Functional Characterization of Mitochondrial Small Heat Shock Protein Genes in Eggplant for Abiotic Stress Tolerance

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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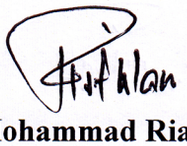
Certificate

This is to certify that the research work embodied in this thesis entitled **"Functional Characterization of Mitochondrial Small Heat Shock Protein Genes in Eggplant for Abiotic Stress Tolerance"** has been carried out in the Department of Biochemistry and Molecular Biology, University of Dhaka, Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India and Plant Biotechnology Division, National Institute of Biotechnology (NIB), Bangladesh. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.



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Dedicated to

My Beloved Family

(For their unconditional love and enormous support)

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Muslima

Abstract

Plants are sessile organisms and have evolved with numerous mechanisms for overcoming various environmental stresses. In response to various abiotic stresses, plants are used to transcribe a class of proteins known as heat shock proteins (HSPs). The mysterious and relatively understudied mitochondrial small heat shock proteins (msHSPs) evolved in the plant lineage, which are the extensive variety of HSPs found in all three domains of life, are intimately linked to protein homeostasis and survival under stress conditions. The present investigation systematically characterized a novel mitochondrial sHSP (SmsHSP24.1) from Eggplant (*Solanum melongena* L.) by transgenic overexpression and CRISPR/Cas9 mediated genome editing method. Differential expression of *SmsHSP24.1* suggested that it plays a constructive role under stressful circumstances. The SmsHSP24.1 overexpressed *Escherichia coli*-BL21 cell line demonstrated exceptional thermo-tolerance, withstanding temperatures of up to 52ºC. The protein had a multimeric structure that was unveiled by spectrometry and electron microscopy assay, acting as a molecular chaperone at high temperatures. In constitutively overexpressed Eggplant lines, overexpression of SmsHSP24.1 dramatically improved resilience to heat, drought, and salt stresses and demonstrated quick germination and seedling vigour in Eggplant. According to the RNAseq data, the glutathione (GHS) pathway's reactive oxygen species (ROS) scavenging enzymes appear to be upregulated. Genes essential to the mitochondrial electron transport chain (ETC) were also subject to transcriptional modifications. Auxin biosynthesis, cell wall transport, and repairs-related genes were also found to be at elevated levels. Quantitative PCR (qPCR), biochemical, and physiological parameters of transgenic Eggplants were found to play a crucial role in a range of stress responses and plant growth and development. Apart from that, approximately 2.0 kb upstream region predicted as *SmsHSP24.1* promoter was cloned, followed by the presence of numerous stress-related cis-regulatory elements has been confirmed by GUS expression assay, indicating that the protein is likely to be expressed as inducible way in transgenic Eggplant lines. Furthermore, CRISPR/Cas9 mediated knocked out of SmsHSP24.1 protein also indicated the indispensable role of this protein in stress response. Our results indicated an entirely novel source of the mitochondrial small heat shock protein (*SmsHSP24.1*) and its positive physiological role in transgenic Eggplant. Consequently, this gene has enormous potential in engineering stress-resilient crop plants to boost agricultural production and ensure our food security.

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List of Abbreviations and Symbols	
%	Percentage
°C	Degree Celsius
E	Extinction coefficient
µ	Micron (micro meter)
µL	Micro liter
µM	Micro molar
β-ME	Beta-mercapto-ethanol
2,4-D	2,4 –di-chlorophenoxy acetic acid
A	Absorbance
APX	Ascorbate peroxidase
AsA	Ascorbate
BAP	Benzyl-amino-purine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
$CaCl_2$	Calcium chloride
cDNA	Complemetary DNA
Cm	Centimeter
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyro-carbonate
DHAR	De-hydro Ascorbate Reductase
DMSO	Dimethyl sulfoxide
dNTPs	Deoxy-nucleoside triphosphates
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DTNB	5,5'-dithiobis -2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tag
EV-1	Entry vector 1
EV-2	Entry vector 2

FAD	Flavin adenine dinucleotide
FW	Fresh weight
G	Gram
GR	Glutathione reductase
GSH	Reduced Glutathione
GSP	Gene specific primer
GSSG	Oxidized Glutathione
GW	Genome walker
h	Hour
H_2O_2	Hydrogen peroxide
hpt	Hygromycin resistant phosphotransferase
IPTG	Isopropyl β-D-thio-galactopyranoside
kb	Kilo base pairs
kDa	Kilo Dalton
L	Liter
LB	Luria-Bertani
LF-PCR	Locus –Finding PCR
m	Meter
M	Molar
mA	Milliampare
MCS	Multiple cloning site
MDHAR	Monodehydro ascorbate reductase
mg	Milligram
min	Minutes
mM	Milli molar
mRNA	Messenger RNA
ms	Milli ssecond
MV	Methyl viologen
MW	Molecular weight
N	Normality
NAA	1-naphthaleneacetic
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NLS	Nuclear localization signal
Ni-NTA	Nickel nitrilotriacetic acid
nM	Nano mole
nm	Nano meter
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pg	*Pennisetum glaucum*
PMSF	Phenyl methane sulphonyl fluoride
PNDR	Pyridine Nucleotide-Disulphide Reductase
PVP	Polyvinyl-polypyrrolidone
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
ssDNA	Single stranded DNA
TE	Tris-EDTA
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N,N-Tetramethylethylenediamine
UV	Ultraviolet
V	Volt

v/v	Volume by volume
wt	Wild type
w/v	Weight by volume

1. Introduction:

1.1. Climate change and its impact on agriculture

Climate change is a factual problem with the uppermost precedence facing humanity nowadays. Agriculture is extremely vulnerable to climate change. Climate uncertainties and the increased occurrence of extreme climate measures are the major challenges to sustainable crop production globally (Malhi et al., 2021). For example, fluctuations in extreme weather proceedings, drops in water availability, anticipated surges in temperatures, and variations in precipitation forms may result in reduced agricultural production. Climate change can disrupt food obtainability and quality as well as reduce access to food. Drought, salinity, and high heat are mostly prevalent in many regions and may cause 50% salinization of all cultivable lands by the year 2050 (Vinocur & Altman, 2005). The spreading of weeds and pests is likely to increase with climate change. Countrywide, drops in agricultural yield or sudden losses of livestock will likely have undulation effects which increased food prices and made uncertainty about food. Expected fluctuations in climatic inconsistency lead to more common extreme situations which will require guided adaptations of crop species on an unprecedented scale to withstand agricultural production (Challinor et al., 2018).

1.2. Climate change-associated abiotic stresses on plants

Climate change is having a severe effect on agricultural production and natural ecosystems as well (Watts et al., 2021). During the last century, mostly manmade actions have augmented CO_2 accumulation and additional greenhouse gases in the atmosphere to a threatening level which leads to global warming and other climatic repercussions (Chaudhry & Sidhu, 2022). The pivotal abiotic stresses expected to increase in response to climate consequences are extreme temperatures, excess light, drought, cold, salinity, UV, ionizing radiation, and nutrient deficiency. Plant growth and development are considerably influenced by abiotic stresses (Xiong et al., 2002).

Up to 70% of production casualties in primary food crops are attributed to abiotic stresses (Reis et al., 2012; Waqas et al., 2019), and it is estimated that 90% of cultivable lands are vulnerable to at least one of these stresses. Plants are rooted in the place where they grow and have to

familiarize themselves with the fluctuating environmental circumstances brought about by the myriad of environmental components, with utmost levels producing abiotic stress. Abiotic stress affects the plant to fluctuating degrees, depending on the duration, onset period, and severity of the stress, and leads to a sequence of structural, functional, molecular, and biochemical changes, eventually affects to the total plant performance and yield. A major challenge in abiotic stress biology is to decode how plants recognize the individual stressors, how the initial signals are converted within the plant, what is the assortment of response mechanisms obtained by them, and how are they genetically decisive (Yoshida et al., 2014).

