Evaluation of Stilbenes biosynthetic gene(s) upon hormonal treatment in Arachis hypogaea

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Dedicated to my beloved parents for whatever I have achieved in life is because of them

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ABBREVIATIONS

Kcal	kilo calorie
STS	Stilbene synthase
CHS	Chalcone synthase
PAL	Phenyl alanine ammonia lyase
C4H	Cinnamate 4 hydroxylase
CO_2	Carbon dioxide
MeJA	Methyl Jasmonate
Nt	*Nicotiana tabaccum*
cDNA	copy of DNA
ESTs	Expressed sequence tags
UDP-glucose	Uridine diphosphate glucose
PDA	Photodiode array
CD	Cyclodextrin
HPLC	High Performance Liquid Chromatography
US	Ultrasound
UV	Ultra violet
SA	Salicylic acid
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetic Analysis
ORF	Open Reading Frame

SMART	Simple Modular Architecture Research Tool
PSIPRED	PSI-blast based secondary structure prediction
CFSSP	Chou and Fasman secondary structure prediction
GO	Gene ontology

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Abstract

Peanuts (*Arachis hypogaea)*, also called ground nut are world's vital oil producing crop. They are consumed all over the world due their high nutritional value. Peanuts contain many compounds like proteins, fibers, polyphenols, antioxidants, vitamins and minerals. In recent times, peanuts are getting higher attention due to their containment of resveratrol and stilbenoids in the kernels and other parts of plant. Stilbenes and stilbenoids are a well-known class of naturally occurring small group of phenylpropanoids which have been identified in at least 72 unrelated plant species. These compounds are stress metabolites produced in leaves and other parts of the plant in response to various biotic and abiotic stresses stresses such as infection, wounding, UV-C exposure and treatment with chemicals. In this study, Structural and functional analysis of stilbenes synthase gene (s) was performed through In-silico analysis. In addition, Real Time PCR evaluated the expression of putative gene upon hormonal treatment in two registered varieties (Golden and BARD) of peanut plant. Differential expression was observed in both the varieties. However, Pattern of expression of putative genes was observed to be different between both the varieties (Inter-Varietal differential expression). The anaylsis showed that hormonal treatment evidently triggers Stilbenes production in *Arachis hypogaea*.

CHAPTER: 1 INTRODUCTION

1.1. The peanut (*Arachis hypogaea L.)*

The peanut (Arachis hypogaea) additionally referred to as groundnut is an crucial oilseed crop and meals legume grown on about 20 million ha in heat tropical or subtropical regions all through the world. (Wynne et al., 1991). The peanut belongs to the botanical family Fabaceae; also called as Leguminosae. (Plant List, 2013).

Peanut is being frequently used for fit to be eaten oil production, direct intake and animal feed. It is a valuable source of protein and oil. (Moretzsohn et al., 2013) (Holbrook and Stalker, 2003). Peanut is cultivated in over a hundred countries, with over 95% of cultivated location in Asia and Africa. World peanut manufacturing totals about 29 million metric lots in line with year. China, India and United States represent the sector biggest producer. Worldwide peanut exports are about 1.25 million metric tons. The United States, Argentina, China, India, Vietnam, and a few African international locations are giant exporters. Canada, Mexico and Europe debts for 80% of peanut product importers in particular from US markets (Soyatech 2011). The marketplace training grown withinside the United States are Spanish, Runner, Virginia, and Valencia. Peanut manufacturing withinside the United States is split into 3 fundamental areas: the southeastern United States vicinity which incorporates Alabama, Georgia, and Florida; the southwestern United States vicinity which incorporates New Mexico, Oklahoma, and Texas; and the 0.33 vicinity withinside the preferred japanese United States which incorporates Virginia, North Carolina, and South Carolina. In Pakistan, groundnut is an crucial coins crop in barani regions of higher Punjab and elements of NWFP. In Sindh, it's miles grown beneathneath irrigated conditions. About 84 % of the full groundnut location lies in Punjab, 13% in NWFP and 3 % in Sindh. During 1998-99, the full location beneathneath groundnut turned into 97,500 hectares with the manufacturing of 104,000 lots and common yield of 1067kg/ha.

World annual manufacturing of shelled peanuts become forty four million tonnes in 2016, led with the aid of using China with 38% of the sector overall. Peanut is one of the 5 maximum crucial oilseeds, with overall international manufacturing in 1990–1991 accomplishing a few 23 086 000 mt.(Zhang et al., 1995).

Like maximum different legumes, peanuts dock symbiotic nitrogen-fixing micro- organism in root nodules. This capability to repair nitrogen method peanuts require much less nitrogen-containing fertilizer and additionally increase soil fertility, making them treasured in crop rotations. Among the super dietary traits of peanut are a protein content material of as much as 30% and a fats content material of as much as 50% this is almost 80% unsaturated. Moreover, numerous wild Arachis species had been used correctly as incredible tropical forages. (Valls and Simpson, 1994) The oil content material of the seed varies from forty four to 50 in line with cent, relying at the sorts and agronomic conditions. Groundnut oil is suitable for eating oil. It fids widespread use as a cooking medium each as delicate oil and Vanaspati Ghee. It is likewise utilized in cleaning soap making, and production cosmetics and lubricants, olein stearin and their salts. Kernels also are eaten raw, roasted or sweetened. They are wealthy in protein and nutrients A, B and a few participants of B2 group. Their calorific fee is 349 in line with one hundred grammes. Phytochemicals in Peanut Plant

Phytochemicals play vital roles in plant safety in opposition to abiotic and biotic stresses, and resistance to microbial infection combines constitutive and triggered protection responses (Dixon and Paiva, 1995). Stilbenes are synthesized in plants when microorganism attack are a part of each essential and inducible defense responses. Function of stilbenes as phytoalexins is involved in response to oxidative stress produced by UV irradiation.or as chemical signals in allelopathy (He et al., 2008) (Reigosa et al., 2006)

Human nutrition comprehends type of macro and micronutrients with many sorts of vitamins, minerals, antioxidants and alternative helpful phytochemicals. Each one of these are necessary for keeping a healthy life. The macronutrients are sources of various kinds of carbohydrates, proteins, and fats (lipids) and the food industry provides these as primary products of foods. (Gunstone, 2011).

Phytochemicals, as plant parts with distinct bioactivities towards animal organic chemistry and metabolism are being wide examined for their ability to supply health benefits. Phytochemicals may offer health benefits, such as: enzymatic reaction cofactors, biochemical reaction substrates, absorbents/sequestrants, enzymatic reaction inhibitors that bind to and eradicate unwanted components in the intestine, reactive or harmful chemical ligands that agonize or antagonize the cell surface or intracellular receptors, selective growth factors for helpful gastrointestinal bacteria, compounds that enhance the absorption and or stability of essential nutrients, fermentation substrates for beneficial oral, stomachic or intestinal bacteria, and, selective inhibitors of harmful intestinal bacteria. Such phytochemicals embody phenolic, terpenoids, alkaloids and flavonoids. (Dillard and German, 2000).

A wide range of antioxdidant phytochemicals have been found in peanut. Studies have found phenolic compound in peanut seeds. Other phenolic resin acids that are known in peanut kernels include, vanillic, m-coumaric, o-coumaric, caffeic gentisic, ferulic, sinapic, and different hydroxybenzoic acids. [Dabrowski and Sosulski, 1984; Phan-Thien et al., 2014; Mattila and Hellstrom, 2007).

The consumption of nuts are beneficial to health due to their appropriate lipid profile, greater in unsaturated fatty acids than in saturated fatty acids. Peanuts, although legume have a high lipid content (ca. 46%) that's made in monounsaturated fatty acids, and they don't contain cholesterol. (Chukwumah et al., 2007 Peanuts also contain bound bioactive compounds that also prevent the risk for the development of chronic diseases like diabetes cancer, and cardiac disorders. (Higgs, 2003; Messina and Barnes, 1991; Hu and Stampfer, 1999). Compounds, like isoflavones and trans-resveratrol, have been identified and quantified in peanuts recently. Composition of peanut oil consists of fatty acid and antioxidants that impart anti-oxidant property. (Chukwumah et al., 2007). (Akhtar et al., 2014).

A vast array of natural products have been found in plants that provide selective advantage against abiotic and biotic stresses. (Chong et al., 2009).

1.3. Stilbenes

Stilbenes are known to belong to a small family of polyphenols discovered in distinct unrelated plant species. Such compounds have different effects for human health and resistance to disease. Plant stilbenes have received noteworthy attention in recent years due to their potential pharmacologic applications and biological activities. Stilbenes are detected in a minimum of seventy two distinct plant species and are produced in response to abiotic and biotic stresses like, wounding, UV-C exposure, infection and treatment with chemicals. Examples of common plant stilbenes isolated from numerous plant families, as well as grape (Vitaceae), pine (Pinaceae), peanut (Fabaceae) and sorghum (Poaceae) (Chong et al., 2009). Stilbenoids have received a great interest over the years because these compounds possess both biological activities and pharmacological applications. (Chong et al., 2009). Stilbenes are detected in a minimum of seventy two unrelated plant species and accumulate in response to biotic and abiotic stresses like infection, wounding, UV-C exposure and treatment with chemicals (Vannozzi et al., 2012).

1.3.1. Stilbene Biosynthesis

Plant stilbenes are derived from the general phenylpropanoid pathway. All higher plants appear to be able to synthesize malonyl-CoA and CoA-esters of cinnammic acid derivatives, however solely few plant species are able to manufacture stilbenes. The first enzymes of the phenylpropanoid pathway are described as, as phenylalanine ammonia lyase (PAL), cinnamate-4hydroxylase (C4H),4-coumarate, CoA ligase (4CL) and Stilbenes Synthase enzyme (Ehlting et al., 2006; Ferrer et al., 1999; Chong et al., 2009). Indeed, stilbene synthase, the important catalyst of this pathway, evolved in an exceedingly restricted variety of plant species, that therefore acquired the capability to produce stilbenes. Stilbenes could then endure differing types of modifications (Chong et al., 2009).

Stilbene synthase (STS) is characteristic of stilbene-producing plants and catalyzes, during a single reaction, the biosynthesis of the stilbene backbone from 3 malonyl-CoA and one CoA-ester of a cinnamic acid by-product (most often cinnamoyl-CoA or p-coumaroyl-CoA. STS macromolecule was first purified from stressed cell suspension

cultures of peanut (Arachis hypogaea) (Shoppner and Kindl, 1984 Cloning of two peanut STS genes revealed intensive similarity to peanut chalcone synthase (CHS) factor throughout the coding region, the position of the only deoxyribonucleic acid was preserved in each genes. (Chong et al., 2009). STS genes exist as a family of related genes in many plant species. (Schroder et al., 1988), grapevine (Melchior and Kindl, 1991; Wiese et al., 1994), scots pine (Preisig-Muller, 1999) and Japanese red pine (*Pinus densiflora*) (Kodan et al., 2002).

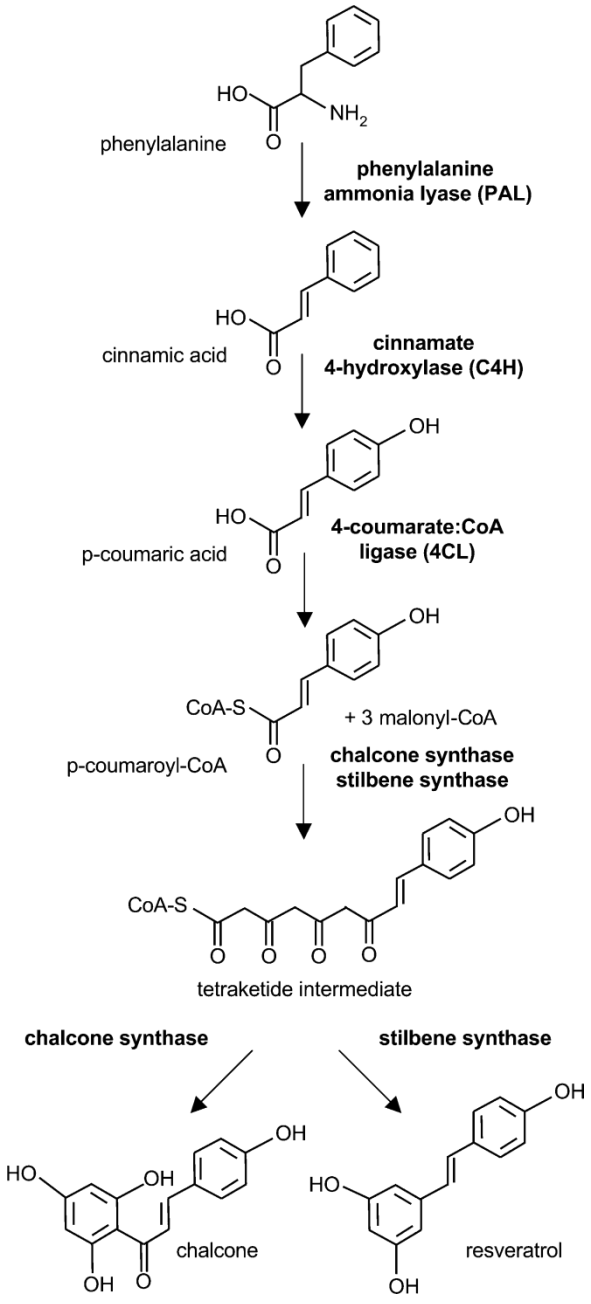

Fig 1.1: Biosynthesis of Stilbene

1.3.2. Biotic and Abiotic Stresses as Regulators of Stilbene Synthesis

Stilbenes act as phytoalexins, constituents of low mass produced de novo in response to microorganisms and accumulate at infection site. (Ahuja et al., 2012 Production of stilbenes in plants according to studies is induced upon infection by many pathogens, and STS gene transfers to the genomes of assorted plants usually led to enhanced or foreign stilbene production and improved resistance to numerous fungal pathogens. Despite having broad information on stilbene activities against plant human microbial pathogens and fungal pathogens, there are limited reports on their activities and production agaisnt phytopathogenic bacteria. (Chong et al., 2009 Stilbenes have also shown to inhibit growth of food-associated bacteria in vitro. For example, a group of wood-related stilbenes possesses antimicrobial activities against some decomposition organisms and food pathogens, as well as both gram-positive bacteria (*Listeria*, *Staphylococcus*) and gram-negative (Salmonella). It is thought that stilbene biosynthesis in plants are often triggered by numerous abiotic stress, including ultraviolet light irradiation, wounding, ozone, salt depletion, drought. ,serious metal stress, and heat stress. (Chiron et al., 2000).

