Identification and analysis of Leucoanthocyanidin reductase (LAR) in *Arachis hypogaea*

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بسم الله الرحمن الرحيم

Identification and analysis of Leucoanthocyanidin reductase (LAR) in *Arachis hypogaea*

A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science

In

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Dedication

Dedicated to my exceptional parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment

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Table of Contents

List of Figures

List of Tables

List of Acronyms

LAR	Leucoanthocyanidin reductase
PAs	Proanthocyanidin reductase
CoA	Coenzyme A
Db	Data base
EST	Expressed Sequence Tags
ORF	Open reading frame
NARC	National Agriculture Research Council
NaOCl	Sodium Oxichloride
NaCl	Sodium Chloride
Mn	Moisture Content
APX	Ascorbate Peroxidase
CAT	Catalase
SOD	Superoxide Dismutase
CTAB	Cetyl trimethylammonium bromide
cDNA	Complementary DNA

FC	Field capacity
ROS	Reactive Oxygen species
OD	Optical density
NCBI	National Centre for Biotechnology Information
TF	Transcription factors
PCR	Polymerase Chain Reaction

ABSTRACT

Drought, salinity and extreme temperatures are the most common abiotic stresses, which affect the growth and productivity of the plant adversely. The plant's exposure to stress activates stress signaling pathways, which induce biochemical and physiological changes essential for stress acclimation. The ground nut, (*Arachis hypogea* L.) a major oilseed crop, is an important source of edible oil and the worlds' third most valuable source of proteins, besides as a dietary source of vitamin E and phytosterols. *Arachis hypogaea* is also a key source of plant's secondary metabolites, especially flavonoids. The important role of these flavonoids as the major blue, purple and red pigments has got these secondary products a great attention over the past years. The major sub groups of these compounds include the flavones, flavandiols, chalcones, anthocyanin, flavonols and proanthocyanidins (the condensed tannins), the aurones, a seventh group is also widespread. The current study will in light the role of proanthocyanidin biosynthetic gene(s) in *Arachis hypogea*. The characterization of respective genes may be helpful to direct the efforts of metabolic engineering in future, specifically to the branch pathway of proanthocyanidins. The goal of our study is to identify the Leucoanthocyanidin reductase (LAR) gene in *Arachis hypogaea* by insilico analysis and to evaluate different growth, physiological and biochemical parameters against salt stress.

INTRODUCTION

Groundnut (*Arachis hypogaea L*.) is one of the most important oilseed self-pollinated crops. It belongs to the family Leguminosae (ancient name) Fabaceae (current name) and sub family Papilionaceae. Arachis is derived from the Greek word "arachos" which means a weed and hypogaea means an underground part or in botanical language, a weed fruit formed beneath the soil. The Bolivian region of South America is believed as the origination of this crop, where the great diversity is found **(Figure 1.1)**.

The groundnut gene pool is composed of two subspecies i.e. fastigiata and hypogaea. Fastigiata is further divided into four varieties, peruviana, fastigiata, vulgaris and aequatoriana while; the sub species hypogaea consists of two varieties hirusta and hypogaea (Krapovickas and Gregory 1994). Peanut was commercially introduced in Pakistan on rain fed (barani) areas in Rawalpindi Division (Agro-ecological zone V) in 1950 (Rasheed, Dawar et al. 2004). From Rawalpindi Division it was then spread to other regions of the country. In the province of Punjab 92.93% peanut crops are grown through natural precipitation, in the Districts of Rawalpindi, Chakwal, Attock and Jhelum. In Southern irrigated plain of Sindh peanut is produced through irrigation including the Districts of Khairpur, Ghotki, Sukhar and Sanghar. While in KPK (Northern irrigated plain– IVb), the main peanut production Districts include Haripur, Karak, Hangu, Kohat and Swabi, where conditions are mainly rain fed. About 1013 hectares land is producing peanut through irrigation in Kurram agency (Kaur 2012).

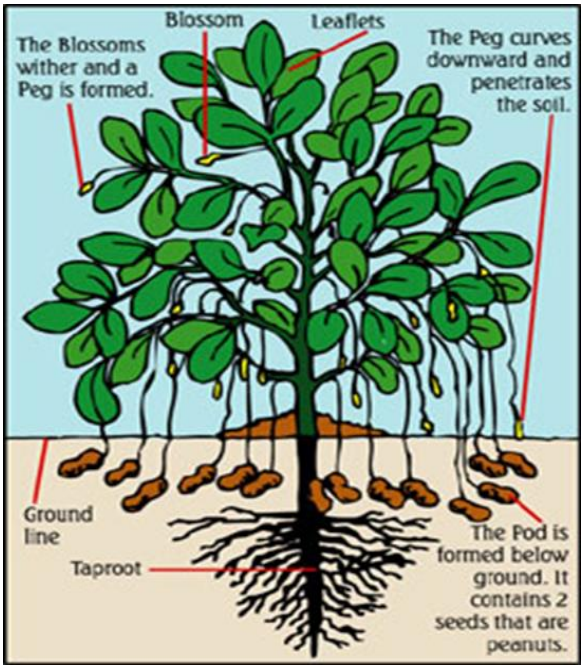


Figure 1.1: Morphology of Arachis hypogaea
(https://www.google.com.pk/morphology+of+arachis+hypogaea)

1.1 Flavonoids

Flavonoids are secondary metabolites of plants, which are polyphenolic having a general three ring chemical structure (C6-C3-C6). They belong to a diverse group of aromatic compounds which arise from malonyl-coenzyme A and Phenylalanine (CoA; through the fatty acid pathway). Flavonoids are basically comprised of six major subgroups, found in higher plants: the flavonols, flavones, flavandiols, chalcones, condensed tannins (the proanthocyanidins) and anthocyanins; the arones, a seventh group is found in abundance, but is not ubiquitous. Special types of flavonoids are also synthesized by some plants, like the isoflavonioids in legumes and some non-legume plants. Similarly, maize (*Zea mays*), gloxinia (*Sinningia cardinalis*), and sorghum (Sorghum bicolor) are some of the plant species which can synthesize 3-deoxyanthocyanins (or polymerized phlobaphenes). The stilbenes, another secondary metabolites which resemble the flavonoids, are produced by another unrelated plant species including peanut (*Arachis hypogaea*), grape

(*Vitis vinifera*) and pine (*Pinus sylvestris*). Hence, it is clear that in the flavonoid pathway branches are evolved many times and may have lost in specific lineages during the span of evolution.

Flavonoids are known for its diverse functions. Flavonol copigments and anthocyanin pigments have a well know physiological function in the attraction of insect pollinators and the dispersal of seeds. Flavonoids are figured in the key breakthroughs of science from 150 years of the past, which includes the Mendel genetics, pigments of the seed coat as his major focus in the trials with the pea (*Pisum sativum*), as well as the Mc Clinktock,s discovery of the transposable elements, which move across the flavonoid biosynthetic pathway genes in maize. More recently the anthocyanins are found helping in the study of the cosuppression, especially in petunia plant (*Petunia hybrid*). Apart from the wonderful pigments in leaves, flowers, seeds and fruits, they also play special signaling roles between microorganisms and plants, in defense process of antimicrobial agents and feeding agents, in male fertility of many species, as well as in UV protection. In some bryophytes (mosses) the initial phases of the pathway have also been found and the synthesis of flavonols, flavanones and flavones, has been suggested to be evolved earlier to work as chemical messengers and later on ultraviolet sun screens (Singh and Singh 1991)**(Figure 1.2)**

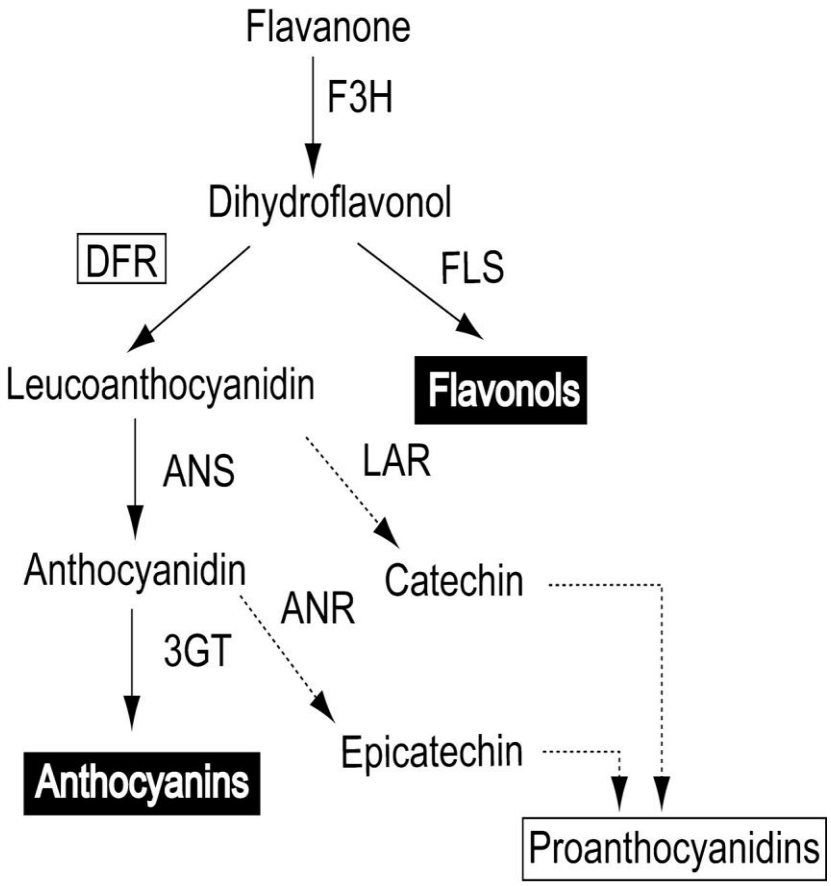


Figure 1.2: Flavonoids biosynthetic pathway. The LAR gene works on Leucoanthocyanidin forming an intermediate product Catechin. This catechin is readily converted into Proanthocayanidin.

1.2 Proanthocyanidins

The Proanthocyanidins (PAs), also called as condensed tannins are the polyphenolic secondary metabolites of plants, which are synthesized through the biosynthetic pathway of flavonoids. These compounds are found in large variety of plants, playing an important role in the defense mechanism against herbivores

(Francisco 1995) (Harborne and Grayer, 1993; Peters and Constabel, 2002). Studies have revealed that PAs are important in protection of ruminants against the pasture bloat (McMahon, McAllister et al. 2000) and act as antioxidants, having enhancing effects on human health such as protection from cardiovascular disease and free radical mediated injury (Bagchi, Bagchi et al. 2000). They also aid to the astringency and taste of various fruits and other plant products, like wine, black tea (*Camellia sinensis*) and some fruit juices. Like in the red wine industry, the PAs contribute in wine quality, enhancing color stability and mouth feel (Glories 1988). Hence, from nutraceutical as well as food quality perspective, the understanding of mechanism leading to PAs polymers formation and its regulation by plant is important. Proanthocyanidins formation and its chemistry has been extensively studied for decades. It is clear from their name that, the extension units are converted into colored anthocyanidins by acid hydrolysis, and it is the basic of the typical mechanism of these compounds (Porter 1989).

Peanut is an important crop worldwide for oil production and human consumption. There are many factors affecting the crop yield and production, including the most common abiotic stresses like salinity, drought, sodacity and cold. Many genes or gene networks have been involved in abiotic stress response. Developing stress tolerant, enhanced flavor and improved oil quality varieties of peanut plants are some of the major challenges of breeding techniques. As peanut is grown in arid and semi-arid areas, so drought and salinity are the most common stresses reducing the crop yield. Therefore, study on the stress responsive genes and its regulation is very much important. The current study will be focused on identification and analysis of Leucoanthocyanidin reductase (LAR) gene in Arachis hypogaea in response to salt stress.

