ISOLATION AND CHARACTERIZATION OF HSP FAMILY GENE FROM CARDAMOM (*Elettaria cardamomum* Maton)

By

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DECLARATION

I hereby declare that this thesis entitled **"Isolation and characterization of Hsp family gene from cardamom (*Elettaria cardamomum* Maton)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Palode **ASWATHY L. B.**

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CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Percentage
(L.)	Linnaeus
Δ	Delta
°C	Degree Celsius
µl	microliter
A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ABA	Abscisic acid
ACD	Alpha Crystallin Domain
AGE	Agarose Gel Electrophoresis
AMV RT	Avian Myeloblastosis Virus Reverse Transcriptase
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatases
BLAST N	Nucleotide BLAST
BLAST	Basic Local Alignment Search Tool
bp	base pair
C terminal	Carboxyl terminal
CD	Conserved Domain
cDNA	complementary DNA
CEO	Cardamom Essential Oil
Clp	Caseinolytic protease
cm	centimeter
Cpn	Chaperonin
Cq	Cycle quantification
Crdm	Cardamom
CS	Citrate synthase
Ct	Cycle threshold
CTAB	Cetyl trimethyl ammonium bromide
CTR	C terminal region

DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosidetriphosphate
ds DNA	Double stranded DNA
EDTA	Ethylenediaminetetraaceticacid
ER	Endoplasmic Reticulum
EST	Expressed Sequenced Tag
et al.	*et alia*
ExPASy	Expert Protein Analysis System
fast QC	fast quality control
FASTA	FAST-All
FRET	Fluorescence Resonance Energy Transfer
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRAVY	Grand Average of Hydropathicity
Grp 78	Glucose related protein 78
HIP	Hsp Interacting Protein
HMW	High molecular weight
HOP	Hsp 70 organizing proteins
hr	hour
HS	Heat stress
Hsc 70	Heat shock cognate 70
HSE	Heat Shock Element
HSF	Heat Shock factor
HSG	Heat shock granules
Hsp	Heat shock protein
ICAR	Indian Council for Agricultural Research
ICH	Indian Cardamom Hills
IDT	Integrated DNA Technology
IISR	Indian Institute of Spices Research
ISH	*In situ* hybridization

JNTBGRI	Jawaharlal Nehru Tropical Botanical Garden & Research Institute
kDa	Kilo Dalton
KSCSTE	Kerala State Council for Science, Technology and Environment
L	Litre
LiCl	Lithium Chloride
LMW	Low Molecular Weight
M	Molarity
MDH	Malate dehydrogenase
MEGAX	Molecular Evolutionary Genetics Analysis X
mg	milligram
min	minute(s)
ml	millilitre
mM	Millimolar
M-MLV RT	Murine Moloney Leukemia Virus Reverse Transcriptase
mRNA	Messenger RNA
MSL	Mean Sea Level
Mtp 70	Mitochondrial heat shock protein 70
MW	Molecular weight
N terminal	amino terminal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnological Information
NCBI SRA	NCBI Sequence Read Archive
ng	Nanogram
NGS	Next generation sequencing
nm	nanometer
NTC	Non Template Control
NTR	N terminal Region
p23	protein 23

PCR	Polymerase Chain Reaction
Phyre^2	Protein Homology/AnalogY Recognition Engine
pI	Isoelectric point
pM	picomolar
PSIPRED	PSI-blast based secondary structure PREDiction
PVP	Polyvinylpyrrolidone
qPCR	Quantitative PCR
qRT-PCR	Quantitative Reverse Transcriptase PCR
RACE	Rapid Amplification of c-DNA ends
rcf	relative centrifugal force
RFC	Relative Fold Change
RNA Seq	RNA sequencing
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	6-carboxy-X-rhodamine
rpm	revolutions per minute
rRNA	ribosomal RNA
RT enzyme	Reverse Transcriptase enzyme
RT	Room temperature
RT-qPCR	Reverse transcription quantitative PCR
s	second(s)
S	Sydberg unit
SAGE	Serial Analysis for Gene expression
sHsp	Small Heat Shock Protein
ß	Beta
SSH	Suppression Subtractive Hybridization
Ta	Annealing Temperature
TAE buffer	Tris Acetate EDTA buffer
Taq	*Thermus aquaticus*
Tm	Melting Temperature
Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
UBCE	Ubiquitin conjugating Enzyme

UniProt	Universal Protein Resource
UV-B	Ultra violet-B
V	Volt
α	alpha
μM	micromolar

1. INTRODUCTION

From ancient times itself, Indians have a unique tryst towards spices. Especially for Keralites, spices have been an indispensable part of their daily cuisines starting from morning tea to dinner dishes. One such inevitable aromatic spice used in day to day kitchen cuisines is small cardamom, generally known as '*Elam'* in Malayalam.

Elettaria cardamomum (L.) Maton, commonly known as green or small cardamom, is one of the most important spice crops with the sobriquet "Queen of Spices". It belongs to Zingiberaceae family and has originated in the moist evergreen forest of the Western Ghats of Southern India. The crop is grown in India, Guatemala, Sri Lanka, Nepal, Indonesia, Costa Rica, Mexico and Tanzania (Madhusoodanan *et al*., 2002).

Cardamom is a herbaceous perennial with underground rhizome and aerial pseudostems (tillers) that grows around 2 – 5 m height and bears 4 – 7 panicles. Flowers are beautiful, tiny and are usually white in colour with pink strips over them. The colour of the fruit is normally green and turns to golden yellow on ripening. It is a shade-loving plant cultivated at an altitude of 600 – 1200 m above mean sea level (MSL) with an annual rainfall of 1500 – 4000 mm and a temperature of 10 to 35 °C (Madhusoodanan *et al*., 2002).

Based on fruit size, small cardamom varieties are broadly classified as *E. cardamomum* var. major consisting of wild varieties and *E. cardamomum* var. minor comprising the cultivars *viz*., Malabar, Mysore and Vazhukka.

The most valued part of cardamom are capsules. Dried ripe fruits of cardamom have high economic value as well as a variety of medicinal properties. For centuries, it has been used for various culinary and traditional medical applications including controlling asthma (Khan *et al*., 2011), teeth and gum infections, digestive and kidney disorders, cataract, nausea, diarrhea and cardiac disorders (Ashokkumar *et al*., 2020). The essential oil and other bioactive molecules in capsules have many therapeutic effects including anti-diabetic, anti-inflammatory, anti-oxidant, anticancer, antifungal, antibacterial, antiviral, analgesic and gastro-protective activities (Al-Zuhair *et al*., 1996; Verma *et al*., 2009; Khan *et al*., 2011; Winarsi *et al*., 2014).

With the increase in demand for cardamom for various uses, the annual production made a drastic leap. Now, the world production of cardamom is around 36,000 tonnes per annum with India being the second leading producer. In India, Kerala leads the production sector constituting 89 % followed by Karnataka (7 %) and Tamil Nadu (4 %) (Pal, 2019).

Though cardamom is having good production potential, unfortunately they are vulnerable to biotic and abiotic stress factors. Being highly altitude specific, it cannot be cultivated at an altitude productively below 600 m. Apart from altitude; temperature plays an important role owing to its direct effect on the productivity of cardamom.

In order to cope up with the stress conditions, plants expresses different classes of heat shock proteins (Hsps). One such class is composed of evolutionarily less conserved small heat shock proteins (sHsps) which act as molecular chaperones by preventing aggregation of misfolded proteins. This diverse molecule has been reported in various plants, playing an important role in thermotolerance (Vierling, 1991). Hence it is imperative for anyone who seeks to tackle the problem of heat stress in cardamom, to carry out a proper study regarding sHsps in cardamom.

The primary objective of this study was to analyze differentially expressed genes belonging to small heat shock (sHsp) family under heat stress. Studying the expression of sHsps might shed light on the molecular aspects of thermotolerance in cardamom.

The present study is to form a foundation to understand the molecular mechanisms involved in thermotolerance in cardamom which can substantiate further studies.

2. REVIEW OF LITERATURE

In this chapter, literature regarding *Elettaria cardamomum* (L.) Maton, Heat shock protein (Hsp) and its classes, small heat shock protein (sHsps), role of small heat shock protein genes in thermotolerance and important techniques associated with the study undertaken have been reviewed.

2.1 SMALL CARDAMOM: THE QUEEN OF SPICES

Elettaria cardamomum (L.) Maton commonly known as small cardamom is the third costliest spice in the world after vanilla and saffron. It is a member of Zingiberaceae family under the order Scitaminae. It is also commonly known by the names 'true cardamom', 'lesser cardamom' or 'green cardamom'.

Cardamom comes under the genera '*Elettaria*' and species '*cardamomum*'. The genus name *'Elettaria'* is derived from the Tamil word "Elettari" which refers to the seeds of cardamom (Retnakaran, 2018). It consists of about six species (Madhusoodhanan *et al*., 2002) of which *E. cardamomum* is the only species found in India. Based on the fruit size, two botanical varieties of true cardamom are known, namely var. *major* Thwaites and var. *cardamomum* (syn. var. *minor* Watt; var. *minuscula* Burkill). The fruit of the var. *major* is larger (2.5 to 5 cm) and less aromatic while compared to var. *minor*. The vast majority of the diversity in cardamom comes from varietal diversity rather than ecological diversity (Madhusoodhanan *et al*., 1994).

Apart from *Elettaria*, the related genus *Amomum* and *Aframomum* also produce aromatic seeds. *Amomum subulatum* Roxburg commonly known as large cardamom is one such. It is indigenous to moist evergreen forests of sub Himalayan tracts and is usually cultivated under cold-humid climate with well uniformly distributed annual rainfall of 2000 to 3500 mm (Retnakaran, 2018).

2.1.1 Taxonomic profile of *E. cardamomum*

Domain	: Eukaryota
Kingdom	: Plantae
Phylum	: Spermatophyta
Subphylum	: Angiospermae
Class	: Monocotyledonae
Order	: Zingiberales
Family	: Zingiberaceae
Genus	: *Elettaria*
Species	: *cardamomum*

2.1.2 General morphology of *E. cardamomum*

Small cardamom is a tall, perennial, monocotyledonous herb that grows 2 – 5 m height by extending out long 10 – 20 erect leafy shoots (tillers) and 4 – 7 panicles. The leaves are lanceolate and are 30 – 35 cm long and 7 – 10 cm wide with adaxial portion dark green and abaxial region light green in colour. Panicles grows around 0.6 – 1.2 m and can be erect, prostrate or semi erect with or without branches. Both tillers and panicles emerge out of subterranean woody rhizome, which is mostly used for its propagation.

Each panicle bears numerous, small, white flowers (4 × 1.7 cm size) characterized by a conspicuous labellum with violet streaks radiating from the center (Reghunath and Bajaj, 1992). Flowers are irregular, bisexual and zygomorphic in nature with inferior ovary. They consist of unequal three lobed corolla arranged on a tubular, light green calyx. There are three stamens in total, of which only one is functional, rest are rudimentary staminoides. Anthers are bilobed and adnate to a short, broad filament and dehisce vertically (Ashokkumar *et al*., 2020). Ovary is composed of three united carpels with numerous ovules. Stigma is funnel shaped, ciliated and attached to the ovary *via* a slender style. Stamens and carpels are arranged in a way to favour cross-pollination rather than autogamy.

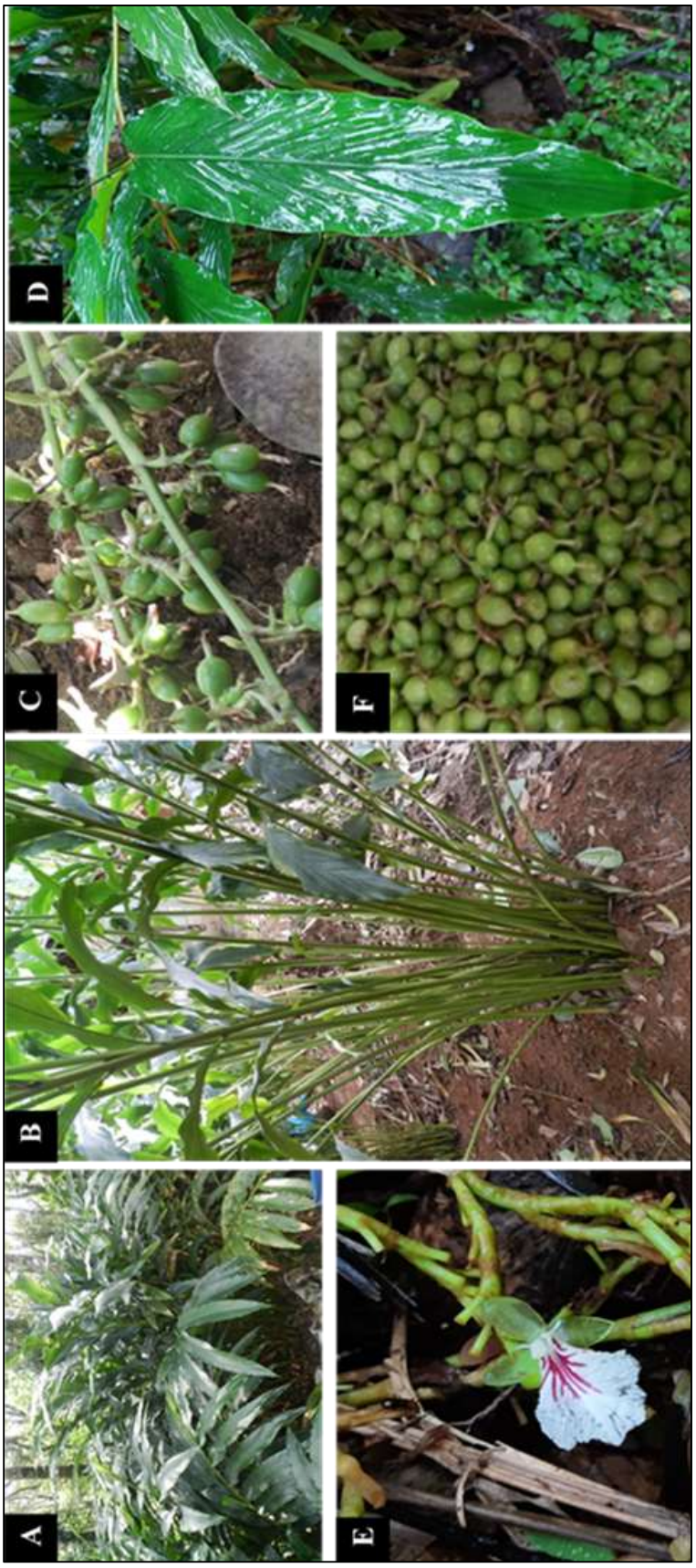


Plate 2.1 Plant profile: Elettaria cardamomum *(A) Field view (B) Habitat (C) Panicle bearing capsules (D) Leaf (E) Flower (F) Capsules*
Photo Courtesy*: Reshma Retnakaran (2018) and Aswani SureshBabu T. (2020)*

The most valued part, capsules are trilocular having 20 – 25 mm length and contain 15 to 32 seeds with a mucilaginous coat (Murugan *et al*., 2016). Seeds are angular and are dark brown in colour with sweet aroma. Fruit colour is green and on maturation turns to golden yellow. The shape of capsules ranges from oval to globose and are non-dehiscent, fleshy and leathery after drying (Ashokkumar *et al*., 2020).

Based on the nature of panicle, there are 3 cultivars *viz*. Malabar (prostrate panicle), Mysore (erect panicle) and Vazhukka (semi-erect panicle). Malabar cultivar which grows around 2 – 3 m height with round capsules is suitable for lower elevations whereas, Mysore cultivar is characterized by longer capsules, seen at higher altitudes. Vazhukka shows characters of both Mysore and Malabar cultivar and is regarded as the natural hybrid of the above two.

2.1.3 Origin and distribution of *E. cardamomum*

Cardamom is a tropical plant native to Western Ghats of Southern India. These surface feeders grow well on high ranges (600 – 1200 m above MSL) with an annual rainfall of 1500 – 4000 mm and a temperature of 10 to 35 °C (Madhusoodhanan *et al*., 2002). Damp, loamy soil rich in humus content is well suitable for its cultivation. The soil must be well drained as it cannot tolerate water logging. As it's a shade lover, 40 to 60 % shade is essential for their proper growth and flowering.

The crop is mostly cultivated in India, Guatemala, Sri Lanka, Nepal, Indonesia, Costa Rica, Mexico and Tanzania. In the global market, Guatemala is the leading producer of cardamom followed by India. According to Indian Spices Board, it has been estimated that the exported quantity of cardamom was around 1,405 tonnes worth Rs. 30,777.00 lakhs during 2019. New Guinea, El Salvador, and the Honduras have also emerged as suppliers of cardamom on the global market.