1.4. Phytohormones in Plant Defense

Plants face numerous threats from environment like cold, drought, salt, heavy metals and pathogenic attacks. Against all these biotic and abiotic stresses, the oxidative state of plants plays a vital role. Many defensive proteins and non-protein compounds are produced through various signaling pathways, in plants as a defense barrier against these stresses. Phytohormones such as Jasmonic acid, Abscisic acid, Salicylic acid (SA) and Ethylene are key components of these signaling pathways, and hence play a decisive role in plant defense. (Bari and Jones, 2009). Salicylic acid mediates the phenylpropanoid pathway, playing an important role against pathogens and some insect pests, and abiotic stresses as well.

1.4.1. Salicylic Acid

Salicylic acid is an important phenolic compound; it is a prominent endogenous plant growth hormone, that generates a large form of metabolic and physiological responses. Besides its role in growth and development, it plays a job in plant innate immunity, as well as resistance in each native and general tissue upon organic phenomenon attacks. (Rivas-San Vicente and Plassencia, 2011). SA additionally triggers the generation of ROS and different defensive processes like cell death and hypersensitive response. (Ding and Ding, 2020).

In plants under stress, detection of phytopathogens regulates the synthesis of Salicylic acid. the basic role of salicylic acid in conferring stress resistance was first confirmed in Arabidopsis and cucumber plants when hormone level was detected before development of local and systemic resistance. (War et al., 2011). Since then its role in biotic stress has been established general plant species. salicylic acid pathway activation at infection site induces a defense response in distal components of plant. exogenous application of SA influences numerous molecular, biochemical and physiological processes in plants also as antioxidative enzyme activities. Besides getting involved in cross talk with pathways mediating plant resistance SA regulates the elements of its own signaling pathway as well. (Checker et al., 2018).

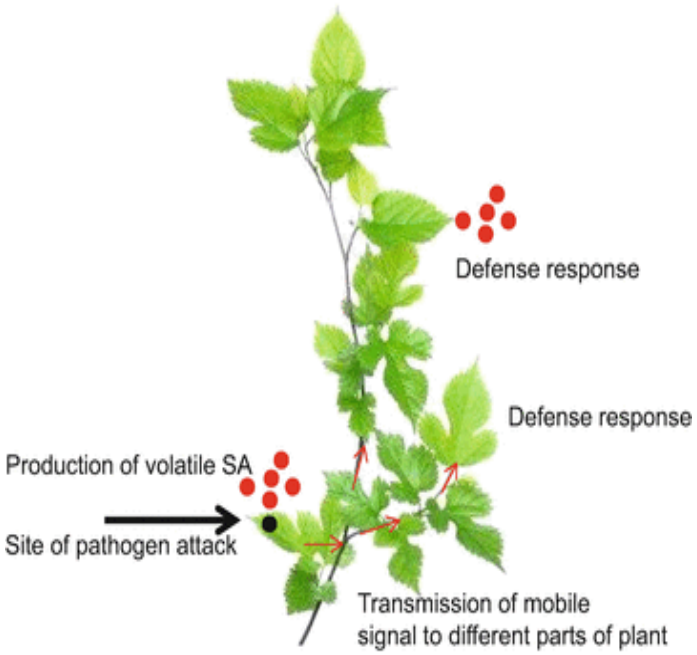

Fig 1.2 Activation of SA pathway at the site of infection triggers a defense response in distal parts of the plant. (Adopted from Check et al., 2018).

CHAPTER: 2 LITERATURE REVIEW

2.1. Arachis hypogaea

Grain legumes (or pulses) are vital food crops that may play a significant role in addressing world food security and environmental challenges and they contribute further in healthy diets. (IONESCU et al., 2017). They help meet food requirements in human diets in different parts of the world. (Roman et al., 2011). Grain legume seeds are excellent sources of vitamins, proteins, fibers, minerals, and polyunsaturated fatty acids. (Tharanathan, 2003; Bouchenak & Lamri-Senhadji, 2013). Depending on species, the protein represents between 20 and 40% of the grain mass. (Roman et al., 2011). . Groundnut additionally referred to as Peanut is an important food crop with its world annual production of about thirty eight million tones. (Bertioli et al., 2011). In general, the grain legumes are poor in methionine however rich in lysine content, thus supplementing the reverse amino acid pattern found in cereals. (Hymowitz, 1990). . Leguminous plants are often utilized in animal feed, green or silage, alone or in mixtures. The by-products of legumes (stems, leaves and husks) resulted when threshing, have a high protein content (8-14%) that is exceeding ten times the protein content of cereal straws (0.71.3%) creating them appropriate for feed purposes. (Roman et al., 2011). Additionally, all grain legumes have nitrogen fixation ability, thus reducing, in many situations, the cost of nitrogen inputs by farmers. (Hymowitz, 1990).

The peanut (Arachis hypogaea L.) is a very important seed crop and food legume grown on some 20 million ha in warm tropical or subtropic areas throughout the world. Peanut stands fifth among the most vital sources of vegetable oils. (FAO, 2010) (Moretzsohn et al., 2013). . The genus Arachis is native to South America. It contains eighty signified species, assembled into 9 sections conferring to their, geographical distribution, morphology and cross compatibility relationships. (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). This genus is widespread in South America (Bertioli et al., 2011). The apparent center of origin of *Arachis hypogeae* was in Gran Panatanal (Mato Grosso, Brazil) and on the eastern slopes of the Bovilian Andes (FAO, 2011). All species

of this genus are uncommon in comparison to different legumes as they produce their fruit below the ground The species of this genus are numerous in environment including open patches of forest associated in quickly flooded areas and grasslands,. based on its sexual compatibilities, morphology and the genus has been divided into eighty species and nine intrageneric taxonomic sections. Cultivated groundnut is tetraploid with an AABB-type genome however, most of the wild species are diploids. Cultivated peanut has very constricted genetic base. (Bertioli et al., 2011).

Among the outstanding nutritionary qualities of peanut are a protein content of up to 30% and a fat content of up to 50% that's nearly 80% unsaturated. Moreover, numerous wild Arachis species are used successfully as high-quality tropical forages. (Valls et al. 1994). . Peanut oil contains a well-balanced antioxidant and fatty acid profile that protects against harmful substances like free radicals. attributable to its distinctive organoleptic properties related to its cardio-protective and anti-inflammatory properties, groundnut oil has found, recently, its place on the enormously competitive global edible oil market. (Akhtar et al., 2014).

2.2. Secondary Metabolites in Peanut

Human diet contains variety of micro and macronutrients with many varieties of minerals, vitamins, antioxidants and alternative useful phytochemicals. The macronutrients are sources of various kinds of carbohydrates, proteins, and fats (lipids) and also the food trade cares to provide these as constituents of a good range of foods or as primary products. (Gunstone, 2011; He et al., 2008). Phytochemicals, as plant parts with distinct bioactivities towards animal organic chemistry and metabolism are being studied for their ability to supply health benefits. Phytochemicals may offer health advantages as: substrates for cofactors of enzymatic reactions, organic chemistry reactions, inhibitors of enzymatic reactions, absorbents/sequestrates that bind to and eliminate undesirable constituents within the intestine, ligands that agonize or antagonize cell surface or intracellular receptors, compounds that enhance the absorption scavengers of reactive or toxic chemicals, and or stability of essential nutrients, fermentation substrates for beneficial oral,

selective growth factors for useful canal bacteria, selective inhibitors of harmful intestinal bacteria and gastric or intestinal bacteria. Such phytochemicals include fiber, terpenoids, alkaloids and phenolics. The three major classes of plant chemicals as phenolic metabolites, alkaloids, terpenoids, and other nitrogen-containing plant constituents. (Dillard et al., 2000).

2.3. Stilbenes

Plants produce a wide variety of natural products, which have developed to provide a selective advantage against environmental stresses. Phenylpropanoids, among them, are a class of secondary metabolites involved in the response of plants to various abiotic and biotic stresses. Many phenylpropanoids have anti-microbial properties synthesized or labeled as phytoalexins in response to a herbivorous attack or infectious agent. For stress-induced phenylpropanoids, however, different functions are represented, related to signaling of protective responses, protection against actinic light damage by radiation, and increase in nutrient bioavailability. (Dixon et al., 1995).

Stilbenes are natural phenolic compounds occurring in a variety of plant families as well as Vitaceae, Dipterocarpaceae, Gnetaceae, Pinaceae, Fabaceae, Poaceae, Leguminoseae, and Cyperaceae. (Jeandet et al., 2010). Stilbenes are natural phenolic compounds occurring in a variety of plant families. Stilbenes are phenylpropanoids characterised by a 1,2-diphenylethylene backbone. Many plant based stilbenes are derivatives of the fundamental unit trans-resveratrol ($3,5,4^0$-trihydroxy-transstilbene). (Chong et al., 2009). Resveratrol and its derivatives, like oxyresveratrol, pterostilbene, and viniferins, are the foremost notable stilbenes because of their various strong biological activities and medicative properties and are extensively studied. (Kalantari and Das 2010; Pangeni et al. 2014; Estrela et al. 2013; Xu et al. 2014; Wang and Yao 2015). Resveratrol has been shown to possess antioxidant, immune-modulatory, anti-inflammatory, and anti-angiogenic effects and a large vary of health benefits, reminiscent of cancer chemoprevention or cardioprotection (Kalantari and Das 2010; Pangeni et al. 2014; Weiskirchen and Weiskirchen 2016). A range of alternative stilbenoids, e.g., piceatannol, pinosylvin, combretastatins, polydatin (piceid), mulberroside, or numerous oligostilbenes,

also are best-known to show valuable biological activities and beneficial effects. (Piotrowska et al. 2012; Du et al. 2013; Wang et al. 2014a; Greene et al. 2015; Laavola et al. 2015; Wang and Yao 2015). Over the last fifteen years, plant stilbenes have received respectable interest, because of their biological activities and potential pharmacologic applications. Recently, stilbenes were much-admired for their anti-wounding properties and acknowledged for their healing and preventive powers as neuroprotective, antitumor, cardioprotective, and inhibitor agents. (Anekonda et al., 2006; Jeande et al., 2009). Stilbenes are considered essential because of their protecting properties against herbivores, nematodes and plant fungal pathogens. (Chong et al. 2009; Jeandet et al. 2010).

Stilbenes are synthesized from a coenzyme A (CoA) activated phenylpropanoid starter unit and 3 malonyl-CoA extender units. the primary step in phenylpropanoid biogenesis is that the chemical change of Lphenylalanine to trans-cinnamic acid, catalyzed by essential amino acid ammonia lyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to form 4-coumaric acid, that is then activated by 4-coumaroyl: CoA ligase (4CL) to make 4-coumaroyl-CoA. a sort III polyketide synthase then consecutive adds three acetate extender units, derived from malonyl-CoA, to one activated 4-coumaroyl-CoA starter unit. resultant folding and cyclization of the generated tetraketide intermediate results either within the production of a chalcone or stilbene ring structure (Watts et al., 2006).