REVIEW OF LITERATURE

2.1 Flavonoids as secondary metabolites

Flavonoids are a highly diversified class of chemical compounds originating from phenylpropanoid and acetogenesis pathway (Boss, Davies et al. 1996). While some flavonoids accumulate in a number of ferns and gymnosperms, an extraordinary multiplicity of variants has evolved among the angiosperms, the variation being due to different substituents on the flavan skeleton (Francisco 1995). An increasing number of flavonoids can be assigned important biological functions (Bogs, Ebadi et al. 2006). Among these is the induction of nod gene transcription in Rhizobium by certain flavonoids released from Phaseolus vulgaris (Hungria, Joseph et al. 1991) and Medicago sativa (Fisher and Long 1992), which in turn promotes the formation of root nodules and N2 fixation. Flavonols have also turned out to be essential for pollen germination in maize (Deboo, Albertsen et al. 1995) and Petunia (Ylstra, Busscher et al. 1994).

The biosynthetic pathway of flavonoids has been thoroughly characterized in *Zea mays*, *Arabidopsis thaliana* and *Vitis vinifera*. The upstream pathway concerns with the synthesis of core component (flavylium ion), which is the key unit of all the flavonoids, that starts from the three malonyl-CoA and one 4-coumaroyl-CoA molecules. The Chalcone isomerase (CHI) and CHS are the main enzymes contributing to the two-step process of condensation, forming a colorless flavanone, Naringenin. Flavone 3-hydroxylase (F3H) oxidizes the latter compound to form dihydrokaempferol (a colorless dihydroflavonol), whose 3' or 5' position of the B-ring can be subsequently hydroxylated through the enzyme flavonoid 3'-hydroxylase (F3'H) or flavonoid 3', 5'-hydroxylase (F3'5'H), forming dihyroquerecetin 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 dihydroflavonols synthesized are converted into anthocyanidins (the colored unstable pigments) through two reactions which are catalyzed by LDOX and dihydroflavonol reductase (DFR). DFR catalyze dihydromyricetin, dihydrokaempferol dihydrokaempferol and dihydroquerecetin to leucodelphinindin, leucopelargonidin and leucocyanidin (colorless flavan-3, 4-cis diols) respectively. While LDOX oxidizes leucodelphinidin, leucopelargonidin and leucocyanidin to delphinidin (purple-mauve, anthocyanidins), pelargonidin (orange anthocyanidins) and cyanidin (red-magenta anthocyanidins) respectively. The above mentioned colors result when anthocyanidins are in the acidic compartment. Also the final common step for the synthesis of stable and coloured compounds (anthocyanins) include the glycosylation of dephinidin, pelargonidin and cyaniding by UDP-glucose; flavonoid 3-O-glucosyl transferase (UFGT). Lastly delphinidin-3-glucodide and cyaniding-3-glucoside can be further methylated by the enzyme methyltransferases (MTs) to form peunidin or mavindin-3-glucoside and petunidin-3-glucoside, respectively.

2.2 Proanthocyanidins (An Important Flavonoid)

The Proanthocyanidins, also termed as condense tannins, are a group of flavonoid polymers which exist in different parts of many plant species. A special characteristic of the PAs is the ability of these compounds to bind to proteins and precipitate it, which reflects that they are multidentate ligands having the ability to bind at more than one point simultaneously (Spencer, Cai et al. 1988). This property can result in the production of colloidal haze in beer when no stabilizing treatment is carried out (Erdal 1986), a problem which can be overcome by consuming PA-free barley as a raw material (Von Wettstein, Jende-Strid et al. 1977). If present in forage legumes, like Onobrychis viccifolia and Lotus corniculatus, the property of the PAs to react with dietary proteins and to produce stable complexes can have significant effects on the animal nutrition. This special ability is the main reason of PAs role as antibloat agents, as these complexes

suppress the formation of stable (Jones, Broadhurst et al. 1976). Moreover, saved against the de-amination of bacteria in the rumen, delivering a protein by-pass mechanism which results in excess duodenal absorption of amino acids (Aerts, Barry et al. 1999). PAs and monomericflavan-3-ols have now been reported to possess antioxidant properties (Hara 1994) and even in some plants their presence has been correlated with insect resistance (Chan, Waiss et al. 1978).

2.3 Proanthocyanidins medicinal value

PAs are found in a large variety of plants and have a key role in the defense mechanism against herbivores (Francisco 1995). They may give protection to ruminants from the pasture bloat (McMahon, McAllister et al. 2000), also can behave as antioxidant to benefit humans against cardiovascular diseases and free radical mediated injury (Bagchi, Bagchi et al. 2000). Proanthocyanidins also play a key role in the taste and astringency of many fruits and plant products, including tea (*Camellia sinensis*), wine and fruit juices. They enhance the quality o.f wines as in case of red wine contributing to color stability and mouth feel (Glories 1988). Hence, from both medicinal and food quality perspectives, understanding the mechanism leading to PAs synthesis is important and also how plants tend to regulate it.

MATERIALS AND METHODS

3.1 In silico analysis

The assembly and organization of *in silico* gene data

NCBI search

cDNA sequencing in high throughput manner is used to obtain EST sequences, based on a study carried out in 1991, in which cDNA clones were chosen randomly and were sequenced from one, or both ends of their inserts. EST as a term was basically used to show this type of sequences. The term EST was introduced to refer to these types of sequence, qualified by its short length (400–600 bases). Therefore ESTs are powerful in searching the genes because they greatly reduce the required time for the gene identification. Primarily the NCBI database is used to get the sequence of the concerned gene. But if the gene sequence is not reported already, then we search for the sequence of gene of related species. In case of peanut the related species are *Medicago sativa (*alfalfa), *Phaseolus* (beans), *Glycine max* (soybean) and *Pisum sativum* (pea). EST sequences of *Glycine max* are obtained by using NCBI EST search tool (http://www.ncbi.nlm.nih.gov/).

3.2 Blast analysis

EST sequence showing the highest similarity and full length sequence is used for further identification. By using the transcriptome database “Peanut DB

(http://bioinfolab.muohio.edu/txid3818v1/)" the obtained EST sequence is Blast against both the peanut genome i.e. *Arachis duranensis* and *Arachis ipaensis.* The resulting contigs are observed by using the MEGA6 software. Contig showing higher similarity are used for further confirmation.

3.3 Open Reading Frame detection (ORF)

The obtained contig sequence is then blast for ORF sequence by using the EXPASY TRANSLATE TOOL (http://web.expasy.org/translate/). The ORF showing the complete length i.e. from start codon to stop codon is selected and used for Domain analysis.

3.4 Identification of putative conserved domain

The ORF sequence showing complete length is used to search for the putative conserved domains of our related gene. For this purpose the NCBI PROTEIN BLAST is used (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 Identification of promoter region

To identify the promoter region, the original sequence was blasted one by one in the Peanut Base (http://www.peanutbase.org/home) against both the genomes. The sequence obtained was then translated and configured. By searching the hits given by the configuration, all the matching sequences were highlighted one by one. Promoter region was identified as 1500 bases upstream of the starting region of the original sequence.

3.6 Primer designing

Primers were manually designed by using the complement and oligo calc tools.

Forward- ATGACTGTGTCGAGCGCC

Reverse - TCAGCCCATAGCTGTGATTG

3.7 Plant material

Different varieties of ground nut were collected from the National Agriculture Research Council (NARC), Islamabad. Out of the total 6 varieties (PG-1058, Photowar, Bari-2000, Bari-2011, Golden, Bard-92, Bard-479) two were selected on the basis of research plan and growth respective, including Golden and Bari-2011.

Plant growth

3.7.1 Soil preparation

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 the seeds in it.

3.7.2 Seeds sterilization

Ground nut seeds were taken and shells were removed. The Seeds were sterilized by rinsing in 70% ethanol (1 min). Proper washing with distill water was done.

Then seeds were rinsed for 5 minutes with 3.5% NaOCl solution (commercial bleach). After sterilization the seeds were thorouthly washed with sterile water, followed by subsequent soaking for about 2 hours before use (Maina, Emongor et al. 2010).

3.7.3 Planting seeds / germination

Seeds were sown in soil at control conditions i.e. 28ºC and 16/8 light/dark hours. All the seeds were sown in triplicates. Germination was observed on 7-10 days after cultivation. About 90% of the plants were successfully germinated **(Figure 3.1).**

Figure (3.1): Showing germination of *Arachis hypogaea* seeds

3.7.4 Stress induction

After 15 days of germination the plants were exposed to salt stress. Salt stress in this case was NaCl solution of 60, 120 and 240 mM. Alternative salt and water treatments were given at regular intervals. Plants were harvested after 10-12 days of treatment and leaves

were taken as sample. These samples were then subjected for the evaluation of different physiological and biochemical characters **(Figure 3.2)**

3.7.5 Harvesting

After 15 days of treatment the plants were harvested. The samples were immediately transferred on liquid nitrogen in order to avoid chemical degradation. These samples were then stored at -80º C for future use.

3.8 Physiological Evaluation

The plant samples were analyzed for the following physiological activities.

3.8.1 Germination percentage

The number of seeds germinated in each treatment was counted on 7th day after sowing. Three replicates were maintained for each treatment. The total germination percentage was calculated by using the following formula.

$$\text{Germination percentage} = \frac{\text{Total number of seeds germinated} \times 100}{\text{Total number of seeds sown}}$$

Figure (3.2): Showing 15 day's old plants

3.8.2 Moisture Content

Moisture content of the plants was calculated by using the formula described by **Nancy Trautmann and Tom Richard**

$$M_n = \frac{W_W - W_d \times 100}{W_W}$$

in which:

M_n = moisture content (%) of material n

W_W = wet weight of the sample, and

W_d = weight of the sample after drying.

3.8.3 Shoot and root length

The shoot and root length were measured one by one with a calibrated scale. Tags were used for each sample and pictures were taken for the sake of identification (**figure 3.3**).

3.8.4 Shoot and root weight

The weight of shoot and root of each plant was measured by using a digital weighing balance. The weight was recorded in grams and comparison was made against the respective treated and control plants.

Figure (3.3): Harvesting of plants after 15 days of stress, figure also indicating the shoot and root length respectively in centimeters.