India is recognized as a rich source of genetic diversity of cardamom (Prasath, 2001). In India, the cultivation is currently mainly concentrated in the southern states: Kerala, Karnataka, and Tamil Nadu extending between 8°30' and 14°30' N latitude and longitude 75-70' E (Prasath *et al*., 2004). This area is an elongated tract from Sirsi of

Karnataka (north) to Thirunelvelli of Tamil Nadu (south). From west to east, it extends over the regions of Western Ghats (Madhusoodanan *et al*., 1994). In Kerala, the major cultivation areas are Nelliampathy, Wynad and Idukki where Njellani constitute the major variety cultivated. In Karnataka, the cultivation land spread across Uttar Kannada, Shimoga, Hassan and Chickmagalur, and the hills of Kodagu (Coorg). Northern and Southern foot hills of Nilgiris, Madurai, Salem and Tirunelveli, Anamalai and parts of Coimbatore districts are the cultivation centres in Tamil Nadu (Prasath *et al*., 2004).

2.1.4 Uses of *E. cardamomum*

From ancient times itself, cardamom has been an indispensable part of kitchen dishes due to its delicious flavour and aroma. The characteristic aroma and flavour are due to the components like α-terpinyl acetate (20 – 53 %), 1, 8-cineole (20 – 60 %), and linalool (8 %), present in the cardamom essential oil (CEO). Indian cardamom is rich in carbohydrate (68.2 %) and protein (10.6 %) (Ashokkumar *et al*., 2020), favoured it to be used in meat dishes and savoury rice dishes like *pulaos, biriyani etc*. It's also an important part of 'garammasala', a blend of various spices.

A variety of cardamom flavoured products are also available in Indian markets including biscuits, tea powder, confectionaries, baked food items *etc*. With the introduction of sophisticated techniques in phytochemistry, cosmetic and perfumery companies have begun to produce extract based perfumes and cosmetics which has already conquered global market. Now a days, instead of chemical additives, spices are used as sources of natural colours, flavours, antimicrobials and antioxidants which are truly safe for the food industry.

Apart from this, cardamom finds application in the indigenous systems of medicine. Evidences suggests that it has been used since 4th century BC by Indian, Greek and Roman doctors to treat bronchitis, asthma, constipation, cough, cold, teeth and gum infections, digestive and kidney disorders (Ashokkumar *et al*., 2020), cataracts, nausea, diarrhoea and cardiac disorders (Khan *et al*., 2011). In Tibetan tradition, it is merged with cinnamom and pepper to treat obesity and glycaemic imbalance. In Chinese

medicinal system, cardamom powdered drink is used as antidote for snake and scorpion venom (Ashokkumar *et al*., 2020).

The phytochemicals present in capsules are actually responsible for its biological properties including antioxidant, antidiabetic, antifungal, anticancer, anti-inflammatory, antiviral and gastro-protective activities (Al-Zuhair *et al*., 1996; Verma *et al*., 2009; Khan *et al*., 2011; Winarsi *et al*., 2014). Mishra (1991) shown that antimicrobial activities of cardamom extract is mainly due to terpenoids. In 2014, Ashokkumar *et al*. (2020) reviewed that terpenoids, flavonoids, anthocyanins, alkaloids and phenolics of capsules are responsible for controlling cardiovascular, pulmonary, kidney and lung associated disorders.

2.1.5 Complexities of adverse climate in small cardamom production

During the past few decades, we had witnessed a tremendous change in our seasons accompanied by irregularity in rainfall as well as extreme temperature. Meteorological reports showed that global temperatures have a hike of approximately 0.4 °C since 1980, with even larger hike observed at Indian Cardamom Hills (ICH). Cardamom being climate sensitive, got easily affected by these changing environmental scenario.

Air temperature, relative humidity and rainfall are the three major climatic elements that directly influence the growth as well as the productivity of these surface feeders. As these monocots are highly temperature sensitive and shade loving, they cannot tolerate a temperature of above 32 °C (Murugan *et al*., 2013). At this temperature, plant withers completely. Early maturation of capsules is also the immediate result of these torrid hotness.

Sweltering heat during the blossoming season (April – May period) causes significant reduction in pollination due to natural abortion of cardamom flowers. Excessive rainfall and extreme drought indirectly affect cardamom by the introduction of minor pests and pathogens. According to Murugan *et al*. (2013), water scarcity and scorching heat are the strongest climatic factors that limit cardamom potential.

According to ICAR-IISR reports, the production loss in Kerala during 2018 – 19 was estimated as 6600 tonnes valued at 6795 million INR. Rough market estimates shows

that the production may fall by 7000 – 8000 tonnes by 2019 – 20 (June – July), due to the shortage of North-East monsoon and deficient summer showers happened during past year.

2.2 HEAT SHOCK PROTEINS (HSPs)

Abiotic stress usually limits plant productivity by inhibiting normal plant metabolism. In order to maintain cellular homeostasis, plants need certain 'caretakers' called molecular chaperones. One such extensively studied molecular chaperone is heat shock proteins (Hsps). They were for the first time discovered from the model organism *Drosophila melanogaster* during heat shock, hence the name 'heat shock proteins'. These are ubiquitous and are observed in all kingdoms of life. As they are chaperones, most of them are found to be expressed constitutively during different stages of plant development (Vierling, 1991; Wang *et al*., 2004).

At molecular level, the most observable response to heat stress is the induction of these sensor proteins. According to Sun *et al*. (2002), plants produce heat inducible Hsps only when there is a temperature shift of 10 to 15 °C from normal. Sachs *et al*. (1986) mentioned that expression of Hsps can be detected within few minutes after heat shock and last only for a few hours regardless of the continuous heat stress. Other than heat stress, these molecules are also expressed under osmotic stress, high salinity, metal toxicity, high concentration of ABA, drought stress, ethanol, heavy metals, amino acid analogues, glucose starvation, calcium ionophores *etc*. (Vierling, 1991; Wang *et al*., 2004). The induction of Hsps have been deeply studied in plants such as pea (Otto *et al*., 1998), wheat (Basha *et al*., 2004), maize (Klein *et al*., 2014), rice (Zou *et al*., 2009) and tomato (Banzet *et al*., 1998) in response to various stresses. During all these adversities, Hsps functions as chaperones by preventing aggregation of denatured proteins and thereby assists in their refolding. In addition to these, they also take part in translocation of proteins, degradation of unstable proteins *via* lysosomal or proteasome degradation pathways and protein assembly.

2.2.1 Classes of Hsps

Based on molecular weight, Hsps are classified as Hsp 100/Clp, Hsp 90, Hsp 70/Dna K, Hsp 60 (chaperonins) and Small Heat Shock Proteins (sHsps).

2.2.1.1 Hsp 100/Clp family

Hsp 100 is a constitutively expressing chaperone belonging to AAA ATPase family (Wang *et al*., 2004). These are hexameric rings which plays huge role in degradation of misfolded proteins formed during stress. Their main function is to solubilize misfolded protein aggregate produced during stress and pass it to Hsp 70 for refolding.

2.2.1.2 Hsp 90

Hsp 90 family include heat shock proteins of molecular weight ranging between 80 to 94 kDa (Vierling, 1991). They are distributed over cytoplasm as well as endoplasmic reticulum (ER). In addition to its major role as a chaperone, they are also involved in protein degradation, cell cycle control and protein trafficking. Evidences showed that these ATP dependent chaperones work in cooperation with Hsp 70 as well as a band of proteins including Hsp Interacting Proteins (HIP), Hsp 70 organizing proteins (HOP), protein 23 (p23) and Hsp 40. Evidences indicated that these housekeeping genes gets upregulated during various stress situations (Wang *et al*., 2004).

2.2.1.3 Hsp 70/ DnaK

Hsp 70 is an ATP dependent chaperone which is produced by both eukaryotes as well as prokaryotes. Prokaryotic homolog is known as DnaK. Hsp 70 family consists of four members: Grp 78, Mtp 70, Hsp 70 and Hsc 70. Among these, Hsc70 is constitutively expressing molecule involved in polypeptide folding as well as its translocation. The structure of Hsp 70 can be dissected as a highly conserved amino terminal ATPase domain of 44 kDa and a carboxy terminal substrate binding domain of approximate size 25 kDa. The major functions of Hsp70 include preventing the aggregation of non-native proteins, assists in refolding, protein import, translocation and signal transduction.

2.2.1.4 Hsp 60/GroEL/ chaperonin family

The first Hsp to be termed as ‘molecular chaperone’ is Hsp 60. It is evolutionarily homologous to prokaryotic counterpart GroEL. Hsp 60 is an ATP dependent chaperone which is further classified on the basis of their subcellular localization. Group I is present in both bacteria and eukaryotic chloroplasts as well as mitochondria. The major

examples are Chaperonin 60 (Cpn 60) and Chaperonin 10 (Cpn 10). Group I chaperonins usually assume a double ring structure composed of back to back stacked rings whereas Group II are 8 – 9 membered ringed structures present in archaea as well as in cytosol of eukaryotes.

2.3 SMALL HEAT SHOCK PROTEIN (sHsps)

Small heat shock proteins (sHsps) are low molecular weight (LMW) Hsps with a size range of 15 kDa to 42 kDa. When compared with other classes of Hsps, sHsps are highly diverse with respect to its sequence, cellular location and functions (Water *et al*., 1996). Due to their high diversification, they are found to be expressed during various stresses such as heat stress (Chen *et al*., 2014), osmotic stress (Sun *et al*., 2001), salinity stress (Wang *et al*., 2015), cold stress (Wang *et al*., 2015) drought stress (Sato and Yokoya, 2008; Wang *et al*., 2015) and UV radiation (Murakami *et al*., 2004) across different plant species. All these reports suggested that sHsps might be associated with general abiotic stress responses in plants. Small Hsps are also found to be expressed in plants during certain stages of development such as pollen development, (Dietrich *et al*., 1991) embryo development (Löw *et al*., 2000), germination (Wehmeyer *et al*., 1996) and fruit maturation (Löw *et al*., 2000).

During stress situations, cellular homeostasis will be highly disrupted, subsequently sHsps are synthesized to protect cellular proteins from further degradation. The major function of small heat shock proteins is to acts as ‘holdases’ during stress by preventing the aggregation of denatured proteins. These universal proteins are synthesized by both eukaryotes and prokaryotes.

Two models are widely followed concerning the chaperone activity of heat inducible sHsps.

a) In normal conditions, sHsps exist in plant cells as large oligomers. After heat shock, they disassemble into smaller oligomers by exposing their substrate binding sites. To these sites, misfolded proteins are held for refolding with the help of ATP dependent chaperones.

b) Under normal conditions, small Hsps remain as inactive oligomers. Due to heat shock, certain conformational changes occur and low affinity state sHsps monomers are converted to high affinity state. Thus, oligomer binds to denatured proteins and form sHsp-denatured protein complex. Later by the action of ATP, denatured proteins are passed to Hsp 70/60 for further folding.

2.3.1 Structure of sHsps

All sHsps share a common primary structure composed of a flexible N terminal region (NTR), a highly conserved 90 to 100 amino acids Alpha Crystallin Domain (ACD) (Jong *et al*., 1993), and a small C terminal region (CTR). In 1982, for the first time Ingolia and Craig reported the presence of α-crystallin domain in *Drosophila* sHsp, a related domain from vertebrate lens protein 'α-crystallin'. ACD is regarded as the signature motif of sHsps which differentiates it from other heat induced proteins. Studies on secondary structures of ACD domain revealed that it has a compact β sandwich structure composed of 3 or 4 β strands (Haslbeck *et al*., 2019).

Quaternary structure is just the assembly of identical sHsp monomers to form a homo oligomer of size up to 350 kDa (Sun *et al*., 2002) and this is due to the distinct properties of C and N terminals (Leroux *et al*., 1997). While studying truncated sHsps, Nakamoto *et al*. (2007) noticed the role of both ACD, C and N terminal in oligomerization. Delbecq and Klevit (2013) during his studies also came to the same conclusion. The oligomerization of sHsp is actually due to weak and dynamic inter subunit contact involving ACD, N and C terminal regions involving strand exchange, helix swapping and loose knots (Sun *et al*., 2002).

Eventhough a large number of sHsp genes have been isolated till now, there is lack of availability of complete structure of plant sHsps due to unavailability of full length cDNA sHsp clones (Haslbeck *et al*., 2019). The most extensively studied is Wheat Hsp 16.9, which is a dodecamer double disc arranged in a trimer of dimer (Van Montforth *et al*., 2001).

2.3.2 Classes of sHsps

Small heat shock proteins are usually encoded by multiple genes devoid of introns (Jong *et al.*, 1993). So far in plants, a total of 12 sHsp gene subfamilies have been identified based on sequence similarity and subcellular localization (Klein *et al*., 2014). Out of these twelve, first seven members are localized on nucleus/cytoplasm designated as Class I (CI) to Class VII (CVII). The rest of them are distributed over cellular organelles such as mitochondria, endoplasmic reticulum (ER), plastid and peroxisomes. The mitochondrial sHsps are named as MI, MII; endoplasmic reticulum targeted as ER, plastid localized as P and finally peroxisomes targeted as Po (Siddhique *et al*., 2008).

2.3.3 Role of sHsps in thermotolerance

Plants usually live in a constantly changing environment. So¸ they have to face a lot of environmental challenges including fluctuating temperature, water scarcity, extreme salinity *etc*. During the past few decades, rise in temperature as part of global warming has created a huge concern. High temperatures can cause devastating damage including disturbance of internal homeostasis, reducing plant growth, development and further leading to plant death. At molecular level, the consequences were evident with repression of synthesis of most of the ordinary proteins involved in plant metabolism, denaturation of enzymes and transient inhibition of untranslated mRNAs.

Normally, plants survive lethal high temperatures by easily acquiring thermotolerance. Substantial works have conveyed that this acquired thermotolerance is due to the accumulation of small heat shock proteins. Sun *et al.* (2002) reviewed that heat shock response is a sustained means of cells to fight against torrid hotness by temporarily reprogramming cellular activities and simultaneous synthesis of sHsps. Using etiolated soybean seedlings, Lin *et al*. (1984) demonstrated that upregulation of LMW Hsps are directly correlated with increased thermotolerance. The protective role of sHsps is a direct indication of their ability to assist in protein damage. The expression of one or more sHsps are sufficient to protect cell from lethal environmental challenges (Morimoto¸ 1998).

Efeoğlu (2009) reviewed that mild heat stress only produces certain mild level sHsps whereas prolonged heat shock demands more and more sHsps and related stressor

proteins. He also deduced that the rate of temperature change as well as duration and intensity of heat determine the extent of accumulation of sHsps. When heat shock persists for a long time it results in the formation of complexes of denatured proteins and sHsps called heat shock granules (HSGs). The MW of HSGs account for about 200 to 400 kDa. Nover *et al*. (1989) reported that the HSGs produced by heat shock applied tomato cell cultures are composed of translation ceased mRNAs, sHsps and ATP dependent chaperones. Finally¸ he claimed that this association is conserving all untranslated proteins during the stress and helps in easy recovery.

Sacchs *et al*. (1986) demonstrated a rise in 2.5 °C per hour is enough for inducing sHsps within 20 min. According to them¸ some might be induced within short period of 3 to 5 min also. Cooper and Ho (1983) reported that sHsps were synthesized up to 4 hrs in maize roots. Later after 4 hrs, production of sHsps declined with the increase in synthesis of additional HMW heat shock proteins indicated the role of large Hsps in folding. Sun *et al*. (2002) reviewed that sHsps have a half-life of 30 to 50 hr after heat stress has been removed. This long half-life indicates the role of sHsps in stress recovery.

In 1988¸ Arrigo *et al*. showed that during heat stress, cytoplasmic sHsps redistributes from cytoplasm to perinuclear region and regained its original position after the heat stress. Lauzon *et al*. (1990) has isolated a sHsp named PsHsp l7.7 from *Pisum sativum* during heat shock treatment at 39 °C for 2 hrs. Using bioinformatics tools they also showed that isolated 17.7 kDa protein is 78.3 % identical to Hsp l7.9 of *Glycine max*. Dietrich *et al*. (1991) characterized a 17.9 kDa sHsp gene from maize seedlings that showed a 50-fold up-regulation during heat stress.

Lee *et al*. (1997) demonstrated that sHsp 18.1 from *Pisum sativum* is involved in combating heat stress by preventing the aggregation of important enzymes citrate synthase (CS), malate dehydrogenase (MDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Murakami *et al*. (2004) reported that the overexpression of sHsp 17.7 induced thermotolerance in rice. He further demonstrated that sHsp 17.7 also evoked UV-B resistance of rice seedlings in parallel with the period of heat treatment. Xue *et al*.

(2010) showed that over expression of heat shock protein gene Hsp 26 in *Arabidopsis thaliana* enhances heat tolerance.

