Influence of Sugar and Photoperiod on Flavonoid Biosynthetic Pathway in *Arachis hypogaea*

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Biotechnology

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MS THESIS WORK

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Copyright Statement

Acknowledgment

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Maryam Khan

“My dissertation is dedicated to my grandparents and parents”

Abstract

Peanut (*Arachis hypogaea*) is an oilseed crop that harbor plethora of flavonoids produced via biosynthetic enzymes of phenylpropanoid pathway. Flavonoids, biologically active phytochemicals with diverse structures, are tightly regulated at transcriptional level by various external and internal stimuli. Expression analysis of flavonoid biosynthetic genes in *Arachis hypogaea* has revealed their genotype dependent regulation in leaves under the influence of sugar and photoperiod treatment. Photoperiod alterations with 8 hr, 12, hr, 16 hr and 24 hr light durations have depicted significant variation in expression of genes at Day 7 and Day 15. In Bari 2011, most of the genes have been found up-regulated with greater extent on Day 15 than on Day 7 while opposite trend has been observed in PG 1247 variety. Sugar treatment has revealed the differential expression of genes using various concentrations (200mM, 400mM, 600mM) of sucrose and glucose, respectively. Moreover, the expression of flavonoid biosynthetic genes has shown specificity for sugar as sucrose has up-regulated while glucose has down-regulated most of the genes in both varieties. The study concluded that flavonoid production in peanut plant depends on genotype, sugar concentrations, specificity of sugar and alternation of photoperiod. Given study will provide better understanding related to transcriptional regulation flavonoid biosynthetic genes under the influence of light and sugar.

Keywords: *Arachis hypogaea*, Sugar, Photoperiod, Expression, Flavonoids

Abbreviations

%	Percent
$(NH_4)_2SO_4$	Ammonium Sulphate
°C	Degree Celsius
ANR	*Anthocynadin reductase*
ANS	*Anthocyanine synthase*
Approx.	Approximately
CHI	*Chalcone isomerase*
CHS	*Chalcone synthase*
DFR	*Dihydroflavonol reductase*
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphate or deoxynucleotides
EDTA	Ethylenediaminetetraacetic acid
et al.	et alia
F3H	. *Flavanone 3-hydroxylase*
FAO	Food and Agriculture Organization
FC	Field Capacity
FLS	*Flavonol synthase*
g	gram
GI	Gastrointestinal
Ha	Hectare
hr	Hour
i.e.	id est means “that is”
Kb	Kilobasepair
KPK	Khyber Pakhtunkhwa
KV	Kilovolt
M	Molar
$MgCl_2$	Magnesium Chloride
min	Minute
ml	milliliter

mM	millimolar
MS	Mass spectrometry
MS media	Murashige and Skoog medium
NaCl	Sodium Chloride
NARC	National Agricultural Research Centre
NF H_2O	Nuclease free water
ng	nanogram
nm	nanometer
NMR	Nuclear magnetic resonance
PARC	Pakistan Agricultural Research Council
PCR	Polymerase Chain Reaction
rpm	Round per minute
RT	Room Temperature
ssDNA	single stranded DNA
TAE Buffer	Tris-Acetate-EDTA Buffer
TE Buffer	Tris-EDTA Buffer
Tris	Trisma
Tris-HCl	(hydroxymethyl)aminomethane- Hydrochloride
USA	Unites States of America
USDA	United States Department of Agriculture
UV	Ultra-violet
V	volt
α	alpha
β	beta
µg	micro gram
µl	microliter

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CHAPTER 1 : INTRODUCTION

1.1- Arachis hypogaea

Arachis hypogaea Linn (*A. hypogaea* L.) is a botanical name for peanut. Commonly, it is, also, referred as groundnut, mongphali, earthnut, goober and monkeynut (Dwivedi et al., 2003). Peanut is an annual legume form Leguminosae or Fabaceae plant family with a unique feature of geocarpy. Peanut is an allotetraploid hybrid (2n = 4× = 40) of *A. ipaensis* and *A. duranensis* (Bertioli et al., 2015). The word of *Arachis hypogaea* is derived from two Greek words, '*Arachis'* that means a legume and '*hypogaea'* that means below ground referring to its fertilization and flowering that take place above ground while its pod formation occurs in the soil (Y. Zhang et al., 2018). Peanut is an oilseed crop with a potential of nitrogen fixation. Peanut is grown in moderately warm, sub-tropical and tropical regions of six continents around the globe and ranks as fourth amongst oilseed crops while thirteenth as food crop (Reddy et al., 2011). Growing period for peanut ranges from 90 to 140 days depending upon variety and temperature for its optimum growth lies between 22 to 28°C. Peanut crop is well-adapted to loose, well-drained and medium textured soils (Henning et al., 1982).

Peanut belongs to herbaceous plants with long and erect stem (Figure 1.1). Roots of peanut are delicate, white and fibrous with root hair at base. Its leaves are sensitive to light while its branches bear bright yellow flowers at time of maturity. Fruit of peanut is found below ground in the soil enclosed in a shell (Steduto et al., 2012). Peanut is rich source of nutrients as its kernels contain 10 to 20% carbohydrates, 20 to 50% protein, 40 to 50% fat, riboflavin, thiamine, vitamin E, falacin, phosphorus, niacin, zinc, calcium, magnesium, potassium and iron (Guchi, 2015). Kernels of peanut are consumed either as roasted, raw or boiled. Extracted oil from peanut used for cooking while, after oil pressing, oilcake find is application as fertilizer, feed for animal and raw material in industry. Besides promising fatty acid profiling and essential micronutrients, peanut plants enclose potential bioactive phytochemicals including saponins, resveratrol, alkaloids, polyphenolics, tocopherols and phenolic compounds that impart beneficial physicochemical and nutritional

properties (Cherif et al., 2010; Duncan et al., 2006; Sim et al., 2012). Multiple applications of peanut in agriculture and industry have made it a potential cash crop for developing and developed countries.

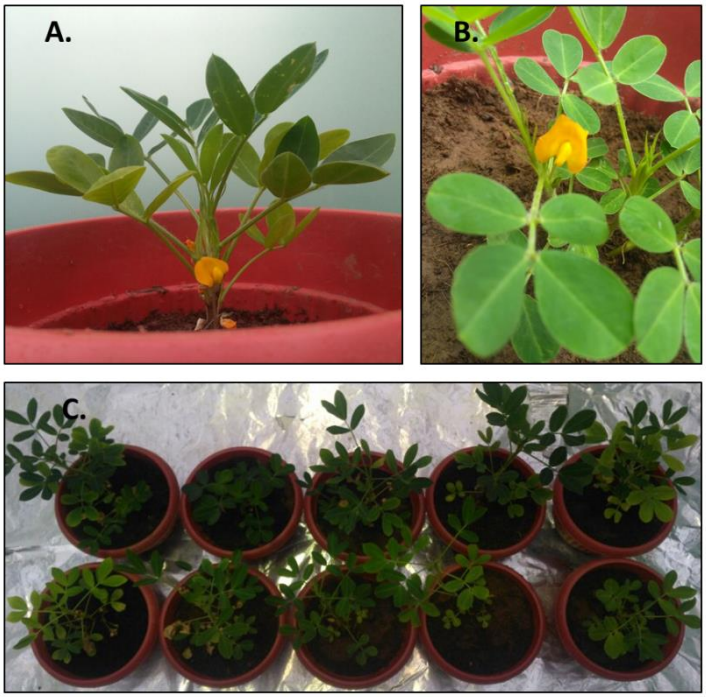

Figure 1.1: *A. hypogaea* Plants

(A) Above ground part of peanut plant showing stem, leaves, flower and branches **(B)** Bright yellow flower **(C)** Vegetative growth of various peanut varieties

1.1.1- Economics of *A. hypogaea* as a Crop Plant

Peanut belongs to legume family and its production is just second to soybean. According to FAOSTAT 2007 database of Food and Agriculture Organization (FAO), (*A. hypogaea*) 34 M tons/annum of peanut produce globally while, according to United States Department of Agriculture (USDA) Data and Statistics 2006, United States of America (U.S.A) nearly produce 2 M tons/annum of peanut (Kottapalli et al., 2009). Peanut productivity in Asia is 1.8 tons/Ha while annual peanut production in western, southern and central Africa is below 1.55 tons/Ha. Peanut crop is grown in Pothwar region of Pakistan covering 81500 Ha with a production of 91400 tones/ annum. Punjab accounts for 85% peanut production in Pakistan, Sindh 5% and Khyber Pakhtunkhwa (KPK) 10%, according to Pakistan Agricultural Research

Council (PARC), while peanut production in the last decade in Pakistan has been given Figure 1.2.

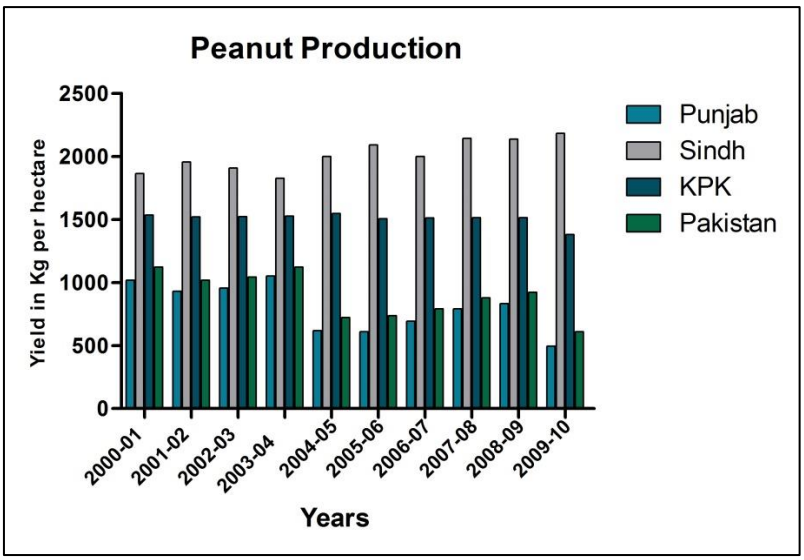

Figure 1.2: Peanut production during last decade in Pakistan (PARC)

Peanut is one of the important cash and food crop for developing countries in Asia and Africa as it contributes to cash income and household food security. Low peanut productivity is attributed to the exposure peanut to abiotic and biotic stresses. Water deficit, salinity, pest and disease incidence are one the severe stresses faced by peanut globally especially in developing countries (R. Varshney et al., 2009). Moreover, contamination of alfatoxin, a mycotoxin that is carcinogenic and highly toxic to humans and animals, in seeds and pods of peanut refrain the producer to sell it in international market and limit the overall market of peanut (Guchi, 2015). Research is being conducted worldwide to eradicate potential hazardous related to peanut while strategies have been devised to promote its usage at various socio-economics levels. In addition to abiotic factors, biotic factors are also one of the important factors, in which plants are exposed in environment of multiple herbivore and pathogenic attacks and affect flavonoid biosynthesis.

1.1.2- Potential Phytochemicals in *A. hypogaea*

Arachis hypogaea other than beneficial fatty acids and micronutrients harbors various bioactive compounds including saponins, terpenoids, quinones, resveratrol, flavonoids, alkaloids, polyphenolics, pholobatannins, tocopherols, tannins, cardiac glycosides and phenolic compounds that impart beneficial physicochemical and nutritional properties to it (Cherif, et al., 2010; Duncan, et al., 2006; Prabasheela et al., 2015; Sim, et al., 2012). These secondary metabolites of plants are the target of many pharmaceutical and nutraceutical due to their health promoting properties (Chung et al., 2016). Among them, flavonoids hold prominent position as they are potential antioxidants in plants and protect them from oxidative stress. Moreover, flavonoids play significant roles in plant physiology and development (Andersen and Markham, 2005).

1.1.3- Stress Signaling in *A. hypogaea*

Plants are the sessile organisms that encounter several biotic and abiotic stresses. They have evolved immunity for these stressors through complex regulation of stress responses thus allaying adverse on their growth and productivity. Basically, plant needs adjustment in its metabolome, trancriptome and proteome profile to acclimatize stress condition through initiating stress metabolism, stress signaling (Shulaev et al., 2008). Peanut like other plants is adversely affected by both biotic and abiotic stresses. Peanut activates stress signaling that regulate physiological and phytochemical changes for stress tolerance. Transcription factors (TFs) are one of those components of signaling pathways that play major roles in stress tolerance and cellular metabolism (Xiao Han et al., 2013; Yokotani et al., 2013). In peanut, *DREB2A, HB7* and *ABF3* are co-expressing transcription factor responsive to abiotic stress and regulate expression of several stress responsive downstream genes (Naika et al., 2013; Pruthvi et al., 2014). Among stress responsive transcription factors, *MYB* superfamily has special position in peanut. *MYB* play significant roles in development of plant and defense related responses (N. Chen et al., 2014). *MYB*s and *MYB* with *bHLH, WRD 40* have been reported to regulate the genes to proanthocyanidin, anthocyanin and flavonoid biosynthesis, moreover, same result were obtained in case of light induced *MYB* (Dubos et al., 2008). Moreover, peanut produces pathogen related proteins through the action of *AhSIPR10* transcription factor in response to

biotic, abiotic stresses. Moreover, A*hSIPR10* mediate the defense signaling of peanut through mediating methyl jasmonate, abscisic acid, hydrogen peroxide and salicylic acid (Jain et al., 2012). Prenylated stilbenoids are the defense molecules that protect the peanut plant during biotic and abiotic stresses. Prenylated stilbenoids are regulated by *Prenyltransferases* enzymes of phenylpropanoid pathway that root production of secondary metabolites in plants (S. Sanders et al., 2018). There is still an ongoing quest to unleash the underline defense mechanisms and signaling in peanut that could be refined by genome annotation of peanut plant.

1.2- Flavonoids

Flavonoids are the structurally diverse group of biologically active compounds that can occur naturally or can be synthesis. They include parent cyclic structures which, also, include C-glycosylated and O-glycosylated derivatives (Andersen and Markham, 2005). Structural Feature of flavonoids include: 1) derivatives of 1-phenylpropane having C15 skeleton, 2) derivatives of 1-phenylpropane having C16 skeleton (rotenoids) and 3) flavonolignan-derivatives of 1-phenylpropane condensed with precursors of C6-C3 lignan (Moss, 2000; Rauter et al., 2018). According to carbon C_6-C_3 -C_6 carbon framework, flavonoids have been classified various group including flavonoids (flavans, flavones, flavonols and anthocyanidins/anthocyanins), neoflavonoids, chalcones, dihydrochalcones, isoflavonoids, aurones, pterocarpans and coumestans as depicted in Figure 1.3. Moreover, flavonoids form complex with other molecules by modifying its structure. Some of these molecules are known as biflavonoid (flavonoid oligomers), flavonolignans (flavonoids with precursors condensed with C_6 -C_3 lignan) and rotenoids while flavonoid in combination with aglycones and glycosides form their derivatives Flavan glycosides (Rauter, et al., 2018). The classification of flavonoid depending upon their structures has been given in Figure 1.3.

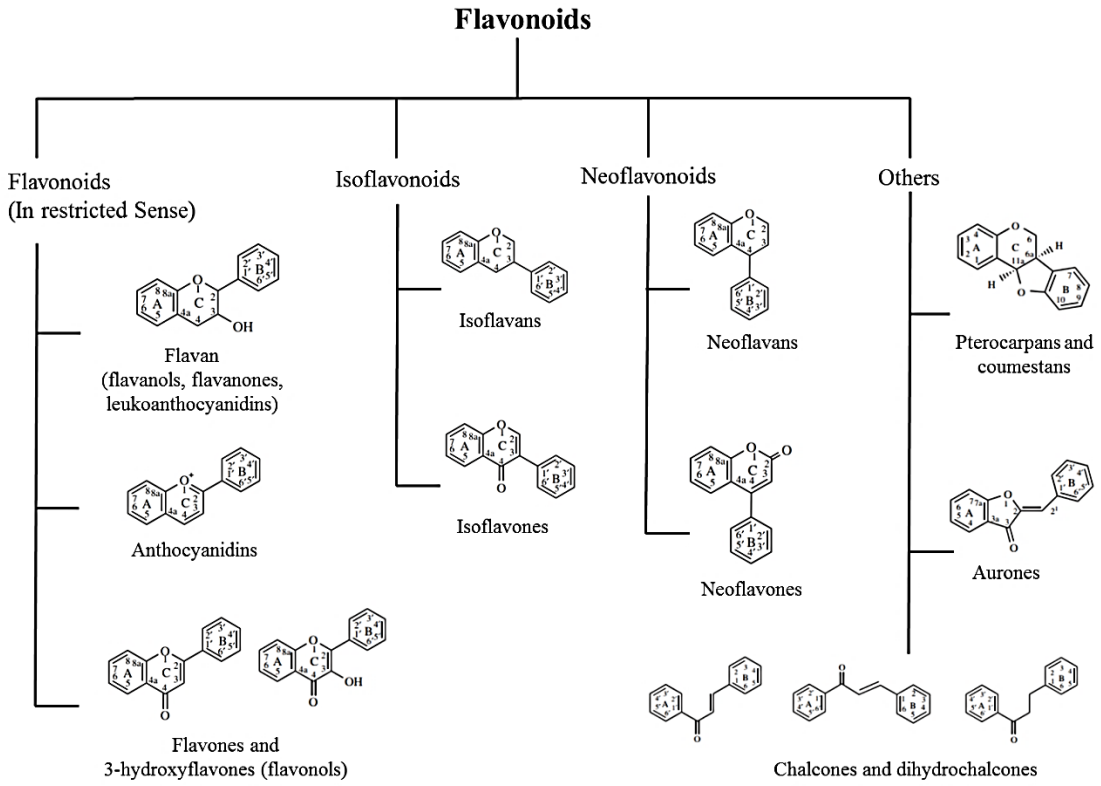

Figure 1.3: Classification of flavonoids based on their structure (Rauter, et al., 2018)

1.2.1- Flavonoids and Stresses

Flavonoids are the diverse group of secondary metabolites that participate in various developmental processes. They have been considered as hallmark for stress as they play role in plant defense against stresses (Winkel-Shirley, 2002). Flavonoids production is controlled by various developmental and environmental stimuli as depicted in Figure 1.4. Regulation of expression of flavonoid biosynthetic genes is one of the renowned models by which plant response to stresses has been studied (Dooner, 1983). Accumulation of flavonoids is one the plant's stress responses that are especially involved in abiotic signaling. They have protective roles against temperature variation, pathogen attack, UV stress, light intensity, limitation of nutrients, drought, humidity and herbicide application (Fini et al., 2011). All the abiotic or biotic stresses lead to the generation of ROS that causes oxidative stress to plants. Plants use anti-oxidant mechanisms that, also, involve accumulation of anti-oxidants that help them to allay ROS (Jovanovic et al., 1994).

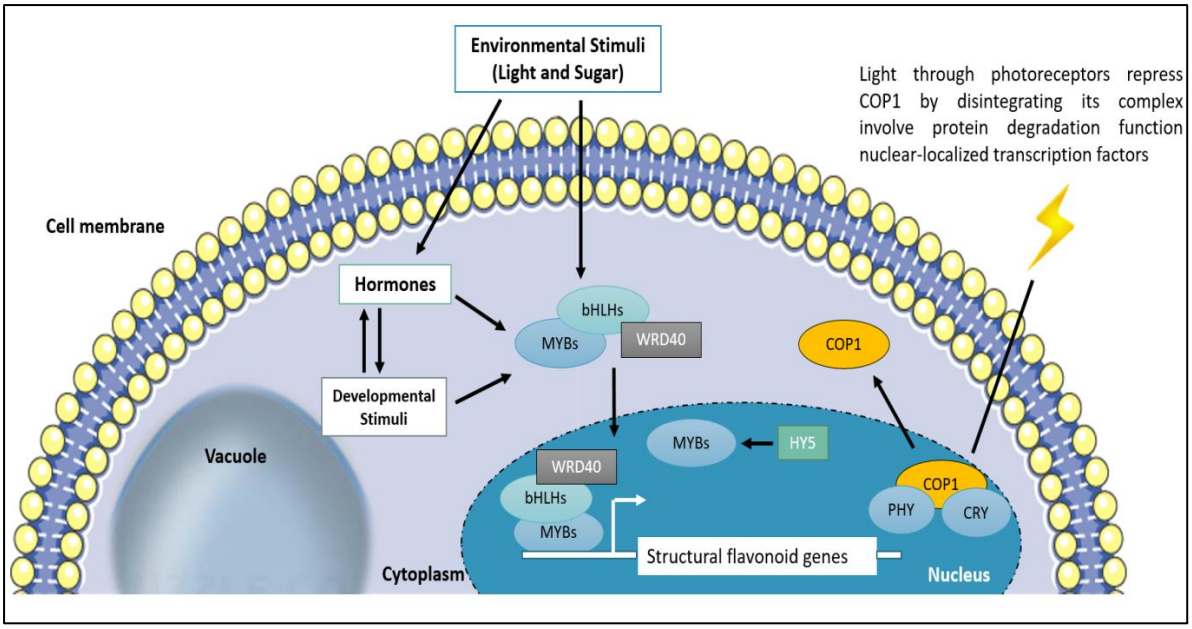

Figure 1.4: Regulation of Flavonoid Biosynthesis under the influence of various external and internal stimuli. Information retrieved from: (W. Xu et al., 2015; Zoratti, Karppinen, Luengo Escobar, et al., 2014)

Various stresses causes fluctuation in flavonoids biosynthesis e.g. light exposure affects flavonoid accumulation that helps the plant to avoid UV radiation in various plants (Singh et al., 2017). Change in temperature affects the biosynthesis of flavonoids e.g. low temperature induce the production of anthocyanin in various species of plants while increase in temperature negatively affects flavonoid production (Chalker-Scott, 1999; Dela et al., 2003). Accumulation of flavonoids derivatives and anthocyanin has, also, been observed in many plant species under drought stress (Ma et al., 2014). Moreover, flavonoids are capable to interact plant with other organism especially microorganisms and help the plant to avoid any environmental stresses (Mierziak et al., 2014). Flavonoids mainly flavonols affects auxin transportation and modulate plant's growth and development and help the plants avoid stress conditions (Di Ferdinando et al., 2012). Precisely, flavonoids, apart from working as UV filter, act as signaling molecule, pytoalexins, allelopathic compounds and detoxifing agents and help the plant acclimatize heat, frost, drought and salinity (Samanta et al., 2011).