1.3. Plant abiotic stress response and tolerance

Abiotic stresses mostly lead to oxidative stress by excess accumulation of free radicals or reactive oxygen species (ROS: $O_2^{\bullet -2}$, $O_2^{\bullet -}$, $^{\bullet}OH$) (Desikan et al., 2001; Gill & Tuteja, 2010; Wang et al., 2003). In a plant cell, ROS are also generated through its normal metabolism that involves various oxidation-reduction cascades located in different cellular compartments (Mittler, 2002). Highly reactive oxygen species t ROS can interrupt various metabolic pathways inside the cell and also attack plant organelles, molecules, and metabolites until cell death happens. On the contrary, plants have evolved different resistance mechanisms for developing antioxidants defense systems or production of ROS scavengers to detoxify the ROS and shield the plant against oxidative damage. Contemporary studies in crop plants discovered that ROS acts as a signaling molecule when it generates at very low levels which induces resistance to environmental immoderations by changing the expression of self-protective genes (Qamer et al., 2021). In plants, various pathways perceive ROS from different organelles such as chloroplast, mitochondria, or peroxisome (Scarpeci et al., 2008). Increasing ROS levels are responded to by the number of antioxidant systems including enzymatic and nonenzymatic methods (Lü et al., 2010). Heat shock proteins (HSPs) are multifunctional proteins that function as a key part in conferring abiotic stress resistance and closely interact with the different antioxidants (Szyller & Bil-Lula, 2021; Ul Haq et al., 2019). An excess and deficit level of antioxidants stimulates the activation of different heat shock transcription factors (HSF) and following biosynthesis of distinct molecular weight HSPs (Bianchi et al., 2014). In all eukaryotic cells, the heat shock response (HSR) also called the cell protective mechanism mostly maintains protein homeostasis (Szyller & Bil-Lula, 2021). A close association between

the heat shock protein expression and redox homeostasis represents that plants have progressed a greater degree of adjustment over ROS toxicity. Alternatively, plants can use ROS as an activator to induce different molecular weight HSPs for better adaptations and cellular homeostasis (Mishra et al., 2018). Consequently, deciphering the plant response mechanisms against numerous stresses and the function of different stress-responsive genes or proteins in acquired stress resistance is highest important to describe their particular role as a part of stress-receptive module.

1.4. Heat shock protein (HSPs) as molecular chaperones

The heat shock proteins (HSPs) are extremely acquainted molecular chaperones, which ardently take part in maintaining protein quality, and cellular homeostasis in normal environmental conditions as well as in stressed conditions. The anti-stress action of HSPs is not restricted to their description. Each unfavorable circumstance can make protein impairment, where HSPs maintained overall cellular homeostasis by restricting the aggregation of stress-imposed denatured proteins and assisting them to maintain in their proper refolding state (He et al., 2018; Kregel, 2002; Wang et al., 2004). Based on the molecular weight, five conserved HSP classes are HSP100 (also known as Clp), HSP90, HSP70 (also known as DnaK), HSP60 (also known as Chaperonin), and small heat shock protein (sHSP) (He et al., 2018). The different HSPs are localized in different organelles in the cell. HSPs can be localized in the nucleus, cytoplasm, mitochondria, chloroplasts, or ER (Park & Seo, 2015; Ul Haq et al., 2019).

Among the HSP subgroups, small HSPs (sHSPs) are part of the highly abundant groups with molecular weights varying from 15 to 43 kDa (Jee, 2016). The sHSPs are universally present in all three kingdoms of life (Bellanger & Weidmann, 2023). Plant sHsps are encoded to the nucleus and then transported to different subcellular compartments according to amino acid sequence resemblances (Kuang et al., 2017). It can be classified into 16 subfamilies, where two are localized in the mitochondria (MI and MII), six (CI, CII, CIII, CIV, CV, CVI) are localized in the nucleus or cytosol, and the others are localized in the endoplasmic reticulum (ER), plastids (P) and peroxisomes (Siddique et al., 2008; Sun et al., 2002). All sHsps share a signature alpha-crystallin domain (ACD) at the C-terminal region which a length of ~90 amino acids long having a conserved β-sandwich like shape (Basha et al., 2010; Sarkar et al., 2009).

Under *in vitro* state, heat shock proteins are often found to accumulate into building blocks of large oligomers of

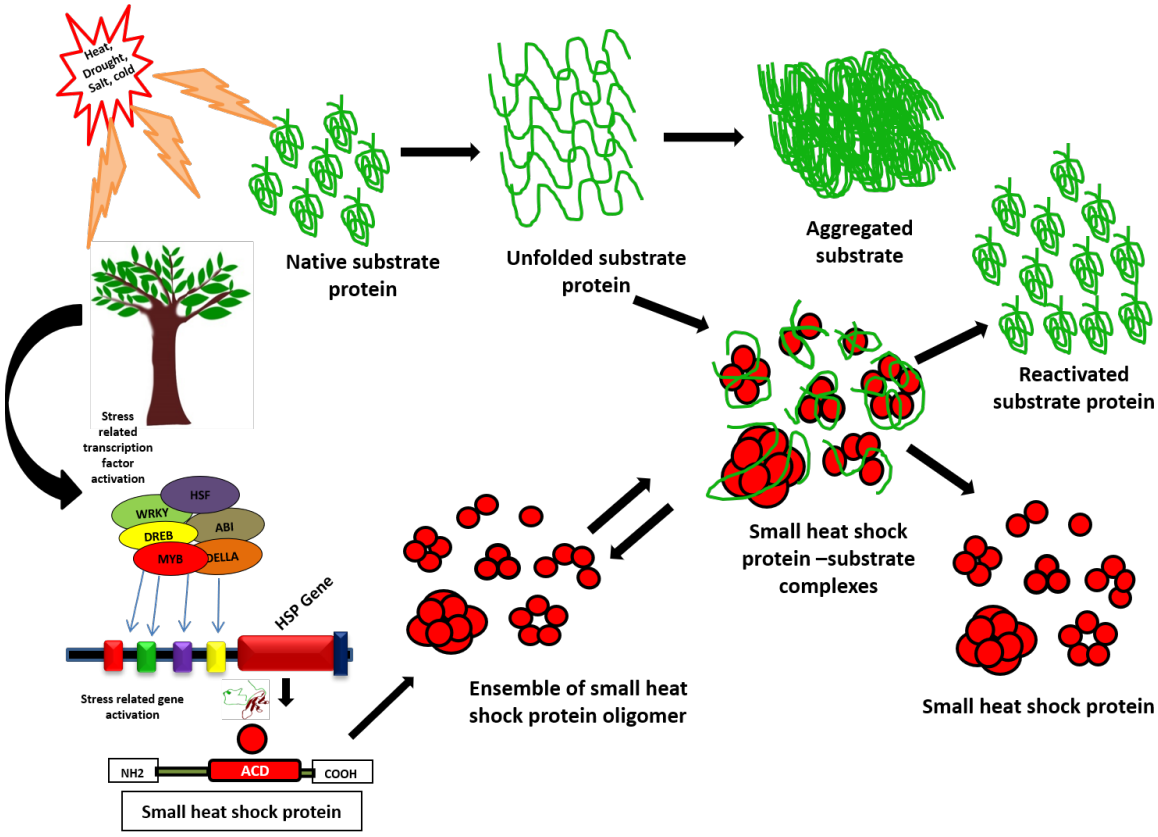


Figure 1.1: Plants respond to several abiotic stresses by activating the synthesis of small heat shock proteins (sHSPs). sHSPs can form different oligomeric structures which bind with the stressed-induced unfolded or aggregated protein and help them back to their normal competent state and make it functional again**.**

