Effect of Photoperiod on the Flavonoid Biosynthesis Pathway Activity in peanut (*Arachis hypogaea*) varieties

By

Irum Nauman

REG#: 119165

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology, Islamabad

2017


بسم الله الرحمن الرحيم

Effect of Photoperiod on the Flavonoid Biosynthesis Pathway Activity in Peanut (*Arachis Hypogaea)* Varieties

A thesis submitted in the partial fullfilmet of the requirement for the degree of Masters of Science

Submitted By

Irum Nauman

REG#: 119165

Supervised By

Dr. Rabia Amir

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology, Islamabad

2017

Dedication

This humble effort is dedicated to

My Beloved Parents

&

Sisters

Acknowledgement

All praise is to Almighty Allah who bestowed upon me a minute portion of His boundless knowledge by virtue of which I am able to accomplish this challenging task.

First of all, I am also thankful to my parents and family who have been a constant source of encouragement for me and brought me the values of honesty & hard work.

I would like to thank principal ASAB Dr. Peter John and Head of Department (Plant Biotechnology) Dr. Muhammad Tahir for providing me with the opportunity to be the part of this prestigious institute.

I am greatly indebted to my supervisor Dr. Rabia Amir. Without her supervision, advice and valuable guidance, completion of this research would have been doubtful. I am thankful to her for encouragement and continual help throughout this work. I am also thankfull to my GEC members Dr. Faiza Munir and Dr. Muhammad Qasim Hayat as he helped me a lot throughout the research work with his inspiring suggestions and provided me with all the possible research facilities.

In the end, I would like to thank my friends Iqra Shah, Tanzeela Bahir, Norina noor, Saman Taufiq, my juniors and lab fellows for their continuous support.

Irum Nauman

ABBREVIATIONS

ANS	Anthocyanin synthase
ANR	Anthocyanin Reductase
CHS	Chalcone Synthase
CHI	Chalcone Isomerase
F3H	Flavanone 3-hydroxylase
DFR	Dihydroflavonols Reductase
LAR	Leucoanthocynadin Reductase
FLS	Flavonole Synthase
ITS	Internal transcribed spacer
ETS	External transcribed Spacer
Matk	Megakaryocyte-Associated Tyrosine Kinase
Rbcl	ribulose 1, 5 bisphosphate carboxylase
mRNA	Messenger RNA
UV	Ultra Violet
PCR	Polymerase chain reaction
CTAB	cetyl Trimethy Ammonia Bromide

RT	Reverse Transcriptase
qRT	Quantitative real time
β	beta
Δ	delta
µL	micro-liter
ML	Milli-liter
CT	Cycle threshold

List of Figures

Abstract

Cultivated peanut, *Arachis hypogaea*, is an important oilseed crop with a total global production area of approximately 59 million acres. Besides oil, peanut seed is rich in protein and a good source of vitamins and minerals. With the great nutrient profile and important role in disease prevention groundnut is needed to be explored to reveal its more beneficial effects. Anthocyanin, proanthocyanidins and flavonols are the major flavonoids that are present in fruits and flowers. Flavonoids are in all parts of the peanut plant. Many environmental factors affect the flavonoids biosynthesis in plants light being the most important one. Out of all flavonoids, flavonols are considered to play important role in photoreception. In this study, expression analysis of a representative flavonoid biosynthetic gene Flavonol Synthase (FLS) was conducted in response to different white light conditions. Highest induction of FLS gene was recorded at 24hour light treatment in both varieties. This study will provide the better understanding of effect of different light durations on the flavonoids biosynthetic pathway genes.

Table of Contents

Introduction

1.1. Peanut

Peanuts or Groundnuts are world's famous edible seeds of legume family. According to USDA report (2015), China has the highest production rate of groundnuts, approximately 45% of worldwide production, India stands second with 16% of production rate and USA has 5 %. In scientific nomenclature groundnuts are termed as "*Arachis hypogaea"*. *Arachis hypogaea* is the botanic name of groundnut which is derived from two Greek words, *Arachis* meaning a legume and *hypogaea* meaning below ground, due to the development of pods in the soil. *Arachis hypogaea* is allotetraploid (AABB 2n = 4x = 40chromosoms) with two main classes; *hypogea* and *fastigiata* (Leal-Bertioli, Bertioli et al. 2012)

1.1.1 Taxonomy and Classification

Groundnut is a member of the genus "Arachis", in the subtribe "Stylosanthinae", of tribe "Aeschynomeneae", of the family "Leguminosae" (Shahzad, Anjum et al. 2011) . Morphologically this genus is well defined and due to its peg and geocarpic reproductive growth it is distinguished from other genera. The genus "*Arachis"* has more than 70 wild species, out of which only *Arachis hypogaea* L. is commonly cultivated (Prasad, Kakani et al. 2010).

1.1.2. Cultivation and Distribution

Groundnut is most commonly cultivated in humid and warm temperature climates (Carley and Fletcher, 1995). Groundnuts are best grown in areas with good rainfall, plenty of sunlight and warm temperatures of about 25^{o} C to 28^{o} C. The groundnut crop is cultivated in almost 108 countries on an area of about 22.2 million hectares out of which 13.69 million

hectares are in Asia (India 8 million ha; China 3.84 million ha), 7.39 million hectares in Africa, and 0.7 million hectares in Central and South America (Carley & Fletcher, 1995). Worldwide production rate of groundnuts has been expected at approximate 39.5 million metric tons (mmt) in 2015-16. In Pakistan groundnut is the most important cash crop of the Pothwar region. Groundnut crop is cultivated over an area of 81.5 thousand hectares with annual production of 91.4 thousand tones. Its cultivation is mostly focused in Rawalpindi division, Jhelum and Attock (PARC).

1.1.3. Morphology

The groundnut plant has stems that grows along the ground and around 0.5 m (20 in) tall or long. It has alternate and compound leaves which are tetra foliate, up to 6 cm (2.25 in) long. The flowers are borne in the axils of the leaves and have golden-yellow petals about 10 mm (0.4 inch) across and are self-pollinated. As pollination occurs, stalks of flower extend, approximately 6 cm long, developing pods are pushed inside the ground, leading to the extraction of legumes from the soil to be harvested. The fruit is a legume (a pod), with rounded ends and are most commonly 25 to 50 mm (1–2 inches), containing 1 to 6 seeds and covered with a reddish brown delicate membrane.

Figure 1: (a) groundnut seed pod containing seeds; (b) tetra foliate groundnut leaves; (c) procumbent groundnut stems; (d) groundnut Flower

1.1.4. Nutritional Importance

Groundnuts are outstanding also inexpensive source of nutrition, providing essential nutrients to human body such as carbohydrates, proteins, fiber, vitamins, lipids and minerals. Groundnuts, when taken in acceptable amount, supplements human body with essential nutrients that can deliver energy, facilitates growth, and plays significant role in the prevention of diseases (Settaluri, Kandala et al. 2012).

It is considered as a 3rd most important vegetable protein source and contains 50% edible oil, 20% carbohydrates and 28% digestible protein(Bhatti and Soomro 1994). Groundnuts are the richest nut sources of folate. Groundnuts, like other legumes, are low in sodium and good sources of calcium, magnesium and potassium, a group that is associated with reduced risk of CVD (cardio vascular diseases) (Higgs 2003). Groundnuts contain important amino acids that are required for proteins synthesis. Carbohydrates are present in adequate amount in groundnuts as they have a fundamental role in providing valuable nutrition to human body. Hence groundnuts along with groundnut oil can serve as a healthy form of nutrition. Groundnuts are a vital source of water soluble vitamins for the human body along with vitamin E which is fat soluble. Another important vitamin which is supplemented by the intake of groundnuts is vitamin B3 (known as Niacin), to an extent of 13.525 mg(Settaluri, Kandala et al. 2012).

Commercially groundnuts are used mainly for oil production as they contain high oil content and is listed under important oil seed crops. In recent studies, it is revealed that groundnuts are also a good source of many bioactive components including phenolic acids, phytosterols, resveratrol and flavonoids. Bioactive compounds are extra nutritional constituents that occur naturally in small quantities in plant and food products(Kris-Etherton, Hecker et al. 2002). Groundnuts are a rich source of flavonoids and their dietary intake is recommended in

order to combat different diseases. These bioactive components are known for their antioxidant properties, disease preventive properties and are thought to promote long life.

1.2. Role of Flavonoids

Groundnuts are rich in secondary metabolites such as phenolic acid, flavonoids, stilbene(resveratrol) and tochopherols (Isanga and Zhang 2007). One of the major secondary metabolite present in groundnuts are flavonoids. Flavonoids are biosynthesized via the phenylpropanoid pathway.

