Expression Profiling of Flavonoid Biosynthetic Genes in *Arachis hypogaea* Under Drought Stress

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Expression profiling of flavonoid biosynthetic genes in *Arachis hypogaea* under drought stress

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Submitted By

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Dedicated to my beloved parents

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ABSTRACT

Arachis hypogaea (peanut) is one of the important crops of family Leguminosae. It is a crop of semi-arid regions and its yield is affected by various environmental stresses specifically drought, leading to serious production losses. Better understanding of stress responses is critical to improve tolerant of crops. Two subclasses of flavonoids, flavonols and chalcones have diverse roles in plant defense and their synthesis is catalyzed by FLS and CHS respectively. CHS is the first enzyme that catalyzes flavonoid biosynthetic pathway while FLS catalyzes late reactions in this pathway. However, a little information about their behavior in peanut is available. In the present study, the expression of these two flavonoid biosynthetic genes is analyzed. Results indicated that RNA transcript level of both genes was up regulated under drought stress in peanut variety BARI-2011. The maximum RNA transcript level was after ten days under severe drought stress while maximum FLS expression was observed after ten days under of mild drought stress in set experimental conditions. Current study suggests the protective role of flavonoids against drought. However, functions of flavonoid biosynthetic genes involved in defense role against drought stress need further validation.

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LIST of ACRONYMS

CHS	Anthocyanin synthase
FLS	Flavonole synthase
ANS	Anthocyanin Synthase
ANR	Anthocyanin Reductase
F3H	Flavanone 3-Hydroxylase
DFR	Dihydroflavonols Reductase
LAR	Leucoanthocyanin Reductase
ITS	Internal transcribed spacer
mRNA	Messenger RNA
UV	Ultra violet
PCR	Polymerase chain reaction
CTAB	Cetyl Trimethyl Ammonia Bromide
RT	Reverse Transcriptase
qRT	Quantitative real time
β	beta
CT	Cycle threshold
Ml	millimeter

1 INTRODUCTION

Arachis hypogaea is commonly known as groundnut, earth nuts, goobers and Mong phalli (Urdu). Technically peanuts are not nuts. They are actually specie of legume family Fabaceae, Leguminosae (ancient name) and sub family Papilionaceae (Ahmad, Anwar et al. 2016). It is an annual herbaceous plant South America is the origin of this crop and now peanut is cultivated around the world mostly in tropical, sub-subtropical climates (Stalker 1997). Mainly peanut is cultivated for its edible seeds and classified as both an oil crop due to its high oil content, and as grain legume (Isleib, Pattee et al. 2004). China is the largest producer in world, accounting for 37.6% of total world production (Isleib, Pattee et al. 2004). Other major producers include Argentina, Brazil, India, Sudan and Northern Nigeria. In Pakistan about 84 % of the total peanut cultivated area lies in rain fed (barani) areas of Punjab, 13% in irrigated areas of NWFP and 3 % in Sindh, while the total production of the country is 609 kg/ha during year 2009-2010 (Agric. Statistics of Pakistan, 2009-10). Among most oil producing crops peanut has ranked fifth worldwide (USDA, 2013a).

Cultivated peanut is tetraploid with chromosome number 2n=40 and genome size is 2813 Mbp while most of the wild species are diploid (2n=2x=20).(DEL PILAR DE SOUZA PEÑALOZA and VALLS 2005)

Peanuts are cultivated from 40°S to 40°N latitude and the appropriate regions for its growth and cultivation includes tropical, sub-tropical and warm temperate zones. Peanut is sensitive to frost and period required for its growth is about 3 1/2–5 months. The type of land required for peanut growth is heavy soils but can also be grown in light, dusty and having less water sandy loams. The annual mean temperature, rainfall and pH which can be tolerated by peanut is 10.5℃ to 28.5℃, 3.1 to 41.0 dm and pH of 4.3 to 8.7 respectively. These annuals were determined on the

basis of its growing zones which range from cool temperate moist through tropical thorn to wet forest life regions (Duke and Wain 1981)

Peanuts used for commercial purpose are all propagated through seed. Different dormancy period is for different type of variety i.e. the Spanish-Valencia type which has successive branching have small or no seed dormancy while the Virginia-type peanuts which are alternately branched have specific seed dormancy duration (Mallikarjuna and Varshney 2014). The production of peanuts yields 11,128,000 MT, averaging 866 kg/ha in Asia alone which can be tripled in the tropic areas where annually three crops are cultivated. (Revoredo and Fletcher 2002)

1.1 Morphology of Peanut Plant

Peanut plant grow 30-50 cm tall and part above ground consists of green-oval shaped leaves. Self-pollinated little yellow flowers appear after about 30-35 days on the bottom of the plant .After pollination the flower produces a stem (called as peg) containing a budding ovary at its end. The peg with peanut embryo on its end grows downward and penetrates the soil. At the end, ovary at the tip of each peg will form a peanut pod. Each pod contains 2 to 3 seeds and overall at maturity one plant produce around 20-40 pods. The plant requires 120-150 days, warm weather, sandy soil, and adequate water (from irrigation or rain) to grow.

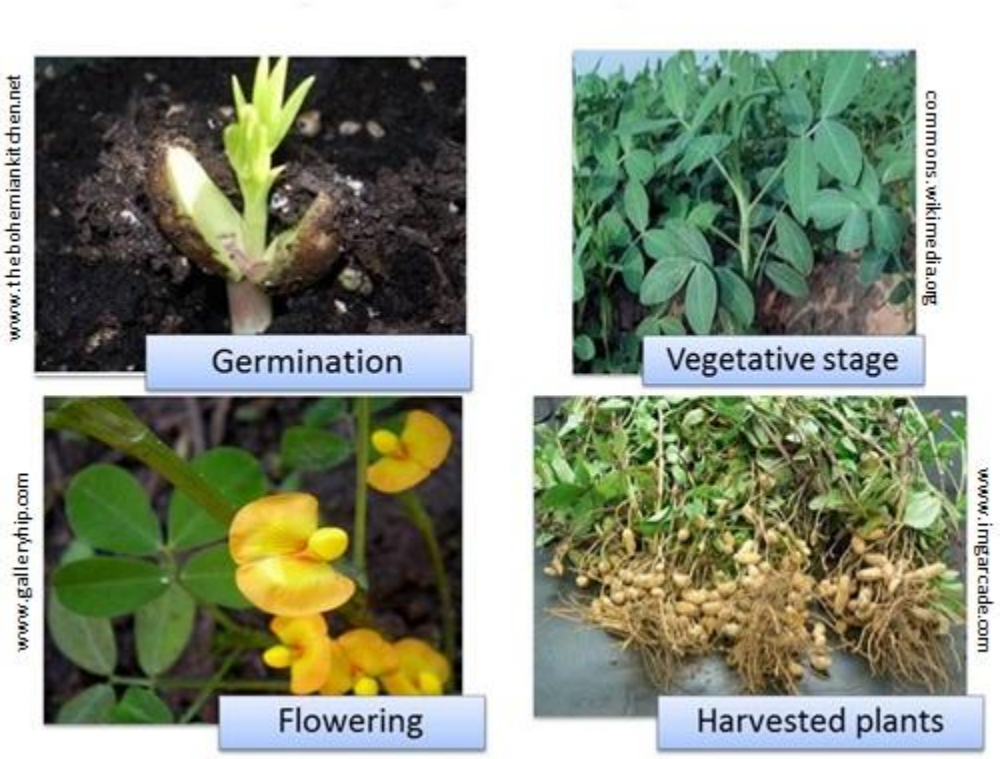

Figure 1:0:1 Different stages of peanut growth

1.2 Nutritional Value

Peanuts have high nutritional value as compared to other animal based foods; they are rich source of fats, proteins, carbohydrates, vitamins and minerals (Singh and Singh 1991). Fatty acid composition in peanut plant ranges from 22-30% and the oil content may reach up to 50%. Peanut is also designated as "The King of Oilseeds" and it is an economical and significant source of important minerals, proteins, vitamins and fats. These minerals and proteins have a substantial role in the nutrition of rural area population, particularly among the children. Commonly this crop is known as poor man's nut (Prabasheela, Venkateshwari et al. 2015).