12–32 subunits, engaging individual dimers for the formation of huge building blocks (Haslbeck et al., 2019). sHSPs have a very high capacity for binding denatured substrates without the help of ATP (Fragkostefanakis et al., 2016; Waters, 2013). Under stress conditions, sHSPs stabilize membranes, and proteins and also assist in protein refolding (Wang et al., 2004). In this context, small heat shock proteins have been named “holdases” proteins. since they bind with the aggregated and unfolded proteins as a first line of protection creating a pool

of proteins competent for separation and refolding after stress endurance (Haslbeck et al., 2019). Under typical circumstances, most sHSPs transcripts are similar, but when exposed to elevated temperatures and other abiotic factors like drought, salinity, and cold, they become easily distinguishable (Park et al., 2013; Yang et al., 2014). Also, the accumulation of diverse types of sHSPs during various environmental stresses and developmental phenomena indicates that each sHSP might have a general as well as a specific adaptive role in protecting cells from stresses (Figure 1.1). Plant cell signaling pathways assisted environmental and developmental processes to regulate seed germination, plant growth physiology, and flowering timing that creates the seeds of the next generation (Meng et al., 2019). Mitochondria are the vital organelles for plant seed germination (Ma et al., 2016). Unfavorable environmental circumstances frequently lead to the generation of ROS in mitochondria and chloroplast due to decreased capabilities in photosynthetic oxidation-reduction processes. It can also alter additional macro-biomolecules including DNA, different membrane proteins, and lipids. To shield the cells, ROS scavenging is extremely crucial (Driedonks et al., 2015). Although all organisms possess sHSPs, the subfamilies localized particularly in mitochondria are evolved solely inside the plant lineage (Waters and Vierling, 2020) with the limited exception of mitochondrial small heat shock protein in Drosophila (HSP22) (Morrow et al., 2000). It has been well known that mitochondrial sHSP22 in Drosophila is tangled in shielding mitochondria and responds to protein degradation. It is also found to be essential for aging regulation and crucial for life span. This exhibits the significant role of mitochondria-localized sHSP proteins under both stressed and normal environmental circumstances (Morrow et al., 2004; Morrow and Tanguay, 2015).

Furthermore, heat-induced plants accumulate significantly more mitochondrial small heat shock protein than heat shock protein HSP60 and HSP70 (Lund et al., 1998), and the accumulation of mitochondrial small heat shock protein is associated with the formation of abiotic stress tolerance in plants (Chou et al., 1989). To date, mitochondrial small heat shock protein has been isolated from peas (Lenne et al., 1995). *Chenopodium rubrum* (Debel et al., 1997) soybean (LaFayette et al., 1996), *Arabidopsis* (Willett, 1996), maize (Lund et al., 1998), tomato (Liu & Shono, 1999), alfalfa (Lee et al., 2012), cotton (Ma et al., 2016) and Many of these putative mitochondrial small heat shock proteins are deposited in databases. However, functional characterization and detailed experimental evidence on their role are rather limited.

1.5. Basis of the present research work

Eggplant is a very nutritious and common domestic vegetable crop in Bangladesh and India, cultivated largely for cash income by resource-poor small and marginal farmers (Kumar, 2020). Despite accounting for almost 25% of Bangladesh's total vegetable growing area, the yield (9.2 t/ha) is determined to be much lower than the worldwide mean of 26.1 t/ha (Khatun et al., 2022). The occurrence of viral, bacterial, and fungal infections, as well as abiotic stressors, are key reasons for the decrease in aubergine output in Bangladesh. As global temperature rises heat stress poses severe threats to crop production, notably vegetable crops like Eggplant in the Indian subcontinent. It is predicted that temperatures would rise by approx. 2.2°C annually across South Asia, which will cause substantial damage to eggplant's growth and performance and also affect the production of other crops as well (Khatun et al., 2021). As a consequence, investigating the impact of abiotic stresses particularly high temperature (HT) on crop plants' growth and performance is essential to boost agricultural production and assure our food security, specifically for those whose livelihoods directly depend on agriculture in the South Asian region.

Therefore, in our study, we aimed to investigate the physiological roles of a mitochondrial small heat shock protein along with its defensive role under various abiotic stress conditions from a novel source of the eggplant.

1.6. Objectives of the study

To investigate the function of Eggplant mitochondrial small heat shock protein in response to various abiotic stresses, the present study was undertaken with the following objectives:

1. Cloning, expression profiling, subcellular localization and biochemical characterization of mitochondrial small heat shock protein (SmsHSP24.1)
2. Functional characterization of SmsHSP24.1 through transgenic overexpression

3. Global transcriptome profiling to evaluate overall molecular changes in transgenic Eggplant
4. Development of mitochondrial small heat shock protein knockout Eggplant lines using CRISPR/Cas9 genome editing system
5. Understanding the transcriptional regulation of mitochondrial small heat shock protein in Eggplant

2. Review of Literature:

2.1. Small heat shock proteins (sHSPs) and their importance in plant growth and development

Small heat shock proteins (sHSPs) are a highly conserved family of molecular chaperones that are ubiquitously expressed across all kingdoms of life, including plants (Haslbeck, 2019). Across the domain of life, sHSPs play a key role in the protection of cells against a wide range of environmental stresses, including high and low temperatures, drought, salinity, and heavy metal toxicity (Ul Haq et al., 2019). As the global climate continues to change, these environmental stresses are becoming more frequent and severe, highlighting the importance of sHSPs in plant adaptation and survival.

As a molecular chaperones, sHSPs play an essential role in maintaining protein homeostasis under normal and stressful conditions in plants (Park et al., 2015). They participate in a variety of physiological activities, such as growth and development and reproduction. Protein homeostasis is maintained by sHSPs by attaching partially folded or misfolded proteins and blocking their aggregation (Verling, 1991; Boston et al., 1996). This chaperone-like activity of sHSPs also plays a crucial role in the regulation of cellular metabolism by modulating enzyme activity and folding (Zhao et al., 2020). For example, sHSPs can interact with enzymes involved in the Calvin cycle, such as Rubisco activase, and prevent their aggregation (Waters et al., 1996; Lopes-Caitar et al., 2016). Plant cytosolic sHSPs are reported to interact with malate dehydrogenase (MADH) enzymes in different plant species like peas, *Arabidopsis*, and wheat on the *in vitro* assays (Carra et al., 2019). sHSPs in *Arabidopsis* have also been reported to interact with the enzyme Fructose-bisphosphate Aldolase (FBA) *in vivo* as well (McLoughlin et al., 2016). Under stress conditions sHSPs chaperon-like activity protects the protein's native structure from thermal/oxidative denaturation or heavy metal toxicity (Sun et al., 2016). sHSPs have been found to play a crucial role in various developmental processes in plants. One of the most studied processes is seed development. sHSPs have been found to accumulate in the seed coat during seed maturation and are involved in protecting the developing embryo from various stresses (DeRocher and Vierling, 1991; Wehmeyer et al., 1996; Chauhan et al., 2009). sHSPs are also reported to be involved in embryogenesis. During

embryogenesis, the developing embryo undergoes various stresses such as heat, dehydration, and oxidative stress. sHSPs have been found to accumulate in developing embryos and are involved in protecting the embryo from these stresses (Young et al., 2001). This has been demonstrated in soybean, sHSPs have been found to accumulate in developing embryos, and their overexpression results in an increase in embryo survival under heat stress (Wei et al., 2020). Furthermore, sHSPs have been found to play a role in organogenesis. In tomatoes, the involvement of sHSPs is play an important role in regulating fruit development and ripening (Arce et al., 2020). The study reported that sHSPs expression increases during fruit development and is associated with an increase in fruit size and weight. Similarly, demonstrated that mitochondrial matrix localized GhHSP24.7 play a crucial role in temperature-dependent seed germination in *Gossypium hirsutum* (Ma et al., 2019). The researchers showed that GhHSP24.7 regulate ROS production during seed germination by modulating mitochondrial electron transport chain protein cytochrome C_1.