Flavonoids are majorly known for their role in pigmentation or coloration of fruits, but their applications are much more than this. Flavonoids are major antioxidant compound. They also play important part as defense compounds or as signaling molecules in reproduction, symbiosis and pathogenesis(Steinkellner, Lendzemo et al. 2007). Due to their efficiency, flavonoids can form a defense system against harmful insects. Flavonoids are nonhazardous substances that display a diverse range of biologically beneficial activities(Semwal, Semwal et al. 2016). Health beneficial role of flavonoids is controlled by its chemical structure. Age related diseases like cancer and cardiovascular diseases can be prevented by the intake of flavonoids in diet(Pandey and Rizvi 2009).

1.2.1. Classification

Variations in aromatic ring substitution patterns in flavonoids leads to seven major flavonoid classes, i.e., flavonols, flavanones, flavones, isoflavones, anthocyanidins and proanthocynadins (Mondal, Phadke et al. 2015).

1.2.2. Biosynthesis pathway

Flavonoids are produced by the combination of polyketide and phenylpropanoid pathways. Upstream pathway is involved in the formation of the basic structure required for synthesize of all flavonoids, starting from coumaroyl-CoA and malonyl-CoA. The first enzyme that play an important role in the biosynthesis of each flavonoid is chalcone synthase (CHS). CHS catalyzes the formation of naringenin chalcone which is a triketide intermediate, from 4-coumaroyl-CoA and three molecules of malonyl-CoA. The second enzyme Chalcone isomerase (CHI) catalyzes the formation of naringenin chalcone to naringenin which is a colorless flavanone. The third enzyme Flavanone 3-hydroxylase (F3H) catalyzes the addition of −OH group at 3 position of flavanone to form dihydroflavonol. Then (F3′H) hydroxylase the 3′ or 5′ position of the B-ring, producing, dihydroquercetin. The fourth enzyme Flavonol synthase (FLS) is the first enzyme involved in the biosynthesis of flavonols which transforms dihydroflavonols into corresponding flavonols like kaempferol, quercetin and myricetin. The fifth enzyme Dihydroflavonol reductase (DFR), catalyze the reaction that leads to anthocyanin and proanthocyanidin production. It acts on dihydroflavonol to form corresponding leucoanthocyanidins, competing for the substrate dihydroflavonol, with FLS. The sixth enzyme Anthocyanine synthase (ANS), it catalyzes the foundation of anthocyanidin from leucoanthocyanidins. Finally, another two enzyme leucoanthocyanidin reductase (LAR) and Anthocynadin reductase (ANR) are accountable for leucoanthocyanidin reduction to catechin (trans-flavan-3-ol) in legumes and formation of proanthocyanidins (condensed tannins), respectively (Fig) (Petrussa, Braidot et al. 2013; Saito, Yonekura-Sakakibara et al. 2013).

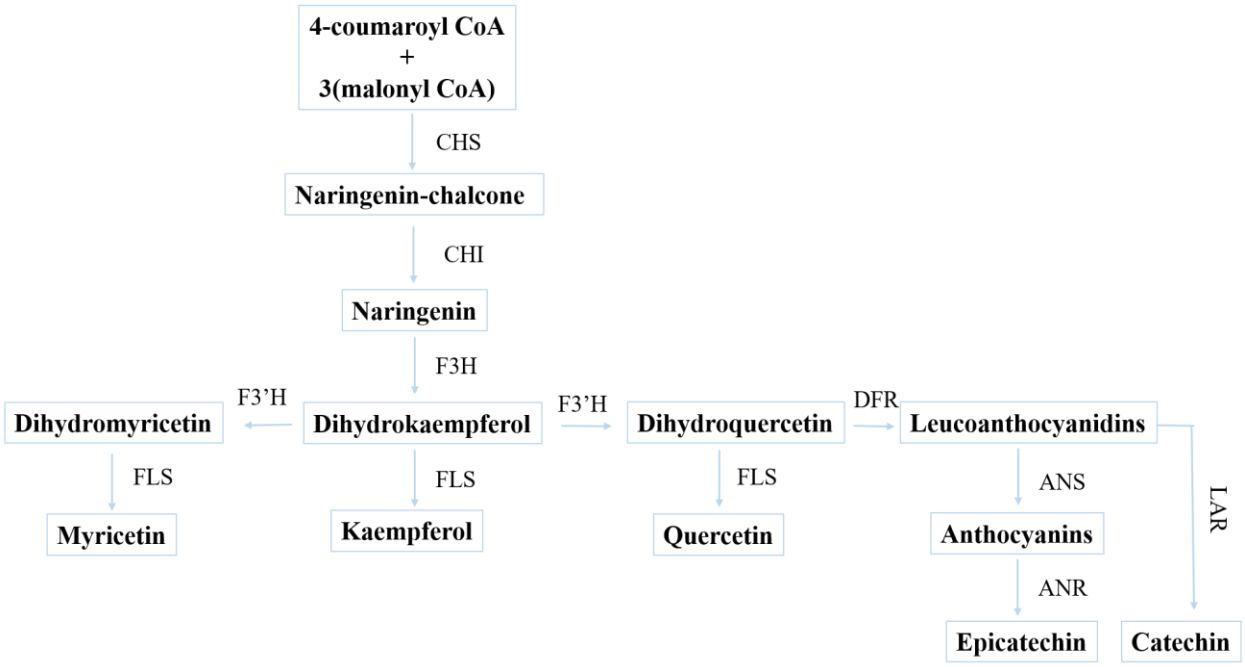

Figure 2: Diagrammatic representation of flavonoid biosynthesis pathway (adopted from (Pandey, Alok et al. 2016))

Enzymes involved in biosynthesis of flavonoids are confined in cytosol (Koes et al. 2005). Flavonoids are transported to vacuoles or cell wall after their biosynthesis. As far as transcriptional regulation of flavonoids is concerned, it place by the synchronized control of transcription of the genes by the interaction of MYC-like basic helix loop helix, DNA binding R2R- MYB transcription factors, and WD-40 proteins (Jaakola and Hohtola 2010).

There are many environmental aspects that can affect flavonoid biosynthesis. In plants, flavonoids are unaffected by oxygen heat, dryness or by mild acidity, nonetheless they can be altered by light (Francisco and Resurreccion 2008). Many studies are available on the effect of light on flavonoid biosynthesis which states positive results. No literature for now is available for its effect on groundnut plant.

1.3. Role of Light

Light is one of the most important environmental factor which regulates plants growth and formation of active constituents present in the plant. For the occurrence of each step-in plant growth and development stimulus of light is required which provides plant the required energy to do its job. Effects of light can be categorized in the form of intensity, quality, duration and direction quality (wavelength) including UV-light. The whole cycle of availability of light on daily and seasonal basis comes under the term "photoperiod". Photoperiod varies with the latitude it is not same all over the world. The biosynthesis of flavonoids can be controlled by similar photoperiodic conditions that also control growth and developmental processes. According to (Taylor 1965) the anthocyanins synthesis in flowers has also been controlled by photoperiodic conditions.

1.4. Motivation

With the great nutrient profile and important role in disease prevention groundnut is needed to be explored to reveal its more beneficial effects. In order to do so studies on groundnut and its components like secondary metabolites are recommended. The role of secondary metabolites and expression of genes involved in their production under different environmental condition which can be either a stress factor or a treatment for their improved production are not well studied in groundnuts. These studies are important because plants secondary metabolites are the most important factor involved in all basic steps of plant growth and development and are considered as major defense system against different stress conditions.

1.5. Objectives

Up to our knowledge no significant literature is present on the outcome of photoperiod on flavonoid biosynthesis in groundnut. Hence understanding the health benefits of groundnuts and their importance as a food crop, this study is designed to investigate the expression of flavonoids Biosynthesis gene(s) under light conditions in *Arachis hypogaea* in hope to get the positive results.

Objectives of the study include:

2. Selection of genetically divers groundnut varieties.
3. Expression analysis of selected genes under light conditions in different groundnut varieties.

Literature review

2.1. Peanut and Flavonoids

Groundnuts are the rich source of flavonoids and they are abundantly present in all parts of groundnut. Flavonoids are not only present in seeds but outer layers of peanut seeds like hull, shell and peel contain a vast variety of polyphenolic compounds that protects seed. Groundnut shell contains eriodictyol and luteolin which are predominant flavonoids and luteolin is a major antioxidant component present in groundnut hull (Daigle, Conkerton et al. 1988). Skin of groundnut contains flavanols and other health elevating compounds (Yu, Ahmedna et al. 2005). According to (Karchesy and Hemingway 1986), 17% by weight procyanidins are present in the mature red groundnut skins. (Lou, Yuan et al. 2004) also analyze the phenolic extracts that are water soluble, from groundnut skins finding eight different flavonoids and six A-type Proanthocyanidins.

2.2. Flavonoids

Flavonoids are secondary metabolites specifically present in plants and an important part of their success in acclimatizing to life as sedentary or inactive organisms living in varied and inconstant surroundings. Major role of flavonoids is related to the pigmentation/ coloration in plants but also play important roles as defense compounds and as signaling molecules in pathogenesis, reproduction, and symbiosis. Groundnuts are also considered as rich source of flavonoids. They are phytochemicals, located in the nucleus of mesophyll cells and within centers of ROS generation (Agati, Azzarello et al. 2012). Flavonoids assemble in plant vacuoles as glycosides (Bohm 1998). Flavonoids are the biggest group of plant phenolics, composed of over half of the 8,000 naturally occurring phenolic compounds.