1.2.2- Flavonoid Biosynthetic Pathway

Biosynthesis of flavonoids involves phenylpropanoid and polyketide pathways as shown in Figure 1.5. *Chalcone synthase* (*CHS*) act on the baseline structure of flavonoids that is formed by coumaroyl-CoA and three molecules of malonyl-CoA and form naringenin chalcone. *Chalcone isomerase* (*CHI*) forms naringenin (a colorless flavanone) from naringenin chalcone. *Flavanone 3-hydroxylase* (*F3H*) add −OH group to flavanone at position 3 to synthesis dihydroflavonol. Moreover, *F3H* hydroxylase B-ring at 3′ or 5′ position to produce dihydroquercetin. *Flavonol synthase* (*FLS*) transforms dihydroflavonols flavonols while *Dihydroflavonol reductase* (*DFR*) catalyzes the production of proanthocyanidin and anthocyanin and competes with *FLS* for dihydroflavonol to synthesis leucoanthocyanidins. *Anthocyanine synthase* (*ANS*) produce leucoanthocyanidins from anthocyanidin while *Anthocynadin reductase* (*ANR*) and *Leucoanthocyanidin reductase* (*LAR*) catalyze the reduction of leucoanthocyanidin to proanthocyanidins and catechin, respectively (Falcone Ferreyra et al., 2012; Petrussa et al., 2013; Saito et al., 2013).

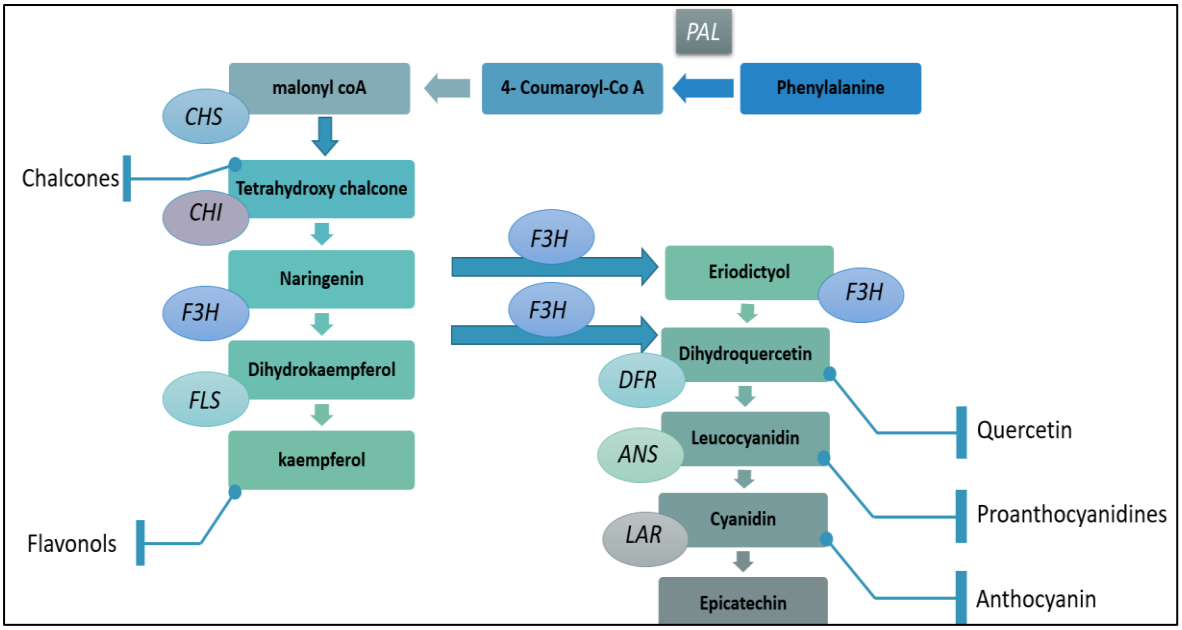

Figure 1.5: General flavonoid biosynthetic pathway in plants (Falcone Ferreyra, et al., 2012)

1.2.3- Significance of Light in the Production of Flavonoids

Plants are autotrophs that drive their energy from light which is, also, act external stimulus to regulate gene expression, plant's growth and development. Light affects plant in term of duration, intensity, type and direction and aid the plant to detect seasonal changes (Zoratti, Karppinen, Escobar, et al., 2014). Photoperiod is defined as total light and dark period in cycle of 24 hr. 12 hr light/12 hr dark photoperiod can be observed at equator but Earth is tilt which causes variation in photoperiod across the globe. Moreover, distance of Sun to Earth that leads to seasonal changes can, also, account for photoperiod variation. Basically, two main photoperiod type exist that include long day and short day. When light period is critical than day length then photoperiod will consider as long day. When dark period is critical than day length then photoperiod will consider as short day. Plants perceive light through the range of photoreceptors that, upon activation, trigger specific signal transduction pathways and regulate gene expression within plants (Arsovski et al., 2012; Lymperopoulos et al., 2018).

Accumulation of flavonoids has been reported with different light conditions in various plants. In normal conditions, flavonoids biosynthesis is adjusted to produce those flavonoids that take part in the flavonoid production. Enhanced phenolic compounds production has been found to associated with longer light duration (A. Taylor, 1965). Several reports indicated that flavonoids accumulation occurs in various plants' tissues in response to light exposure to reduce UV penetration in vulnerable parts of the plants. Dihydroxy B-ring-substituted forms of flavonoids react with light and reduce oxidative stress by UV radiation (Singh, et al., 2017). Photoperiod is one of the essential mediators of plant's growth and development. It is, also, one the keen regulators of plant flavonoid biosynthesis. Generally, it is believed that longer light period in photoperiod duration can induce flavonoid production because it increases light related energies in plants (Koyama et al., 2012; A. Taylor, 1965).

1.2.4- Sugar mediated Regulation of Flavonoid Biosynthesis

In a plant life, sugars are necessities that modulate nutrients, osmotic homeostasis, carbon fixation, molecular synthesis, metabolism, signaling and oxidative stress (Bolouri-Moghaddam et al., 2010). Sugar are energy molecules

formed through light energy captured via the process of photosynthesis and keep the flow of energy within living organism (Dokulil and Kaiblinger, 2009). Sugars mostly exist in two free forms: 1) Monosaccharides mostly including fructose and glucose; and 2) Disaccharides mainly sucrose and maltose. Sucrose which is a disaccharide composed of fructose and glucose is an abundant and majorly transported sugar that is indigenous to all plant tissues while glucose is the most plenty monosaccharide within a plant (J. Moreno and Peinado, 2012).

Sugar is distributed throughout plant via transporters mainly include two families: monosaccharide transporters and disaccharide transporters. Sugars either act as importers (carbon sink) that help in organ development and supply of nutrients in reproductive and heterotrophic cells or exporters (carbon source) that take part in photosynthesis and carbon fixation in mesophyll cells (L. E. Williams et al., 2000). Plant senses sugar through special sensor. Amongst sucrose sensors and glucose sensors are most prominent. Glucose sensors (known as *Hexokinases*) were first reported in *Arabidopsis* then in other plants including maize, tobacco and rice and signified glucose signaling to modulate metabolism of plants (Jang et al., 1997; Moore et al., 2003; Sheen, 2014).

Sugars have been found to regulate expression of genes involved in conversion, storage and utilization of nutrients and imposed their regulatory roles in plant primary and secondary metabolism (Paul and Pellny, 2003; Rolland et al., 2002). Glucose has been reported to take part in three signal transduction pathways including glycolysis dependent pathway, hexokinase dependent and independent pathway (Xiao et al., 2000). Moreover, these pathways through glucose and sucrose might be involved cell cycle phase G1 to provide nutrients and regulate cyclins (Riou-Khamlichi et al., 2000). Sucrose functions same as that of glucose and takes part in gene regulation but sucrose mediated pathways are complex (Huijser et al., 2000). *Hexokinases* are crucial for sugar sensing but regulate ROS level and glucose-6-phosphate to activate flavonoid biosynthesis and anti-oxidant defense mechanism. So, high level of soluble sugars in plant tissue and organelles can encourage flavonoid accumulation apart from their stimulation through ROS system (Bolouri-Moghaddam, et al., 2010).

1.2.5- Significance of Flavonoids in *A. hypogaea*

Flavonoids are the secondary metabolites that are produced during specific stages of plant development. They, also, take part in various defense responses to protect plants against biotic and abiotic stresses. They can kill plant pathogens or inhibit their reproduction and growth. Flavonoids can exist either in bounded or free form (Havsteen, 2002; Sobolev et al., 2008). Peanut, like many plants, harbors rich content of flavonoids with a major portion of dietary antioxidants. Nutritionists recommend consumption of whole seeds of peanut (Shem-Tov et al., 2012). Globally, breeders are trying to develop peanut varieties as functional food with enhanced flavonoid contents. Peanut was reported with presence of various forms of flavonoids including C-glycoside flavone, flavonol, dihydroflavonol, flavonone and 5,7-dimethoxyisoflavone and dihydroquercetin. The presence of these flavonoids can improve the tolerance of peanut to fungus by inhibiting fungal (*Aspergillus flavus* and *Trichoderma viride)* growth that include. Moreover, various genotype of peanut showed variable content of flavonoid (Daigle et al., 1983; Mabry et al., 1970).

1.3- Significance of Flavonoid in Pharmaceutical Industry

Flavonoids are the potential bioactive compounds reported with anti-oxidant and anti-cancer activity. Moreover, they protect heart by avoiding cardiovascular diseases (Andersen and Markham, 2005). Accumulation dietary flavonoids can be neuroprotective and can have chemopreventive actions (R. J. Williams et al., 2004). Flavonoids help the body to control oxidative stress due the production of reactive oxygen species (ROS) in stress conditions. They have found to have anti-aging, anti-inflammatory, anticancer, anti-atherogenic and anti-apoptosis effects (Han et al. 2007). Peanut is a legume with bio-active flavonoids that impart antioxidant, anti-mutagenic and anti-proliferative activity to them (Cardador-Martinez et al., 2002; Dong et al., 2007; Heimler et al., 2005). That is why; flavonoids are the target for pharmaceutical and nutraceutical industries. Various techniques based on modulation of flavonoid biosynthetic pathway have been in used for their mass production. But the limited knowledge of flavonoid biosynthetic pathway is main hurdle in success of mass production flavonoids for pharmaceutical and nutraceutical industries.

1.4-Objectives of the Study

Peanut has great potential for flavonoids that finds their need in pharmaceutical and nutraceutical industries. Moreover, flavonoids play vital roles in combating biotic and abiotic stress in peanut plants. Techniques including pathway engineering and bioreactor are being tried for mass production of flavonoids but limited knowledge for understanding flavonoid biosynthetic pathway poses hurdle in this process. One gene one enzyme hypothesis is not enough for understanding biosynthesis each type of flavonoids (Chung, et al., 2016). That is why; understanding of flavonoid biosynthetic pathway genes under the influence of sugar and photoperiod has been developed by given studies. Objectives of the study titled "Influence of sugar and photoperiod on flavonoid biosynthetic pathway in *Arachis hypogaea*" are as follow:

1. Study the effect and specificity of sugars on the expression of genes encoding flavonoid biosynthetic enzymes in *A. hypogaea*.
2. Expression analysis of flavonoid biosynthetic genes under the influence of photoperiod alterations in various varieties of *A. hypogaea.*

CHAPTER 2 : LITERATURE REVIEW

2.1- *A. hypogaea* in Agriculture

Over 3800 years ago, *Arachis hypogaea* (Peanut or Groundnut) originated in Bolivia, South America. World colonists, explorers (especially Spanish and Portuguese), missionaries were the cause of worldwide distribution of peanut from America. Now, peanut is grown in tropical, sub-tropical and moderately warm regions around the globe (Reddy, et al., 2011). USA, China and India are the leading producer of peanut crop for over 25 years. According to FAO 2006, peanut production has been documented as total production 35.9 M tons from 25.2 MHa areas while statistics of peanut remained same till FAOSTAT 2011. According to ethnological studies, domestication of peanut took place before Spanish conquest of South America. Peanut spread from USA to Europe and from there it reached Asia and Africa by the traders (Wright and Rao, 1994). By now, peanut has gained importance for poor-income farmers in Africa and Asia being as both cash and food crop. In 1940 to 1950, peanut was introduced and planted in Rawalpindi Division on 400 Ha. It is mainly grown in rain fed areas known as barani areas. According to PARC, Punjab is the major contributor of peanut production while Sandhar, Sukkhar, Peshawar, Mardan, Kurram agency and Kohat are one of the major areas of its cultivation.

Peanut that is, also, known as as poor man's nut, is grown globally for its edible vegetable oil and proteins (Hammons, 1973). It can be used directly while peanut cake after oil extraction is perfect source of animal feed. Peanut kernels is rich source of vitamins and micronutrients including riboflavin, thiamine, vitamin E, falacin, phosphorus, niacin, zinc, calcium, magnesium, potassium and iron (Guchi, 2015). Peanuts are used for oil production especially in Asia while the major portion of it is consumed directly as confectionery, peanut butter, salted peanut (Dudhal, 2017). Among legumes, peanuts are the good source for unsaturated fatty acids and contain various phytochemicals that positively affect human health (Bankole et al., 2005). According to epidemiological survey, peanuts promote human health by lowering cholesterol and low density lipoproteins due to presence of beneficial fatty acids and phytochemicals (Alper and Mattes, 2003; Jiang et al., 2002) .

2.1.1- Current Hurdles in Peanut Production

Peanut can be imperative cash crop because of its foreign trade and domestic use in developing and developed countries but its yield and quality is affected by climate changes, biotic and abiotic stresses. Other reasons for its lowers productivity especially in developing countries are: 1) Cultivation of crop under rainfed areas, 2) Biotic stress due to climate changes, 3) Increased biotc attack especially that of fungus, 4) Socio-economics factors and 5) low input use (Steduto, et al., 2012).

Peanut breeders are working across the globe to improve its yield under abiotic stresses and to avoid aflatoxins contamination due fungus attack (R. Varshney, et al., 2009). Peanut is affected by many diseases including early leaf spot, late leaf spot, rust, bud necrosis, collar rot but among them infection of *Aspergillus* species is the most disastrous one as they cause accumulation of acutely poisonous carcinogenic compound known as aflatoxins and quantitative yield loss (Guchi, 2015; Waliyar, 2006). Moreover, drought is another hurdle in its production as it is grown in semi-arid tropics environment that is categorized by erratic and short rainfall with long period of no rain (R. Varshney, et al., 2009). Recent advances in the production of crops and crop genomics are opening new ways to overcome hurdles in the production and trade of peanut (R. K. Varshney et al., 2006).

2.1.2- Gene Pool of *A. hypogaea* harboring various Pyhtochemicals

Nuts are beneficial due to the presence of desirable lipid profile, although peanut is a legume but contain high amount of unsaturated fatty acids. Peanut, in addition to beneficial nutrients, contains various bioactive compounds which provide health benefits not only to plants but to human health upon their consumptions (Higgs, 2003). Peanut is used for oil production commercially but the presence of by-product that includes fiber, protein, polyphenol, vitamins, antioxidants and minerals makes it a functional food. It has been reported as great source of bioactive compounds including flavonoids, phytosterols, phenolic acids and resveratrol. These compounds have disease preventive characteristics and promote health of their consumers (Arya et al., 2016). (Prabasheela, et al., 2015) through phytochemical analysis, have detected pholobatannins, flavonoids, tannins, quinones, cardiac glycosides, saponins and terpenoids in the ethanolic extract of runner and spanish and runner peanut varieties. (Kim et al., 2013) have found saponins, triterpenoid, β-

Sitosterol, ursolic acid, 3-O-β-D-glucopyranoside, resveratrol, quercetin and kaempferol in ethyl acetate, hexane, methanol and chloroform root extracts of peanut.

Resveratrol is a phytoalexin belongs to biologically active compounds that is expressed under stress condition in plants and protect them from pathogens (Nayak, 2018). It is, also, linked to the cure of cardiovascular disease and cancer. It reported by (T. H. Sanders et al., 2000) in the aqueous ethanol extract of *A. hypogaea.* (Adhikari et al., 2018) has positively screened peanut shells with polyphenols, flavonoids and antioxidants. (Mazur et al., 1998) has reported isoflavones, saponins, total phenolic compounds and alkaloids in peanut whereas (Sim, et al., 2012) has reported resveratrol in various peanut varieties. (Aftab and Vieira, 2010) have compared the antioxidant activity of peanut's resveratrol and quercetin with that of curcumin. (Ku et al., 2005) have induced piceatannol and resveratrol production under control conditions in peanut's callus.

There are many reports indicating the presence of flavonoids in peanut, for example, luteolin which is a flavonoid has been reported by (D. A. Moreno et al., 2006) in ethnolic extract of peanut's nutshells. (Daigle, et al., 1983) have detected bunch of flavonoids including quercetin, aglycones, rhamnetin and isorhamnetin in the extracts of leaves and testa of peanuts. Moreover, (Sobolev, et al., 2008) have isolated and investigated four conjugates of flavonoid along with triamides, spermidine in flowers of peanut flowers through nuclear magnetic resonance (NMR) and mass spectrometry (MS). (Elsorady and Ali, 2018) have identified pro-anthocyanidins and flavan-3-ols in peanuts whereas (Yu et al., 2014; G. Zhang et al., 2013) have identified isosaponaretin, carotene and luteolin along with flavonoids and polyphenols in peanut shells.

2.2- Flavonoids

Flavonoids are biologically active compounds that include parent cyclic structures and their derivatives. They have been reported in the literature in the field of organic synthesis, natural product chemistry, biochemistry, plant biology, toxicology, medicinal and food chemistry (Rauter, et al., 2018). Flavonoids are basically plant's pigments while they can, also, play roles as phytoalexins, protectants against UV, pigments, disease resistance agents and signals for nodulation (Petrussa,

et al., 2013; van Tunen et al., 1989). Expression of flavonoid biosynthetic genes has been studied extensively at various levels and allowed to understand the route for flavonoid biosynthesis. Enzymes of flavonoid biosynthetic pathway that include *hydroxylases, syntheses* and *reductases* have been thought to be evolved from enzymes belong to primary metabolism. Flavonoid biosynthetic pathway was evolved before lignin pathway but its evolution is proceeded by phenylpropanoid pathway (Stafford, 1991). In plants, flavonoids have been found in epidermal cell of leaves tissues, skin of fruits or in that parts which are susceptible to UV radiations. Flavonoids could be found be plant cell vacuole as soluble pigments that include flavonols, flavones, chalcones, anthocyanins, condensed tannis, proanthocyanidins and flavandiols (Petrussa, et al., 2013).