3.9 Biochemical Assays

3.9.1 Enzyme assays

(i) Ascorbate Peroxidase (APX)

The peroxides (POD) activity was performed by the technique described by (Reddy, Subhani et al. 1985) with some modifications. The POD reaction (3 ml) contained 50mM phosphate buffer (pH 6.8) 20 mM guaialcol, 40 mM H_20_2 and 0.1 ml enzyme extract. The absorbance difference of the reaction mixture at 470 nm on UV-visible spectrophotometer (BMS, Biotechnology Medical Services)

was recorded after 2 minutes. The APX value was calculated by using the following formula.

$$\textbf{APX (Mm/gFW) activity} = \frac{\textbf{AB change} \times \textbf{total volume}}{\textbf{Sample vol} \times \textbf{EC} \times \textbf{mins} \times \textbf{gFW}}$$

(ii) Catalase (CAT)

For Catalase activity 0.01 ml of 10 mM H_2O_2 enzyme extract and 25 mM potassium phosphate buffer (pH 7.0) were used in the reaction. Absorbance was recorded at 240 nm on UV-visible spectrophotometer (BMS, Biotechnology Medical Services) (Lee, Choi et al. 2010). The CAT value was calculated by the following formula.

$$\textbf{CAT (Mm/gFW) activity} = \frac{\textbf{AB change} \times \textbf{total volume}}{\textbf{Sample vol} \times \textbf{EC} \times \textbf{mins} \times \textbf{gFW}}$$

(iii) Superoxide Dismutase (SOD)

For SOD determination, a reaction of 3ml was prepared in glass breaker containing 50mM potassium phosphate buffer (pH 6.8), 75mM EDTA, 13mM methionine, 0.05 mM NBT, 40 mM H_2O_2 and 0.025 mL enzyme extract. The glass beakers containing these constituents were placed under fluorescent lamp

(30 W) for 20 minutes and absorbance was recorded at 560 nm using UV-visible spectrophotometer (BMS, Biotechnology Medical Services) (Beauchamp and Fridovich 1971). SOD was determined by using the following formula.

$$\textbf{SOD activity (units/g)} = \frac{R_3 - R_2}{R_1 \times 50/100}$$

Where;

R_1 =Absorbance of control

R_2 = Absorbance of blank

R_3 =Absorbance of sample

3.10 Total Soluble Proteins

Fresh wheat leaves (500 mg) were homogenized with 10ml phosphate buffer (pH 7.8) and centrifuged at 14000 rpm at 4 ºC for 20 minutes. The top aqueous layer was then transferred into another sterile tube and stored at 4 ºC for further analysis.

The supernatants of the total soluble proteins content was determined by using Bradford assay. For assay reaction, the solution containing (0.5 ml Bradford reagent and 2 ml distilled water were mixed in a falcon tube). 20µl protein extract

was added to the reaction mixture, shaken vigorously and kept for 5 minutes at room temperature.The absorbance was measured at 595 nm on UV-visible spectrophotometer (BMS, Biotechnology Medical Services) while using distilled water as blank. Protein concentrations were calculated from the standard BSA (Bovine Serum Albumin) curve (Yadegari, Heidari et al. 2007).

3.10.1 Chlorophyll content

Plant leaves weighing about 1gm were taken in fresh condition from each plant. The leaves material was incubated at 80 ^{o}C for 48 hours in order to get dry mass and was manually grinded into powder. For the determination of Photosynthetic pigments (chlorophyll a, chlorophyll b, and Carotenoids) 25 mg of powdered plant material was taken in a 15 ml Falcon tube. Magnesium oxide of equal amount (25 mg) was added and was thoroughly mixed for few minutes. The extraction of pigments was determined in 5ml methanol and homogenized by shaker at 200 rpm for 2 hours. The resulted sample was subjected to 5 minute centrifugation at 4000 rpm at room temperature. Then 3 ml of supernatant was taken in a cuvette and absorbance was measured at three wavelengths 470 nm, 653 nm and 666 nm on UV-visible spectrophotometer (BMS, Biotechnology Medical Services) against methanol as solvent blank. The concentrations of pigments were determined by the formulas stated by (Lichtenthaler 1987) **.**

3.10.2 Soluble sugar content (Dubois Method)

Fresh leaves weighing 50 mg was taken and grounded to a fine powder in pre-warmed 3ml solution of 90% ethanol. The powdered sample was then incubated at 80^{o} C 1 hour. The aqueous layer at the top was contained in a sterile tube. The remaining solid material was crushed again in 3ml pre-warmed 90% ethanol

solution and incubated again at the same conditions. Supernatant was taken and mixed with the first one, followed by final volume of 15 ml with distilled water. Plant extract of 1 ml was mixed with the same amount of 5% phenol. Then 5ml of concentrated H_2SO_4 was added gently and the final volume of 10 ml was made with distill water. The resulting mixture was then incubated for 30 minutes with periodic shaking. While using UV-visible spectrophotometer (BMS, Biotechnology Medical Services) and filtered distilled water as blank, the absorbance was recorded at 485nm. Then soluble sugars content was calculated from glucose standard curve (Qayyum, Razzaq et al. 2011).

3.11 DNA extraction

Genomic DNA was isolated from *Arachis hypogaea*, cultivar, GOLDEN, by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Richards, Russell et al. 1998). For this purpose up to 1 gm of plant green tissues was uniformly ground in autoclaved pestle and mortar by adding 4-5 mL of CTAB buffer preheated at 65ºC. The mixture was transferred to autoclaved 1.5 mL eppendorf tubes, vortexed briefly and incubated in water bath adjusted at 65ºC for forty minutes. Tubes were again vortexed and centrifugation was done for 10 minutes at 10,000 g. Supernatant was transferred to a new set of autoclaved eppendorf tubes and equal volume of chloroform was added. Vigorous vortexing and centrifugation was done at 10,000 g for 10 minutes. This step was repeated until supernatant became clear. Clear supernatant was again transferred to new autoclaved eppendorf tubes and ice cold iso-propanol (100%) was added in equal volume. Contents of tubes were mixed by gently inverting them. After this tubes were placed at -20ºC for at least 30-40 minutes followed by centrifugation at

12,000 g for 15 minutes. The supernatant was discarded this time and the pellet was washed twice with 70% ethanol. After washing, maximum ethanol was removed from tubes and pellet was dried in concentrator for removing any leftover ethanol. Dried pellet

was resuspended in about 200 µL distilled water containing RNase A, incubated for 1 hour at 37ºC and stored at -20ºC.

3.12 RNA extraction

RNA was extracted by TRIZOL method. About 100mg tissue sample was taken and grinded with liquid nitrogen by using autoclaved motor and pestle. 1ml Trizole was added to make slury and the homogenate was transferred to a 2ml eppendorf tube. The sample was then incubated for 5 minutes at room temperature and centrifugation was done at 12000 rpm for 10 minutes at 4◦C. The supernatant wash then transferred into a fresh eppendorf tube and 270ul chloroform was added to it. Shaking vigorously by hand for 15 seconds the tubes were incubated for 5 minutes at room temperature. The tubes were then centrifuged at 12000rpm for 15 minutes at 4◦C and the aqueous phase was transferred to a new eppendorf. 0.65ml of isopropanol was added and incubation was done at -80°C for 2 hours. The tubes were again centrifuged at 12000rpm for 10 minutes at 4° C and supernatant was removed. 1ml of 75% ethanol was added and the pellet was broken by vortex. Centrifugation was performed at 9500rpm for 10 minutes at 4°C. Ethanol was discarded, the pellet was air dried and stored at -80°C for future use.

(W.M. Keck Foundation Biotechnology Microarryay Resource Laboratory at Yale University)

3.13 Reverse transcription/ cDNA synthesis

cDNA was prepared by using reverse transcriptase enzyme. Two master mix reactions were used i.e. Master mix 1 (containing PCR water, 5mMdNTPs and oligo dTs) and Master mix 2 (containing 5x RT buffer, 0.1MDTT and Rnase Out).

Reverse transcriptase enzyme and total RNA were contained in a tube having already prepared master mix. The tube was then subjected to different temperatures and the cDNA was stored at -20°C for PCR use.

RESULTS

4.1 Germination percentage

The seeds were grown on already prepared peat moss and the germination percentage was recorded. About 90% of the seeds were successfully germinated. The germination percentage was calculated by using the following formula.

$$\text{Germination percentage} = \frac{\text{Total number of seeds germinated} \times 100}{\text{Total number of seeds sown}}$$

$$= \frac{72 \times 100}{80}$$

$$= 90\%$$

4.2 Stress induction

The plants were exposed to salt stress after 15 days of germination. NaCl treatment with alternative watering was performed at regular time periods. The amount of salt solution was given according to the FC (Field capacity) of the growth medium (peat moss). The FC was calculated by dry oven method using the following formula.

$$\text{Field capacity (FC)} = \text{Saturated weight of peat moss} - \text{dry weight of peat moss}$$

$$= 46-6$$

$$= 40$$

Plants were harvested after 10-12 days of treatment and leaves were taken as sample. These samples were then subjected for the evaluation of different physiological and biochemical characters.

4.3 Moisture Content

Moisture content of the plants was calculated by using the formula described by **Nancy Trautmann and Tom Richard**

$$M_n = \frac{W_W - W_d \times 100}{W_W}$$

In which:
M_n = moisture content (%) of material n
W_W = wet weight of the sample, and
W_D = weight of the sample after drying.

The moisture content (MC) was found declining with increasing concentration of salt stress (**Figure 4.1).**

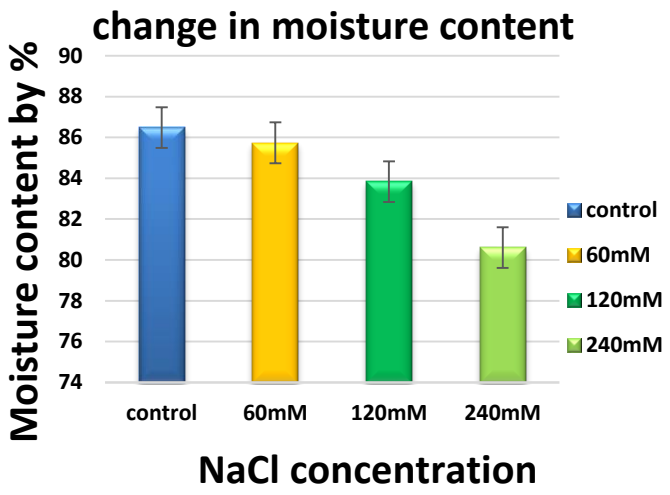


Figure (4.1): showing the MC decreasing as the concentration of salt increases. Control having more MC than 60mM and so on.

4.4 Shoot and root length

The shoot and root length was measured by using a graduated scale. It was observed that the growth of both the parameters decreases with increasing concentration of salt stress. The results are shown in **figure (4.2) and (4.3).**

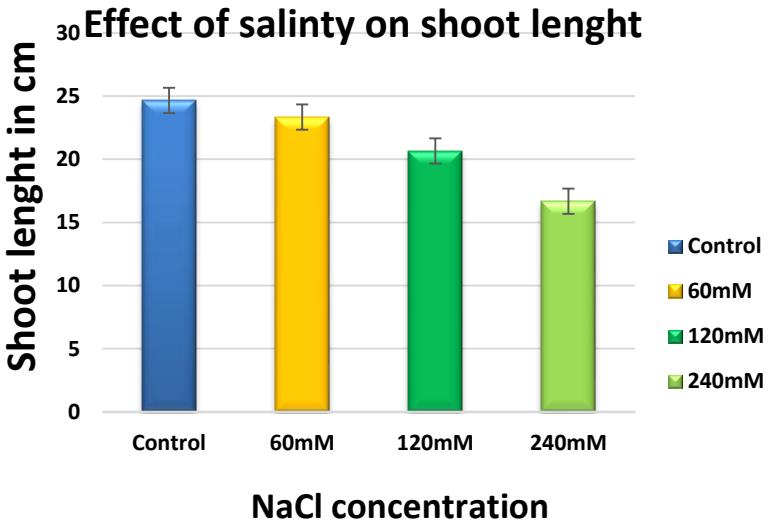


Fig (4.2) showing the decrease of shoot length in response to increasing salt stress