Peanut plants are a rich source of many secondary metabolites including flavonoids. Secondary metabolites are a vast and diverse assortment of natural organic compounds, the great majority of which do not appear to participate directly in growth and development. Due to complex structures, the secondary metabolites are difficult to synthesize. One of the inexpensive and most widely used vegetable oil in India is peanut oil, which is not only used for nutritional purpose but also for fuel and radiance. The calories present in peanut oil are 549 cals/100 g, according of WOI and which is five times more than that of beef. The stalk of peanut is used as fodder for animals, as silage or green fertilizer(Anonymous 1948)

1.3 Flavonoids:

Flavonoids are secondary metabolites and found in all parts of peanut plant. They are a group of phenolics having a general three ring chemical structure (C6-C3-C6)(Tsao 2010). More than nine thousand different types of flavonoids have been revealed due to modifications in flavonoids biosynthetic reactions (Tsao 2010). All flavonoids are derivatives of one general phenylpropanoid pathway but perform a diversity of functions. Flavonoids are also known as 'specialized metabolites' due to their species-specific metabolism in plants (Lattanzio, Kroon et

al. 2008) . One reason of immense importance of these metabolites for crop breeding is their role in allelopathy, this trait can affect the growth of target organism positively or negatively for example in plants, fungi and insects (Nakabayashi, Yonekura-Sakakibara et al. 2014)

Flavonoids have long been recognized as playing diverse functions in plants. From 150 years of past, these metabolites are considered as fundamental breakthroughs of science including the Mendal genetics, pigments of the seed coat as his focus in the trials with the pea (Pisum sativum), as well as discovery of transposable elements by Barbara McClintock, which move across the flavonoid biosynthetic pathway genes in maize plant. Recently an important subclass of flavonoid anthocyanins are found helping in the study of cosuppression, particularly in petunia plant (Pentunia hybrid).

Flavonoids were early proposed as developmental regulators(Stafford 1991).A well-known function of flavonoids in plants is the presence of anthocyanins as pollinator attractants via flower color and for seeds dispersal agent through brightly color food. Besides this, they play a critical role in responses of higher plants to a wide range of environmental constraints (de Alencar Filho, Teixeira et al. 2016). Flavonoids absorb the harmful UV radiation inducing cellular damage in Plants as well as play an important part as signal molecules in plant-microorganism symbiosis. Currently Flavonoids are figured to play a significant role inside the root during nodule meristem formation. Flavonoids may act as detoxifying agent. Under the elicitation of toxic metals, flavonoids are accumulated in plants and not only detoxify the ROS but also the toxic metals by chelating depending on the diversity of molecular structures.

1.3.1 Subclasses of Flavonoids

Flavonoids have been classified per their chemical structure. More than 5000 naturally occurring flavonoids have been characterized from various plants(Bravo 1998). They have been widely

studied in a range of land plants and have been divided into nine subclasses (Ferreyra, Rius et al. 2012)and aurones(Martens, Preuß et al. 2010). Flavonols, Anthocyanins and proanthocyanidins are three major subclasses of flavonoids, flavonols are plentiful and play pivotal roles in a variety of plants (Jaakola, Määttä-Riihinen et al. 2004).

1.3.2 Flavonoid biosynthetic pathway

Several genes involved in the flavonoid biosynthetic pathway have been recognized and characterized. Entry point of this metabolic pathway is CHS gene that encodes for an enzyme which play role in catalyzing the reaction that turn 4-Coumaroy-CoA and Malonlyl-CoA into a metabolite chalcone. This conversion leads the phenylpropanoids pathway to subsequent reactions of flavonoids biosynthesis (Shih et al., 2008). Key enzymes contributing in the pathways leading to major flavonoids subclasses have been investigated including CHS (Chalcone synthase), CHI (chalcone isomerase), F3H (flavanone 3- hydroxylase), DFR (dihydroflavonol 4reductase), FLS (flavonol synthase), FNS (flavone synthase) and ANS (anthocyanidinsynthase).

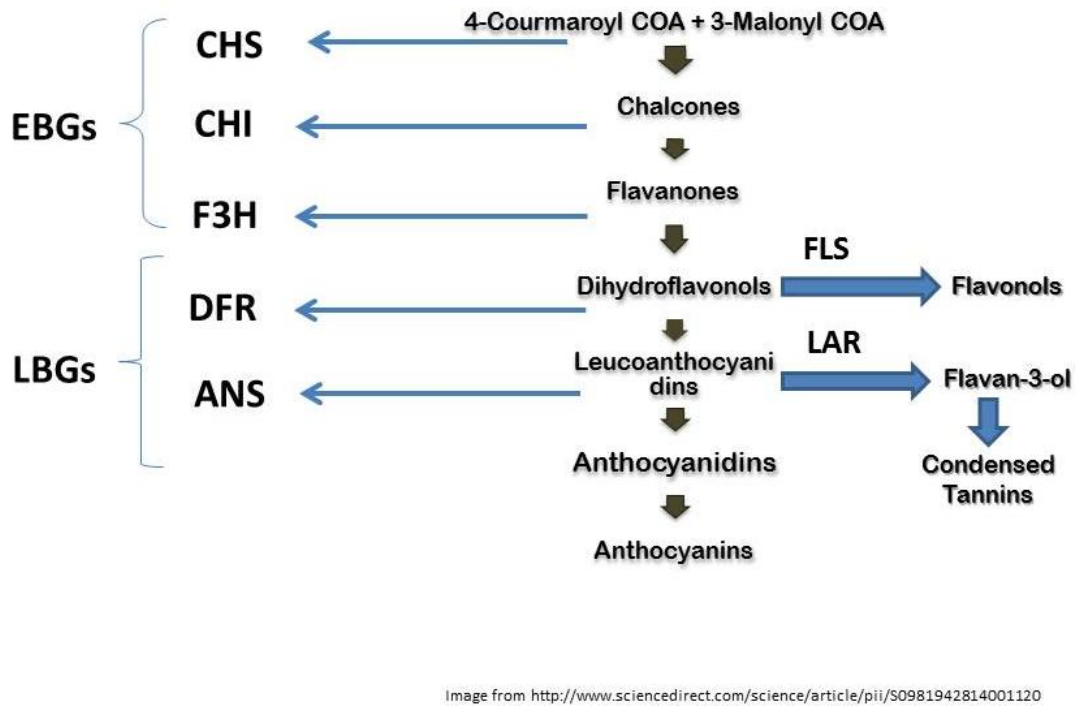

Figure 1:2 Overview of flavonoid biosynthetic pathway

1.3.3.1 Chalcone synthase (CHS)

CHS is the first enzyme initiating flavonoid pathway (Rubin, Tohge et al. 2009). That is related to the chalcones production. Chalcones is a type compounds that are of organic origin and produced generally in plants and play role in natural defense mechanisms (Heller and Forkmann 1988). CHS enzyme catalyzes the step in flavonoid biosynthetic pathway that convert 4coumaroyl-CoA and malonyl CoA to naringenin-chalcone (Kreuzaler and Hahlbrock 1972). It is reported that CHS act as a chief enzymes involved in the production of flavonoids (Crosby, Pietraszewska-Bogiel et al. 2011). Some studies showed the protein-protein interactions of these enzymes. It has investigated the interaction of CHS interacts with CHI (an enzyme catalyzing consecutive stage) and with other non-consecutive enzymes. (Crosby, Pietraszewska-Bogiel et al. 2011) Chalcone synthase serves as the preliminary step for flavonoid biosynthesis.