UV protection was the first role that was assigned to flavonoids in experiments with *Arabidopsis* mutants (Landry et al., 1995). Flavonoids including morin have, also, to have metal chelating agents in roots apoplast (Kidd et al., 2001). Flavonoids, also, regulate plant growth by modulating hormonal signaling. Auxin is plant hormone that is stress responsive and control opening/closing of leaves stomata and allocate nutrients under stress conditions (Dietrich et al., 2001; Palme and Gälweiler, 1999). Flavonoids resembles auxin in their structure and bind to protein that carries auxin and disturb auxin transportation (Jacobs and Rubery, 1988). Flavonoids have shown their accumulation in root tip, hypocotyl root, cotyledonary node, root apical end cortex cells and signified that the accumulation/synthesis of specific flavonoids can change auxin transportation in plants (Peer et al., 2001; Saslowsky and Winkel-Shirley, 2001). A glutathione transporter have been found to participate in flavonoids distribution in subcellular compartments but it is not true for every plant species (Marrs et al., 1995).

2.3- Regulation of Flavonoids under various Stresses

Flavonoids find their roles in both plant development and defense. Flavonoids were first reported in ferns and mosses and can be found in all plants of Kingdom Planta (Stafford, 1991). The synthesis of flavonoids is affected by both developmental and environmental stimuli. Flavonoids are basically plant's pigments while they can, also, play roles as phytoalexins, protectants against UV (van Tunen, et al., 1989). Flavonoids have protective roles against temperature variation, pathogen attack, UV

stress, light intensity, limitation of nutrients, drought, humidity and herbicide application (Fini, et al., 2011). Accumulation of flavonoids is one the plant's stress responses that are involved in plant defense against stresses. Apart from working as UV filter, act as signaling molecule, pytoalexins, allelopathic compounds and detoxifing agents and help the plant acclimatize heat, frost, drought and salinity (Samanta, et al., 2011).

Biosynthesis of flavonoids is highly dependent upon temperature and light e.g. low temperature induce the production of anthocyanin in various species of plants while increase in temperature negatively affects flavonoid production (Chalker-Scott, 1999; Dela, et al., 2003). Evaluation of functions of flavonoids in responses to various external stimuli is of great interest these days. Accumulation of flavonoids have been observed in leaves of wheat under stress which suggested the role of flavonoids in stress tolerance (Ma, et al., 2014). Moreover, (del Valle et al., 2015) proved that flavonoids accumulate in genotype, spatial and temporal dependent manner in stressed plants.

2.3.1- Flavonoids Regulation under the Influence of Photoperiod

Accumulation of flavonoids has been reported with different light conditions in various plants. In normal conditions, flavonoids biosynthesis is adjusted to produce those flavonoids that take part in the flavonoid production. Enhanced phenolic compounds production has been found to associated with longer light duration (A. Taylor, 1965). Several reports indicated that flavonoids accumulation occurs in various plants' tissues in response to light exposure to reduce UV penetration in vulnerable parts of the plants. Photoperiod is one of the essential mediators of plant's growth and development. It is, also, one the keen regulators of plant flavonoid biosynthesis. Generally, it is believed that longer light period in photoperiod duration can induce flavonoid production because it increases light related energies in plants (Koyama, et al., 2012; A. Taylor, 1965).

(Carvalho et al., 2010) have indicated accumulation of catechins and flavonols and enhanced expression of flavonoid biosynthetic genes in leaves of sweet potato growing at 16 hr light duration as compared to 8 hr. (Reyes et al., 2004) have observed higher anthocyanin content in potato with 15 hr light treatment as compared

to 12 hr, however, total phenolic compounds were unaffected. Expression of flavonoid genes under the influence of light has been well documented in grapes. (Koyama, et al., 2012) have reported induced production of flavonoids including flavonols and anthocyanins in grapes acclimatized to longer light conditions. (Zoratti, Karppinen, Escobar, et al., 2014) have observed accumulation of flavonols in grape affected by various light conditions, moreover, they observed higher expression of *FLS* in plants with longer light exposure. (Landry, et al., 1995) have proved the role of flavonoids as UV protectants. They have, also, observed UV hypersensitivity in plant containing mutated *CHI* and *CHS*.

Flavonoids production was seen to be affected by light in apples, strawberries and bananas. (Feng et al., 2013) have observed higher accumulation of flavonol and anthocyanin in peels of apples with higher exposure of sunlight. (Pandey et al., 2016) have observed decrease in expression of flavonoid biosynthetic genes in plants of banana treated with longer duration of dark. (Azuma et al., 2012) have concluded that accumulation of flavonoid depended upon light conditions in strawberries.

2.3.2- Effect of Sugar on Flavonoid Production

Sugar is an important factor that controls the growth and development of plants. Plants respond to stresses by forming various sugars that participate in the synthesis of cellulose, proteins and starch and; also, act as fuel for growth of plant and signaling molecule to regulate cellular gene expression. Various studies have reported the effects of available sugars on growth and development and suggested increasing amount of sugar can stimulate flowering in some plants while senescence in other species while delay in the germination of seeds has, also, observed in some species (J. Moreno and Peinado, 2012). High level of sugar has been found to encourage the growth of tuber in potatoes and enhance adventitious roots formation in *Arabidopsis*. Sugar responsive pathway has been studied through analyzing sugar mediated gene expression microarray experiments.

Sugar is distributed throughout plant via transporters mainly include two families: monosaccharide transporters and disaccharide transporters. Sugars either act as importers (carbon sink) that help in organ development and supply of nutrients in reproductive and heterotrophic cells or exporters (carbon source) that take part in

photosynthesis and carbon fixation in mesophyll cells (L. E. Williams, et al., 2000). (Rolland et al., 2006) have found that high concentration of sugar activated sink activities while low concentration of sugar down regulated source activities. Control mechanism is needed to regulate flux of sugar related to sink and source interaction during germination of seeds, development of seedlings and pollen (Truernit et al., 1999; Weber et al., 1997). Sufficient data is not available on how the sugar regulates various processes but various studies indicated certain aspects related to sugar signaling. (Finkelstein and Gibson, 2002; León and Sheen, 2003) have studied the role of sugar and hormonal signaling in seedling development and regulation of hexokinases. (Huijser, et al., 2000) has studied the significance of sugar in signaling pathways by identifying mutants with differential sugar responses.

Several studies have reported flavonoids regulation under influence of sugar. (Solfanelli et al., 2006) has reported up-regulation of *DFR* amd ANS in grape and accumulation of anthocyanin after sucrose treatment while (Gollop et al., 2002; Gollop et al., 2001) have observed same under the non-specific induction of glucose, fructose and sucrose. (Solfanelli, et al., 2006) have, also, reported specific up-regulation of genes of flavonoid biosynthesis.

2.6- Medicinal use of Flavonoids

Flavonoids are the plant's secondary metabolites with great anti-oxidant capacity. In human, disease condition causes the generation of ROS which go beyond antioxidant ability of body and cause oxidative stress that plays roles in disease pathogenesis and progression. Oxidative stress is main culprit for the development of cancer, aging, heart and neurodegenerative diseases. Dietary anti-oxidants such as flavonoids have potential to prevent oxidative stress due to disease thus help the body to cure it (Mandloi et al., 2014). Many dietary flavonoids have been reported to have a preventive role against cancer and heart disease that might be caused by oxidative damage by ROS in disease conditions (Shad et al., 2009). Apart from anti-oxidant activities, flavonoids impart positive effects on human by having anti-apoptosis, anti-inflammatory, anti-aging, anti-atherogenic and anticancer roles (Xiuzhen Han et al., 2007).

Peanut is dry legume and a good source of bioactive compounds e.g. polyphenol (Cheng et al., 2009). Peanut's flavonoids have been reported to have anti-mutagenic activity (Cardador-Martinez, et al., 2002), antioxidant capacity (Heimler, et al., 2005) and anti-proliferative effects (Dong, et al., 2007). Moreover, (Aparicio-Fernández et al., 2008) has reported anti-oxidative activity of flavonoids extracted from *Phaseolus vulgaris*. (B. Xu et al., 2007) has reported anti-oxidative activity of legume seed against oxidative stress generated by low-density lipoprotein of human. (Andersen and Markham, 2005) have reported cardio-protective property of flavonoids while (R. J. Williams, et al., 2004) have informed about the neuro-protective and cardio-protective of dietary flavonoids.

(García et al., 2018) has used flavonoids extracted from green tea to cure hepatocellular carcinoma as they have previously reported to have antimetastatic, antiangiogenic, anti-inflammatory, antioxidant activities. (Khan et al., 2018) have showed antiplatelet effect of flavonoids in preclinical trails through various mechanisms. (Garcia Larsen et al., 2018) have shown that presence of anthocyanin can inhibit inflammation and found to cure chronic obstructive pulmonary disease. (Oteiza et al., 2018) have investigated the systemic effects of flavonoids on gastrointestinal (GI) and found that flavonoids have potential to neutralize toxic effects of drugs, regulate gut secretion, maintain integrity of intestinal barrier, control immune system, maintain microflora, avoid colorectal cancer and help in the absorption of lipid and carbohydrates. (Muller et al., 2018) have illustrated the role of flavonoids in lipid and glucose metabolism and their capacity to prevent metabolic syndrome/diseases.

CHAPTER 3 : MATERIALS AND METHODS

3.1- Seed Collection and Germination

Qualified and healthy seeds of two different varieties of *A. hypogaea* (PG 1247 and Bari 2011) were collected from department conducting Oilseed Research Program at National Agricultural Research Centre (NARC), Islamabad, Pakistan. Seeds of each variety were dehusked and surface sterilized by soaking them in 70% ethanol for 2 min followed by washing through autoclaved distilled water, separately. Excessive ethanol was vaporized by spreading the seeds on cleaned filter paper sheet in a surface sterilized safety cabinet. Completely dried seeds of selected peanut varieties were wrapped separately in an aluminum foil sheet and kept at 4°C for 48 hr to break dormancy of seeds. In the sterilized conditions, seeds were transferred onto the wet cotton layered in a breaker for sake of germination. Each layer contained separately placed five to six seeds while forceps were used to transfer seeds. Beakers that are used for germination were properly labeled and wrapped in an aluminum sheet to avoid penetration of light. Then, beakers were placed in a dark cabinet with temperature ranges 22-28°C for time till germination.

3.2- Plant Growth and Stress Induction

Seedling with equal morphology and well-developed roots of each peanut variety were divided into two halves for light and sugar treatment, respectively. Germinated seeds were sown in soil containing coconut fibers for light treatment while cigar roll method followed by treatment in Murashige and Skoog medium (MS media) was used for sugar treatment.

3.2.1- Light Treatment

Flavonoid expression in two selected varieties of *A. hypogaea* under 8/16 hr, 12/12 hr, 16/8 hr and 24/0 hr photoperiod (light/dark) conditions were evaluated at day 7 and day 15 of light treatment. For light treatment, seedlings of each variety were sown in autoclaved/sterilized potting media containing soil and coconut fibers in 1:1 ratio in 250mL disposable paper cup with capacity to hold 100g of potting media. Sowed seedlings of each variety were divided into four groups. Approximately 10 to 12 plants were assigned for each group including plants for both day 7 and day 15.

Plants of all groups were provided with temperature ranges 25 to 30°C and are kept in growth chamber with 16/8 hr light and dark condition. Plants were watered according to field capacity (FC) measured through method explained by (Lopez and Barclay, 2017). Seedlings were grown to, at least, to growth stage with two sets of leaves and then subjected to light treatment. One of the groups was provided with 8/16 hr photoperiod, one with 12/12 hr, one with 16/8 hr and one with 24/0 hr. Amongst, plants with photoperiod 16/8 hr were considered as control group while other as treated groups. Temperature range for all the groups was kept same i.e. 25 to 30°C and watered according to FC. Treatment starting day was considered as day 0 while sampling was carried out at day 7 and day 15.

3.2.1.1- Field Capacity Measurement

Field capacity is the measure of upper limit of available water or moisture content contained in pores after water drainage by gravitational force (de Oliveira et al., 2015). The FC of potting media was evaluated through two methods as mentioned. According to one method, 100g of potting media which was a mixture of soil and coconut fibers in 1:1 ratio were put in Whatman filter paper no. 1 placed in funnel tube with a stand. 100 mL water was added slowly into the funnel containing potting media. Whole media got over flowed with water which started tripping through stem of funnel tube. Excessive water flowing though funnel was collected carefully into graduated cylinder until it stopped tripping. The difference between 100mL and volume of collected water in the graduated cylinder measured in mL gave FC which was ≈28 mL/100g. Moreover, the media at this stage is known as saturated media which was used to calculate FC by other method.

In the second method, saturated media with water was weighed to get wet weight in g. Then, this saturated media was placed in drying oven at 70°C for 1 to 2 days until the media was left with no moisture and weighed. The difference between weight of saturated media and dried weight gave FC of potting media which was ≈28 mL/100g.

3.2.2- Sugar Treatment

Sucrose and glucose were chosen to study the influence of sugar on the transcription of flavonoid biosynthetic genes in peanut plant. Seedlings of peanut

were grown for 10 to 15 days to get ample sets of leaves in filter paper rolls placed vertically in a beaker with simple tap water. Then, plants were transferred to MS media containing sugar solution of different concentrations. Total seven study groups were formulated including control group with no sugar treatment, 200 mM sucrose (S 200 mM), 400 mM sucrose (S 400 mM), 600 mM sucrose (S 600 mM), 200 mM glucose (G 200 mM), 400 mM glucose (G 400 mM) and 600 mM glucose (G 600 mM) solution containing groups for each of the two varieties of peanut. All the experiments were performed in form of triplicate.

3.2.2.1- Cigar Roll Method

Cigar roll culture system (cigar roll method) is paper based plant culturing system for early stage seedling growth in which uniformly grown seedlings are wrapped between two filter paper sheets to make rolls which are placed vertically in a water supplied beaker (Zhu et al., 2005). For early stage growth of peanut seedling before sugar treatment in MS media, cigar roll method was selected. Equal sized germinated seeds of peanuts are selected and wrapped into cigar rolls between two filter paper sheets of A5 size in the sterilized conditions. These rolls were vertically kept in a 1L beaker. Beakers were kept in controlled environment conditions that are 16/8 hr light and dark condition and temperature range 23 to 26°C. Approximately 8-10 rolls were placed in each beaker and each beaker is provided with 100 mL autoclaved/filtered tap water every day. Seedlings were allowed to grow for 10 to 14 days to enough leaf sets. To avoid depletion of nutrients, seedlings are provided with 1/10 Hoagland's solution in tap water occasionally.

3.2.2.2- Sugar Treatment in MS Media

Plants grown in cigar roll culturing system were transferred to MS liquid media for sugar treatment. MS media was prepared in 1L reagent bottle by following recipe given in Table 3.1. MS media was autoclaved and divided into Erlenmeyer 250 mL flasks. 1M stock solutions of glucose and sucrose were prepared and autoclaved, separately. For sugar treatment, total seven groups for each variety were formed including control, S 200 mM, S 400 mM, S 600 mM, G 200 mM, G 400 mM and G 600 mM group. Sugar solutions of sucrose (200mM, 400mM and 600mM) and glucose (200mM, 400mM and 600mM) were prepared from stock solution according to required amount. 20 mL of sugar solution of each concentration was added in each

250mL flask of each designated group containing 100 mL MS media in sterilized condition while autoclaved distilled water was added into flasks containing media for control group. All the prepared flasks of each group of two varieties were cotton plugged and autoclaved. Plants of Bari 2011 and PG 1247 grown in cigar rolls especially their roots were surface sterilized by soaking into 1% bleach solution for 1min followed by washing into autoclaved distilled water for 5min before their transfer to MS media containing sugar solutions. Each group contained at least three to five plants. Plants were kept in shaking incubator with speed of 100 rpm, at 25°C with continuous light exposure for 48 hr. Experiment was performed in highly sterilized conditions. Sampling was carried out after 48 hr of incubation while experiment was performed three times.

Table 3.1: Preparation of MS media

Sr.#	Ingredients	Quantity
1.	MS media	4.43g
2.	Sucrose	30g
3.	Distilled water	Amount required to make total volume of media equal to 1L

3.3- Total RNA Extraction from Leaves

3.3.1- Sampling for RNA Extraction

Expression analysis of flavonoid genes was carried out in the leaves samples of peanut plants of all the studied groups. Almost two to three leaves weighing ≈0.2g from each plant as tissue sample were plucked with help of sterilized forceps and collected into properly labeled autoclaved DNase and RNase free 1.5 mL Eppendorf tube followed by immediate freezing into liquid nitrogen. Three to five tissue samples were obtained from each studied plants. Tissue samples were either directly subjected to RNA extraction or stored at -80°C until RNA extraction. Thawing of tissue samples was highly discouraged to protect integrity and quality of extracted RNA.

3.3.2- TRIzol Method for RNA Extraction

TRIzol/Tri reagent RNA extraction, also known as guanidinium thiocyanate-phenol-chloroform extraction, was used to extract total RNA from leaves sample as described by (Jaakola et al., 2001). Frozen stored/fresh tissue samples were finely ground in liquid nitrogen by using sterilized autoclaved mortar and pestle. Samples were immediately transfer to liquid nitrogen to avoid thawing while retrieving samples from -80°C freezer and kept in it until grinding. Tissue sample per eppendorf tube was processed one by one. After grinding, 1mL Invitrogen™ TRIzol™ Reagent was directly added into mortar to make slurry that transferred into 1.5 mL Eppendorf tube. Slurry was homogenized by hand for 10s and incubated for 10 min on ice. Vigorous mixing was avoided. Incubated sample was centrifuged at 14,000 rpm for 10 min at 4°C. 200 µL of chloroform was added into collected supernatant into a 1.5 mL Eppendorf tube and mixed gently by turning the tube up and down for 15s. Mixture was incubated on ice for 5 min and centrifuged at 14,000 rpm for 5 min at 4°C. Two phases with a middle whitish layer of protein was formed. Amongst upper aqueous layer was collected into a new 1.5 mL Eppendorf tube followed by addition of 500µL ice-cold isopropanol. Samples were incubated at -20°C for 2 hr and then, centrifuged at 14,000 rpm at 4°C for 10 min. A white pellet was formed and supernatant was discarded carefully without losing pellet. 1mL 75% ethanol was used to wash the pellet. Pellet was retrieved by centrifugation at 9500 rpm at 4°C for min while supernatant was discarded once again. Pellet was air dried in surface sterilized fume hood to remove residual ethanol. Pellet was dissolved in 50 µL Tris-EDTA buffer (TE buffer), 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl, and evaluated through gel electrophoresis and spectrophotometry. RNA was stored at -80°C for further usage.

3.3.3- Gel Electrophoresis

Integrity and quality of extracted RNA was evaluated through agarose gel electrophoresis. 1% agarose gel was prepared by dissolving 0.7g Agarose 1-Biotechnology grade from bioWORLD into 70 mL 1X Tris base-acetic acid-EDTA buffer (TAE buffer). Microwave was used to completely dissolve agarose. Casting tray was set by placing spacers and combs. 4 µL ethidium was added into mixture and mixed properly. Mixture was added into preset caster and allowed to solidify. Combs

and spacer were removed without breaking the gel/gel well. Gel was placed into a gel medium sized gel tank filled with 1X TAE buffer. Gel was completely dipped in the buffer. 3 µL RNA was mixed with 0.5 µL 6X loading dye, blue by Thermo Fisher Scientific. Mixture was carefully loaded into designated well. Negative control (TE buffer use to dissolve RNA), positive control (confirmed RNA sample) and Quick-Load® 1 kb DNA Ladder-NEB were loaded into designated well, respectively. Gel electrophoresis was performed by providing electrical potential of 90 volt for 25 min. Gel was analyzed by using UV-transilluminator Biotop® to check presence/absence, size, intensity and quality of RNA. Photograph was taken for record.

3.3.4-Spectrophotometric Analysis

Concentration of RNA and impurity in the extracted sample was evaluated through spectrophotometric analysis by using NanoDrop™ 2000/2000c Spectrophotometer of Thermo Fisher Scientific. 1 µL TE buffer that was used to dissolve RNA was used for blanking the spectrophotometer while 1 µL of sample was used for analysis. Sample absorbance was recorded which provided sample concentration in ng/µL, 260/280 ratio and 260/230 ratio. 260/280 absorbance ratio was used to evaluate purity of nucleic acids (for DNA, generally accepted 260/280 ration is ~1.8 while, for RNA, it is ~2). 260/230 absorbance ratio was evaluated to check presence of contamination in the sample which expected to be in range of 2 to 2.2.