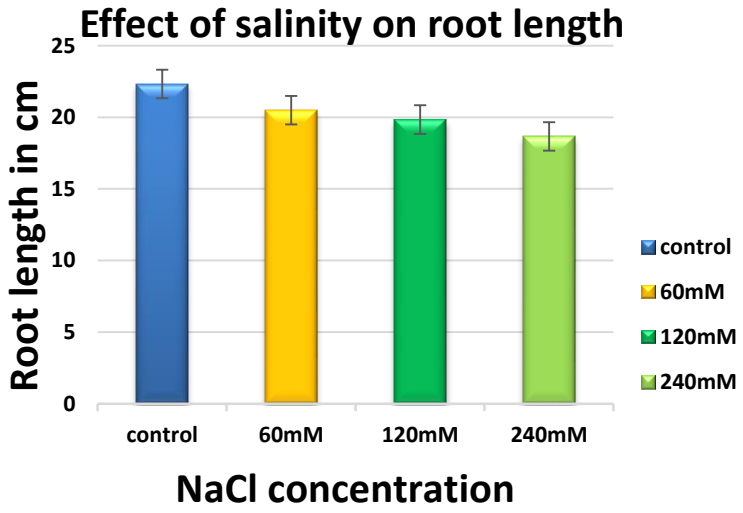


Fig (4.3) showing the decrease of root length in response to increasing salt stress

4.5 Shoot and root weight

The shoot and root weight was measured by using a digital balance. In both cases the weight was found declining as the concentration of salt increases. The respective shoot and root weight is shown in **Figure (4.4) and (4.5).**

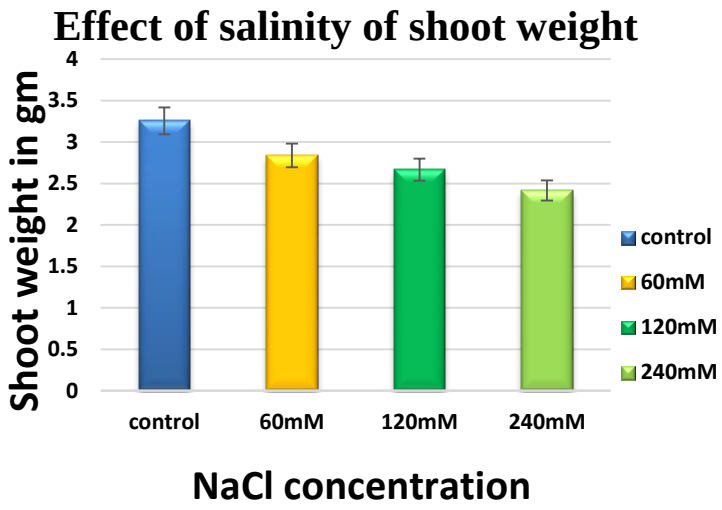


Figure (4.4) showing the decrease of shoot weight in response to increasing salt stress.

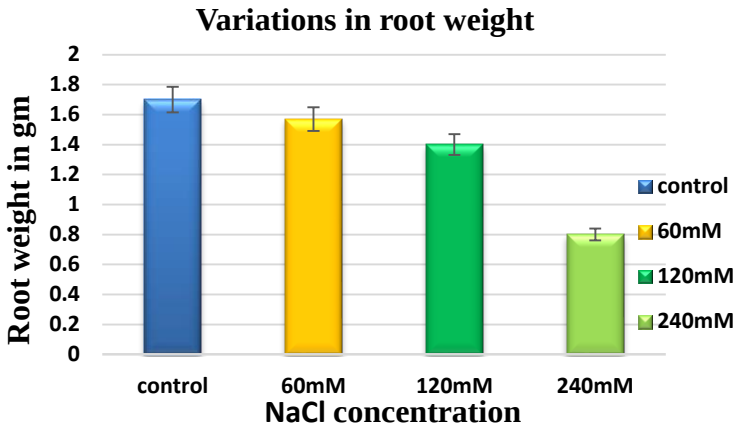


Figure (4.5) showing the decrease of shoot and root weight respectively, in response to increasing salt stress.

4.6 Biochemical assays

Biochemical analysis of the ground nut plants were performed by using the leaf samples collected earlier and stored at -80°C. The assays included chlorophyll content, total soluble sugar content, total soluble protein and enzyme assays i.e., Peroxidase (POD), Catalase (CAT), Ascorbate (APX) and Superoxide dismutase (SOD).

4.6.1 Chlorophyll content

Chlorophyll content was determined by using the method previously derived by **(Lichtentaler and Wellburn, 1985).** The total chlorophyll content of the control plants was found higher than the treated plants. Plants treated with 60mM showed higher content than that of 120mM and 240mM respectively. Chlorophyll content was calculated according to the following formula

$$\text{Chlorophyll (mg/g Fwt)} = \frac{(\text{OD}-0.01) \times 10}{92.6474 \times \text{Fwt of sample}}$$

Where Fwt= Fresh weight of sample

The total chlorophyll content according to mass spectrophotometric absorption is shown in **figure (4.6).**

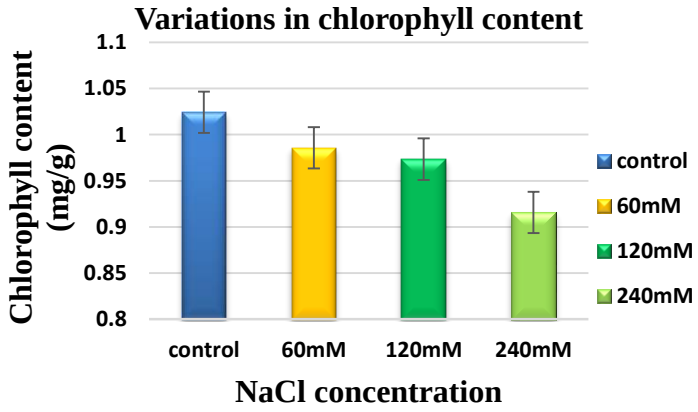


Figure (4.6) showing the value of chlorophyll content declining as the salt concentration increases

4.6.2 Total soluble proteins

The quantitative analysis of total soluble proteins was confirmed by UV spectrophotometry analysis at 595nm. The protein content was found increasing with increasing salt concentration from 60mM to120mM to 240mM respectively. The control plants showed lower content of protein than the treated plants (**Figure (4.7).**

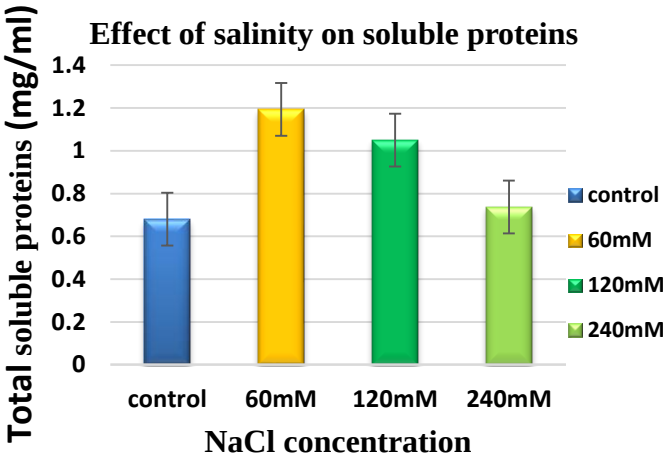


Figure 4.7 showing variation in total soluble protein content under control (0Mm NaCl) and stress conditions (60mM, 120mM and 240mM)

4.6.3 Total soluble sugar (Dubois Method)

The sugar content of *Arachis hypogaea* leaves was measured by using a UV spectrophotometer at 485nm. The relative sugar content was recorded and found to be increasing with increasing concentration of salt stress. The control plants (0mM NaCl) showing decreased sugar content as compared to treated plants (60Mm 120mM and 240mM) **(Figure 4.8)**.

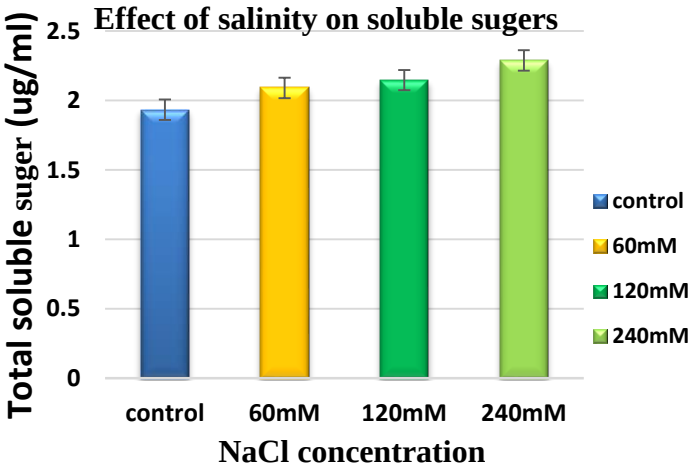


Figure (4.8) showing variation in total soluble sugar content under control (0Mm NaCl) and stress conditions (60mM, 120mM and 240mM)

4.7 Enzyme assays

Reactive oxygen species (ROS) are produced in plants in response to biotic and abiotic stresses. These ROS cause damage to the plant cells like cell membrane damage, enzyme inhibition, peroxidation, protein oxidation and nucleic acid degradation. But plants have their internal system which activates the production of antioxidant enzyme that helps the plant to reduce and withstand stressful conditions (Allen 1995). Major ROS scavenging enzymes include Super oxide dismutase (SOD), Ascorbate peroxidase (APX), Glutathione reductase (GR) and Catalase (CAT). In our study we have studied some of these antioxidant enzymes.

4.7.1 Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) activity was performed by using the method of **(Reddy, Subhani et al. 1985)**. UV spectrophotometer analysis was performed (umol/unit time/g) at 470nm and the readings were recorded with an interval of 10 seconds. APX was found increasing with the increasing concentration of NaCl .The control plants (0mM NaCl) showed lower value as compared with the treated plants (60mM, 120mM and 240mM).The OD was found changing after each 10 seconds because of the decomposition of Ascorbic acid (**Figure 4.9)**.

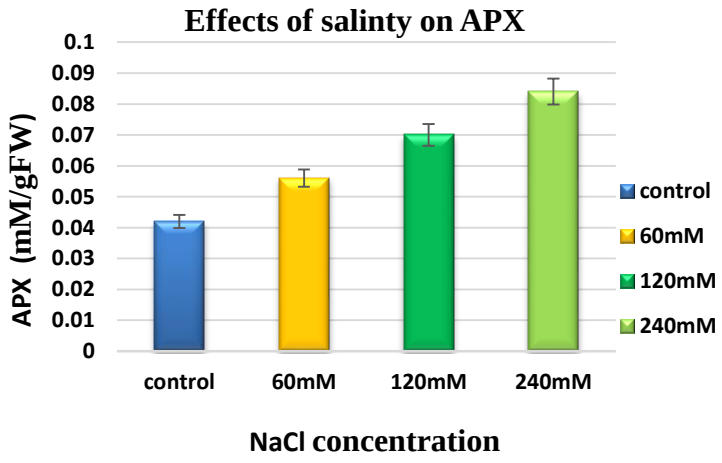


Figure (4.9) showing gradual increase in APX value from control (0mM NaCl) to treated plants (60mM, 120mM and 240mM.

