diluted cDNA, and 3.5µL RNase-free water. The following protocol was used to set up a cycling condition for the qRT-PCR:

qRT-PCR profile

Cycling Step	Temperature	Time	Cycles
Initial denaturation	95° C	30s	1
Denaturation	95° C	5s	30-40
Annealing/Extension	60° C	10s	
Melt curve analysis after final cycle	65-95°C (Increment 0.5° C)	5 sec/step	1

In the content of each run, controls without template were included to check contaminations in the reagent and two technical replicates were used for qRT-PCR. qMSI1 gene (Musashi RNA binding protein 1) was selected as a reference gene for normalization of data and for calculating relative fold changes in gene expression, the $2^{-\Delta\Delta Ct}$ method was used. All the primer sequences are listed below:

Table 1: Primers for qRT-PCR.

Gene's name	5` Forward primers	3` Reverse primers
FvCHS 13	GCCTTTGTTTGAGCTGGTCT	CCCAGGAACATCTTTGAGGA
FvDFR	CACGATTCACGACATTGCGAAATT	GAACTCAAACCCCATCTCTTTCAGCTT
FvF3H	CCCTAAGGTGGCCTACAACCAAT	CTTCTTGCAAATCTCAGCGC
FvANS	TGACATCAAGGTGAGGGAGAAA	TGCTCAATGGGAAGATCAAAGA
FvPAL	TCAAGGAATGCAGGTCTTATCC	CGTTGAACACCTTGTCACATTC
FvMYB1	ATGAGGAAGCCCTGCTGCGA	AACGACGCAACCCTGCAGCC
FvMYB10	TCAAATCAGGTTAAACAGA	TTAAAGACCACCTGTTTCCT
**qMSI1*	TCCCCACACCTTTGATTGCCA	ACACCATCAGTCTCCTGCCAAG

*House-keeping gene.

FvCHS- F. vesca chalcone synthase 13, *FvDFR- F. vesca* dihydroflavonol 4-reductase, *FvF3H- F. vesca* flavonoid 3 hydroxylase, *FvANS- F. vesca* anthocyanidin synthase, *FvPAL- F. vesca* phenylalanine ammonia-lyase.

3.7 Total anthocyanin determination

To determine total anthocyanin content in strawberries, quantification was carried out according to the pH differential method by UV-visible spectrophotometer at 520nm and 700nm (Lee et al., 2005). Frozen strawberry samples were ground into fine powder in liquid nitrogen and approximately 1.0g was added into 1.5mL of extraction solution containing methanol with a mixture of 0.1% hydrochloric acid. Samples were then placed on a shaker for 1h and centrifuged at 15,000 x g for 15 minutes at 12° C. 1mL of supernatant was collected in a fresh tube and stored at -80° C for the later analysis. Extractions were done in triplicate.

Total anthocyanins were determined using two different buffer; 0.025 M potassium chloride pH = 1.0 (1.86g KCl was measured into a beaker and 980mL distilled water was added, the pH was then measured and adjusted with HCl to 1.0 (±0.5). Afterwards, the buffer was transferred into a volumetric flask and diluted with distilled water to 1L), and 0.4 M sodium acetate pH = 4.5 (54.43g $CH_3CO_2Na.3H_20$ was measured into a beaker, and 960mL distilled water was added. The pH was subsequently measured adjusting with HCl to 4.5(±0.5) and transferred to 1L volumetric flask diluting to volume with distilled water). Measurements were carried out using visible spectrophotometer (SmartSpec Plus, Bio-Rad, USA. 250µL of the extract was diluted with 1mL of buffer (1:4) in a 1cm path-length disposable cuvette and incubated at room temperature in the dark for 20 minutes. After, absorption (A) was measured at λ = 520nm and λ = 700nm. All extracts were analyzed in three replicates.

The calculations of anthocyanin pigment concentration were expressed as cyanidin-3-glucoside equivalents, as follows;

$$\left(\frac{A}{\varepsilon \text{ x1}}\ x\ DF1\ x\ DF2\ x\ MW\ \right)/m$$

Equation 1: Equation for determining Total Anthocyanin concentration

Where A = $(A_{520nm} - A_{700nm})$pH 1.0 – $(A_{520} - A_{700})$pH 4.5; ε =26.9 L $mmol^{-1}cm^{-1}$, molar extinction coefficient for cyanidin-3-glucoside; 1=path-length in cm; DF1= 1:4 extracts and buffer dilution factor; DF2= volume of extraction solution; MW= 449.2 mg $mmol^{-1}$, molecular weight for cyaniding-3-glucoside; m=amount per gram fruits. Results are reported as monomeric anthocyanins, expressed as cyaniding-3-glucoside equivalents in mg/L.

3.8 Statistical analysis

For qPCR analysis of strawberry leaf samples and fruits, data are presented as means (± SE) of three biological replicates. For the analysis of total anthocyanin, data are also presented as means (± SE) of three biological replicates.

4 Results

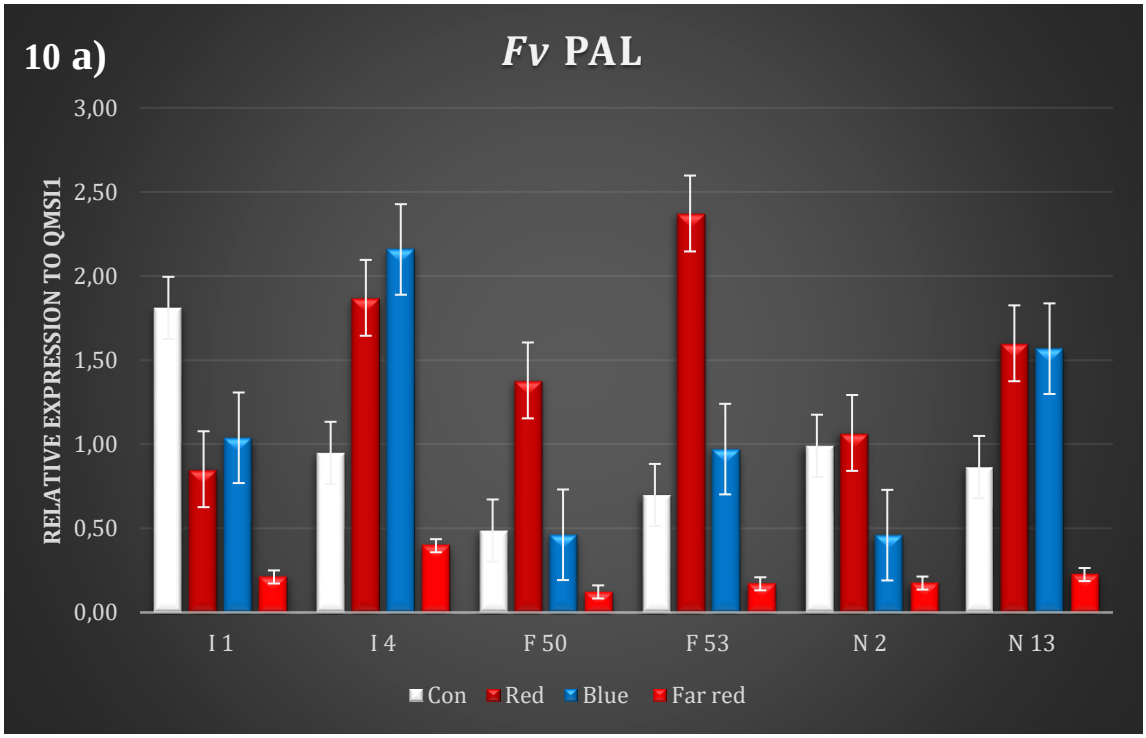
4.1 The expression levels of genes involved in the flavonoid pathway of *Fragaria vesca* leaves

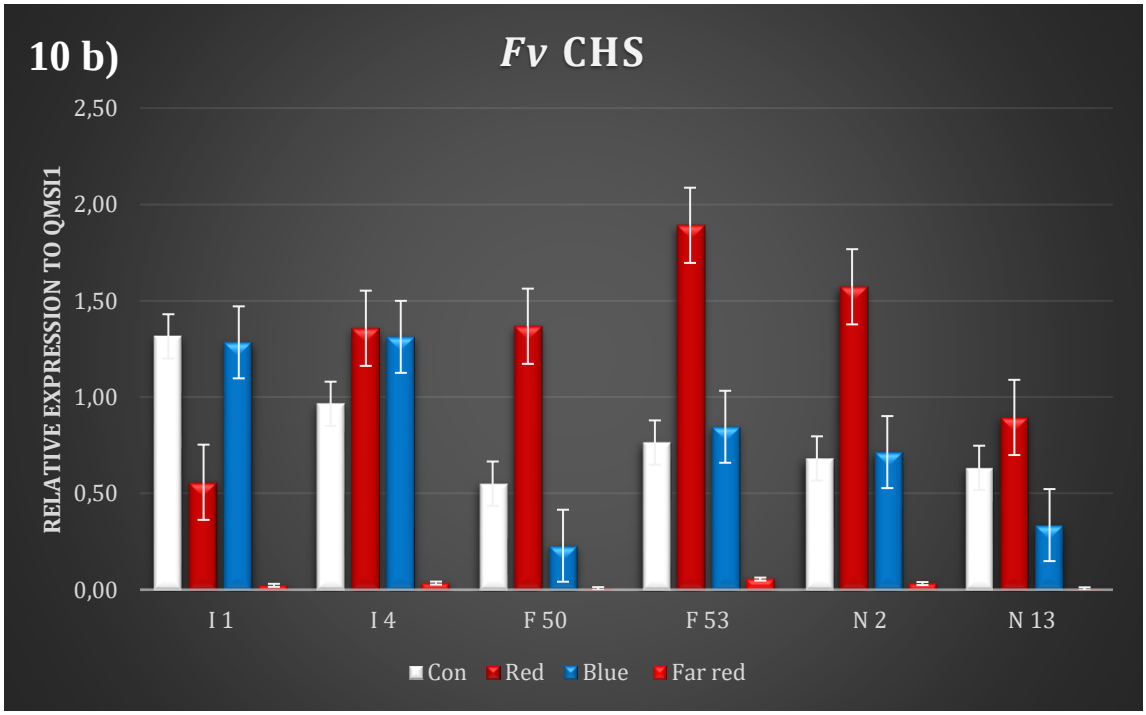
Transcriptional activities of five structural genes; *FvPAL*, *FvCHS*, *FvF3H*, *FvDFR*, *FvANS*, and two regulatory genes; *FvMYB10* and *FvMYB1* that are involved in flavonoid biosynthesis pathway of *Fragaria vesca* leaves, clones from six different latitudes (Italia 1 and 4, Finland 50 and 53, Norway 2 and 13) were investigated under LED light treatments of red, blue, far-red and white light as control. The expression levels were examined based on different time points (0 hour, 24 hours, 28hours, 4 days, 7 days and 11 days) after the light treatments. The results showed that expression levels of the genes were mainly up-regulated under LED light qualities after 48 hours of exposure (Figure 10). In the other time points, flavonoid pathway genes were expressed at highest level under the white light (control) treatment.

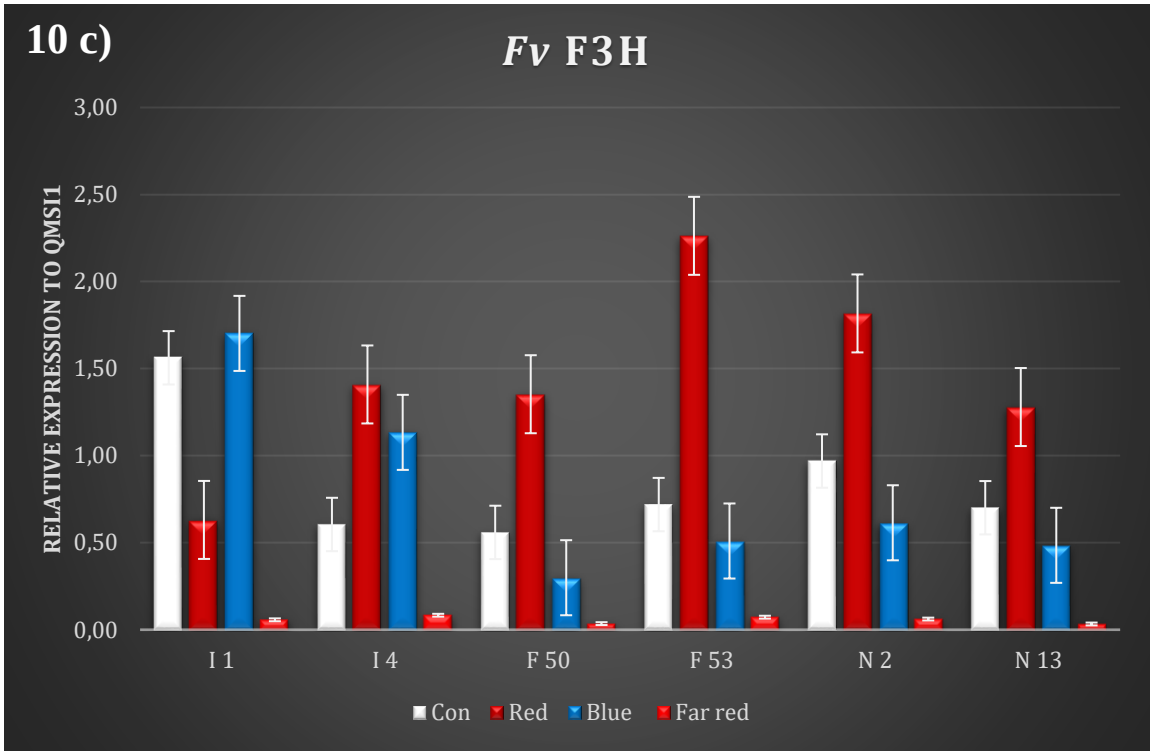
All the pathway genes examined responded almost similarly to all the light treatments. However, there were variations among different latitudes. The genes of Italian *F.vesca* clones had the highest expression when exposed to blue light, while Finnish and Norwegian clone's genes were up-regulated and responded under red light (Figure 10). This is an exception to Italian 4 clones where red light seems to have activated the overexpression of *FvCHS* and *FvF3H* which are early biosynthesis genes in the flavonoid pathway. Far-red light had little or no influence on the expression of genes in all the clones. The effect of Far-red was only observable in *FvPAL* (Figure 10a), the first gene in the general phenylpropanoid pathway, although the impact was the lowest when compared to other light treatments. It was noted that almost all the genes in Italian 1 clones responded more to white light exposure, nevertheless among LED light treatments, the genes had higher expression under blue light.

FvMYB10 acts as a positive regulator of biosynthesis genes in the flavonoid pathway of strawberry. In the experiment, *FvMYB10* was tested in all clones under the four light treatments (white light, red light, blue light, far-red light), and the results showed that the gene's activities were highly affected by the treatments. The expressions were up-regulated in the blue light treatment and the most striking elevation of the expressions were seen in the Italian clones (Figure 10f). The expression of *FvMYB1*, a transcriptional repressor, was also up-regulated. There was a highly variable increase in the transcriptional levels across all clones in all the

treatments (Figure 10g), and the transcription pattern is almost consistent with the expressions of all the structural genes tested.






10 c)
Fv F3H
RELATIVE EXPRESSION TO QMSI1
3,00
2,50
2,00
1,50
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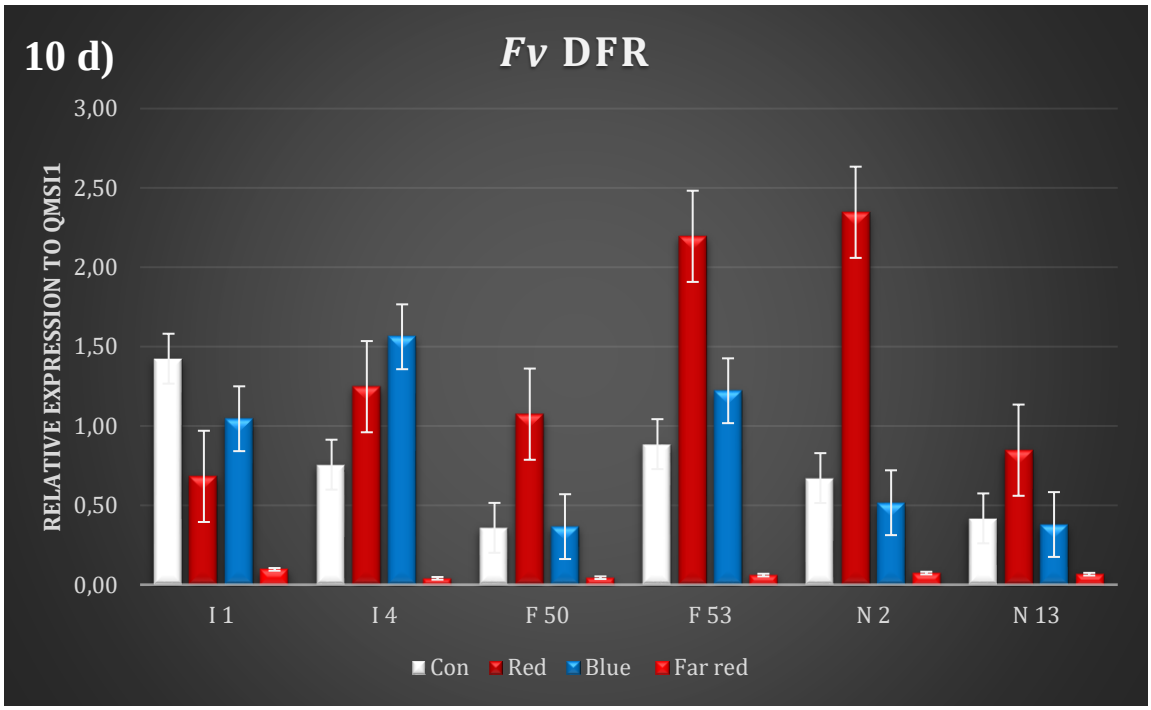
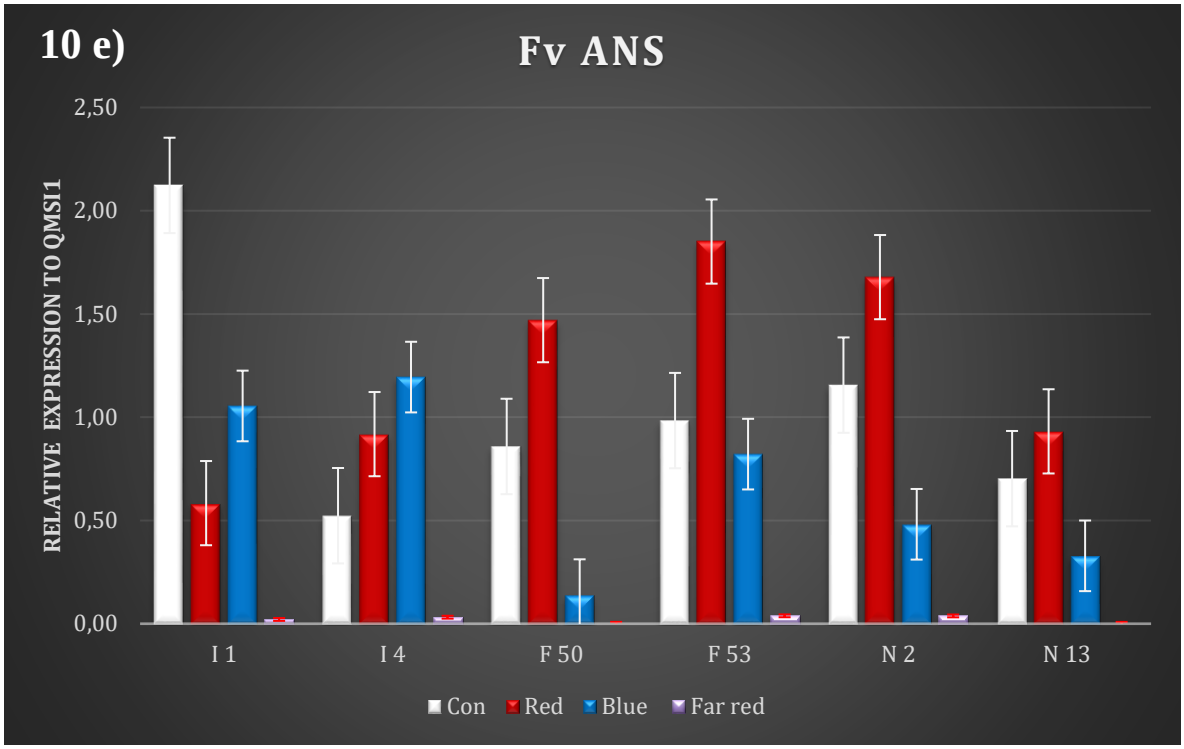
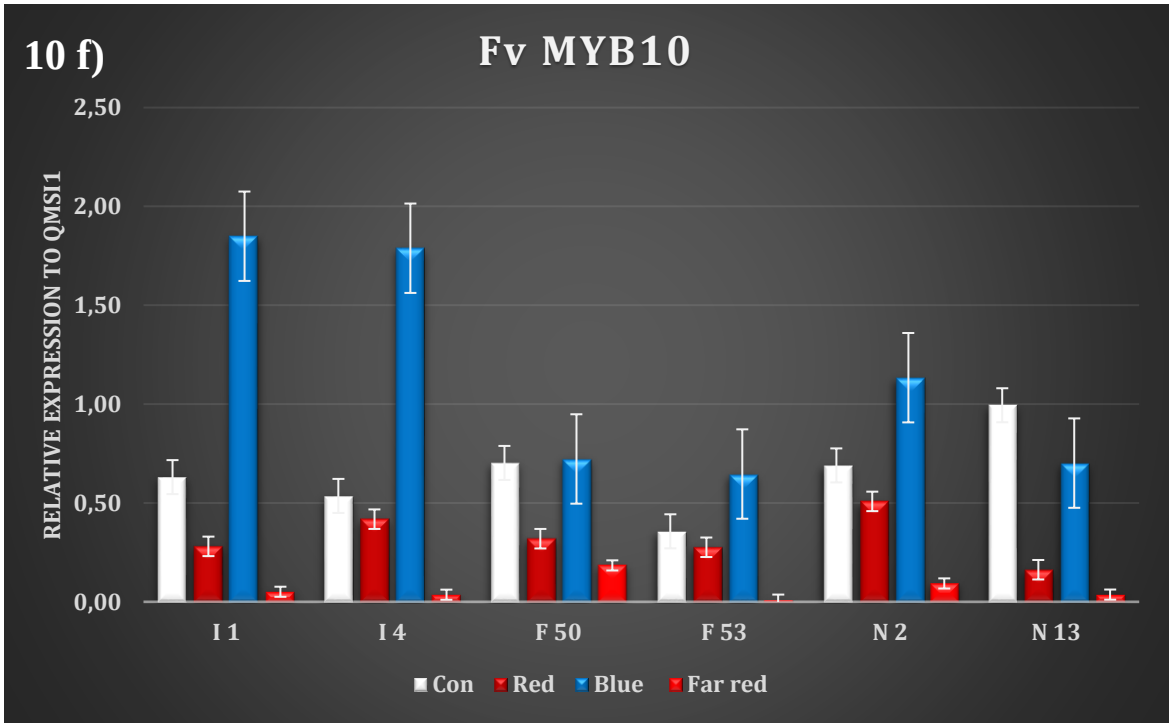
0,50
0,00
I 1
I 4
F 50
F 53
N 2
N 13
Con
Red
Blue
Far red