3.4- Conversion of mRNA into Single Stranded DNA (cDNA)

RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher Scientific with oligo dT primers was used to convert mRNA into single stranded complementary DNA. For 20 µL reaction, 1 µg of total RNA with 260/280 ratio of ~2 and 260/230 in the range of 2-2.2 was used. Reagents and their quantity used to synthesis cDNA has been given Table 3.2. NF water, primer and RNA was added in a labeled sterile 0.2 mL Eppendorf tube which was spin down and incubated in Applied Biosystems thermal cycler at 65°C for 5 min followed by chill on ice for at least 2 min. After addition of RNase inhibitor, dNTPs, 5X buffer and RT-enzyme (*Reverse tanscriptase*), tube was placed in thermal cycler, once again, for 60 min at 42°C followed by reaction termination at 70° C for 5 min. Tubes containing reaction mixtures were kept at -20°C for storage after confirmation of cDNA synthesis through

polymerase chain reaction (PCR) by using actin-housekeeping gene primers. Negative control for the reaction included all reagents except RNA.

Table 3.2: Reagents and their quantity used to synthesis cDNA

Sr.#	Ingredients	Quantity
1.	Nuclease Free water (NF water)	To 12.5 µL
2.	10 µM Oligo $(dT)_{18}$ primer	1
3.	RNA	1 µg
4.	5X reaction buffer	4 µL
5.	10 mM dNTPs	2 µL
6.	RiboLock RNase inhibitor (20U/µL)	0.5 µL
7.	RevertAid 200U/µL (*Reverse tanscriptase*)	1 µL
	Total Volume	**20 µL**

3.4.1- Confirmation of cDNA Synthesis through Actin PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed to confirm the synthesis of cDNA by using primers for housekeeping gene that was actin. All the PCR mentioned in Table 3.3 was added in labeled sterilized/autoclaved 0.2 mL PCR graded tube and spin down. Tubes containing reaction mixture for respective cDNA were placed in the Applied Biosystems thermal cycler with set conditions given in Figure 3.1 while primer sequence for reverse and forward actin primers has been provided in Supplementary Table 1. Negative control included all PCR reagents excluding cDNA while positive control has cDNA of confirmed sample. After completion of reaction, amplification was detected through gel electrophoresis using 2% agarose gel.

Table 3.3: PCR reagents

Sr.#	Ingredients	Quantity
1.	NF water	14.5 µL
2.	10X $(NH_4)_2SO_4$ buffer	2.5 µL
3.	25 mM $MgCl_2$	2.5 µL
4.	2.5 mM dNTPs	2 µL
5.	10 µM Actin forward primer	1 µL
6.	10 µM Actin reverse primer	1 µL
7.	cDNA	1 µL
8.	Taq DNA *Polymerase* (5 U/ µL) Thermo Fisher Scientific	0.5 µL
	Total Volume	**25 µL**

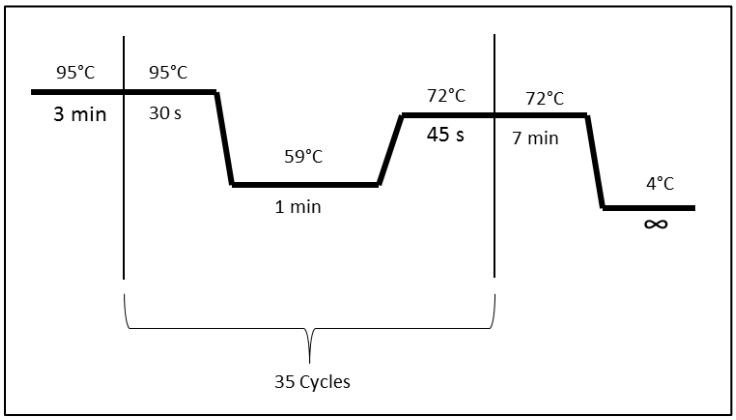

Figure 3.1: Conditions for RT-PCR using actin primers

3.4.1.1- Gel Electrophoresis

Amplification products including positive and negative controls were analyzed using 2% agarose gel which was prepared by dissolving 1g agarose in 50 mL 1X TAE buffer. 4 µL was of ethidium bromide has been used while preparing gel. Electric potential was set at 120 volt for 25 min for gel electrophoresis. Gel was analyzed in UV and picture was taken a record.

3.5- Expression Profiling of Flavonoid Biosynthetic Genes

Real-time PCR due to its sensitivity and reproducibility has become a preferred method for both absolute and relative gene expression. Relative gene expression of flavonoid biosynthetic genes was carried out to access the effect of light and sugar on their regulation in peanut leaves. Confirmed cDNA samples of all the studied were subjected real-time PCR analysis.

3.5.1- Primer Designing and Optimization

Primers sets for seven gene including actin, *AhCHS, AhCHI, AhFLS, AhDFR, AhF3H, AhLAR* and *AhANS* were designed from conserved region. Sequences for all the primer sets have been given in Supplementary Table 1. Primers were designed as to give the amplification of ~200 bp to enhance real-time PCR efficiency. The Tm for all sets of primers was optimized through RT-PCR by following above explained method for actin primers in section 3.4.1.

3.5.2- Real-time PCR

In real-time PCR method uses fluorescent reporter either specific or non-specific that accumulates with each amplification cycle to monitor real-time progress of PCR. Real-time PCR involving SYBR® Green-non-specific DNA binding dye was used to evaluate copy number of genes in form of Ct-value which is an intersection between threshold line and amplification curve. All the reagents except cDNA that are given in Table 3.4 were used to prepare master mix in an Eppendorf tube on ice. 13.5 µL master mix was added into each designated 7300 Real-Time PCR System PCR tube. 1.5 µL of diluted cDNA in NF water in 1/5 ratio was added in respective tube. Tubes were capped and placed in Applied Biosystems 7300 Real-Time PCR System for analysis with set condition given for each gene in Figure 3.2. Ct-value with dissociation and amplification curve was analyzed and recorded for relative gene expression by Livak method. Real-time PCR for each sample was performed in triplicate. Working dilution of cDNA for real-time was optimized though standard curve analysis by using actin primer.

Table 3.4: Reagents and their quantities used in real-time PCR

Sr.#	Reagents	Quantity for 1X
1.	NF water	5 µL
2.	Maxima SYBR Green/ROX qPCR Master Mix	7.5 µL
3.	Forward Primer	0.5 µL
4.	Reverse Primer	0.5 µL
5.	1/5 dilution of cDNA	1.5 µL
	Total Volume:	**15 µL**

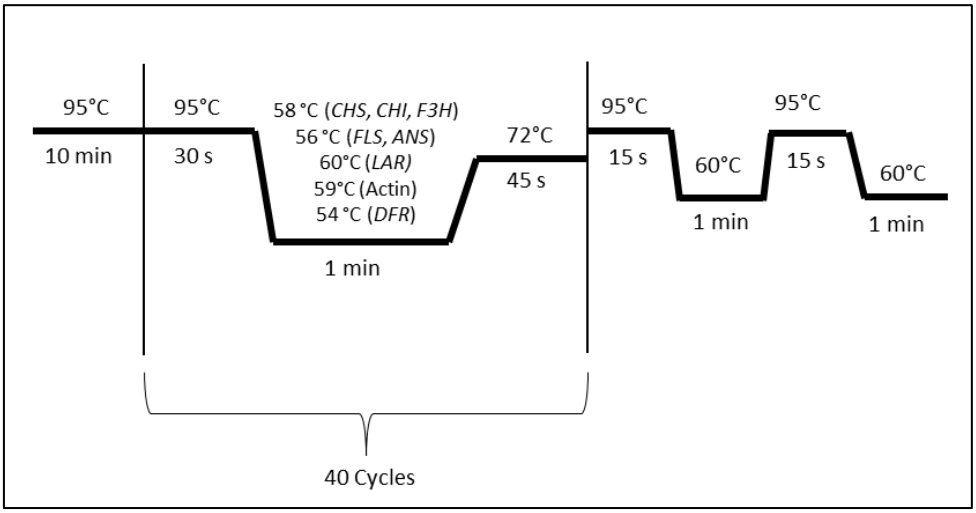

Figure 3.2: Conditions used for real-time PCR

3.5.1- Livak Method

Relative gene expression represents the expression of gene of interest in relation to internal control gene (Schmittgen and Livak, 2008). $2^{-\Delta\Delta CT}$ method, also known as Livak method (Livak and Schmittgen, 2001), has been used to calculate relative gene expression of gene of interest in correspondence to expression of actin. To calculate relative gene expression of each treated group following steps have been followed:

Step #1: Calculation of Mean and variance

In this step, mean with variance of Ct-value of gene of interest and their respective actin of treated group, control group were calculated, separately. For triplicate, standard deviation (S.D) was taken as variance while for replicate or more than that samples per group standard error mean (S.E.M) was taken s variance.

Step #2: Calculation of ΔCT

ΔCT is the difference of CT-value of gene of interest and actin of same sample i.e. ΔCT (Gene of interest – actin). Variance was calculated by taking square root of ($S1^2$-$S2^2$) while S1 is variance of gene of interest while S2 is variance of actin.

Step #3: Calculation of ΔΔCT

ΔΔCT is the difference of CT-value of treatment group and control group i.e. ΔΔCT (Treatment group – Control group). Variance was considered same as that ΔCT.

Step #4: Calculation of Mean Fold

Mean fold which is relative gene expression was calculated by following $2^{-\Delta\Delta CT}$ formula. For control group, 1 is considered to be mean fold. Variance was considered same as that ΔCT.

3.6- Statistical Analysis

Data was arranged and organized using Microsoft® office 2010 Excel. Inferential statistics was applied to calculate the significance of collected data by using GraphPad Prism® version 5.01, USA. Student t test was applied to calculate difference between control and treated group while overall variation was calculated through analysis of variance (ANOVA test). 0.05 *p*-value was considered to significant in the given analysis.

CHAPTER 4 : RESULTS

4.1- Plants Growth under Light and Sugar Treatment

Two peanut varieties, Bari 2011 and PG 1247, were selected for both treatments. Bari 2011 is an overall better performer than PG 1247. Among them, Bari 2011 is the commercially available peanut variety that is renowned for its sweeter seeds. Moreover, Bari 2011 is a resistant variety while PG 1247 is stress sensitive according to Oil Seed Program, NARC, Islamabad. Germination rate for both of the varieties were same. Seeds per pod were higher for Bari 2011 while seeds of PG 1247 were bigger than Bari with fragile shell.

4.1.1- Plants under Photoperiod Treatment

Plants grown under variable photoperiod durations have shown quite difference in growth. At day 7 of treatment, observed growth pattern was same for both varieties as shown in Figure 4.1. At day 15 of treatment, etiolation was observed in plants with 8 hr and 12 hr light treatment in both varieties while more lateral growth was observed in plant with 24 hr light treatment as compared to control as depicted in Figure 4.2 and 4.3.

4.1.2- Plants under Sugar Treatment

Plants for sugar stress were grown for at least two weeks after their transfer to in vertical cigar rolls. Seedlings were transferred to cigar roll after appearance of apical root and shoot. When plants were transferred to MS media containing sugar solutions they have ample sets of leaves as shown in Figure 4.4. After 2 day of treatment, plants were harvested for further processing.

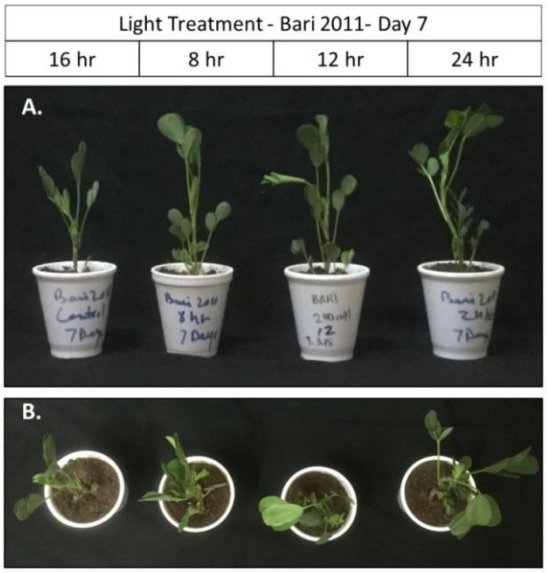

Figure 4.1: Peanut variety Bari 2011 growth at Day 7 under various durations of light exposure

(A) Side view of peanut plants that are showing approximately same growth **(B)** Ariel view of peanut plants that are showing approximately same growth

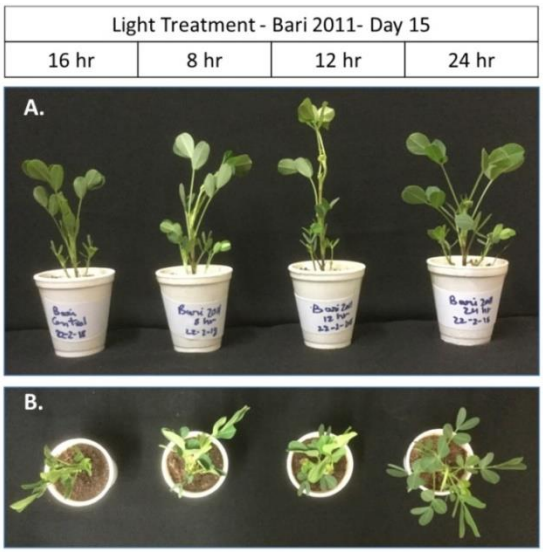

Figure 4.2: Peanut variety Bari 2011 growth at Day 15 under various durations of light exposure

(A) Side view of peanut plants that are showing etiolation in plants grown under 8 hr and 12 hr treatment **(B)** Ariel view of peanut plants that are showing more lateral growth in 24 hr treated plants compared to control

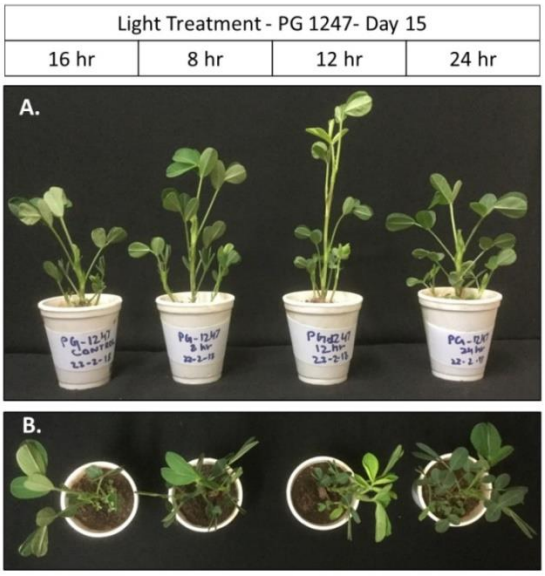

Figure 4.3: Peanut variety PG 1247 growth at Day 15 under various durations of light exposure

(A) Side view of peanut plants that are showing etiolation in plants grown under 8 hr and 12 hr treatment **(B)** Ariel view of peanut plants that are showing more lateral growth in 24 hr treated plants compared to control

Figure 4.4: Peanut variety Bari 2011 growth under sugar stress

(A) Peanut plant grown in cigar roll of filter paper **(B)** Plants transferred in MS media for sugar treatment

4.2- Analysis of Gel Electrophoresis

Agarose gel electrophoresis was performed to evaluate and visualize RNA isolated from leaves from both varieties under sugar and photoperiod, respectively. Moreover, amplicons of RT-PCR was, also, investigated through gel electrophoresis.

4.2.1- Evaluation of RNA integrity through Gel Electrophoresis

For RNA evaluation and visualization, 1% agarose gel was used for electrophoresis in TAE buffer. Three distinct bands were appeared including 5s RNA and 18s RNA band. 28s RNA band was absent this might be due to presence of break that is mostly present in some plant species. mRNA band was observed just below 18s RNA band in positive control and samples. Negative control showed zero band 1 Kb NEB ladder was used for comparison (Figure 4.5).

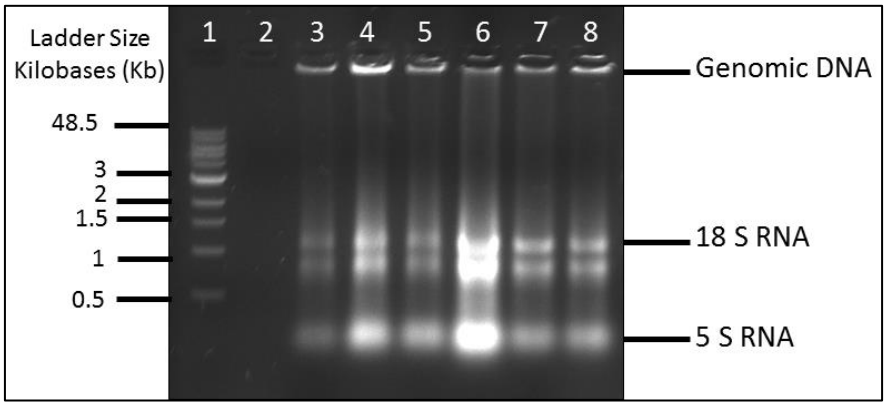

Figure 4.5: 1% agarose gel showing integrity of RNA Extracted from leaves of peanut varieties

Lane 1 contains 1Kb NEB ladder, Lane 2 has negative control, Lane 3 has positive control while lane 4,5,6,7,8 are representing genomic DNA, 18S and 5S RNA band with mRNA band below 18S.

4.2.2- Confirmation of Single Stranded DNA Synthesis

cDNA synthesis from RNA samples of both control and treated groups were used as template in RT PCR using primer of actin (housekeeping gene) for confirming cDNA synthesis. 2% agarose gel was used to evaluate amplification of actin gene. Band of approximately 200 bp was observed in positive control while no band was appeared in negative control (Figure 4.6. The samples which have shown distinct band of approximately 200 bp were considered as positive and while their respective cDNA template were selected for real time PCR analysis.

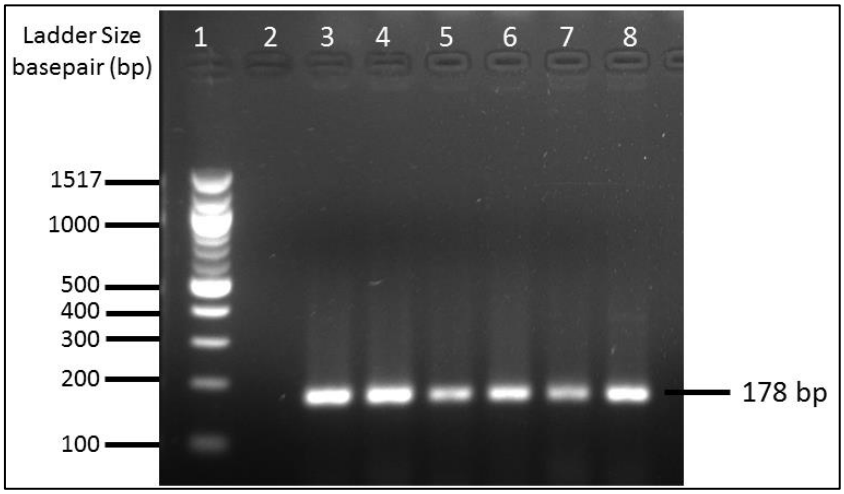

Figure 4.6: 2% agarose gel confirming first strand DNA synthesis from total RNA through RT-PCR of housekeeping gene i.e. Actin

Lane 1 contains 100 bp NEB ladder, Lane 2 has negative control, Lane 3 has positive control while lane 4,5,6,7,8 are representing successful amplification of actin and confirm cDNA synthesis

4.3- Expression Analysis of Flavonoid Biosynthetic Genes

Expression analysis of flavonoid biosynthetic genes in leaves of *A. hypogaea* has revealed differential expression of genes in different varieties. Real-time PCR analysis has, also, shown that expression of flavonoid genes is depend upon the type and intensity stimulus i.e. light and sugar.

4.3.1- Expression profiling of Flavonoid Biosynthetic Genes under Light Treatment

Differential expression of flavonoid biosynthetic genes has been observed in both selected varieties i.e. Bari 2011 and PG 1247. Even expression of genes has been found time dependent as genes at day 7 and day 15 have different pattern of fold changes. In Bari 2011, most of the genes showed higher fold changes in their expression on day 15 than day 7 while opposite expression pattern was observed in PG 1247.

4.3.1.1- Relative Gene Expression of Early Pathway Genes CHS

Expression analysis of *CHS* was performed for all the studied groups of selected two varieties i.e. Bari 2011 and PG 1247. It has shown very high expression in all treated group of PG 1247 at day 7. At day 15, expression of *CHS* became normal in group treated with 12 hr and 24 hr light while it was down regulated in group with 12 hr light treatment. Significant up-regulation of *CHS* was observed in all treated groups of Bari 2011 at day 15. At day 7 in Bari 2011, very high expression of CHS was observed in 24 hr light treated as compared to control while expression *CHS* was approximately equal to control group for 8 hr and 12 hr light treated groups (Figure 4.7).