1.3.3.2 Flavonol synthase (FLS)

FLS gene is responsible for the production of flavonols during flavonoid biosynthetic pathway.Flavonols are one of the ancient and most common flavonoid having wide variety of activities (Ferreyra, Rius et al. 2012), ranging from pollen fertility (Taylor and Hepler 1997), to free radical scavenging (Rice-Evans, Miller et al. 1997), antimicrobial activity(Koes, Quattrocchio et al. 1994), co-pigments in fruits and flowers (Scheffeldt and Hrazdina 1978) and as UV-protectant (Ryan, Swinny et al. 2002). They also play crucial role in regulating the development of either whole plant or single organ, and maintain the homeostasis of cellular reactive oxygen species. Flavonols play key role in controlling fertility of plant, pollen germination and seed development. Even very low flavonol concentration (mostly in terrestrial plants) can accomplish these tasks efficiently (Pollastri and Tattini 2011). Flavonols have various

roles in health promoting effects as they have activities like antioxidant, antiinflammatory, antiangiogenic,anti-proliferative and neuro-pharmacological (Lim and AG Koffas 2010).

1.4 Drought

Drought is one of the major environmental stresses that limits agriculture production. It is a meteorological term and is commonly defined as a period without significant rainfall. Almost all plants show drought stress tolerance but its extent differs from species to species and even within species. Annual production losses due to water shortage vary in nature depending on the timing of stress, intensity and period (Farooq, Wahid et al. 2009).

Different plants use several approaches to overcome water shortage for their survival e.g. drought avoidance and drought tolerance. Plant species select a strategy depending on the two parameters, intensity and exposure time to drought and it also includes the aptitude of that plant to accomplish molecular, biochemical, and physiological variations (Xoconostle-Cázares, Ramirez-Ortega et al. 2010). Being a crop of Arid regions, peanut crop is relatively tolerant under drought stress.

An increase in reactive oxygen species (ROS) in plants is due to water shortage (Cruz de Carvalho 2008). Studies have reported the buildup of flavonoids acting as powerful scavengers of reactive oxygen species in defense against stress (Treutter 2006, Bartwal, Mall et al. 2013, Nakabayashi, Yonekura-Sakakibara et al. 2014). About 70% areas of peanut cultivation worldwide are semiarid regions, where drought is one of the major environmental constraints, adversely effecting photosynthesis (Kambiranda, Vasanthaiah et al. 2011) mineral nutrition, plant metabolism, development and ultimately production of peanut (Reddy, Reddy et al. 2003). According to a recent calculation, worldwide peanut annual production suffered loss of approximately 6 million ton over US $520 million due to drought (Sharma and Lavanya 2002,

Sarkar, Thankappan et al. 2014). It is critically important to understand the mechanism of stress response in plants for agricultural as well as economic performance for the betterment of tolerance of crops against such abiotic stresses using breeding and phytochemical genomics (Mir, Zaman-Allah et al. 2012).

2 REVIEW OF LITERATURE

The peanut plant scientifically known as Arachis hypogaea is a grain legume which is grown extensively around the world and reproduces via self-pollination. The word Arachis is a derivative of Greek word “arachos” meaning weed while the botanic meaning of hypogaea is an underground part i.e. a weed fruit forming underneath the soil(Prasad, Kakani et al. 2010). The origin and domestication of peanut crop was about 3500 years ago, in areas of central and Southern parts of America. Now this crop is broadly cultivated around the world particularly in warm temperate and tropical regions(Evans 1996).

Peanut seed coat color varies due to the content of a flavonoid i.e. anthocyanin in seed coat. Its color can be from white to pink, brown, purple and black(Shem-Tov, Badani et al. 2012). Various studies have shown that wild species of groundnut can tolerate various stress factors which includes two major ones which are drought and fungus(Guimarães, Brasileiro et al. 2012) These two have greatly affected the yield and production of cultivated peanuts. In the vegetative organs and tissues of peanut the buildup of flavonoids is also affected by several biotic and abiotic stress conditions. The accumulated flavonoids not only help in adapting with surrounding environment moreover in overcoming stress conditions.(De Micco and Aronne 2012). It is apparent that abiotic stress factors effect development and subordinate metabolite production in higher plants (Shen, Zhou et al. 2010).

2.1 Flavonoids as secondary metabolites

Flavonoids are found throughout the plant kingdom and belong to diverse group of aromatic compounds(Harborne and Williams 1998). Mainly these compounds are found in high concentrations in epidermis of leaves and the skin of fruits and play various important roles as secondary metabolites. Many biological functions have been assigned to an increasing number of

flavonoids(Bogs, Ebadi et al. 2006). Specific flavonoids released from *Phaseolus vulgaris* (Hungria, Joseph et al. 1991) and *Medicago sativa* (Dakora, Joseph et al. 1993) cause the induction of food nod gene transcription in Rhizobium which subsequently promotes the formation of root nodules and in turn N2 fixation. Flavonols play an essential role for pollen germination in maiz plant (Deboo, Albertsen et al. 1995) and Petunia (Ylstra, Busscher et al. 1994).

In plants, flavonoids have been proposed as playing significant role in antioxidation under stressed conditions. The antioxidant property of these metabolites is supported by their chemical structure, location of OH groups, double C bonds as well as glycosylation, prenylation, methylation are modifications in structure that controls antioxidant properties (Schijlen, De Vos et al. 2004). Although in plants the mediation of ROS reduction by flavonoids is still unclear. In plant cell , flavonoids in the chloroplast playing antioxidant activities suggest their important part as scavenging agents of singlet oxygen and their role in stabilizing chloroplast outer cover membrane under stress conditions (Agati, Azzarello et al. 2012). Dihydroxy B-ring substituted flavonoids hinder generation of ROS creating complexes with iron and Copper ions and exist in the nucleus of mesophyll cells. In liverworts and mosses antioxidant flavonoids biosynthetic genes are present and are typically up-regulated in response to severe stress(Yeo and Shahidi 2015). It has been reported that peanut kernels contain antioxidant flavonoids, dihydroquercetin (Hussain, Chatha et al. 2012).These mechanisms propose that the ability of flavonoids to antioxidize is a distinctive quality of all terrestrial plants.

2.2 Genes involved in flavonoid biosynthesis

Flavonoids play a key role in pigmentation as red, blue and purple colors in plants, this property of flavonoids have increased attention of researchers. From1664 when Robert Boyle explained

acid and base effects on plant pigments to the description of genes involved in structure and regulation of several processes in the 20th century, important regarding to structural, chemical and biosynthetic pathways has been collected. Specialized forms of flavonoids are synthesized specially by legumes and a few non-legumes is known as is flavonoids, same as in Sorghum bicolor, *Zea mays* and *Sinningia cardinalis*, a polymerized form 3-deoxyanthocyanins is produced (Winkel-Shirley 2001). The stilbenes, related complexes to flavonoids, are formed by a different group of distinct species that contains grape, peanut and pine (Winkel-Shirley 2001). So, it shows the evolution of this pathway and suggest that it may missing from definite plant lineages due to multiple times evolution over a long period of evolution.

Flavonoids synthesis pathway is secondary metabolic pathway that has foremost attention of researchers. (Mane, Robinet et al. 2008, Lenka, Katiyar et al. 2011). It has been thoroughly characterized in *Zea mays, Arabidopsis thaliana* and *Vitis vinifera* (Martens, Preuß et al. 2010). Flavonoids are synthesized by the combination of the phenylpropanoid and polyketide pathways .The phenylpropanoid pathway provides p-coumaroyl-CoA(Ferrer, Austin et al. 2008). The polyketide pathway is responsible for C2 chain elongation by utilizing malonyl-CoA as the condensing unit. The phenylpropanoid pathway initiates from the aromatic amino acids phenylalanine and tyrosine, which are synthesized by the shikimate pathway(Saito, Yonekura-Sakakibara et al. 2013).

The Chalcone isomerase (CHI) and CHS are the main enzymes contributing to two-step process of condensation, forming a colorless flavanone, Naringrnin. Flavone 3-hydroxylase (F3H) oxidizes the later compound to form dihydrokaempferol (a colorless dihydroflvanol), whose 3’ or 5’ position of the B-ring can be subsequently hydroxylated through the enzyme F3’H or F3’5’H , forming dihydroquerecetin and dihydromyricetin respectively.