2.4. Effect of Salicylic Acid on Stilbenes Biosynthesis

Salicylic acid (SA) is a phytohormone involved in plant development and growth, transpiration, photosynthesis, transport Pathogenesis-related (PR) proteins and ion uptake. (Hayat and Ahmad 2007; Vicente and Plasencia 2011 Salicylic acid (SA) plays a vital role in plant defense mechanisms against pathogens. SA infusion into V. vinifera berries increased the buildup of PAL mRNA, further as and also the synthesis of latest PAL macromolecule and protein activities. (Durner et al. 1997). SA infiltration into entire V. vinifera berries increased the buildup of PAL mRNA, further as and also the synthesis of latest PAL macromolecule and protein activities (Chen et al. 2006 SA plays a vital role in plant defense and is usually concerned within the activation of defense responses against

hemi-biotrophic and biotrophic pathogens also the activation of systemic acquired resistance (SAR). Grant and Lamb 2006; Bari and Jones 2009; Vicente and Plasencia 2011). Recent analysis noticeably shows upregulation of VaSTS2, VaSTS3, VaSTS5, VaSTS6, VaSTS8, and VaSTS10 genes upon SA treatment. VaSTS2 and VaSTS10 genes upregulated the most. (Kiselev et al., 2010) SA has shown to induce the expression of particular genes in VaPAL and VaSTS multigene families: VaPAL3, VaSTS2, VaSTS3, VaSTS4, VaSTS5, VaSTS6, and VaSTS8. Moreover, the effect of SA treatment on PAL and STS, the key genes involved in resveratrol biosynthesis, was quite different from the effect of the rolB transformation. Based on these results SA and the rolB protein product induce biosynthesis and accumulation of resveratrol, although along different regulatory pathways. (Kiselev et al., 2010). The peanut is amongst the restricted variety of plant species that produce resveratrol, that has anti-microbial, anti-oxidant and antifungal activity and a also reduces cardiovascular disease and cancer risk. It has been proven that signal molecules like jasmonic acid (JA), ethylene and salicylic acid (SA), play important roles in plant responses to varied abiotic and biotic stresses (Dong et al., 1998 SA is responsible for the induction of genes involved in the systemic acquired resistance (SAR) response. (Dempsey et al., 1995). RS messenger RNA expression was elicited in peanut leaves in response to JA etthylene and SA. Ethylene, SA and JA are observed to be involved in various plant defense mechanisms after a pathogen attack. SA is invloved in the establishment of SAR native defense responses against pathogen attack (Feys et al., 2000).

On the other hand, ethylene and JA play a crucial role in the establishment of nonspecific disease resistance through signaling pathways distinct from the classic SAR response pathway regulated by SA. (Kiselev et al., 2010. In addition to its role in plant pathogenesis, ethylene, SA and JA play a major role in plant responses to abiotic stresses such as ozone, light and UV radiations. (Yalpani et al., 1994) (Boller et al., 1980). Therefore, induction of RS expression by ethylene, SA and JA raises the chance that these molecules share a typical sign pathway within the regulation of RS organic phenomenon in the peanut (Chung et al., 2003).

AIMS AND OBJECTIVES

The aim of his study is to perform expression analysis on stilbene biosynthetic gene(s) upon Salicylic acid treatment with following objectives:

1) In-silico structural and functional analysis of Stilbenes Synthase gene(s)
2) Investigate the expression level of stilbene synthase gene(s) upon hormonal treatment i.e. Salicylic Acid.

CHAPTER: 3 MATERIALS AND METHODS

3.1. In-silico Analysis

3.1.1. Identification of gene

Stilbene synthase genes in *Arachis hypogaea* were identified and sequences were retrieved from NCBI. The databases used for this study were:

EST database: (http://www.ncbi.nlm.nih.gov/)

PeanutDB: (http://bioinfolab.muohio.edu/txid3818v1/)

Peanut Base: (http://www.peanutbase.org/)

3.1.2. Multiple Sequence Alignment and Phylogenetic Analysis

Stilbene synthase sequences were retrieved from NCBI and aligned by using MEGA-X Software. Both the nucleotide and amino acid sequences were aligned. A phylogenetic tree or evolutionary tree is a branching diagram or tree showing the evolutionary relationships among various biological species based upon similarities and differences in their physical or genetic characteristics. MEGA-X software was used for constructing phylogenetic tree.

3.1.3. Open Reading Frame and Conserved Domain identification

ExPASy translate tool was used to obtain open reading frames of the three stilbene synthase genes (https://web.expasy.org/translate/) and used to find the conserved domain through conserved domain database (https://www.ncbi.nlm.nih.gov/cdd).

3.1.4. Primary Sequence Analysis

The nucleotide and amino acid sequence of gene and protein reveals important information about the isoelectric point (pI), molecular weight (Mw), extinction coefficient (protein-protein and protein-ligand) interactions, instability index, aliphatic index (AI-relative volume of protein occupied by aliphatic amino acids), and Grand Average of Hydropathicities (GRAVY-sum of all hydropathicity values of all amino acids divided by number of residues in a sequence). The molecular weight and isoelectric point of the three stilbene synthase enzymes were calculated through compute pI/Mw tool of ExPASy (https://web.expasy.org/compute_pi/). The physical properties of the three stilbene synthase enzymes were analyzed using ExPASy protparam tool (http://web.expasy.org/protparam) (Gastieger et al., 2005). The transmembrane region for STS4, STS5 and STS6 was predicted using SOSUIsignal (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuisignal/sosuisignal_submit.html). SignalP 3.0 server was used to predict the signal peptide cleavage sites for STS4, STS5 and STS6.

3.1.4. Subcellular localization of protein

Subcellular localization of protein helps predict the role of protein and its interaction with several other molecules. WoLF PSORT tool was used (https://www.genscript.com/wolf-psort.html).

3.2. Homology Modelling

3.2.1. Secondary structure prediction

The secondary structure of protein is comprised of helices, sheets and amino acid turns and is responsible for the protein folding. PSI-blast based secondary structure PREDiction (PSIPRED) and Chou and Fasman secondary structure prediction (CFSSP) server (Ashok & Kumar, 2013).

3.2.2. Protein model prediction

The 3D structure of protein can be predicted through SWISSMODEL (https://swissmodel.expasy.org/). The detailed structure of protein with amino acid residues can be studied in PyMOL viewer (https://pymol.en.softonic.com/).

3.2.3. Evaluation of protein quality

The protein quality was evaluated and verified through ERRAT (https://servicesn.mbi.ucla.edu/ERRAT/), QMEAN (https://swissmodel.expasy.org/qmean/) (Benkert et al., 2011), and Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) (Lovell et al., 2002). The Z-score is calculated by comparing the QMEAN score of model and reference. The Z-score is the normalized to mean 0 and standard deviation 1. The QMEAN Z-score is an indication for the standard deviation of the models QMEAN score from the experimental structures.

3.2.4. Secondary structure analysis of protein

The secondary structures of stilbene synthase protein were analyzed through SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

3.2.5. Protein-protein interaction and functional analysis

To find the interaction of stilbene synthase with other closely related protein STRING v10.0 was used (http://string-db.org/). Stilbene synthase of *Arachis hypogaea* was used as query sequence to generate the functional association network of protein. The protein family of stilbene synthase was also determined by finding the motif through motif finder server (http://www.genome.jp/tools/motif/). Motif is a highly conserved region of amino acids in a protein in a protein sequence with in the domain. The active site of enzyme was predicted through COFACTOR (http://zhanglab.ccmb.med.umich.edu/COFACTOR/).

3.3. Docking

3.3.1. Enzyme substrate interaction

In order to find the interaction between enzyme and substrate, docking was performed between stilbene synthase and substrates identified through literature review. Two of the candidate substrates used for the analysis were p-coumaryl CoA and malonyl CoA. The docking was performed using Patchdock server. The interaction of ligand with active site residues and bond length was analyzed through PyMOL.

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3.4. Seed collection and germination

Healthy and qualified seeds were collected from the department of oilseed research program at National Agricultural Research Centre (NARC), Islamabad, Pakistan. Seeds were de-husked and sterilized by soaking them in 70% ethanol for 2 minutes followed by washing through autoclaved water, separately. Excessive alcohol was vaporized by spreading the seeds on a cleaned filter paper in a surface sterilized safety cabinet. Then completely dried seeds were wrapped up in aluminum foil sheet and kept at 4°C for 48 hours to break the dormancy of the seeds. Later, seeds were transferred to a wet cotton layer under sterilized conditions for the sake of germination. Each layer contained five to six seeds placed at a good distance. Petri dishes were labeled and placed in a dark cabinet with temperature ranges from 22-28°C for time till growth.

3.5. Soil Preparation

Soil was taken from the groundnut field of NARC, Islamabad and was autoclaved in order to avoid any fungal infection in the plants.

3.6. Seed Plantation

Seeds with equal morphology and well developed roots were sown in soil under control conditions i.e. 28ºC and 16/8 light/dark hours. Germination was observed on 7-10 days after cultivation. About 90% of the plants were successfully germinated.

3.7. Hormonal Treatment

When the plants reached trifoliate stage, they were given hormonal treatment by foliar spray method. And leaf samples were collected at several time-points and placed in liquid nitrogen. Samples were collected in triplicates for accurate studies. For later studies the samples were stored at -80°C.

3.8. Total RNA Extraction

Total RNA from stored leaf samples was extracted by TRIZOL method. 3 to 4 young leaves were ground in liquid nitrogen and homogenized in 1ml TRIZOL in autoclaved mortar. The sample was centrifuged at 14000rpm for 5 min at 4^{o}C. The supernatant was carefully transferred to a clear tube without transfer of debris particles and incubated for 5 min on ice to permit complete dissociation of nucleo-protein complex. Then 200µl chloroform was added in the Eppendorf per 1 ml Trizol reagent. The mixture was incubated for 2-3 min on ice and then centrifuged for 20 min at 14000rpm at 4ºC. After this step three phases appeared in the Eppendorf upper aqueous phase containing RNA, middle phase containing proteins and lower red-phenol phase containing DNA. Aqueous phase was transferred to a new Eppendorf. Then 500µl ice-cold isopropanol was added to the aqueous phase and incubated on ice for 10 min. The mixture was centrifuged at 14000 rpm for 10 min. White pellet was formed. Supernatant was discarded. Pellet was re-suspended in 1ml 75% ethanol. Sample was vortexed briefly so as to avoid RNA degradation., then centrifuged

for 5 min at 9500 rpm. The supernatant was discarded and excess ethanol was evaporated to avoid contamination in the RNA. The pellet was air dried on sterilized working area for 5-10 min. The pellet was re-suspended in 20-50 µl RN-ase free water. RNA was stored at -80^{o}C for further use.

3.9. Gel Electrophoresis (RNA)

The integrity and quality of isolated RNA was evaluated by running it on 1% agarose for 30 minutes at 90 V. 0.7g of molecular grade agarose was melted in 70 ml 1% TAE buffer to completely dissolve it. Agarose was completely dissolved by microwave. Combs were placed in the casting tray with appropriate space. 4 µL Ethidium Bromide as added in the gel mixture in fume hood under safety conditions and mixed properly. The gel was poured in the caster and allowed to solidify properly. As the gel solidified, the combs were removed carefully and placed in the gel tank filled with 1X TAE buffer. The gel was completely immersed in TAE buffer. 3 µL RNA sample was mixed with 2 µL 6X loading dye to make a 5 µL mixture and then loaded in the well. Blank (solution used to dissolve RNA in this case RNase free water) and ladder were also loaded in the well. The sequence was ladder (100kb), blank, sample of RNA. The gel was analyzed under UV trans-illuminator Biotrop® for the presence/absence, size and quality of RNA. The image of gel was taken through gel doc for record.

3.10. RNA Quantification

The purity and integrity of RNA was evaluated through Thermo Fisher Scientific Nano drop ^{TM} 2000/2000c. The spectrophotometer was blanked using 1 µL RNase free water three times to avoid calibration error. Then 1 µL sample was placed on the Nano drop and absorbance was taken for nucleic acid. For each sample absorbance was recorded providing concentration of RNA in ng/ µL, 260/280 ratio, and 260/230 ratio.

3.11. cDNA Synthesis

cDNA was synthesized using thermofisher scientific kit. 1µl oligo dT primers were added to PCR tube containing 1µg of total RNA and 12.5 µl Nuclease-Free water. It was gently mixed with brief centrifugation followed by incubation at 65°C for 5 minutes. After

incubation, it was chilled o ice and spinned down. Then, following components were added in a PCR tube; 4µl 5X buffer, 0.5µl Ribolock RN-ase Inhibitor, 2µl 10mM dNTP Mix and 1µl RevertAid RT. Total volume of the mixture was kept 20µl. PCR was set at conditions 42°C for 60 minutes and 70°C for 5 minutes.

3.12. Confirmation PCR

A conventional PCR using housekeeping gene (beta actin) was performed for confirmation of cDNA synthesis. 1µl of cDNA, forward and reverse primers were added to master mix (2µl) and nuclease free water (5µl). The reaction conditions are mentioned in Table 3.1.

Table 3.1. Reagents and their quantities used for Actin PCR

Sr. No.	**Reagents**	**Quantity**
1.	NF water	14.5 µL
2.	10X $(NH_4)_2SO_4$ buffer	2.5 µL
3.	25mM $MgCl_2$	2.5 µL
4.	10 mM dNTPs	2 µL
5.	10 µM Actin Forward Primer	1 µL
6.	10 µM Actin Reverse Primer	1 µL
7.	cDNA	1 µL
8.	Taq DNA Polymerase (5 U/10 µL) Thermo Fisher Scientific	0.5 µL
	Total Volume	**25 µL**

Table 3.2. Actin PCR Conditions

Sr. No.	PCR steps	Temperature	Time
1.	Initial denaturation	94ºC	4 min
2.	Denaturation	94ºC	30 sec
3.	Annealing	58ºC	1 min
4.	Extension	72ºC	45 sec
5.	Final Extension	72ºC	7 min

3.13. Gel Electrophoresis (DNA)

2% agarose gel was used to confirm the amplification of PCR product including positive and negative control. Gel was prepared by dissolving 1.4g agarose in 70 ml 1X TAE buffer using microwave. 4 µL Ethidium Bromide was added to the gel for visualization of bands. Samples were loaded and run at 90V for 30 minutes. The gel was visualized under UV trans-illuminator and gel doc images were kept for record.