10 d)
Fv DFR
RELATIVE EXPRESSION TO QMSI1
3,00
2,50
2,00
1,50
1,00
0,50
0,00
I 1
I 4
F 50
F 53
N 2
N 13
Con
Red
Blue
Far red


10 e)
Fv ANS
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
I 1
I 4
F 50
F 53
N 2
N 13
Con
Red
Blue
Far red


10 f)
Fv MYB10
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
I 1
I 4
F 50
F 53
N 2
N 13
Con
Red
Blue
Far red

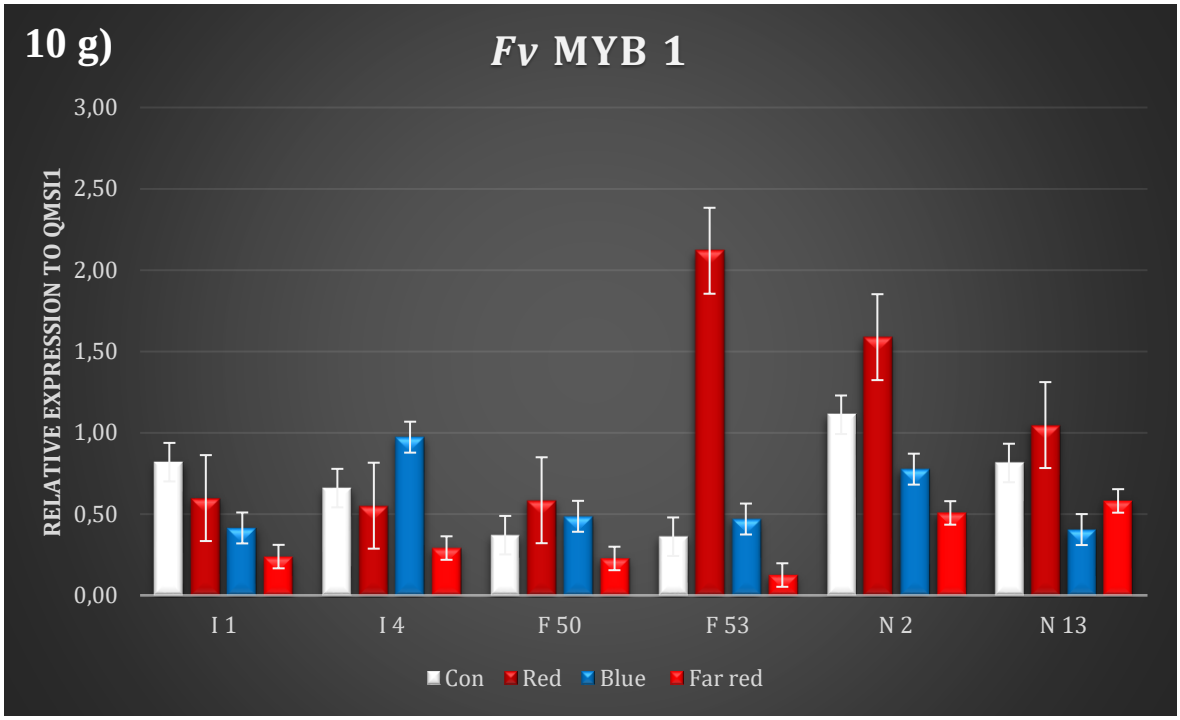


Figure 10: Expression levels of flavonoid pathway genes in clones of *F. vesca* leaves from different latitudes after 48 hours of LED light treatments. a) *FvPAL*, b) *FvCHS* c) *FvF3H*, d) *FvDFR*, e) *FvANS* f) *FvMYB10*, g) *FvMYB1*. The letters with a number represent the latitude of each clone; Italia 1 (I1), Italia 4 (I4), Finland 50 (F50), Finland 53 (F53), Norway 2 (N2), Norway 13 (N13). Different colours represent different wavelengths of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

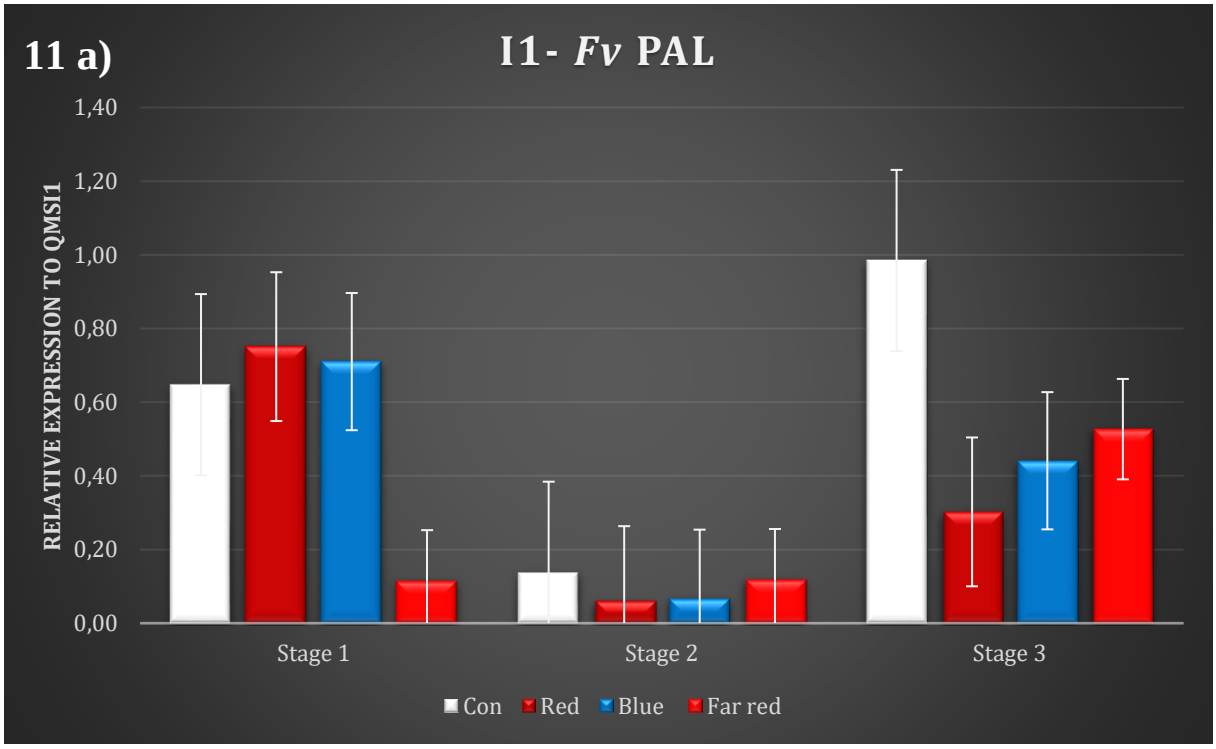
4.2 Expression of flavonoid pathway structural and regulatory genes in *Fragaria vesca* berries.

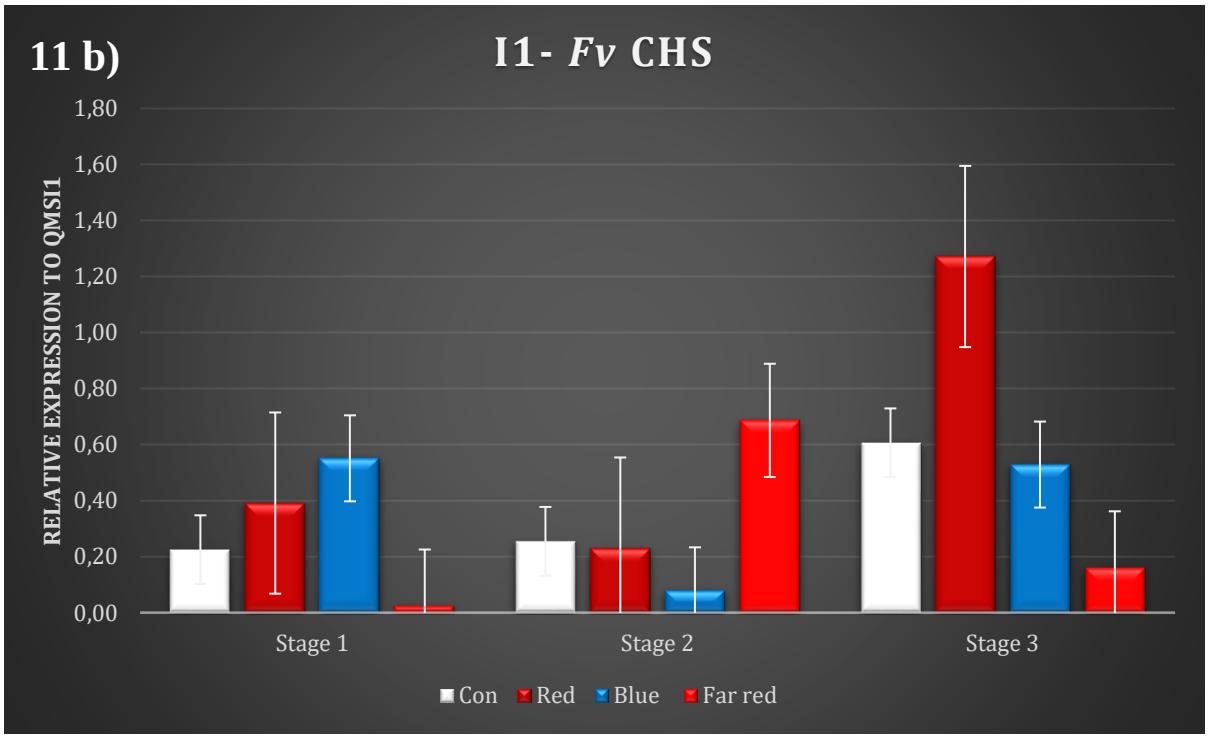
The expression of five structural and two regulatory genes involved in the steps of flavonoid biosynthesis was studied in three developmental stages; the green stage (1), the white stage (2) and the red ripe stage (3) of *F. vesca* berries under LED light treatments (red, blue, far-red), white light as control. The experiment was conducted to investigate the transcriptional level of

these genes across the light treatments and across clones from different latitudes. The structural genes are *FvPAL*, *FvCHS*, *FvF3H*, *FvDFR* and *FvANS*. Regulatory genes *FvMYB10* and *FvMYB1* were also examined.

The gene expressions were up-regulated in all light treatments, including far-red light, which had little influence on the genes examined in *F. vesca* leaves. However, the genes responded differently to LED light treatments, and the differences can be seen between berries developmental stages and across all clones. In the third stages of berry development, *FvCHS* (Figure 11a*), FvDFR* (Figure 11d), and *FvANS* (Figure 11e), expressions were upregulated under red light in I1 clones (Figure 11), *FvPAL* (Figure 13a), and *FvDFR* (Figure 13d) genes were up-regulated under blue light in I4 clones (Figure 13), *FvF3H* gene (Figure 14c) was over-expressed under red light in F50 clones (Figure 14), *FvPAL* (Figure 15a), *FvF3H* (Figure 15c), and *FvDFR* (Figure 15d) genes were up-regulated under red light in F53 clones (Figure 15). *FvDFR* (Figure 16d) and *FvANS* (Figure 16e) responded more to blue and red light respectively in N2 clones (Figure 16) and in N13 clones (Figure 16), *FvDFR* (Figure 16d) and *FvANS* (Figure 16e) were more active under blue light. Irrespective of the light treatments, most of the early biosynthetic genes in all clones, for instance, *FvPAL* and *FvCHS* had increased expressions in the first and third berry developmental stages, and the late genes *FvANS* showed increasing pattern from the first stages to the third stages across all clones. Conversely, only N2 clones (Figure 16) had the highest expression in the first stage and the expression decreased from the first stage to the third stage in all the genes.

In the experiment, irrespective of light treatments, the expression of *FvMYB10* was very low in the early fruit developmental stages of all clones but increased substantially in the ripening stages (Figure 11f). Conversely, *FvMYB1* expression under LED light treatments was decreased with the progressive developmental stages of fruits in all clones (Figure 11g).


11 a)
I1- Fv PAL
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


11 b)
I1- Fv CHS
RELATIVE EXPRESSION TO QMSI1
1,80
1,60
1,40
1,20
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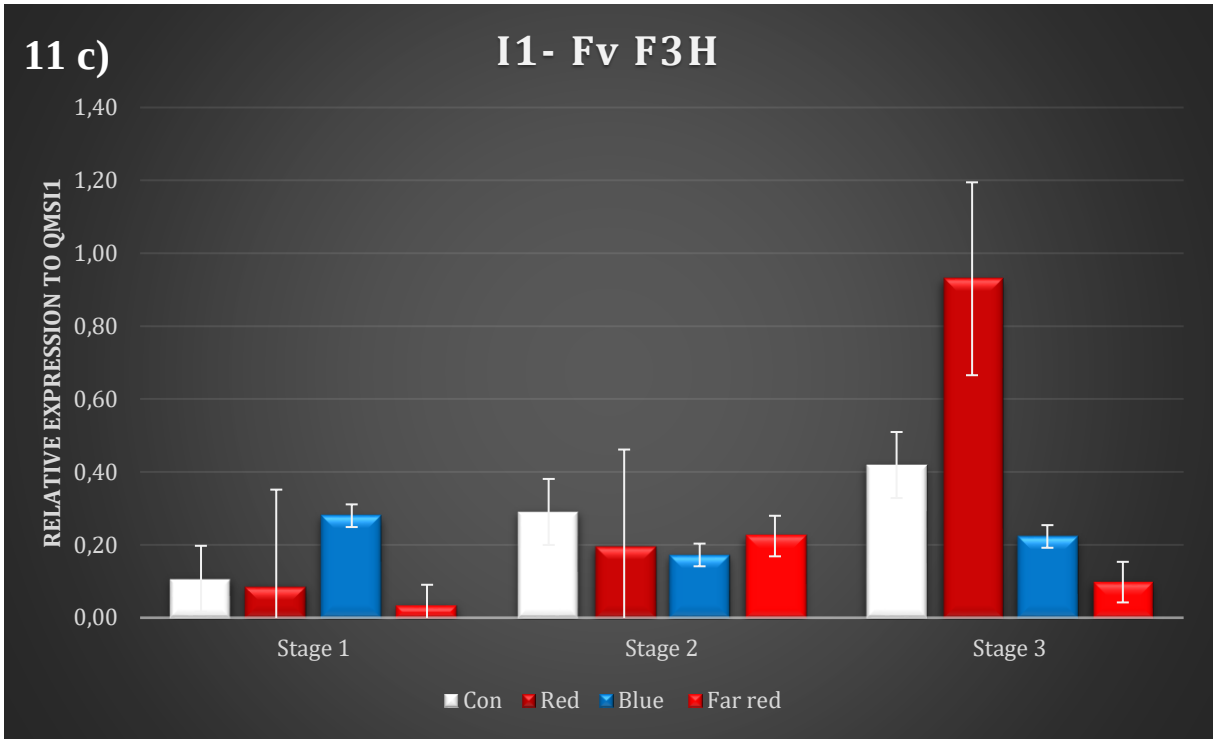
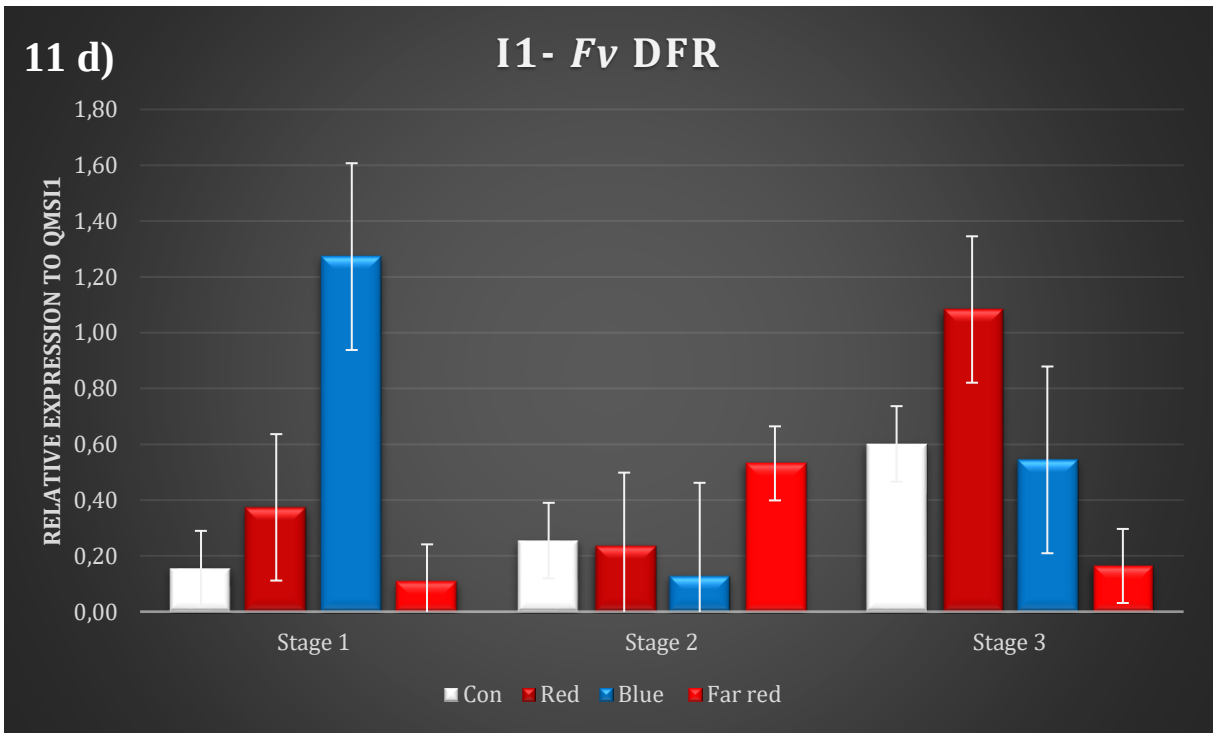
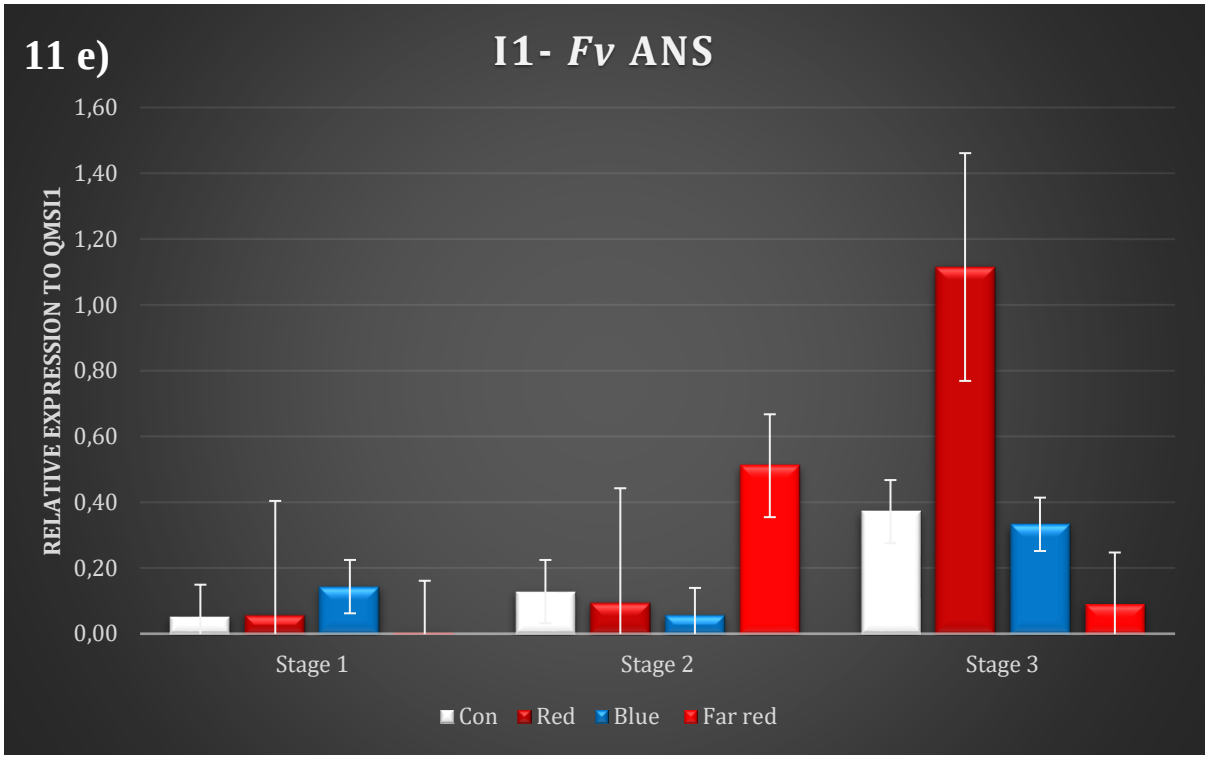
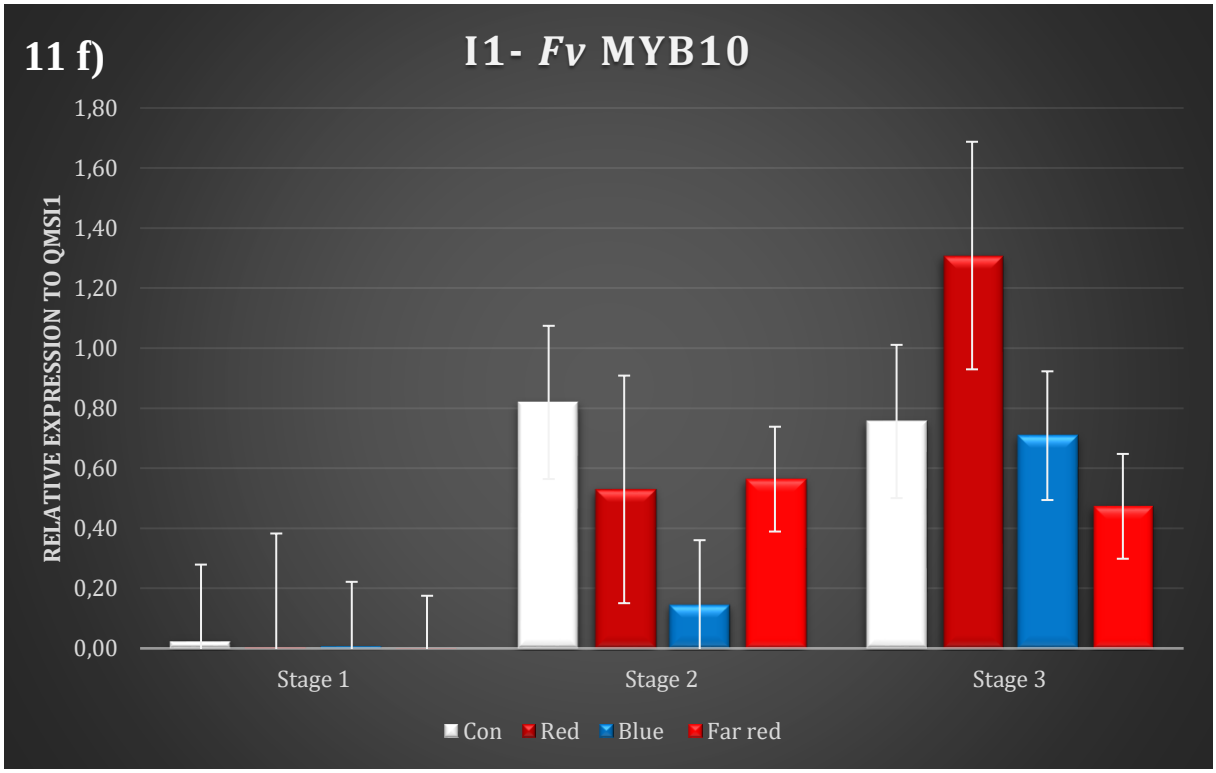
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