4.3.1.2- Relative Gene Expression of Early Pathway Genes CHI

Real time PCR analysis of *CHI* gene showed its early expression. At day 7, *CHS* has shown significant positive regulation in all treated groups of Bari 2011 and PG 1247 as compared to control group (Figure 4.8). Group with 24 hr light treatment of Bari 2011 has shown maximum expression at day 7 while 12 hr light treated group of PG 1247 has depicted maximum expression. At day 15, expression of *CHS* was dropped to normal in all treated group of Bari 2011 and 24 hr light treated group of PG 1247 while expression was found to be significantly down regulated in 8 hr and 12 hr light treated groups of PG 1247.

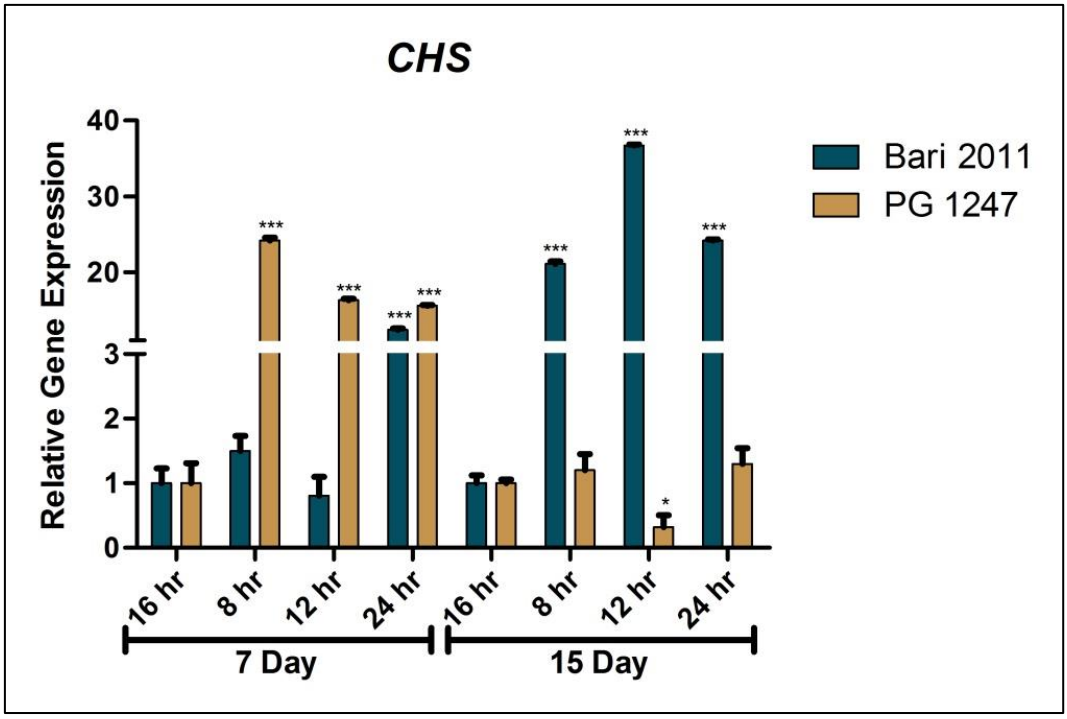

Figure 4.7: Expression analysis of *CHS* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting *p*-value less than 0.05, '**' *p*-value less than 0.01, '***' *p*-value less than 0.001.

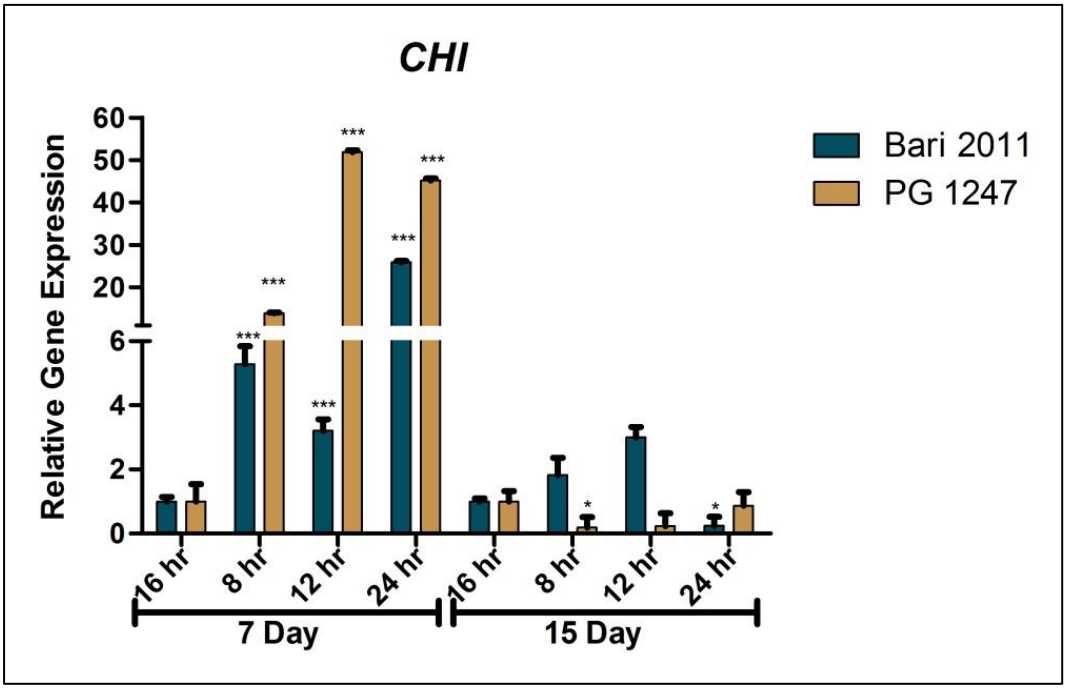

Figure 4.8: Expression analysis of *CHI* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting *p*-value less than 0.05, '**' *p*-value less than 0.01, '***' *p*-value less than 0.001.

4.3.1.3- Relative Gene Expression of F3H

F3H is an intermediate gene of flavonoid biosynthetic pathway whose expression was found to be up-regulated in all treated group of PG 1247 as compared to control group at day 7 while at day 15, its expression became normal in group with 8 hr light treatment and lower than control in groups with 12 hr and 24 hr light treatment. In Bari 2011, *F3H* was found down-regulated in group with 8 hr and 24 hr light treatment groups while at day 15, expression of *F3H* was up-regulated in same pattern as depicted at day 7 (Figure 4.9).

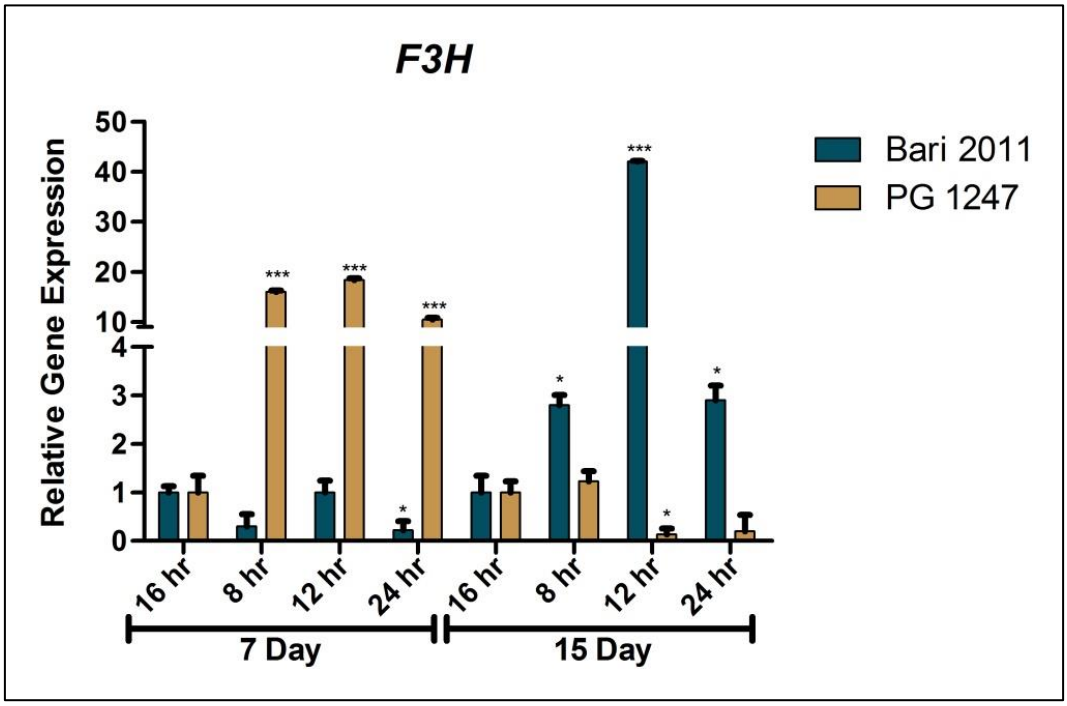

Figure 4.9: Expression analysis of *F3H* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting *p*-value less than 0.05, '**' *p*-value less than 0.01, '***' *p*-value less than 0.001.

4.3.1.4- Relative Gene Expression of FLS

FLS under varying photoperiods have showed differential expression in both varieties (Figure 4.10). In Bari 2011 at day 7, gradual up-regulation of *FLS* was observed with gradual increase in light among treated groups while at day 15, this expression was increased in groups with 8 hr and 12 hr light treatment and was decrease in 24 hr light treated group. In PG 1247, up-regulated *FLS* gene was observed in all treated groups as compared to control at day 7 while down regulation of it was observed in 8 hr and 12 light treated groups at day 15. 24 hr light treated group of PG 1247 has shown higher expression as compaed to control at day 15.

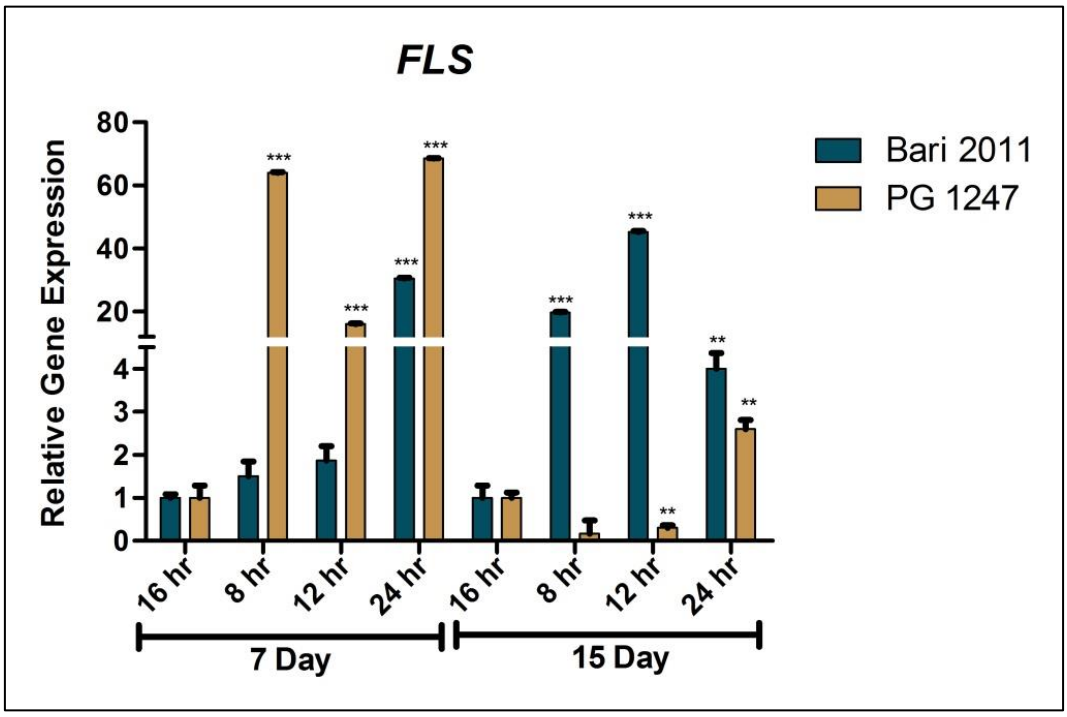

Figure 4.10: Expression analysis of *FLS* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting *p*-value less than 0.05, '**' *p*-value less than 0.01, '***' *p*-value less than 0.001.

4.3.1.5- Relative Gene Expression of DFR

Differential expression of *DFR* under 8 hr light treated group has revealed its higher expression in both varieties at day 7 and day 15 as compared to control group. At day 7, up-regulation of DFR was observed in 12 hr and 24 hr light treated group of PG 1247 while normal group like expression was observed in 12 hr and 24 hr light treated group of Bari 2011. A day 15, highest expression of DFR was observed in 12 hr light treated group of Bari 2011 while 12 hr light treated group of PG 1247 has showed same expression as that of control. Up-regulation was observed in both 24 hr light treated groups of Bari 2011 and PG 1247 (Figure 4.11).

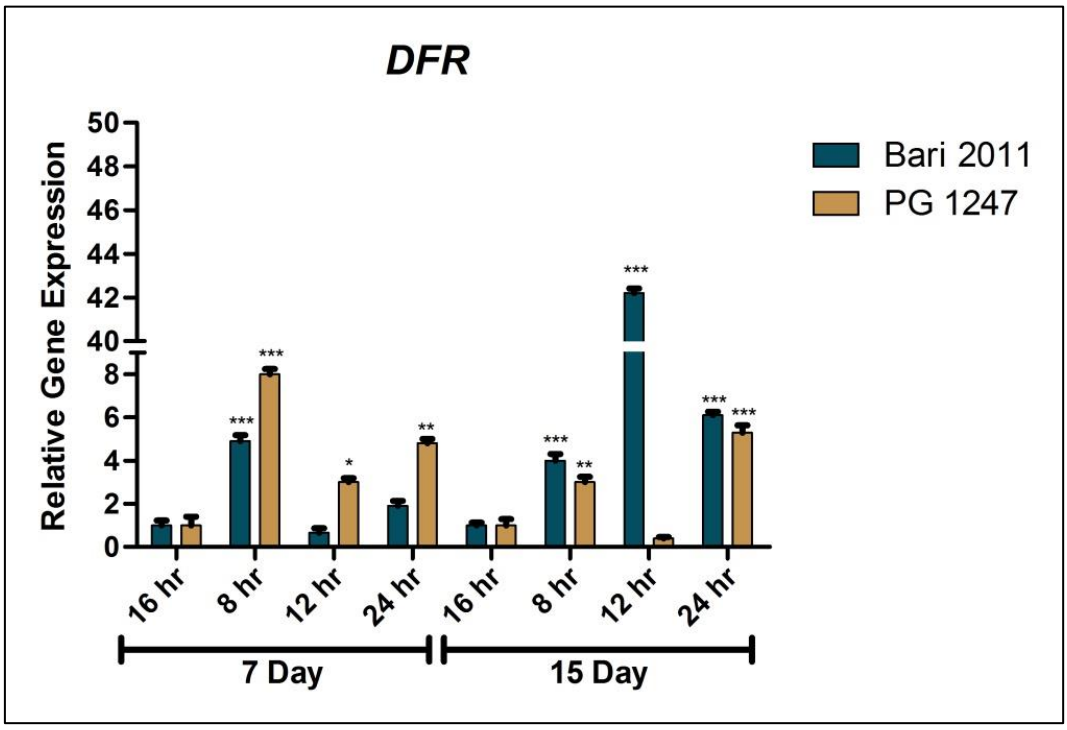

Figure 4.11: Expression analysis of *DFR* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting *p*-value less than 0.05, '**' *p*-value less than 0.01, '***' *p*-value less than 0.001.

4.3.1.6- Relative Gene Expression of LAR

Expression analysis of *LAR* under the influence of photoperiod has revealed higher expression of *LAR* in all treated group of PG 1247 at day 7 while all treated groups of Bari 2011 at day 15 as compared to control group (Figure 4.12). Maximum expression of *LAR* was observed at day 7 in 8 hr light treated group of PG 1247 while Bari 2011 has showed maximum expression at 12 hr light treatment at day 15. In Bari 2011, expression of all treated groups was found approximately equal to control at day 7 while same was observed for all treated group of PG 1247 at day 15.

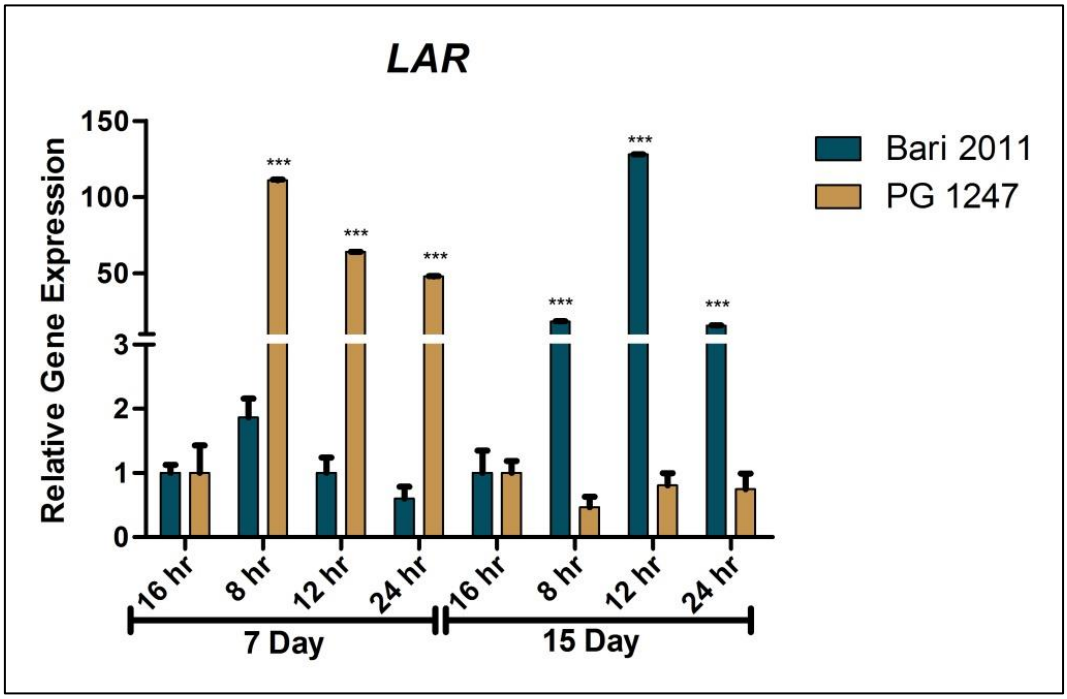

Figure 4.12: Expression analysis of *LAR* gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. p-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.1.7- Relative Gene Expression of ANS

Expression analysis of *ANS* in Bari 2011 and PG 1247 has revealed differential expression at both days in all treatment groups (Figure 4.13). At day 7, all treatment groups of PG 1247 have shown higher expression of ANS as compared to control while 8 hr and 12 hr light treated groups have depicted up-regulation in Bari 2011. At day 15, up-regulation of ANS was only observed in 8 hr and 12 hr light treatment of Bari 2011.

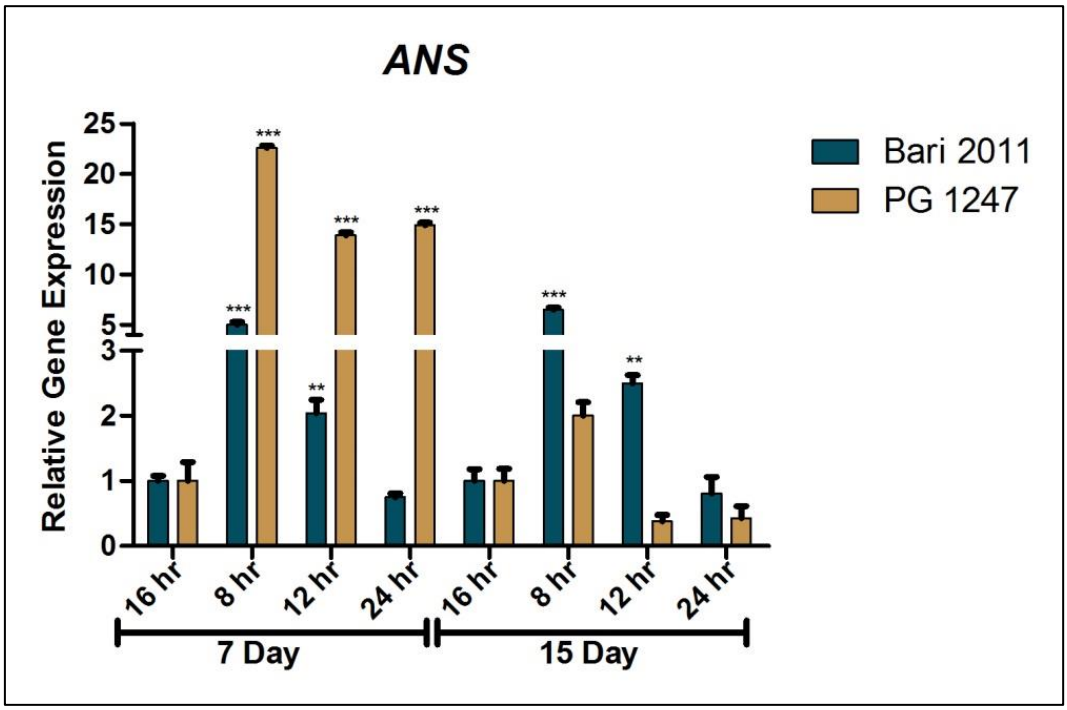

Figure 4.13: Expression analysis of ANS gene under photoperiod treatment in peanut varieties, Bari 2011 and PG 1247, at day 7 and day 15.