3.14. Primer Designing

Primer 3 software was used to design primers for the six stilbene synthase genes. The conserved region of gene was taken and primer set giving product up to 200 nucleotide sequence was taken keeping the GC content to 50%. The Tm for the primers was optimized using the protocol of Actin PCR with varying values for melting temperature. The primer details are given in supplementary Table.

3.15. Real Time PCR

Real Time PCR method utilizes a fluorescent dye for detection of amplification of gene in each cycle. SYBER® Green fluorescent dye that binds non-specifically to the gene sequence was used in the Real Time experiment to evaluate the copy number of the gene

through the Ct value. The Ct (threshold value) is an intersection of threshold line and amplification curve. Reagents and their quantities used in Real Time experiment were as given in Table 3.3. Mastermix was prepared in a sterilized Eppendorf tube containing all reagents except cDNA on ice. 13.5 µL mixture from master mix was added into the designated PCR tubes. 1.5µL of cDNA mixed in NF water in the ratio 1:5 was added in the designated PCR tubes and capped. The PCR tubes were placed in the Real time PCR by Applied Biosystem for analysis under specific conditions. The Ct values for the genes were analyzed and recorded through Livak method. The PCR was performed in triplicates for validity of results.

Table 3.3. Reagents and their quantities used in Real Time PCR

Sr. No.	**Reagents**	**Quantity**
1.	NF water	5 µL
2.	Maxima SYBR® Green Master mix	7.5 µL
3.	Forward Primer	0.5 µL
4.	10 µM Actin Reverse Primer	0.5 µL
5.	1/5 dilution of cDNA	1.5 µL
	Total Volume	**15 µL**

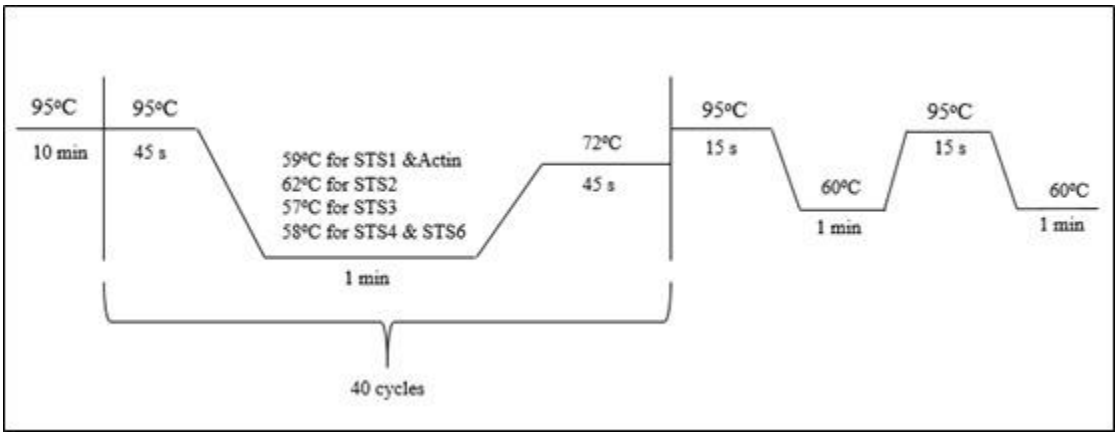


Fig.3.1. Real Time PCR conditions

3.16. Livak Method

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

Step #1: Calculation of Mean and variance

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

Step #2: Calculation of ΔCT

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

Step #3: Calculation of ΔΔCT

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

Step #4: Calculation of Mean Fold

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

3.16. Statistical Analysis

Microsoft Excel 2010 and GraphPad Prism® was used for arranging and organizing data and analyzed through inferential statistics. The difference between control and treated group is calculated through student t test and variation was performed through variance analysis (ANOVA test). The significant *p*-value considered for the analysis was 0.05.

CHAPTER: 4 RESULTS

4.1. Multiple Sequence Alignment and Phylogenetic Analysis

Total of 10 sequences for stilbene synthase in different variants and cultivars of groundnut were obtained from NCBI. The variation in cDNA length was not notable with difference of only few nucleotides. The predicted amino acid products were composed of approximately 389 amino acids. To evaluate the evolutionary relationship among the proteins, the amino acid and nucleotide sequences were completely aligned. The aligned sequences were then used to construct a tree based on neighbor-joining method (Fig 1) with bootstrap analysis of 1000. The optimal tree obtained was with a branch length = 0.52145274. The bootstrap values on the branch shows the percentage of replicate trees in the evolutionary analysis. The evolutionary tree distances were computed using Maximum Composite Likelihood method and are in the units of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}+$ non-coding. All ambiguous positions were removed for each sequence (pairwise deletion). There were a total of 1927 positions in the final dataset. The analysis was conducted in MEGA X. Chalcone synthase of glycine max was kept as reference.

Alignment of the stilbene synthase from various cultivars and variants of groundnut along with chalcone synthase of glycine max revealed three common motifs conserved across all sequences. The location of motif 1 on the sequence starts at base 29 and ends at base 78, while motif 2 starts at 116 and ends at 165. The third motif starts at base 292 and ends at base 341.

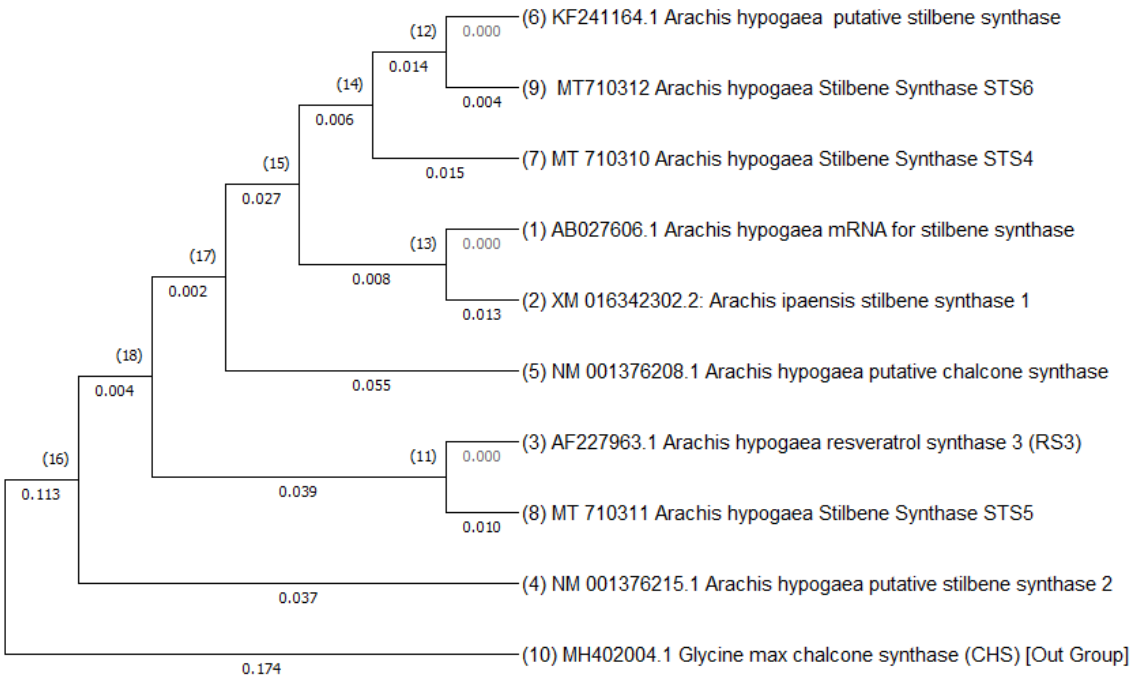
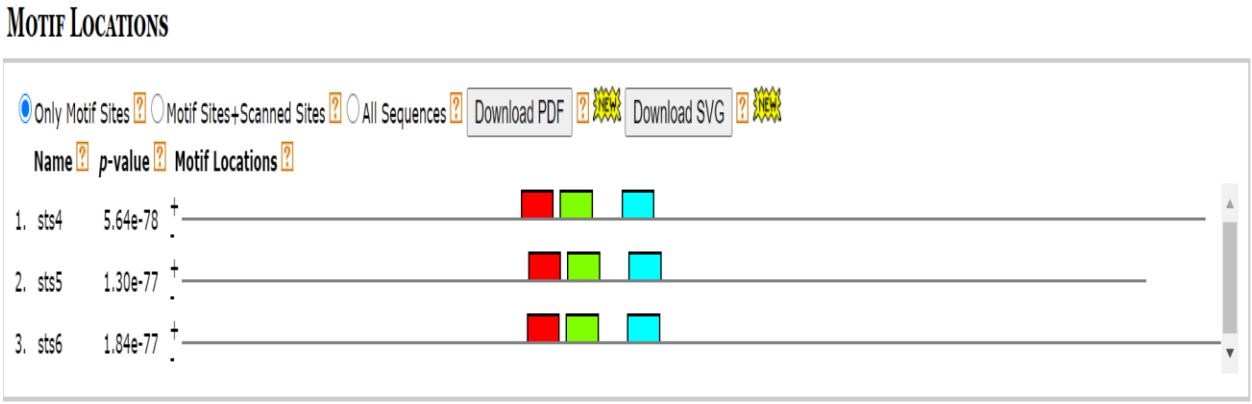


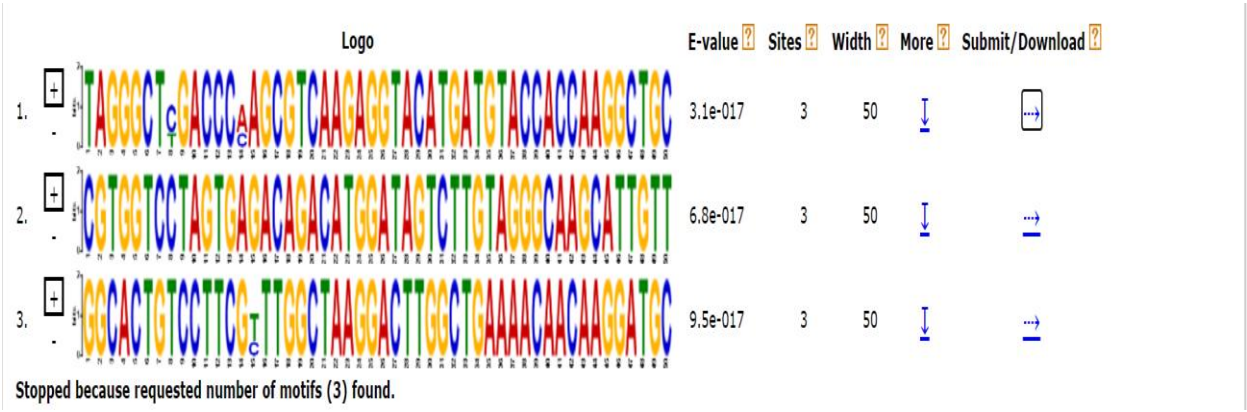
Fig 4.1. Phylogenetic tree of stilbene synthase of different variants and cultivars of groundnut. Glycine max Chalcone synthase is kept as outgroup. The tree was constructed using 11 sequences with Neighbor-joining method and bootstrap values show on the nodal branch.

4.2. Open Reading Frame and Conserved Domain Identification

The suitable open reading frames for our three candidate genes, STS4, STS5 and STS6 were obtained from ExPASy translate tool. The predicted protein product from the ORF for the three candidate genes suggested that it comprises of about 389 amino acids each.



(a)



(b)

Fig 4.2. (a) Alignment of the 10 sequences for stilbene synthase shows the presence of 3 motifs conserved across all sequences. (b) Motif sequence analysis

SMART database was used to find conserved domain for our candidate genes, using their amino acid sequences. The parameters used were kept at default mode. Two conserved domains were identified in all three candidate genes. The N terminal domain comprises of Chalcone/stilbene synthetase domain starting from amino acid position 4 and ends at 228. The e-value for the domain is quite low 3.9×10^{-126}. The lower e-value depicts the quality of assay while there were few matches available.

4.3. Primary Sequence Analysis and Physiochemical Characterization

The characterization of a protein is incomplete without assessing its physical and chemical properties. Table 1 in annexure depicts the physiochemical properties of three stilbene synthase from *Arachis hypogaea.* Isoelectric point pI is the condition when the protein produces equal number of positive and negative charges in solution and overall charge on the protein is zero. The pI values of STS4, STS5 and STS6 were 6.71, 5.93 and 5.56 which showed that the three enzymes have slightly acidic character. The instability index for all three genes was lowers than 40 showing a stable protein. The high values of aliphatic index show that all the three proteins are thermostable. The values of GRAVY range between -0.107 to 0.106. The extremely low values of GRAVY for the three candidate proteins suggested that they form stable interaction with water. The results for predicting the location of protein in the cell suggested that STS4, STS5 and STS6 are all located in the cytoplasm.