11 c)
I1- Fv F3H
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


11 d)
I1- Fv DFR
RELATIVE EXPRESSION TO QMSI1
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


11 e)
I1- Fv ANS
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


11 f)
I1- Fv MYB10
RELATIVE EXPRESSION TO QMSI1
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

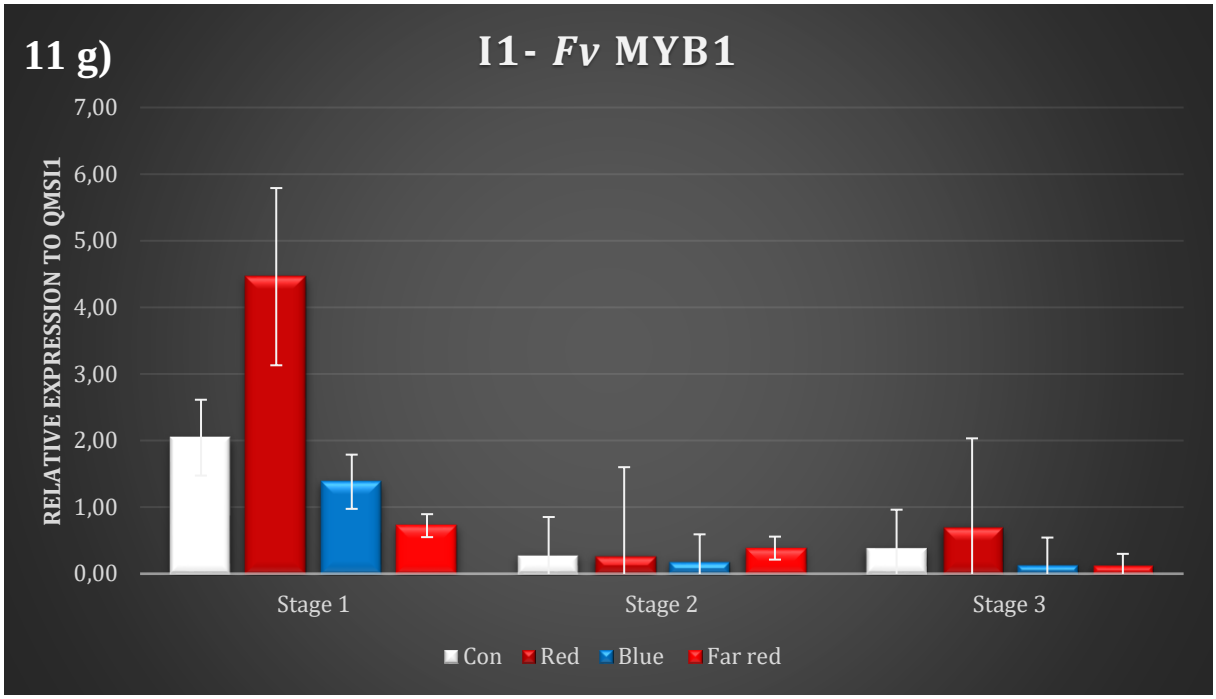
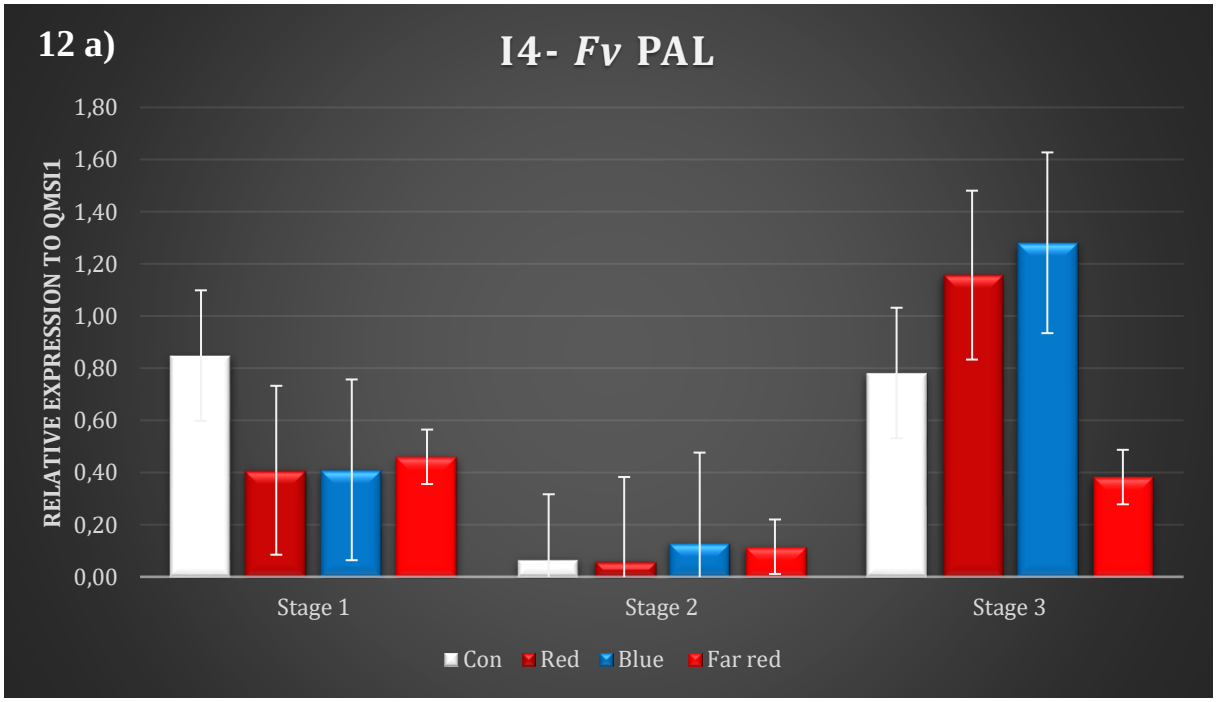
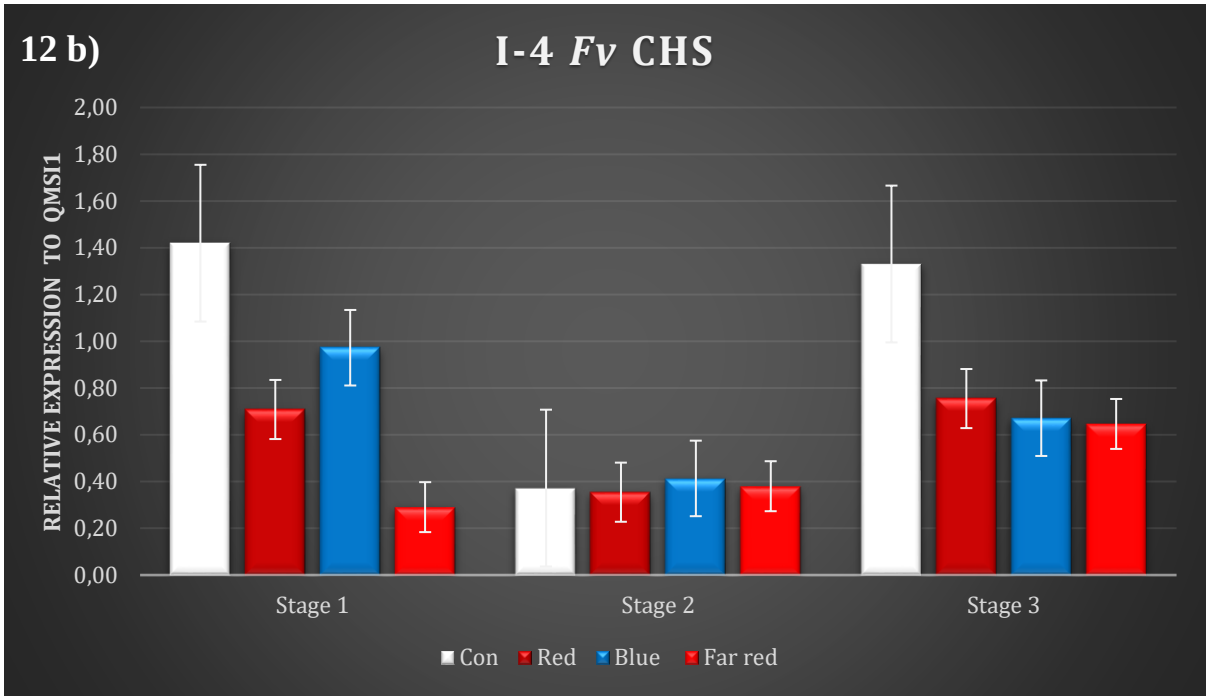


Figure 11: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*, d) *FvDFR*, e) *FvANS*, f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Italia 1 (I1) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

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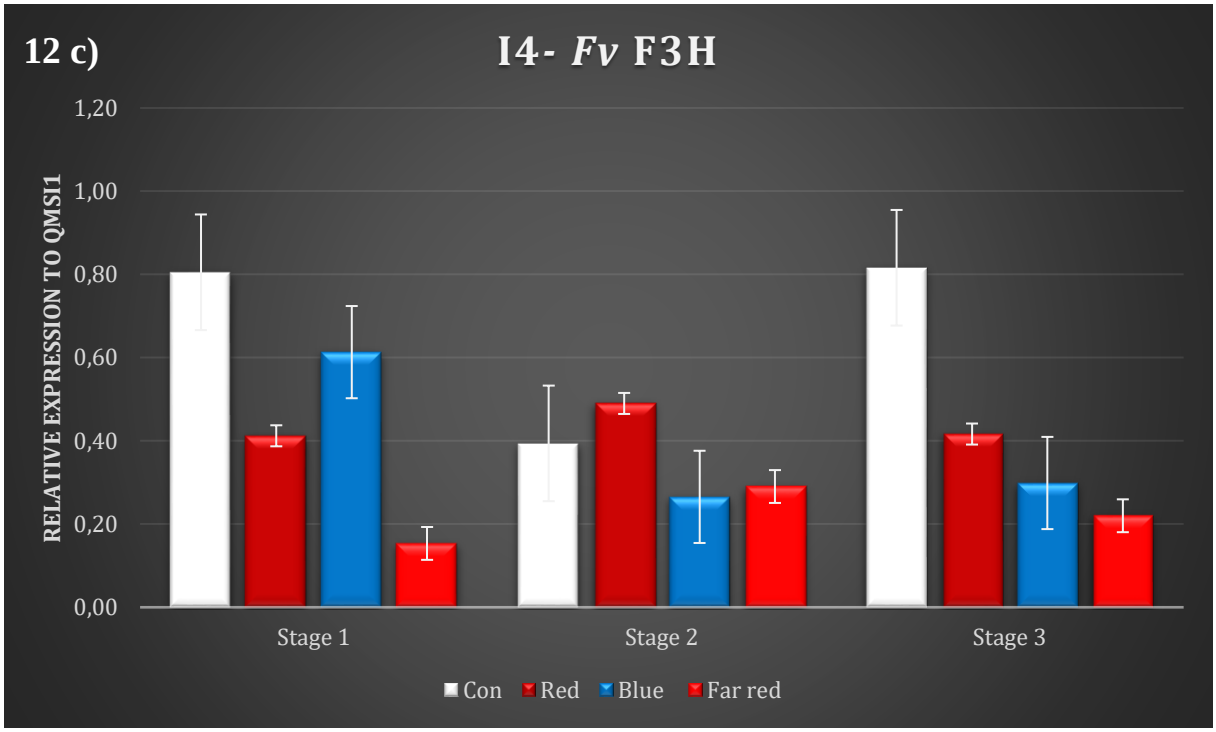
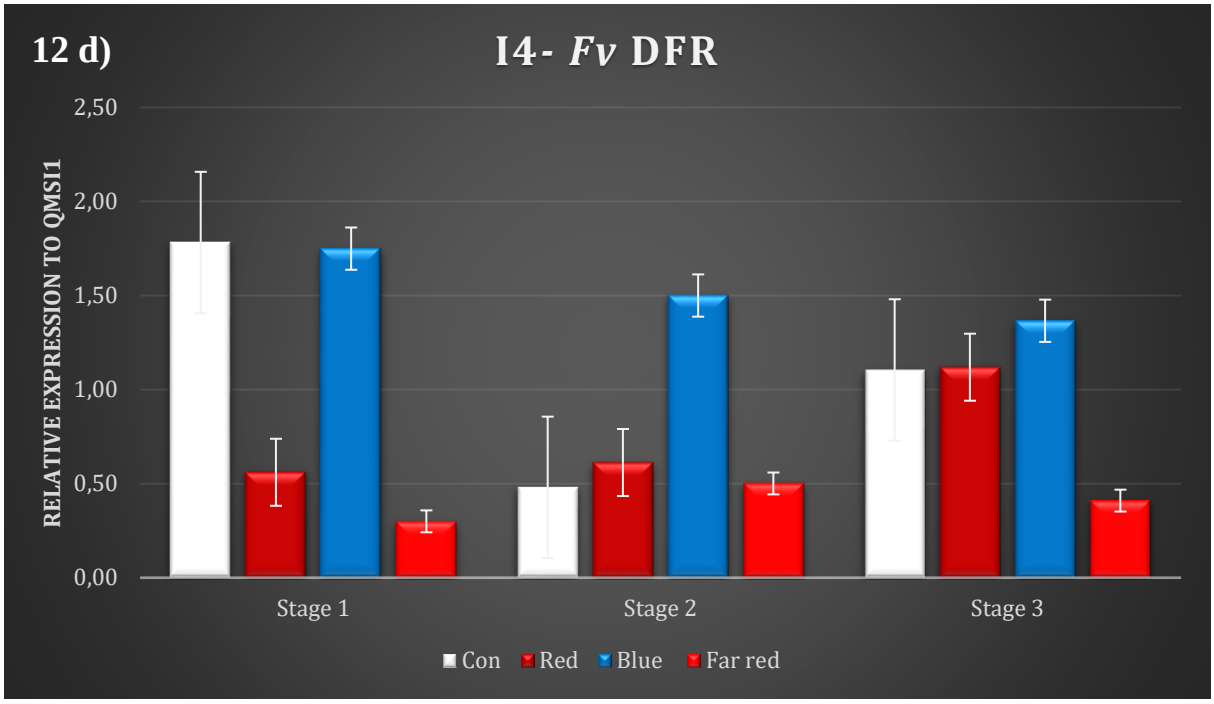
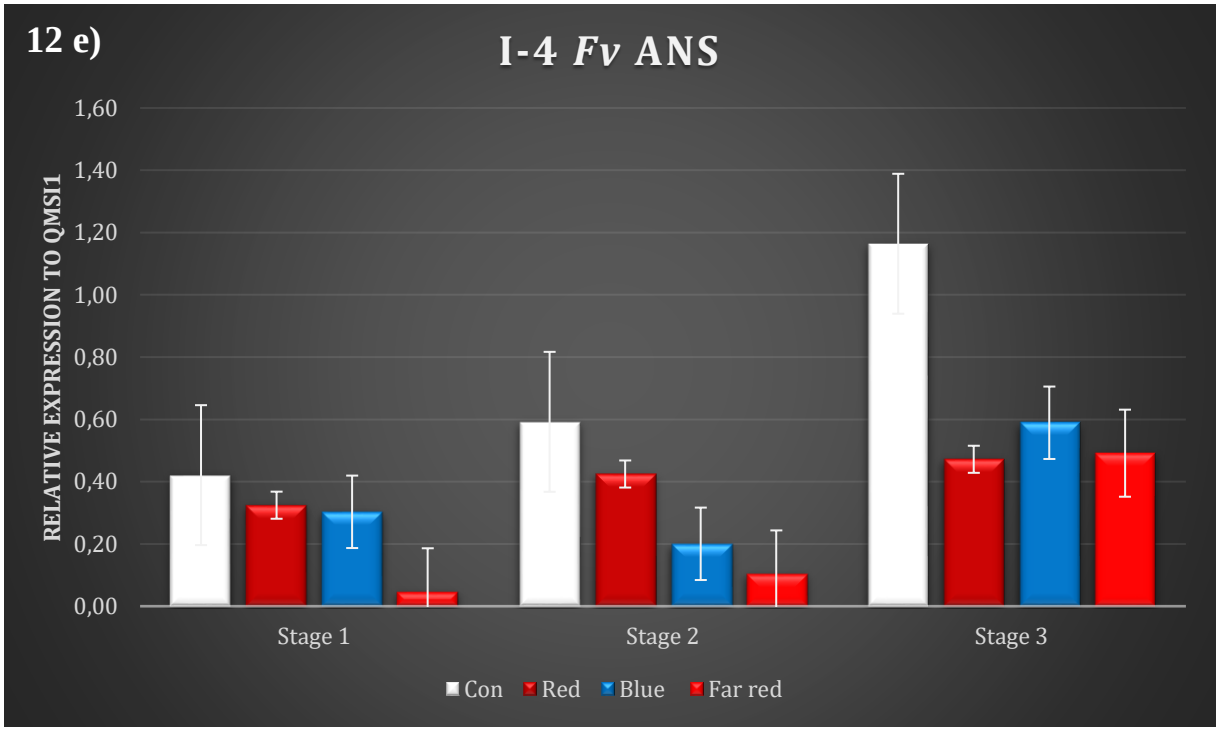
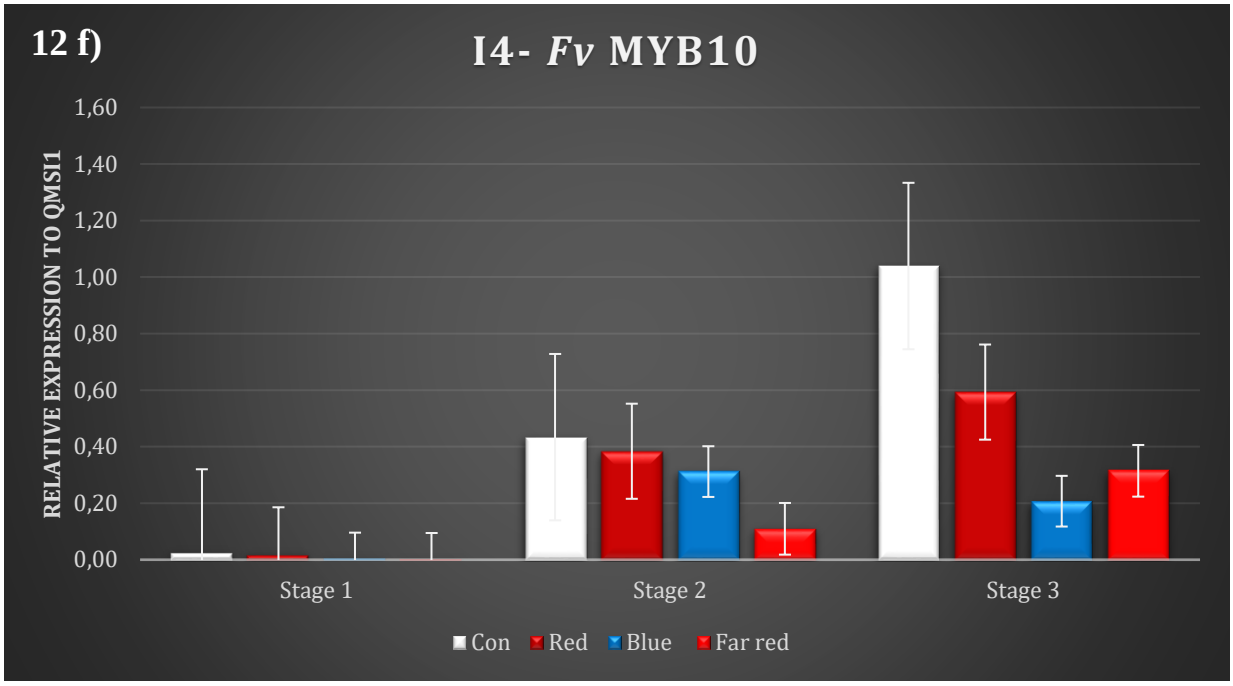
I-4 Fv CHS
RELATIVE EXPRESSION TO QMSI1
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