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.1.8- Expression of Flavonoid Biosynthetic Genes under Light Treatment in Bari 2011 at Day 7 and Day 15

Expression analysis of flavonoid biosynthetic genes in Bari 2011 at day 7 and day 15 has revealed the behavior of these under varying light duration (8 hr, 12hr, 16hr and 24 hr). 16 hr light treated group was termed as control group for analysis. Most of the genes were found up-regulated at day 15 than at day 7 as depicted in (Figure 4.14 and 4.15). *CHI, F3H* and *ANS* were found to be up-regulated at day 7 while *CHS, F3H, FLS, DFR, LAR* and *ANS* were found up-regulated at day 15 in 8 hr light treated group. In 12 hr group, *CHI* and *ANS* were up-regulated at day 7 while all other genes except *CHI* were up-regulated at day 15. In 24 hr group, *CHI, CHS* and *FLS* have increased expression as compared to control at day 7 while at day 15, expression of *CHS, F3H, FLS, DFR* and *LAR* were up-regulated and expression of *CHI* was significantly lowered.

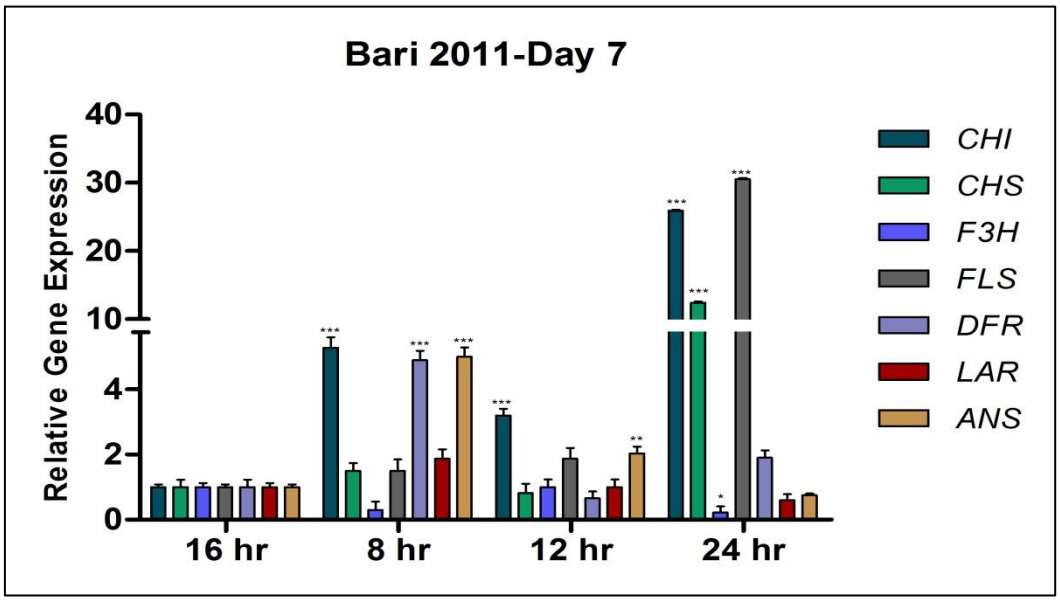

Figure 4.14: Expression analysis of flavonoid biosynthetic genes under photoperiod treatment in Bari 2011-a peanut variety at day 7

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

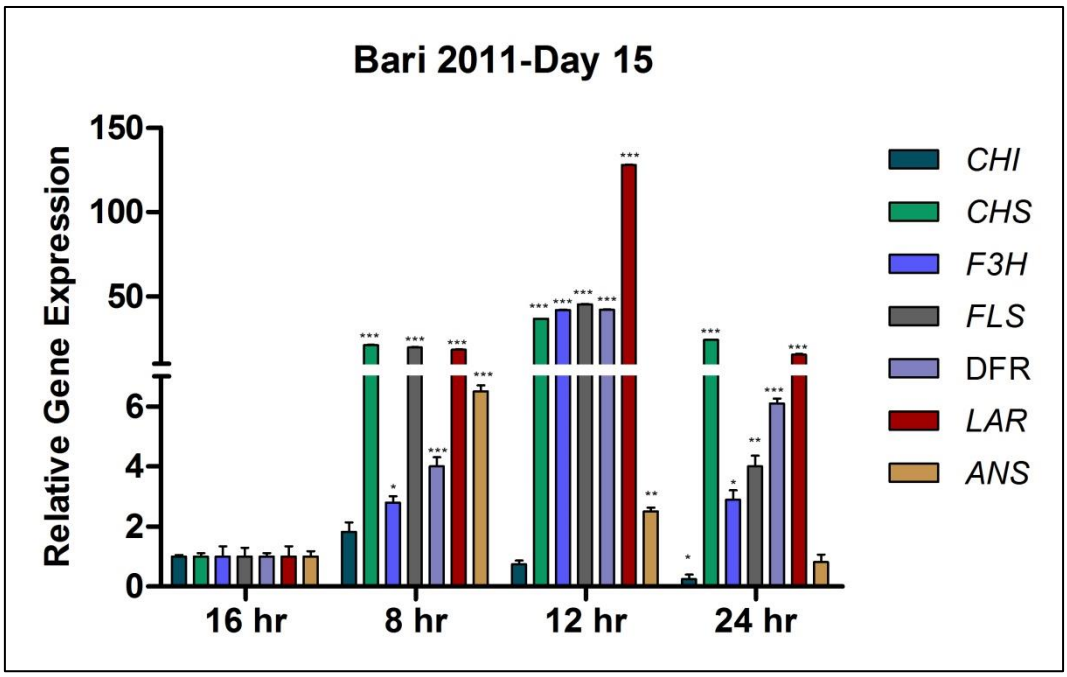

Figure 4.15: Expression analysis of flavonoid biosynthetic genes under photoperiod treatment in Bari 2011-a peanut variety at day 15

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.1.9- Expression of Flavonoid Biosynthetic Genes under Light Treatment in PG 1247 at Day 7 and Day 15

Expression analysis of flavonoid biosynthetic genes in PG 1247 at day 7 and day 15 has revealed the behavior of these under varying light duration (8 hr, 12hr, 16hr and 24 hr). 16 hr light treated group was termed as control group for analysis. Most of the genes were found up-regulated at day 7 than at day 15 as depicted in (Figure 4.16 and 4.17).

All the genes of flavonoid biosynthetic pathway genes (*CHI, CHS, F3H, FLS, DFR, LAR* and *ANS)* have shown up-regulation in all treated groups at day 7. Among them, *DFR* has shown lowest expression in all treated groups but it expression was higher as compared to control group. At day 15, expression of *CHS, F3H* and *ANS* was approximately equal to normal in 8 hr light treated group while expression of *CHI, FLS* and *LAR* was down-regulated. Expression of *DFR* in 8 hr and 24 hr treated group was up regulated at day 15. Approximately all genes were down-regulated in group with 12 hr light at day 15. In 24 hr light group, most of the genes have same expression as that of control except *ANS* and *DFR* that have higher expression.

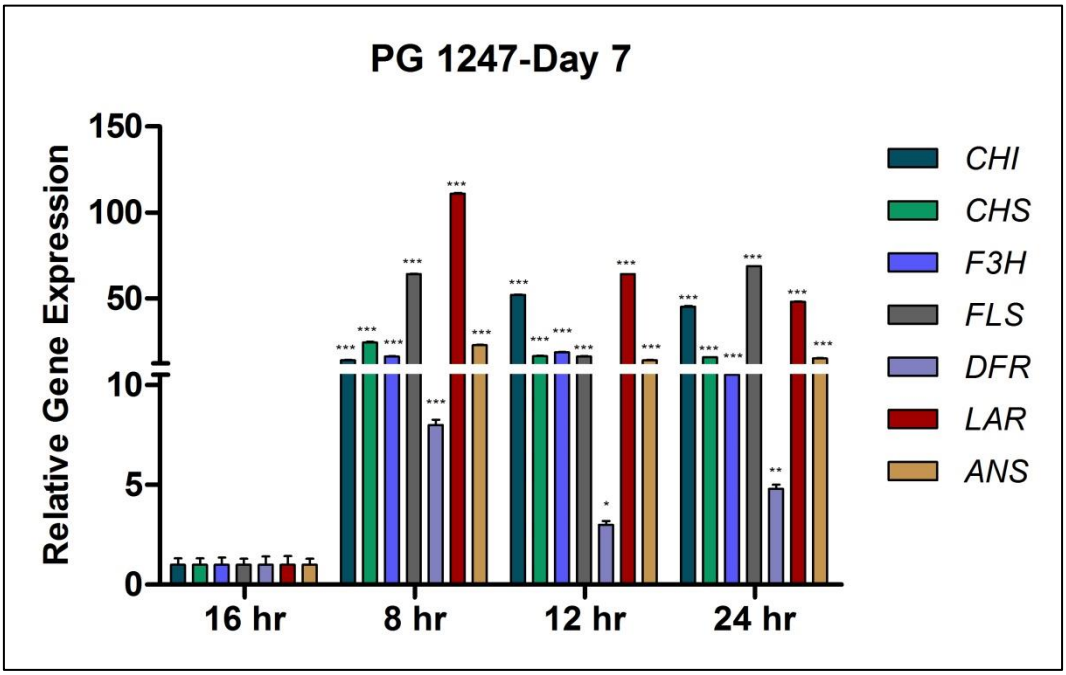

Figure 4.16: Expression analysis of flavonoid biosynthetic genes under photoperiod treatment in PG 1247-a peanut variety at day 7

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

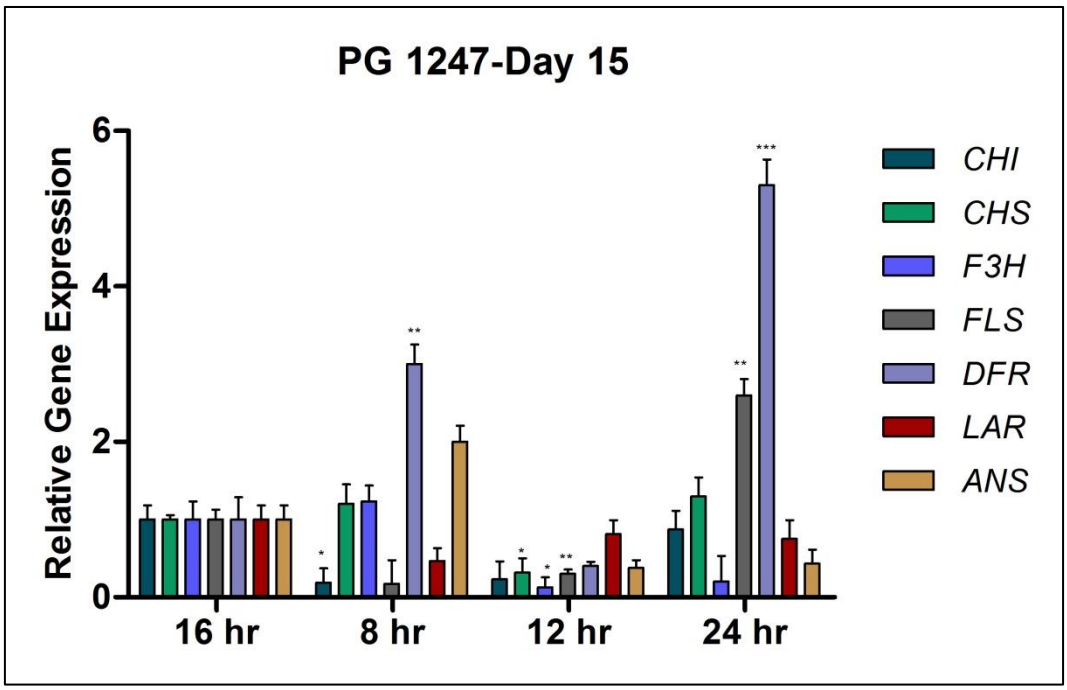

Figure 4.17: Expression analysis of flavonoid biosynthetic genes under photoperiod treatment in PG 1247-a peanut variety at day 15

Expression analysis for each group has been given as mean ± standard error mean. Group with 16 hr light has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2- Sugar influencing Transcription of Flavonoid Biosynthetic Genes

Expression analysis of flavonoid biosynthetic genes in Bari 2011 and PG 1247 under the influence of sucrose and glucose at various concentrations has been evaluated. Analysis showed that expression of these genes is not as much genotype dependent as that was in case of photoperiod. Moreover, expression was found to be specific for specific sugar treatment.

4.3.2.1- Relative Gene Expression of CHS

CHS expression analysis in Bari 2011 has revealed its up-regulation in 200 mM and 400 mM sucrose treated plants as compared to control while sucrose at 600mM and glucose at all concentrations have down-regulated its expression. In PG 1247, *CHS* was highly expressed in 400 mM and 600 mM sucrose treated plants while for all the concentrations of glucose and 200 mM sucrose its expression was approximately equal to control group (Figure 4.18).

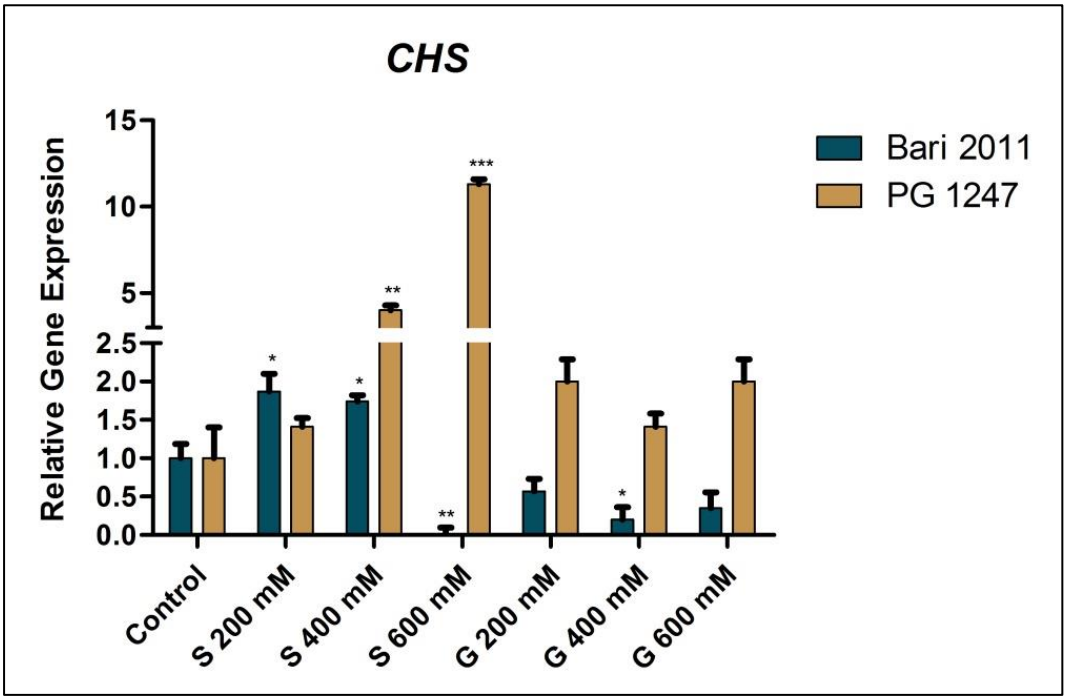

Figure 4.18: Expression analysis of *CHS* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247.

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.2- Relative Gene Expression of CHI

CHI expression was found to up regulated in plants of Bari 2011 treated with 600 mM sucrose (Figure 4.19). In PG 1247, *CHI* enhanced expression was observed in plants treated with 400 mM and 600 mM sucrose. Expression was remained as that of control group in plants of PG 1247 treated with 200 mM sucrose, 200 mM and 400 mM glucose. Down regulation of *CHI* was observed in plants of Bari 2011 treated with 200 mM, 200 mM sucrose and 200 mM, 400 mM, 600mM glucose while same was observed for PG 1247 plants treated 600 mM glucose.

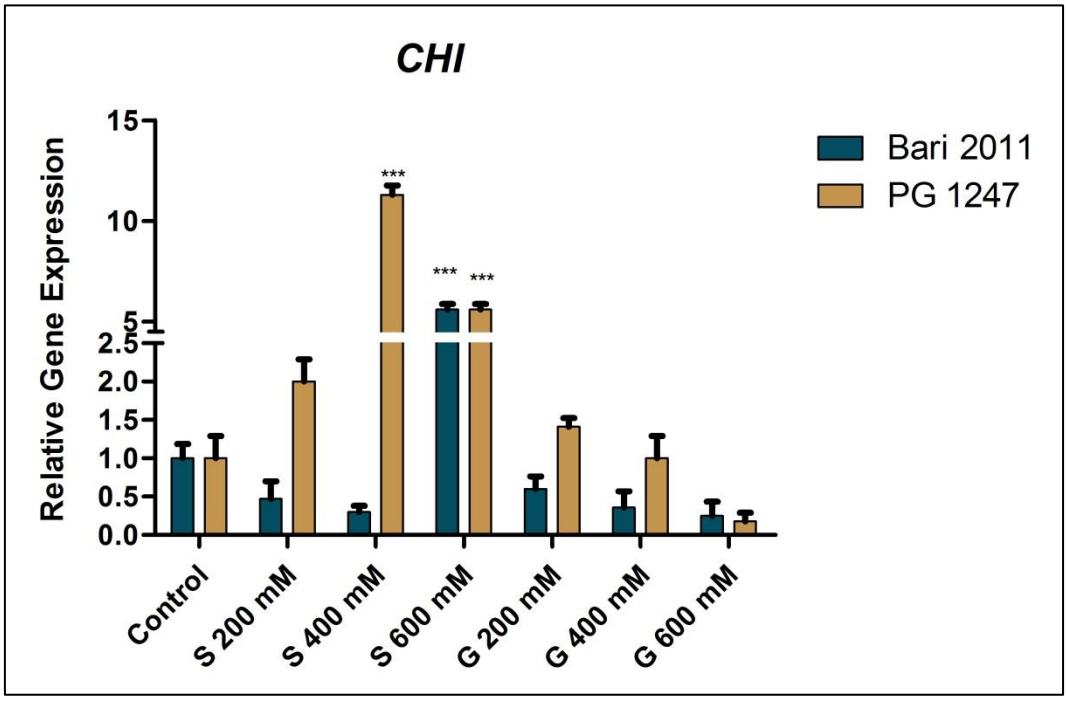

Figure 4.19: Expression analysis of *CHI* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247.

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.3- Relative Gene Expression of F3H

F3H expression pattern was found quite opposite in treated groups of Bari 2011 and PG 1247 (Figure 4.20). Higher expression of *F3H* was observed for all the concentrations of sucrose and 200mM glucose as compared to control in PG 1247. Maximum expression of *F3H* was observed in PG 1247 plants treated with 200 mM glucose. In Bari 2011, *F3H* showed down regulation in all treated groups. Maximum down regulation was in plants treated with 600 mM glucose in Bari 2011.