2.2.1. Environmental factors affecting flavonoids biosynthesis

Flavonoid biosynthesis is affected under the influence of different environmental factors.

The most important environmental factor affecting flavonoid biosynthesis in plants is **light**. The utter dependency of plant growth and development on light has determined sophisticated mechanisms to detect and understand multiple characteristics of the light signal. Light effects can be categorized in photoperiod(duration), intensity(quantity), direction and quality(wavelength) including UV-light (Zoratti, Karppinen et al. 2014).

Another important environmental factor is temperature. **Temperature** alone or in relation with light affects the flavonoid biosynthesis in plants. Out of all flavonoids anthocyanins production is most affected by temperature. Low temperatures increase the induction of anthocyanins in many plant species (Chalker-Scott 1999) whereas high temperatures cause the decrease in the expression of anthocyanin synthase gene (Dela, Or et al. 2003). Too high temperature has negative impact on flavonoid biosynthesis while the low temperature can enhance the production of flavonoid.

Soil is the most important factors for the plants growth, mainly run by weathering and climatic conditions. **Soil nutrients** are directly linked with biosynthesis of secondary metabolites as well as their abundance or limited availability has effects on secondary metabolites (flavonoids) in plants. In continuation of effect of soil nutrients, soil water availability is also one of the important factor. Less water availability refers to the **drought** conditions which affect flavonoid biosynthesis. Soil salinity or salt stress is effect flavonoid biosynthesis.

In addition to abiotic factors, biotic factors are also one of the important factor, in which plants are exposed in environment of multiple herbivore and pathogenic attacks and affect flavonoid biosynthesis.

2.3. Photoperiod

As discussed earlier, Light has been concerned as an important environmental factor regulating gene expression and plant development. Plants are always dependent on the sun for their energy sources, and there can also be the different characteristics of light involved in their growing environment, including the direction of light, strength, wavelength and duration (cycle). Plants are basically dependent on sunlight for their source of energy and they can also sense the different features of light in their growth environment including light direction, intensity, wavelength and duration (photoperiod). Plants have developed mechanisms aiding them to detect seasonal transformations through response to changes in duration of light. The photoperiod is the total amount of light and darkness in a24h daily cycle. At equator, the photoperiod is of 12 h dark and 12 h light but with the tilt of the earth's axis towards the sun, the lengths of the light and dark and periods change, to develop unequal partitions of the 24-h cycle. There are two main photoperiod types: long day, in which light period is more than the critical day length; short-day in which dark period is more than a critical day length.

Plants have a complex range of photoreceptors to manage their response with specific light conditions. As light signals are detected by plants, photoreceptors trigger numerous signal transduction cascades to control light dependent gene expression and responses in plants.

2.3.1. Effect of light on Flavonoid Biosynthesis

Numerous studies have demonstrated the importance of the accumulation of flavonoids and biosynthesis in different light conditions. Biosynthesis of biologically active compounds such as flavonoids can be adjusted by same photoperiod conditions that are involved in development processes. Studies on several plants have revealed that generally longer light durations increases the contents of phenolic compounds (Taylor 1965). Carvalho and co-workers spotted an increased expression in transcripts of flavonoid biosynthetic genes and accumulation of flavonols and catechins in sweet potato leaves growing under 16 h light conditions than short 8 h light conditions. (Carvalho, Cavaco et al. 2010). In another study on red and purple potatoes also showed that the anthocyanin content of the treated potato was more in the longer day of 14-15 hours compared to that of the short days, 12-14 hours of light but the total phenolic content was not affected by the treatment(Reyes, Miller et al. 2004).

Non-climacteric grape vine fruits are most extensively studied for their flavonoids gene expression under light conditions. Grape acclimatize to high light conditions by inducing expression of flavonoids especially of anthocyanins, PAs and flavonols(Koyama, Ikeda et al. 2012). Flavonol levels in grape berries are most affected by different light conditions and appear to be sensitive to variations in light conditions. Enhanced expression of flavonol synthase (FLS) gene and high accumulation of flavonlos has been reported by longer light durations in different cultivars like merlot, Shiraz, Cabernet Sauvignon and Pione (Zoratti, Karppinen et al. 2014)

In other fruits like apples, bananas and strawberries flavonoids biosynthesis is affected by light conditions. In apple skin, most significant environmental factor that activates flavonoid biosynthesis is sunlight, specifically anthocyanin and flavonol biosynthesis. Flavonols and anthocyanin levels are high in those fruits whose peels are exposed to sun than those fruits

grown in shaded areas (Feng, Li et al. 2013). Banana plant are also studied for the expression of flavonoid biosynthesis genes under dark exposures. The qRT-PCR expression analysis of gene transcripts suggests that dark exposure causes significant decrease in the expression of genes that are involved in flavonoid biosynthesis(Pandey, Alok et al. 2016). In Rosaceae family like in strawberries flavonoid accumulation is much dependent on light conditions. Other genes involved in the flavonoid pathway are also controlled by light conditions as stated in the study by (Azuma, Yakushiji et al. 2012), light treatment significantly induced the higher expression levels of CHI, CHS, F3'5'H F3H AND UFGT.

From the above cited literature, all genes of flavonoid pathway are up regulated but FLS (flavonols synthase) gene has light sensitive nature.

2.4. Flavonol synthase (FLS) Gene

Flavonoids are famous natural plant products who have vast range of physiological functions in plants, though they also contribute in plant foods for their health promoting properties. flavonols are the Well-known subclass of flavonoids and perform many role in plants such as regulation of auxin transport, UV protection flower pigmentation, and signaling (Böhm, Boeing et al. 1998; Harborne and Williams 2000)

Flavonols are basically produced by dihydroflavonols desaturation, reaction is catalyzed by flavonol sunthase (FLS) enzyme. Flavonol Synthase belongs to 2-oxoglutarate iron – dependent oxygenese family(Xu, Li et al. 2012). The first activity of FLS was reported in treated parsley cells which were then categorized in vitro as soluble 2-oxoglutarate-dependent dioxygenase (2-ODD) (Britsch, Heller et al. 1981). Large number of FLS cdna have been cloned from different plant species such as Arabidopsis thaliana, solanum tuberosum, petunia hybrid,

strawberry and soya bean (Holton, Brugliera et al. 1993; Eldik, Reijnen et al. 1997; Pelletier, Murrell et al. 1997; Almeida, D'Amico et al. 2007; Takahashi, Githiri et al. 2007).

Many properties are associated with flavonols such as antiproliferative, antioxidant, neuropharmalogical and antiangiogenic(Lee, Jung et al. 2005; Kim, Liu et al. 2006). As we know that flavonols also protect plants from UV (B) radiations, such as in soybeans cultivars with UV-B resistant properties are comprise of more flavonols than the susceptible cultivars(Reed, Teramura et al. 1992).

Main FLS by products includes quercetin, kaempferol, and myricetin, which plays many biologically important activities in plants(Li, Kim et al. 2013). In grapevine different derivatives of flavonols such as quercetin, kaempferol, mayricetin and isohaemnetin are produced(Price, Breen et al. 1995). Other flavonols such as rutin is an efficient nitric oxide scavenger, this property amend the nitric-oxide induced damage in plant tissue(Vanacker, Tromp et al. 1995).

Material and Methods

3.1. Seed Collection

Seeds of 10 Groundnut (*Arachis hypogaea)* varieties namely PG 1198, PG 1247, PG 1051, PG 1026, PG 1252, PG 1090, PG 1248, PG 1163, PG 1162, pothwar and PG1195 were collected from Oil Seed Department, NARC, Islamabad.

3.2. Plant Material

Before sowing, all seed were placed at 4^0C for 3-5 days to break their dormancy. Groundnut seeds were sown in pots filled with peat moss in growth room under controlled conditions. The optimum conditions for groundnut growth is 16/8h light and dark conditions and 25-30^0C temperature. The Groundnut seed takes 7-10 days for germination.

3.2.1. Light Treatment

To study the photoperiod effect 8h, 12h, and 24h photoperiod was given to plants for 7 and 15 days under controlled temperature conditions of 26 $\pm$ 2^0 C. Right after germinations of seedlings pots were shifted to growth chambers with specific light conditions. For each treatment and time point 10 plants were grown. Whole experiment was replicated three times.

3.2.2. Sample Collection:

Tissue sample was collected in RNAase and DNAase free autoclave Eppendorf's. 7 days and 15 days' leaf tissue sample was collected from treated plants. 4-6 leaves were collected in each tube. During harvesting samples were snap freeze in liquid nitrogen and then moved to -80^0 c.