Chen *et al*. (2014) performed heat shock study in rice revealing the role of five sHsps (Hsp 26.7, Hsp 23.2, Hsp 17.9A, Hsp 17.4 and Hsp 16.9A) in thermotolerance. They finally concluded that the above five sHsps can be used as biomarkers for screening rice cultivars with varied thermotolerance.

Wang *et al*. (2015) isolated and characterized a small heat-shock-protein gene Hsp 18.6 from rice and communicated its role in diverse stresses such as drought, salt, heat and cold.

Huang *et al*. (2018) identified the expression signature of CaHsp 16.4 in thermo-tolerant and thermo-sensitive pepper (*Capsicum annuum* L.) lines under both heat and drought stress. During heat stress the expression level of CaHsp 16.4 in thermotolerant line was higher than the thermo-sensitive line. Whereas under drought stress the expression pattern was highly dynamic. Cellular localization studies showed that CaHsp 16.4 localizes in cytoplasm and nucleus.

Yang *et al*. (2020) isolated a class II sHsp called LimHsp16.45 from *Lilium davidii* and has shown that it is similar to 17.6 II sHsp of *Arabidopsis thaliana* in structure and function. They also suggested the role of LimHsp16.45 in stomatal regulation due to its overexpression in stomatal guard cells than other epidermal cells. In addition to this they also demonstrated the involvement of LimHsp16.45 in heat stress¸ salinity and osmotic stress. By comparing the functions of 17.6 II sHsp of *Arabidopsis thaliana* and LimHsp 16.45 of *Lilium davidii*, they came to the conclusion that better thermotolerance is provided by LimHsp 16.45.

2.4 RNA ISOLATION

High quality RNA is an important prerequisite for cDNA library construction¸ gene expression studies¸ Reverse transcription quantitative PCR (RT-qPCR), transcriptome analysis using next generation sequencing (NGS), Rapid Amplification of cDNA ends (RACE), array analysis, northern blot analysis and gene isolation. However¸ obtaining good quality RNA is arduous task because it is an easy prey for RNases. Thus the first

and foremost thing is to avoid the traces of ubiquitous RNases from contaminating the surroundings and consumables used at work site. So, whatever be the method for isolation, a few precautions have to be taken to avoid the contamination with RNases (Sambrooke *et al*., 1989; Sharma *et al*., 2004; Nadiya *et al*., 2015). They are:

- Collect the samples for RNA isolation *via* flash freeze method.
- Solutions for RNA isolation were prepared using 0.01 % DEPC (Diethylpyrocarbonate) water or RNase free water and incubated overnight at RT. The treated solutions were autoclaved for 20 min to degrade the traces of DEPC.
- All the glass wares used for RNA isolation must be cleaned properly and autoclaved.
- Pipette tips must be immersed in DEPC water overnight and autoclave twice before use to remove the remains of DEPC.
- Mortar pestle and spatula were also treated with DEPC, incubated overnight, wrapped in aluminium foil and baked at 180 °C for 5 hrs.
- The workbench for RNA isolation, all plastic wares and centrifuge were wiped twice with RNaseZap (Ambion) to remove RNase present on the surface. Frequent swabbing of gloves with RNaseZap solution avoids RNase contamination.
- Electrophoresis gel tank, gel comb and gel tray were soaked in a buffer (0.1 M NaOH and 100 mM EDTA) for 2 hrs and rinsed well with DEPC treated water.

So far there is no universally accepted standard protocol for RNA isolation across plant species. From the protocols available till now, single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform extraction developed by Sacchi and Chomcynzki (1987) is the most accepted one. Depending up on the constituents and nature of plant sample used, people modified the conventional Trizol method to obtain high integrity RNA.

Small cardamom tissues are marked by a large number of polyphenols and polysaccharides which directly affects the quality of RNA isolated and interferes with further downstream processes. Nadiya *et al*. (2015) attempted a comparative study on

different methods for isolating RNA from various small cardamom tissues and have found that RNeasy Kits combined with CTAB method yielded RNA with good purity and higher yield than either using conventional methods or kit methods individually. The RNA thus obtained is highly amenable for transcriptome and small RNA analysis.

2.5 REVERSE TRANSCRIPTION PCR (RT-PCR)

RNA cannot serve as a template for PCR reactions. So some steps has to be done prior to PCR for converting RNA to complementary DNA (cDNA). This is done using a specific RNA dependent DNA polymerase enzyme called Reverse Transcriptase. Usually total RNA or Poly A^+ RNA is used as a template and primed with either target specific primers or hexamers and oligo dT (Pathak and Rastogi¸ 2009). Primer selection is mainly based on our intended purpose.

Once the cDNA is synthesized it is used for normal PCR reactions by addressing reverse transcription specific factors. Two most commonly used RT enzymes are Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). MMLV-RT is preferred for longer transcripts as it displays reduced RNAse H activity (DeStefano *et al*., 1991) with an add on proofreading 3'- 5' exonuclease activity, whereas AMV-RT become a choice when our transcript is having complex secondary structure (Merck, 2020).

2.6 PRIMER DESIGNING

PCR is a revolutionary scientific technique used widely for the *in vitro* amplification of DNA. The essential requirements for running a PCR reaction includes template DNA¸ primers¸ Taq polymerase¸ PCR buffer¸ dNTPs and an instrument called thermocycler. Eventhough the steps and stages of a PCR reaction are relatively simple there are a lot of complexities associated with it. One such is specificity of a PCR reaction.

For a successful and specific PCR reaction¸ good quality primers are essential. During primer designing¸ it is imperative to strictly adhere on the criteria for designing primers. Here are some important design considerations described by Etebu (2013) aimed to obtain specific amplification with high yield.

- Primer length is critical and must lie between 18 to 30 bp.

- Ensure that Tm of both reverse and forward primers are always same. According to Dieffenbach *et al*. (1993) Tm of both primers must be higher than 54 °C. Suggs and co-workers (1981) suggested an easy method to compute the oligonucleotide Tm using the equation 2 °C (A+T) + 4 °C (G+C).
- Primer dimers (both self and cross dimers) must be avoided.
- Primers should not be complementary at 3' ends and possibility of forming secondary structures (hairpins) must be avoided.
- Primer sequences should not have more than 4 GC residues.
- At 3' end, one GC clamp is enough to ensure specificity.

Designing primers manually is a laborious task thus tending us to use various computational tools which are user friendly. Each of the platforms have their own design guidelines. Primers can be designed either from known sequences already deposited on nucleotide sequence databases or from unknown sequences. In case of known sequences primer designing is quite easy as short oligos can be designed from the flanking ends of our target. But for unknown sequences the things are different. Here primer designing is based on conserved sequences (Sibhatu, 2003). The conserved sequences are identified using multiple sequence alignment programme such as MEGAX, CLUSTAL *etc*. Based on these conserved sequences, primers are generated using primer designing softwares like IDT, primer 3 plus *etc*. After primer designing, Etebu (2013) recommended BLAST analysis to confirm specificity of primers.

With the advent of NGS method transcriptome sequencing has become easier and unremarkable. But still we lack proper primer designing platforms suitable for transcriptome data. Recently in 2015, Mořkovský *et al.* presented a new pipeline called Scrimer that automates preliminary steps such as adaptor removal, read mapping *etc*. and in turn generate primers.

2.7 GENE EXPRESSION ANALYSIS

Gene expression studies are aimed at detecting the presence as well as quantity of functional gene product(s) at transcriptional or translational level. It is a sensitive touchstone of biological activity wherein a variation in gene expression signature can

be easily identified. This difference in signature pattern reflects a change in biological process. Gene expression profiling goes beyond the static information of the genome sequence into a dynamic functional view of an organism's biology. Hence, is a widely used approach in research, clinical and pharmaceutical settings to better understand individual genes, gene pathways or greater gene activity profiles (https://www.bioline.com/workflows/gene-expression-analysis). Several techniques and protocols have been developed for this purpose. But all these follows a common procedure starting with RNA/protein isolation succeeded by amplification¸ quantitation-detection methods. Northern blotting¸ Microarray analysis¸ Serial analysis of gene expression (SAGE), qRT- PCR, *In situ* hybridization (ISH), Next Generation Sequencing are some among them.

The very first such study was started by Alwine *et al*. in 1977 with the introduction of Northern blotting which is used for single transcript detection. Later for large scale gene expression analysis microarray has been introduced. They became popular among scientific community due to its ability to compare same set of genes between test sample and reference sample. In this way differential expression of thousands of genes of two populations can be calculated. During the past few decades¸ gene expression analysis using next generation sequencing (NGS) technology-RNA-Seq, and RT-PCR is gaining ground as it enables the measurement and comparison of millions of random position reads that are subsequently mapped and aligned to each gene, providing a measure of gene expression at an unprecedented level of detail (https://www.bioline.com/workflows/gene-expression-analysis).

2.7.1 Real -Time PCR

The introduction of Real-Time PCR (qPCR) has dramatically changed the outlook of gene expression studies. Speed, simplicity, convenience, tremendous sensitivity, specificity, robustness and high performance are some of the attributes that makes qPCR different from normal PCR.

It became popular due to its potential to collect the data on real time basis throughout the process thus allowing the researcher to avoid the gel preparation step at the end.

The instrument uses either fluorescent dyes or probes for quantification where emitted fluorescence is measured to calculate the product concentration.

SYBR GREEN, a fluorescent dye binds non-specifically to dsDNA and produce fluorescence that is directly correlated with the amount of DNA present in the sample. This is highly flexible as the same dye can be used for different assays. At the same time multiplexing will result in false positives due to non-specificity. Here accurate results are ensured using Melt curve analysis. On the other hand, fluorescence emitting probes like Taqman probe, FRET probes, molecular beacons and Scorpions probes are highly specific and binds in a sequence specific manner (Pathak and Rastogi, 2009).

2.7.2 Differential gene expression studies using Real-Time PCR

Gene quantification using Real-Time PCR can be done in two ways: Absolute quantification and relative quantification. Absolute quantification gives us a direct picture of initial number of copies of our target sequence present whereas relative quantification analysis displays a change in gene expression in a given sample relative to reference sample. When compared to absolute quantification method, relative quantification doesn't requires any standard curve for analysis (Pfaffl, 2001).

Recent studies are mostly aimed towards relative quantification using qPCR as it's an adequate tool to investigate small changes in gene expression. It is used in comparative gene expression studies between normal and diseased sample, mutant versus wild-type sample, before and after treatment, time course during development or across treatment regime, spatial variation within organs, tissues or other sample types *etc*. (https://www.bioline.com/workflows/gene-expression-analysis).

While studying differential gene expression, difference in expression were calculated based on a standard or reference known as calibrator (Livak and Schmittgen,2001). Selection of appropriate reference genes are important because it directly affects the quality of Real-Time data (Qu *et al.*, 2019). There are various mathematical methods available for computing the relative quantification. Some of them are Comparative Cq method, LinRegPCR, Liu and Saint method, qBase software and Pfaffl method. Depending on the methods used, they can yield slight variations in results and thus discrepant measures of standard error (Wong and Medrano, 2005). Regier and Frey

(2010) made an experimental comparison of relative qPCR quantification approaches for gene expression studies in poplar and came to the conclusion similar to Wong and Medrano (2005).

A widely used method to measure relative gene expression is the Comparative Ct method also known as $2^{-\Delta\Delta CT}$ method or Livak method (Livak and Schmittgen¸ 2001). In this method, the Ct values obtained from two different experimental RNA samples are directly normalized to a housekeeping gene (reference gene) and then compared.

The below given are some studies which conveyed the use of qPCR in heat stress related relative gene expression studies:

Zhang *et al*. (2005) conducted a differential gene expression study in Festuca (thermotolerant and thermosensitive lines) under heat stress conditions using SSH and qRT-PCR. From the differentially expressed ESTs identified by SSH method¸ the expression patterns of 17 randomly selected differentially expressed genes with known functions were successfully verified using qRT-PCR. Similar type of study has also been conducted by Pavli *et al*. (2011) to reveal the mode of expression of Hsp 90 genes during heat stress in different sorghum genotypes. The study revealed that the peak expression of Hsp 90 was observed upon exposure to heat at either for 60 min or 30 min and this variation in peak time is due to genotypic variation.

Reddy *et al*. (2015) has cloned genes belongs to cytosolic Class I (CI) sHsp gene family in *Pennisetum glaucum* and studied its expression under various abiotic stresses such as cold¸ drought¸ heat and salinity. The results revealed that CI sHsps have relatively faster response kinetics than the Hsps of other species. All the sHsps he studied showed a common pattern of expression during heat stress consisting of a peak induction period followed by a declining trend. This observation was in line with the findings of Woods *et al*. (1998) and Pavli *et al*. (2011).

Song *et al*. (2017) reported 68 differentially expressed genes in soybean under heat stress through transcriptome sequencing. Later by using qPCR they successfully validated the expression of selected ESTs including HMW Hsps (Hsp 90 and 70) and Hsp 20.

Vishwakarma *et al*. (2018) studied the differentially expressed genes in Indian wheat under heat stress by combining techniques such as Northern Hybridization, qRT-PCR and SSH. He suggested that ESTs that showed a clearly defined coupling of plant stress tolerance under heat stress can be further used as HS tolerance linked markers.

3. MATERIALS AND METHODS

The study entitled "Isolation and characterization of Hsp family gene from cardamom (*Elettaria cardamomum* Maton)" was conducted at the Biotechnology and Bioinformatics Division, KSCSTE - Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, during 2019 – 2020. In this chapter, details regarding the experimental materials used and methodology adopted for the study are presented.

3.1 DIFFERENTIAL EXPRESSION OF SHSPS UNDER HEAT STRESS USING REAL-TIME PCR

3.1.1 Maintenance of cardamom plants

Wild cardamom plants (at six month old stage) collected from Edamalayar, Ernakulam and maintained at Cardamom Conservatory of JNTBGRI, were used for the study. Potted cardamom plants (triplicates) were maintained inside the growth chamber for two weeks at a temperature of 22 °C, relative humidity 80 % and 12 hr photoperiod. Once in three days, the plants were well watered.

Figure 3.1 Cardamom plants maintained inside the growth chamber

3.1.2 Sample collection

For total RNA isolation, 100 mg leaf tissue pooled from three biological replicates were used. The plants were subjected to heat shock at 40 °C by raising the temperature of the growth chamber at the rate of 0.9 °C/min from normal. The heat shock temperature was maintained for 3 hr and samples were collected at different time intervals *viz*. 30 min, 1 hr, 2 hr and 3 hr. Leaf tissues thus harvested were immediately wrapped in aluminium foil, labelled and frozen in liquid nitrogen. Leaf tissues collected before heat treatment were taken as control.

3.1.3 RNA isolation

RNases are the biggest foes during RNA isolation. They are highly reactive molecules which are cosmopolitan in nature, showing their presence even in our fingertips. So, RNA isolation has to be done carefully by taking certain precautions from the very first step of sample collection till the end. Work benches and instruments were made RNase free by wiping it with RNaseZap (Ambion, USA). Gloves were intermittently wiped with spirit to avoid contamination. Diethylpyrocarbonate (DEPC) (Sigma, USA), a strong RNase inhibitor was used for removing the RNases from glass wares and solutions. 0.1 % DEPC was prepared by adding 1 ml of DEPC in 1000 ml of autoclaved distilled water and kept overnight in Tarsons digital spinot (Tarsons Products Pvt. Ltd., India) for dissolving completely. Accessories such as mortar and pestle, spatula, scissors and forceps were made RNase free by treating it with 0.1 % DEPC for 24 hr at dark. Later, these were sterilized by baking at 180 °C for 5 hr in a hot air oven (NSW India Limited, India), after wrapping in aluminium foil. DEPC water was double autoclaved to remove the traces of DEPC, as it has the ability to inhibit the activity of RNA. All the reagents for RNA isolation were also prepared using autoclaved DEPC treated water. New autoclaved pipette tips as well as tubes were used. Gel apparatus was soaked in a soaking buffer (Appendix II) for 2 hr and rinsed well with DEPC treated water. TAE buffer used for (Appendix II) agarose gel as well as tank buffer preparation were made in DEPC treated water. All the pipettes and chemicals used in RNA isolation were reserved for RNA works and separated from the rest.

RNA isolation was performed using modified CTAB method combined with RNeasy Plant Mini Kit method (Nadiya *et al*., 2015). The protocol followed was briefly discussed below:

- 1 ml extraction buffer (Appendix I) and 20 µl β-mercaptoethanol were pre-warmed at 65 °C on ThermoStat plus (Eppendorf, Germany).
- The 100 mg sample was ground using liquid nitrogen and transferred the powdered material into a labelled 2 ml microfuge tube.
- The pre-warmed extraction buffer was added to the above tubes and vortexed for about 1 min to mix the contents thoroughly.