4.7.2 Catalase (CAT)

CAT activity was performed by the method described by (Lee, Choi et al. 2010)**.** The readings were taken by using a UV spectrophotometer at 240nm at different time points of 10 seconds interval. The CAT level was found increasing gradually with increasing concentration of NaCl stress. The control plants (0mM NaCl) showed declined level of CAT as compared to the treated plants (60mM, 120mM and 240mM NaCl) **(Figure 4.10).**

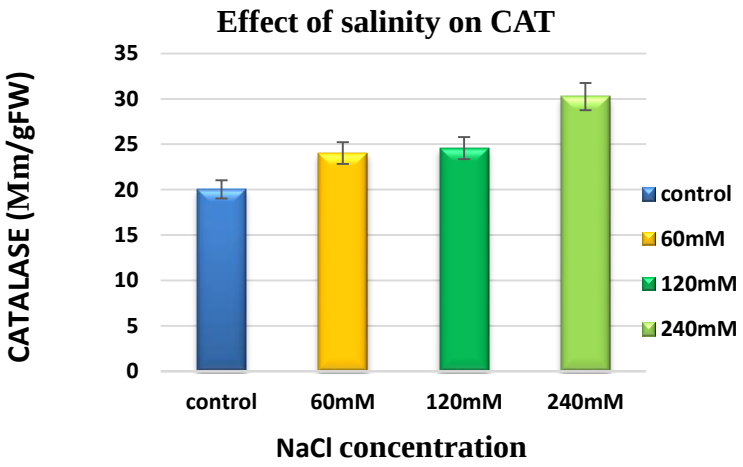


Figure (4.10) showing increases in Catalase level as the NaCl concentration increases from control (0mM NaCl to 60mM, 120mM and 240mM respectively.

4.7.3 Superoxide dismutase (SOD)

The SOD activity was performed by the method used by (Beauchamp and Fridovich 1971). UV spectrophotometer analysis was done at 560nm. The SOD values were recorded and found to be increasing while increasing the NaCl stress. The control plants (0mM NaCl) showed lower level of SOD as compared to the treated plants (60mM, 120mM and 240mM NaCl) (**Figure 4.11).**

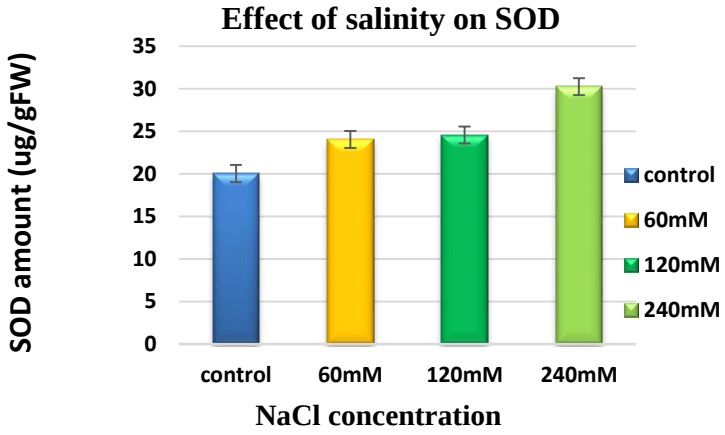


Figure (4.11) showing increases in SOD level as the NaCl concentration increases from control (0mM NaCl) to 60mM, 120mM and 240mM respectively.

4.8 In silico data analysis

Leucoanthocyanidin reductase (LAR) gene has been reported in Soybean (*Glycine max*), Silverleaf desmodium (*Desmodium uncinatum*), Sainfoin (*Onobrychis viciifolia*) and other legumes. Peanut (*Arachis hypogaea*) being a legume is predicted to be a source of LAR. To confirm this prediction we used the following bioinformatics tools and molecular biology techniques.

- Peanut Base (http://www.peanutbase.org/)
- Peanut DB (http://bioinfolab.muohio.edu/txid3818v1/)
- NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- Expasy Translate Tool (http://web.expasy.org/translate/)
- Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

4.9 Retrieval of Nucleotide sequence

In order to retrieve the nucleotide sequence of LAR, the NCBI SEARCH TOOL was used (http://www.ncbi.nlm.nih.gov/). Hence the LAR sequence of *Arachis hypogaea* was not reported therefore we searched for the sequence of the close relative i.e. *Glycine max* **(Figure 4.12).**

#t3818.cdna.v1.contig21922 LAR, glycine max

GGGGGGATGCACAACATAGTCACTCTTCTTTAGTACTTATTTTTGTGAGGGAGACAAAACAAGT
ATTAGGTTTCTAACATGACTGTGTCGAGCGCCACCATTCCTTCAGCCACCAAGGCTCGGATTCTG
GTCATCGGAGCGACCGGCTTTATGGGTCAATTTGTGACAAAGGCGAGTCTTGCCTTCGGACATC
CCACTTGTTTGCTGGTTCGGCCTTCCCATAAAATGCACTCCAAGGCTGCCCTTCTCAAATCCTTTC
AGGATAAAGGTGCCACACTCATTCATGGGGTAATAAATGATAAGGATCAAATGGAGAAAATTT
TGAAAGACAATGAGATAGAGATTGTCATTTCTCTTGTTGGAGGGGGCAATGTTCTGGACCAGAT
TACTTTAATAGATGCCATGAAATCTGTGAAGACTATCAAGAGGTTTTTGCCATCAGAGTTTGGG
CATGACGTGGACAGGGCAGAGCCTGTGGAGCCAGGGCTAACAATGTACAAAGAGAAACGCGT
AGTTAGACGCATGGTTGAAGAGAGTGGGATCCCCTTCACCAACATCTGTTGCAATTCCATTGCTT
CTTGGCCTTACTATGACAATTGTCATCCTTCTAAGCTCCCTCCTCCCACGGACCAATTTCAAATTT
ATGGTGATGGCAACATTAAAGCTTACTTTGTTGATGGCAATGATATTGGAAGGTTGACAATGAA
AGCAGTTGATGATGTTAGAACACTGAACAAAAACGTTCATTTTAGACCCTCAATGAATTGTTACA
GCGTCAACGAGCTTGCTTCTTTATGGGAAAAGAAAATTGGTCGAACAATTCCCAGAGTTACCAT
CTCAGAAGATGATCTTCTTGCTGCAGCTGCAGCGAACTTGATACCTGAAAGCATTGTAGCCTCAT
TCACGCATGACATTTTCATCAAGGGTTGCCAAGTTAACTTCAGCACAGATGGCTCAAATGATGTT
GAAATTGGAACACTGTACCCTGATGAAAAGTTCCGATGCTTGGAGGATTGCTTTGAGGATTTTG
TTCCCATGATCCGTGACAAGATTCTTGGAGGCCCAAATGATGAACTTGCCAAAACCAATAATAA
GTCATTGCTAGAAGCTGTGCCAATCACAGCTATGGGCTGAAAATGACAAATCTAAATAAACATG
TGACCTGAGAATCCATATGTATTAATTCACCATAAATTTGGTGGTGGAGGCAGAGAGAATTATT
GGTCTAATTTATATAATAATTTATGGAGTAAAATAGACCTTATTTTCGTATGTACTGATTTATTAT
TGCTAGGTCAAGAATATACGTACTCCTTCAATATATATGTTTCAGCCTAGAAATTACGCTGTGTT
TTCCGGAGCAGAATTAAAAAATAATGCTTCAAGAGAGATGTAATAAAGTTTAATACTACTCCTG
GTGGTACTCTTGATGTATTT

Figure 4.12: Nucleotide sequence of Leucoanthocyanidin reductase gene, having a size of 1437 base pairs (retrieved from NCBI)

4.10 Analysis on Peanut DB

Peanut DB is the transcriptome database which includes the predictive gene data from the two parent genomes of *Arachis hypogaea* (i.e. *Arachis duranensis* and *Arachis ipaensis*). This data base is a public genomic source which integrates the transcriptome data of peanut from different reservoirs (*i.e.*, Sanger, 454 and Illumina sequences). Currently the Peanut DB focuses on the transcriptomics analysis of *Arachis hypogaea.* This source was used to obtain the Enzyme Commission (EC annotation) for the concerned contig by filter search for Leucoanthocyanidin reductase. A number of contigs were analyzed but only five were found having our respective domain for LAR i.e. NADB_Rossmann superfamily **(Table 1)**.

S.NO	Contig	Conserved domain
1	**t3818.cdna.v1.contig21922**	√
2	t3818.cdna.v1.contig22227	√
3	t3818.cdna.v1.contig21790	√
4	t3818.cdna.v1.contig18314	√
5	t3818.cdna.v1.contig3879	√

Table 1: List of contigs showing positive results for the conserved domain (NADB_Rossmann super family). A large number of contigs were analyzed for the candidate domain, but only 5 were found to have positive results.

4.11Open reading frame (ORF) detection

The contig showing the most significant E-value was selected (**t3818.cdna.v1.contig21922)** and ORF analysis was performed by using **EXPASY TRANSLATE TOOL** (http://web.expasy.org/translate/). The ORF showing the

complete length i.e. from start codon to stop codon was selected and used for Domain analysis (**Figure 4.13).**

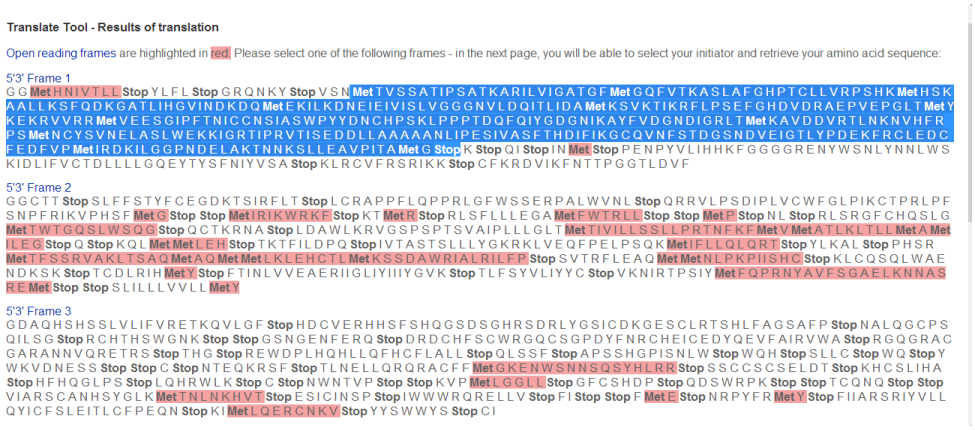


Figure (4.13): Showing the complete Open Reading Frame analyzed by EXPASY TRANSLATE TOOL for the contig **t3818.cdna.v1.contig21922.**

4.11.1 Identification of putative conserved domain

The conserved domain for the LAR gene was confirmed by using the NCBI PROTEIN BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved domain for the respective gene was identified as NADB_Rossmann Superfamily domain. The conserved domain for the contig **t3818.cdna.v1.contig21922** is shown in the **Figure (4.14).**

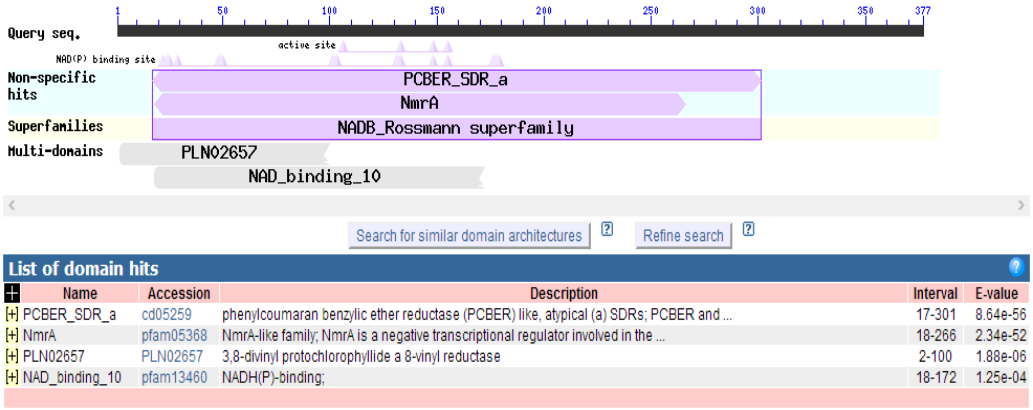


Figure (4.14): Showing the conserved domain **NADB_Rossmann superfamily** against the contig **t3818.cdna.v1.contig21922**