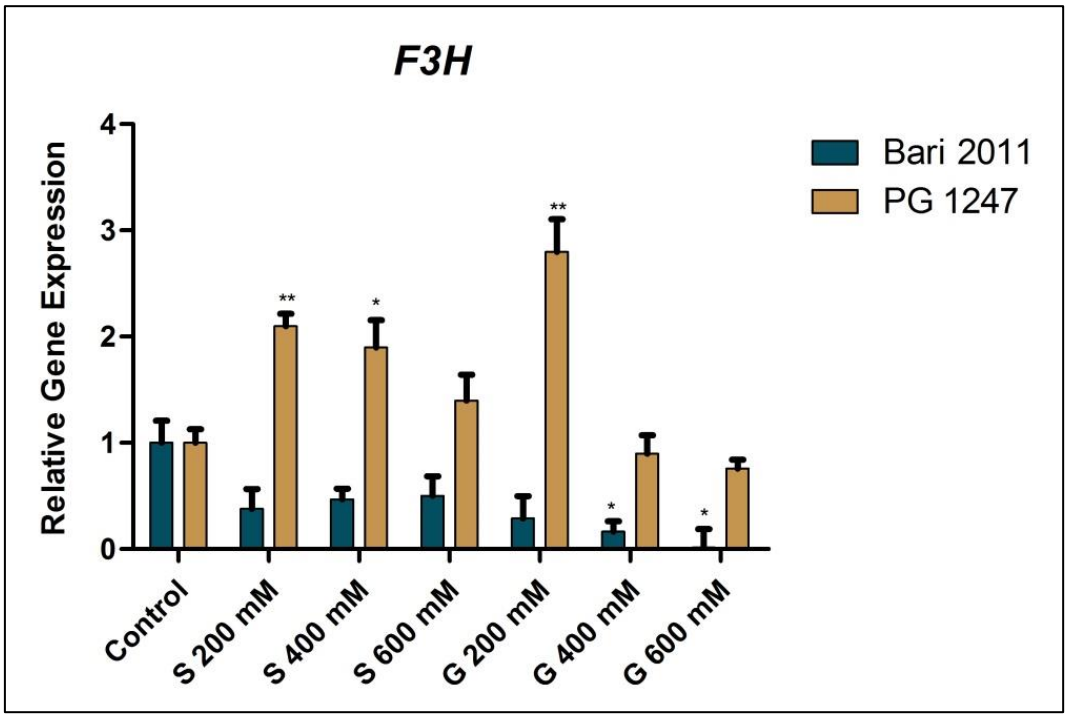

Figure 4.20: Expression analysis of *F3H* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.4- Relative Gene Expression of FLS

FLS expression pattern for glucose treated groups were same for both varieties while quite distinct pattern of expression was observed in sucrose treated plants (Figure 4.21). In PG 1247, gradual increase in expression of *FLS* was observed with increase in sucrose concentration while significant down regulation was observed in all plants treated with glucose for all concentrations. In Bari 2011, plants treated with 400 mM sucrose have same expression as that control group while all other treated groups have showed decreased expression of *FLS* as compared to control group.

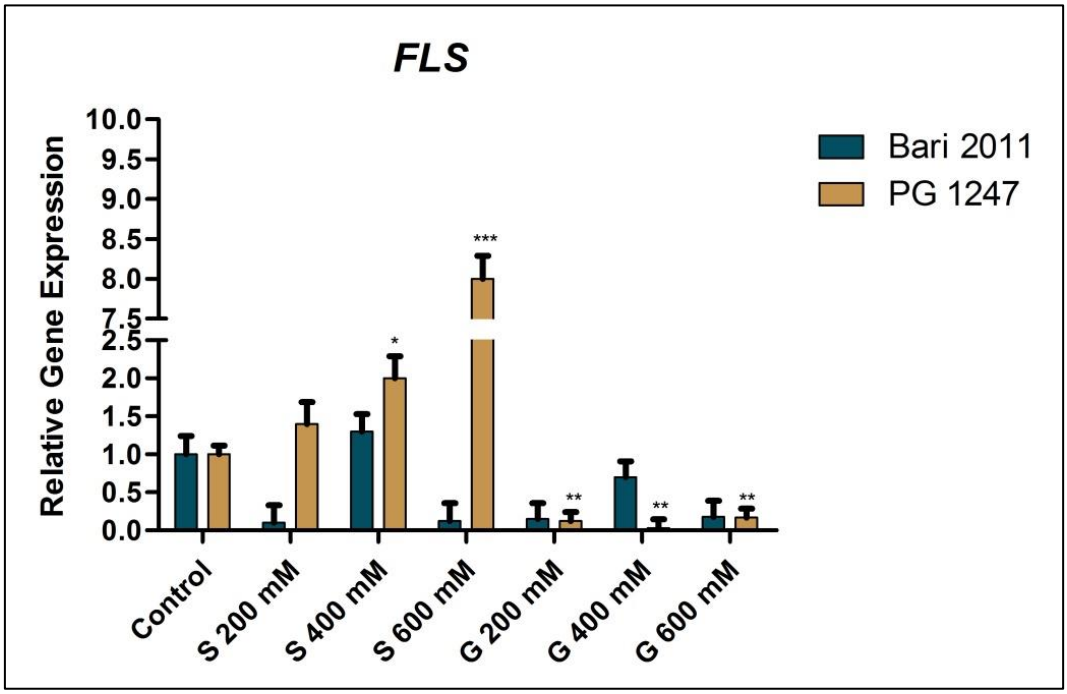

Figure 4.21: Expression analysis of *FLS* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.5- Relative Gene Expression of DFR

Bari 2011 and PG 1247 have depicted quite distinct pattern of *DFR* expression among studied groups (Figure 4.22). In PG 1247, increased expression of *DFR* was observed in plants treated with 400 mM, 600 mM sucrose and 600 mM glucose while *DFR* expression was found unaffected in plants treated with 200 mM sucrose. Down regulation of DFR was observed in 600 mM sucrose, 400 mM glucose treated plants of PG 1247. In Bari 2011, *DFR* expression was increased in plants treated with 200 mM and 400 mM glucose while its decreased expression was in all sucrose treated groups.

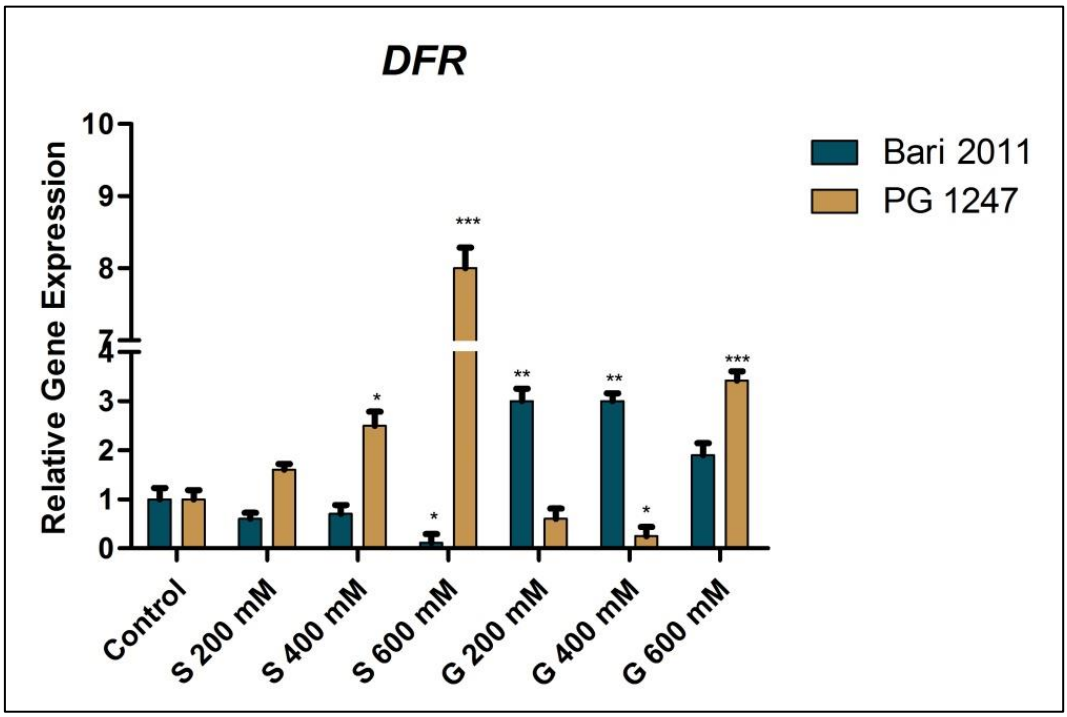

Figure 4.22: Expression analysis of *DFR* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.6- Relative Gene Expression of LAR

LAR expression was enhanced by treatment sucrose while inhibited by glucose in plants of PG 1247. In Bari 2011, 600 mM glucose and 400 mM sucrose have enhanced the expression of *LAR* while all other sucrose and glucose treatments have down-regulated the expression of *LAR* as compared to control plants (Figure 4.23).

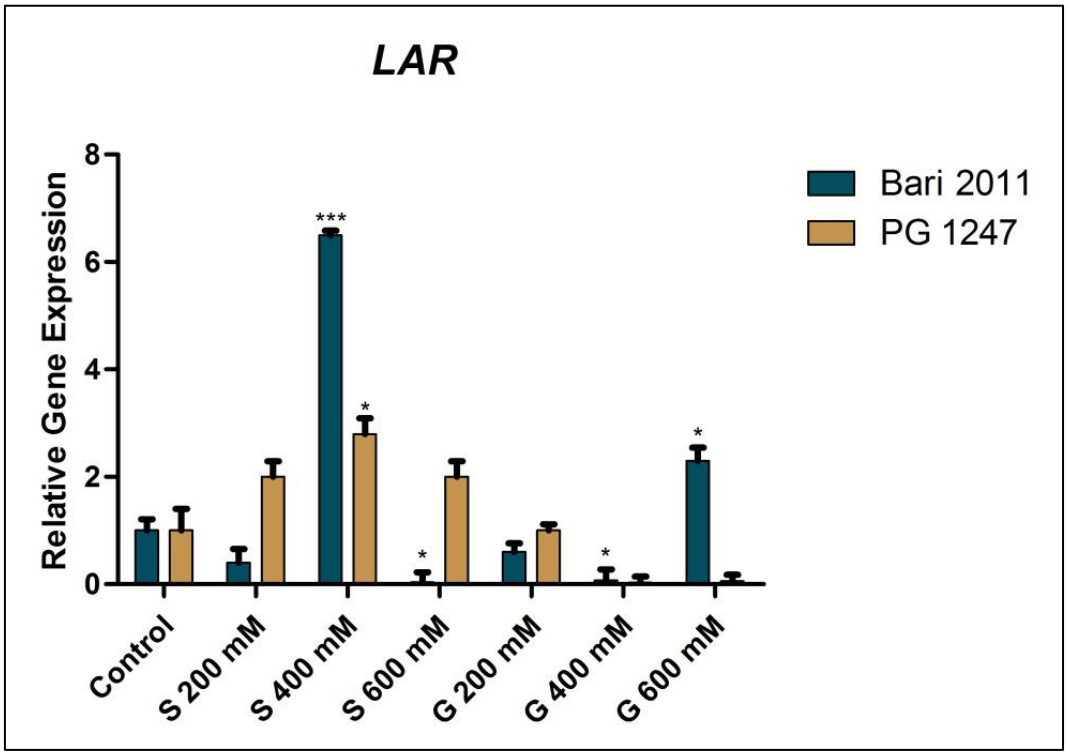

Figure 4.23: Expression analysis of *LAR* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.7- Relative Gene Expression of ANS

ANS expression was up-regulated for all the concentrations of sucrose in both varieties, Bari 2011and PG 1247 (Figure 4.24). Expression was highest in plants treated with 400 mM sucrose in Bari 2011 and 200 mM sucrose in PG 1247. Expression *ANS* was mostly was remained unaffected in case of glucose treated plants while slight up regulation of *ANS* was observed plants treated with 400 mM glucose as compared to control group.

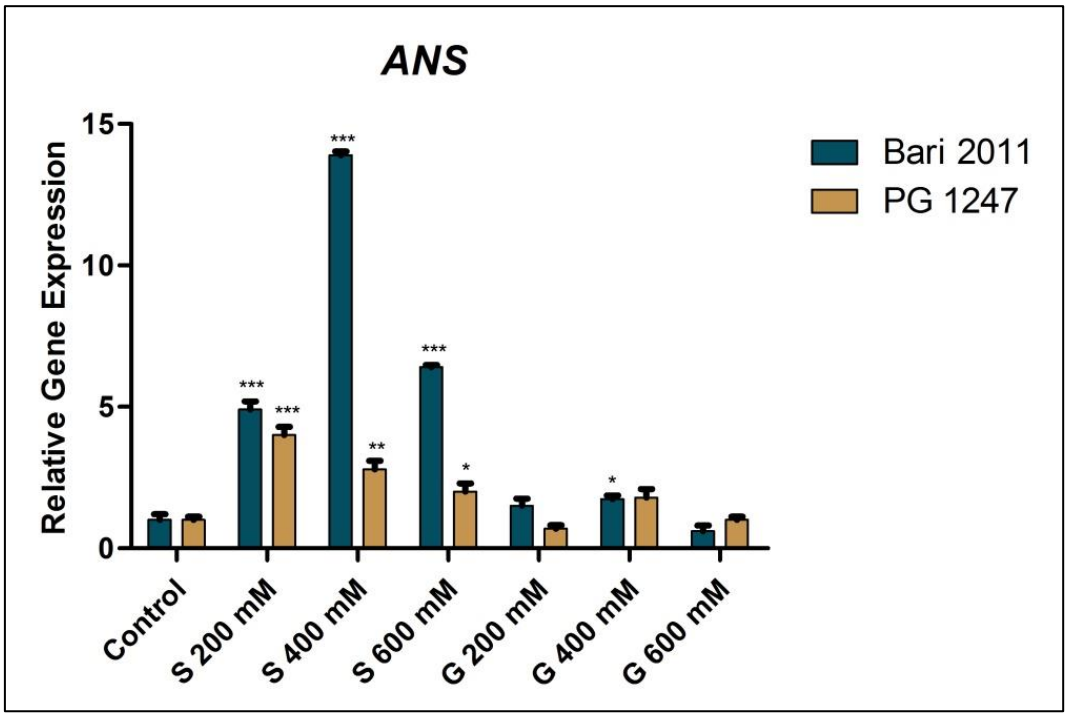

Figure 4.24: Expression analysis of *ANS* gene under sugar stress in peanut varieties, Bari 2011 and PG 1247

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.8- Expression of Flavonoid Biosynthetic Genes under Sugar in Bari 2011

Effects of sucrose and glucose on flavonoid biosynthesis genes of Bari 2011 are quite evident by analyzing Figure 4.25 and 4.26. Both sugars control the pathway in quite distinct manner. In sucrose treated Bari 2011 plants, concentration 400 mM was found to be more influential for flavonoid production as up regulated expression of CHS, LAR, ANS. At 200 mM concentration of sucrose, CHS and ANS was observed up-regulated while FLS was found down regulated. Most of the genes (CHS, LAR, DFR, F3H and FLS) have down regulated while expression of ANS and CHI was up- regulated by 600 mM sucrose treatment. Only the expression of ANS and DFR were up-regulated by glucose at all concentrations while LAR was only expressed at 600 mM glucose treatment. CHI, CHS, FLS and F3H were down regulated by glucose. Expression of flavonoid genes were found to specific for sugar in Bari 2011 as shown in Figure 4.27.

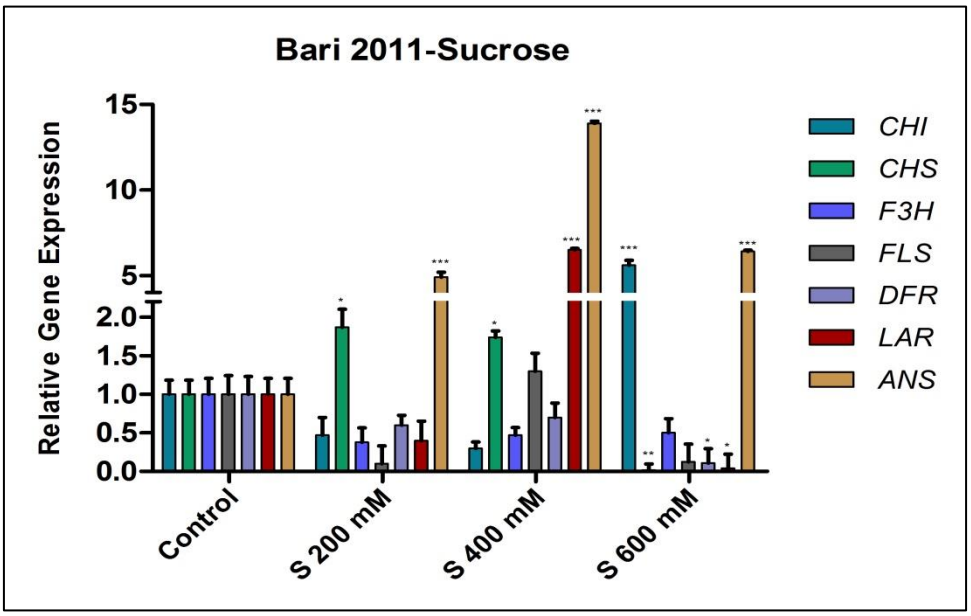

Figure 4.25: Expression analysis of flavonoid biosynthetic genes under sugar treatment in Bari 2011-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

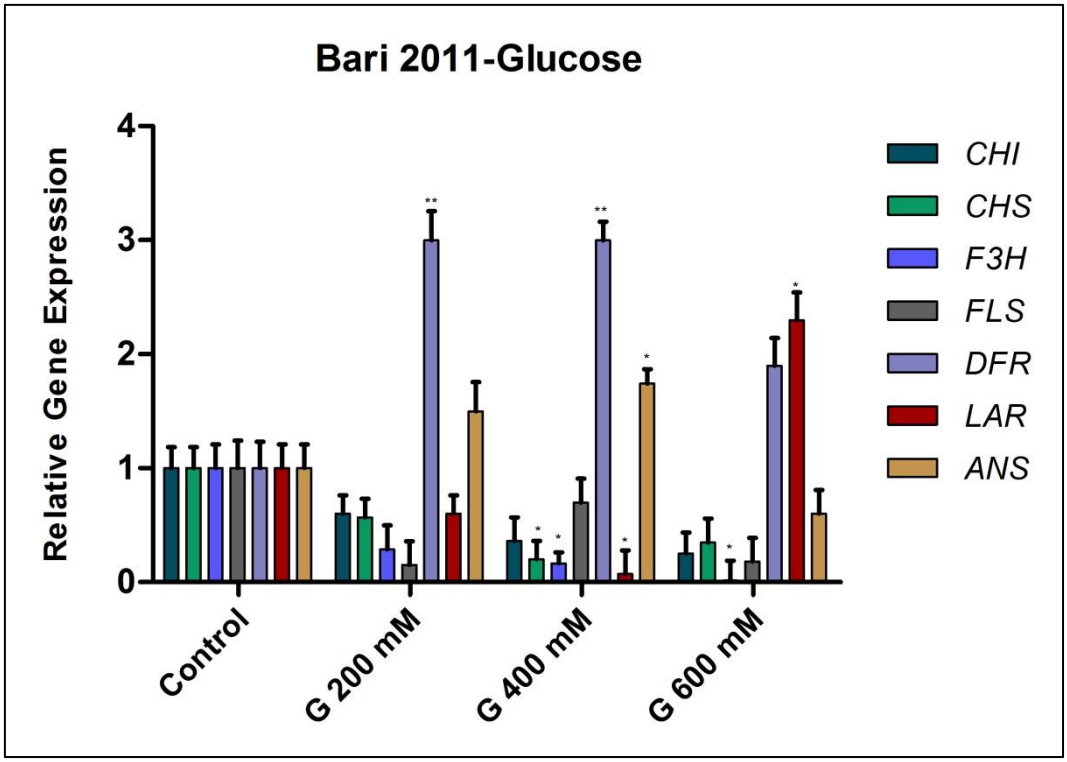

Figure 4.26: Expression analysis of flavonoid biosynthetic genes under sugar treatment in Bari 2011-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

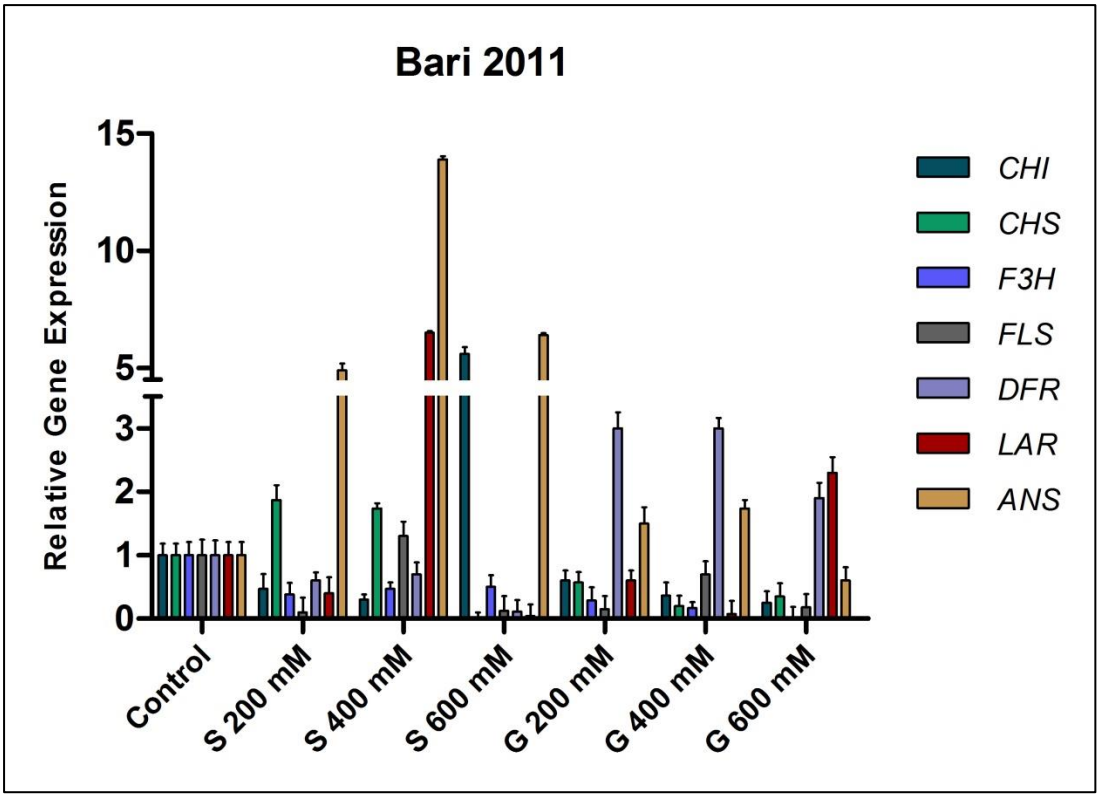

Figure 4.27: Expression analysis of flavonoid biosynthetic genes under sugar treatment in Bari 2011-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

4.3.2.9- Expression of Flavonoid Biosynthetic Genes under Sugar in PG 1247

Effects of sucrose and glucose on flavonoid biosynthesis genes of PG 1247 were quite evident by analyzing Figure 4.28 and 4.29. In plants treated with 200 mM sucrose, F3H and and ANS was up- regulated while expression of other genes was remain unaffected. All the genes were regulated in plants with 400 mM sucrose while CHI, CHS, FLS, DFR and ANS were observed to be up-regulated in plants treated with 600 mM sucrose.