12 c)
I4- Fv F3H
RELATIVE EXPRESSION TO QMSI1
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


12 d)
I4- Fv DFR
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


12 e)
I-4 Fv ANS
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red



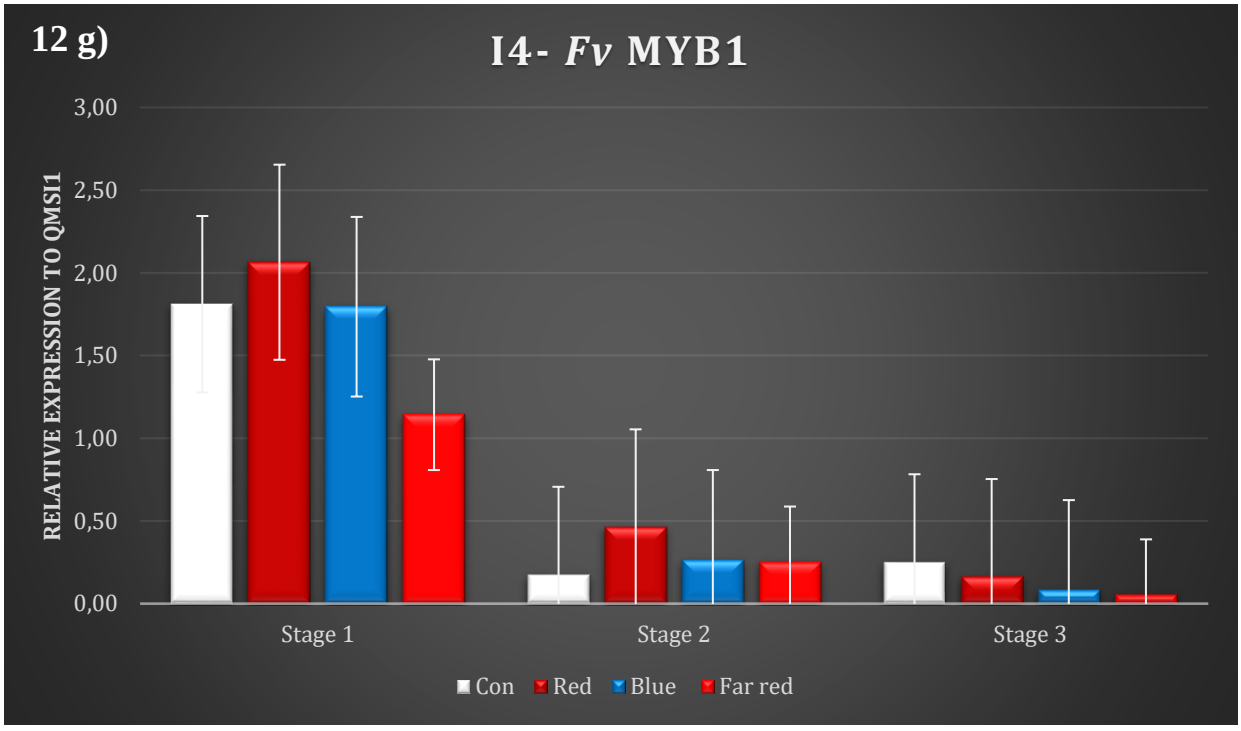
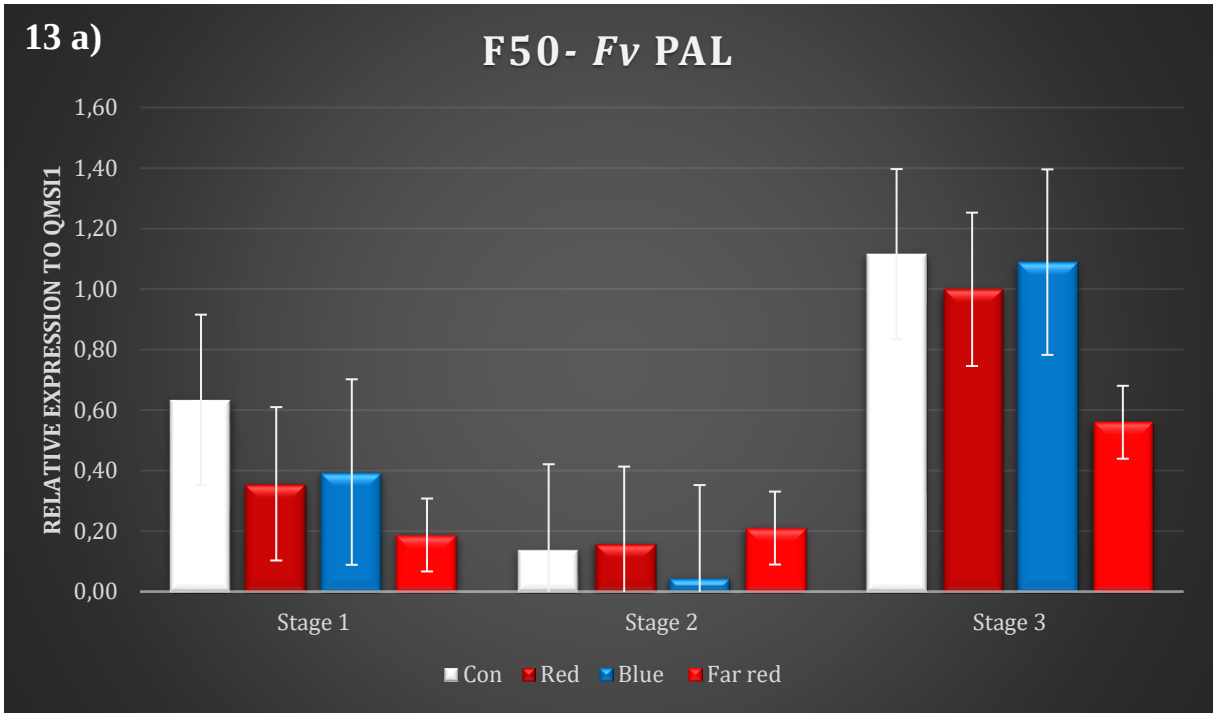
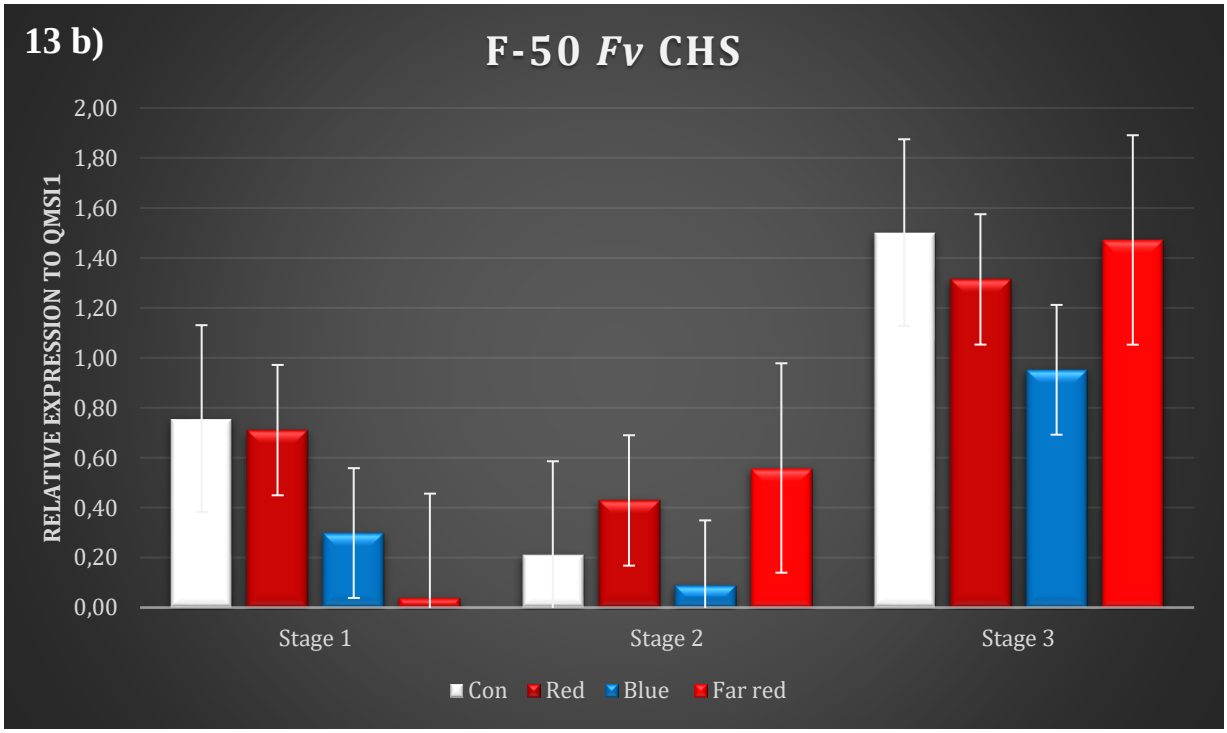


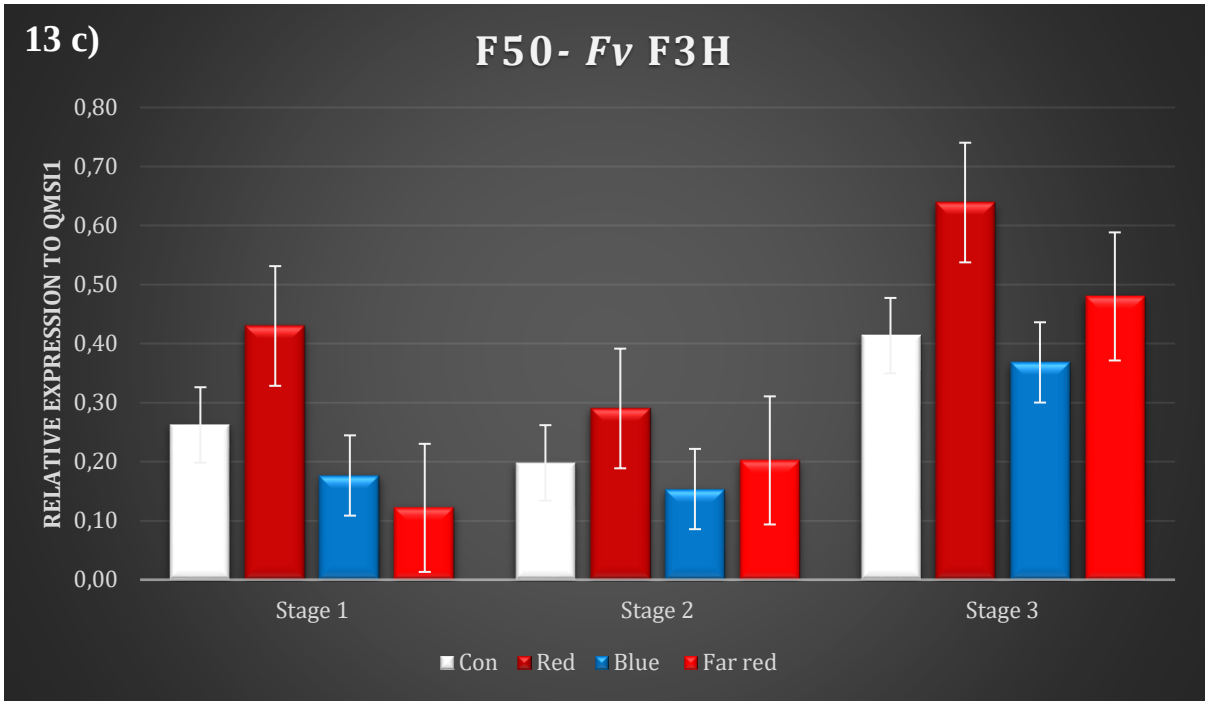
Figure 12: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*, d) *FvDFR*, e) *FvANS*, f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Italia 4 (I4) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

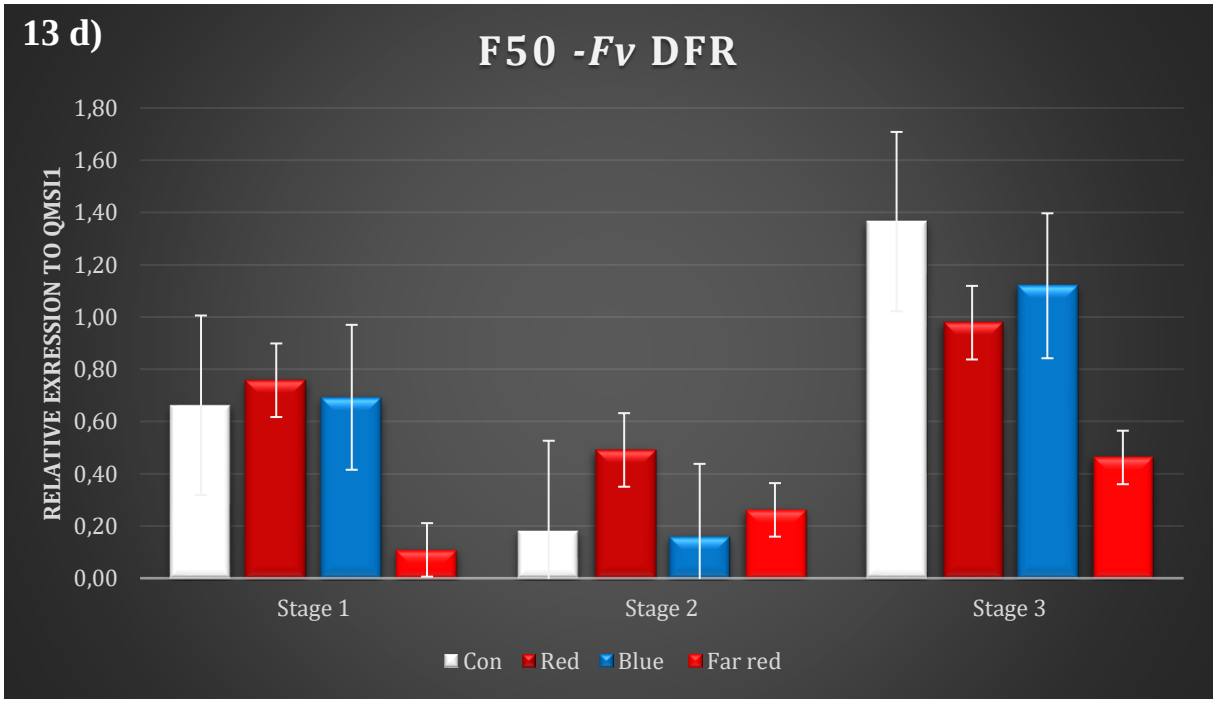
13 a)


F50- Fv PAL
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

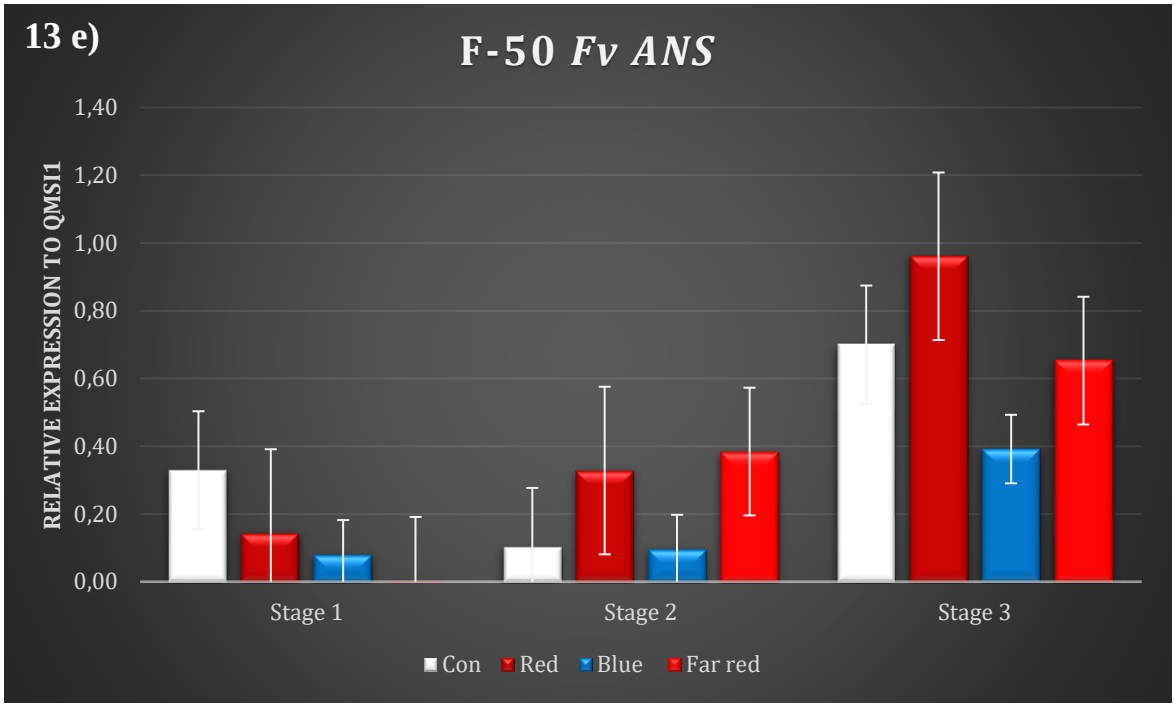
13 b)