Naringenin can be directly hydroxylated by F3’5’H or F3’H, to form pentahydroxy flavanone and eriodictyol respectively, that can be further hydroxylated to deliver dihydromyricetin and dihydroquerecetin. These three dihydroflavanols synthesized are converted into anthocyanidins (the colored unstable pigments) through two reactions which are catalyzed by LDOX and dihydroflvanol reductase (DFR) catalyze dihydromyricetin, dihydrokaemferol and dihydroquerecetin to leucodelphinindin , leucopelargonodin and leucocyanidin to delphinidin (purple-mauve, anthocyanidins), pelargonidin (orange anthocyanidins) and cyaniding (red-magenta anthocyanidin) respectively. The above mentioned colors result when anthocyanidins are in acidic compartments.

Even in the mosses/bryophytes the initial steps of this pathway are found and it recommends the possible evolution of processes producing flavones, flavanones, and flavanols, which play role as chemical signaling and then ultraviolet sunscreens (Stafford 1991). Flavonoids have potential health benefits for humans. Flavonoids are considered to have significant activities when animals ingest these compounds, and a great interest in medicinal value of these metabolites for example is flavonoids linked to have anti-cancer benefits, and presence of secondary metabolite ‘stilbenes’ in red wine supposed to contribute to treat heart diseases.

In last few years’ considerable effort has been focused at clarifying the flavonoid synthetic pathway at molecular or genetic level. Based on alterations in flower and seed color or pigmentation, mutant plants have been isolated based on alteration in flavonoid synthesis in a variety of plants. As first major trial models, three plants as petunia, maize, snapdragon were established in experimental system, and research work with these species directed to the identification and isolation of several genes involved in structural and regulatory activities of flavonoid pathway (Tanaka, Tsuda et al. 1998). Arabidopsis as a model plant, recently has

facilitated in analyzing the regulation and organization at subcellular level of the flavonoid biosynthetic process. (Ferreyra, Rius et al. 2012).

2.3 Flavonoid Biosynthetic pathway under Abiotic Stress

In recent years, flavonoids have become the point of attention for scientists about abiotic stress conditions. Warren et al. (2003) anticipated that levels of kaempferol and quercetin (derivatives of flavonoids) in leaves of *Populus trichocarpa* boosted under exposure of UV-B irradiation (Pietta, Simonetti et al. 1998, Warren, Bassman et al. 2003) related C glycosylflavones level of different rice cultivar under UV-B light. UV-B light enhanced the C glycosylflavones in a tolerant cultivar of rice but was not detected in a cultivar that was susceptible (Caasi-Lit 2005).

A variety of other environmental stresses like salinity, cold and water deficit also cause the up-regulation of flavonoids biosynthetic genes. Anthocyanin biosynthesis highly up-regulated in response to drought stress in ripening fruit (Castellarin, Pfeiffer et al. 2007).Yang et al. (2007) stated that flavonoid production upsurges under water deficit conditions in *Glycyrrhiza inflata* Batal in its suspension culture (Yang, He et al. 2007)

Experiments of Ghanzanfar and Walid (2006) advocated the existence of anthocyanins, flavones and phenolics are connected to amplified the tolerance to salinity in sugarcane crop(Ma, Sun et al. 2014). Currently, mechanism of secondary metabolites at molecular level to investigate the response to environmental stresses is being studied. Yuan et al. (2012) stated that under water deficit conditions, the expression of numerous flavonoids biosynthesis genes is augmented in Scutellaria baicalensis Georigi roots (Yuan, Liu et al. 2012). Ithal and Reddy reported flavonoids biosynthetic pathway rice genes OsAns and OsDfr, are induced by factors like drought, salinity and Abscisic acid, in 2004 (Ithal and Reddy 2004). The chief cause to worldwide crop loss is considered as abiotic stresses which are responsible for the reduction of average yield for most

crops by more than 50% (Zhu 2002). These stresses amend metabolic function in plants because of up regulation of various genes leading to plant adaptation and the mitigation of the stress effects.

More phenolics and anthocyanins can result from early season water deficiency to improve berry and wine quality by regulating the deficit irrigation. Flavonoid production in Glycyrrhiza inflate in cell suspension culture is also increased due to water insufficiency (Wang, Qi et al. 2010). Phenylpropanoid antioxidant concentrations enhanced in response to water deficit (Guidi, Degl'Innocenti et al. 2008).

Winkel-Shirley (2002) stated that priorities for research to understand flavonoids functions at molecular level in improving responses of plants to stress, as well as the amounts and types of flavonoids produced under stress conditions with an aim of engineering improved stress response in crops (Winkel-Shirley 2002). The hypothesis that flavonoids act as antioxidant agents in higher plants that are confronted with several of environmental stresses, has been validated by numerous lines of evidence (Chaves, Maroco et al. 2003). Plants develop effective mechanisms as to protect them from such stresses (i.e. biotic and abiotic).

One of the stress response mechanisms examples is specialized/secondary metabolism. Currently, it has been reported that flavonoids contributed to the mitigation of oxidative and drought stress in Arabidopsis thaliana (Nakabayashi, Yonekura-Sakakibara et al. 2014). However, there is no proper documentation for flavonoids behavior during drought stress. Flavonoids as important secondary products in the model plant Arabidopsis thaliana. More than 5000 number of metabolites (metabolome) in A. thaliana are estimated in it. Genomic resources in A. thaliana are far outstripping those of other plant species which make linking metabolome to genome a challenging issue (Saito and Matsuda 2010).

An improved cellular metabolism under abiotic stress by osmotic modification maintains the turgor and is regulated by a drought-adapted legume *Arachis hypogeae*, L. Leaf area retention, enhanced efficiency of water usage and better survivability has been described in peanut under stress, on a whole plant level (Nautiyal, Rachaputi et al. 2002, Govind, ThammeGowda et al. 2009).

Tolerance level of crops varies among different plant species and plant genotypes, and is a complex mechanism for plant response to drought stress. Crop species can adapt different strategies to tolerate drought stress despite a few common stress responses. Crop plants may have numerous well-organized mechanisms of adaptation in environment which let them complete their life cycle and make their survival possible under stressful environmental conditions (Rascio and Rocca 2005).

Thus, system for investigating genes linked with tolerance to drought is an excellent plant due to these characters. A little information is available about the regulatory mechanisms and molecular signaling of response to water deficit stress in peanut as Sequence of whole genome of peanut has not been interpreted so far and it has complex genetic background (Seijo, Lavia et al. 2007). Studies suggest that flavonoids play an important role under drought stress. Progress in breeding for tolerant varieties has been slow in peanut (Hall 2004). The possible reasons for this include valuable traits of tolerance against drought such as transpiration, harvest index, or efficiency of transpiration does not have additive effect and a partial description of linked traits (Passioura 1977).

2.3.1 Role of Chalcones and Flavonols

Chalcone synthase (CHS) gene is not only play important role in plant development but its expression may induce under stress conditions in plants. Studies have shown that CHS

expression contribute in the salicylic acid defense pathway by increasing level of flavonoid and is flavonoid phytoalexins (Dao, Linthorst et al. 2011). In plants, CHS expression is elicited by an extensive range of environmental and developmental stimuli (Martin 1993).

There are three main derivatives of flavanols as myricetin, quercetin, and kaempferol that have various biological activities (Miean and Mohamed 2001). These products differ chemically only by a single OH group that is present in B ring of flavonoids. Flavonols play a key role in stress signaling pathways and considered are one of the most important flavonoids thus its ratio also fluctuate in response to different environmental stresses (Winkel-Shirley 2002, Pruthvi, Narasimhan et al. 2014). Enhanced production of Quercetin derivatives specifically was observed in Petunia by UV-B light so they considered acting as one of the major sunscreen with developed antioxidant activity (Ryan, Swinny et al. 2002)

These metabolites also play crucial role in regulation and development of either whole plant or single organ, and in maintaining the homeostasis of cellular ROS. Flavonols play an important role in regulation of different processes in plant like fertility of plant, seed development and pollen germination (Ylstra, Busscher et al. 1994). Even very especially in terrestrial plant even a very low level of flavonol concentration is enough to complete these cellular tasks proficiently (Pollastri and Tattini 2011). Flavonols can play several roles and have health supporting effect, for example they have activities like antioxidant, anti-inflammatory, neuro-pharmacological anti-angiogenic and anti-proliferative (Martens, Preuß et al. 2010). Stress tolerance in plants is supposed to be not possibly regulated by a single gene. Expression level of certain genes is up regulated when a plant combats an environmental stress (Bray 1993). As cultivated peanut has a low genetic diversity and due to its multi gene nature it is difficult to breed by conventional techniques (Pandey,Monyo,etal.2012).