3.3. DNA and RNA isolation

3.3.1. Genomic DNA Extraction

DNA extraction was performed for DNA barcode analysis. Isolation of DNA was performed on finely grounded powder of leaf samples by using CTAB extraction protocol. 20 mg of plant sample was grinded using liquid nitrogen to powder form. After that 500 micro-liters of CTAB was added to the grounded samples.10 micro-liters of RNASE (10mg/ml) was added and mix by inverting. Samples were incubated at 65^0c water bath for 15 min. centrifugation was done at 14000 rcf for 10 minutes and supernatant was transferred to new tubes. 400 micro-liters CIA (chloroform-isoamylalcohol, 24:1 Raito) was added to all samples. Again, centrifuged for 5min at 14,000 rcf and transfer the supernatant to clean tubes. Then 500 micro-litres of cold isopropanol was added and tubes were placed in freezer for 1 hour or overnight. After incubation samples were centrifuged for two minutes at 14,000 rcf, Supernatant was discarded and pallet was suspended in 500 micro-liters 70% ethanol incubated in freezer for 15 min. then centrifuged for 2 minutes at 14,000 rcf, and supernatant was removed. 500 micro-liters 90% ethanol was added and placed in freezer for 15 minutes. Again, centrifuged for 2 minutes at 14,000 rcf. Supernatant was removed and pallets were air dried. Pallets were suspended in 100 micro-liters of TE buffer and stored at -20^0C.

3.3.2. RNA Extraction:

RNA extraction was performed to analyze the gene expression analysis. Isolation of RNA was performed on finely grounded powder of leaf samples by using Trizole extraction protocol. First, tissue samples were grinded into fine powder using liquid nitrogen. Immediately after grinding samples were shifted into eppendrofs and 1ML Trizole was added. Incubated on ice for 5minutes. Then centrifuge was set at 4^0C and samples were centrifuged at 13000rpm for 10 minutes. Supernatant was then transferred to new tubes. 270micro-liter chloroform was added to

the tubes and incubated on ice foe 3 minutes. Again, samples were centrifuged for 15 minutes at 13000rpm for phase separation. Upper phase was transferred to new tubes and 650 micro-litre isoprponol was added. Samples were incubated on ice for 10 min and samples were again centrifuged for 10 mins at 13000 rpm. Supernatant was discarded and pallets were first washed with 1ml 75% ethanol by centrifugation at 9500rpm for 10 minutes at 4^0C. Again, supernatant was discarded and pallets were then air dried and suspended in 50 micro-liter TE buffer. To dissolve the pallets, incubation was done for 55^0C for 5 minutes. And then RNA was stored at -80^0C

3.3.3. Nano drop Quantification

For accurate determination of concentration and purity of extracted samples nanodrop analysis was done using Thermo fisher scientific NanoDrop Spectrophotometer. 1 micro-liter RNA/DNA sample was used for this purpose. Concentrations of samples were measured in ng/µl. The raito of 260/280 wavelengths was used to assess the purity of RNA. Raito of 1.8 to 2.0 indicates pure RNA/DNA.

3.3.4. First Strand cDNA synthesis

First strand cDNA synthesis was done using two master mixes by using Invitrogen first strand cDNA synthesis kits.

Master Mix 1:

Master Mix 1 was prepared in PCR tubes using 10 µl PCR water, 1µl 5mM dNTPs and 1µl oligo dT primer. 1 µl RNA was added in Master Mix 1 and incubated on 65^0 C for 5 minutes and then incubated at ice, for 1 minute.

Master Mix 2:

Master Mix 2 was prepared by using 1µl 5X RT buffer, 1µl of 0.1 M DTT and 1µl Rnase out. Then Mater mix 2 was added in initial tubes and 1µl RT enzyme was added. PCr tubes were then given a short spin to mix all the reaction components. Tubes were then incubated at 50^0C for 30 minutes and at 70^0C for 15 minutes.

3.4. PCR based techniques

3.4.1. Basic PCR

Basic PCR was performed to amplify rbcl and matk gene regions in extracted DNA samples. PCR was performed ina total voloume of 25 µl in a thermos cycler. PCR cycles consisted of an initial denaturation for 2 min at 95°C, 35 cycles of denaturation (1 min 30 s at 94°C), annealing (2 min at 50^0C) and extension (3 min at 72°C), and a final extension at 72°C for 10 min.

3.4.2. Reverse Transcriptase PCR

Reverse transcriptase PCR (RT-PCR) amplification of β-actin gene was performed to confirm the integrity of constructed cdna library in a total volume of 25 µL in a Thermo cycler. PCR reagents from NEB were used. PCR cycles consisted of an initial denaturation for 2 min at 95°C, 35 cycles of denaturation (1 min 30 s at 94°C), annealing (2 min at 50^0C) and extension (3 min at 72°C), and a final extension at 72°C for 10. Primers used for RT-PCR are mentioned in Appendix I.

3.4.3. Real time PCR (qRT-PCR)

qRT-PCR was done to determine the expression of genes. For qRT-PCR invitrogen's Syber green kit was used. Recipe used for real time PCR includes 12.5 µl syber green, 0.5µl rox dye, 0.5-1µl primersF/R, 9.5µl PCR water and 2µl cdna. PCR conditions consisted of initial denaturation for 10 min at 95 ^{0}C, 40 cycles of denaturation (15 s at 95°C), annealing (1 min at 56^0C)

and extension (45s at 72°C) and a final dissociation stage was added in the end. Primers used for qRT-PCR are mentioned in Appendix I.

3.4.3.1 Fold Change and statistical Analysis

Fold change values were calculated using comparative ct method or $2^{-\Delta\Delta CT}$ method. This method is used to determine the relative difference between control and treated sample. For statistical analysis student t test was applied. Statistical analysis is performed to determine the significance of the results. If the value of t test is less than 0.05 then result is considered significant. T test was applied using "prism 7" software.

3.4.4. Gel Electrophoresis:

PCR based gene amplification was checked on 1% agarose gel. 1% agarose gel was prepared by adding 1gm agrose, to 50ml 1XTAE buffer. To boil the solution, placed it in a microwave oven for 1 minute. The boiling gel was cooled down to room temperature. 5µl Ethidium Bromide was used to facilitate visualization of PCR bands after electrophoresis. Afterward the solution was poured directly into the casting tray (sealed already), ensuring that no bubbles get into the gel. The gel was allowed to cool, when it solidifies and slightly become opaque within 20-30 minutes, removed the seal. Carefully removed the combs. Flooded the gel by addition of approximately 1L of 1X TAE running buffer. 2µl of loading dye along with 2 µl of PCR product was loaded and then ladder was loaded in the first well of gel.

3.5. Sanger Sequencing

All the PCR products of 10 varieties against 3 genes were then purified for sequencing. An enzyme EXOSAP-IT was used for purification. For every 5 micro-liters of PCR sample 2 micro-liters of EXOSAP-IT is added. Then the samples were first incubated at 800C for 15

minutes and then at 150C for 15 minutes. The purpose of using this enzyme is to remove any remaining nucleotides in samples. For sequence analysis, purified PCR samples were then sent to DNA Macrogen, Korea. PCR products were directly sequenced in 2 directions of each fragment

Results

4.1. DNA Barcode Analysis

DNA barcoding is a technique in which short DNA sequences of the standard segment of the genome are used for large scale species identification. In most plants rbcL and matK gene are used for barcoding and when these two regions remain unobserved then ITS and ETS regions are used for barcoding. Peanut barcode is not present until now therefore we did the Peanut DNA barcode analysis to identify the genetically diverse peanut varieties. For this purpose, we selected two genes "matk" and "rbcl".

4.1.1. Agarose Gel Quantification of PCR Products

PCR was performed using primers obtained from literature for rbcl and matk genes. Band with expected size of 1500 for rbcl and 1570 bp for matk was amplified and analyzed on 1%agarose gel. The band size was confirmed by comparing it size with 1 kb DNA ladder as shown in fig 7. The results showed the existence of particular genes in all 10 *Arachis hypogaea* varieties. (supplementary information).

4.1.2. Bioinformatics Analysis

Sequencing results of the PCR products were analyzed with "Geneious" software.

4.1.2.1. Multiple Alignment

The sequences of all 10 varieties for matk and rbcl gene were aligned by multiple alignment tool. Alignment results showed that the sequences of all 10 varieties for both genes are highly conserved. Figure 4, 5, 6 and 7 shows the multiple sequence alignment of 10 *Arachis hypogea* varieties. Different colors in the chromatogram shows the nucleotide bases (A, T, G and C). The

identity graph is shown at the top of each alignment and represents the conserved regions. The representative sequences are mentioned on the left. They cannot be used for barcoding the *Arachis hypogea* (Peanuts) varieties. It is because the breeding program for developing these varieties have not changed or mutated the gene region.

Figure 3: Multiple sequence alignment of rbcl sequences of cpDNA of 10 peanut varieties

Figure 4: Multiple sequence alignment of matK sequences of cpDNA of 10 Peanut varieties

4.2. FLS Gene Expression Analysis

Seeds of two varieties were selected on phenotypic bases such as seed color for the expression analysis of FLS gene under light conditions of 8 Hours, 12 Hours and 24 Hours.