- After vortexing, the tubes were incubated on the thermostat at 65 °C for 15 min. Vortex the tubes 6 – 8 times during incubation at 3 – 4 min intervals.
- The tubes were refrigerated for 2 min. 640 µl of chloroform: isoamyl alcohol (24:1) was added and vortexed vigorously for 1 – 2 min.
- The Z 216 MK cold centrifuge (HERMLE, Germany) was precooled and the samples were spun at 13,000 rpm for 15 min at 4 °C. After centrifugation, the upper aqueous layer was immediately transferred to a fresh tube.
- To the above aqueous supernatant, 720 µl 8 M Lithium Chloride was added and mixed thoroughly.
- Tubes were stored at ⁻80 °C for 1 hr.
- After 1 hr, the samples were spun at 16,000 rpm for 15 min at 4 °C and poured off the supernatant.
- 700 µl qiazol lysing reagent was added to the tube containing the pellet and pipetted gently to allow the pellet to dissolve.
- After vortexing, the tubes were kept on the bench top at room temperature for 5 min.
- 140 µl chloroform was added to the tube containing the homogenate and shaken vigorously for 15 s.
- The tubes were again kept at room temperature for 2 – 3 min.
- The samples were centrifuged at 12000 rcf for 15 min at 4 °C.
- The aqueous layer was transferred to a new collection tube and 1.5 volume of 100 % ethanol was added and mixed thoroughly by pipetting several times.
- 650 µl of sample was transferred to RNeasy mini spin column in a 2 ml collection tube.
- The samples were centrifuged at 10,000 rpm (Eppendorf, Germany) and discarded the flowthrough.

- Repeated the above two steps with the remaining sample.
- 700 µl RW 1 buffer was added to the same column and centrifuged at 10,000 rpm for 15 s. The flowthrough was discarded.
- A 500 µl RPE buffer was added to the RNeasy spin column and centrifuged at 10,000 rpm for 15 s. The flowthrough was discarded.
- The RNeasy spin column was transferred to a new 2 ml collection tube and centrifuged at full speed for 1 min to dry the membrane.
- The RNeasy spin column was again transferred to a new 1.5 ml eppendorf tube.
- 30 – 50 µl RNase free water was added directly to the spin column membrane to elute the RNA.
- After a while, centrifuged at 10,000 rpm for 1 min.
- Previous two steps were repeated with another 30 – 50 µl RNase free water.

The final product thus obtained was aliquoted and stored at -80 °C.

3.1.3.1 Agarose gel electrophoresis

The integrity and quality of RNA thus isolated were checked using Agarose gel electrophoresis. 3 µl sample was loaded on 1.2 % agarose gel. Electrophoresis was carried out at 70 V using 1 X TAE tank buffer. After half an hour, the gel was analyzed using UVP EC 3 Chemi HR 410 Imaging system (UVP, California) using Geli software.

3.1.3.2 Nanophotometric analysis

The concentration as well as purity (A_{260}/A_{280} and A_{260}/A_{230}) of RNA were determined using N50 NanoPhotometer (IMPLEN, USA).

3.1.4 cDNA synthesis

Since RNA cannot be used as a template for PCR studies, it was converted to cDNA using Takara PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan). First strand cDNA synthesis was performed in accordance with the instructions provided in the kit manual. 40 µl reaction mixtures were prepared and incubated at 37 °C for 15 min. The

inactivation of RT enzyme was performed by incubating the tubes at 85 °C for 5 s. In order to minimize the intervention of RNases, all the steps were carried out in RNase free condition by wearing gloves as well as using new sterile tips and tubes.

Table 3.1 Reaction mix used for cDNA synthesis

Reagent	Volume (µl)
Prime script buffer (5 X)	2
Primescript RT	0.5
Oligo dT primer (50 µM)	0.5
Random hexamer (100 µM)	0.5
RNA (150 ng/µl)	2.5
RNase free water	4
Total volume	10

3.1.5 Primer designing

Transcriptome of cardamom leaf was obtained from NCBI SRA (Accession No's: SRX4183672, SRX4183673). Quality analysis was done using fast QC (Andrews, 2010), a bioinformatics tool for generating statistics from raw data such as total read count, sequence quality score, GC content, adapter content *etc*. After that the adapter sequences were trimmed with BBduk (Bushnell, 2014). The required reference sequences were collected using the keyword 'plantae viridae rRNA' from NCBI and thus filtered out the unwanted sequences with BBmap (Bushnell, 2014). The unmapped sequences were then assembled into contigs using Trinity. This assembled sequence file was used as the raw data for primer designing. In the beginning, small heat shock protein sequences were selected based on the keyword 'small heat shock protein' from the annotated bulk data and sequences were saved as a separate file according to their molecular weight in Notepad ++ in FASTA file format. Later sequences were aligned using the software 'MEGAX' (Kumar *et al*., 2018) with a reference sequence. Reference sequence is the sequence which shows maximum similarity and identity with our desired target sequence, obtained *via* BLAST tool (Altschul *et al*., 1990). After alignment, unwanted 5' as well as 3' flanking end sequences were trimmed out to get

more aligned conserved sequences. Based on these conserved sequences thus obtained, Real-Time primers as well as normal PCR primers were designed online using IDT's Primer quest tool (https://www.idtdna.com). Since all small heat shock proteins are characterized by a conserved α-crystallin domain (ACD), primers were designed excluding this region. For finding the conserved region, NCBI conserved domain search tool (Lu *et al*., 2020) was used.

Primers were designed by following the criteria such as:

- Primer length: 17 to 30 bases
- G:C content: 45 to 60 %
- Primer Tm between 57 and 65 °C
- GC clamp: not more than 2 G or C residues in the 3' most 5 bases
- Avoid secondary structure
- No runs and repeats
- Avoid primer dimers (self and cross dimers)

The efficiency of the newly designed primers were verified by using the online tool 'Net Primer' (https://www.premierbiosoft.com/netprimer). Those primer sets having ratings more than 80 were selected. The selected primers were checked for their specificity using an *in silico* PCR tool called NCBI Primer-BLAST (Ye *et al*., 2012) and omitted primers with non-specific products. Primer sets which passed the quality criteria were sent to EUROFINs Scientific Pvt. Ltd., Bangalore for synthesis.

3.1.5.1 Annealing temperature standardization

Since some of the primer pairs had significant differences in their melting temperature, a gradient PCR was carried out for all primer sets at 54 °C, 56 °C and 58 °C to standardize the annealing temperature. 15 µl reaction mixture was prepared. PCR amplification was performed using Veriti Thermal Cycler PCR (Applied Biosystems, USA). The amplified products thus obtained were resolved using 1.5 % agarose gel along with a suitable ladder. The gel was visualized using UVP EC 3 Chemi HR 410 Imaging system (UVP, California).

Table 3.2 Reaction mix used for Ta standardization

Reagent	Concentration	Volume (µl)
EmeraldAmp GT PCR Master mix	2 X	7.5
Forward primer	5 pM	1
Reverse primer	5 pM	1
cDNA (1:5)	---	1
DNase free water	---	4.5
Total volume	---	15

Table 3.3 Thermal profile used for Ta standardization

	Stage	Repeat	Temperature (°C)	Time (s)
Initial denaturation	1	1	95	300
Denaturation	2	35	95	45
Annealing			54/56/58	30
Primer extension			72	45
Final extension	3	1	72	300

3.1.6 Real-Time PCR analysis

In order to validate the expression of selected small heat shock protein genes under heat stress during different time frames, qRT-PCR analysis was performed using 1 µl of diluted cDNA (1:5) as template. ROX (6-carboxy-X-rhodamine) reference dye was added to the reaction mixture for normalization of the fluorescent signals. Normalization of gene expression was done using Ubiquitin Conjugating Enzyme (UBCE) as reference gene. Each reaction was performed in triplicate with a volume of 20 µl. Along with that, an NTC was set for each primer by replacing the cDNA with 1 µl DNase free water.

Amplification was carried out at 95 °C for 30 s, followed by 40 cycles (95 °C for 15 s, 58 °C for 34 s). Directly after the amplification step, melt curve analysis was performed to monitor primer-template specificity. Experiments were performed in

StepOnePLus™ Real-Time PCR system (Applied Biosystems, USA). After amplification, the relative fold change in gene expression was analyzed using Comparative Ct method (Livak and Schmittgen, 2001). Data analysis was done using MS Excel software 2013.

Table 3.4 Reaction mix used for qPCR reaction

Reagent	Concentration	Volume (µl)
TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus)	2 X	10
ROX	50 X	0.4
Forward primer	5 pM	2
Reverse primer	5 pM	2
cDNA (1:5)	---	1
DNase free water	---	4.6
Total	---	20

3.2 GENE ISOLATION

The sHsp genes studied under differential expression experiment were further isolated using gene specific primers particularly designed for normal PCR (See Table 4.3). For each primer set, PCR reaction was performed in triplicate with a volume of 15 µl each.

Table 3.5 Reaction mix used for gene isolation

Reagent	Concentration	Volume (µl)
Master mix	2 X	7.5
Forward primer	5 pM	1
Reverse primer	5 pM	1
cDNA (1:5)	---	1
DNase free water	---	4.5
Total	---	15

PCR amplification was marked by the initial denaturation at 95 °C for 5 min, followed by 40 cycles (95 °C for 45 s, 58 °C for 35 s, 72 °C for 45 s) and final extension step covering 5 min at 72 °C using Veriti Thermal Cycler PCR (Applied Biosystems, USA). The amplified products were resolved on 1.5 % agarose gel and were analysed using UVP EC 3 Chemi HR 410 Imaging system (UVP, California). The resultant bands were compared using Orion X 100 bp DNA ladder (Origin, India) loaded along with the sample.

3.2.1 Gel elution and PCR clean-up

From the gel, our desired bands were eluted to a final volume of 30 µl using NucleoSpin® Gel and PCR Clean-up Midi Kit (Macherey-Nagel, Germany). The final products were aliquoted and stored at -20 °C. 2 µl of eluted products were resolved on 1.5 % agarose gel to confirm the quality and quantity of products along with Orion X 100 bp ladder (Origin, India). The bands with less intensity were further amplified using 1 µl eluted product as PCR template. PCR reaction was performed in triplicate using Veriti Thermal Cycler (Applied Biosystems, USA). The thermal profile discussed above was the same used here. The amplified products were resolved in 1.5 % agarose gel and were analysed using UVP EC 3 Chemi HR 410 Imaging system (UVP, California). The replicates for each product were pooled and PCR clean-up was performed with NucleoSpin® Gel and PCR Clean-up Midi Kit (Macherey-Nagel, Germany) and eluted out to 30 µl. Finally, 2 µl PCR clean-up product was resolved on 1.5 % agarose gel along with Orion X 100 bp DNA ladder (Origin, India) to confirm the band quality.

3.2.2 Sequencing of purified PCR products

From the final stock of PCR clean-up products, 10 µl of 50 ng samples were labelled and sent for sequencing (outsourced) along with 5 pM gene specific primers (See Table 4.3). Sanger sequencing was performed from 5’ end of the products in 3500 Genetic Analyzer (Applied Biosystems, USA).

3.2.3 Sequence analysis

For the obtained sequences, NCBI BLASTN (Altschul *et al*., 1990) was performed to identify the most similar hit from the nucleotide database. The corresponding amino acid sequences of sequenced products were deduced using ExPASy (Expert Protein Analysis System) Translate tool (Gasteiger *et al*., 2005) and performed UniProt BLAST analysis (UniProt Consortium, 2019). Various physico-chemical properties of peptides were predicted using the ExPASy Protparam tool (Gasteiger *et al*., 2005). NCBI CD search (Lu *et al*., 2020) was performed to map the conserved domain of the obtained amino acid sequences. The secondary structure of translated sequences were predicted using PSIPRED (Jones, 1999) and Phyre^2 (Kelly *et al*., 2015) was used to analyze the percent of confidence of secondary structure predicted.

4. RESULTS

This chapter includes the results of the study entitled "Isolation and characterization of Hsp family gene from cardamom (*Elettaria cardamomum* Maton)" carried out at the Biotechnology and Bioinformatics Division of KSCSTE-JNTBGRI, Palode during 2019 – 2020.

4.1 DIFFERENTIAL EXPRESSION OF SHSPS UNDER HEAT STRESS USING REAL-TIME PCR

4.1.1 RNA isolation

Total RNA was isolated from flash frozen leaf tissues of *E. cardamomum* using modified CTAB method combined with RNeasy Plant Mini Kit method. Samples were eluted out to a final volume of 80 µl and stored at -80 °C.

The isolated RNA was seen as two intact bands (28 S and 18 S) with slight degradation in 1 % AGE (Figure 4.1). No apparent genomic DNA contamination was noted. Concentration as well as purity values of RNA isolated were represented in Table 4.1.

Table 4.1 Yield and purity of isolated RNA

Sl. No.	Sample	Concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
1	C	176.4	2.15	2.17
2	T_1	358.88	2.171	2.004
3	T_2	311.56	2.167	1.844
4	T_3	248.56	2.152	2.381
5	T_4	319.32	2.174	2.432

******T_1, T_2, T_3 and T_4 represents the sample collected at 30 min, 1 hr, 2 hr and 3 hr after heat shock. C - Control, sample collected before heat treatment.***

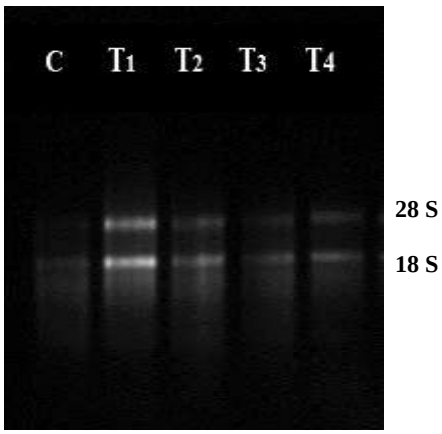


Figure 4.1 Gel image of Total RNA isolated from cardamom leaf tissues. *Lane C represents the RNA isolated from control. Lane T_1, T_2, T_3 and T_4 - RNA isolated from leaf tissues after heat shock (40 °C) at 30 min, 1 hr, 2 hr and 3 hr*

4.1.2 cDNA synthesis

The isolated RNA samples (labelled as C, T_1, T_2, T_3 and T_4) were diluted to a volume of 15 µl with a final concentration of 150 ng/µl. Once again, the concentration was confirmed using Nanophotometer. First strand cDNA synthesis was carried using oligo dT primers and random hexamers in the presence of Primescript RT enzyme (Takara). For each 40 µl cDNA reaction performed, 10 µl of RNA was used. The cDNA synthesized were diluted to a final volume of 50 µl in 1:5 ratio using autoclaved double distilled water.

4.1.3 Primer designing

A total of 14 sets of primers, seven for qPCR studies and remaining seven for gene isolation studies were designed using IDT's Primer quest tool after aligning the transcriptome sequences using MEGAX software.