3 MATERIAL & METHODS

3.1 Plant Material

Different varieties of groundnut were collected from the National Agriculture Research Council (NARC), Islamabad. Mature and healthy seeds of ten varieties named PG1198, PG1162, PG1026, PG1195, PG1051, PG1247, PG1263, PG1248, PG Potohar, PG 1133 of A. hypogea were subjected to DNA barcoding.

Ground nut seeds were taken and shells were removed. Dehusked seeds were surface sterilized by 70% ethanol followed by soaking in 3.5% bleach (NaOCl) solution (Iqbal, Nazir, Iqbal, Tehrim, & Zafar,2011). Cold treatment was given to break seed dormancy. Peat moss was used as growth medium instead of soil. Already prepared peat moss was taken from the market and was put in sterile pots. The pots were sprinkled with water before putting soil in it. Pots were kept in growth room under controlled conditions. The optimum conditions were 16/8 hr light and dark conditions and 25-30°C temperature (Pruthvi, et al., 2014). Seed germination occurred about 6-10 days after sowing. All the seeds were sown in triplicates.

3.1.1 Drought Stress Induction

All plants were grown in controlled environment and watered to field capacity till one week after germination. Three replicates of variety (BARI 2011 of A. hypogea) were grown for different levels of drought stress. Drought stress was induced by watering plants to ½ and ¼ field capacity while control plants were watered to field capacity. Field capacity of the soil was measured on volume bases.

3.1.1.1 Experiment Design

Drought stress in this case was generated using three regimes:

- A well-watered control which was irrigated to field capacity
- A mild drought treatment where plants were exposed to water stress to ½ field capacity
- A severe drought treatment watering plants to ¼ field capacity.

Plants were watered after every two days. And leaf sample was collected after 5, 10 and 15 days of stress induction. Samples were frozen in liquid nitrogen immediately following the treatments. Leaf samples were stored at -80 degrees C for further analysis.

3.2 Molecular Analysis

3.2.1 Genomic DNA Extraction for Barcoding

Genomic DNA was extracted from young leaf samples of peanut plant by modified cetyl trimethyl ammonium bromide (CTAB) method (Cullings, 1992; Doyle, 1987). The extracted DNA was assessed using 1% agarose gel electrophoresis.

3.2.2 Target Gene Amplification

PCR amplification of targeted genes ITS2 was done. All reactions were adjusted to a volume of 25 microliters. Each reaction mixture contained the following; 1.5 µL MgCl2, each primer one microliter,2.5 µL of Taq, 1 µl of template, 1 µL of dNTP‘s and 16 µL of water. Amplified products were run on 1% agarose gel.

3.2.2.1 Purification of Amplified Products

An enzyme EXOSAP-IT was used for purification. For every 5 micro liter of PCR sample 2 micro liter of EXOSAP-IT is added. Then the samples were first incubated at 370C for 15 minutes and then at 800C for 15 minutes. The purpose of using this enzyme is to remove any remaining nucleotides in samples for sequencing.

3.3 RNA Extraction Leading to cDNA Synthesis

RNA from leaf samples of peanut plant was isolated using Trizol method (Jaakola, Pirttilä, Halonen, & Hohtola, 2001). The extracted RNA was stored at -80°C after nanodrop quanification through Thermo Scientific NanoDrop spectrophotometer (Desjardins & Conklin, 2010). Extracted RNA was converted into cDNA using two master mixes (Krug, 1987). Master Mix 1 (1X) was prepared in PCR tube by adding 10 µl DEPEC water, One microliter of 5 mM dNTPs and 1 µl of RT primer/oligo dT. 1 µl RNA template was added in Master Mix 1 and first incubation at 65°C for 5 min followed by second incubation on ice for 1 min. Master Mix 2 (1X) was prepared in another PCR tube by adding one microliter of 5X RT buffer, one microliter of 0.1 M DTT and one microliter RNase out. Then Master Mix 2 was added in first PCR tube and 1 µl RT enzyme was added. Reaction components were mixed gently via micro centrifugation and incubated at 50°C for thirty minutes. In last step, sample was heated at 70°C for fifteen minutes to stop the reaction. cDNA was stored at -20°C for further use in amplification via Polymerase chain reaction

3.3.1 Reverse Transcriptase PCR

RT-PCR was done for ß actin gene to confirm success of cDNA synthesis. Products were run on 2.5% agarose gel.

3.4 Real Time PCR

cDNA from control and drought stress treated samples were subjected to real time analysis of CHS nad FLS gene to check stress response. Invitrogen kit SYBR® GreenERTM was used for given analysis on the set condition of RT PCR for candidate genes.

3.5.5.1 Data Analysis

Data were evaluated by double delta Ct method ($2^{-\Delta\Delta CT}$ method). The results were showed by mean ± standard error. $P < 0.05$ was statistically significant.

4 RESULTS

4.1 Barcoding Analysis

4.1.2 Sanger Sequencing Results

Sequences of ITS region were analyzed using Genius software by multiple sequence alignment tools. Fig shows the alignment of sequences of ten varieties of Arachis hypogaea.100% sequence identity was found in all sequences. ITS2 sequence was conserved in all ten varieties that show this region is highly conserved in peanut varieties and lack sufficient variability to be used as a barcode.

Figure 4:1Multiple sequence alignment of ITS2 sequences of nrDNA of Pakistani *Arachis hypogea* varieties .Green line shows the conservation of ITS region in all varieties

Figure4:0:2 Peanut plants under drought stress

4.2. Expression Analysis

4.2.1 Agarose Gel Quantification

Gel electrophoresis for RNA extraction for treated and controlled samples was done and checked by nano-drop quantification. RNA samples were also confirms on 1% agarose gel electrophoresis. Gel was run for 45 minutes at 65 Volts.

4.2.2 cDNA library Construction

cDNA was synthesized from RNA extracted from samples collected at different time points and RT-PCR for actin gene was performed to confirm the success of cDNA synthesis protocol. RT-PCR products were run on 2% agarose gel and equal bands in fig indicate that cDNA has been synthesized.