Figure 5: Plants grown under different light conditions. (a) plants at germination (b) 8Hour light condition plants (c) 12Hour light condition plants.

Figure 6: (a) 24 Hour light condition plants (b) control plants.

4.2.1. Agarose Gel quantification.

Extracted RNA from control and treated plants was run on 1% agarose gel to confirm the integrity of RNA (fig). And to confirm cdna synthesis reverse transcriptase PCR of β-Actin gene was performed on all control and treated samples. PCR products were run on 2.5% agarose gel and expected band size of 178 bp was confirmed with the 100 bp ladder.

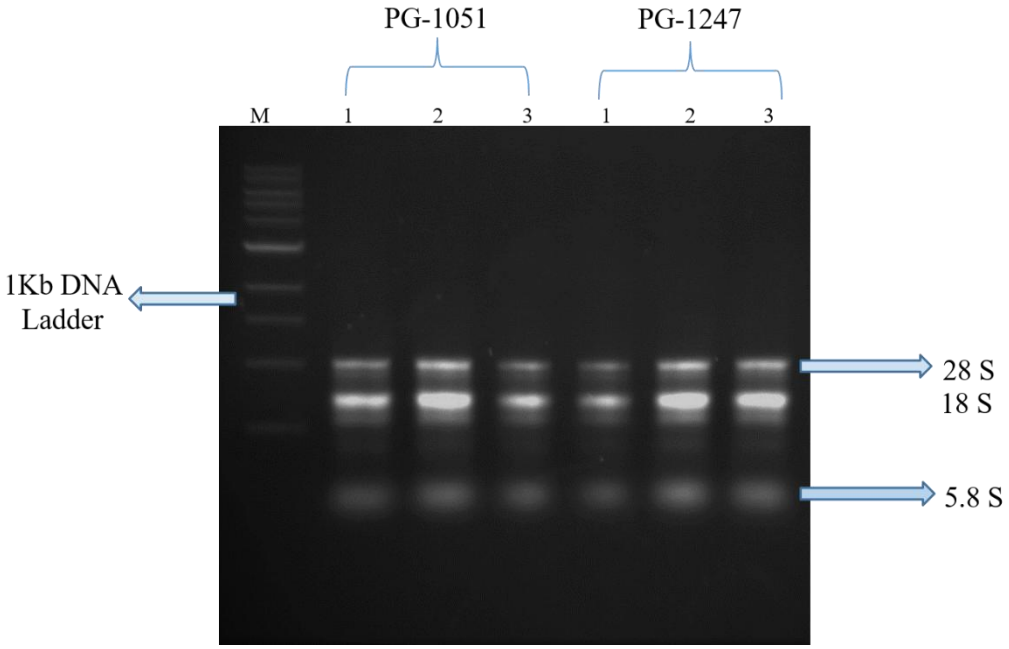

Figure 7: Representative Gel of total RNA extracted from both varieties on 1% agarose gel

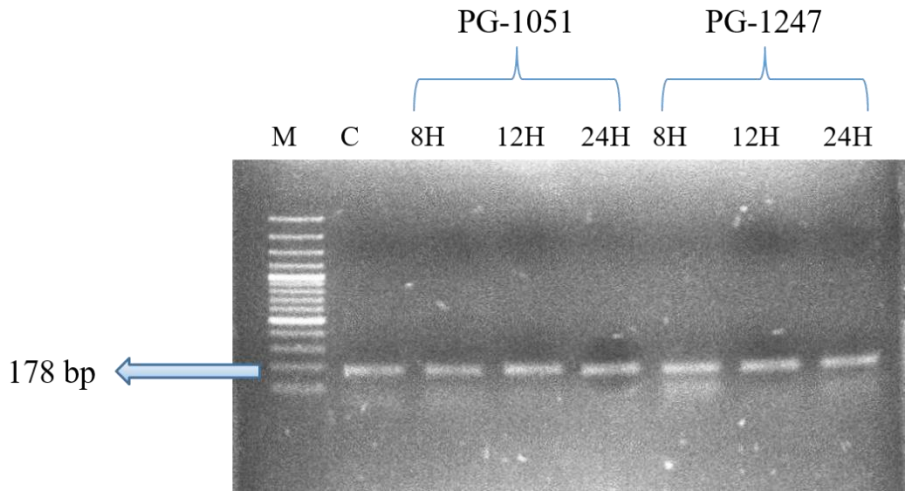

Figure 8: Representative Gel of RT on actin to confirm cDNA synthesis on both varieties on 2.5% agarose gel

4.2.2. qRT-PCR Analysis

Quantitative real-time PCR is the most specific and sensitive method for quantifying gene expression at mRNA level. SYBR Green is used as a detection system. SYBR Green binds to DNA during the product amplification in qRT-PCR.

4.2.2.1. Melt Curve Analysis

The melt curve analysis is significant to show the integrity of the qRT-PCR reaction. In a representative melt curve result, single peak is present at same dissociation temperature for each sample. If any extra peak is present it means that nonspecific amplification or primer dimers are formed in the reaction (supplementary information)

4.2.2.2. Amplification Plot Analysis

Amplification Plot helps in the determination of ct value for each sample. C_t (threshold cycle) is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction. If the concentration is higher it will be detected early than low concentration which will be detected in later cycles. Low ct value shows higher expression of gene in particular sample. (supplementary information)

4.2.3. Expression of FLS gene in variety PG-1051

Expression of FLS gene was analyzed in control and light treated samples of 7 days and 15 days' plants of variety PG-1051. qRT-PCR analysis shows that expression of FLS gene is increasing with increase in light exposure with highest expression in 24 hours 15 days' samples. There is 2.5-fold increase at 7 days in 24-hour plants and 3.8-fold increase at 15 days 24-hour plants. In 8-hour and 12-hour plants expression is less than control because control plants are grown at 16-hour light conditions. As the light exposure decreases the expression of FLS gene also decreases.

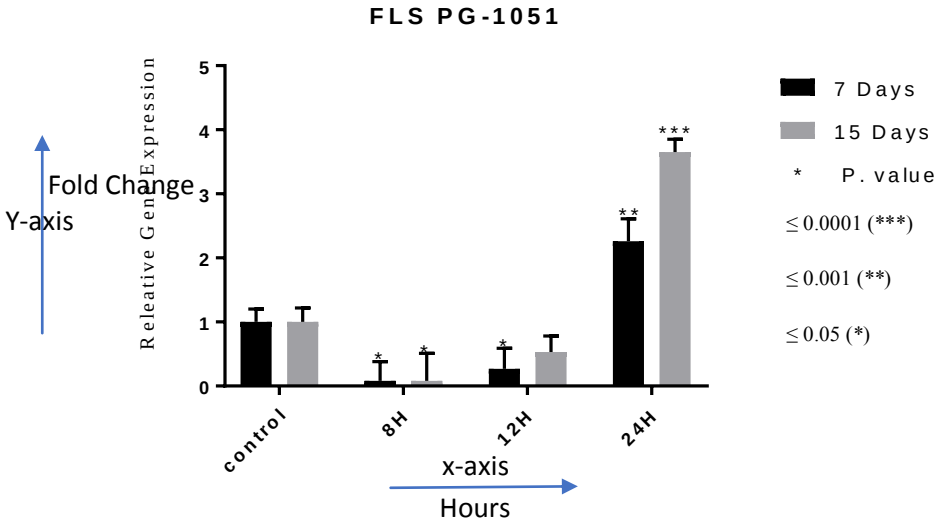

Figure 9:Expression pattern of FLS gene under different light conditions in peanut variety PG-1051

4.2.4. Expression of FLS gene in variety 1247

Expression of FLS gene was analyzed in control and light treated samples of 7 days and 15 days in variety PG-1247. qRT-PCR analysis showed that expression of FLS gene is increasing with light exposure with highest expression in 24-hours 7 days' samples. There is 8.2-fold increase at 7 days 24-hour plants and 2.5-fold increase at 15 days 24-hour plants compared to the control samples. In 8-hour and 12-hour plants expression is less than control because control plants are grown at 16-hour light conditions. As the light exposure decreases the expression of FLS gene also decrease. In this variety, alternate behavior is expressed by FLS gene with high induction at 24 hour 7 days and then expression starts decreasing with a drop of approximately 5.7-fold. From this result, we can speculate that FLS gene expression is variety dependent.