Apart from their significant role in normal growth and development, sHSPs are one of the major armor of plants against different biotic and abiotic stresses. The upregulation of sHSPs in response to different biotic stress has been widely reported in various plant species (Dodds and Rathjen,2010; Khan et al., 2021). For instance, *AtsHSP17.4C1* was reported to be upregulated in *Arabidopsis thaliana* in response to *Pseudomonas syringae* infection, a bacterial pathogen that causes leaf spot disease in plants (Sewelam et al., 2019). Interestingly in another study, the downregulation of several sHSPs genes has been reported in *Arabidopsis* during *Spodoptera littoralis* herbivory (Bricchi et al., 2012). *NtsHSP17* was found to be differentially expressed in *Nicotiana* plants and played a significant role in the resistance against *Ralstonia solanacearum,* a bacterial pathogen that causes wilt disease in plants (Miambo et al., 2007). Similar upregulation of sHSPs upon fungal, insect, or viral infection has also been reported in different plants (Mayer et al., 2013; Dev, 2021; Li et al., 2015). In tomatoes, the sHSP gene was found to be induced by fungal infection with *Botrytis cinerea*, which causes grey mold disease in plants (Cantu et al., 2009) as well as viral infection with *Turnip mosaic* virus in Chinese cabbage (Lyu et al., 2020). Similar fungal infection-mediated upregulation has also been reported in wheat plants in response to *Fusarium graminearum* infection, a fungal pathogen that causes Fusarium head blight disease in crops (Wang et al., 2017). sHSPs were also found to be induced during a viral infection such as in potato plants, sHSPs were reported

to be upregulated in response to Potato virus Y infection, a plant virus that causes potato tuber necrotic ringspot disease. Apart from that, the involvement of sHSPs in plant defense against insects has been also reported in several plant species. For example, in tomato plants, sHSPs were upregulated in response to whitefly infestation, a major pest that causes crop damage worldwide. Similarly, induce upregulation of sHSPs was also reported in *Arabidopsis thaliana* upon infestation with aphids, a common pest that causes significant damage to crops.

Other than biotic stress, the significant role of sHSPs in different abiotic stresses such as heat, cold, drought, salinity, heavy metals, oxidative stress, etc. has been extensively studied in different plant species (Table 2.1). One example of the importance of sHSPs in plant adaptation to climate change is the response of *Arabidopsis thaliana* to high-temperature stress. In the most recent research, it appeared that overexpressing the Arabidopsis sHSP gene AtHsp17.6 improved the plants' ability to withstand heat stress and lifted their thermotolerance. (Bartling et al., 1992). The scientists went on to demonstrate that AtHsp17.6's capacity to stabilize the photosynthetic apparatus and inhibit the buildup of ROS (reactive oxygen species) under heat stress was what caused the improved thermotolerance. Similar to how sHSPs have been demonstrated to be crucial in plants' responses to drought stress. Wang et al. (2020) demonstrated that the overexpression of the sHSPs gene OsHSP18.0 in rice improved the plant's drought tolerance by increasing its ability to maintain leaf water potential and protect against oxidative damage. The authors further showed that OsHSP18.0 protected the photosynthetic machinery from damage caused by drought stress.

One of the unique features of sHSPs is their localization to specific organelles, including the mitochondria, chloroplasts, and endoplasmic reticulum. These organelle-localized sHSPs play a critical role in protecting these organelles against different stresses. For example, several recent reports suggest that mitochondrial sHSPs are essential for the maintenance of mitochondrial protein and ROS homeostasis and thus maintain mitochondrial functions under different stress conditions. However, their modus operandi at the molecular level is not yet clear and needs more investigation to understand their interactions with different pathways. Mitochondrial sHSPs also possess evolutionary significance as they specifically evolved in plant linage except for *Drosophila melanogaster* and *Toxoplasma gondi*, the only other two eukaryotes currently known to possess mitochondrial sHSPs (de Miguel et al., 2005; Morrow et al., 2000). To date, only a few studies demonstrated the physiological role of mitochondrial

sHSPs in different plants. For instance, a study conducted by Downs, and Heckathorn (1998) demonstrated the role of mitochondrial sHSPs as a thermo-protectant of NADH: ubiquinone oxidoreductase complex (complex I) and essentially the electron transport chain during heat stress in plants. Similar thermotolerance was also reported in different plant species such as tobacco (Sanmiya et al., 2004), tomato (Banzet et al., 1998), maize (Hu et al., 2010), rice (Kim et al., 2020), and pea (Avelange-Macherel et al., 2019). Mitochondrial sHSPs have also been implicated in the regulation of ROS (reactive oxygen species) homeostasis, which plays a crucial role in mitochondrial function. A study conducted by Wang et al., (2019) demonstrated that the overexpression of the tomato mitochondrial sHSPs, LeHSP21 in tobacco plants resulted in increased antioxidant enzyme activity and reduced ROS levels under heat stress (Wang et al., 2019).

Despite the importance of mitochondrial sHSPs in maintaining mitochondrial function and cellular homeostasis under stress conditions, their molecular mechanisms of action remain unclear. Further investigation is required to understand the interactions between mitochondrial sHSPs and different pathways involved in mitochondrial protein and ROS homeostasis. Apart from maintaining ROS homeostasis under stress conditions, recent studies showed the importance of mitochondrial sHSPs in temperature-dependent seed germination via regulation of ROS production. Ma et al., (2019) demonstrated that mitochondria localized GhHSP24.7 modulates cytochrome C/C1 production in the mitochondrial electron transport chain and induces the generation of ROS and consequently accelerates endosperm rupture and promotes seed germination in a temperature-dependent manner. They further validate their finding by overexpressing the homolog of *GhHSP24.7* in *Arabidopsis* and tomato indicating the critical physiological role of mitochondrial sHSPs in plants. Another study conducted by Wang et al., (2021) revealed that the *Arabidopsis* mitochondrial sHSPs (HSP23.6) interact with the mitochondrial protein FIS1, a key player in mitochondrial fission and fusion, suggesting a potential role of mitochondrial sHSPs in regulating mitochondrial dynamics (Wang et al., 2021). Although, few studies in recent times elucidate the molecular mechanism of mitochondrial sHSPs-mediated plant development under normal and stress-induced conditions; many investigations are still needed to further understand the significant role of mitochondrial sHSPs in the plant lineage. In conclusion, sHSPs especially mitochondria-localized sHSPs play a critical role in the adaptation and survival of plants under changing

environmental conditions. With the ongoing changes in the global climate, the importance of sHSPs in plant stress responses is likely to increase. Research on sHSPs in plants is therefore essential for the development of new strategies to improve crop productivity and resilience under changing environmental conditions.