Figure 4.2 Notepad ++ window showing assembly file of cardamom transcriptome data

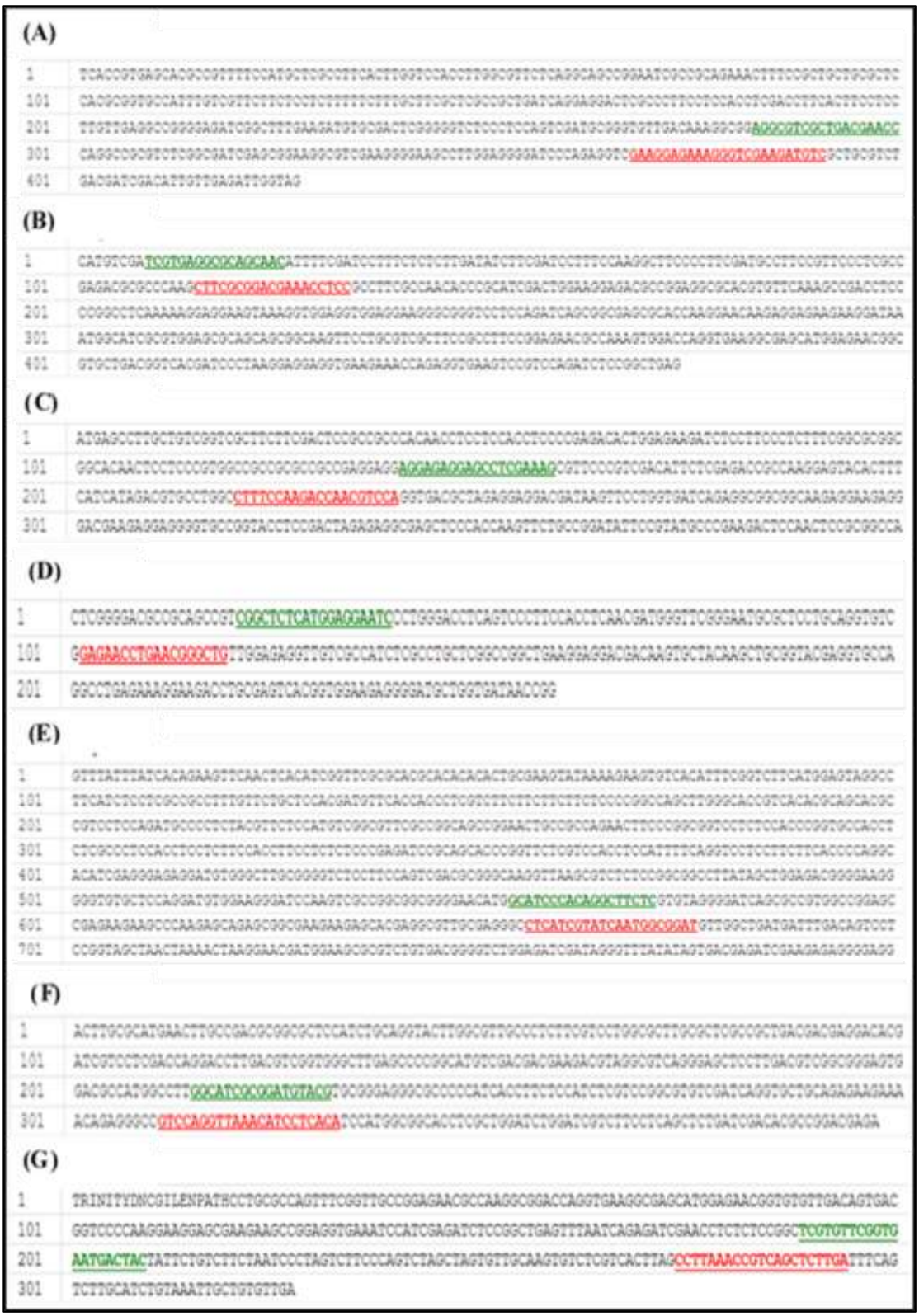


Figure 4.3 IDT window showing position of forward and reverse primers of sHsp genes designed for Real-Time PCR studies: *(A) sHsp 16.9 (B) sHsp 18.1 (C) sHsp 18.6 (D) sHsp 25.6 (E) sHsp 22.7 (F) sHsp 17.9 (G) sHsp 17.8 ***Highlighted green and red oligos represents forward and reverse primers respectively*

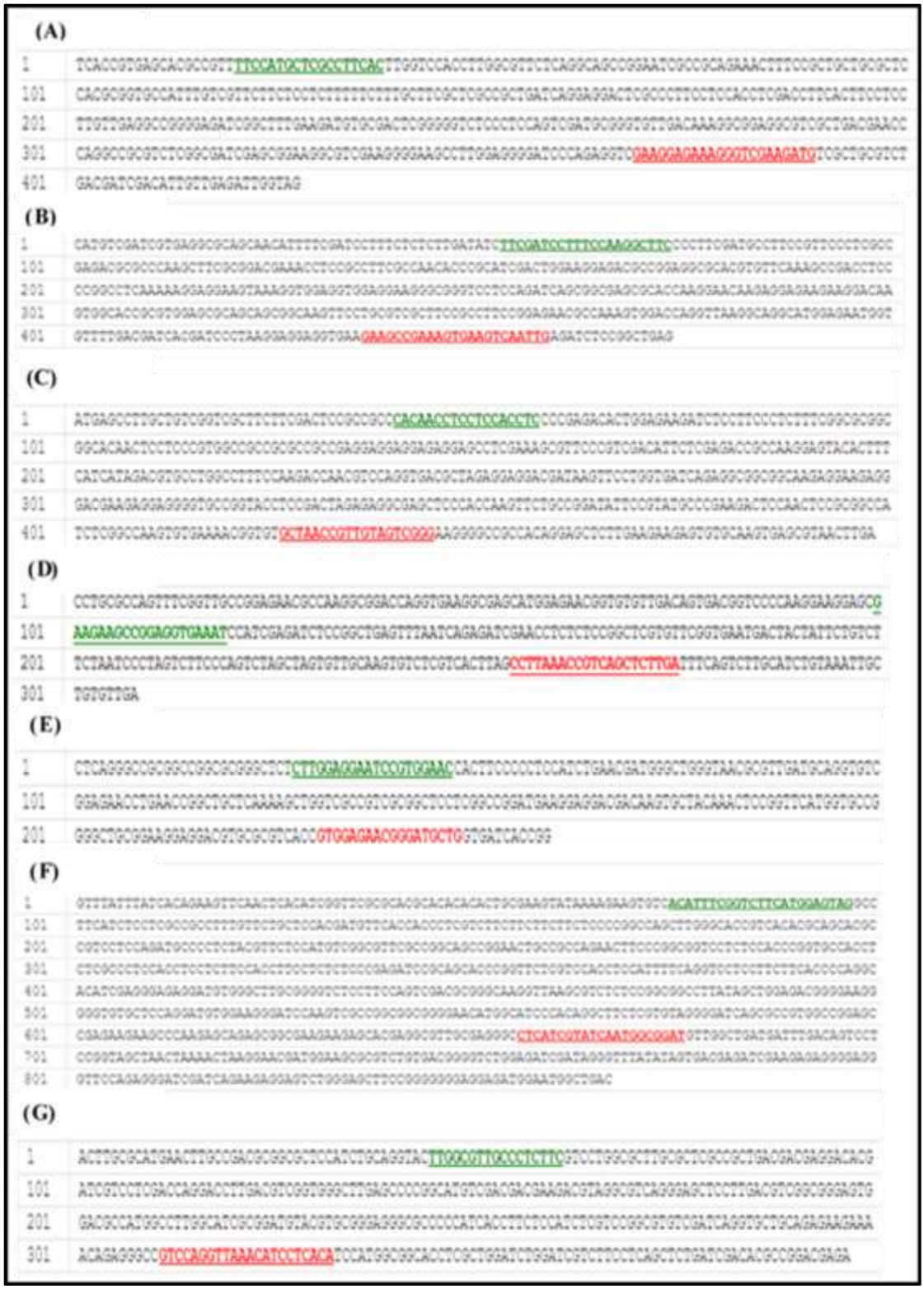


Figure 4.4 IDT window showing position of forward and reverse primers of sHsp genes designed for gene isolation studies: *(A) sHsp 16.9 (B) sHsp 18.1 (C) sHsp 18.6 (D) sHsp 17.8 (E) sHsp 26.5 (F) sHsp 22.7 (G) sHsp 17.9 ***Highlighted green and red oligos represents forward and reverse primer respectively*

Table 4.2 List of primers designed for Real-Time PCR studies

Sl. No.	Primer name	Sequence 5' to 3'	Length (bases)	Tm (°C)	Amplicon length (bases)
1	sHsp 16.9 F	AGGCGTCGCTGACGAAC	17	57.60	109
2	sHsp 16.9 R	GACATCTTCGACCCTTTCTCCTTC	24	62.72	
3	sHsp 18.1 F	TCGTGAGGCGCAGCAAC	17	57.60	125
4	sHsp 18.1 R	GGAGGTTTCGTCCGCGAAG	19	60.99	
5	sHsp 18.6 F	AGGAGAGGAGCCTCGAAAG	19	58.82	100
6	sHsp 18.6 R	TGGACGTTGGTCTTGGAAAG	20	57.30	
7	sHsp 26.5 F	GGGCTCTCTTGGAGGAATC	19	58.82	94
8	sHsp 26.5 R	GGTTCAGGTTCTCCGACAC	19	58.82	
9	sHsp 22.7 F	GCATCCCACAGGCTTCTC	18	58.24	123
10	sHsp 22.7 R	ATCCGCCATTGATACGATGAG	21	60.25	
11	sHsp 17.8 F	TCGTGTTCGGTGAATGACTAC	21	57.87	106
12	sHsp 17.8 R	TCAAGAGCTGACGGTTTAAGG	21	57.87	
13	sHsp 17.9 F	GGCATCGCGGATGTACG	17	57.60	118
14	sHsp 17.9 R	TGTGAGGATGTTTAACCTGGAC	22	58.39	

Table 4.3 List of primers designed for gene isolation studies

Sl. No.	Primer name	Sequence 5' to 3'	Length (bases)	Tm (°C)	Amplicon length (bases)
1	sHsp 16.9 F	TTCCATGCTCGCCTTCAC	18	55.97	370
2	sHsp 16.9 R	CATCTTCGACCCTTTCTCCTTC	22	60.25	
3	sHsp 18.1 F	TTCGATCCTTTCCAAGGCTTC	21	57.87	406
4	sHsp 18.1 R	CAATTGACTTCACTTTCGGCTTC	23	57.87	
5	sHsp 18.6 F	CACAACCTCCTCCACCTC	18	58.24	405
6	sHsp 18.6 R	CCCGACTACAACGGTTAGC	19	58.24	
7	sHsp 26.5 F	CTTGGAGGAATCCGTGGAAC	20	59.35	221
8	sHsp 26.5 R	CAGCATCCCGTTCTCCAC	18	58.24	
9	sHsp 22.7 F	ACATTTCGGTCTTCATGGAGTAG	23	58.87	602
10	sHsp 22.7 R	ATCCGCCATTGATACGATGAG	21	57.37	
11	sHsp 17.8 F	TCAAGAGCTGACGGTTTAAGG	21	55.18	178
12	sHsp 17.8 R	GACATTAGATTGGCAGTGATTCG	23	58.39	
13	sHsp 17.9 F	TTGGCGTTGCCCTCTTC	17	55.18	228
14	sHsp 17.9 R	TGTGAGGATGTTTAACCTGGAC	22	58.87	

4.1.4 Primer reconstitution and dilution

All the primer sets (seven for qPCR studies and remaining seven for gene isolation studies) were reconstituted according to manufacturer's instructions. This was kept as stock and stored at -20 °C. From these stocks, working standards of 5 pM concentration were prepared for subsequent studies.

4.1.5 Annealing temperature standardization

Annealing temperature was selected as 58 °C after performing PCR at 54 °C, 56 °C and 58 °C.

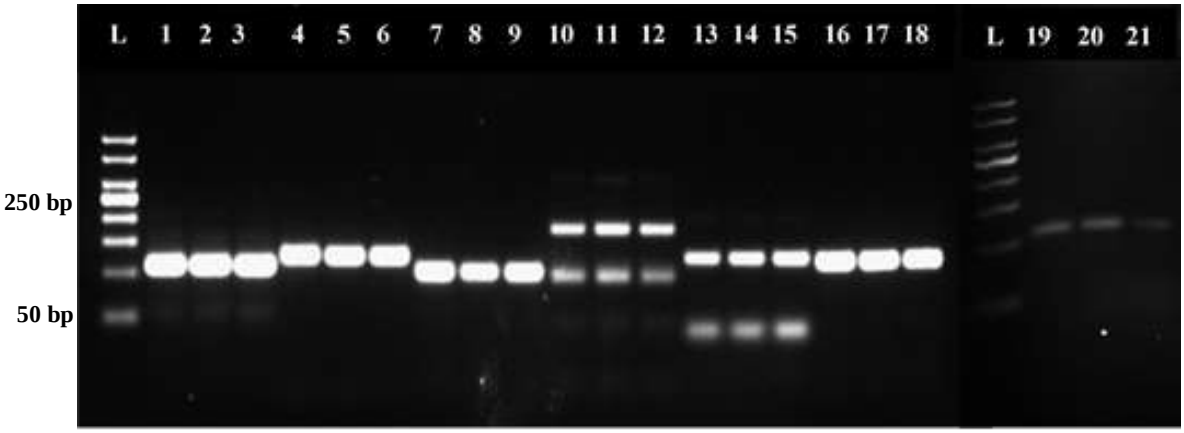


Figure 4.5 Standardization of annealing temperature of Real-Time primers. *Lane L - Orion X 50 bp DNA ladder (Origin, India); Lane 1,2,3 - sHsp 16.9 ; Lane 4,5,6 - sHsp 18.1; Lane 7, 8,9 - sHsp 18.6; Lane 10, 11, 12 - sHsp 26.5; Lane 13, 14, 15 - sHsp 22.7; Lane 16, 17, 18 - sHsp 17.9 and Lane 19, 20, 21 - sHsp 17.8. Lane 1, 4, 7, 10, 13, 16, 19 represents PCR products amplified at Ta 54 °C; Lane 2, 5, 8, 11, 14, 17, 20 represents PCR products amplified at Ta 56 °C; Lane 3, 6, 9, 12, 15, 18, 21 represents PCR products amplified at Ta 58 °C*

Figure 4.6 Standardization of annealing temperature of gene isolation primers. *Lane L - Orion X 100 bp DNA ladder (Origin, India); Lane 1,2,3 - sHsp 16.9 ; Lane 4,5,6 - sHsp 18.1; Lane 7, 8,9 - sHsp 18.6; Lane 10, 11, 12 - sHsp 17.8; Lane 13, 14, 15 - sHsp 26.5; Lane 16, 17, 18 - sHsp 22.7 and Lane 19, 20, 21 - sHsp 17.9. Lane 1, 4, 7, 10, 13, 16, 19 represents PCR products amplified at Ta 54 °C; Lane 2, 5, 8, 11, 14, 17, 20 represents PCR products amplified at Ta 56 °C; Lane 3, 6, 9, 12, 15, 18, 21 represents PCR products amplified at Ta 58 °C*

4.1.6 Selection of primers for Real-Time studies

Since some of the primer sets designed for Real-Time studies showed multiple products, those were omitted and four sets of primers corresponding to sHsp genes sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 were selected (See Table 4.4).

Table 4.4 List of primers selected for Real-Time studies

Sl. No.	Primer name	Sequence 5' to 3'	Length (bases)	Tm (°C)	Amplicon length (bases)
1	sHsp 18.1 F	TCGTGAGGCGCAGCAAC	17	57.60	125
2	sHsp 18.1 R	GGAGGTTTCGTCCGCGAAG	19	60.99	
3	sHsp 18.6 F	AGGAGAGGAGCCTCGAAAG	19	58.82	100
4	sHsp 18.6 R	TGGACGTTGGTCTTGGAAAG	20	57.30	
5	sHsp 17.8 F	TCGTGTTCGGTGAATGACTAC	21	57.87	106
6	sHsp 17.8 R	TCAAGAGCTGACGGTTTAAGG	21	57.87	
7	sHsp 17.9 F	GGCATCGCGGATGTACG	17	57.60	118
8	sHsp 17.9 R	TGTGAGGATGTTTAACCTGGAC	22	58.39	

4.1.7. Real-Time PCR analysis

Differential expression analysis of sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 were performed using UBCE as endogenous control. By using the resultant Ct values, relative mRNA expression at different time period were calculated using Comparative Ct method. For all the sHsps studied, it was observed that maximum mRNA accumulation occurred in the first 30 min when subjected to heat stress at 40 °C (See Table 4.6). Later after the peak expression, a decreasing trend was observed for all sHsps.

Among the four sHsp genes studied, sHsp 18.1 and sHsp 17.8 showed a tremendous fold change of 5571 and 5000 respectively in the initial 30 min after subjecting to heat shock at 40 °C (See Table 4.6). At the same instant, the RFC values of sHsp 18.6 and sHsp 17.9 were 198.8 and 435.35 respectively.