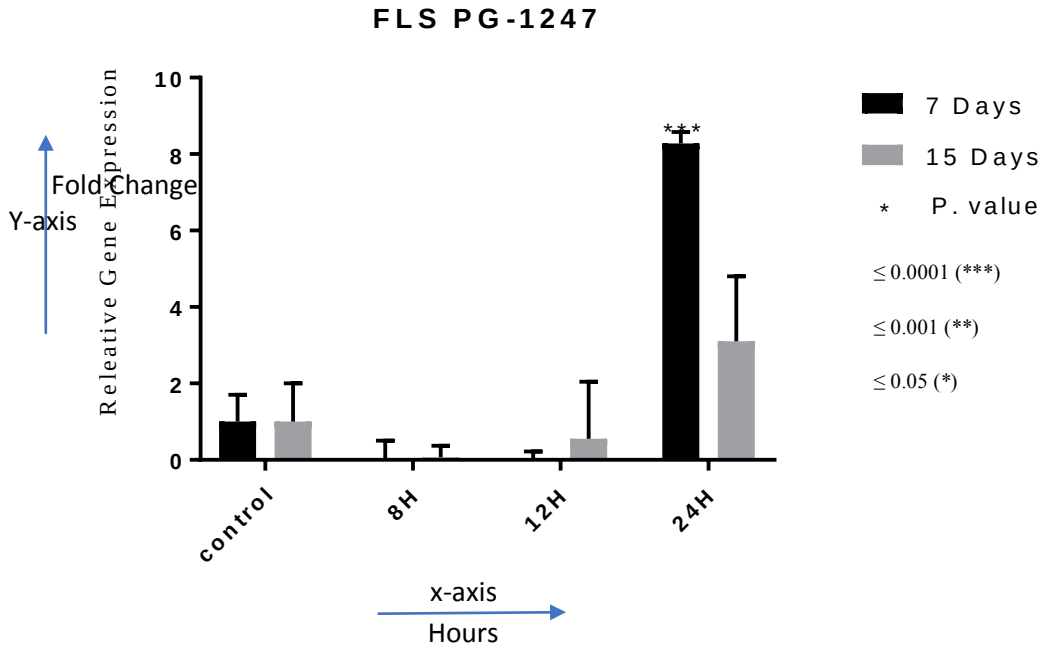

Figure 10: Expression pattern of FLS gene under different light conditions in peanut variety PG-1247.

Discussion

The flavonoid biosynthesis has been considered as a significant target for manipulation at genetic level in plants due to their role in, providing insect resistance, pigmentation in flowers as well as their health promoting properties(Butelli, Titta et al. 2008; Luo, Butelli et al. 2008). Flavonoids are tissue-specific compounds and their accumulation in tissue specific manner is vastly studied for gene regulation in plants as their genes are responsive to many stress conditions. (Dixon and Paiva 1995), and during development (Taylor and Grotewold 2005). The biosynthesis of the various group of flavonoids is strongly regulated by several spatial and sequential signals that can bound the accumulation of these compounds in plants. A complete knowledge about the enzymes and genes that are involved in flavonoid biosynthesis of this crop would be crucial in the selection of gene(s) to enhance flavonoids in this crop through biotechnological approach.

Light being the major aspect important for synthesis of flavonoids. The light signals are received by several receptors, that control acclimation and avoidance responses to limited or excess light (Chen, Chory et al. 2004; Neugart, Krumbein et al. 2016)thus creating a complex network of light response (Casal 2013). Majorly in fruits, Flavonoid composition is strongly under influence of neighboring light conditions. In general, higher the solar radiation higher will be the flavonoid content in fruits. The composition of flavonoids in fruit tissues can also be altered by specific wavelengths of light.(Zoratti, Karppinen et al. 2014). Light intensity variations also may affect flavonoid content because the A and B rings containing flavonoid hydroxyl groups vary in position and number. In various studies it is shown that high light promotes the biosynthesis of flavonoids, such as dihydroxy B-ring-substituted flavonoids

(luteolin 7-O- and quercetin 3-O-glycosides)(Pan, Chen et al. 2017). Flavonol glycosides gather in the epidermal cell's vacuoles in a light-dependent manner, FLS gene activity is expected to be light-sensitive (Wisman, Hartmann et al. 1998).

Considering the health promoting properties of flavonoids and the role of light in their synthesis, in the present study, we have studied the expression of FLS gene under different light conditions.

In this study, light exposure resulted in the increased expression of FLS gene with maximum induction at 24 hours in 15 days' plants in PG-1051 variety and in PG-1247 variety maximum induction was in 7 day old plants grown under continuous light. As the dark exposure increases it resulted in the decreased expression of FLS gene in peanut plants. This clearly suggests that light controlled factors can also be involved in transcriptional regulation of flavonoid biosynthesis in peanut. It is confirmed from previous studies that the trend followed in this study is similar to the reports available. In some Previous studies it is demonstrated that light treatment influence gene expression, as in grape and kale, that could lead to the accumulation of specific flavonol glycosides (Neugart, Krumbein et al. 2016).Further studies in grape berries also confirmed that flavonol levels are sensitive to changes in light conditions; flavonols accumulate with increased expression of FLS(Matus, Loyola et al. 2009). FLS expression under light exposure is also cultivar dependent like in Shiraz (Downey, Harvey et al. 2004) and Merlot (Fujita, Goto-Yamamoto et al. 2006).

At low light conditions plants need to absorb adequate amount of light for photosynthesis, survival, and growth, while at excess light conditions plants have to prevent photo-oxidative damages(Neugart, Krumbein et al. 2016). The phenotypic observation was made

during this study tells that when plants are grown under continuous light for 15 days their growth was stunted approximately half to that of control plants. This can be due to the photoprotection effect of flavonoids preset in peanut.

The effect of different lighting conditions on the synthesis of flavonoids is mainly mediated by the R2R3-MYB transcription factor (Zoratti, Karppinen et al. 2014). In recent studies, R2R3 MYB transcription factors been recognized that are involved in flavonoid biosynthesis and categorized in several fruit producing species and some of them have been found to respond to light(Liu, Long et al. 2016). R2R3-MYB transcription factor for the flavonoid biosynthesis coordinatively regulate the inhibition or activation of structural gene to formulate their expression (Czemmel, Heppel et al. 2012).

Conclusion

Light is one of the most important modulator of flavonoid Biosynthesis in plants. In present study, we have analyzed the expression of FLS gene under different light conditions. Increase in light exposure leads to the enhanced expression of FLS gene. Maximum expression of FLS gene is induced at 24H photoperiod in both varieties. But, alternate behavior by FLS is displayed in both varieties which makes the expression of FLS variety dependent. These observations suggest that light- regulated factors are involved in transcriptional regulation of FLS in peanut.

Future Prospect

Arachis hypogaea is the very important cash crop of Pakistan. Studying the expression of gene involved in flavonoid biosynthesis in response to different biotic and abiotic factors can be a step forward towards the improvement of this crop. Light is the main environmental factor that regulates flavonoid biosynthesis. In relation to this study, more enhanced research can be done by analyzing all other genes involved in flavonoid biosynthesis under light conditions. Furthermore, study of role of transcription factors involved in flavonoid biosynthesis under light conditions is also very important.

Supplementary Information

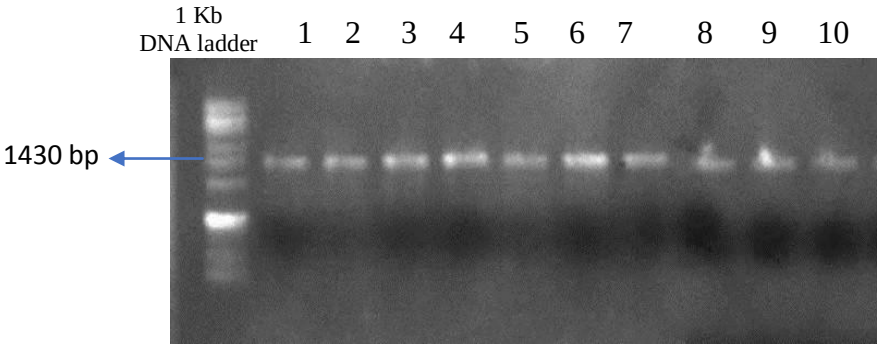

Figure: Amplification of 10 Peanut varieties employing rbcl gene specific primers on 1 % agarose gel.

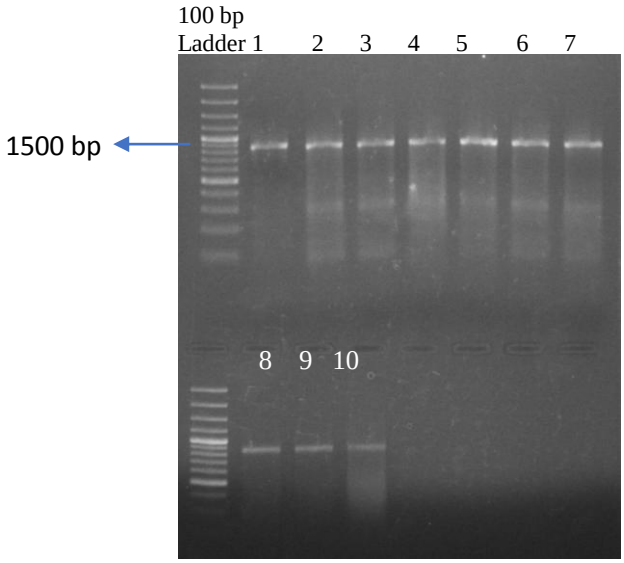

Figure: Amplification of 10 Peanut varieties employing matk gene specific primers on 1 % agarose gel.