4.4. Functional Analysis

The three candidate genes have transferase activity, transferring acyl groups other than amino-acyl group. It catalyzes Claisen-like condensation reaction and production of group of compounds called polyketides. It was further confirmed by COFACTOR analysis which showed high confidence score of approximately 0.95 for STS 2, 0.8 for STS3 to be present in cytoplasmic region while score of 1 (highest possible score) as component of cell.

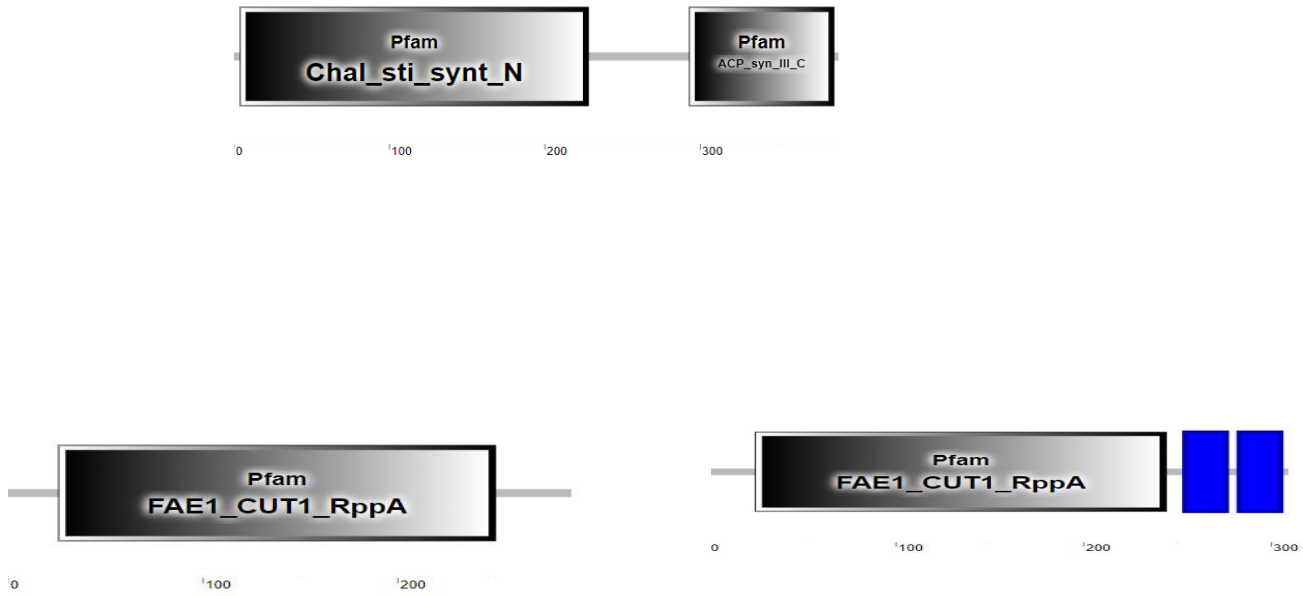


Fig 4.3. Two conserved domains (Chalcone/stilbene synthase and ACP synthase) for stilbene synthase enzymes.

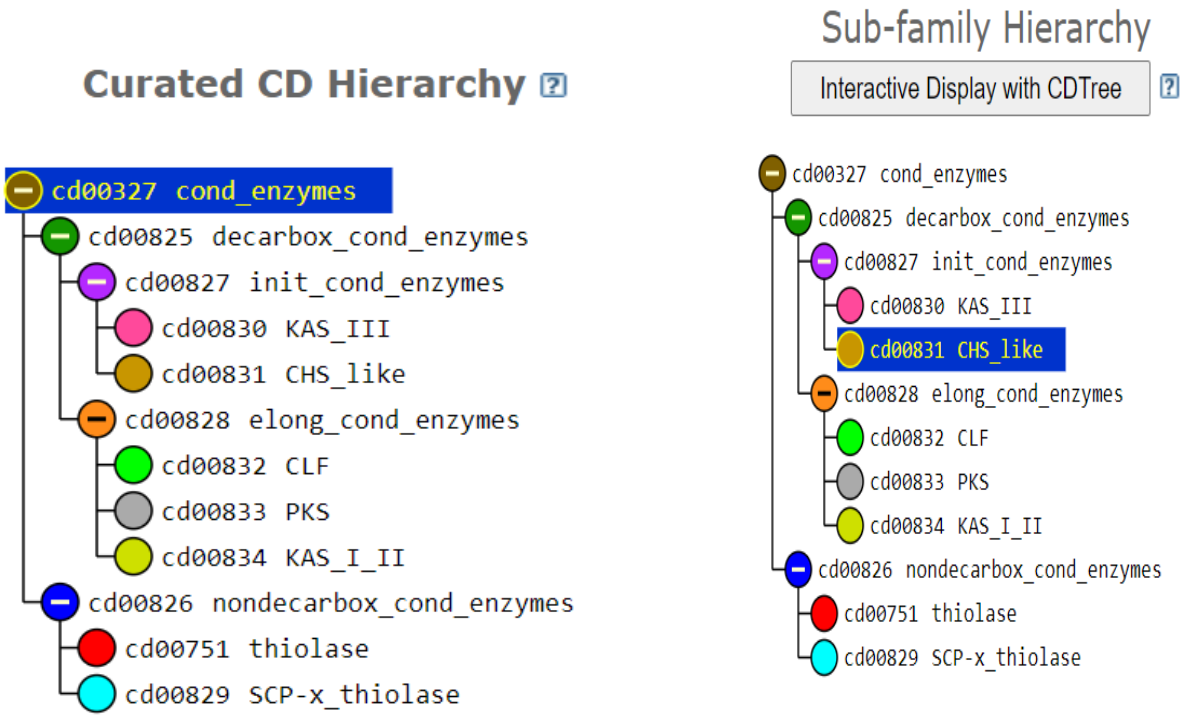


Fig 4.3.1. The two conserved domain in stilbene synthase enzyme

4.4. Homology Modelling

4.4.1. Protein Model Prediction

The structures were predicted through PSIPRED and CFSSP which showed that the proteins consist of alpha helices, beta sheets and coils. 3D model of the enzymes was predicted using SWISS-Model software keeping stilbene synthase from *Arachis hypogaea* 1z1e.1.A as template. The software generated 10 models out of which the structure with suitable QMEAN score and sequence identity to the model template was selected.

4.4.2. Secondary Structure Prediction

The secondary structure prediction results indicated the presence of alpha helices more than other secondary structures in the three enzymes. The percentage of alpha helices in *Arachis hypogaea* STS4 was 43.19%, Beta turns 5.66% and random coils to be 35.8%. In *Arachis hypogaea* STS5 the percentage of alpha helices was 44.73%, while the beta turns were 6.43% and 33.98% were random coils. And the last *Arachis hypogaea* STS6 44.73% alpha helical structure were present, with 5.14% beta turn and 34.19% random coils. There was no disordered arrangement or state found for all three proteins.

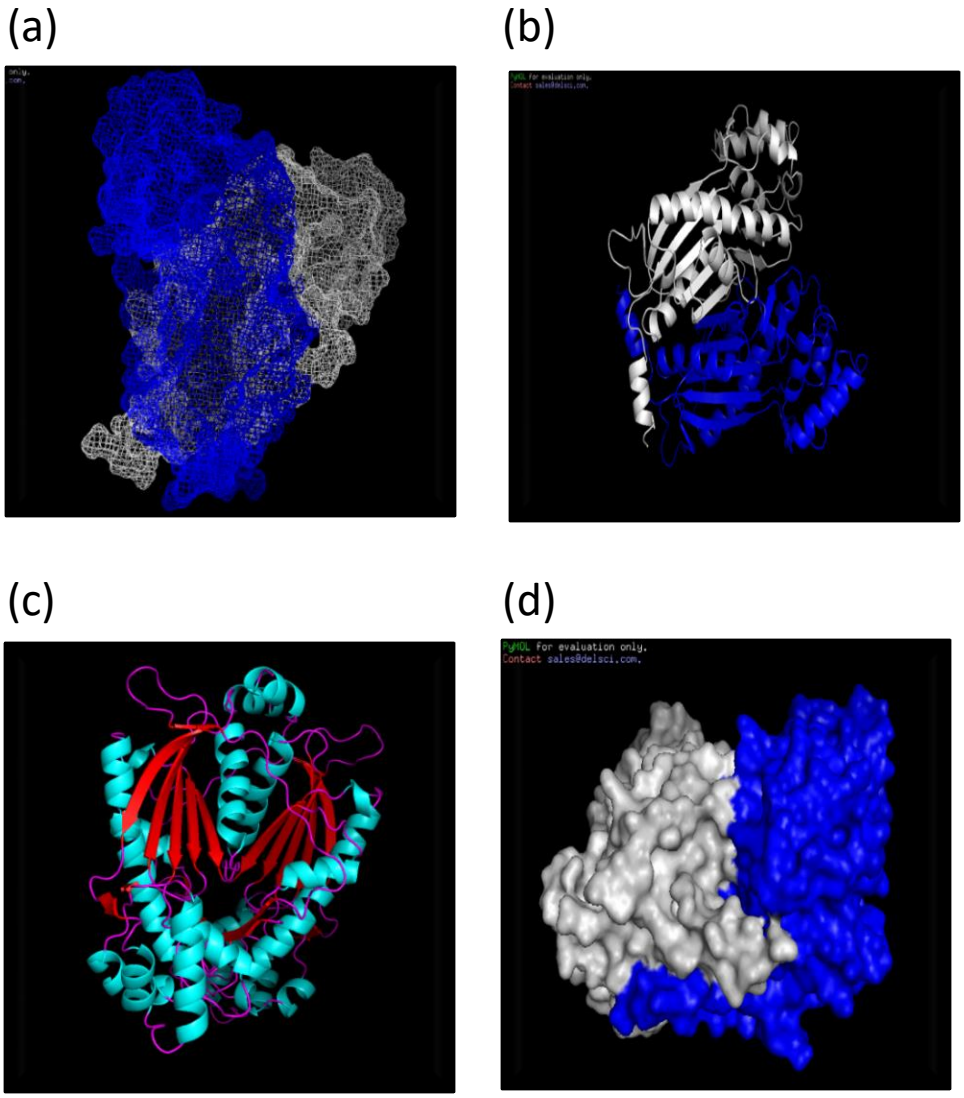


Fig 4.4. Three dimensional models of stilbene synthase IV in *Arachis hypogaea* showing (a) and (b) helices and beta sheet (c) distinct chains (d) surface view of protein (blue= helices, red= beta-sheets, purple= loop).

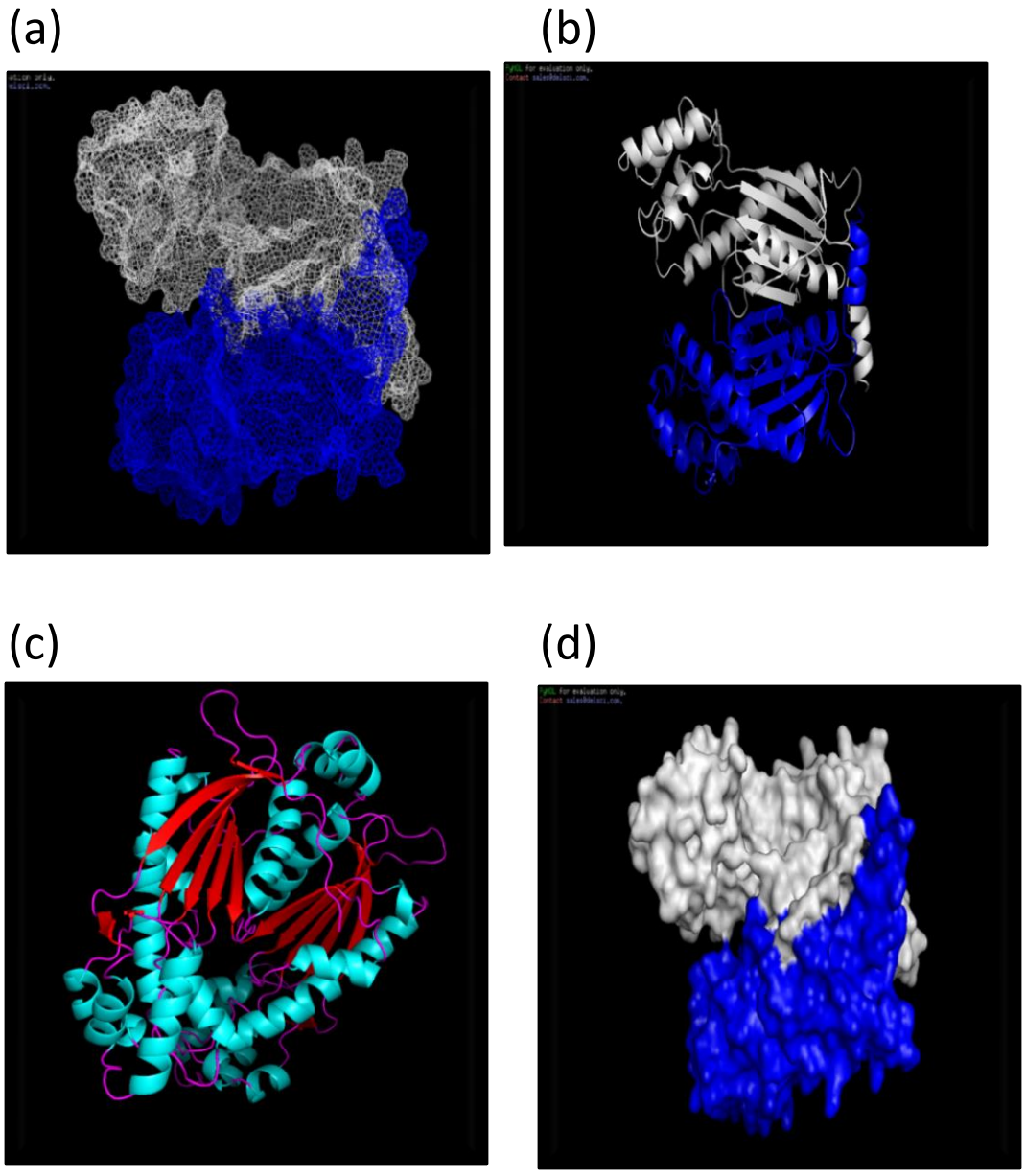


Fig 4.5. Three dimensional models of stilbene synthase V in *Arachis hypogaea* showing **(a)** and **(b)** helices and beta sheet **(c)** distinct chains **(d)** surface view of protein (blue= helices, red= beta-sheets, purple= loop).