4.11.2 Identification of promoter region

The promoter region of the gene was identified by using Peanut Base (http://www.peanutbase.org/home). The nucleotide sequence was blast against both the genomes (i.e. *Arachis duranensis* and *Arachis ipaensis*) and the obtained results were then translated and configured. By searching the hits given by the configuration, all the matching sequences were highlighted one by one. Promoter region was identified as 1500 bases upstream of the starting region of the original sequence (**Figure 4.15 and 4.16).**

PROMOTER REGION (PARENT ARADU)

TTTTCCTTCAACGGTGTGATAATTTTGGAATGTATCTTTTCAATTAATAAAGCTAATTAAGTACAATTTTGTATAAATTA
ACGTTCTATTTTATTCTTGTAAAATATATATACCATTTTAACTTTCGCAAGTTTTAGCGAGTTAAAATAGTTTCTATAAA
AAAAAAATGGGGTTATATTAATTCTTGATGAAGTGAGATAAGGATATAAAAATATAAAATTATATTTGATAGGTAAAA
TATGGTGGACAGAGACATTATATTTAAAGATATTAAATTAATGTATTTTGCGTTTATTCTGATAAAAAAGACATAGAGA
CACTAATAAAAAATATAATTTATTTTTTGTTTTTTCATTATTCTTATTAATTTTTTATAATTATATTTTTTATTGTTATATT
TTTTAAAAATTTTTAAATGAAAAAAATAAAAATGAATTAGATTTTCAAATTTTATTTTACTTTATCACCAAACAAAATAT
AAAAATATTAAATTTTGTATCTTTATTCTTTATATTTTATTCTCAATATTTTATCTTATTCTATTTTCAAAAACAAATACA
CCTTATAAATCAAGTTGTCTCTTAAAAATCTAATGAAAACTAATGGGACTTATATTTTTTACAAATAAAGACTATATTTT
GATTCGTTAAAATATACGAGAATTAAAATAGTTTACTCATTATTATATGCCTAAACTGATTAAAATAAACATTAAAAGT
AATAAATTGATTGTTAAATAATATGGTTAAAAAAGAAAAACTATTGGAGCAATCAGTTTTTAATAATTTTGACTATCAT
AAAATATTAAATTGTGTTTTATATGTATAAATTTTAAAAAATATGAGTATAGATTATATTGATTTATGTATGTAAACTCT
GATAAATATATAGATATAAATTATTTTTTGTGTAGAAATACATATAAATATAAGTATAAATTATTATTGTCAAATACTAT
CCTAAAATTATATGTACAAATTTTAAAAAATATAAATACAAATTATACTGATTTATGTATACAAATTCTAATAAATATA
TAAATACAAATTATATATTTTTTATATGTAAACACATATAAATATAAATATAAATATAAATTATTGCTAACCAAATAAT
AACAAAAAATAATAAATGTTGATAATTACTTCACATTGCTCATAAAAAAGACACACTTAATCATTTGAAAGAAAAATT
AAAATTGAGAAAATTCATCCCAGTTTAAGCGCTTGATTATACAAGATGCTACCCAGCTATCAGTCAAAGAAAGAAAGT
TGAGAACTAAACTTCAAAAATTAAAATTCCGGTGTCGAATTCTTCATTCACTTGATGAGTTTAAGTTCCTGCACGTGTTG
AAGAGTAGCTGAAAAGTGGAAACTATTAGCGGGGTCAATGTAAGAGACTAAGAGTCAACAATTATATTTTATTTTTTAT
CCATAATATTTTTATAGTAAGAGCGCTCTTTTTTCCTATAAAAACTAAATACCACCCGGTGGTATTGC

Figure (4.15): Promoter region of the LAR gene

with respect to *Arachis duranensis* (ARADU) (PLANT CARE database)

PROMOTER REGION (PARENT ARAIP)

TCGTTAATTGTGAACCAAATTCTCAATTTTTTATAATTGAGCACTACGTTGTGATTCCAATGAACAAGACT
AAACTCTTGGTTATTGATTTAAATAGTAATATTTTCTAACTAAACTTTGAAAATGTTAAATTCTGATTTAA
AGGTATAATGAACTTAAACTTTTAAACTTGTAAATTTTCTAAGCTAGTACTTAGCAATGTTGTTATGGTGG
GTAAGTTAGTGTGGCAAATACTTAATTGTAGAGACAAGCTGTGGGTGGTTAGACAAATATATATCTGATTG
TGGTACTAAAAATATAAATTCTTTTTGTACATAAATAATTTTAAGAATGACCTACAAATATGGCCAAATAA
GAGTCAAGTTCCTCTTAAAAATATTGGGTAAAAGGATGGAACCCAAAAGAACACCTTGAAAAGTTGAATTT
CCCTTTGGTAGGAACTAAATTGGTGAAAGTGGATTTGTTGGATTTGGAGTGTAAAGAGTGTTATCTAGTTG
AAAGAAAACCAAACTAGATGTCCTTCACTAATGTCACATGTCATAAGCTTTAGTTTAACTTGTGAAAATCA
ACTTCTTCCATTGAACATATACAGTTGAAAAGACTTGAAGTGACGGGAATACCTAAAAAATATTTGGAGTA
AAAGGAGTAAACCAAATATCAAACTATAACTATTGGACAATAGATTTCTTCTGTAAAATGATTTTACACTA
TAAAAGGACCTTAAGCCACCTTTTTAAAGCCTCTTTTTTTCTGAAAATTTGGAGGCTAAAAAATCCATCTT
TTTTTCTCCCCAAAATTTCTCTCATTGTTATTCATTTTATTCATCACCTTCACGAGTAAAACTGGCATTCG
AGTGTTCCAACAGAACACTCGACAACCATTAATTAACCCATGTCCACTTTAAACAAAATTCATTCATCCTC
TTGTGACATTAGTGGAAGTCTCAATACTTGTTCTCCACCTCTATTCCTCAATATTAGTTCTTGTATTGTTT
ATTTGCCATTAACAAAGTATTTTCCTCACAAGTTTACTTACCCCAAATCTCTTATCTTAATCATTTTACAC
TTAATCTATTTTCACATAACTCACCCGAATCAATGTCAAATCAGAGAGGGTTAAATTTTTACTCCATGAGA
AATGCTTCAAATTGCTGGAGAAGCATCAGTGGGGCTTTTGATCACATTAAAGAATGATTTTCTTGGTGCAT
TGAGGAGATTAATCAATCCTTTTGGTACGACACCTGGTCTCCCCTTGGTAAGGTTTGTGATTTGATTCCTT
ATGTTCATATTTATGATAGTGAATTAAATATTGATGATGCCTGGGTTTATGTTGATTAGTATATTGAGTGG
TTAGAACTCGAGTGATATTTGTGCTCTACTTCACAGACAGAGATGTTGAAGGCTAGTCTTGTCTAGGTTTT
TTTTTTTTTTCAGACACCAAAAAATAGATATAATCTAATGCTACACAATGGTAGTAATTACTTCCGAAAGTGCTACAC

Figure (4.16): Promoter region of LAR with respect to Arachis ipaensis (ARAIP).(PLANT CARE database)

4.11.3 Promoters alignment

Both the promoters were then aligned and the results obtained showed 100% similarities with 0.0

E-value **(Figure 4.17)**

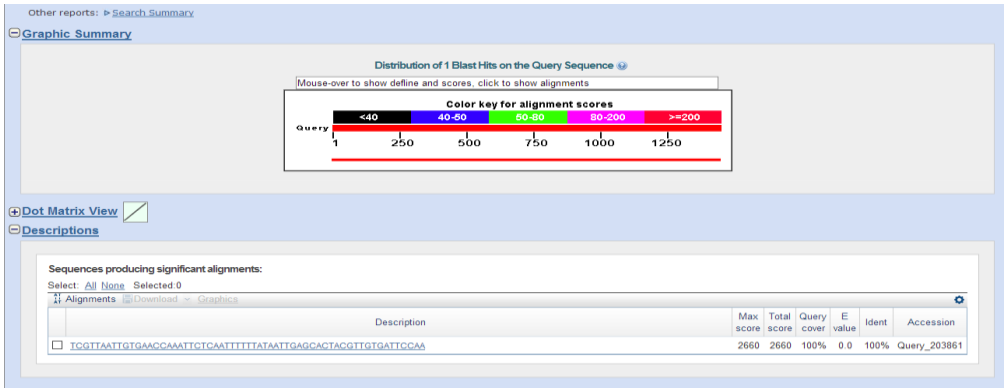
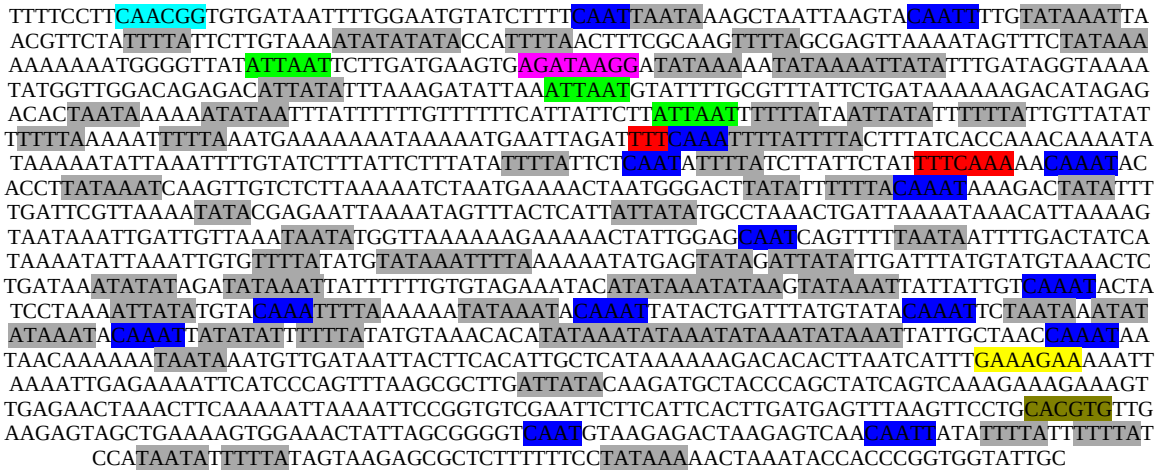


Figure 4.17: Alignment results of both the promotors (ARADU and ARAIP) showing 100% similarity with 0 E-value.

4.11.4 Identification of Cis-regulatory elements

Sequence motifs are short, recurring patterns in DNA that are presumed to have a biological function. Often they indicate sequence-specific binding sites for proteins such as nucleases and transcription factors (TF). Others are involved in important processes at the RNA level, including ribosome binding, mRNA processing (splicing, editing, polyadenylation) and transcription termination. The motifs were identified by using the online database of Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). **Figure (4.18) and (4.19)** shows the sequence motifs of the promoter region of ARADU and ARAIP respectively.



CAAT BOX	CAAT, CAAT, CAATT, CAAAT	
CCAAT BOX	CAACGG	
G BOX	CACGTG	
I-BOX	AGATAAGG	
TATA BOX	TTTTA, TAATA, ATATAT, TATA, TATAAAT, TATAAATTTA	
BOX 1	TTT, TTTCAAA	
BOX 4	ATTAAT	

Figure 4.18: Cis-regulatory elements in the promoter region retrieved against parent ARADU. Different colors indicate different cis-regulatory elements.