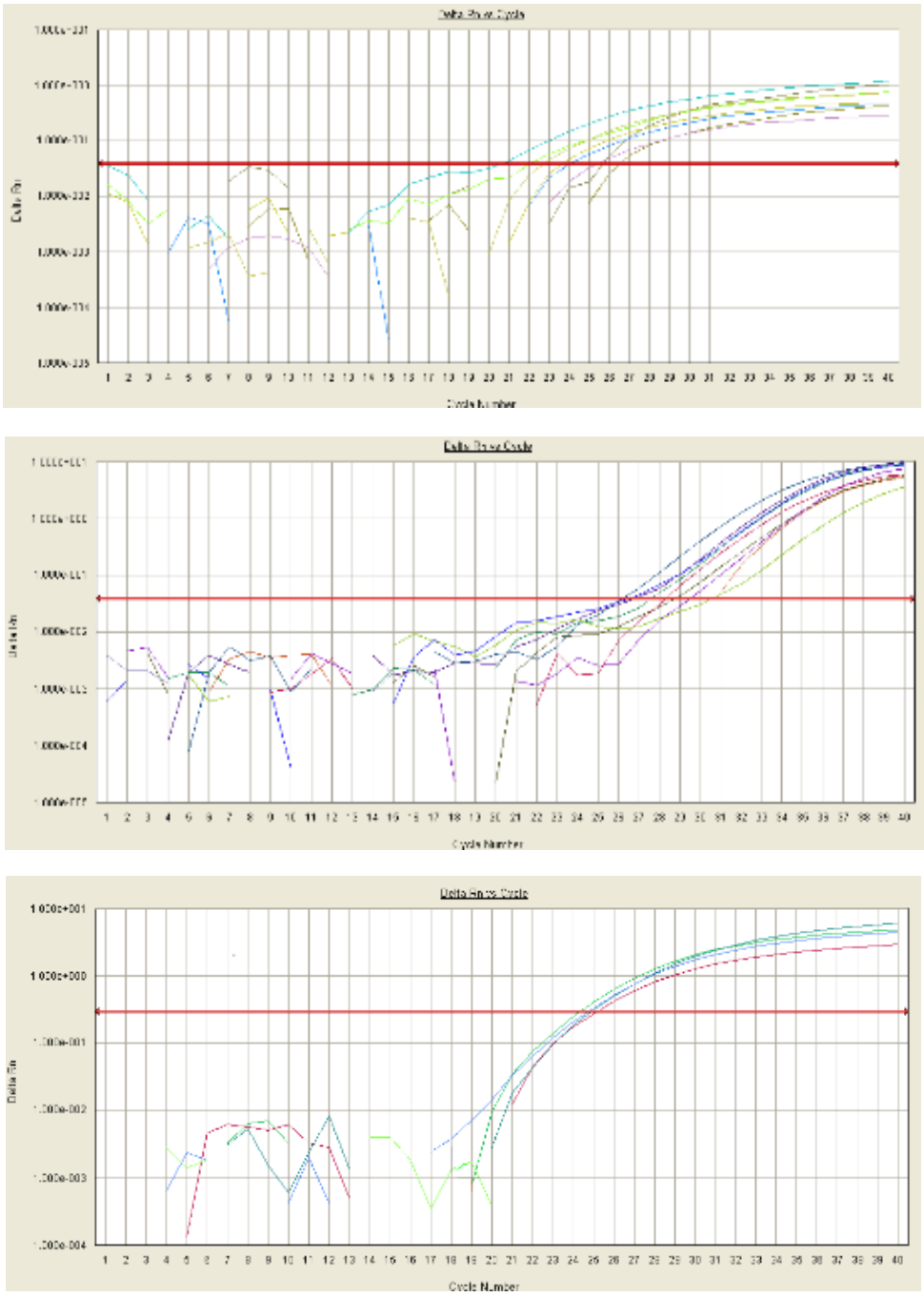

Figure: Representative Amplification Plots of FLS gene in control and treated samples and of β-Actin gene.

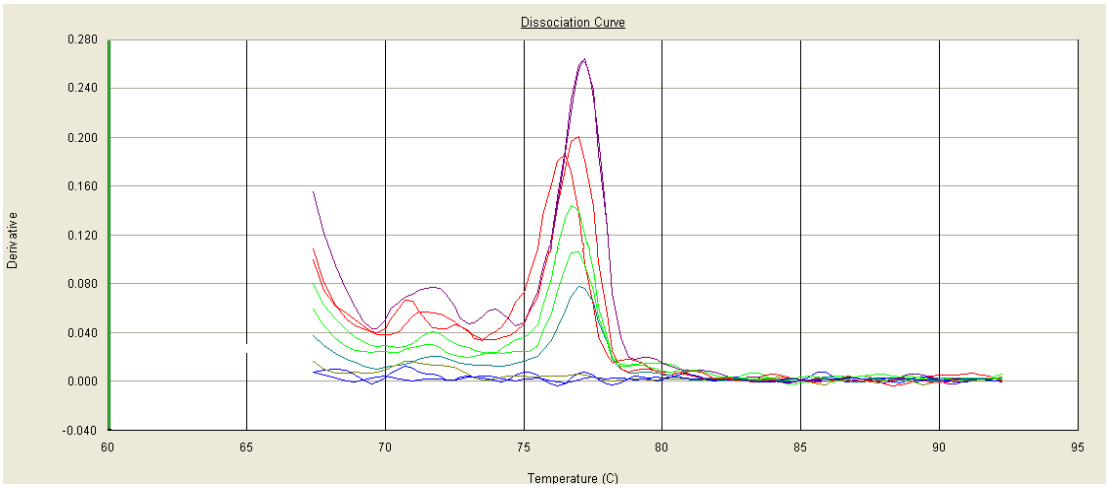

Figure: Representative melt curve of control and treated samples.

Reference

1. Agati, G., E. Azzarello, et al. (2012). "Flavonoids as antioxidants in plants: location and functional significance." Plant Science **196**: 67-76.
2. Almeida, J. R., E. D'Amico, et al. (2007). "Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (Fragaria× ananassa)." Archives of Biochemistry and Biophysics **465**(1): 61-71.
3. Azuma, A., H. Yakushiji, et al. (2012). "Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions." Planta **236**(4): 1067-1080.
4. Bhatti, I. and A. H. Soomro (1994). Agricultural inputs and field crop production in Sindh, Directorate of Agriculture Research Sindh.
5. Bohm, B. A. (1998). Introduction to flavonoids, Harwood academic publishers.
6. Böhm, H., H. Boeing, et al. (1998). "Flavonols, flavone and anthocyanins as natural antioxidants of food and their possible role in the prevention of chronic diseases." Zeitschrift fur Ernahrungswissenschaft **37**(2): 147-163.
7. Britsch, L., W. Heller, et al. (1981). "Conversion of flavanone to flavone, dihydroflavonol and flavonol with an enzyme system from cell cultures of parsley." Zeitschrift für Naturforschung C **36**(9-10): 742-750.
8. Butelli, E., L. Titta, et al. (2008). "Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors." Nature biotechnology **26**(11): 1301-1308.

9. Carvalho, I. S., T. Cavaco, et al. (2010). "Effect of photoperiod on flavonoid pathway activity in sweet potato (Ipomoea batatas (L.) Lam.) leaves." Food chemistry **118**(2): 384-390.
10. Casal, J. J. (2013). "Photoreceptor signaling networks in plant responses to shade." Annual review of plant biology **64**: 403-427.
11. Chalker-Scott, L. (1999). "Environmental significance of anthocyanins in plant stress responses." Photochemistry and photobiology **70**(1): 1-9.
12. Chen, M., J. Chory, et al. (2004). "Light signal transduction in higher plants." Annu. Rev. Genet. **38**: 87-117.
13. Czemmel, S., S. C. Heppel, et al. (2012). "R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine." Protoplasma **249**(2): 109-118.
14. Daigle, D., E. Conkerton, et al. (1988). "Peanut hull flavonoids: their relationship with peanut maturity." Journal of Agricultural and Food Chemistry **36**(6): 1179-1181.
15. Dela, G., E. Or, et al. (2003). "Changes in anthocyanin concentration and composition in 'Jaguar'rose flowers due to transient high-temperature conditions." Plant Science **164**(3): 333-340.
16. Dixon, R. A. and N. L. Paiva (1995). "Stress-induced phenylpropanoid metabolism." The plant cell **7**(7): 1085.
17. Downey, M. O., J. S. Harvey, et al. (2004). "The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes." Australian Journal of Grape and Wine Research **10**(1): 55-73.