F-50 Fv CHS
RELATIVE EXPRESSION TO QMSI1
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

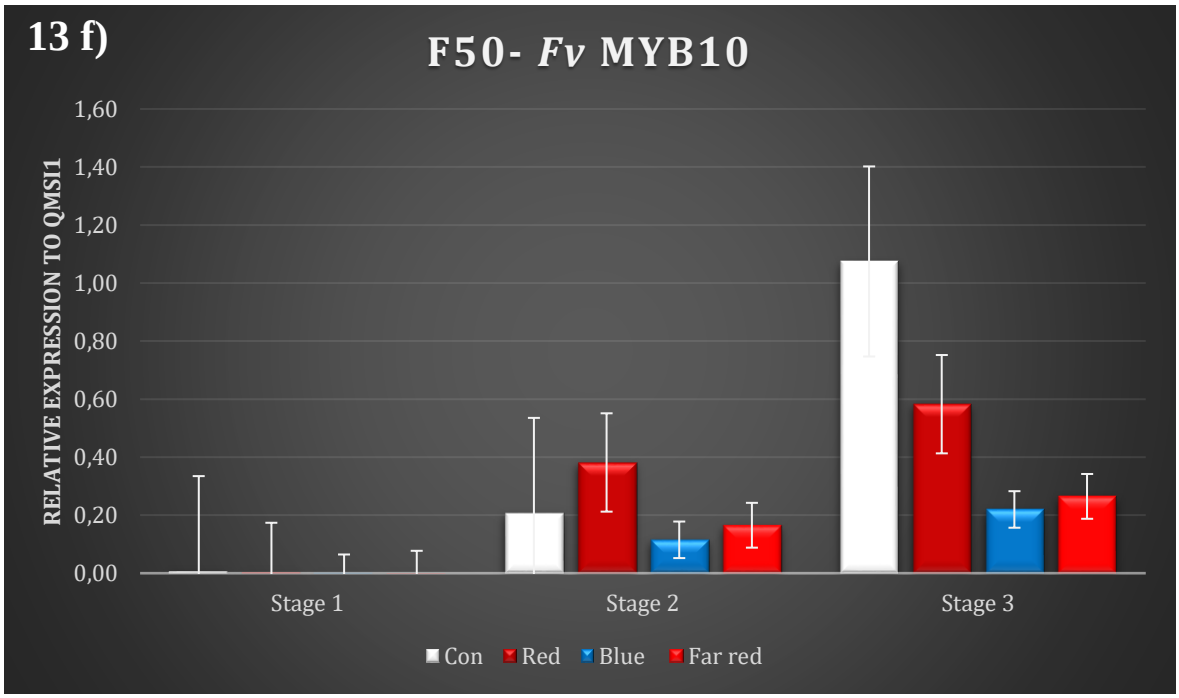

13 c)
F50- Fv F3H
RELATIVE EXPRESSION TO QMSI1
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


13 d)
F50 -Fv DFR
RELATIVE EXRESSION TO QMSI1
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

13 e)


F-50 Fv ANS
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

13 f)


F50- Fv MYB10
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

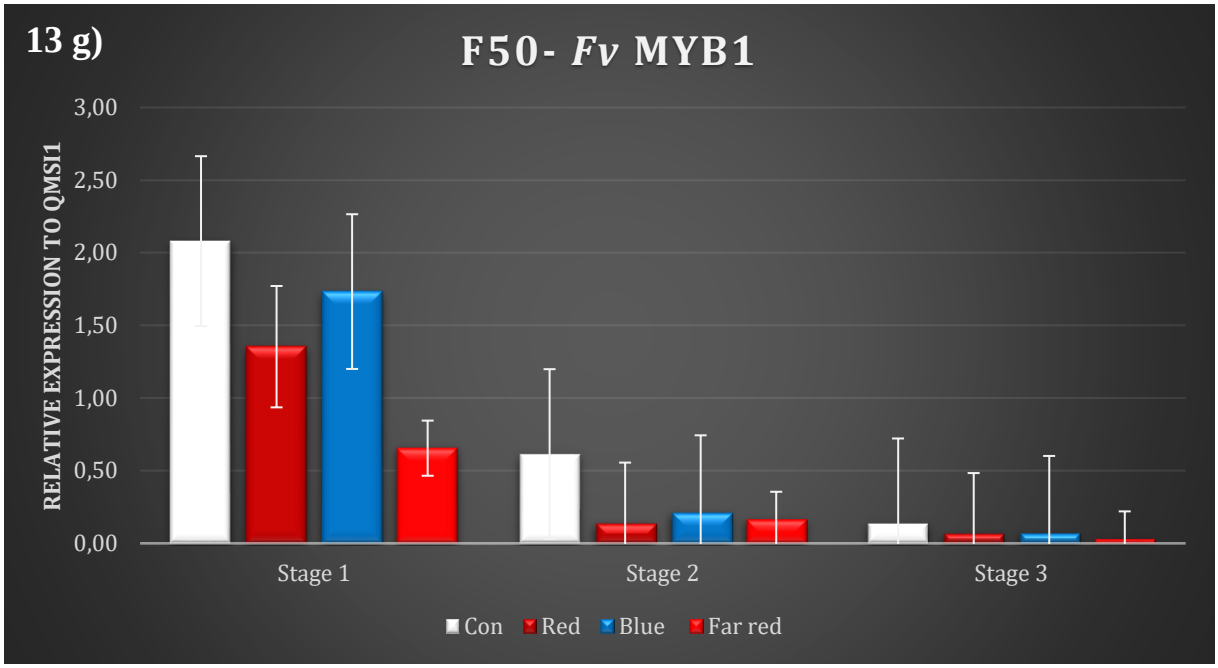
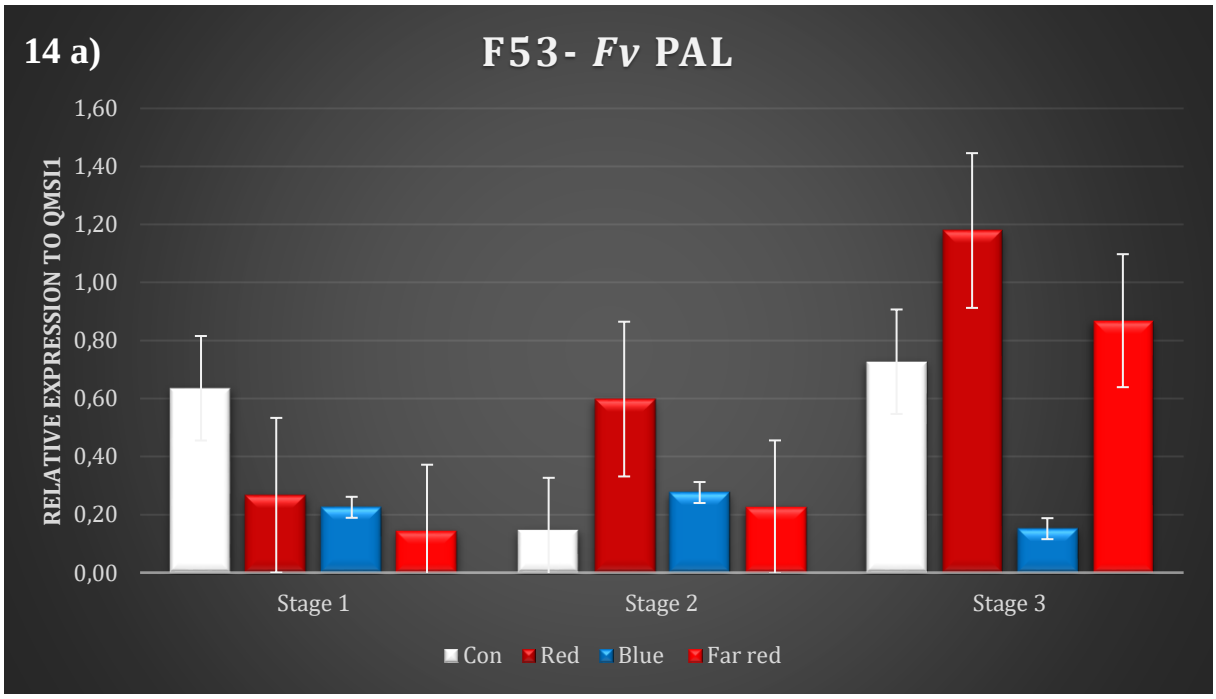
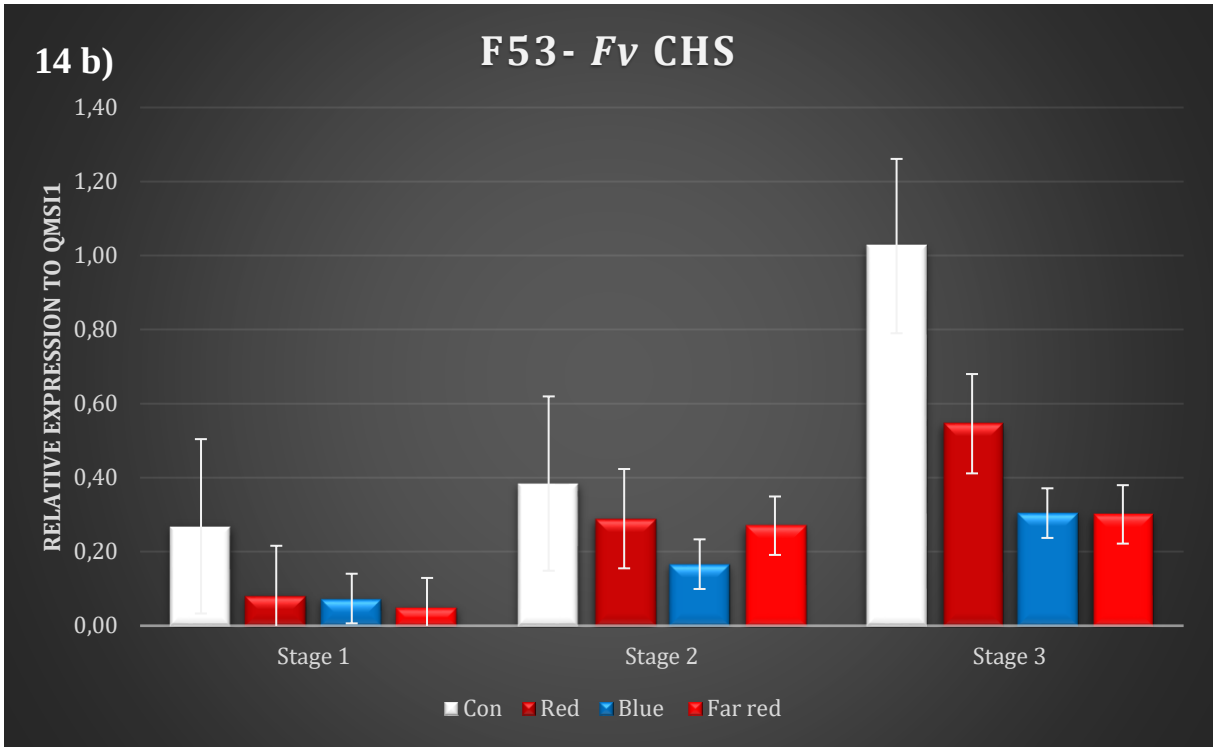
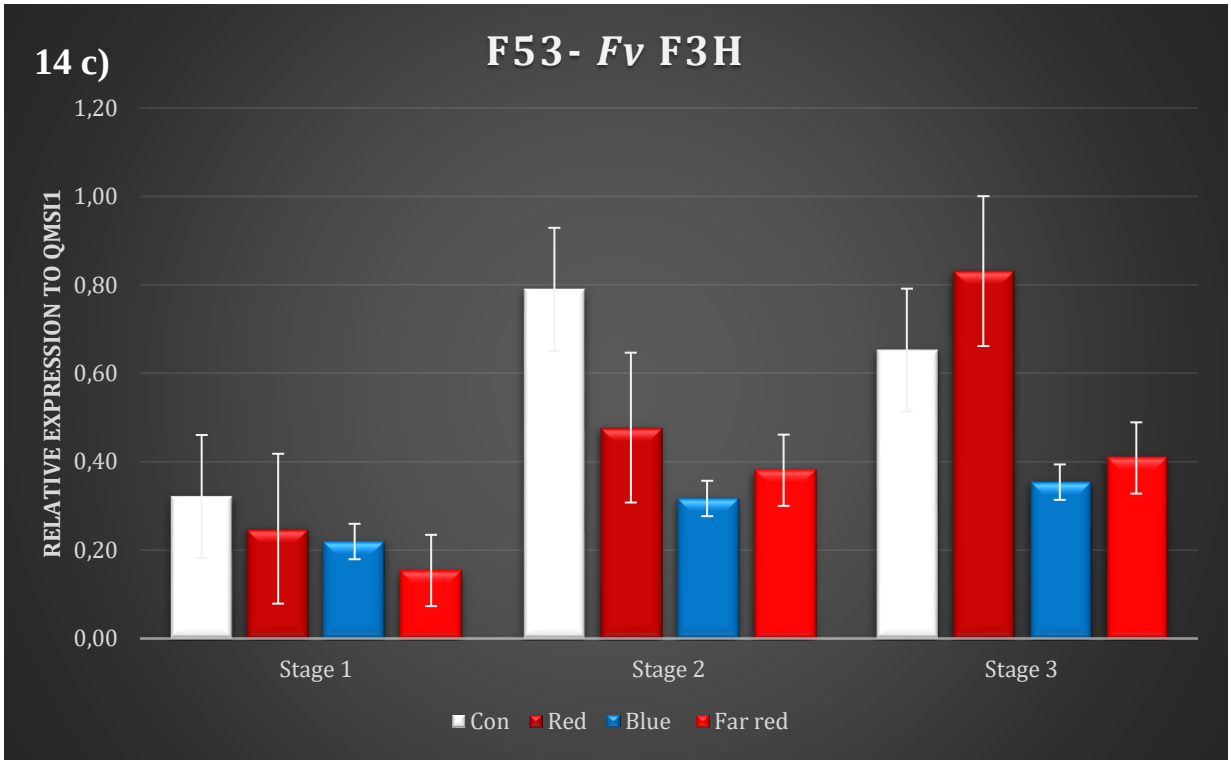
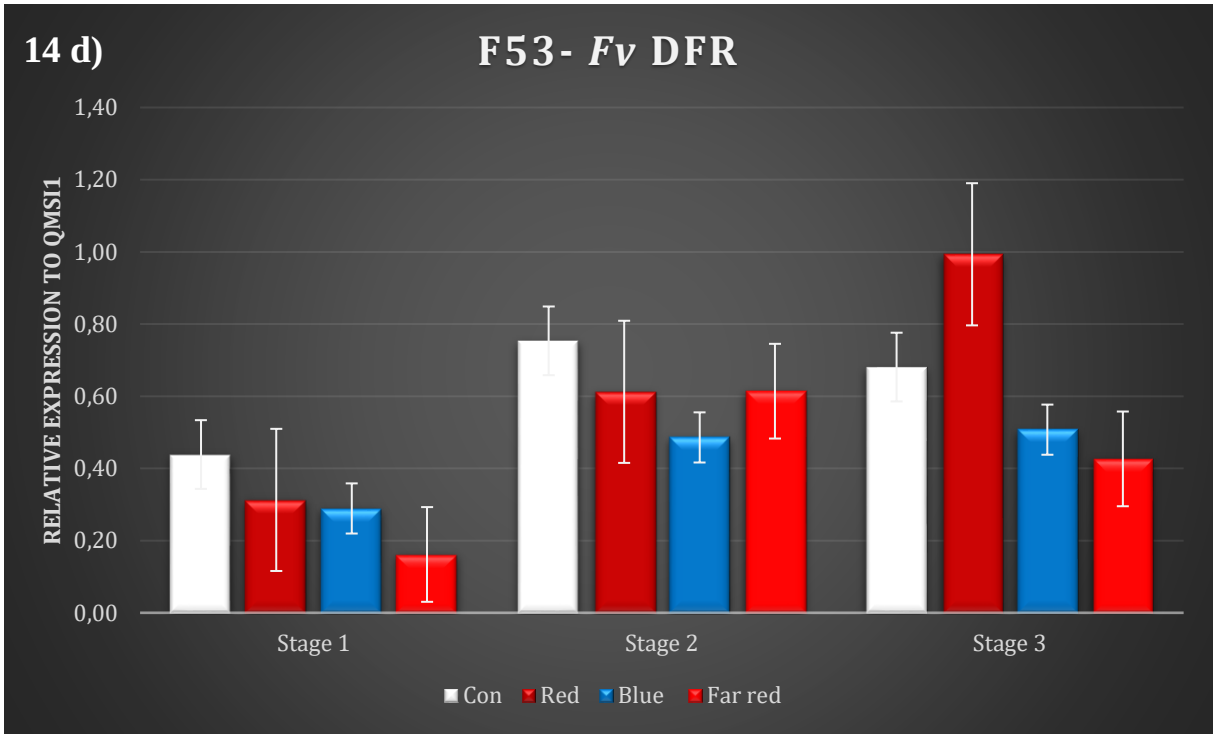


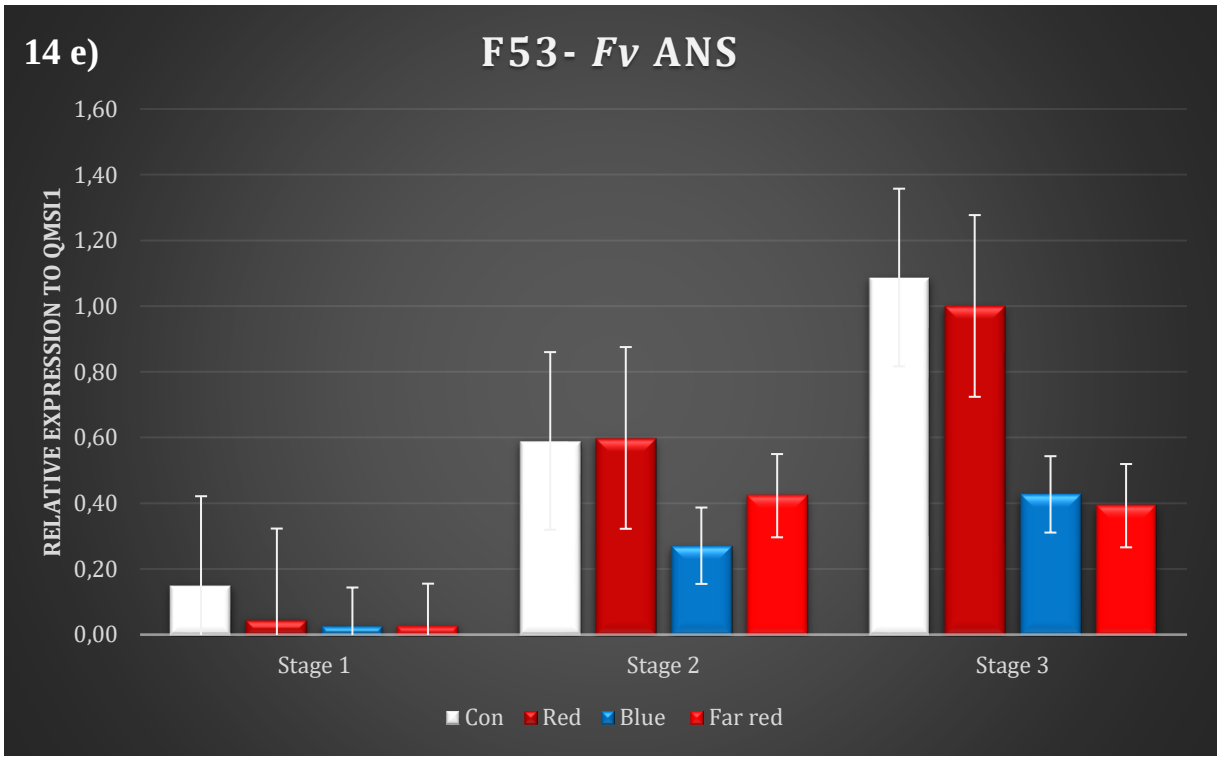
Figure 13: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*, d) *FvDFR*, e) *FvANS*, f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Finland 50 (F50) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