Table 2.1 Type of different small heat shock proteins (sHSPs) and their source under abiotic stresses

Type of small heat shock proteins (sHSPs)	Nature of Stress	Source	Reference
sHSP17.2	Heat stress	*Rosa chinensis*	(Jiang et al., 2009)
sHSP17.8	Heat stress	*Camellia sinensis*	Wang et al., 2017)
sHSP17.6	Heat stress	*Brassica napus*	(Young et al., 2004)
sHSP17	Heat stress	*Creeping bentgrass*	(Sun et al., 2016)
sHSP20	Dehydration stress	*Oryza sativa*	(Bhushan et al., 2011)
sHSP24.1	Dehydration stress	*Oryza sativa*	(Agrawal et al., 2016)
sHSP17.7	Dehydration stress	*Oryza sativa*	(Sato & Yokoya, 2008)
sHSP17.5	Dehydration stress	Barley	(Reddy et al., 2014)
sHSP17.7	Heavy metal	Carrot	(Park et al., 2013)
sHSP23.1	Heavy metal	Popular	(Yang et al., 2015)
sHSP16.9	Salt stress	*Oryza sativa*	(Yu Jin et al., 2014)
sHSP6.45	Salt stress	*Lilum davidii*	(Mu et al., 2013)
sHSP17.8	Salt stress	*Rosa chinensis*	(Jiang et al., 2009)
sHSP23.6	Flood stress	*Solanum lycopersicum*	(Hüther et al., 2016)
sHSP17.6	Oxidative stress	*Arabidopsis thaliana*	(Scarpeci et al., 2008)
sHSP18.6	Combined stress	*Oryza sativa*	(Wang et al., 2015)
sHSP16.9	Combined stress	*Oryza sativa*	(Yu Jin et al., 2014)
sHSP23.7	Combined stress	*Oryza sativa*	(Zou et al., 2012)
sHSP17.6	Combined stress	*Arabidopsis thaliana*	(Sun et al., 2001)
sHSP17.5	Combined stress	*Nelumbo nucifera*	(Zhou et al., 2012)
sHSP17.7	Cold stress	Peach	(Zhang et al., 2011)
sHSP17.5	Cold stress	Chestnut	(Soto et al., 1999)
sHSP17.4 and 17.6	Cold stress	Tomato	(Sanchez-Bel et al., 2012)

2.2. Evolution and classification of sHSPs in plants

In the last few years, there has been a lot of research done on the evolution of sHSPs in plants. Since the sHSPs gene family is largely conserved among various plant species, it likely has a long evolutionary history (Waters, 2008). According to a phylogenetic study, conserved domains are shared by sHSPs across all phyla. The major chaperone function of sHSPs is mediated by the presence of a conserved -crystallin domain (ACD) of roughly 80–100 amino acids (de la Fuente and Novo, 2022). The ACD is comprising a compact, seven-stranded β-sandwich that forms the structural core of all sHSPs (Figure. 2.1 A, B).

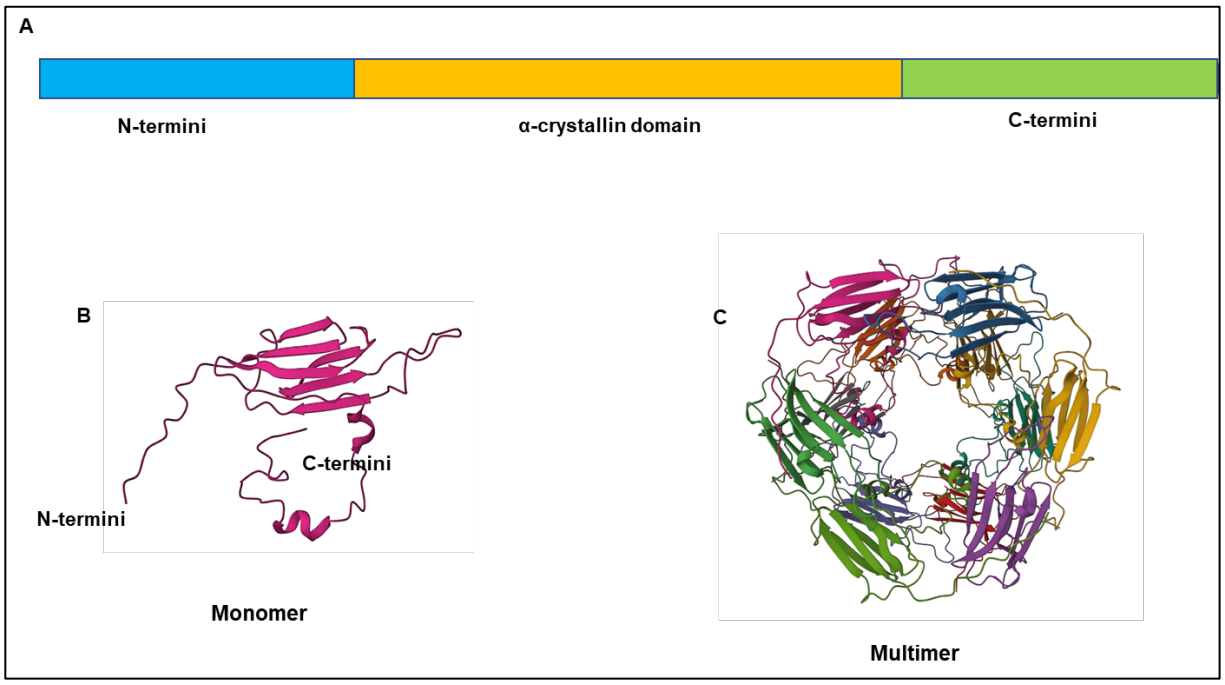


Figure 2.1: Structural appearance of plant small heat shock proteins (sHSPs). (A) The N-terminal domain (NTD) of sHSPs is labeled as blue, the C-terminal extension (CTE) is labeled as green and the Alpha-crystallin domain (ACD) is labeled as yellow. (B) Monomeric assembly of plant sHSPs. (C) Multimeric assembly of plant sHSPs. Structures based on AtHSP21 are retrieved from PDB (Yu et al., 2021).

The ACD is flanked by divergent variable length N- terminal domain (NTD, typically <85 amino acids) and short C-terminal extensions (CTE) that are thought to be involved in sHSPs oligomerization, substrate binding, and subcellular localization (Waters and Vierling, 2020)