18. Eldik, G. J., W. H. Reijnen, et al. (1997). "Regulation of flavonol biosynthesis during anther and pistil development, and during pollen tube growth in Solanum tuberosum." The Plant Journal **11**(1): 105-113.
19. Feng, F., M. Li, et al. (2013). "Phenylpropanoid metabolites and expression of key genes involved in anthocyanin biosynthesis in the shaded peel of apple fruit in response to sun exposure." Plant physiology and biochemistry **69**: 54-61.
20. Francisco, M. L. D. and A. Resurreccion (2008). "Functional components in peanuts." Critical reviews in food science and nutrition **48**(8): 715-746.
21. Fujita, A., N. Goto-Yamamoto, et al. (2006). "Organ-specific transcription of putative flavonol synthase genes of grapevine and effects of plant hormones and shading on flavonol biosynthesis in grape berry skins." Bioscience, biotechnology, and biochemistry **70**(3): 632-638.
22. Harborne, J. B. and C. A. Williams (2000). "Advances in flavonoid research since 1992." Phytochemistry **55**(6): 481-504.
23. Higgs, J. (2003). "The beneficial role of peanuts in the diet–Part 2." Nutrition & Food Science **33**(2): 56-64.
24. Holton, T. A., F. Brugliera, et al. (1993). "Cloning and expression of flavonol synthase from Petunia hybrida." The Plant Journal **4**(6): 1003-1010.
25. Isanga, J. and G.-N. Zhang (2007). "Biologically active components and nutraceuticals in peanuts and related products." Food Reviews International **23**(2): 123-140.
26. Jaakola, L. and A. Hohtola (2010). "Effect of latitude on flavonoid biosynthesis in plants." Plant, cell & environment **33**(8): 1239-1247.

27. Karchesy, J. J. and R. W. Hemingway (1986). "Condensed tannins:(4. beta.. fwdarw. 8; 2. beta.. fwdarw. O. fwdarw. 7)-linked procyanidins in Arachis hypogea L." Journal of Agricultural and Food Chemistry **34**(6): 966-970.
28. Kim, J.-D., L. Liu, et al. (2006). "Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion." The Journal of nutritional biochemistry **17**(3): 165-176.
29. Koyama, K., H. Ikeda, et al. (2012). "Light quality affects flavonoid biosynthesis in young berries of Cabernet Sauvignon grape." Phytochemistry **78**: 54-64.
30. Kris-Etherton, P. M., K. D. Hecker, et al. (2002). "Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer." The American journal of medicine **113**(9): 71-88.
31. Leal-Bertioli, S. C., D. J. Bertioli, et al. (2012). "The effect of tetraploidization of wild Arachis on leaf morphology and other drought-related traits." Environmental and Experimental Botany **84**: 17-24.
32. Lee, B.-H., S.-M. Jung, et al. (2005). "Quercetin Inhibits the 5-Hydroxytryptamine Type 3 Receptormediated Ion Current by Interacting with Pre-Transmembrane Domain I." Molecules & Cells (Springer Science & Business Media BV) **20**(1).
33. Li, X., Y. B. Kim, et al. (2013). "Differential stress-response expression of two flavonol synthase genes and accumulation of flavonols in tartary buckwheat." Journal of plant physiology **170**(18): 1630-1636.
34. Liu, C., J. Long, et al. (2016). "Characterization of a citrus R2R3-MYB transcription factor that regulates the flavonol and hydroxycinnamic acid biosynthesis." Scientific reports **6**: 25352.

35. Lou, H., H. Yuan, et al. (2004). "Polyphenols from peanut skins and their free radical-scavenging effects." Phytochemistry **65**(16): 2391-2399.
36. Luo, J., E. Butelli, et al. (2008). "AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol." The Plant Journal **56**(2): 316-326.
37. Matus, J. T., R. Loyola, et al. (2009). "Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of Vitis vinifera." Journal of Experimental Botany **60**(3): 853-867.
38. Mondal, S., R. R. Phadke, et al. (2015). "Genetic variability for total phenolics, flavonoids and antioxidant activity of testaless seeds of a peanut recombinant inbred line population and identification of their controlling QTLs." Euphytica **204**(2): 311-321.
39. Neugart, S., A. Krumbein, et al. (2016). "Influence of light and temperature on gene expression leading to accumulation of specific flavonol glycosides and hydroxycinnamic acid derivatives in kale (Brassica oleracea var. sabellica)." Frontiers in plant science **7**.
40. Pan, J., H. Chen, et al. (2017). "Understanding the molecular mechanisms underlying the effects of light intensity on flavonoid production by RNA-seq analysis in Epimedium pseudowushanense BL Guo." PloS one **12**(8): e0182348.
41. Pandey, A., A. Alok, et al. (2016). "Genome-wide expression analysis and metabolite profiling elucidate transcriptional regulation of flavonoid biosynthesis and modulation under abiotic stresses in banana." Scientific reports **6**.
42. Pandey, K. B. and S. I. Rizvi (2009). "Plant polyphenols as dietary antioxidants in human health and disease." Oxidative medicine and cellular longevity **2**(5): 270-278.

43. Pelletier, M. K., J. R. Murrell, et al. (1997). "Characterization of flavonol synthase and leucoanthocyanidin dioxygenase genes in Arabidopsis (Further evidence for differential regulation of" early" and" late" genes)." Plant physiology **113**(4): 1437-1445.
44. Petrussa, E., E. Braidot, et al. (2013). "Plant flavonoids—biosynthesis, transport and involvement in stress responses." International journal of molecular sciences **14**(7): 14950-14973.
45. Prasad, P. V., V. G. Kakani, et al. (2010). "Growth and production of groundnut." UNESCO Encyclopedia: 1-26.
46. Price, S., P. Breen, et al. (1995). "Cluster sun exposure and quercetin in Pinot noir grapes and wine." American Journal of Enology and Viticulture **46**(2): 187-194.
47. Reed, H. E., A. H. Teramura, et al. (1992). "Ancestral US soybean cultivars characterized for tolerance to ultraviolet-B radiation." Crop science **32**(5): 1214-1219.
48. Reyes, L., J. Miller, et al. (2004). "Environmental conditions influence the content and yield of anthocyanins and total phenolics in purple-and red-flesh potatoes during tuber development." American Journal of Potato Research **81**(3): 187-193.
49. Saito, K., K. Yonekura-Sakakibara, et al. (2013). "The flavonoid biosynthetic pathway in Arabidopsis: structural and genetic diversity." Plant Physiology and Biochemistry **72**: 21-34.
50. Semwal, D. K., R. B. Semwal, et al. (2016). "Myricetin: A dietary molecule with diverse biological activities." Nutrients **8**(2): 90.
51. Settaluri, V., C. Kandala, et al. (2012). "Peanuts and their nutritional aspects—a review." Food and nutrition Sciences **3**(12): 1644.

52. Shahzad, T., F. M. Anjum, et al. (2011). "Characterization of different groundnut varieties grown in Pakistan." Pakistan Journal of Food Sciences **21**(1-4): 52-55.

53. Steinkellner, S., V. Lendzemo, et al. (2007). "Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions." Molecules **12**(7): 1290-1306.

54. Takahashi, R., S. M. Githiri, et al. (2007). "A single-base deletion in soybean flavonol synthase gene is associated with magenta flower color." Plant molecular biology **63**(1): 125-135.

55. Taylor, A. (1965). "Some effects of photoperiod on the biosynthesis of phenylpropane derivatives in Xanthium." Plant physiology **40**(2): 273.

56. Taylor, L. P. and E. Grotewold (2005). "Flavonoids as developmental regulators." Current opinion in plant biology **8**(3): 317-323.

57. Vanacker, S. A., M. N. Tromp, et al. (1995). "Flavonoids as scavengers of nitric oxide radical." Biochemical and biophysical research communications **214**(3): 755-759.

58. Wisman, E., U. Hartmann, et al. (1998). "Knock-out mutants from an En-1 mutagenized Arabidopsis thaliana population generate phenylpropanoid biosynthesis phenotypes." Proceedings of the National Academy of Sciences **95**(21): 12432-12437.

59. Xu, F., L. Li, et al. (2012). "Isolation, characterization, and function analysis of a flavonol synthase gene from Ginkgo biloba." Molecular biology reports **39**(3): 2285-2296.

60. Yu, J., M. Ahmedna, et al. (2005). "Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics." Food chemistry **90**(1): 199-206.

61. Zoratti, L., K. Karppinen, et al. (2014). "Light-controlled flavonoid biosynthesis in fruits." Frontiers in plant science **5**.

Appendix I

Primers for DNA barcode analysis.

Sr#	Primer ID	Sequence	Reference
1.	Rbcl-F	ATGAGTTGTAGGGAGGGA	(gong et al., 2014)
	Rbcl-R	TTACAAAGTATCCATTGCTGGG	
2.	matK-F	CCTATCCATCTGGAAATCTTAG	(li et al., 2013)
	matK-R	GTTCTAGCACAAGAAAGTCG	

Primers for RT_PCR and qRT-PCR

Sr#	Primer ID	Sequence
1.	FLS-F	CACCCAACCCTTCTTTCCAT
	FLS-R	GTAAGGACTGGGGCATGTTC