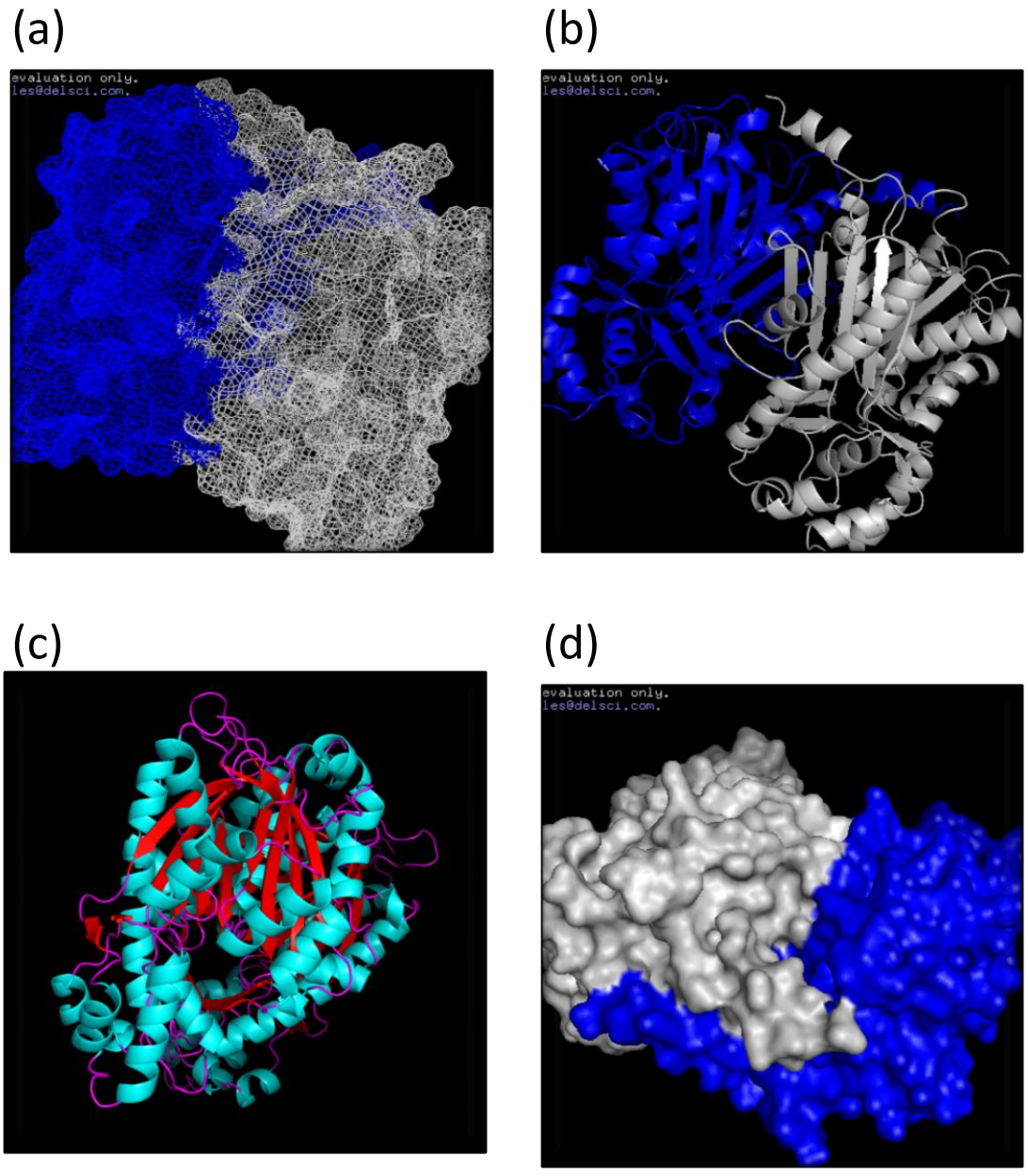


Fig 4.6. Three dimensional models of stilbene synthase VI in *Arachis hypogaea* showing **(a)** and **(b)** helices and beta sheet **(c)** distinct chains **(d)** surface view of protein (blue= helices, red= beta-sheets, purple= loop).

4.4.3. Protein Quality Estimation

QMEAN score for STS4, STS5 and STS6 calculated from QMEAN server has small negative values. Small negative values indicate a good quality structure.

The dots plotted in the graph in different shades of grey represent the QMEAN score of the reference template. The Z-score relates to the favorable states of the protein. A higher QMEAN Z-score gives better agreement with predicted features. The QMEAN Z-score ranges from 0 - 1.

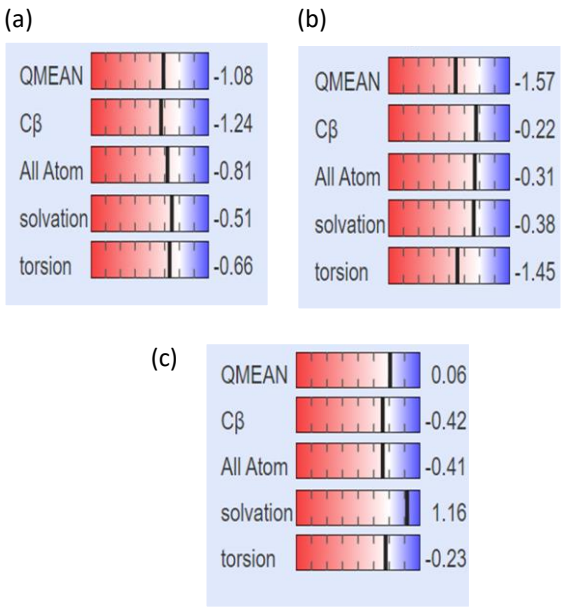


Fig 4.7. QMEAN score for **(a)** STS 4 **(b)** STS 5 and **(c)** STS 6. For the given enzymes the QMEAN score lies in light red to blue region which indicates a good structure.

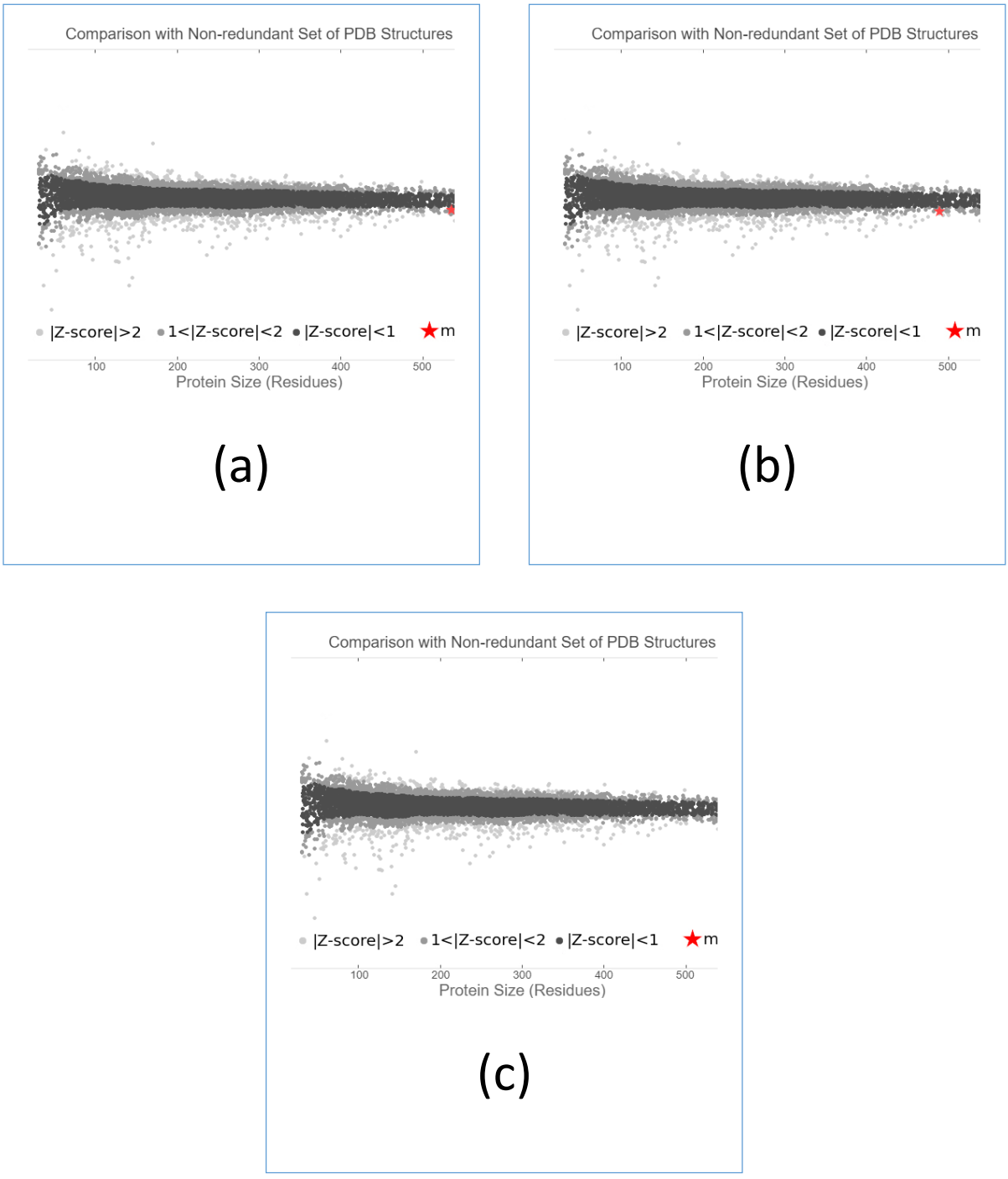


Fig 4.8. Evaluation of protein quality through Z-score **(a)** STS4, **(b)** STS5 **(c)** STS6. Most of the residues have QMEAN Z-score less than 1. Red steric sign gives the location of model residue.

4.5. RNA Extraction and Polymerase Chain Reaction

RNA was successfully extracted for the given time points for BARD and GOLDEN with distinct bands of 28SrRNA, 18SrRNA and 5SrRNA. RNA was converted to cDNA and the PCR product amplification was confirmed with actin on 2% agarose gel electrophoresis.