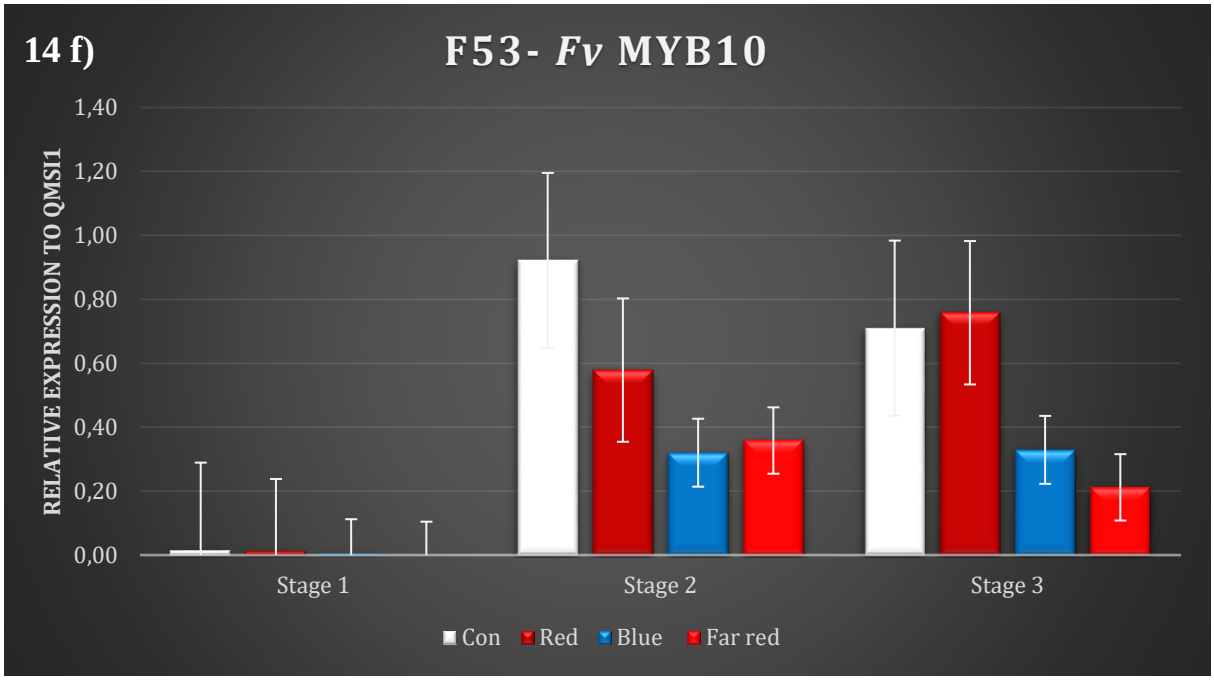



14 b)
F53- Fv CHS
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


14 c)
F53- Fv F3H
RELATIVE EXPRESSION TO QMSI1
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


14 d)
F53- Fv DFR
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


14 e)
F53- Fv ANS
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red



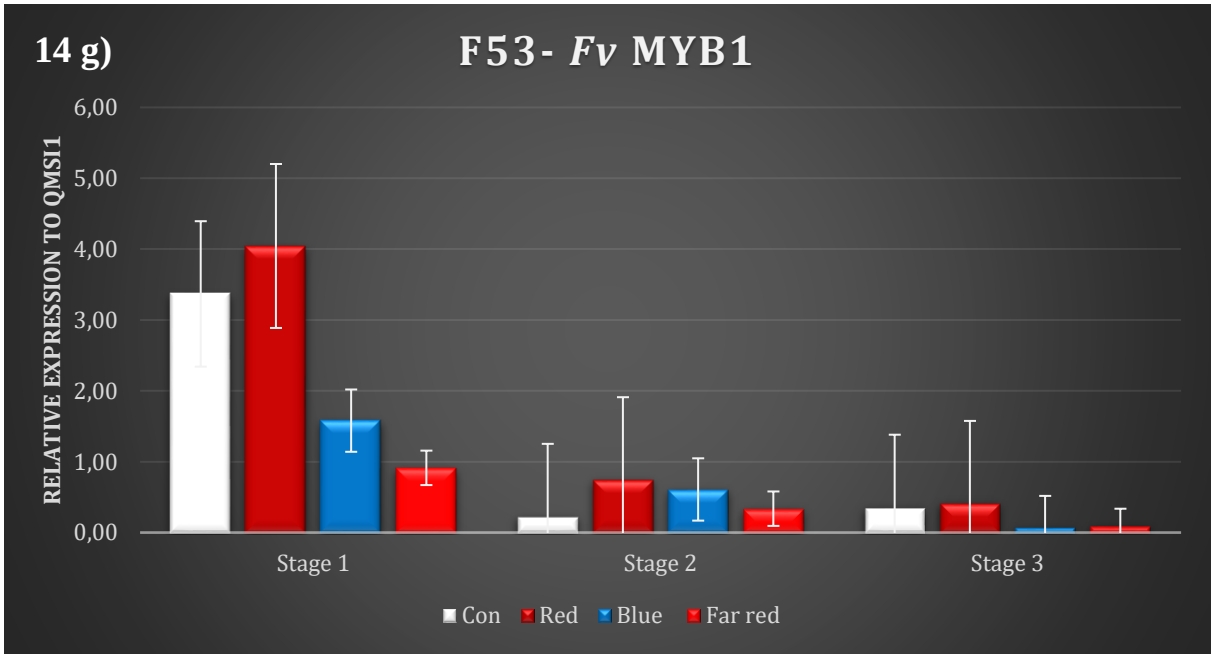
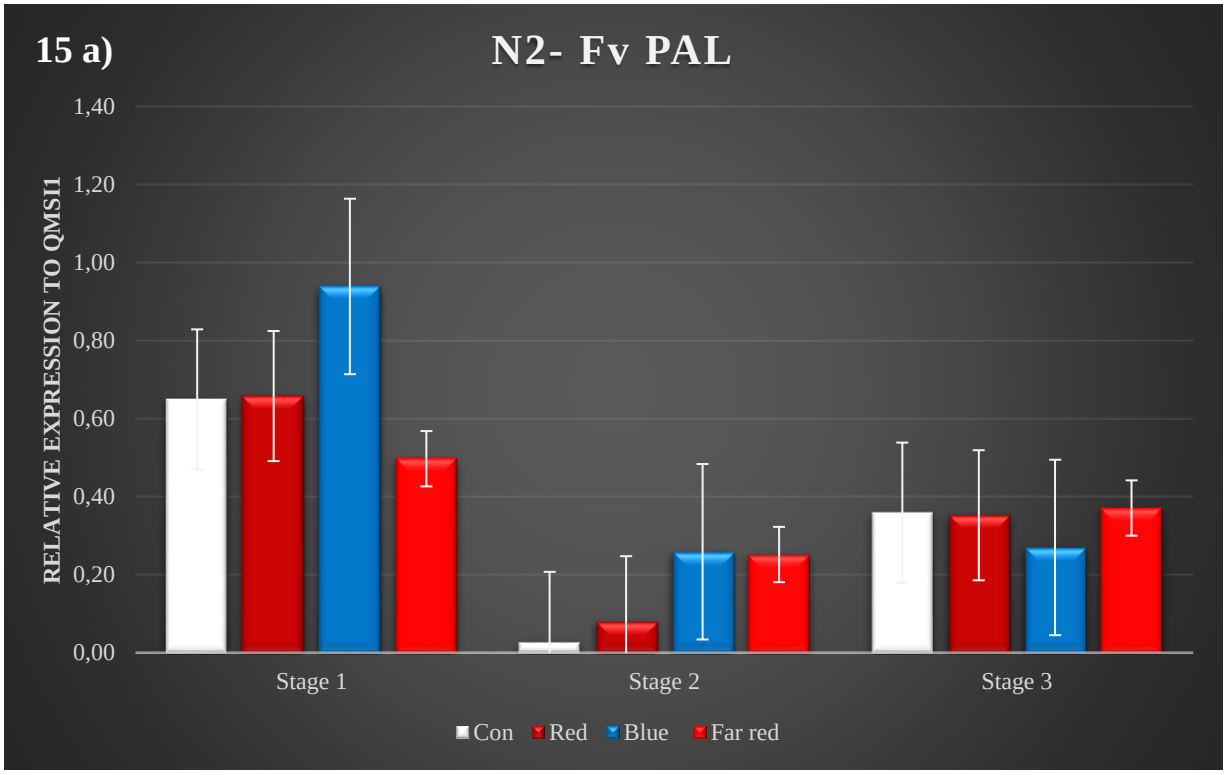
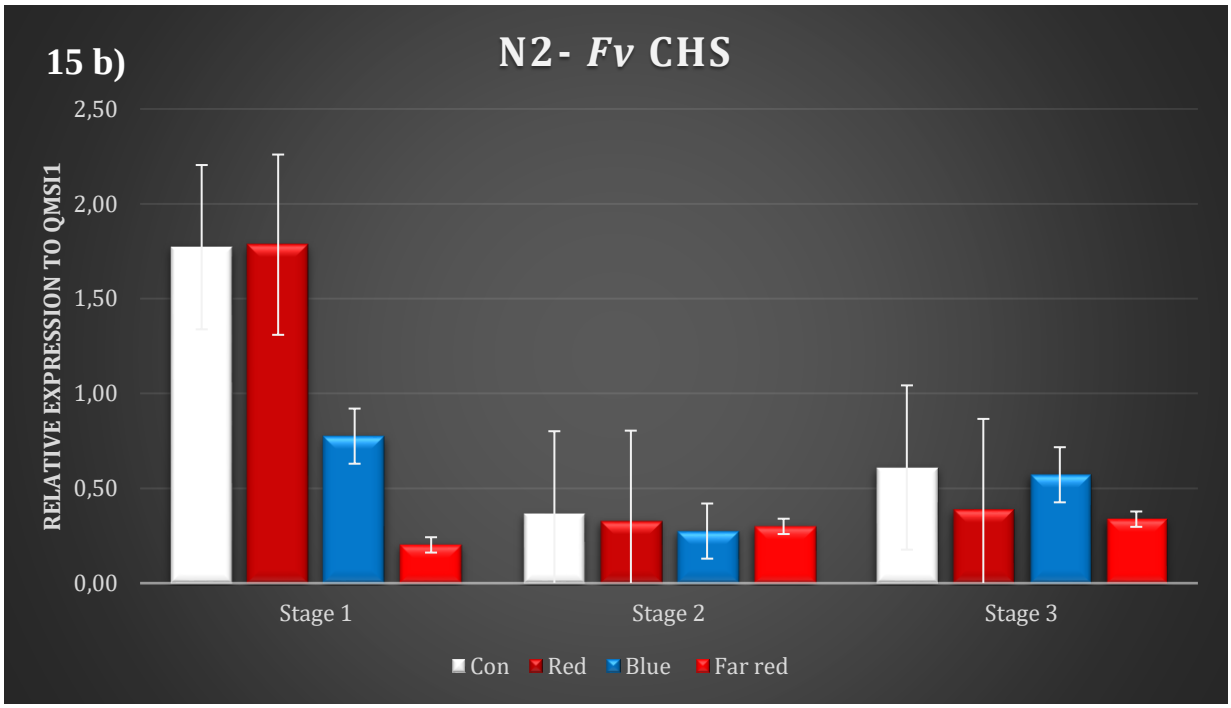
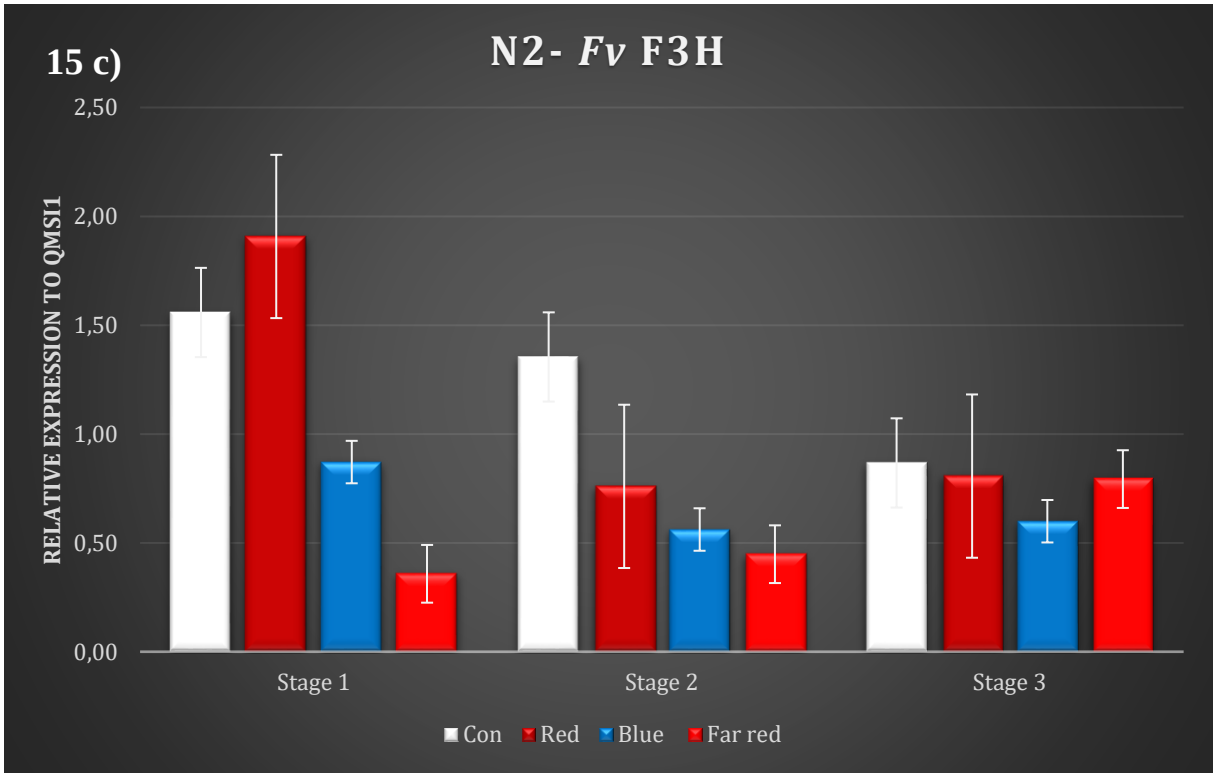
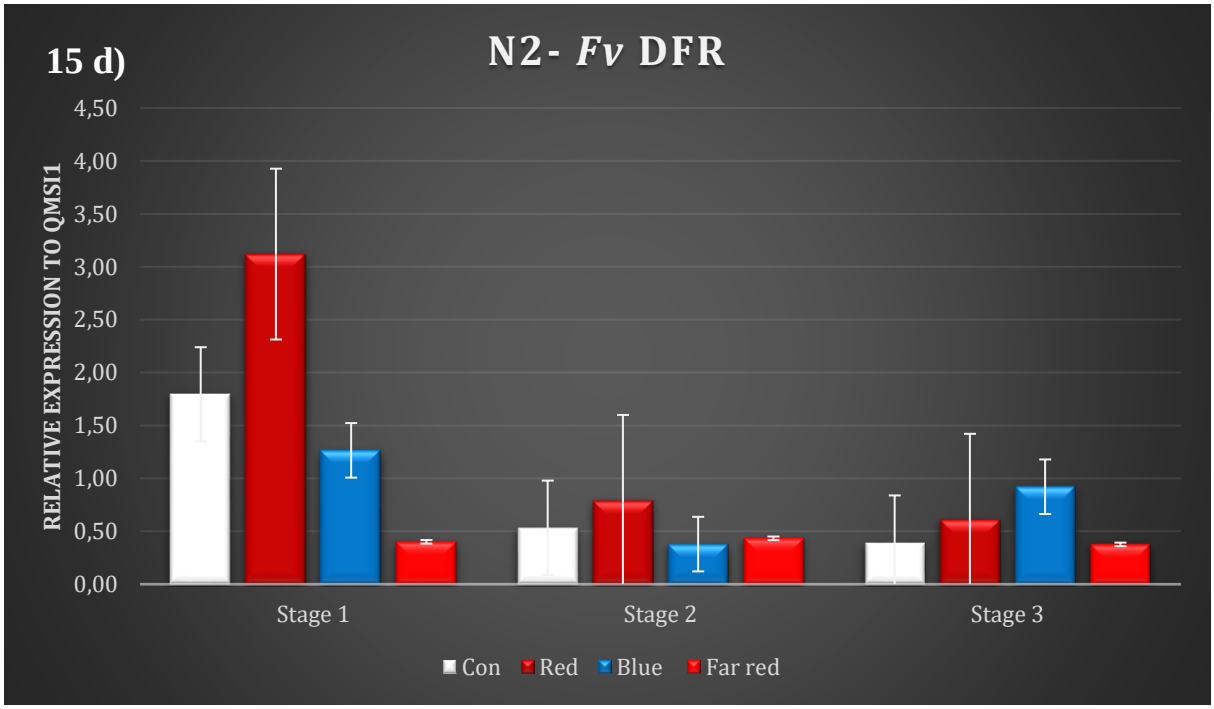


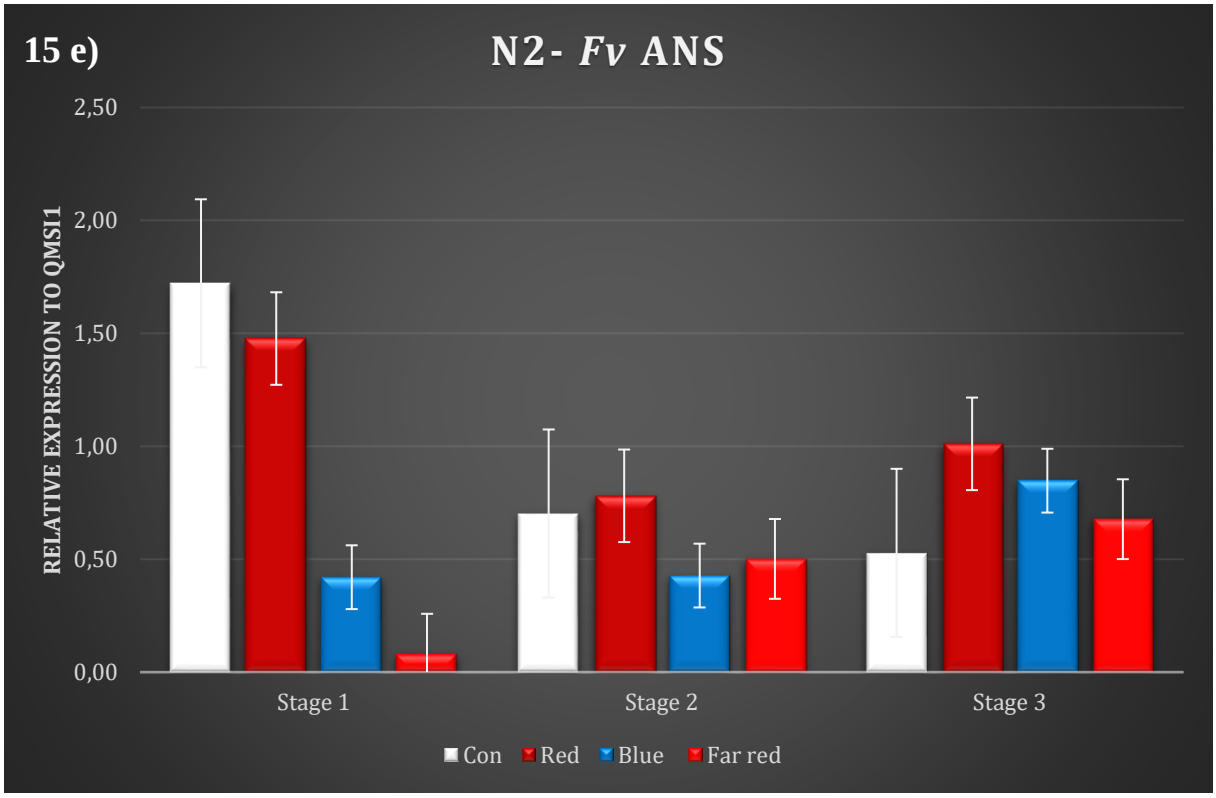
Figure 14: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*,, d) *FvDFR*, e) *FvANS*, f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Finland 53 (F53) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