(Figure. 2.1 C). Cryo-electron microscopy studies in recent times proved that several monomers of the same sHSPs can form different multimeric oligomerizations under different stress conditions (Yu et al., 2021) (Figure 2.2). The sHSPs gene family has undergone multiple gene duplication and loss events during plant evolution, which has contributed to the diversity of sHSPs in different plant species. One of the earliest studies on the evolution of sHSPs in plants was conducted by Waters et al. (2008). The study analyzed the sHSPs gene family in a wide range of plant species, including mosses, ferns, gymnosperms, and angiosperms. The study found that sHSPs can be divided into 10 subfamilies, which have different evolutionary origins. The study also found that sHSPs are highly conserved within each subfamily, but there is considerable sequence divergence between subfamilies. Another study by Yu et al., (2016) investigated the evolution of sHSPs in the *Solanaceae* family, which includes tomato, potato, and pepper. The study found that sHSPs in the *Solanaceae* family can be divided into 13 with different evolutionary origins. These studies suggest that sHSPs genes have undergone multiple gene duplication and loss events during plant evolution, which has contributed to the diversity of sHSPs in different plant species. Based on this, sHSPs can be divided into several subfamilies based on their sequence similarity. These subfamilies have evolved independently in different plant lineages. Earlier Verling et al., (1991) reported two classes of sHSPs in plants, *i.e.*, class I and Class II both as cytosolic. Later, Waters et al., (1996) classified plant sHSPs into six broad groups from class I to Class VI where Class I, Class II, and Class III are cytosolic or nuclear and Class IV, Class V, and Class VI are targeted to plastids, the endoplasmic reticulum and the mitochondria respectively. The Class I sHSPs are the most abundant and widely distributed sHSPs in plants (Guan et al., 2004). They are characterized by the presence of a conserved IXI/V motif in the C-terminal domain and have a molecular weight of around 15-30 kDa. Class I sHSPs are typically located in the cytoplasm and nucleus and have been extensively studied in various plant species, including *Arabidopsis*, rice, maize, and wheat (McLoughlin et al., 2016). In *Arabidopsis*, there are 17 Class I sHSPs, which are further divided into four subclasses based on their sequence similarity (Waters et al., 2008). Class I sHSPs are involved in various stress responses, including high temperature, drought, salt, and oxidative stress (Sun et al., 2002; Waters et al., 2008; Wang et al., 2017). Class II sHSPs are characterized by the presence of a conserved I/LWE motif in the C-terminal domain and have a molecular weight of around 20-30 kDa. Class II sHSPs are mainly localized

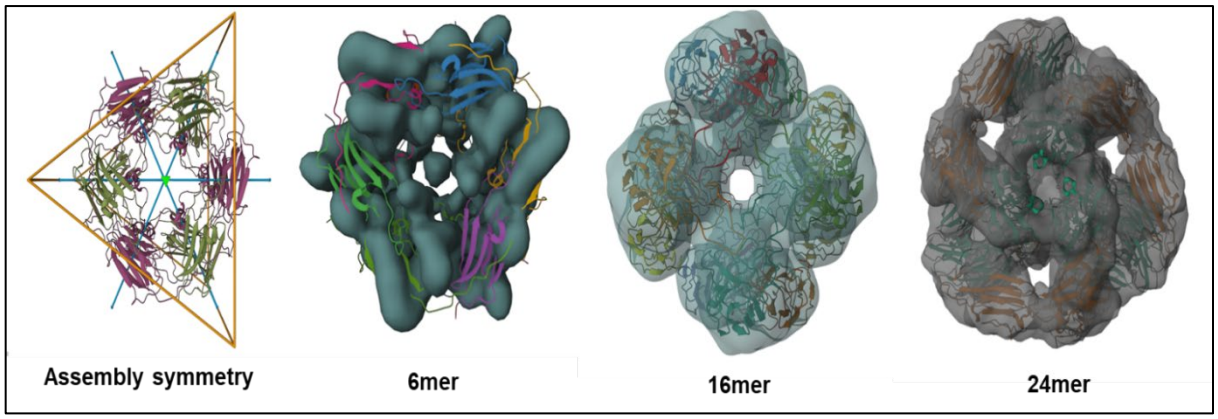


Figure 2.2: Different Polyhedral assembles or multimeric structures of plant small sHSPs observed under a cryo-electron microscope (cryo-EM). Structures based on AtHSP21 are retrieved from PDB (Yu et al., 2021).

in the cytoplasm, and have been identified in various plant species, including rice, *Arabidopsis*, and tomato (Kuang et al., 2017; Zhang et al., 2018; Löw et al., 2000). In rice, there are 23 Class II sHSPs genes are reported, which are further divided into four subclasses based on their sequence similarity (Sarkar et al., 2009). Class II sHSPs are involved in various stress responses, including high temperature, drought, and salt stress (Sarkar et al., 2009). Class III sHSPs are characterized by the presence of a conserved PWI motif in the C-terminal domain and have a molecular weight of around 17-25 kDa. Class III sHSPs are mainly localized in the cytoplasm and have been identified in various plant species, including *Arabidopsis*, tomato, and rice (Wan et al., 2016; Siddique et al., 2003; Mani et al., 2015). The CIV subfamily is characterized by the presence of the V/IIP motif in the ACD and includes sHSPs from *Hevea brasiliensis* (Pasaribu et al., 2021). Interestingly the subfamily V (CV) is reported to be found only in some eudicots and not yet functionally characterized well (Waters and Vierling, 2020). The CVI subfamily is characterized by the presence of the L/M and G/Y motifs in the ACD and includes sHSPs from Arabidopsis (Basha et al., 2010).

2.3. Classification based on subcellular localization

sHSPs can also be classified based on their subcellular localization. sHSPs are present in various subcellular compartments, including the cytosol, chloroplasts, mitochondria, endoplasmic reticulum, peroxisomes, and nucleus. The subcellular localization of sHSPs is

closely linked to their function in stress response and protein quality control. With the ever-increasing well-annotated plant genome sequence in recent times, Waters and Vierling (2020) further classified plant sHSPs into a total of 11 different groups. They reported six classes of sHSPs that are targeted to a different membrane-bound organelle in the plant cell such as chloroplasts (CP), mitochondria (MTII), the endoplasmic reticulum (ER), peroxisomes (PX), and the nucleus (CIII), as well as proteins reported to be dual-targeted to both mitochondria and chloroplasts (MTI/CP) (Basha et al., 2010). The remaining five classes are reported to be cytosolic; they lack a specific targeting signal, although they also may be found at times in the nucleus (CI, CII, CIV, CV, and CVI) (Siddique et al., 2008; Waters et al., 2008).

2.4. Molecular mechanism of mitochondrial sHSPs in plants

Like other small heat shock proteins, mitochondrial small heat shock proteins also act as molecular chaperones that play a critical role in maintaining protein and organelle integrity under various abiotic stresses, including heat stress. In addition to preventing protein aggregation and promoting protein refolding, mitochondrial sHSPs have been shown to modulate the redox state and regulate the transcriptional stress response. Under stress conditions, proteins may become unfolded or misfolded, leading to their aggregation and dysfunction. Mitochondrial sHSPs bind to unfolded or misfolded proteins, preventing their aggregation and promoting their refolding (Avelange Macherel et al., 2019). This prevents the accumulation of damaged proteins, which otherwise can lead to organelle dysfunction and cell death. Mitochondrial sHSPs have also been shown to modulate the redox state under stress conditions. Reactive oxygen species (ROS), which can oxidatively damage proteins, lipids, and DNA, are mostly produced by mitochondria (Lambert and Brand, 2009). For example, in rice (*Oryza sativa*), the mitochondrial sHSPs OsHSP24.1 was found to protect mitochondrial function and improve heat tolerance by reducing ROS accumulation (Kim et al., 2020). Studies have shown that mitochondrial sHSPs have been shown to interact with other proteins involved in a complex network of stress-responsive genes that help to protect against stress-induced damage. Mitochondrial sHSPs have been shown to regulate the expression of stress-responsive genes by interacting with transcription factors and co-regulators. For example, in *Arabidopsis thaliana*, the mitochondrial sHSPs AtHSP23.6 was found to interact with a transcription factor that regulates the expression of stress-responsive genes, promoting their expression and