TCGTTAATTGTGAACCAAATTCTCAATTTTTTATAATTGAGCACTACGTTGTGATTCCAATGAACAAGACTAAACTCTTG
GTTATTGATTTAAATAGTAATATTTTCTAACTAAACTTTGAAAATGTTAAATTCTGATTTAAAGGTATAATGAACTTAAA
CTTTTAAACTTGTAAATTTTCTAAGCTAGTACTTAGCAATGTTGTTATGGTGGGTAAGTTAGTGTGGCAAATACTTAATT
GTAGAGACAAGCTGTGGGTGGTTAGACAAATATATATCTGATTGTGGTACTAAAAATATAAATTCTTTTTGTACATAAA
TAATTTTTAAGAATGACCTACAAATATGGCCAAATAAGAGTCAAGTTCCTCTTAAAAATATTGGGTAAAAGGATGGAAC
CCAAAAGAACACCTTGAAAAGTTGAATTTCCCTTTGGTAGGAACTAAATTGGTGAAAGTGGATTTGTTGGATTTGGAGT
GTAAAGAGTGTTATCTAGTTGAAAGAAAACCAAACTAGATGTCCTTCACTAATGTCACATGTCATAAGCTTTAGTTTAA
CTTGTGAAAATCAACTTCTTCCATTGAACATATACAGTTGAAAAGACTTGAAGTGACGGGAATACCTAAAAAATATTTG
GAGTAAAAGGAGTAAACCAAATATCAAACTATAACTATTGGACAATAGATTTCTTCTGTAAAATGATTTTACACTATAA
AAGGACCTTAAGCCACCTTTTTAAAGGACCTCTTTTTTTCTGAAAATTTGGAGGCTAAAAAATCCATCTTTTTTTCTCCC
CAAAATTTCTCTCATTGTTATTCATTTTATTCATCACCTTCACGAGTAAAACTGGCATTCGAGTGTTCCAACAGAACACT
CGACAACCATTAATTAACCCATGTCCACTTTAAACAAAATTCATTCATCCTCTTGTGACATTAGTGGAAGTCTCAATACT
TGTTCTCCACCTCTATTCCTCAATATTAGTTCTTGTATTGTTTATTTGCCATTAACAAAGTATTTTCCTCACAAGTTACTT
ACCCCAAATCTCTTATCTTAATCATTTTACACTTAATCTATTTTCACATAACTCACCCGAATCAATGTCAAATCAGAGAG
GGTTAAATTTTTACTCCATGAGAAATGCTTCAAATTGCTGGAGAAGCATCAGTGGGGCTTTTGATCACATTAAAGAATG
ATTTTCTTGGTGCATTGAGGAGATTAATCAATCCTTTTGGTACGACACCTGGTCTCCCCTTGGTAAGGTTTGTGATTTGA
TTCCTTATGTTCATATTTATGATAGTGAATTAAATATTGATGATGCCTGGGTTTATGTTGATTAGTATATTGAGTGGTTA
GAACTCGAGTGATATTTGTGCTCTACTTCACAGACAGAGATGTTGAAGGCTAGTCTTGTCTAGGTTTTTTTTTTTTTCA
GACACCAAAAAATAGATATAATCTAATGCTACACAATGGTAGTAATTACTTCCGAAAGTGCTACACT

ACE	**ACTACGTTGT**	
CAAT BOX	**CAAT, CAAAT, CCAAT, CAATT**	
CATT-motif	**GCATTC**	
GAG-motif	**AGAGATG**	
LTR	**CCGAAA**	
P-BOX	**CCTTTTG**	
SKN-1_motif	**GTCAT**	
TAT-BOX	**TATA, ATTTTATTCATCA, TTTTA, TACATAAA, ATATAT**	
TGACG-motif	**TGACG**	
TCA-element	**CCATCTTTTT**	

Figure 4.19: Cis-regulatory elements in the promoter region retrieved against parent ARAIP. Different colors indicate different cis-regulatory elements.

4.12 Phylogenetic analysis

For phylogenetic analysis of the candidate gene the nucleotide sequence already retrieved was used as query. NCBI blast tool was used to get the nucleotide and protein sequence alignments. The obtained data was then analyzed by using Geneious software.

4.12.1 Nucleotide sequence alignment

Nucleotide sequence of the candidate gene was blast against the NCBI nucleotide blast. A total of 103 Blast hits were recovered, including a large number of species from the Leguminosae family to be aligning with our query. The species with best alignment results were selected and multiple sequence alignment was performed through Geneious software. The species selected for multiple sequence alignment included *Arachis duranensis, Arachis ipaensis, Desmodium, Glycine max, Onobrychis, Onobrychis 2* and *Vigna angularis*. The black regions in the figure shows 100 % similar regions, the grey color indicated 80-100% similarity, while the white regions shows no similarity (**Figure 4.20**).

4.12.2 Amino acid sequence alignment

The amino acid or protein sequence alignment was performed by using the NCBI protein blast tool. The sequence of candidate gene was used as query against the protein blast and a number of plant species were retrieved having similarity. The plant species with more specific results were selected and the amino acid sequences were obtained. These sequences were then aligned by using the multiple alignment tool of the Geneious software. The plant species showing

higher similarity included the two parents i.e., *Arachis duranensis* and *Arachis ipaensis*, *Cajanus cajan, Glycine max LAR1*, *Lotus corniculatus LAR1-2*, *Lotus uliginosus*, and *Vigna angularis.* The black regions in the figure shows 100 % similar regions, the grey color indicated 80-100% similarity, while the white regions shows no similarity (**Figure 4.21**).

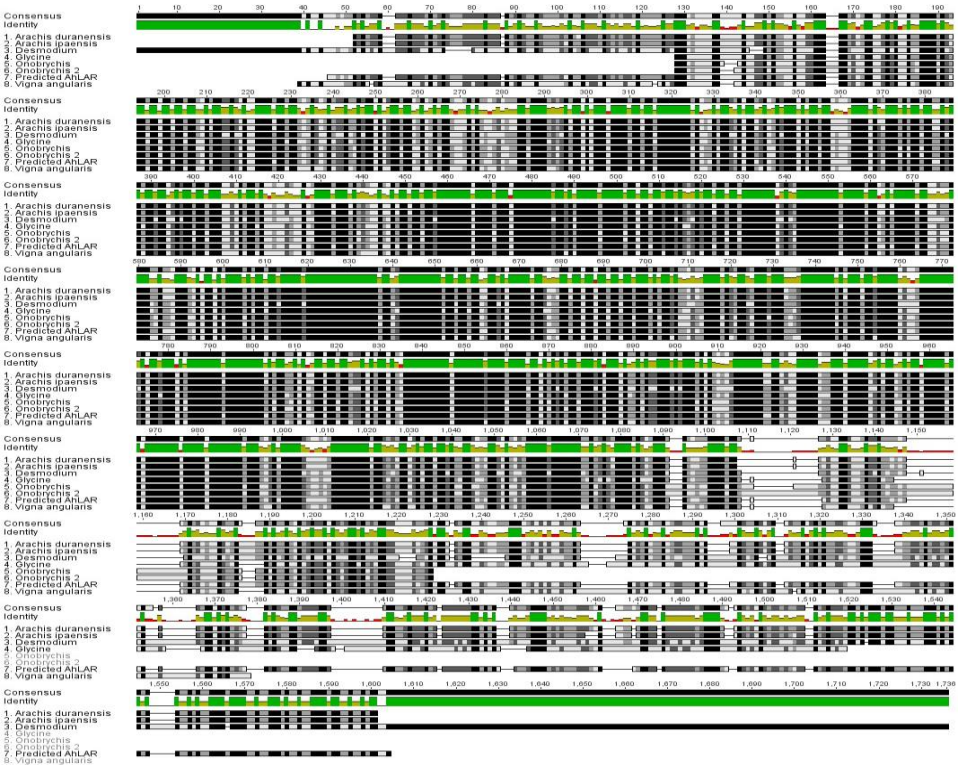


Figure 4.20: Multiple sequence alignment of nucleotide sequence of the candidate gene LAR. The represented sequences are mentioned on the left. The identity graph is shown at the top of alignment. The black and grey regions indicate the conserved sequences among different species. While the white regions show non-conserved sequences.

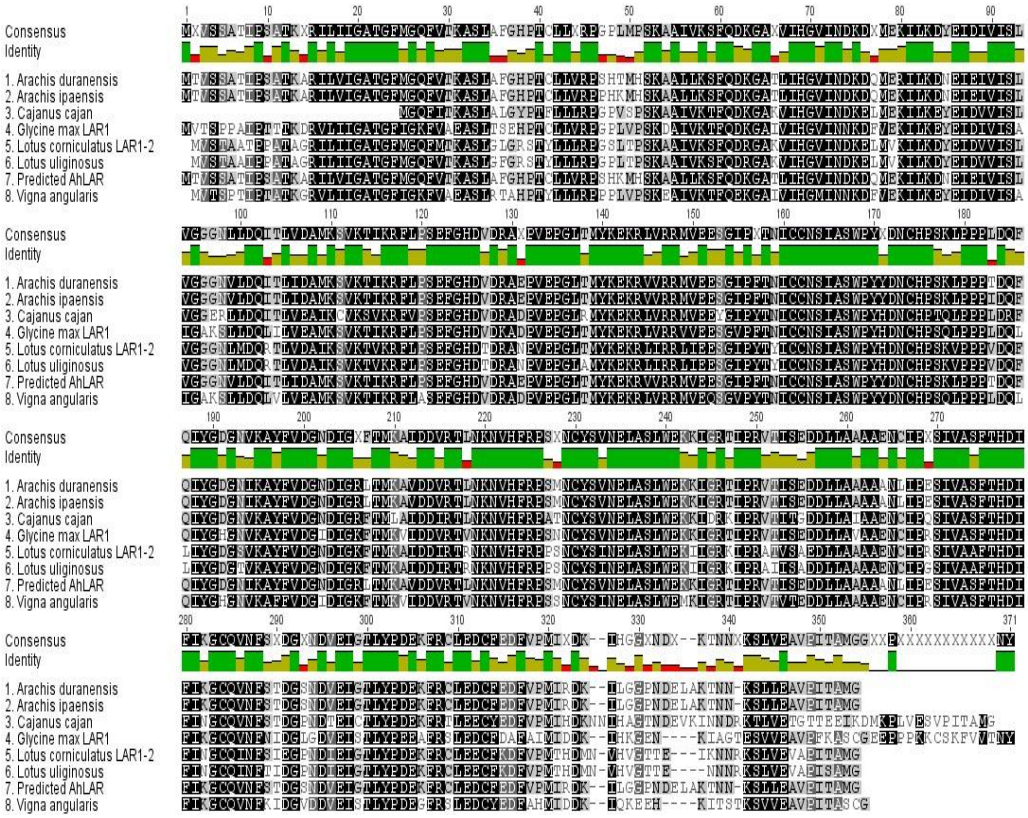


Figure 4.21: Multiple sequence alignment of amino acid sequences of candidate gene LAR. The represented sequences are mentioned on the left. The identity graph is shown at the top of alignment. The black regions show 100% conserved sequences, while the grey regions show 80-100% similar sequences. The non-conserved regions are shown in the white color.