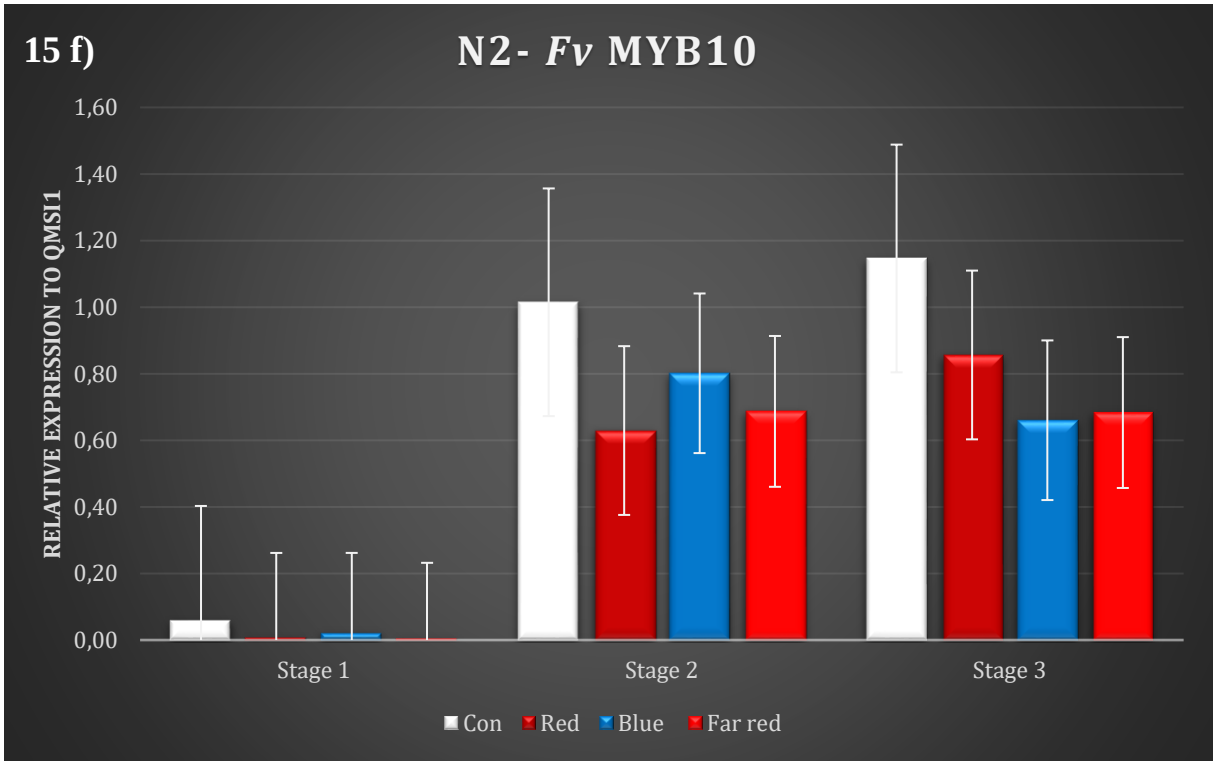

15 a)
N2- Fv PAL
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


15 b)
N2- Fv CHS
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


15 c)
N2- Fv F3H
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


15 d)
N2- Fv DFR
RELATIVE EXPRESSION TO QMSI1
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


15 e)
N2- Fv ANS
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


15 f)
N2- Fv MYB10
RELATIVE EXPRESSION TO QMSI1
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

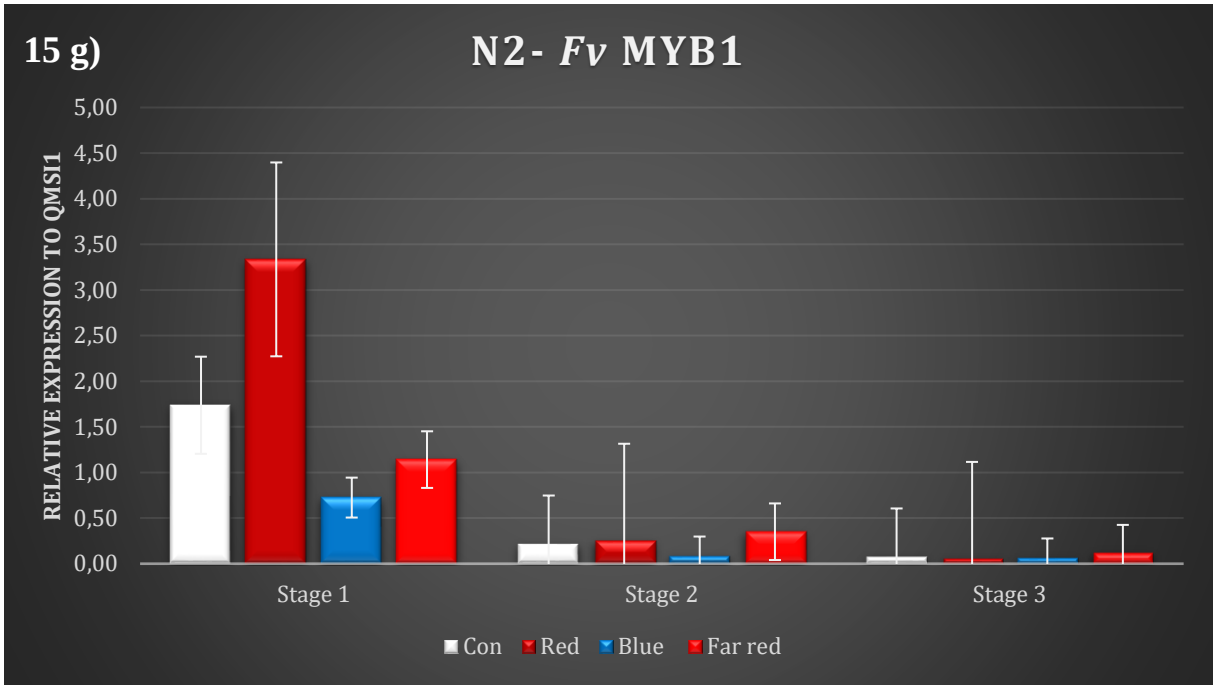
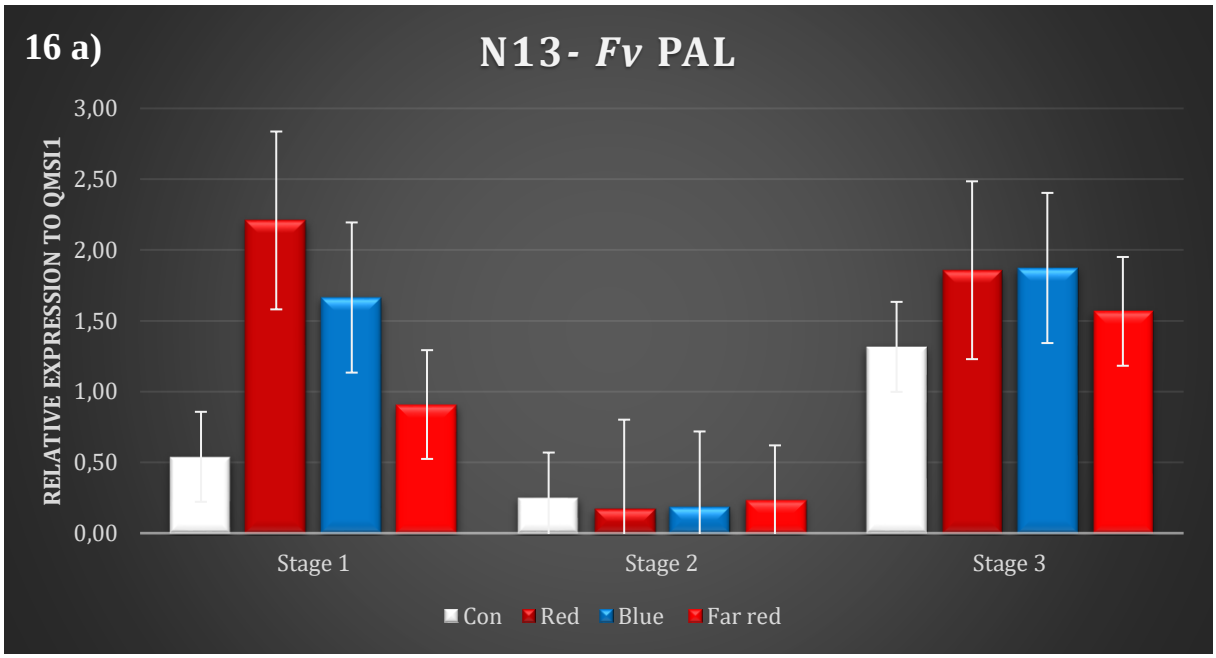
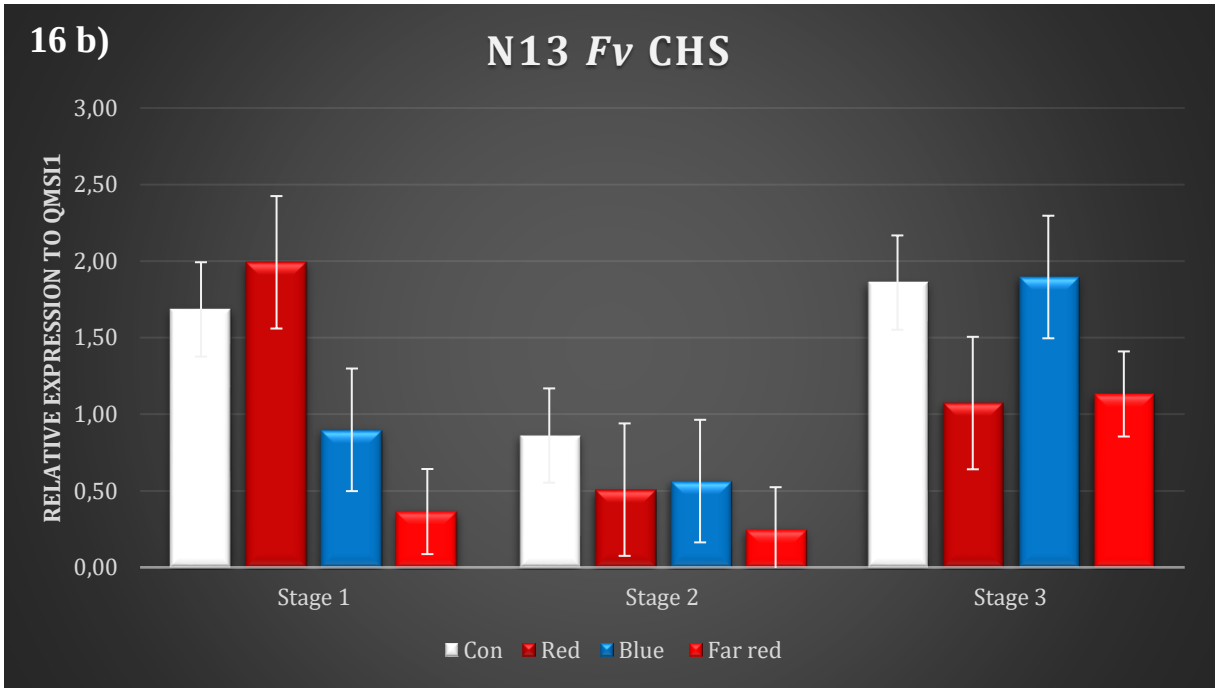


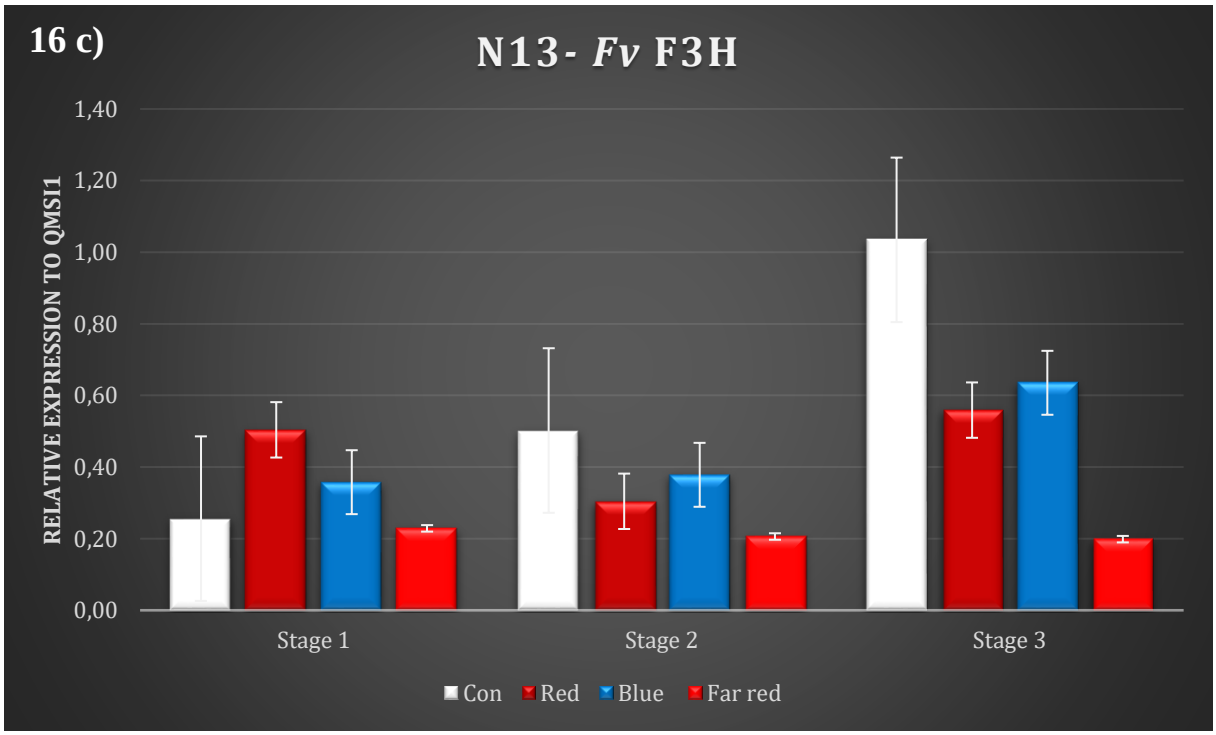
Figure 15: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*, d) *FvDFR*, e) *FvANS* f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Norway 2 (N2) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

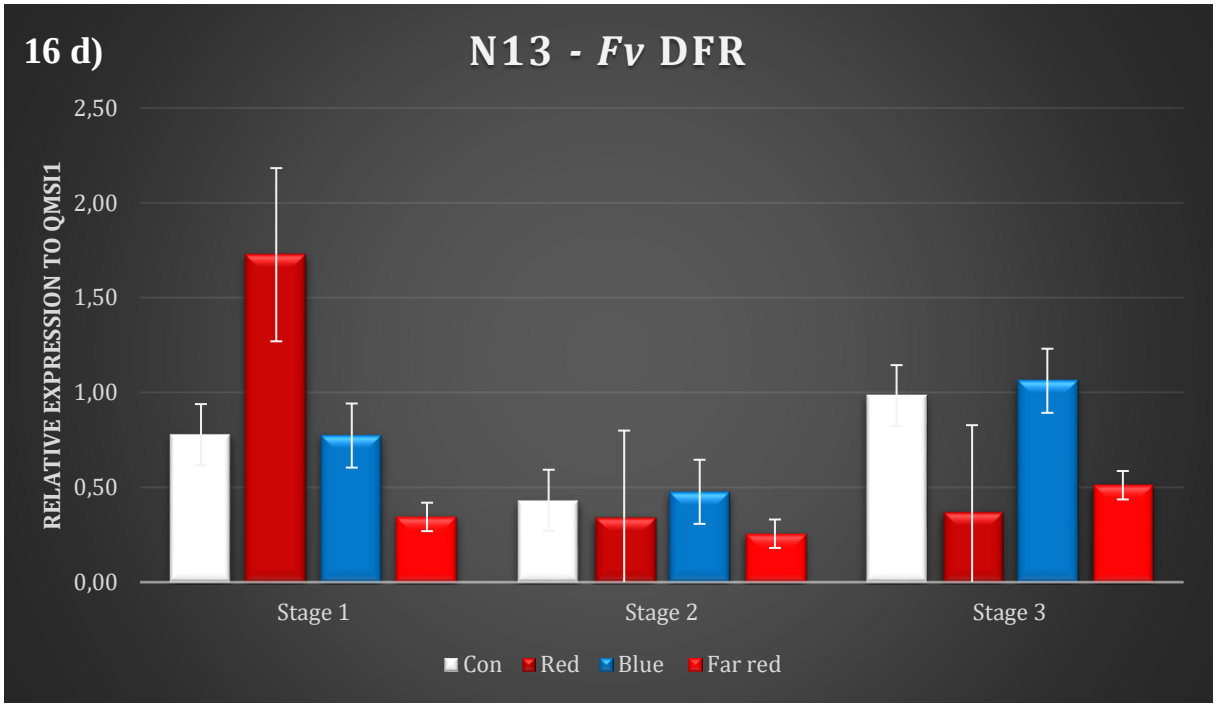


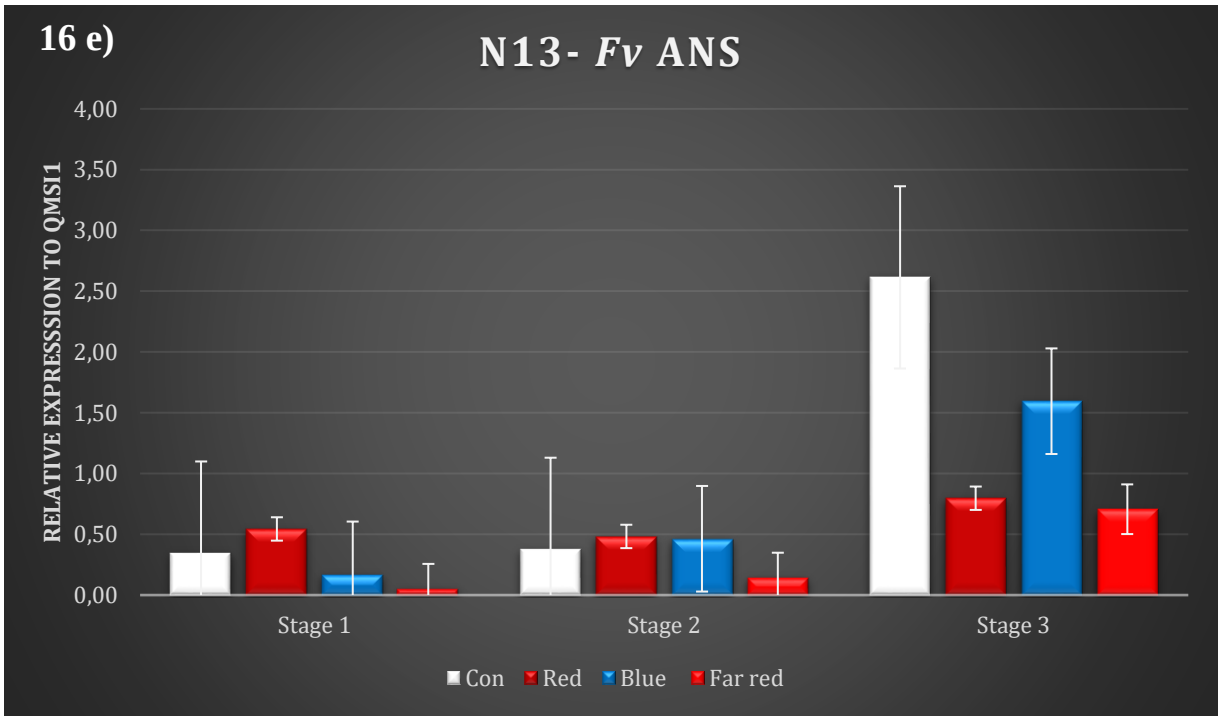
16 b)