improving heat tolerance (Nishizawa et al., 2006). Recently, genome-wide identification and characterization of mitochondrial sHSPs have been extensively studied in different plants, however; further investigation is still needed to reveal their evolutionary significant functional and physiological role in higher plants. The mechanism by which mitochondrial sHSPs prevent protein aggregation is thought to involve their ability to bind to unfolded or misfolded proteins, preventing their aggregation and promoting their refolding. This mechanism has been demonstrated *in vitro* using purified recombinant mitochondrial sHSPs and model substrate proteins such as citrate synthase and malate dehydrogenase. In addition, studies in yeast have shown that expression of plant mitochondrial sHSPs can prevent the aggregation of a misfolded mitochondrial protein and improve mitochondrial function. In *Arabidopsis thaliana*, the mito-AtHSP23.6 and AtHSP23.7 have been shown to form oligomeric assembles *in vitro*, consisting of 12 to 16 subunits (Krüger et al., 2012). Studies revealed that these oligomers have a dynamic structure, often poly-hexagonal to dodecamer with subunits exchanging with each other over time (Figure 2). Upon heat stress, the oligomeric structure of mitochondrial sHSPs changes, with the formation of larger oligomers consisting of up to 48 subunits. Several studies demonstrated that these larger oligomers have been proposed to have a greater ability to prevent protein aggregation and protect mitochondrial proteins from stress-induced damage (Haslbeck et al., 2015). Thus, it has been proposed that mitochondrial sHSPs prevent the toxicity of unfolded proteins by sequestering them into large oligomeric complexes under stress conditions in plants. Studies also suggest that these oligomeric complexes of mitochondrial sHSPs are flexible which allows them to respond to different cellular stresses and maintain mitochondrial function. In conclusion, the molecular mechanisms underlying mitochondrial sHSPs' function are complex and involve the formation of large oligomeric complexes that interact with unfolded or misfolded proteins. These oligomers are dynamic, and their size and composition can change in response to stress or other cellular signals, allowing mitochondrial sHSPs to respond to different cellular stresses and maintain mitochondrial function. This concept is still evolving and further research on mitochondrial sHSPs will deepen our understanding of their molecular mechanisms and their role in plant physiology.

2.5. ROS as a signaling molecule in plants

Highly reactive chemicals known as reactive oxygen species (ROS) are produced by plants during routine metabolic activities as well as in reaction to environmental stressors including heat, drought, and pathogen attacks. Recent studies have demonstrated that ROS can function as signaling molecules that regulate a variety of cellular functions, including growth, development, and stress responses (Figure. 2.3) (Mittler, 2017). However, more investigation needs to explore the role of ROS as signaling molecules in plants. Studies suggest that ROS can act as signaling molecules by modifying the redox state of proteins, which leads to changes in protein activity, stability, and localization (Shao et al., 2012). Moreover, ROS can also modulate the expression of genes involved in various biological processes. Several studies have reported the involvement of ROS in different signaling pathways, including hormonal signaling pathways (Huang et al., 2019), calcium signaling pathways (Mori and Schroeder, 2004; Marcec et al., 2021), and MAP kinase pathways (Jalmi and Sinha, 2015). are well demonstrated in recent years. Several studies suggest that hormonal signaling pathways are regulated by ROS in plants. For example, ROS can modulate the levels of abscisic acid (ABA) and ethylene, which are key regulators of plant growth and development (El-Maarouf-Bouteau et al., 2015). In *Arabidopsis*, the application of exogenous ROS has been shown to induce ABA biosynthesis, which leads to the upregulation of stress-responsive genes (Pei et al., 2000). Kwak et al., (2003) also reported similar regulation of the ABA signaling pathway specifically the expression of ABA-responsive genes such as *ABI1*, *ABI2*, and *ABF3* by ROS under drought conditions. Similarly, ROS can also regulate the levels of auxin hormone, which plays a critical role in plant growth and development (Yuan et al., 2013). Studies further suggest that cytosolic ROS accumulation can lead to altering *PIN* gene expression important for auxin transport and distribution in plants (Grunewald and Friml, 2010). Furthermore, ROS can also modulate the levels of Jasmonic acid (JA), which is a key regulator of plant defense responses. ROS have been reported to regulate the expression of genes involved in ethylene and JA signaling pathways, including *ERF*, *MYC*, and *JAZ* genes (Mittler et al., 2011). In *Arabidopsis*, the application of exogenous H2O2 has been shown to induce the expression of JA-responsive genes, which leads to the activation of defense responses (Davletova et al., 2005). The calcium (Ca^{2+}) signaling pathway is known as one of the well-established secondary messengers in

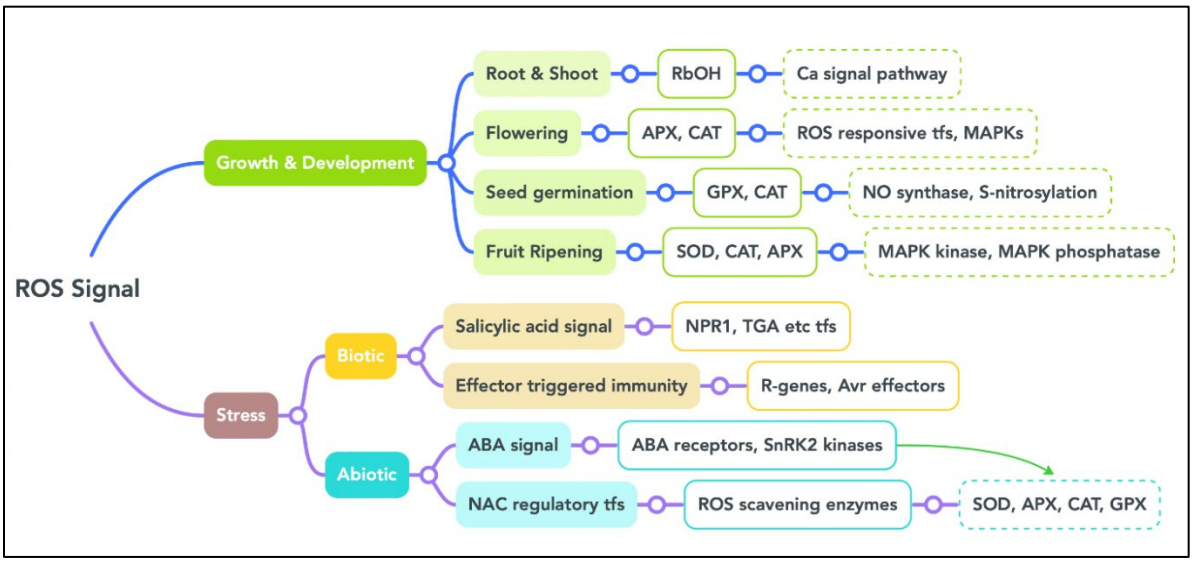


Figure 2.3: Reactive oxygen species (ROS) signaling in plant cells. ROS is a common signal produced in normal metabolic processes and response to different biotic and abiotic stresses in plant cells. This signal is subsequently transmitted to the cell and activated different antioxidant enzymes. ROS also involved in different signaling pathways, including hormonal signaling, calcium signaling, and MAP kinase pathways which lead to the activation of transcription reprogramming and ultimate defense responses in the plant**.**

plant cells under both stress and normal growth conditions (Tuteja and Mahajan, 2007). Studies demonstrated the crucial role of Ca^{2+} ions as signaling molecules in plants. ROS can modulate calcium signaling by activating calcium channels and pumps, which leads to an increase in intracellular calcium levels. In *Arabidopsis*, the application of H2O2 has been shown to induce an increase in intracellular calcium levels, which leads to the activation of calcium-dependent protein kinases (CPKs) (Kobayashi et al., 2007). In rice, the application of H2O2 has been shown to induce the expression of *ORS1*, which is a NAC transcription factor binding protein and regulates senescence (Balazadeha et al., 2011). Like the Ca^{2+} signaling pathway, MAP kinase pathways are also regulated by ROS in plants. MAP kinases are key signaling molecules in plants, which play a critical role in regulating various cellular processes. ROS can modulate MAP kinase pathways by activating MAP kinase cascades, which leads to changes in gene expression (Son et al., 2011). In *Arabidopsis*, the application of H2O2 has been shown to activate *MPK6* and *MPK6*, which are members of the MAP kinase family (Rentel et al., 2004).