Table 4.5 Change in Ct value of cardamom sHsps with respect to sample

Sample	Time (min)	Ct average			
		sHsp 18.1	**sHsp 18.6**	**sHsp 17.8**	**sHsp 17.9**
Control	0	28.285	26.044	29.48	25.669
T_1	30	15.993	18.561	17.208	16.919
T_2	60	16.75	19.976	18.176	17.501
T_3	120	17.887	21.847	19.99	18.411
T_4	180	17.627	21.358	19.916	18.686

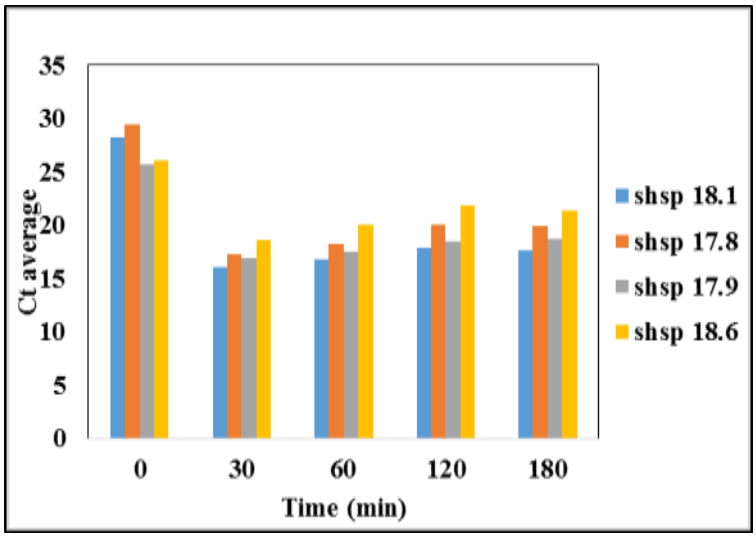


Figure 4.7 Change in Ct value of sHsps with respect to sample. **** 30, 60, 120 and 180 min represents the samples collected at different instants after heat stress. 0^{th} min represents untreated sample.*

Table 4.6 sHsp gene expression at different instants after subjected to heat stress

Time (min)	Relative fold change			
	sHsp 18.1	**sHsp 18.6**	**sHsp 17.8**	**sHsp 17.9**
0	1	1	1	1
30	5571.024	198.832	5000.217	435.352
60	4013.226	90.765	3344.158	380.066
120	1630.264	22.165	912.231	194.117
180	1227.596	19.562	563.803	94.224

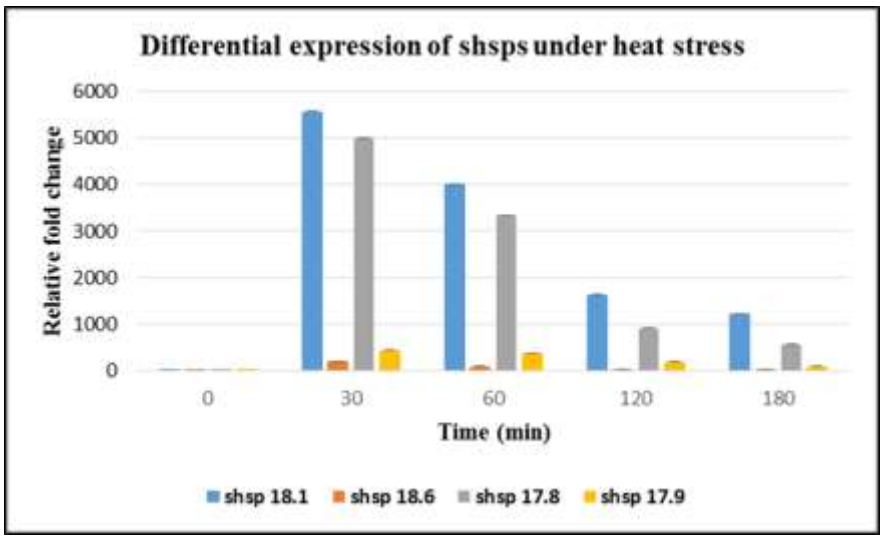


Figure 4.8 Accumulation of sHsp gene transcripts in cardamom after subjected to thermal stress for four different time periods. **********30, 60, 120 and 180 min represents the samples collected at different instants after heat stress. 0th min represents untreated sample Bars show Relative fold change value with standard error*

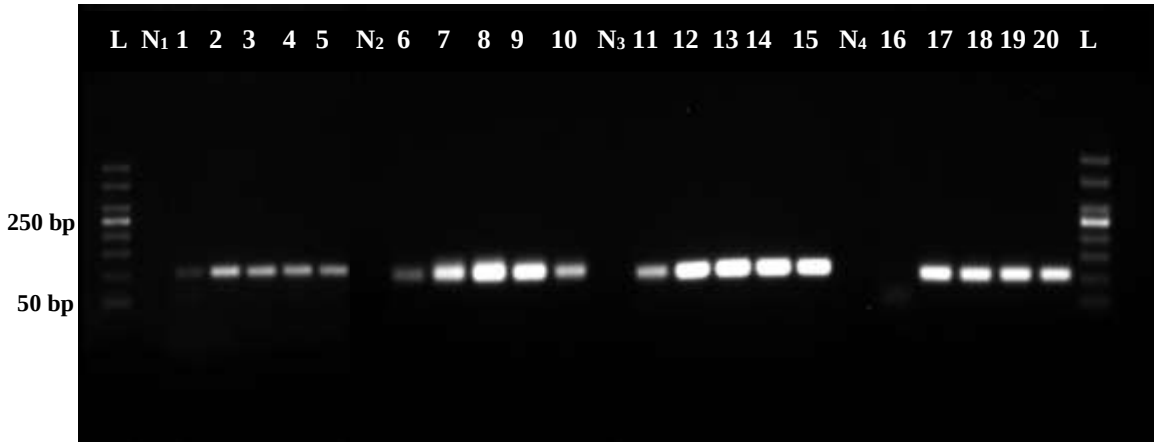


Figure 4.9 mRNA abundance of different sHsps (sHsp 18.1, sHsp 17.8, sHsp 17.9 and sHsp 18.6) in cardamom under heat stress at different instants. *Lane L - Orion X 50 bp ladder (Origin, India); Lane 1 to 5 - sHsp 18.1; Lane 6 to 10 - sHsp 17.8; Lane 11 to 15 - sHsp 17.9; 16 to 20 - sHsp 18.6. N_1, N_2, N_3 and N_4 are the NTCs of sHsp 18.1, sHsp 17.8, sHsp 17.9 and sHsp 18.6 respectively*

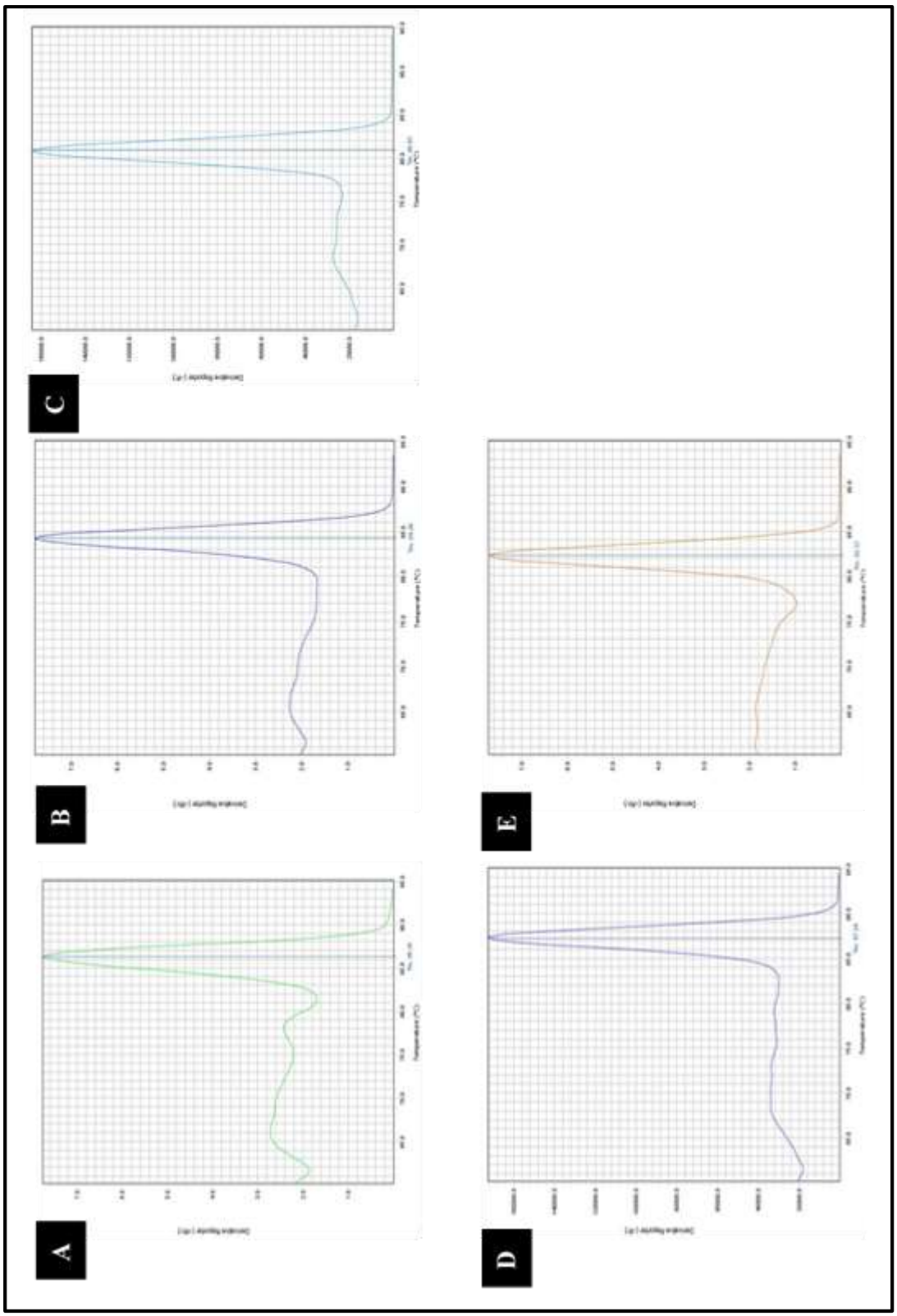
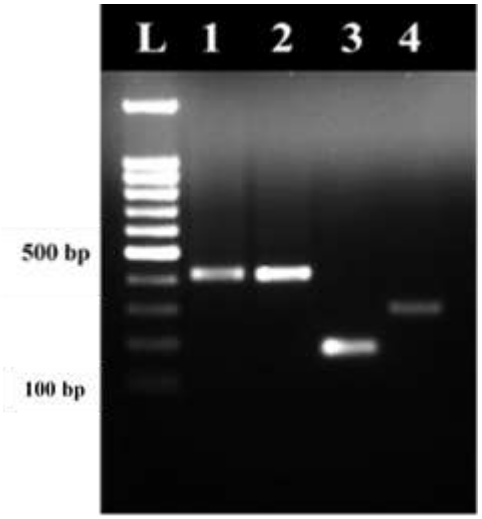


Figure 4.10 Melt curve analysis: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.8 (D) sHsp 17.9 (E) UBCE*

4.2 GENE ISOLATION



All the four sHsps studied were isolated using gene specific primers (see Table 4.3). After PCR clean-up, a volume of 2 µl product was resolved on 1.5 % gel. Amplified products corresponding to band size 405 bp, 406 bp, 178 bp and 288 bp were obtained for sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9.

Figure 4.11 PCR clean-up products resolved on 1.5 % agarose gel. *Lane 1 - sHsp 18.1; Lane 2 - sHsp 18.6; Lane 3 - sHsp 17.8; Lane 4 - sHsp 17.9*

4.2.1 Sequence analysis

After single direction sequencing (5' to 3'), a sequence length of 233 bp, 371 bp, 157 bp and 160 bp were obtained for sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 respectively. NCBI BLASTN results (See Figure 4.14) showed that sHsp 18.1, sHsp 18.6 and sHsp 17.9 of cardamom have 86.54 %, 70.24 %, 76.06 % identity with sHsp 18.1, sHsp 18.6 and sHsp 17.3 of *Musa acuminata* subsp. *malaccensis* respectively. Small Hsp 17.8 showed an identity value of 74.16 % with sHsp 17.8 of *Nymphaea colorata* with 56 % query coverage. Nucleotide sequences of sHsps were converted to amino acid sequences using ExPASy Translate tool. UniProt BLAST results (See Figure 4.15) showed that the deduced amino acid sequence of crdm sHsp 18.1 showed 75 % identity with sHsp 17.8 of *Phoenix dactylifera*. Similarly, for sHsp 18.6 and sHsp 17.9 UniProt BLAST, most similar hit obtained was 'sHsp domain containing protein' and 'sHsp protein' from *Musa* with 52.5 and 52.4 % identity. Crdm sHsp 17.8 showed an identity of 76.5 % with sHsp domain protein of *Camellia sinensis* var *sinensis.*

Physico-chemical properties such as amino acid composition, total number of amino acids, pI, molecular weight, instability index, aliphatic index, Grand Average of Hydropathicity (GRAVY) were determined using ExPASy Protparam tool (See Table 4.7 and 4.8). 'Alpha crystallin sHsp 23 family like' conserved domain was obtained for both sHsp 18.1 and sHsp 18.6 during NCBI CD search. Secondary structure was predicted for four sHsps using PSIPRED with coils dominated among all. The

confidence level in secondary structure prediction was calculated as above 90 % for 18.6 and 18.1 sHsps and below 50 % for 17.8 and 17.9 sHsps.

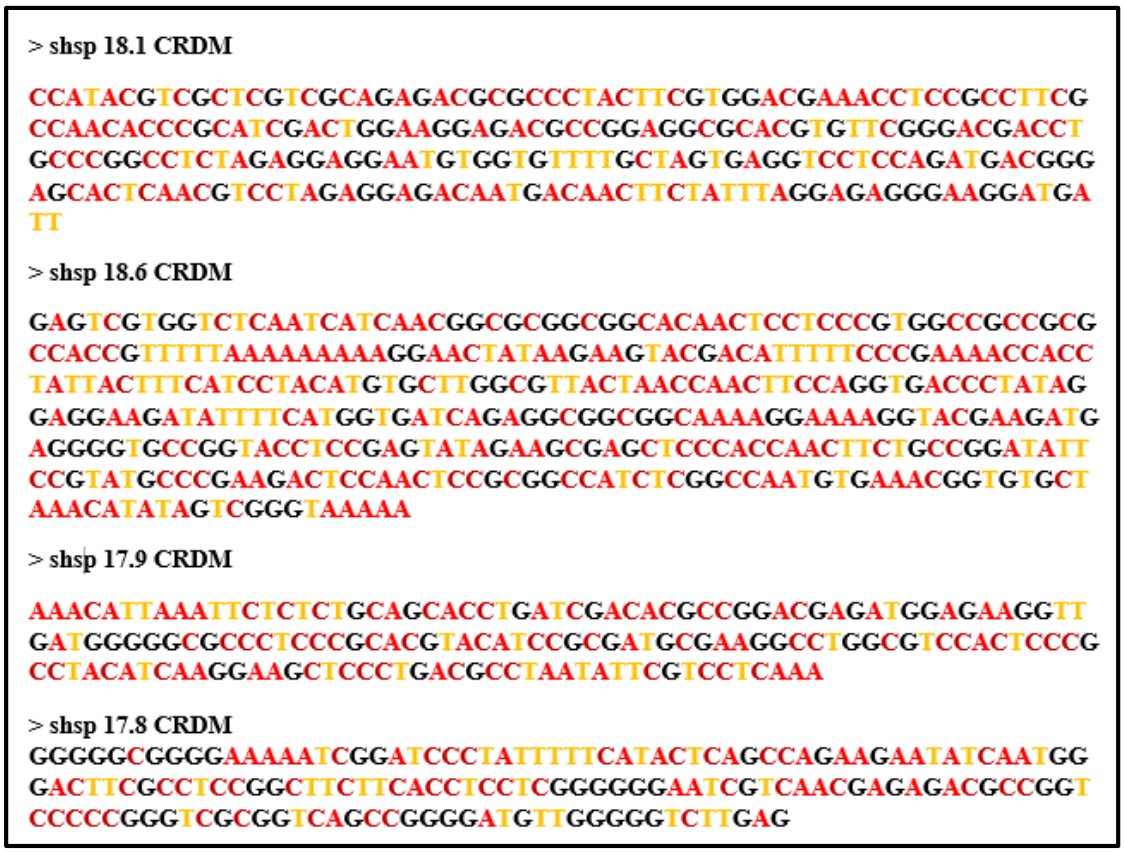


Figure 4.13 Sequence of purified sHsp products

Figure 4.14 Translated amino acid sequences of sHsp genes (using Translate tool)