4.12.3 Phylogenetic tree

Following the compilation of sequences data set and alignment, phylogenetic tree was constructed by using the Geneious software. The phylogenetic tree showed high evolutionary relationships of the candidate gene among various species. The analysis of the phylogenetic tree revealed that LAR gene in *Arachis hypogaea* had an early origin in the evolutionary history as compared to similar genes in other plant species. The candidate gene is closely related to *Arachis duranensis, Arachis ipaensis, Lotus corniculatus LAR1-2, Lotus uliginosus, Cajanus cajan, Glycine max LAR1* and *Vigna angularis* **(Figure 4.22).**

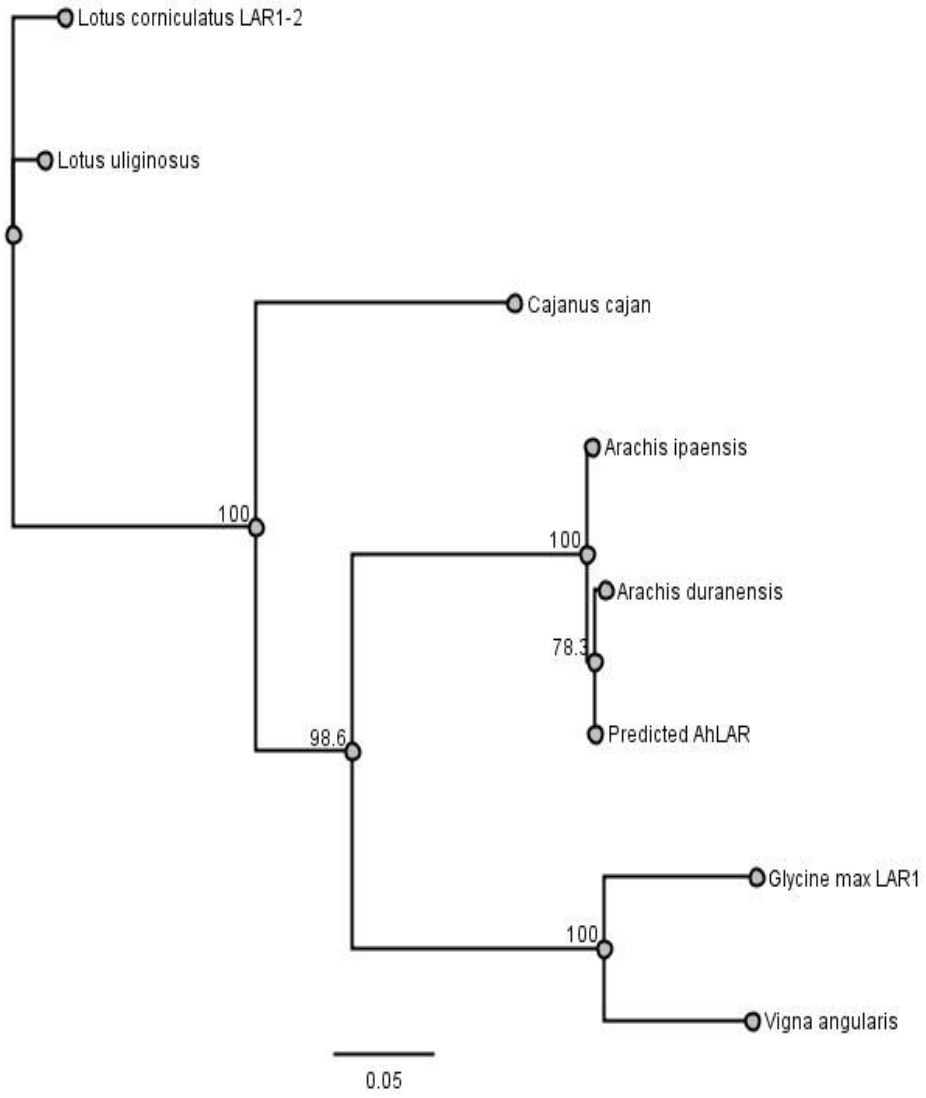


Figure 4.22: Maximum likelihood phylogeny of the candidate gene among various plant species

4.12 PCR assay

PCR amplification was done by using the designed primers of Leucoanthocyanidin reductase. Both Genomic DNA and cDNA were separately used and PCR amplification was performed. The PCR product was analyzed by running on a 1% agarose gel for 40 minutes. Gel image was taken under UV gel documentation unit. The band size was confirmed by comparing with the ladder and was found 1kb plus **(Figure 4.23).**

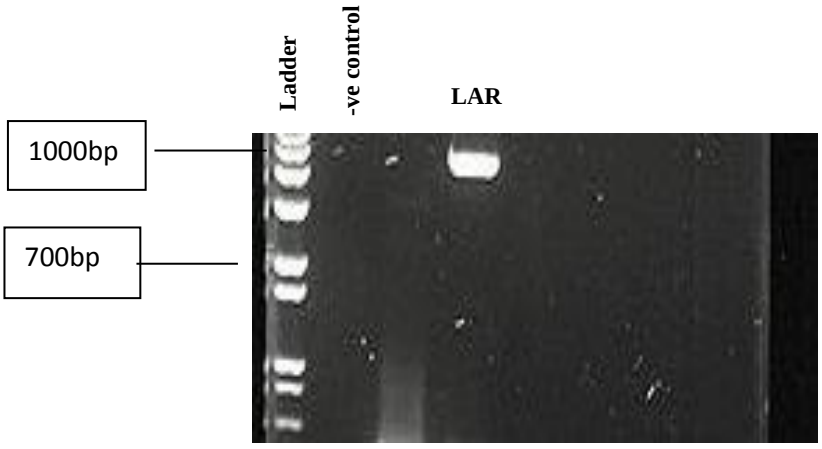


Figure (4.23): PCR results showing the DNA bands of the candidate gene

DISCUSSION

Arachis hypogaea is considered as one of the most important crops in the world. It is a key source of oil production and serves among the top oil producing crops. It is widely cultivated in Asia, America and Africa (Guimarães, Brasileiro et al. 2012). There is limited study about the transcriptome and gene regulation in response of salt stress in peanut. Therefore this study is aimed to identify the leucoanthocyanidn reductase (LAR) gene in *Arachis hypogaea* and evaluation of different physiological and biochemical parameters in response to different concentration of NaCl stress. Since LAR is among the major enzymes, which are involved in the flavonoid biosynthetic pathway, much genes encoding LAR from other plants have been characterized at the genetic, chemical and enzymatic levels. However, there is no such report on the functional characterization of LAR gene in *Arachis hypogaea*. Moreover, the flavonoids are one of the most important medicinal components in *A. hypogaea*. In the current study, LAR gene was successfully identified from *Arachis hypogaea* via in silico analysis and confirmed its sequence through PCR amplification. The identification and characterization of *AhLAR* will be helpful in greater understanding of the role of LAR in the synthesis of flavonoids at the molecular level. *A. hypogaea* LAR has 1056bp ORF encoding a 351 amino acid protein. The predicted nucleotide sequence is highly similar to previously reported Leucoanthocyanidin reductase from other plants, for example, *Desmodium*, *Glycine max*, *Onobrychis*, *Onobrychis 2*, *Vigna angularis* and to its wild parents *Arachis ipaensis* and *Arachis duranensis* (NCBI Nucleotide BLAST). The deduced LAR protein shared

Identity with *Arachis duranensis* and *Arachis ipaensis*, *Cajanus cajan*, *Glycine max LAR1*, *Lotus corniculatus LAR1-2*, *Lotus uliginosus* and *Vigna angularis* (NCBI Protein BLAST).

During the study the nucleotide sequence of LAR was retrieved by using NCBI. The sequence was then aligned on Peanut transcriptome database and the contig showing significant value was selected for further analysis through EXPASY SEARCH TOOL, NCBI PROTEIN DATA BASE and Peanut Base. Gene specific primers were designed and efforts were carried out for PCR amplification of the candidate gene sequence.

Salinity is amongst the key environmental factors that cause reduction in plant growth, development and productivity worldwide. Salt stress affects the morphological, biochemical and physiological responses of plants (Amirjani 2010).

Salinity (salt stress) is among the major environmental factors which tend to cause decline of plant growth, productivity and development throughout the world. Salt stress affects the physiological, morphological and biochemical parameters of plants (Siringam, Juntawong et al. 2011) (Amirjani 2010). The peanut plants after 15 days of germination were exposed to different concentrations of salt stress i.e. 0mM (control), 60mM, 120mM and 240mM. The plants were harvested after 15 days of treatment and samples were taken for different physiological and biochemical activities.

The root and shoot weight was found decreasing as the salt stress increased. The control plants (0mM NaCl) were found to have more weight than the treated ones (60mM, 120mM and 240mM NaCl). The shoot and root length were also found decreasing with the increasing concentration of salt. It has already been reported that the salinity injury

cause decline and retardation of the plant growth by inhibiting the elongation of the cells (Yasar, Ellialtioglu et al. 2008).

The chlorophyll content was measured by UV spectrophotometric method. The amount of chlorophyll (mg/g) was observed to be higher in the control plants (60mM NaCl) as compared to the treated plants i.e. 60mM, 120mM and 240mM. The content was found continuously decreasing with increasing concentration of salt stress from 60mM to 240mM. The results are very much in accordance to the work done by (Yasar, Ellialtioglu et al. 2008) (Kusvuran 2010) and (Nazarbeygi, Yazdi et al. 2011).

The percentage of moisture content was also found decreasing with the increasing NaCl concentration. The control plants under zero salt stress showed higher moisture content followed by the treated plants i.e. 60mM, 120mM and 240mM respectively. The progressive decline in moisture content lowers the osmotic potential of the cells necessary for maintaining the turgor pressure (Navarro, Garrido et al. 2003).

The total soluble sugar and total soluble protein content is also affected by the salt stress. The results calculated for both the contents showed a direct relation to the concentration of salt stress induced. The plants with 0mM NaCl concentration (control) have lower values of sugar and protein contents as compared to the plants exposed to NaCl stress i.e. 60mM, 120mM and 240mM respectively. The results were in accordance to the report of (Watanabe, Kojima et al. 2000). The results obtained for the protein activity in general agree with what (Chao, Gu et al. 1999) had presented. They noticed an increase of protein content of the tomato plant *Lycopersicon esculentum* (L.) in response to salt treatment. Further, (Sibole, Cabot et al. 2003), discovered that the treating of clover plant (*Medicago citrna* L.) for about 30 days with different concentrations of NaCl i.e. 0, 1, 50, 100, 200mM increased soluble protein content in the seedlings, as compared with control plants.

The antioxidant enzymes (Ascorbate peroxidase, Catalase and Superoxide dismutase) tend to degrade the ROS produced, which cause cell dysfunction, membrane damage and cell death (Chookhampaeng 2011).During the study the value of antioxidant enzymes was found increasing with the increasing salt concentration. The control plants (0mM NaCl) showed higher concentration of antioxidant enzymes as compared to the treated plants (60mM, 120mM and 240mM NaCl). The results were similar to the study performed by (Yasar, Ellialtioglu et al. 2008).

5.1 Future perspective

The identification of Leucoanthocyanidin reductase gene in *Arachis hypogaea* will lead to investigate more and more facts about its role in the stress response and flavonoid biosynthetic pathway. Where, the study of salt stress on the peanut

cultivar can enhance our knowledge about the variety specific response to the stressful environment. The current study will be a positive step for the expression analysis and different abiotic stress studies in the future, more likely it will further explore the way of molecular mechanism of the flavonoid biosynthetic pathway in *Arachis hypogaea*. The evolutionary linkage of the candidate gene through phylogenetic approaches will broaden the knowledge about the origin of LAR gene and its key role as an intermediate component of the flavonoid biosynthetic pathway.

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CHAPTER 6