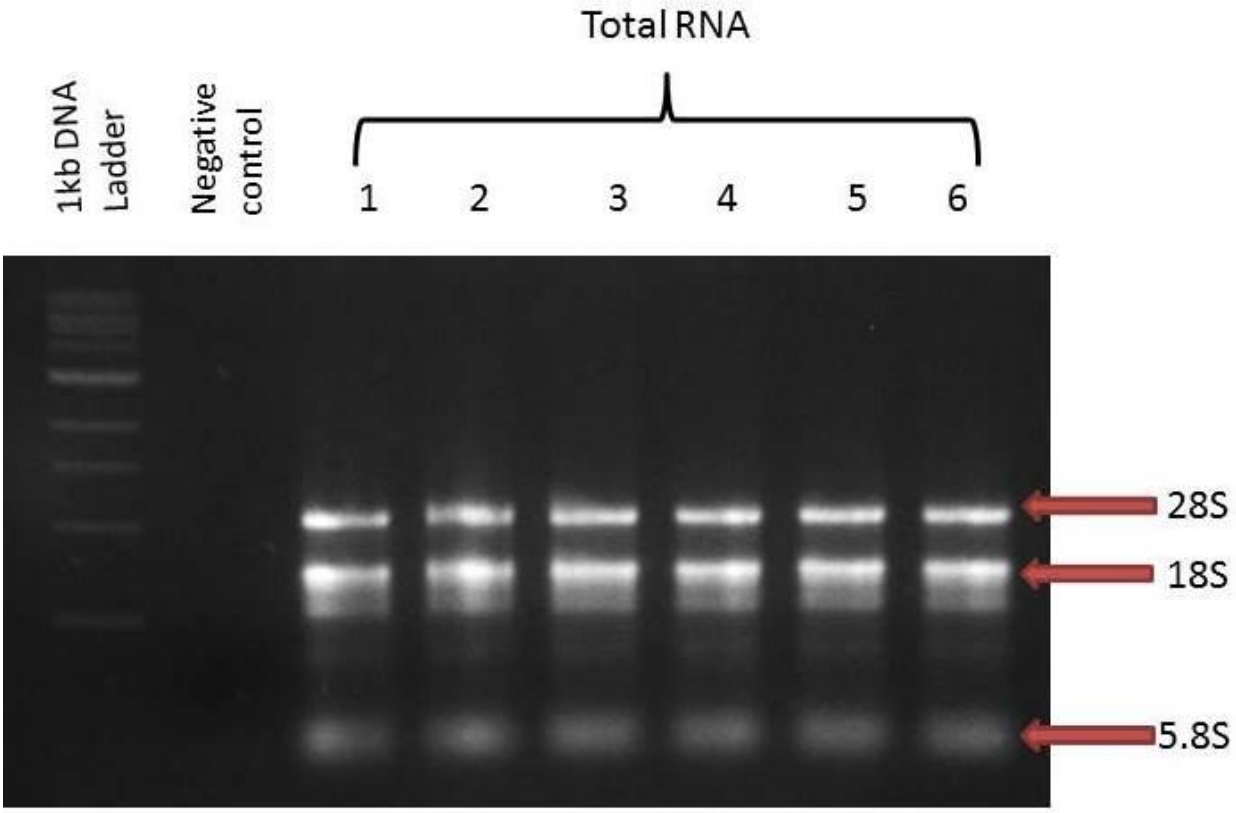

Figure 4:0:3 Gel electrophoresis of total RNA extracted from leaves of *A. hypogaea* RNA samples were electrophoresed on a 1% agarose gel in 1× TAE buffer.Lane1-3 control plant samples, Lane 4-6 treated plant samples.

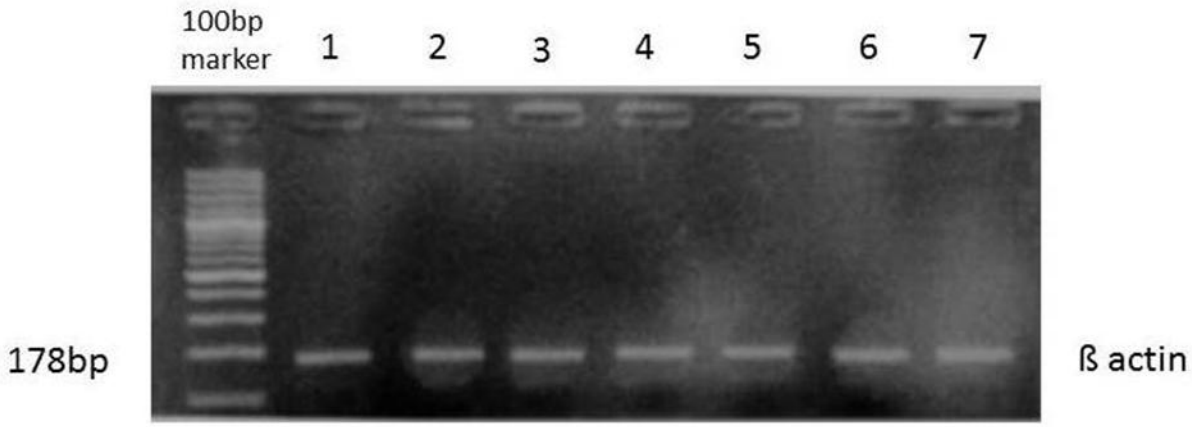

Figure 4:4 Representative agarose gel electrophoreses of RT PCR products of beta actin

4.2.3 qRT-PCR Analysis of Candidate Genes

qRT-PCR analysis for control and treated plant samples was conducted and results show a significant increase in expression of CHS gene. The maximum RNA transcript level was after ten days under severe drought stress. CHS expression was also enhanced after five days of stress in both cases ½ and ¼ field capacity. After twenty days of stress the CHS gene expression level was down regulated.

Expression level of FLS gene for control and treated sample was analyzed using qRT-PCR .Up regulation of FLS gene expression was observed under drought stress. Maximum expression incurred at ten days in case of mild drought stress. Expression of gene was also enhanced after five days of stress and in contrast to CHS gene the expression level of FLS gene was not down regulated at twenty days in both ½ field capacity and ¼ field capacity cases.

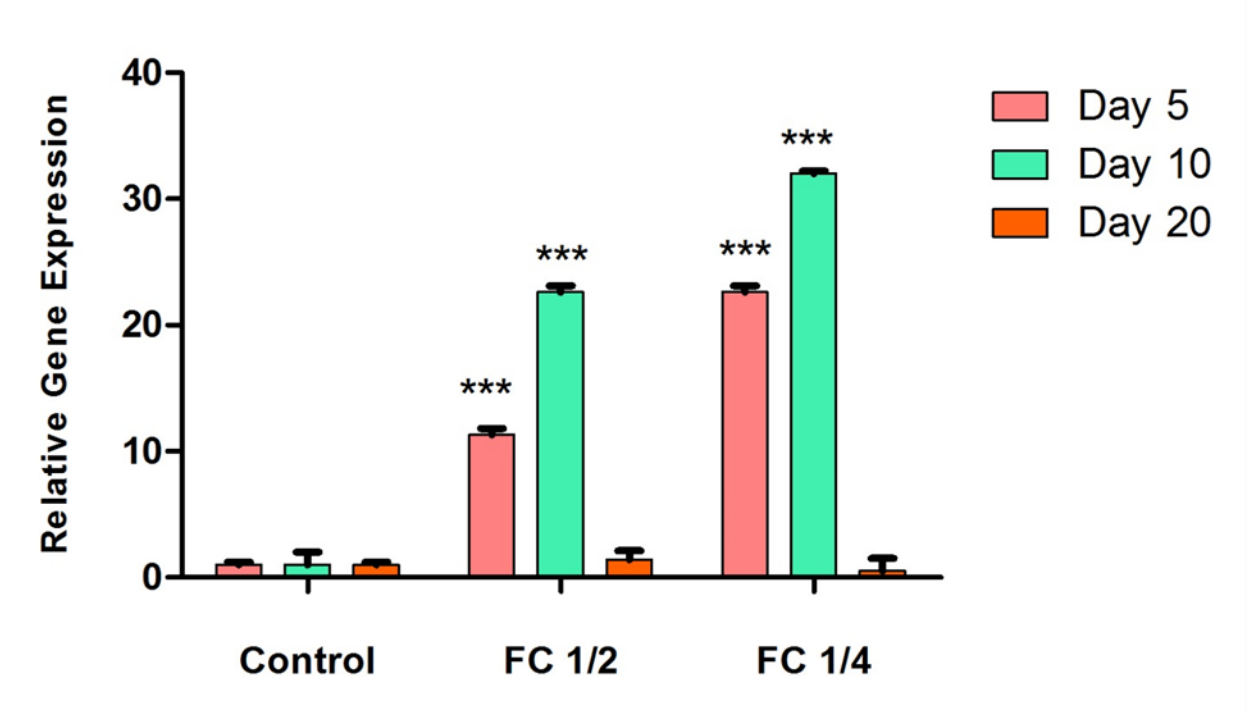

Figure 4:5 Expression analysis of CHS gene under different drought conditions in variety BARI-2011.qPCR was performed to determine the expression of FLS at 5, 10 and 20 days of stress induction. Significance of data was determined by "t" test. ''*'' indicting P <0.05, ''**'' P<0.01, ''***'' P<0.001