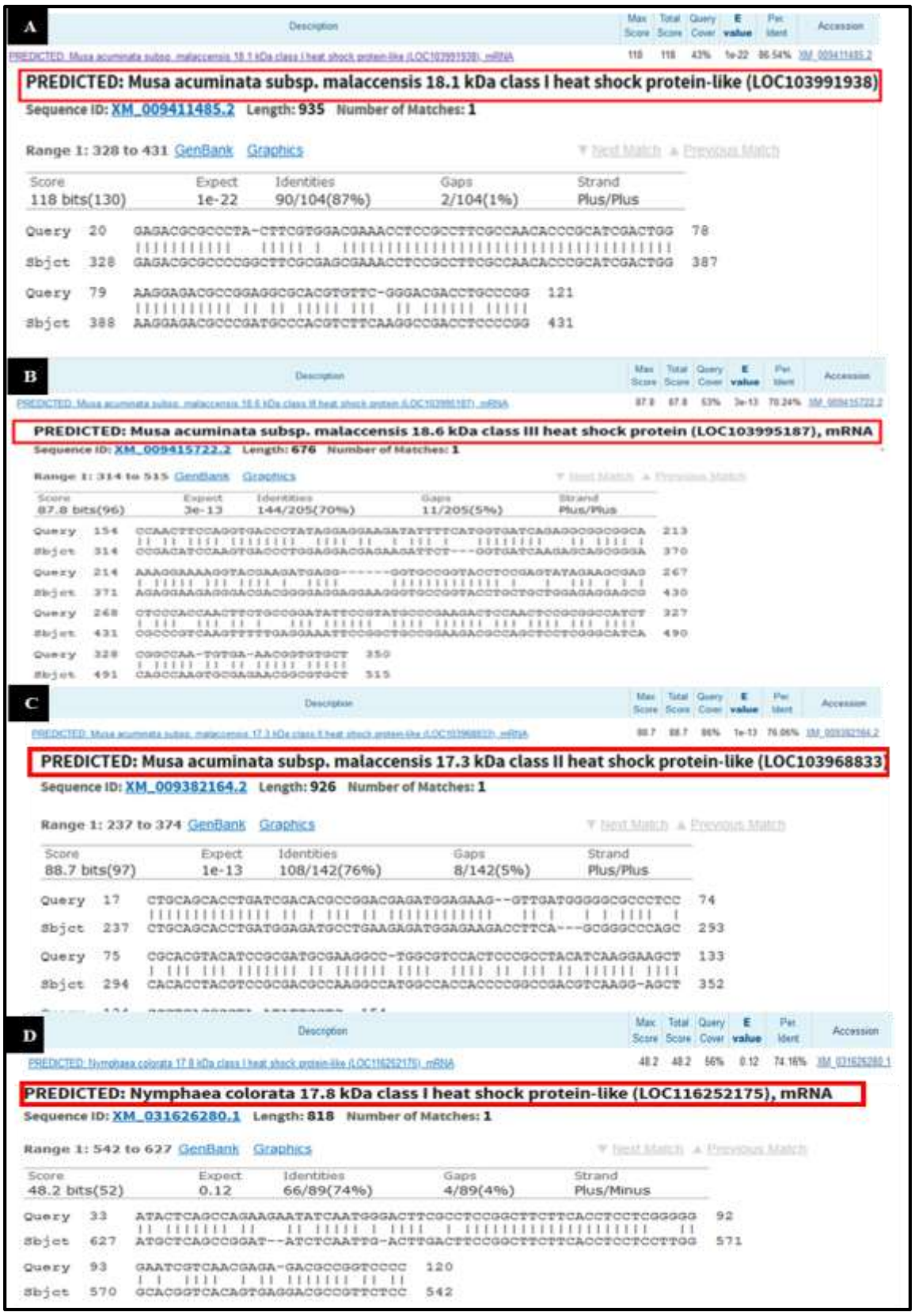


Figure 4.14 BLASTN results of sequenced products: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.9 (D) sHsp 17.8*

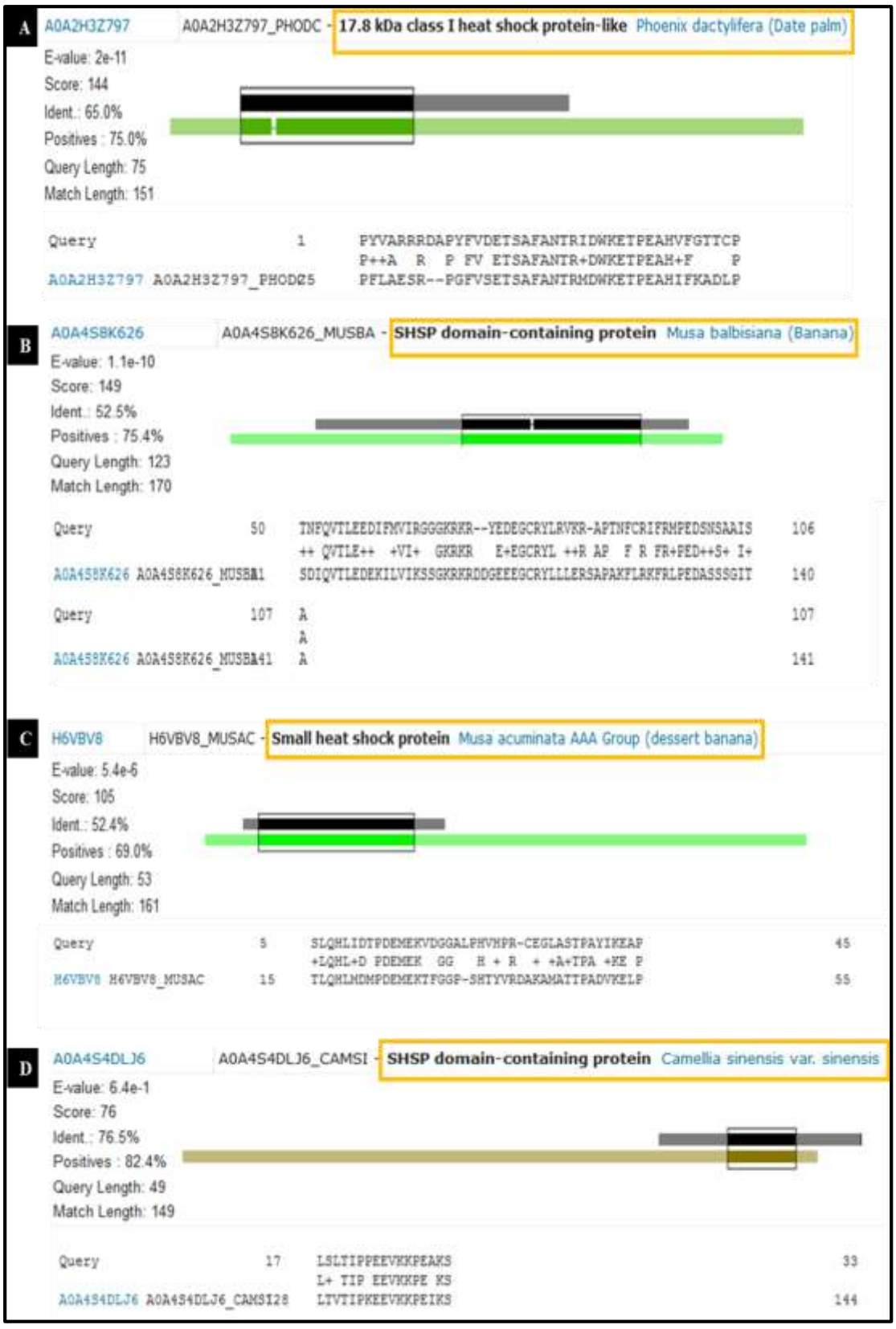


Figure 4.15 UniProt BLAST results of sHsps: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.9 (D) sHsp 17.8*

Table 4.7 Physico-chemical properties of translated sHsp sequences

. **PROPERTIES**	**sHsp 18.1**	**sHsp 18.6**	**sHsp 17.9**	**sHsp 17.8**
No. of amino acids	75	119	52	49
Molecular weight	8404.40	13787.19	5796.75	5386.12
pI	4.78	9.97	6.29	9.81
Molecular formula	$C_{367}H_{561}N_{101}O_{116}S_5$	$C_{618}H_{989}N_{181}O_{163}S_7$	$C_{262}H_{418}N_{70}O_{74}S_2$	$C_{240}H_{384}N_{70}O_{71}$
Total no. of atoms	1150	1958	826	765
Total number of negatively charged residues (asp + glu)	11	10	7	6
Total number of positively charged residues (arg + lys)	7	22	6	9
Instability index	46.93	48.41	19.16	76.97
Aliphatic index	56.00	86.05	99.42	57.76
GRAVY	-0.369	-0.329	-0.165	-0.916

Table 4.8 Amino acid composition of sHsps (%)

Amino acid	sHsp 18.1	sHsp 18.6	sHsp 17.9	sHsp 17.8
Ala (A)	10.7	7.6	7.7	6.1
Arg (R)	8.0	**10.1**	3.8	10.2
Asn (N)	2.7	5.9	1.9	0.0
Asp (D)	4.0	3.4	5.8	6.1
Cys (C)	2.7	3.4	1.9	0.0
Gln (Q)	1.3	1.7	1.9	0.0
Glu (E)	10.7	5.0	7.7	6.1
Gly (G)	6.7	5.0	5.8	6.1
His (H)	1.3	2.5	5.8	2.0
Ile (I)	2.7	6.7	7.7	6.1
Leu (L)	4.0	8.4	**11.5**	4.1
Lys (K)	1.3	8.4	7.7	8.2
Met (M)	4.0	2.5	1.9	0.0
Phe (F)	5.3	5.0	3.8	4.1
Pro (P)	5.3	5.0	9.6	**18.4**
Ser (S)	5.3	5.0	3.8	12.2
Thr (T)	**12.0**	2.5	3.8	4.1
Trp (W)	2.7	0.8	0.0	0.0
Tyr (Y)	2.7	4.2	1.9	2.0
Val (V)	6.7	6.7	5.8	4.1
Pyl (O)	0.0	0.0	0.0	0.0
Sec (U)	0.0	0.0	0.0	0.0

*** ***Highlighted values indicates highest contribution***

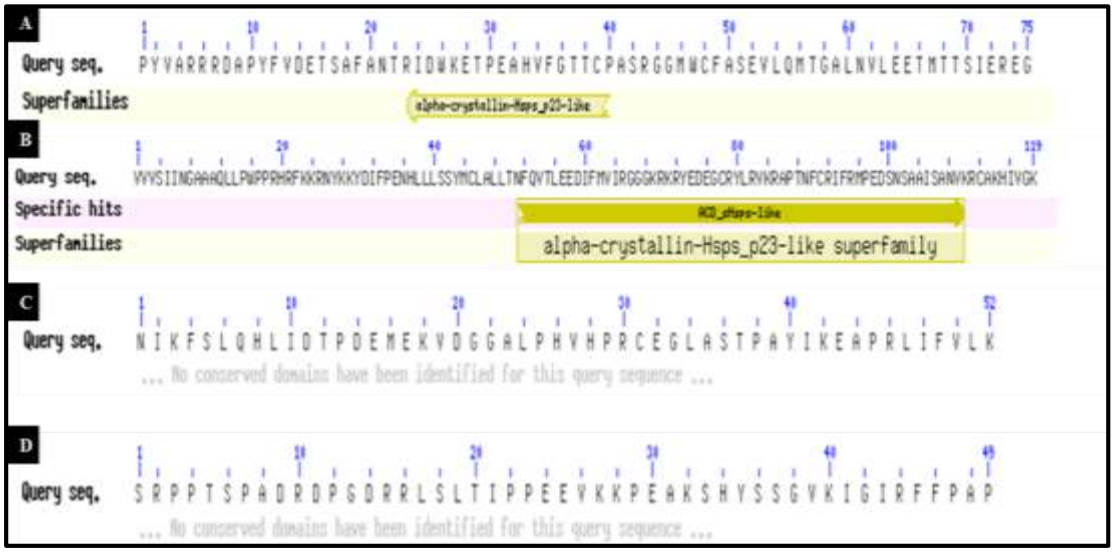


Figure 4.16 NCBI CD search window showing conserved domain of sHsps: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.9 (D) sHsp 17.8*

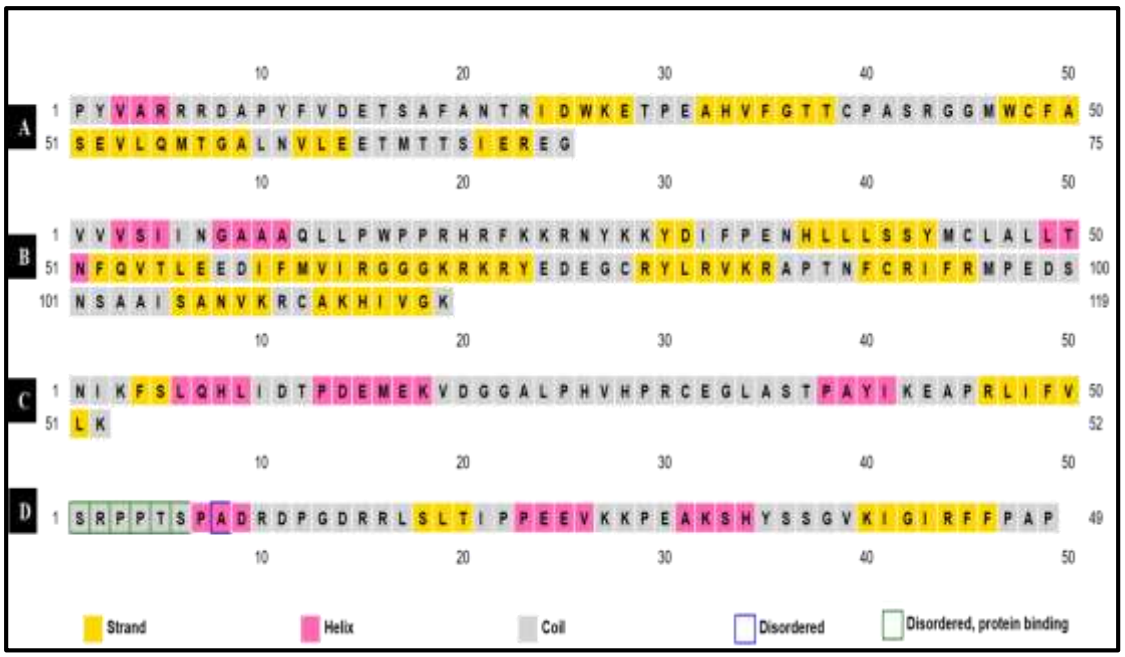


Figure 4.17 Secondary structure predicted using PSIPRED: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.9 (D) sHsp 17.8*

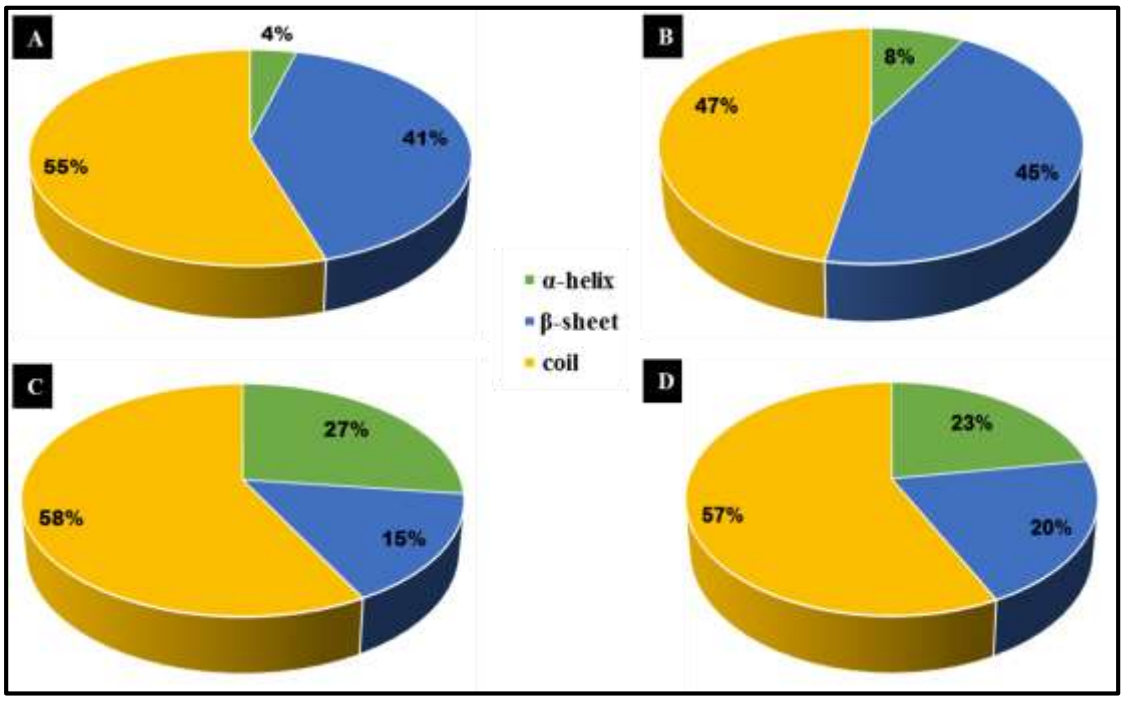


Figure 4.18 Percentage distribution of secondary structures: *(A) sHsp 18.1 (B) sHsp 18.6 (C) sHsp 17.9 (D) sHsp 17.8*

5. DISCUSSION

Cardamom is a large herbaceous perennial which is highly valued for its aromatic capsules. These sciophytes usually come up well in areas of low temperature and high humidity with a mean rainfall of 1500 to 4000 mm. Common people have a unique tryst with this spice due to its wide range of cuisine and medicinal attributes. With the increase in demand for cardamom for various uses, the annual production made a drastic leap but got limited by various environmental factors. According to Murugan *et al*. (2013), water scarcity and scorching heat are the uttermost environmental factors that limits cardamom production potential.