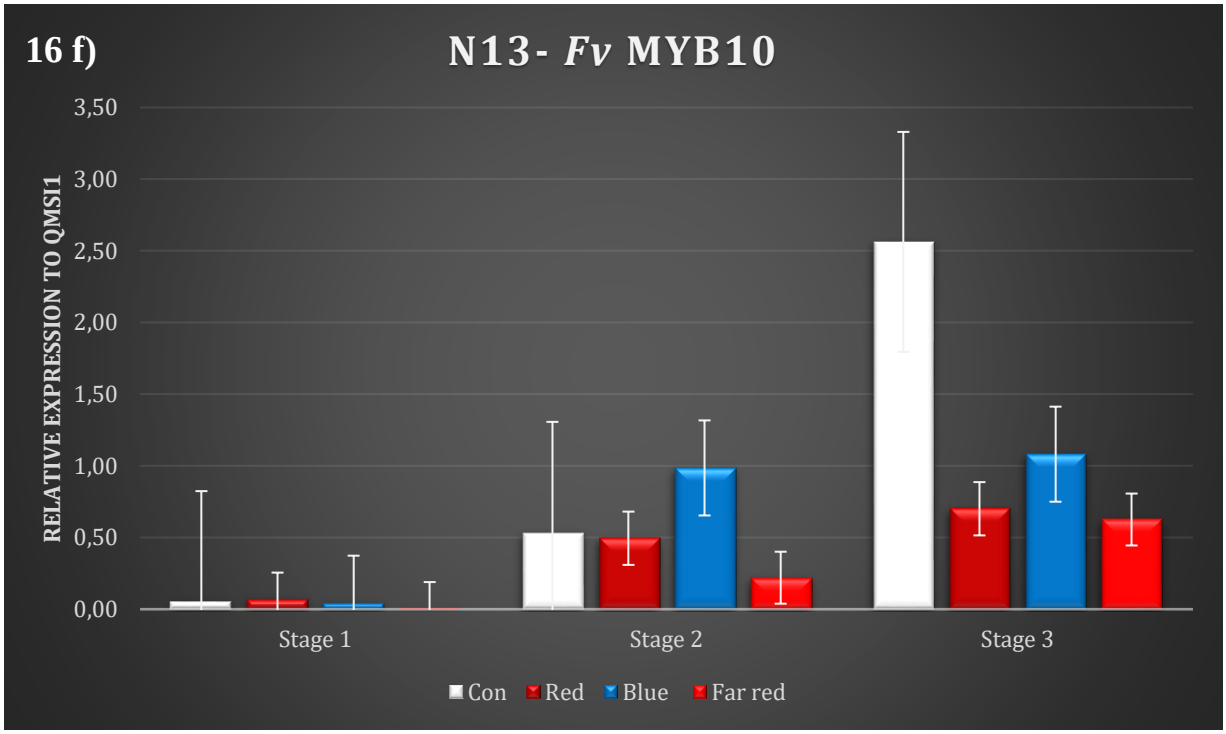

N13 Fv CHS
RELATIVE EXPRESSION TO QMSI1
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red

16 c)


N13- Fv F3H
RELATIVE EXPRESSION TO QMSI1
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


16 d)
N13 - Fv DFR
RELATIVE EXPRESSION TO QMSI1
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red


16 e)
N13- Fv ANS
RELATIVE EXPRESSSION TO QMSI1
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Stage 1
Stage 2
Stage 3
Con
Red
Blue
Far red



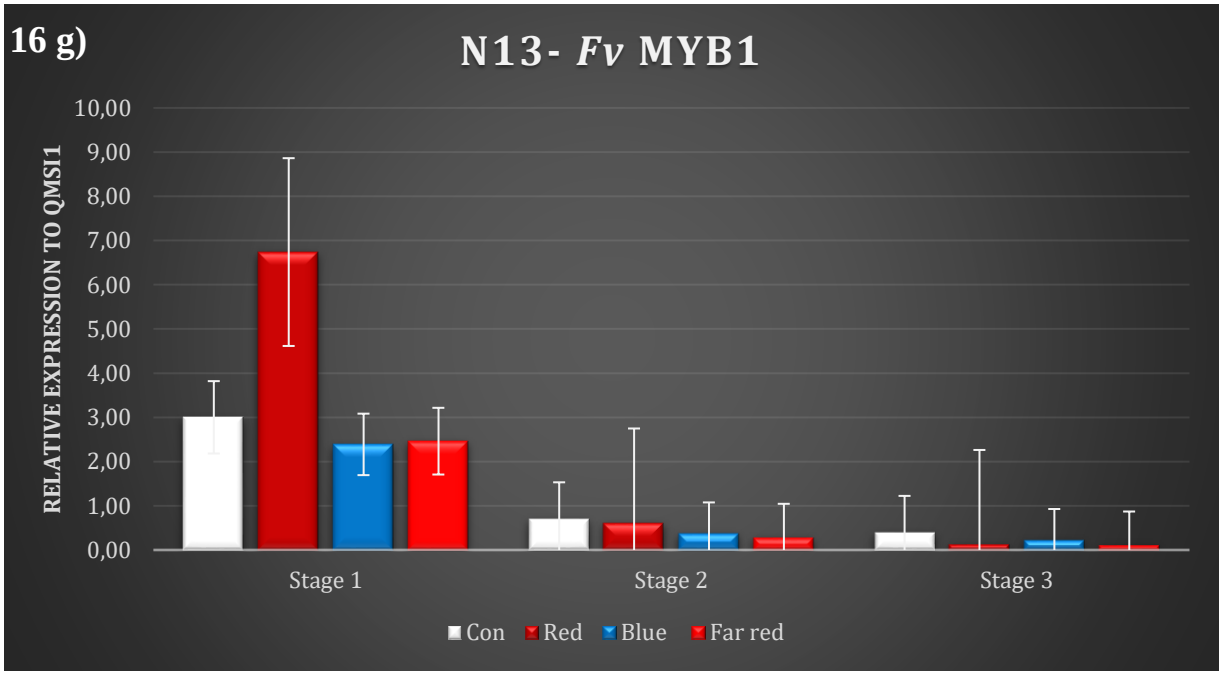


Figure 16: The expression levels of flavonoid pathway genes a) *FvPAL*, b) *FvCHS*, c) *FvF3H*, d) *FvDFR*, e) *FvANS*, f) *FvMYB10*, g) *FvMYB1*, in three berry ripening stages; Stage 1(S1), Stage 2 (S2) and Stage 3 (S3) of Norway 13 (N13) clones under LED light treatments. Different colours represent the different wavelength of the LED light. qMSI 1 gene was used to normalize the expression of the genes under identical conditions. All data are presented as means of three replicates. Error bars show standard error (SE).

4.3 Total anthocyanin concentration

The total anthocyanin concentration in ripe strawberries was expressed as cyanidin-3-glucose, which is the most common anthocyanin in nature, also common in *F. vesca* (Lee et al., 2005). All the clones had higher total anthocyanin content in all the LED light treatments than the control. However, the concentration in the control treatment of I1 and N2 clones was higher when compared to other light treatments. The red light seems to have stimulated the additional accumulation of total anthocyanin in Italy and Finland clones, Norwegian clones responded more to Blue light. When compared to other light treatments, Far-red light had a lesser influence on anthocyanin accumulation in Finland and Norwegian clones, but the effect was similar to blue light in Italian clones (Figure 17).

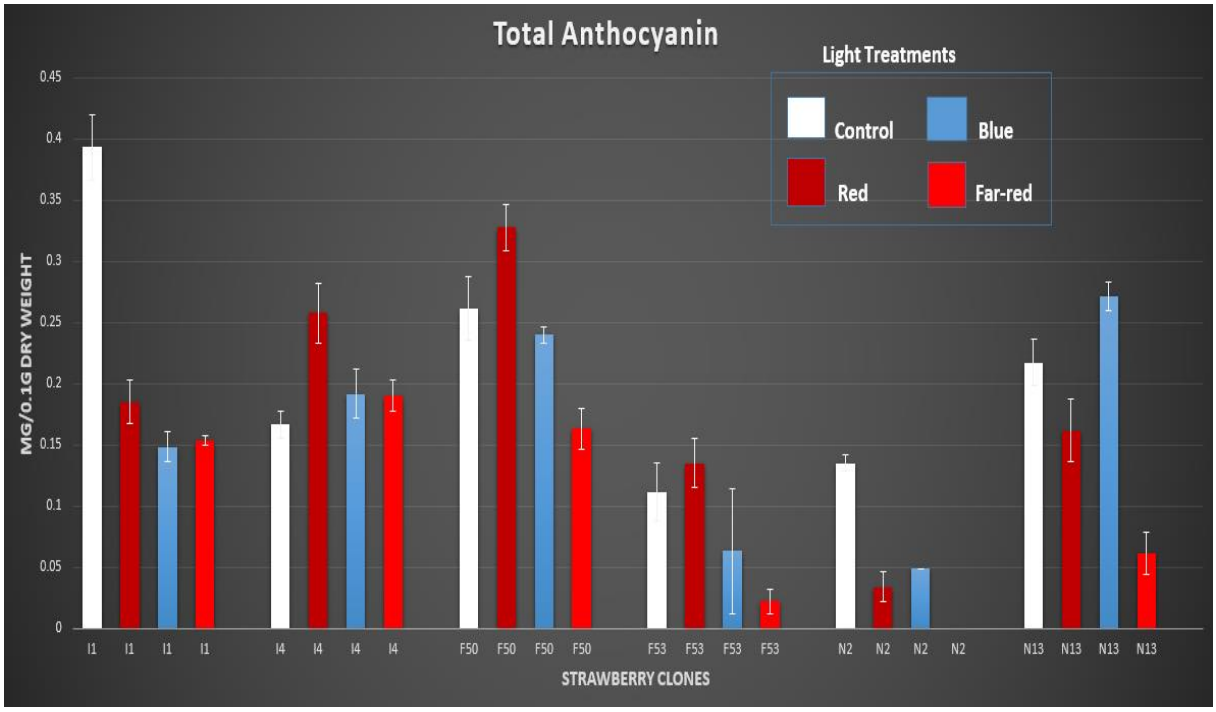


Figure 17: Total monomeric anthocyanins (mg/L, cyanidin-3-glucose equivalents) concentrations in ripe berries (Stage 3) of *F. vesca* clones from different latitudes Italia 1 (I1), Italia 4 (I4), Finland 50 (F50), Finland 53 (F53), Norway 2 (N2), Norway 13 (N13) under LED light treatments. Different colours represent the different wavelength of LED lights.

5 Discussion

Flavonoids are important secondary metabolite that have been found to have several health benefits and also involved in many crucial functions in plants (Mozaffarian & Wu, 2018; Winkel-Shirley, 2001; Winkel-Shirley, 2002). The great values of flavonoids make them target of numerous studies, which concern the developments of food with a high concentration of these compounds. Woodland strawberry is a rich source of flavonoids, and its small genome and short generation time make it a model plant for the diverse *Rosaceae* family (Dias et al., 2016; Shulaev et al., 2011). There have been several studies which aimed at improving the health beneficial properties of strawberry, and light conditions have been found to be one of the most important environmental factors affecting the accumulation of bioactive compounds in plants (Zoratti, Karppinen, et al., 2014).

Light quality is one of the main factors of light conditions that have positive effects on growth and accumulation of plant secondary metabolites, (Bian et al., 2015; Saito Y. et al., 2010; Song et al., 2017; Wang et al., 2009; Yoshida et al., 2016). In higher plants, flavonoid pathway genes are basically induced by light, resulting in anthocyanin accumulation (Kadomura-Ishikawa et al., 2013), and responses to different light qualities differ in different plants species (Cominelli et al., 2008; J. H. Cheng et al., 2015; Zhang et al., 2018). LED lights are recently adopted as artificial plant lighting over the others due to their unique advantages and their ability to match their wavelength to plant photoreceptors, to improve production and influence plants morphology and metabolism (Hasan et al., 2017; Rini et al., 2018). In our present study, we focused on the effect of different light qualities in the regulation of flavonoid biosynthetic genes in clones of woodland strawberry plants from different latitudes.

Environmental interactions with plant genes have an influence on flavonoid biosynthesis and their total concentrations. A long-lasting influence of the environment can cause local adaptations in plants of the same species to accumulate flavonoids differently due to their origin differences (Jaakola & Hohtola, 2010). Our study shows that when treated with different light qualities, there are differences in the gene expressions levels and the total anthocyanin accumulation in each clone of *F. vesca* originating from a different latitudes. A study by Oleszek et al., (2002) shows the significance of plant origin and growth site to the profile of secondary metabolites. The relationship between flavonoid level and latitude was reported in

P. sylvestris where the needles of the tree that originated from northernmost latitude had the lowest level of taxifolin 3`-O-glucoside, quercetin, taxifolin and total flavonoid than the ones from southernmost latitude (Jaakola & Hohtola, 2010). Also, Lätti et al., (2008) show the variation of anthocyanin content in 179 *V. myrtillus* clones from 20 populations growing on a south-north axis (60°21′N–68°34 N) that spanned 1000 km in Finland. Southern populations exhibited significantly lower content of anthocyanin profile and there were differences in the profile between berries growing in different latitudes (Jaakola & Hohtola, 2010). Comparable to the present study, the genetic differences of strawberry clones due to environment effect on their mother plants from different latitudinal origin, potentially have influenced the variations in their responses to the light treatments.

Differences exist between the expressions of flavonoid pathway genes of the leaves and berries under the light treatments. In the leaves, relatively similar expression patterns were observed among all the genes studied (Figure 11). The Italian clones were mostly up-regulated under the blue LED light, while the red LED light increased the expression levels of the Finnish and Norwegian clones. Conversely, a number of different expression patterns exist among the genes studied in the berries. Red and blue of the spectrum light induced an increase in the transcriptional levels of most genes and clones (Figure 12, Figure 13, Figure 14, Figure 15, Figure 16, Figure 16). The early stage berry samples showed mostly the similar expression patterns compared with the leaves, i.e. the Italian clones responded more to blue light and the Finnish and Norwegian clones to red light. Interestingly, the shift in this pattern was detected in the ripening (stage 3) berries, in which the Italian and the Finnish clones responded mostly more to red light and the Norwegian clones to red light. This pattern was also consistent with the total anthocyanins results. The Italian and the Finnish clones had higher concentrations of anthocyanins under spectrum of red light, and the Norwegian clones accumulated higher contents under blue light (Figure 17). This was an opposite result to the gene expression pattern of the leaf tissues, where Italian clones were up-regulated under blue light, and Finnish and Norwegian clones under red light (Figure 11). However, Finnish clones' gene expressions under red light are consistent in both the leaves (Figure 11) and mostly in the berries (Figure 14, Figure 15) and this also reflected in the anthocyanin accumulation where the highest contents were detected in the berries grown under red light (Figure 17). These results indicate very interesting latitude of origin related adaptations in the light responses of *F. vesca* plants

which require deeper analysis of the light signalling pathway upstream of the flavonoid pathway.

Our results suggest that far-red light had little influence on *F. vesca.* The effect of far-red on total anthocyanin accumulations in ripe berries is lower compared to other LED lights treatments (Figure 17), also in the leaf tissues, expression of genes under far-red light was almost undetected (Figure 11). This finding agrees with Li & Kubota, (2009) study, where baby leaf lettuces' total anthocyanin concentration was decreased significantly under supplemental far-red light compared to supplemental blue and UV-A. In this study, red light and blue light seem to be the prominent wavelength imparting increase in the total anthocyanin content and transcriptional levels of genes compared to far-red and white light. Various effect of specific wavelength of LED light on the regulation of flavonoid pathway genes and flavonoid accumulation has also been reported in other plant species, for example in bilberry, the total anthocyanin content in ripe berries was significantly increased by monochromatic lights of blue, red and far-red, in comparison to fruits treated with white light or kept in darkness (Zoratti et al., 2014). In Gerbera, anthocyanin accumulation in flowers was particularly stimulated by blue light (Meng et al., 2004), in cranberry fruits, red and far-red light increased the anthocyanin accumulation (Zhou & Singh, 2002). In grapefruits (Koyama et al., 2012) and cultivated strawberry (Kadomura-Ishikawa et al., 2013), blue light significantly increased anthocyanin biosynthesis. In our study, the lowest concentrations of total anthocyanins was detected in N2 clones under the LED light treatments (Figure 17). This result was consistent with the related gene expression which had decreasing expression pattern from the first stages of berries to the third stages, and their highest expressions were seen in the first stages whereas at the third ripening stages expressions were down-regulated (Figure 16). Other clones had a variable increment in their total anthocyanin accumulations under LED light treatments, with the highest accumulation in F50 under red light (Figure 17).

MYB-R2R3 transcription factors play a pivotal role in the regulation of structural genes of the flavonoid pathway at transcriptional level (Zhang et al., 2015), and transcription of these genes is under the control of light (Zoratti, Karppinen, et al., 2014). In strawberry, *MYB10* has been reported to effectively regulate the expression of the early and late flavonoid biosynthetic genes. It plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during the ripening of strawberry fruits (Lin-Wang et al., 2014). Over-expression of *MYB10* in 'Alpine'

strawberry *F. vesca* resulted in plants with elevated leaves, petioles, stigmas and fruit anthocyanin concentrations (Zhang et al., 2015), and *MYB1* has been described as a suppressor related to the regulation of ripening of strawberry fruits (Aharoni et al., 2001). Combined, interactions of the two transcription factors regulate expressions of individual or set of a structural flavonoid pathway genes (Hossain et al., 2018). This evidence is consistent with our study where *FvMYB10* gene expression was upregulated in *F. vesca* leaves (Figure 11). Italian clones had high transcriptional levels under blue light and this correlates with the expression of related genes studied. However, the increased expression of Finnish and Norwegian clones under blue light is in contrast with the transcription pattern of the structural genes as they have transcriptional peaks under the red light treatments. Also, *FvMYB10* gene was highly expressed in *F, vesca* third stages of berries (Figure 12f, Figure 13f, Figure 14f, Figure 15f, Figure 16f, Figure 16f). The gene expression was down-regulated in the first stages and as well as in the second stages. There was a strong relationship with *FvMYB10* expression and fruit ripening. This probably indicates the important role of *FvMYB10* in the anthocyanin biosynthesis in ripe fruits. Moreover, the expression trends in all clones are consistence with the expression patterns of *FvANS* (Figure 12e), the main structural gene of anthocyanin biosynthesis.

FvMYB1 gene expressed in the contrasting pattern to *FvMYB10*, the expression was higher in the first stages of berry development and down-regulated in the third stages (Figure 12g, Figure 13g, Figure 14g, Figure 15g, Figure 16g, Figure 16g). However, *FvMYB1* did not act as a repressor in the leaf tissues, the expressions of *FvMYB1* gene in the leaves were equally upregulated (Figure 10g). There was a highly variable increase in the transcriptional levels across all clones in all treatments, and the transcriptional pattern is almost consistent with the expression of all the structural genes studied. The role of *FvMYB1* as a repressor of flavonoid pathway genes in *F. vesca* leaves is still unclear. A possible explanation could be that *MYB1* only expresses in the early stages of development, in the leaves and in the green berries but at the ripening stage, its expression is suppressed. Aharoni et al., (2001) described and characterized *FaMYB1*, and this study suggests that *FaMYB1* may play a key role in regulating the biosynthesis of anthocyanins and flavonols in strawberry. It may act to repress transcription in order to balance the levels of anthocyanin pigments produced at the latter stages of strawberry fruit maturation, and/or to regulate metabolite levels in various branches of the flavonoid biosynthetic pathway. Further studies are needed in order to understand the specific roles of *FvMYB1* in leaf tissues.

In the leaves of *F. vesca*, the changes in the expression of the flavonoid biosynthesis genes can be clearly seen only after 48 hours of LED light exposure. In the other time points, most of the genes expression were lower compared to the samples under white light. This effect can also be seen in the expression levels of genes studied in *F. vesca* berries, where most genes were up-regulated under the white light in some of the berry developmental stages. This suggests the possibility that expression of biosynthesis genes may be time-based under light treatments and expressed at early stages after exposure. A similar result was recorded in Thwe et al., (2014), where the phenylpropanoid pathway genes generally showed higher numbers of transcripts at 2 days after light exposure (DAE), compared with 4, 6, 8, and 10 DAE. The gene expression peaked at 2 DAE, and after that, it suddenly decreases and plateaued until 10 DAE.

6 Conclusion

Light qualities (specific wavelengths) have a complex effect on the biosynthesis of flavonoids in plants, and spectral wavelengths of red and blue have been found to be the prominent lights wavelengths influencing the biosynthesis of flavonoids. This study shows that *F. vesca* clones from different latitudes responded differently to LED light treatments. The southernmost (Italian) clones responded most to blue light regarding the leaves and the early developmental stage fruits, whereas leaves and early stage fruits of the Finnish and North Norwegian clones responded most to red light. Most interestingly, a shift in this pattern was detected at the end of fruit ripening, when the Italian clones responded more to red light, similarly to the Finnish clones, and the Norwegian clones responded more to blue light. The differences can both be seen in the gene expression and the total anthocyanin accumulations.

The results give novel evidence on the role of the latitude related adaptions in the light perception affecting the flavonoid biosynthesis, and offer new tools for deeper understanding of this regulation. Therefore, further studies will be carried on the metabolic profiling of the *F. vesca* ripe berries using HPLC/MS to characterise different metabolite accumulated during the LED light treatment. The photoreceptor signaling network will also be analyzed to understand the molecular mechanism behind the latitude related differences in perception of different wavelengths of light in the signaling pathway.

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