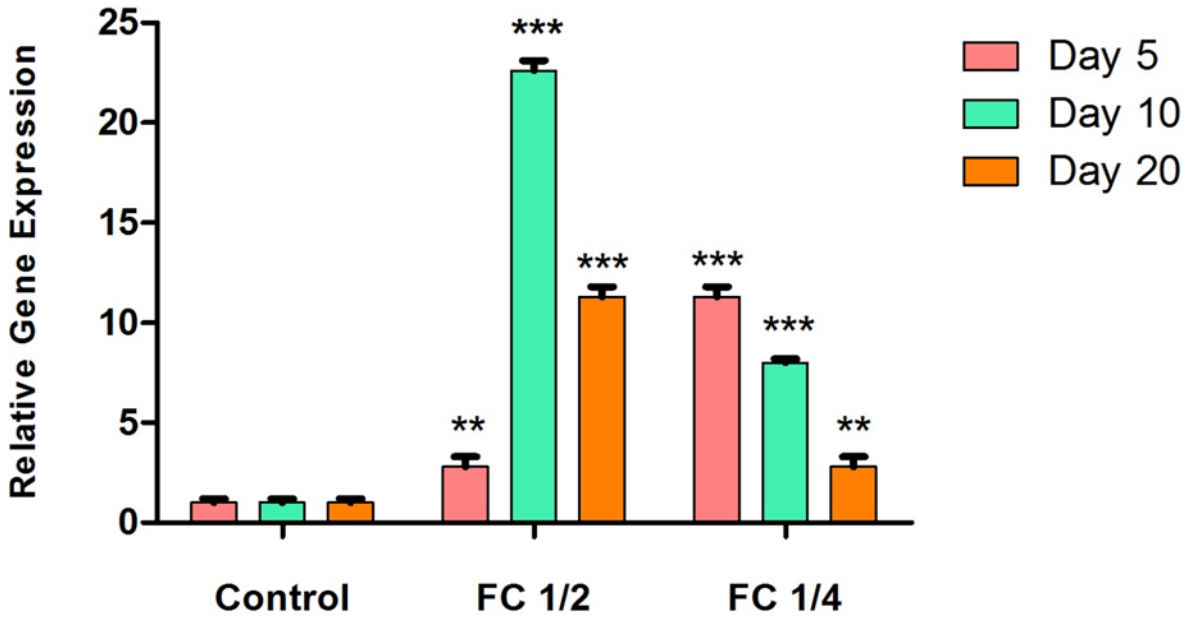

Figure 4:6 Expression analysis of FLS gene under different drought conditions In variety BARI-2011.qPCR was performed to determine the expression of FLS at 5, 10 and 20 days of stress induction. Significance of data was determined by "t" test. ''*'' indicating P<0.05, "**" P<0.01, "***" P<0.001

5 DISCUSSION

Arachis hypogaea is commercially important source of oil production and serves among the top oil producing crops. It is widely cultivated in Asia, America and Africa (Guimarae, Brasileiro et al.,2012). Most of the peanut growing areas are in semiarid region a major environmental limitation is drought. Recently it is reported that an yearly loss of peanut production was around 6 million ton because of drought only (Sarkar et al, 2014). Studies show that water shortage has adverse effect on metabolism, mineral nutrition, photosynthetic reactions (Bhagsari et al,. 1976), development and ultimately yield or total production of peanut (Suther&Patel 1992). Better understanding of stress response in plants is critical to improve production of peanut crop. It is also reported that in flavonoids contributed to the mitigation of drought stress in *Arabidopsis thaliana* (Nakabayashi et al; 2014). In past few years, various studies have focused on secondary metabolites of plants and the molecular mechanism behind their responses to different abiotic stresses (Yuan et al. 2012). Water shortage directly effects directly flavonoid gene expression and metabolic pathway (Simone D. Castellarin et al. 2007). It has reported that in *Scutellaria baicalensis Georigi* roots the expression of different biosynthetic genes was enhanced in water deficit environment (Yuan et al. 2012). There is limited study about the transcriptome and gene regulation in peanut under drought stress. Therefore, the study is aimed to analyze the expression genes involved in flavonoid biosynthetic pathway in peanut. In many plants, several major flavonoid pathway genes regulating their expression under environment stresses have been studied. (Lenkat al.,2011; Vasquez-Robinet et al., 2008). In *Glycyrrhiza inflata Batal*, drought is reported to boost flavonoids level in cell suspension culture (Yang et al., 2007). As drought stress increase level of ROS in plant cells, these metabolites have been proposed as antioxidant under stressed conditions in several plants. The antioxidant properties of these metabolites are

determined by specific position of hydroxyl groups and carbon double bonds as well as methylation, glycosylation and prenylation (Rice-Evans et al. 1997).

CHS catalysis first step of flavonoid pathway. It was reported that CHS showed maximum gene expression under severe drought stress (Vasquez-Robinet et al. 2008) Similarly in *Scutellaria baicalensis*, water deficit upregulated the RNA transcript level of CHS in leaves and roots (Yuan et al., 2012). In current study, upregulation of the CHS gene have been found in BARI 2011 variety leaves under drought stress, which recommends for CHS a major protective role against drought. These results are also in agreement to the report in which increased expression of pathway gene CHS was observed in potato under drought stress (André et al. 2009) In our experiment, it is noticed that the expression levels of FLS is also enhanced under drought stress. These results indicated that under abiotic stress FLS also play an important role in peanut plant, but the mechanism of expression of different genes involved in biosynthesis of flavonoids in response to stress may be different from each other. As compared to early part of the pathway, FLS is a late gene of this metabolic pathway. Reports on grape berries and *Populus euramericana* suggested that the expression level of the genes was boosted under abiotic stress (Castellarin et al., 2007, Kim et al., 2012). In current experiment, the transcript levels of FLS was enhanced in leaves in candidate peanut variety under drought. In rice comparable results were reported as increased expressions of flavonoid pathway genes were observed under stress treatment (Ithal and Reddy, 2004).

Drought is considered as one of the major environmental stresses that adversely affects growth and ultimately decrease peanut yield. A high priority of researchers is to understand the molecular basis of metabolic function of flavonoids and also the levels and types of flavonoids produced under environmental stresses in crops (WinkelShirley, 2002) The results of present

study suggest that flavonoids have an important protective role against drought. It is notable that different cultivar may have different mechanism molecular level to combat stress. Of course, detailed functions of these genes associated to the defense role under drought stress need further validation in peanut.

6 FUTURE PROSPECTS

Medicinal use of plants and their extracts is becoming an important concern in health industry. Peanuts are rich source of medicinally important compounds that are products of flavonoid biosynthetic pathway. Flavonols and chalcones are among these products and a little is known about their synthesis in Arachis hypogaea. In addition they also have roles in various stress signaling pathways. So flavonoid biosynthetic pathway in Arachis hypogaea can be explored in order to find key enzymes and genes that play role in synthesis of these compounds. As these compounds play a crucial role against defense mechanism so understanding of plant responses to different environmental stresses at molecular level is crucial to improve tolerance of crops. The expression analysis of flavonoid biosynthetic genes needs further validation in peanut plant. As well as comparison of stress response behavior of different peanut varieties can be done to unravel set of genes involve in tolerance behavior. This in turn, will help in agriculture industry in understanding of drought tolerance of crops and development of drought tolerant

7 SUPPLIMENTARY INFORMATION

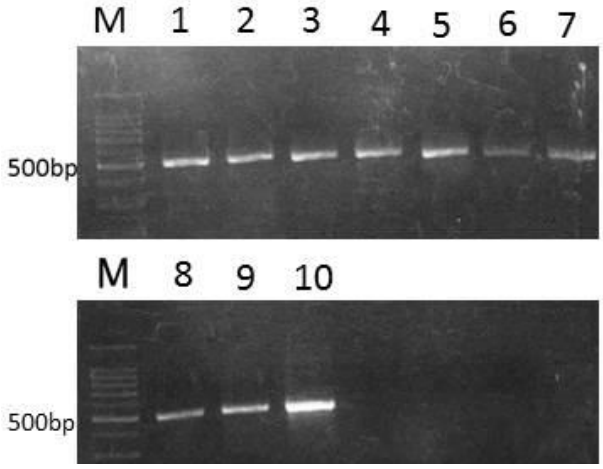

Figure 7:1 Agarose gel of PCR products of ITS2, M is 100bp ladder, Lane1-10 varieties of *A.hypogaea* subjected to sequencing