In plants treated with 200 mM glucose, F3H was only one that was significantly up-regulated while FLS was significantly down regulated. In plants with 400 mM glucose treatment, expression of FLS, DFR and LAR were significantly decreased as compared to control. In plants with 600 mM glucose treatment, DFR was up-regulated while CHI, FLS and LAR were down regulated. Expression of flavonoid genes were found to specific sugar with respect to type and quantity in PG 1247 as shown in Figure 4.30.

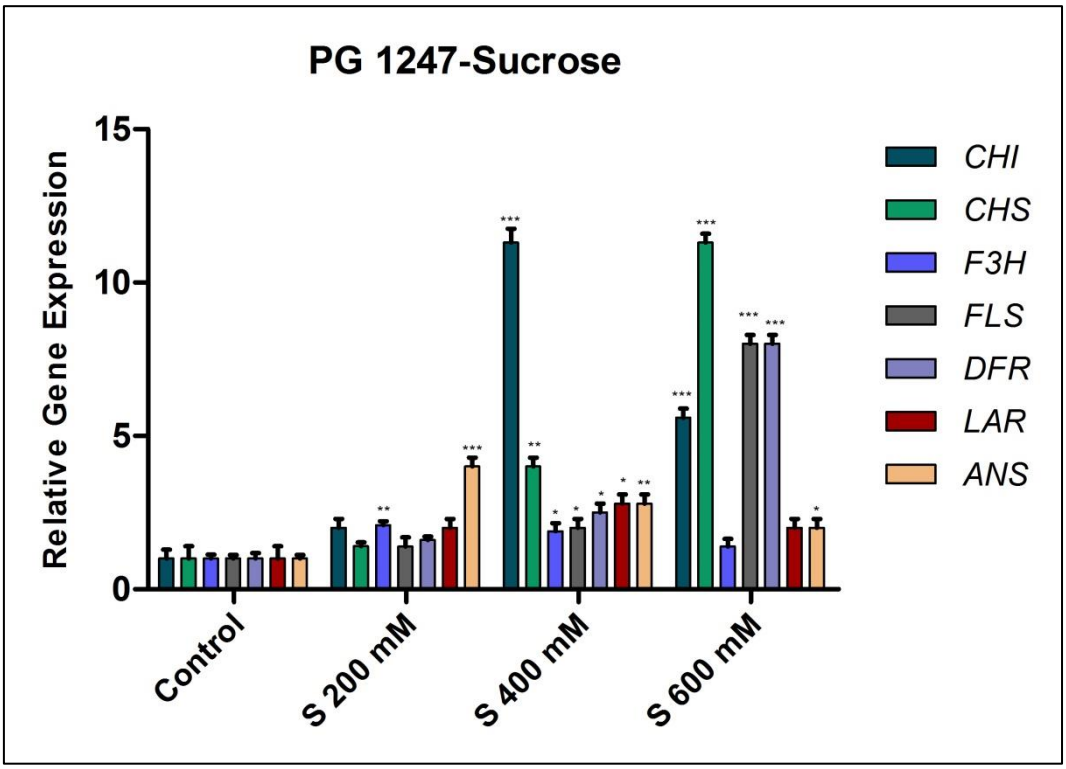

Figure 4.28: Expression analysis of flavonoid biosynthetic genes under sugar treatment in PG 1247-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

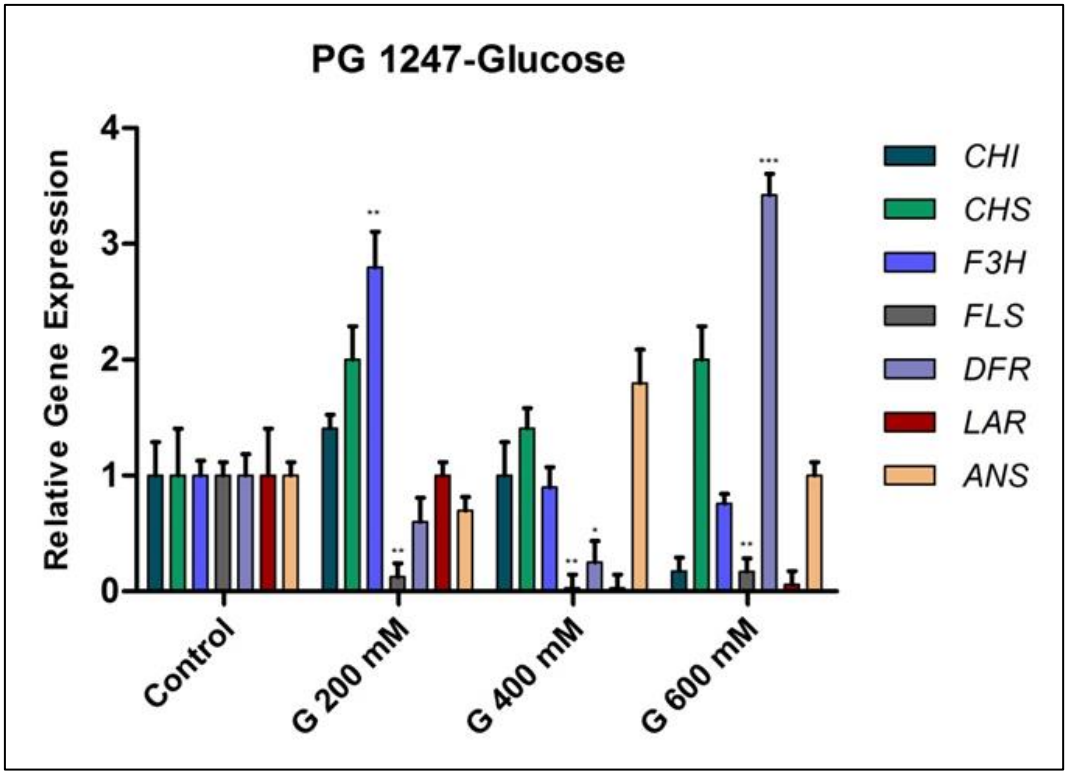

Figure 4.29: Expression analysis of flavonoid biosynthetic genes under sugar treatment in PG 1247-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group. *p*-value smaller than 0.05 will considered as significant. '*' is depicting p-value less than 0.05, '**' p-value less than 0.01, '***' p-value less than 0.001.

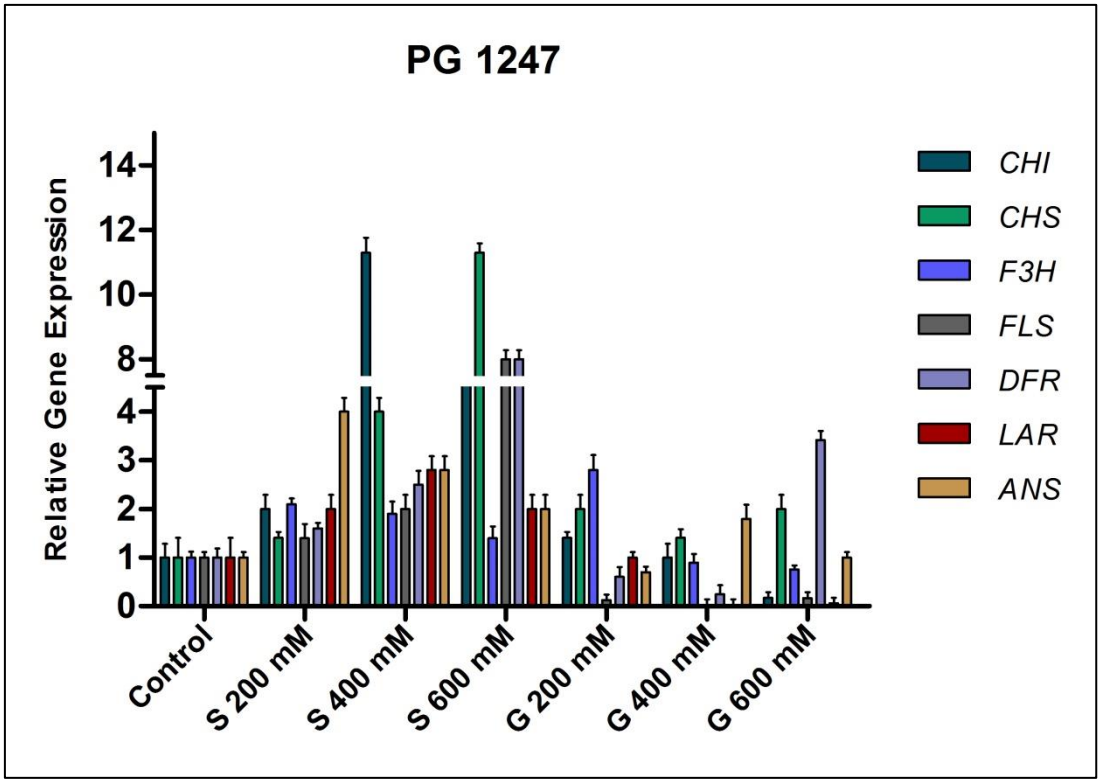

Figure 4.30: Expression analysis of flavonoid biosynthetic genes under sugar treatment in PG 1247-a peanut variety

Expression analysis for each group has been given as mean ± standard error mean. Group with no sugar treatment has been designated as control group while all other groups considered as treated group.

CHAPTER 5 : DISCUSSION

Arachis hypogaea (peanut) is a allotetraploid hybrid (2n = 4× = 40) that belongs to legume family (Bertioli, et al., 2015). Peanut is an oilseed 'Kharif crop' and used in crop rotation program due to its nitrogen fixation ability (Reddy, et al., 2011). Besides promising fatty acid profiling and essential micronutrients, peanut plants enclose potential bioactive phytochemicals including flavonoids (Guchi, 2015). Kernels of peanut are consumed either as roasted, raw or boiled. Extracted oil from peanut used for cooking while, after oil pressing, oilcake find is application as fertilizer, feed for animal and raw material in industry. Multiple applications of peanut in agriculture and industry have made it a potential cash and functional crop for developing and developed countries (Cherif, et al., 2010; Duncan, et al., 2006; Sim, et al., 2012).

Peanut production, like other plants, is adversely affected by both biotic and abiotic stresses. Peanut activates stress signaling that regulate physiological and phytochemical changes for stress tolerance. Accumulation of phytochemicals during stress conditions is an important stress tolerance mechanism in plants (Winkel-Shirley, 2002). Among all the phytochemicals, flavonoids held a prominent position to protect the plant during stress as they have protective roles against temperature variation, pathogen attack, UV stress, light intensity, limitation of nutrients, drought, humidity and herbicide application (Fini, et al., 2011). In a plant, flavonoids production is controlled by various developmental and environmental stimuli. Flavonoids accumulation in plants has been widely studied in tissue specific manner at both transcriptome and metabolome level under the influence of sequential and spatial and signals (Dixon and Paiva, 1995; L. P. Taylor and Grotewold, 2005). Comprehensive understanding of flavonoid biosynthetic pathway including its genes and enzymes has been focused in crop development due its obligatory benefits (Butelli et al., 2008; Luo et al., 2008).

Light and sugars play crucial roles in flavonoid production. In normal conditions, flavonoids biosynthesis is adjusted to produce those flavonoids that take

part in the flavonoid production. Enhanced phenolic compounds production has been found to associated with longer light duration (A. Taylor, 1965). Generally, it is believed that longer light period in photoperiod duration can induce flavonoid production because it increases light related energies in plants (Koyama, et al., 2012; A. Taylor, 1965). Moreover, photoreceptors create complex light response and regulate flavonoid production under limited and excess of light (Casal, 2013; M. Chen et al., 2004; Neugart et al., 2016). Sugars act as both energy and signaling molecules and help the plant to endure stress by regulating phytoprotectants. Furthermore, high level of soluble sugars in plant tissue and organelles have, also, been found to encourage flavonoid accumulation apart from their stimulation through ROS system (Bolouri-Moghaddam, et al., 2010).

Expression analysis of flavonoid biosynthetic genes under the influence of photoperiod and sugar in various varieties of *A. hypogaea* has been conducted to have a detailed insight of the flavonoid production in peanut. Leaves were selected for the analysis as they are primary site for crucial chemical reactions related to biosynthesis of phytochemicals. Variety selection was carried on basis of their performance in the field. Bari 2011 is a commercially important peanut variety of Chakwal region of Pakistan with small sized striated seeds. It is renowned for its better performance in stress conditions and nicely flavored seeds. On the other hand, PG 1247 has bigger and brown colored seeds with low performance under stress. So, differently behaving varieties were selected to analysis the effects of genotype onto flavonoid accumulation. Moreover, selection of two different varieties for analysis has been carried out to check the potential of flavonoid biosynthetic genes as marker for screening better performing peanut varieties.

Expression analysis of flavonoid biosynthetic genes in leaves of *A. hypogaea* has revealed differential expression of genes in different varieties. Real-time PCR analysis has, also, shown that expression of flavonoid genes is depend upon the type and intensity stimulus including light and sugar. In Bari 2011, most of the genes have been found up-regulated with greater extent on Day 15 than on Day 7 while opposite trend has been observed in PG 1247 variety. This signified that PG 1247 was more sensitive to light alteration than Bari 2011. On the other hand, expression of flavonoid

biosynthetic genes has shown specificity for sugar type and quantities as sucrose has up-regulated while glucose has down-regulated most of the genes in both varieties.

At day 7, Bari 2011 has showed induction in proanthocyanidines and anthocyanin related genes i.e. *CHI, DFR, ANS* in plants treated with 8 hr light while plants treated with 12 hr light elevated expression of anthocyanin related genes (*CHI* and *ANS*). Induction of proanthocyanidines and anthocyanin in plant treated with lesser duration of light might be due to ROS production. Plants treated with shorter duration of light face nutrient deficiency due limitation in photosynthesis, thus, creating stress that lead to ROS production (Tripathy and Oelmüller, 2012). To reduce this stress plants might triggered their defense response involving proanthocyanidines and anthocyanin production (Pérez-Díaz et al., 2016). Plants of Bari 2011 with all-time light exposure showed higher expression of flavonol producing genes (*CHS, CHI,FLS*). In PG 1247, up-regulation of all flavonoid biosynthetic genes signifies that the variety is responsive to variation in light/dark cycle.

At day 15, all the flavonoid biosynthetic genes except *CHI* were found to be induced by all photoperiod treatments and signified flavonoid accumulation in leave of in Bari 2011. Down-regulation of *ANS* and *CHI* signified the shift in pathway that favors flavonol production. In PG 1247, expression of most the genes were normalized and down-regulated at all light treatments except late pathway genes (DFR and ANS) that are involved in anthocyanin production. Increased expression of genes under increased light is attributed to transcriptional machinery. Light through photoreceptors represses COP1 (a proteasome complex that degrade regulators involve in the production of flavonoid in the dark) by disintegrating its complex and encourages flavonoid production in presence of light (Czemmel et al., 2012; Zoratti, Karppinen, Luengo Escobar, et al., 2014).

Sugar treatments were given in MS media to avoid interference from other molecules. Expression analysis of flavonoid biosynthetic genes in Bari 2011 and PG 1247 under the influence of sucrose and glucose at various concentrations has been evaluated. Analysis showed that expression of these genes is not as much genotype dependent as that was in case of photoperiod. Moreover, expression was found to be specific for specific sugar treatment. In Bari 2011 plants, 400 mM sucrose was found

to be more influential for the production of proanthocyanidines and anthocyanin as it up-regulated expression of *CHS, LAR, ANS*. At 200 mM sucrose up-regulated the expression of *CHS* and *ANS* while down-regulated *FLS*. Most of the genes (*CHS, LAR, DFR, F3H* and *FLS*) have down regulated while expression of *ANS* and *CHI* was up- regulated by 600 mM sucrose treatment. In PG 1247, *F3H* and *ANS* expression in the plants treated with 200 mM sucrose have signified the accumulation of anthocyanin. All the genes were regulated in plants with 400 mM sucrose while *CHI, CHS, FLS, DFR* and *ANS* were observed to be up-regulated in plants treated with 600 mM sucrose and indicated the production of various type of flavonoid in it.

In Bari 2011, the expression of proanthocyanidines and anthocyanin related genes *ANS* and *DFR* were up-regulated by glucose at all concentrations while *LAR* was only expressed at 600 mM glucose treatment. *CHI, CHS, FLS* and *F3H* were down regulated by glucose. In PG 1247, the plants treated with 200 mM glucose, *F3H* was only one that was significantly up-regulated while *FLS* was significantly down regulated. In plants with 400 mM glucose treatment, expression of *FLS, DFR* and *LAR* were significantly decreased as compared to control. In plants with 600 mM glucose treatment, *DFR* was up-regulated while *CHI, FLS* and *LAR* were down regulated. Up-regulation of gene under influence of sucrose has been reported previously in Grape and *Arabidopsis* (Solfanelli, et al., 2006). In contrast, down regulation of flavonoid biosynthesis genes under glucose treatments might be due to its poor translocation in a plant (X. Chen et al., 2013). Moreover, positive effect of sucrose on flavonoid biosynthesis might be due its electrical potential and water solubility (Hazarika, 2003; Shimizu and Kennedy, 1994).

Precisely, flavonoid production in peanut plant depends on genotype, sugar concentrations, specificity of sugar and alternation of photoperiod. But expression analysis in relation to transcriptional regulatory genes is needed for comprehensive understanding of the flavonoid biosynthetic pathway. Moreover, metabolomic profiling of flavonoids in relation to the expression of flavonoid biosynthetic genes in various plant tissues at various developmental stages is demanded to explore their potential in variety development.

APPENDICES

Supplementary Table 1:

Sr.	Primer	Sequence 3’ to 5’
1.	Actin- F	GAGCTGAAAGATTCCGATGC
	Actin- R	GCAATGCCTGGGAACATAGT
2.	CHS-RT-F	GCTGCACTCATTGTTGGTTC
	CHS-RT-R	CCAGGGGATCAAAAGCTTCA
3.	CHI-RT-F	GAGCACAGTATGGGGTTCAG
	CHI-RT-R	TTCCTTCCCCTCCAAAGACA
4.	F3’H-RT-F	
	F3’H-RT-R	
5.	FLS-RT-F	CACCCAACCCTTCTTTCCAT
	FLS-RT-R	GTAAGGACTGGGGCATGTTC
6.	LAR-RT-F	AAAGTTCCGATGCTTGGAGG
	LAR-RT-R	AATTCTCTCTGCCTCCACCA
7.	DFR-RT-F	
	DFR-RT-R	
8.	ANS-RT-F	AGGAAGTTGGAGGGATGGAA
	ANS-RT-R	CCAATGTGCATGAGGATGGA

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