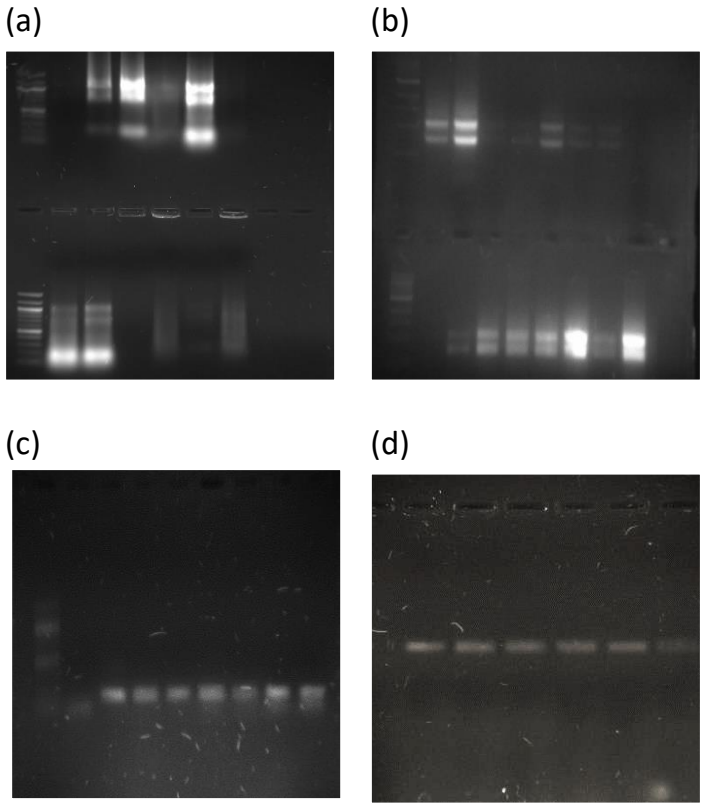


Fig 4.9. Gel electrophoresis images for the 28SrRNA, 18SrRNA, 5SrRNA **(a)** BARD **(b)** Golden **(c)** BARI **(d)** Gel electrophoresis image of Actin gel

4.6. Expression Profiling of Stilbenes Synthase genes

To evaluate the effect of Salicylic acid treatment on the expression of stilbene synthase genes AhSTS1, AhSTS2, AhSTS3, AhSTS4, AhSTS6 along with ß-actin (house-keeping) genes in the leaves of *Arachis hypogaea* equi-molar amount of cDNA was used as a template for real-time PCR. The expression of AhSTS1, AhSTS2, AhSTS3, AhSTS4, and AhSTS6 didn't follow a specific pattern of expression.

In BARD, expression of STS1 increased after half an hour of Salicylic acid treatment and remained upregulated till 3 hours. After 6 hours, the expression down regulated abruptly. Expression level increased a bit after 8hr and 24hr of treatment. However, in Golden variety, expression level of AhSTS1 gene was low. In comparison to control, expression was seen after 3hr of treatment.

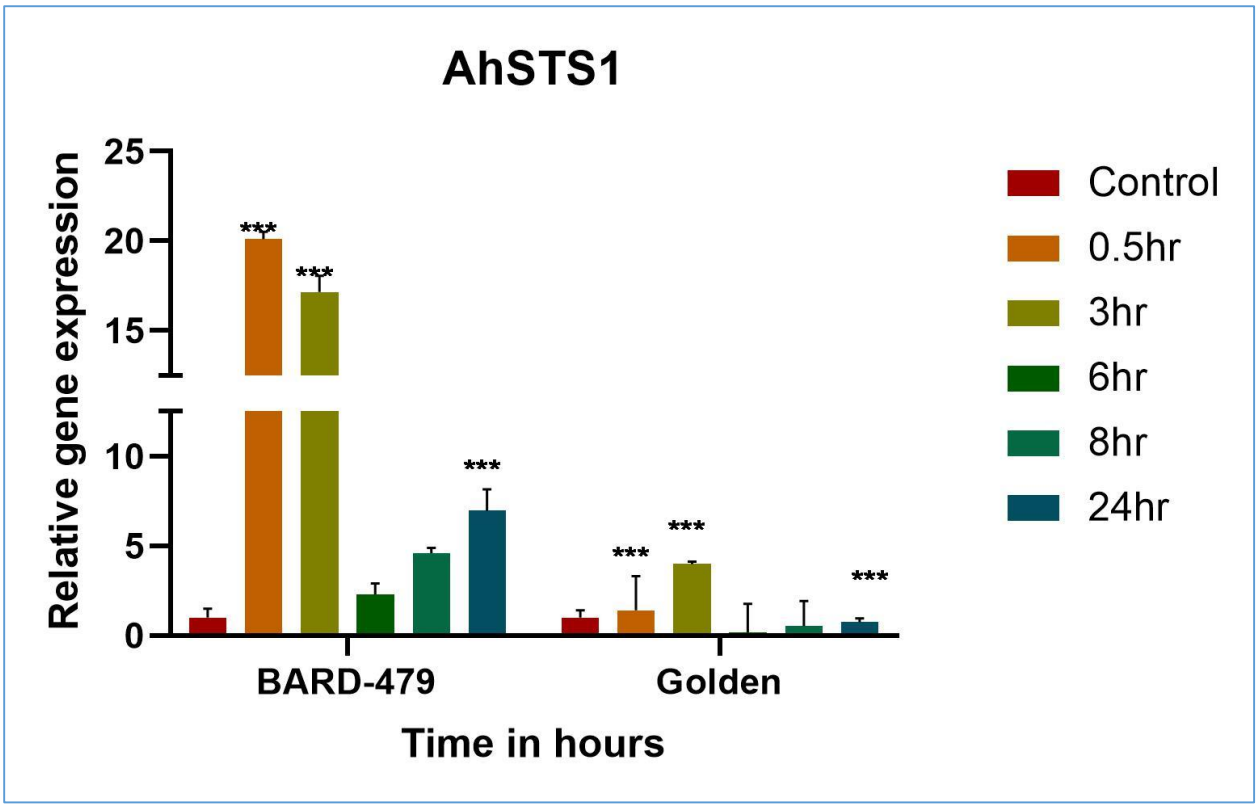


Fig 4.10. Expression analysis of AhSTS1 gene upon Salicylic acid treatment in BARD and GOLDEN. Real-Time PCR was performed to determine the expression of both genes at different hours after hormonal treatment.

Expression pattern of AhSTS2 in BARD was quite interesting. Expression increased abduptly after half an hour of treatment. Then almost no expression was observed after 3hr of treatment, then again the gene expressed at 6hr of treatment. Then no expression at 8hr, finally AhSTS2 expressed again at 24 hr. while in Golden, AhSTS2 only expressed at 8hr of treatment.

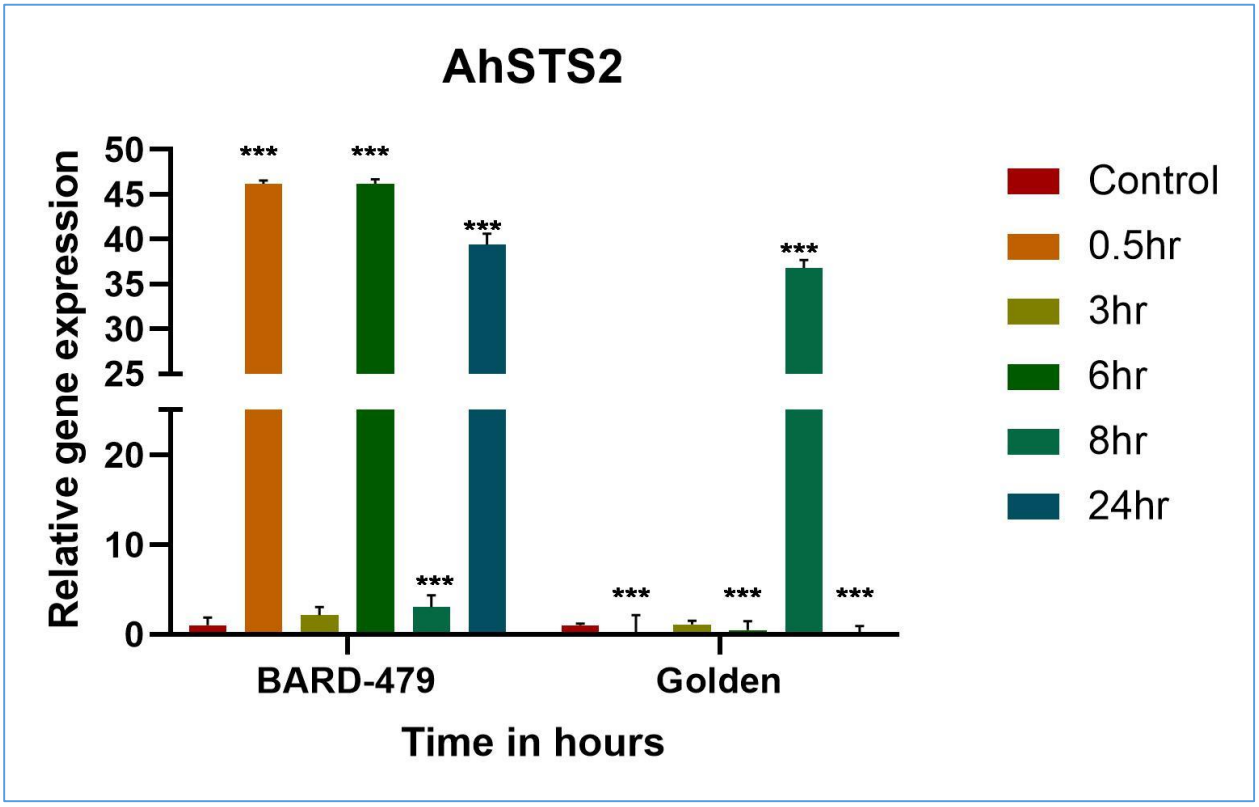


Fig 4.11. Expression analysis of AhSTS2 gene upon Salicylic acid treatment in BARD and GOLDEN. Real-Time PCR was performed to determine the expression of both genes at different hours after hormonal treatment

Expression pattern of AhSTS3 in BARD was as such that the gene expressed at half an hour of treatment and 6hr of treatment. In Golden, AhSTS3 expressed at 8hr of treatment.

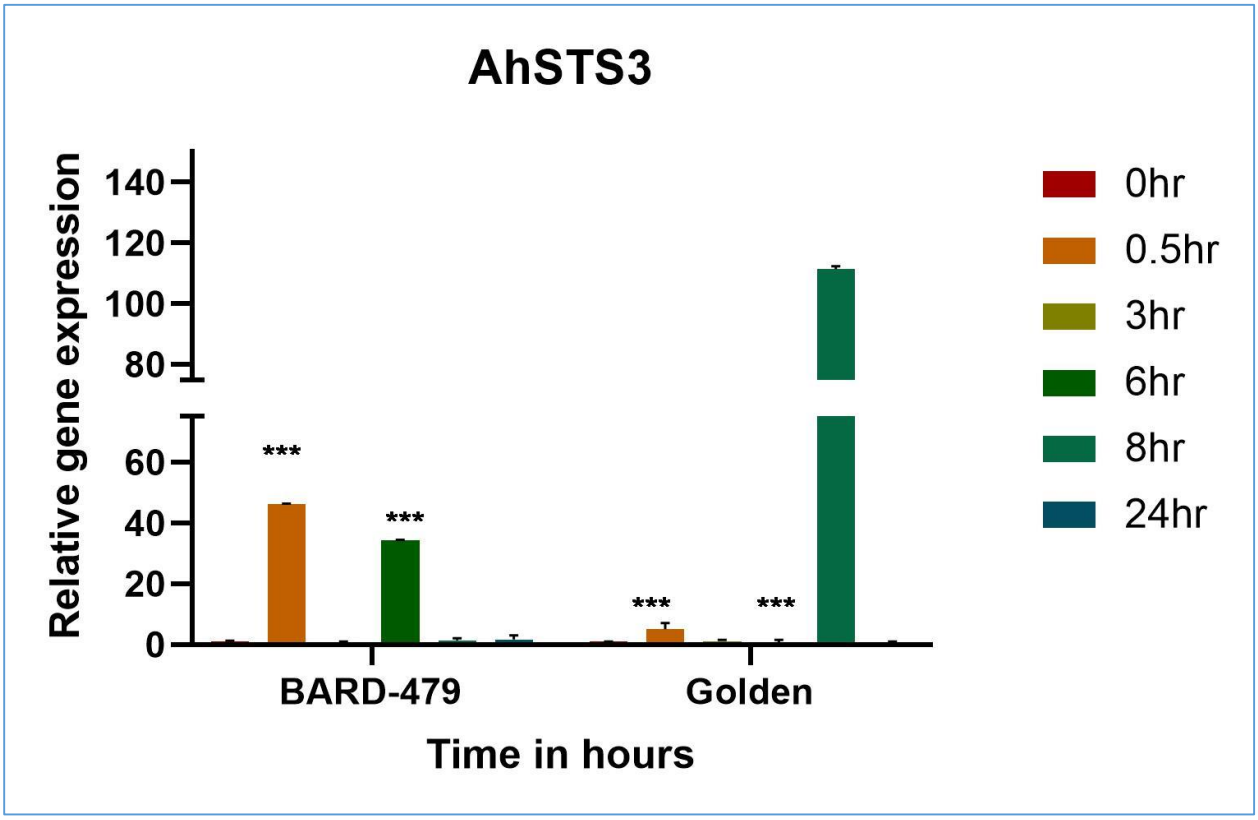


Fig 4.12. Expression analysis of AhSTS3 gene upon Salicylic acid treatment in BARD and GOLDEN. Real-Time PCR was performed to determine the expression of both genes at different hours after hormonal treatment

AhSTS4 in BARD expressed itself in the form of a curve. Expression gradually increased until 8hr and then induction decreased at 24hr. in Golden, however the expression level of AhSTS4 was lower as compared to BARD. Conversely, the pattern was quite similar. It was observed that the gene first down regulated after half an hour then the induction

increased after 3hr of treatment. Later, expression decreased gradually till 24hr of treatment.