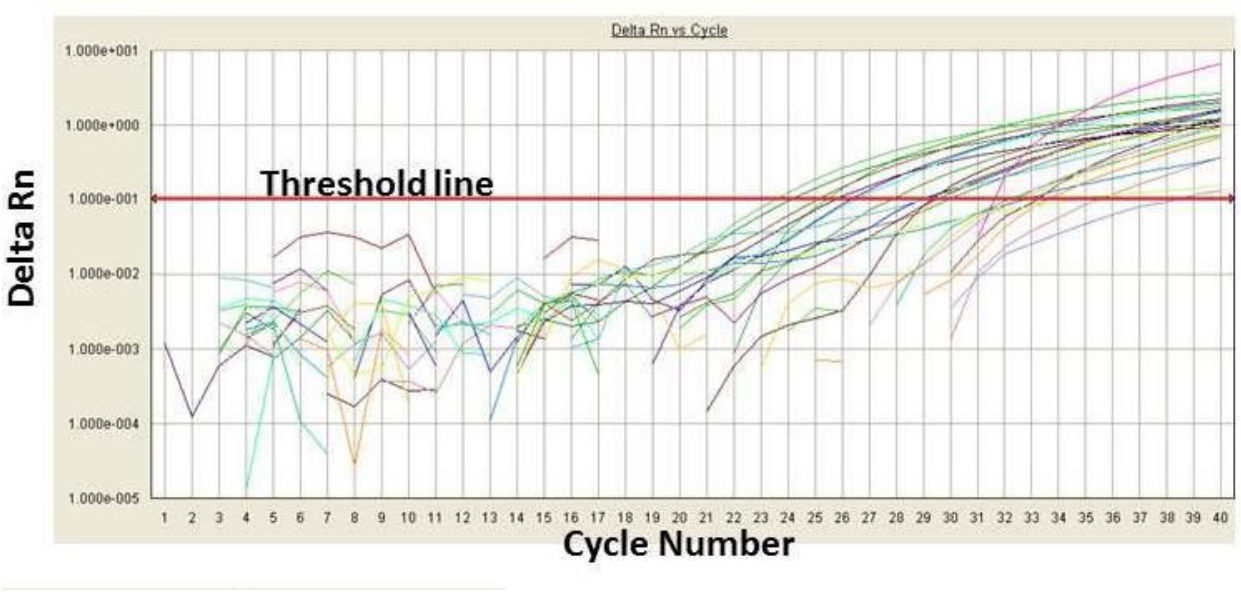

Figure7:2 Representative qRT-PCR amplification plot for FLS gene

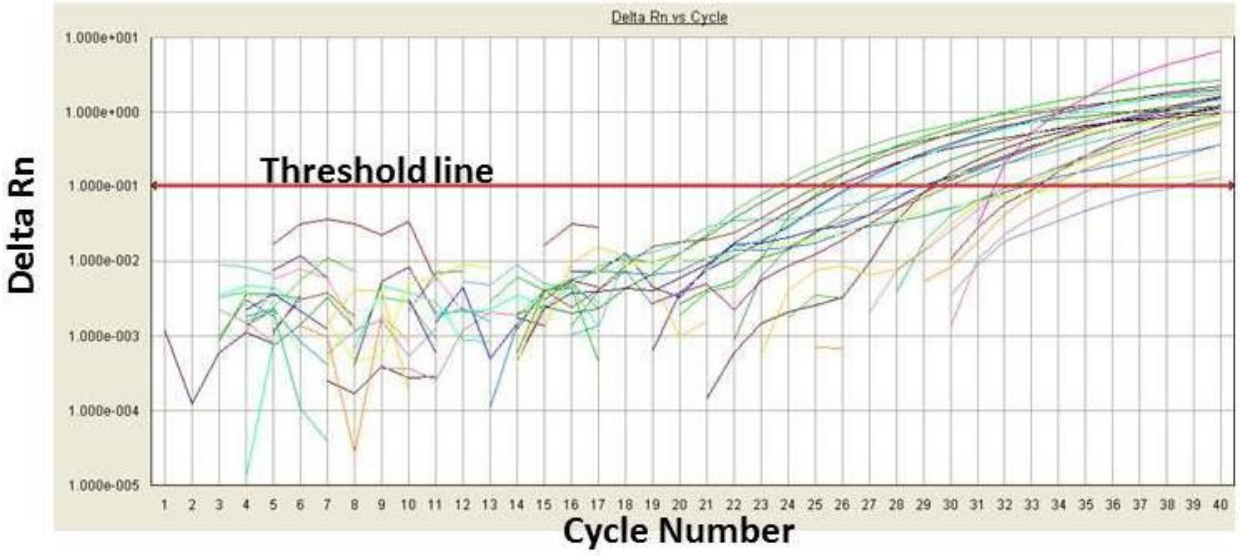

Figure7:3 Representative qRT-PCR amplification plot for CHS gene

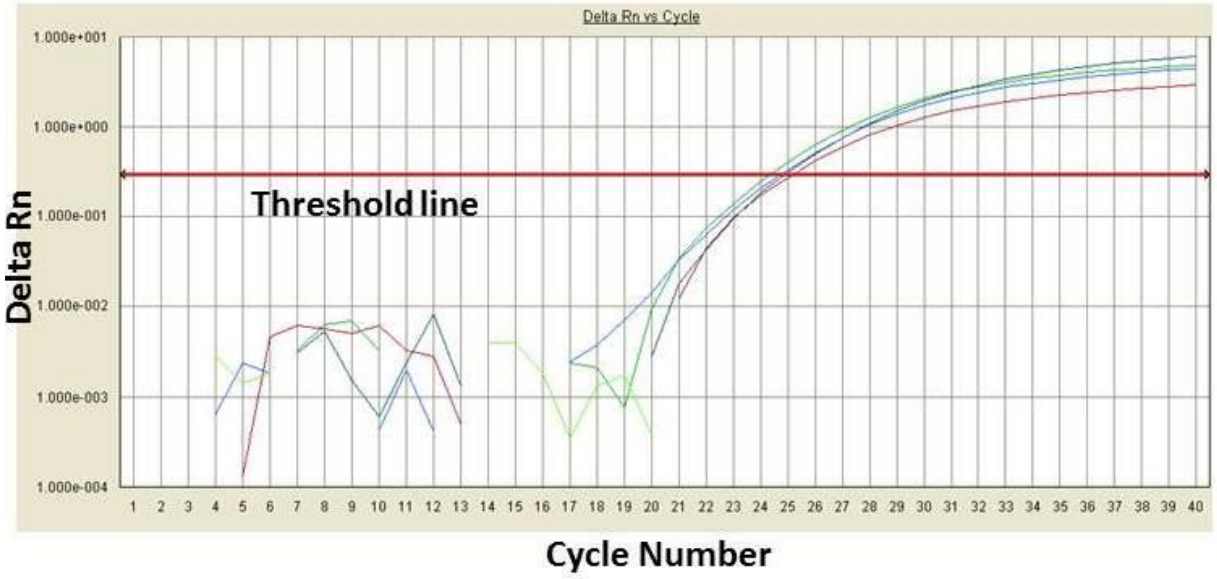

Figure7:4 Representative qRT-PCR amplification plot for beta actin

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9 APPENDICES

Primer Designing

PCR primers sets were designed for the amplification of the coding sequence of the selected gene from cDNA samples from peanut plants using primer3.

(http://www.simgene.com/Primer3)

For DNA Barcoding;

Gene	Primer name	Sequence 5'-3'
ITS2	ITS-F1 ITS-R1	ATGCGACTTGGTGTGAAT GACGCTTCTCCAGACTACAAT

For Expression analysis by qRT-PCR;

Gene	Primer name	Primer 5'-3'
CHS	CHS-F1 CH-R1	GCTGCACTCATTGTTGGTTC CCAGGGGATCAAAAGCTTCA
FLS	FLS-F1 FLS-R1	CACCCAACCCTTCTTTCCAT GTAAGGACTGGGGCATGTTC