In order to thrive in such adverse situations, they need to activate their defense system. Generally in plants such functions are taken care of by a group of universal proteins known as heat shock proteins (Hsps). Now studies are focused on the evolutionarily less conserved divergent group of Hsps called small heat shock proteins (sHsps) which act as molecular holdases by preventing aggregation of misfolded proteins during stress. The significance of this diverse molecule in tackling thermotolerance (Vierling, 1991) has been already reported in plants such as pea (Otto *et al*., 1998), wheat (Basha *et al*., 2004), maize (Klein *et al*., 2014), rice (Zou *et al*., 2009) and tomato (Banzet *et al*., 1998). Hence it is imperative for anyone who seeks to tackle the problem of heat stress in cardamom, to carry out a proper study regarding sHsps in cardamom. The primary objective of this study was to analyze differentially expressed genes belonging to the small heat shock (sHsp) family under heat stress. Studying the expression of sHsps might shed light on the molecular aspects of thermotolerance in cardamom.

In this study, we have analyzed the expression pattern of sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 during four different instants under heat stress and revealed that there exists a common sHsp expression pattern during heat stress similar to one reported by Pavli *et al*. (2011).

For this study six month old wild cardamom plants (triplicates) were subjected to heat stress at 40 °C after gradually increasing temperature from normal at the rate of 0.9 °C/min. According to Sun *et al*. (2002), plants produce heat inducible Hsps only when there is a temperature shift of 10 to 15 °C from normal. That's why for this study a

temperature of 40 °C was chosen. Altschuler and Mascarenhas (1985) reported that the pattern of sHsp expression varies with the way we are giving the heat treatment i.e. sudden heat shock or gradual heat stress. He has also shown that the gradual rise from the normal environmental temperature to heat shock temperature is a better way to understand the thermotolerance in plants as it might be more representative of our field conditions.

After subjecting to heat stress at 40 °C, leaf tissues were collected at intermittent periods like 30 min, 1 hr, 2 hr and 3 hr via flash freezing method to inactivate the action of RNases. The time periods for collection of samples were decided based on previous reports of Sachs *et al*. (1986) and Lenne and Douce (1994).

Since small cardamom tissues are marked by a large number of polyphenols and polysaccharides, RNA isolation was carried out using combined CTAB and RNeasy kit method developed by Nadiya *et al*. (2015). In this method large RNA molecules (rRNAs and mRNAs) precipitated using LiCl were further purified through a column to obtain high quality RNA.

In 1% gel, the isolated RNA was observed as two intact bands (28S and 18S rRNA) with slight degradation which may be due to handling issues or some delay happened during the steps. No apparent genomic DNA contamination was observed on the gel. In contrast to the normal band ratio of 2:1, bands were observed with almost similar intensity.

Concentration as well as purity values displayed on Nanophotometer implied that RNA isolated were amenable for cDNA synthesis. cDNA was prepared using 150 ng/µl of RNA with the assumption that 150 ng/µl of RNA is completely converted to cDNA. The cDNA synthesized was diluted to 1: 5 ratio and used for subsequent studies.

Primers are trivial components which determine the extent of success of a PCR reaction (Dieffenbach *et al*., 1993). For our study, two sets of primers were designed: one set is for Real time PCR studies and the other for gene isolation. Primers were designed from leaf transcriptome data of cardamom using IDT's Primer quest tool after strictly following the criteria of primer design put forward by Etebu (2013). Small Hsps are regarded as the less evolutionarily conserved group of Hsps but featured by a unique α-

crystallin conserved domain (Sun *et al*., 2002). So in order to avoid the redundancy in primer sequences between primer sets during synthesis, NCBI CD search was done before primer designing. According to Dieffenbach *et al*. (1993), the prime factors that affect primer quality are Tm and cross reactivity between primers. Once the IDT's computer programme has given a probable set of primers, primer quality and efficiency were determined via Net Primer tool and NCBI Primer BLAST (Etebu, 2013) respectively.

After passing all quality criteria, a total of seven sets of sHsp primers namely sHsp 16.9, sHsp 17.8, sHsp 17.9, sHsp 18.6, sHsp 22.7, sHsp 18.1 and sHsp 26.5 were selected. Preliminary selection of primers for qPCR studies was carried out by running a normal PCR using cardamom cDNA as template. Primers which showed desired products only (sHsp 17.8, sHsp 17.9, sHsp 18.1 and sHsp 18.6) were selected for subsequent studies. Annealing temperature for all these primers were standardized as 58 °C through gradient PCR.

Proper selection of reference genes is necessary for producing reliable, accurate and reproducible quantitative data (Qu *et al*., 2019). After preliminary screening of reference genes, expression patterns of selected sHsps (sHsp 17.8, sHsp 17.9, sHsp 18.1 and sHsp 18.6) were investigated using UBCE as endogenous control. The expression of sHsps in stressed plants with respect to control under four different time periods were analyzed using Comparative ΔΔCq method (Livak and Schmittgen, 2001). The expression pattern revealed that all these sHsps were expressed in considerably high level in response to heat stress, especially during first 30 min after heat shock at 40 °C. Similar expression pattern has been reported by Viswakarma *et al*. (2018) after subjecting gradual heat shock in different wheat genotypes. He has shown that maximum accumulation of Hsp 90 transcripts occurred in the first hour after attaining heat shock temperature. Pavli *et al.* (2011) has also done similar kind of study on different sorghum genotypes, but the method of giving stress was different and the results revealed that Hsp peak expression instants vary with genotype of the plant. Woods *et al*. (1998) also had similar inference that there exists a tight correlation between the early sHsp accumulation and genotype. In line with the observations of Pavli *et al*. (2011) and Woods *et al*. (1998) it can be inferred that the early peak

expression of sHsps in our study may be due to the genotype studied. This early sHsp accumulation might help the cells in early recovery and continue the growth process. However the fact is that still we are lacking studies on these aspects.

When comparing our results to those of earlier studies, it can be pointed out that all sHsp heat stress related studies follow a common trend of sHsp expression pattern consisting of initial gene induction and peak expression followed by a declining trend. Studies have already reported that Hsp mRNA level did not increase with a prolonged heat exposure, but a decrease could be noticed after a peak value. Two possible explanations for this result can be given: one is the transient binding nature of HSFs with HSEs. i.e. The stability of binding of HSFs on HSEs determines the extent of transcription of sHsp mRNAs. If the HSFs bind the HSEs for a longer time with increased processivity more transcripts will be produced and it will be continued for a longer period. The other explanation is the increased removal of adenine residues from poly A tail of mRNAs. Poly A tail shortening reduces the mRNA half-life thereby resulting in early degradation. Osteryoung *et al*. (1992) demonstrated that sHsp mRNAs produced during gradual heat stress have shorter Poly A tails when compared to abrupt stress producing sHsp transcripts. He conveyed that the difference in poly A tail length, may therefore reflect not only a gradual versus an abrupt stress treatment, but more generally the overall severity of the stress treatment. The effect of gradual heat stress in preventing an increase in poly A tail length parallels the ability of a gradual or prior heat stress treatment to confer thermotolerance in eukaryotes. Hence we can conclude that once sufficient amounts of sHsp mRNAs are produced for conferring tolerance the cell begins to regulate the sHsp genes at transcript level which is evident with decrease in sHsp mRNA with prolonged exposure. Nevertheless this decrease is not necessarily followed by a reduction in the protein level (Lenne *et al*., 1995). Eventhough the sHsp mRNAs have disappeared after 3 or 4 hrs, the proteins will remain for around 20 hrs to 3 days depending on plant species (Altschuler and Mascarenhas, 1985). Actually they are the real cavalries who fought against torrid hotness.

Among the four sHsp genes studied, sHsp 18.1 and sHsp 17.8 showed a tremendous fold change of 5571 and 5000 respectively in the initial 30 min after subjecting to heat

shock at 40 °C (See Table 4.6). At the same instant, the RFC values of sHsp 18.6 and sHsp 17.9 were 198.8 and 435.35 respectively. These four differentially expressed sHsps were isolated, purified and sequenced. A sequence length of 233 bp, 371 bp, 157 bp and 160 bp were obtained for sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 respectively. These sequences correspondingly yielded amino acid sequences of length 119, 75, 49 and 52 using ExPASy Translate tool. At nucleotide and amino acid level, BLAST results got most similar hits with 'sHsps of different plant species' which confirmed their identity. But query coverage was low due to the short size of our sequences. During NCBI CD search also, presence of 'Alpha crystallin sHsp 23 family like' conserved domain obtained for sHsp 18.1 and 18.6 confirmed their identity again (Sun *et al*., 2002). Since the read length was too small for sHsp 17.8 and sHsp 17.9, we were unable to obtain any CD. In case of predicted secondary structures of sHsps, coil structures were dominated. Secondary structure was predicted for sHsp 18.1 and sHsp 18.6 with 90 % confidence and sHsp 17.8 and sHsp 17.9 with below 50 % confidence.

An apparent limitation of our study is the lack of knowledge on 'at what point of heat treatment sHsp gene induction started'. Some research reports communicated that sHsps expression can begin immediately after 3 to 5 min of heat stress (Sun *et al*., 2002). Sample collection at that point may be helpful to sort out that limitation. The average gene size of sHsp is determined as 600 bp (Mogh *et al*., 2019). Since our sequence reads were short (especially for sHsp 17.8 and 17.9), we were unable to go for a complete gene characterization study. Further studies such as RACE need to be done for obtaining full length sequences.

On the basis of our results, it can be speculated that 17.8 and 18.1 sHsps could be used as potential biomarker candidates for screening tolerance to heat stress in cardamom. The findings obtained from this study can be used as a foundation for future lines of research related to rational improvement of cardamom against thermal stress. But, transcriptional level studies only cannot be considered as the end of gene expression studies. So studying these molecules at translational level is a good choice to learn the expression pattern more vividly. Extended studies on the different sHsp families in cardamom and the molecular mechanism of conferring thermotolerance are potential

areas of research in future. This information could form the basis for overexpression studies and other genetic manipulations to create climate resilient cardamom with better thermotolerance.

6. SUMMARY

Investigations on “Isolation and characterization of Hsp family gene from cardamom (*Elettaria cardamomum* Maton)” was conducted at the Biotechnology and Bioinformatics Division, KSCSTE-JNTBGRI, during 2019 – 2020.

Small cardamom, popularized around the world with the sobriquet “Queen of Spices”, is commonly cultivated in high ranges of Southern India. Being a psychrotroph, it is easily affected by the fluctuating climatic scenario which in turn affects its growth and productivity pattern. In order to mitigate such situations, plants usually come up with cavalries called heat shock proteins. One such defender is a highly divergent, less conserved category of heat shock proteins called small heat shock proteins (sHsps) which have a significant role in tackling heat stress. This study is primarily focused on determining the changes in expression pattern of selected sHsp genes at transcriptional level under heat stress in cardamom.

In order to study the variations in expression of sHsps at the mRNA level during heat stress, the cardamom plants were subjected to heat shock at 40 °C by raising the temperature of the growth chamber at the rate of 0.9 °C/min from normal. After attaining 40 °C, the temperature was maintained for a period of 3 hrs and samples were collected at instants viz. 30 min, 1 hr, 2 hr and 3 hr intervals. Untreated samples were left as control. Total RNA isolation was carried out using modified CTAB method combined with RNeasy Plant Mini Kit method. After quantity and quality determination, the RNA was reverse transcribed.

Primers for real time PCR as well as gene isolation studies were designed from cardamom leaf transcriptome data obtained from NCBI SRA. Primer designing was performed using IDT’s primer quest tool. Later, primer quality and specificity were probed using Net Primer and NCBI Primer BLAST respectively. After passing all the quality criteria, a total of seven sets of sHsp primers namely sHsp 16.9, sHsp 17.8, sHsp 17.9, sHsp 18.6, sHsp 22.7, sHsp 18.1 and sHsp 26.5 were selected. Preliminary selection of primers for qPCR studies was carried out by running a normal PCR using cardamom cDNA as template. Primers which showed desired products (sHsp 17.8, sHsp 17.9, sHsp 18.1 and sHsp 18.6) only were selected for subsequent studies. Annealing temperature for all these primers were standardized as 58 °C through

gradient PCR. Expression patterns of selected sHsps (sHsp 17.8, sHsp 17.9, sHsp 18.1 and sHsp 18.6) were investigated using StepOnePLus™ Real Time PCR system. The resultant Ct values obtained from Real time analysis were later used for calculating relative fold change in gene expression of sHsps using Comparative ΔΔCq method.

The expression kinetics varied between each sHsps; peak expression was observed during the first 30 min after heat shock at 40 °C. Small Hsps 17.8 and 18.1 showed considerable fold change value of 5571 and 5000 respectively during the initial 30 min. Compared to 17.8 and 18.1 sHsps, 18.6 and 17.9 showed less expression (198.8 and 435 times). With prolonged exposure to heat, a sharp decline was observed in the expression pattern.

These four differentially expressed sHsps were isolated, purified and sequenced. A sequence length of 233 bp, 371 bp, 157 bp and 160 bp were obtained for sHsp 18.1, sHsp 18.6, sHsp 17.8 and sHsp 17.9 respectively. DNA sequences of all sHsps were translated using ExPASy Translate tool. NCBI BLASTN results showed that sHsp 18.1, sHsp 18.6 and sHsp 17.9 of cardamom have 86.54 %, 70.24 %, 76.06 % identity with sHsp 18.1, sHsp 18.6 and sHsp 17.3 of *Musa acuminata* subsp. *malaccensis* respectively. Small Hsp 17.8 showed an identity value of 74.16 % with sHsp 17.8 of *Nymphaea colorata* with 56 % query coverage. UniProt BLAST results also gave most similar hits as sHsps from different plant species. From the predicted amino acid sequences, physico-chemical properties and secondary structure were determined using ExPASy Protparam and PSIPRED tool respectively. The percentage of confidence in determining secondary structure was predicted with Phyre^2 software. Small Hsp 18.1 and sHsp 18.6 showed 'Alpha crystallin sHsp 23 family like' conserved domain during NCBI CD search which confirmed their identity again.

The results indicated that the sHsp genes studied have a direct link with thermotolerance in cardamom. On the basis of the results, it can be speculated that 17.8 and 18.1 sHsps could be used as potential biomarker candidates for screening tolerance to heat stress in cardamom. In addition to that, the present work could form the basis of future lines of studies in molecular aspects of thermotolerance in cardamom.

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8. APPENDICES

APPENDIX I

Reagents required for RNA isolation

1. ***RNA extraction buffer*** **(pH: 8)**

Tris HCl	100 mM
CTAB	2 g
EDTA	0.5 M
NaCl	2 M
PVP	2 %

Dissolve all reagents and adjust pH to 8. Finally make the volume up to 1 L using deionized water. Store at RT.

APPENDIX II

Reagents used for Agarose gel electrophoresis

1. ***Gel tray soaking buffer***

NaOH	0.1 M
EDTA	100 mM

2. ***TAE buffer (50X)*** **(pH: 8)**

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	0.5 M

Dissolve all reagents and adjust pH to 8. Finally make the volume up to 1 L using deionized water. Store at RT.

Stored at RT.

3. ***Gel loading dye (6X)***

Bromophenol Blue	0.2 g
50 % glycerol	6 ml
Milipore water	4 ml

9. ABSTRACT

The study entitled “Isolation and characterization of Hsp family gene from cardamom (Elettaria cardamomum Maton)” was conducted at the Biotechnology and Bioinformatics Division, KSCSTE - Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, during 2019 – 2020. The primary objective of this study was to analyze differentially expressed genes belonging to small heat shock (sHsp) family under heat stress from cardamom.

During the study, six month old cardamom plantlets were exposed to thermal stress by gradually increasing the temperature from 22 °C to 40 °C to understand the mode of expression of small heat shock protein (sHsp) transcripts namely sHsp 17.8, sHsp 17.9, sHsp 18.1 and sHsp 18.6. After attaining 40 °C, the heat stress treatment was continued for a period of 3 hrs and leaf tissues were collected at instants viz. 30 min, 1 hr, 2 hr and 3 hr intervals. Total RNA was isolated from control as well as treated samples and converted to cDNA. Real-Time PCR analysis was performed using gene specific primers designed from cardamom transcriptome data.

The highest level of transcript accumulation was evident after the exposure of plants to thermal stress at 40 °C for 30 min. Among the four sHsps studied, sHsp 18.1 and sHsp 17.8 showed the highest expression at 30 min with a fold change of 5571 and 5000 respectively compared to the control. The two remaining sHsps also showed considerable upregulation during the initial period of heat stress. After 30 min, a sharp reduction in the expression of all sHsp transcripts has been observed. In addition to this, the overall transcript kinetics were different among the sHsp genes studied. These differentially expressed genes were isolated, purified and sequenced. Sequence analysis using various bioinformatics tools confirmed their identity as small heat shock proteins. Physico-chemical parameters as well as secondary structure were predicted using Protparam and PSIPRED respectively.

In line with our results, it can be speculated that 17.8 and 18.1 sHsps could be used as potential biomarker candidates for screening tolerance to heat stress in cardamom. The findings obtained from this study can be used as a foundation for future lines of research related to rational improvement of cardamom against thermal stress.