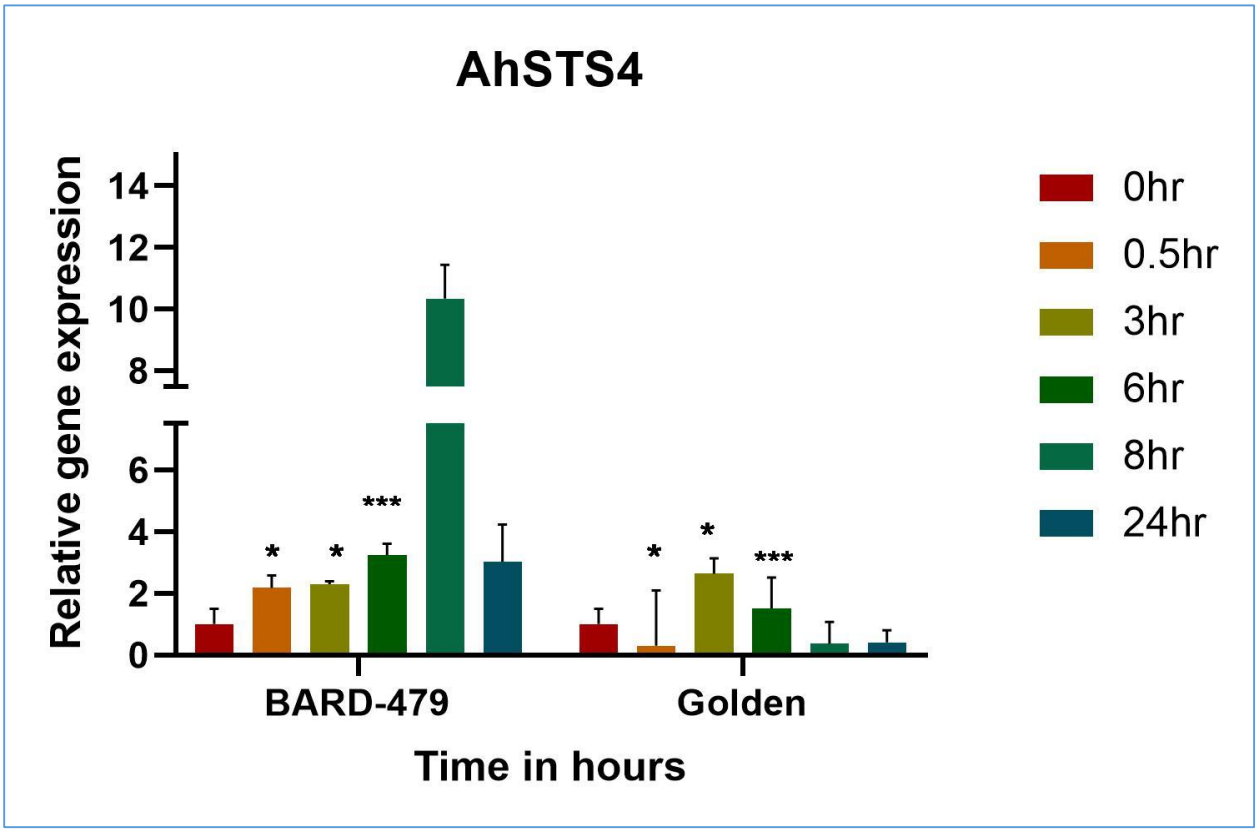


Fig 4.13. Expression analysis of AhSTS4 gene upon Salicylic acid treatment in BARD and GOLDEN. Real-Time PCR was performed to determine the expression of both genes at different hours after hormonal treatment

Nearly no expression of AhSTS6 was observed in Golden variety. Whereas in BARD, the gene highly expressed itself at 8hr of treatment.

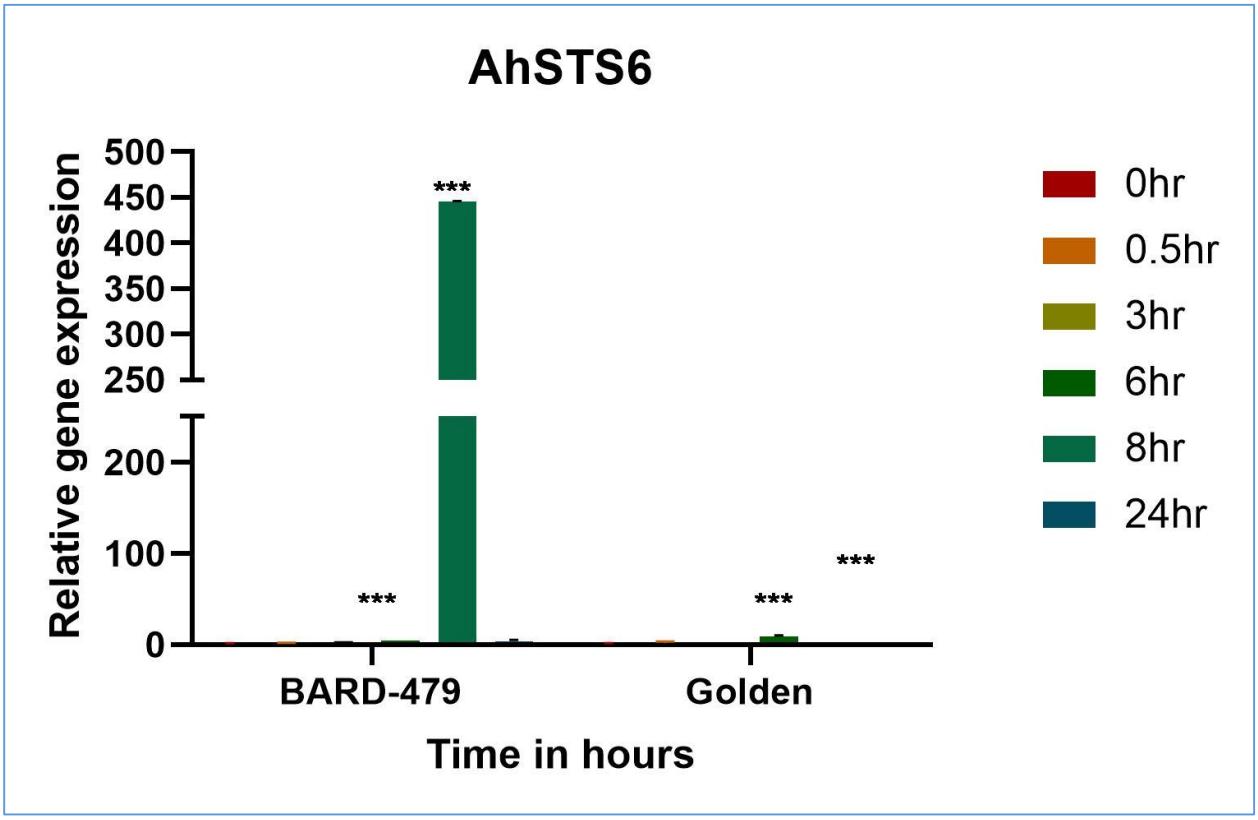


Fig 4.15. Expression analysis of AhSTS6 gene upon Salicylic acid treatment in BARD and GOLDEN. Real-Time PCR was performed to determine the expression of both genes at different hours after hormonal treatment

Discussion

Arachis hypogaea is one of the vital crops that produces phytochemicals important for plant defense against biotic and abiotic stresses not only that, these compounds hold pharmacological properties that help combat several human diseases. One of the unique phytochemicals produced by Arachis hypogaea is Stilbenes/ Stilbenoids. This compound is produced by a few unrelated species of plant. Stilbenes are produced by Stilbene Synthase enzyme, specifically when plant is under some environmental stress. Stilbenes are derived from the general phenylpropanoid pathway. In *Arachis hypogaea* six stilbene synthase genes have been isolated from the leaves. The sequencing results of genes show a maximum sequence similarity with difference in amino acid at few positions. Structure prediction of putative Stilbene Synthase enzyme showed presence of alpha helices, beta sheets and loops. The predicted 3D model of Stilbene Synthase enzyme may be used as a template to model structures of ligand and other binding proteins. This will help researchers develop better understanding of structural properties of the enzyme. 3D model prediction is one of the very few useful method for studying the structure-function aspects of protein. The outcome from this research may be useful in the field of bioinformatics research. Expression profiling of putative Stilbene synthase genes showed differential gene expression. It was observed that the pattern of expression of genes was different among two varieties of Peanut (Inter-Varietal Differential expression). In BARD, the expression was high compared to Golden Variety. As observed in another study, differential gene expression pattern of stress-responsive genes to drought was observed in two cultivars of Peanut. (Khady Nani Drame et al., 2007). Treatment of Salicylic Acid on Peanut Plant induces expression of some of the Stilbene Synthase genes of STS gene family as in Golden the expression of AhSts6 was not observed. And expression AhSTS1 was comparatively low. In a study, SA was found to enhance the expression of VaSTS2, VaSTS3, VaSTS4, VaSTS5, VaSTS6, and VaSTS8 of multigene family of 21 to 43 STS genes in *Vitis amurensis*. (K. V. Kiselev et al., 2009). Expression of VvSTS gene

family (48 STS genes) is diferentially regulated by biotic and abiotic stresses, pathogen infection, mechanical wounding or hormones (Almagro et al. 2014). Among which only VvSTS7 and VvSTS36 responded to SA. Recent study reported that several VvSTS genes in grapevine cell suspension cultures are diferentially expressed in response to different elicitors such as MeJA and ethylene (Chialva et al. 2018).

ANNEXURE

1. ORFs

StS4:

MVSASDIRKVQRAEGPATVLAIGTANPPNCVDQSTYADYYFRVTNSEHMTDLK
KKFQRICERTQIKNRHMYLTEEILKENPNMCAYKAPSLDAREDMMIREVPRVG
KEAATKAIKEWGQPMSKITHLIFCTTSGVALPGVDYELIVLLGLDPSVKRYMM
YHQGCFAGGTVLRLAKDLAENNKDARVLIVCSENTSVTFRGPSETDMDSLVGQ
ALFADGAAAIIIGSDPVPEVENPLFEIVSTDQQLVPNSHGAIGGLLREVGLTF
YLTRVFRILFHKTSMMHSVKLLIH

STS5:

MVSVSEIRNVERAEGPATVLAIGTANPSNCVDQSTYADYYFRVTNSEHMTDLK
KKFQRICERTQIKNRHMYLTEEILKENPNICAYKAPSLDAREDMMIREVPRVG
KEAATKAIKEWGRPMSEITHLIFCTTSGVALPGVDYELIVLLGLDPSVKRYMM
YHQGCFAGGTVLRLAKDLAENNKDARVLIVCSENTSITFRGPSETDMDSLVGQ
ALFADGAAAIIIGSDPVPEVEKPLFEIVSTDQNLSLVAMEPSVVSFVKLALHS
ILTKVFLILFHKISTTHSVKLLILWVYLIITQYFGLHILVDVQF

STS6:

MVSASDIRKVQRAEGPATVLAIGTANPPNCVDQSTYADYYFRVTNSEHMTDLK
KKFQRICERTQIKNRYMYLTEEILKENPNMCAYKAPSLDAREDMMIREVPRVG
KEAATKAIKEWGQPMSKITHLIFCTTSGVALPGVDYELIVLLGLDPSVKRYMM
YHQGCFAGGTVLRLAKDLAENNKDARVLIVCSENTSITFRGPSETDMDSLVGQ
ALFADGAAAIIIGSDPIPDVENPLFEIVSTDQQLVPNSHGAIGGLLREVGLTF
YLNKSVPDIISQNINDALTKAFDPLGISDYNSIFWIAHPGGRAILDQVEEKVN

LKPEKMKATRDVLSNYGNMSSACVFFIMDLMRKKSLEAGLKTTGEGLDWGVLF GFGPGLTIETVVLRSVAI

Table 1: Physiochemical properties of stilbene synthase								
Gene ID	**aa**	**pI**	**Mw**	**II**	**AI**	**EC**	**GRAVY**	**Half life**
STS1	389	5.96	42952.7	35.03	91.23	33390	-0.088	>10 hours
STS2	540	6.84	59343.75	40.66	93.70	54360	-0.070	>10 hours
STS3	389	5.66	42840.39	35.17	91.98	33390	-0.078	>10 hours

Real Time PCR Primers

Supplementary Table 1:		
Sr.	**Primer**	**Sequence 5' to 3'**
1.	Actin- F	CAGGCCGTTCTCTCCCTTTAT
	Actin- R	CATCAAGGCATCGGTGAGAT
2.	AhSTS1-F	ACCAGGTTGAACAGAAGGTG
	AhSTS1-R	ACCCCAATCAAATCCTTCTC
3.	AhSTS2-F	CACGTGATGTTCTTAGCGAT
	AhSTS2-R	TTCAATAGTGAGGCCAGGTC
4.	AhSTS3-F	TCACACATTTGATCTTCTGC

	AhSTS3-R	TGTTATTTTCAGCCAAGTCC
5.	AhSTS4-F	AGCACATACGCAGATTACTA
	AhSTS4-R	CAAGGACGGTGCTTTATATG
6.	AhSTS6-F	TGGTTCTGATCCTATTCCAG
	AhSTS6-R	AAAGCTTTAGTGAGTGCATC

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