2.6. Impact of ROS in transcriptome reprogramming

Transcriptional reprogramming is a key response to counter various stresses in plants and involves the differential expression of stress-responsive genes crucial for growth and development. ROS can directly or indirectly affect transcriptional reprogramming through a range of mechanisms, including the regulation of transcription factors, epigenetic modifications, and alternative splicing of pre-mRNA. ROS can directly regulate transcription factors by modifying their activity or stability. For example, the heat shock transcription factor HsfA1a can be activated by ROS in response to heat stress, leading to the upregulation of heat shock genes under heat stress conditions (Mittler, 2006). Similarly, the transcription factor WRKY33 can be activated by ROS in response to pathogen infection, leading to the upregulation of pathogen-responsive genes (Marchive et al., 2013). In *Arabidopsis thaliana*, ROS have been shown to activate the transcription factor WRKY33, which in turn regulates the expression of stress-responsive genes such as *CBF3*/*DREB1A* and *COR15A*/*B* under cold stress conditions (Sun et al., 2020). Similarly, in tomatoes, ROS generated by salt stress activates the transcription factor *SlNAC4*, which binds to the promoter of stress-responsive genes and enhances their expression during stress conditions (Zhu et al., 2014). Studies suggest that *Arabidopsis* basic leucine zipper (bZIP) TF, *AtbZIP10*, is activated in response to oxidative stress induced by hydrogen peroxide (H2O2). The activation of *AtbZIP10* further leads to the upregulation of several downstream stress-responsive genes, including ROS scavenging enzymes (Miao et al., 2006). Similarly, the *Arabidopsis* NAC (NAM/ATAF/CUC) TF, *AtNAC019*, is reported to be induced by ROS, and overexpression of *ANAC019* enhances the tolerance of plants to oxidative stress (Tran et al., 2010). Other plant species have also been linked to the engagement of ROS in the controlling of TFs. For example, in rice, the bZIP TF, *OsbZIP23*, is upregulated in response to H2O2, and overexpression of *OsbZIP23* enhances the tolerance of plants to salt stress (Xiang et al., 2008). These findings suggest that ROS plays a critical role in the regulation of TFs in response to different abiotic stresses. Apart from directly regulating the activity of TFs, ROS can also indirectly modulate transcriptional reprogramming through the activation of *DNA methyltransferases*, *histone acetyltransferases*, and *histone deacetylases*, thereby affecting the chromatin structure and gene expression (Sewelam et al., 2016). Significant regulators of gene expression in plants include epigenetic alterations such

as DNA methylation and histone modifications. For instance, it has been revealed that ROS controls the expression of the DRM2 and DME genes, which are involved in DNA methylation and demethylation pathways (Kim et al., 2019). Studies have also shown that the generation of ROS under drought stress may alter DNA methylation patterns, which may impact gene expression and aid in the development of drought resistance (Ding et al., 2014). These findings suggest that ROS can affect epigenetic modifications in different plant species, leading to changes in gene expression in response to different abiotic stresses. Alternative splicing of pre-mRNA is another mechanism by which ROS can affect transcriptional reprogramming. Alternative splicing of a gene can generate several mRNA transcripts from a single pre-mRNA molecule, leading to the production of different protein isoforms with distinct functions (Miura et al., 2012). Few recent studies suggest that ROS can affect alternative splicing by regulating the activity of splicing factors, which are proteins that bind to pre-mRNA and determine which exons are included or excluded from the final mRNA transcript. For example, in *Arabidopsis*, treatment with the ROS-generating compound methyl viologen was shown to alter the expression of genes involved in alternative splicing (Wang et al., 2019). Similarly, in tomatoes, ROS was found to regulate alternative splicing of genes involved in responses to abiotic stress (Srivastava et al., 2009). These studies suggest that although overproduction of ROS might be detrimental to plant cells but in a reduce basal level production, ROS may act as a crucial signal molecule in plants and can change the overall transcriptome. Further studies necessary to understand this new concept of ROS will enlighten new mechanisms of ROS in plant growth and development under both normal and stress conditions. These mechanisms may act in concert to regulate gene expression in response to stress, allowing plants to adapt to changing environmental conditions. Understanding the mechanisms by which ROS regulates gene expression will be critical for developing strategies to enhance plant stress tolerance and improve crop yields.

2.7. Role of ROS scavengers

Overall, ROS also plays a critical role in mediating various physiological and developmental processes in plants, including growth, and development. However, the severity of response to different biotic and abiotic stresses often leads to over production of various ROS, such as superoxide (O2.-), hydrogen peroxide (H2O2), and hydroxyl radical (OH.), and

ultimately leads to programmed cell death (PCD) (Petrov et al., 2015). Since ROS production is tightly controlled in plant cells, a variety of ROS scavenging systems, including enzymes like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), as well as non-enzymatic antioxidants like ascorbate (AsA), and glutathione (GSH), work to keep ROS levels within a specific range. The essential enzyme Superoxide Dismutase (SOD) controls the amount of ROS in plants. Superoxide radicals are changed into hydrogen peroxide by SOD, which is then cleaned up by the enzymes catalase and ascorbate peroxidase (APX) (Mittler, 2017). According to the different metal cofactors in the catalytic site, SODs can be divided into four major types: Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD (Abreu & Cabelli, 2010). Fe-SOD and Mn-SOD occur mainly present in lower plants, whereas Cu/Zn-SOD is mainly found in higher plants (Xia et al., 2015). In *Arabidopsis thaliana*, a total of seven cDNAs and genes for SOD were reported which were further classified as three *Cu/Zn-SODs* (*CSD1*, *CSD2*, and *CSD3*), three *Fe-SODs* (*FSD1*, *FSD2*, and *FSD3*), and one *Mn-SOD* (*MSD1*) (Kliebenstein et al., 1998). Similarly, in a recent study, twenty-six *SOD* genes were identified from the whole genome of wheat, including 17 Cu/Zn-SODs, six Fe-SODs, and three Mn-SODs. Studies have shown that SOD plays a critical role in plant responses to various biotic and abiotic stresses. For example, overexpression of SOD genes has been shown to improve plant tolerance to salt stress in *Arabidopsis* (Wang et al., 2004). Similarly, SOD overexpression in rice has been shown to enhance resistance to blast fungus (Li et al., 2019). CAT is another important enzyme that regulates ROS levels in plants. Hydrogen peroxide is broken down by CAT into water and oxygen. It has been demonstrated that CAT activity rises in response to a variety of abiotic stressors, including heat, salt, and drought (Sofo et al., 2015). In addition, CAT has been shown to play a critical role in the regulation of plant growth and development (Mhamdi et al., 2010). Ascorbate peroxidase (APX) is a heme-containing enzyme that reduces hydrogen peroxide to water using ascorbate as an electron donor (Asada, 1992). APX is localized in the cytosol, chloroplasts, and mitochondria and plays a critical role in scavenging hydrogen peroxide in these organelles (Mittler, 2017). In *Arabidopsis*, there are eight APX isoforms (APX1-APX4) that have different subcellular localizations and are regulated differently (Panchuk et al., 2002; Leng et al., 2021). Studies have revealed that APX is essential for plants to respond to a variety of environmental stresses, including salt, heavy metals, and drought (Koussevitzky et al., 2008). For instance, it has been demonstrated that overexpressing

APX increases sweet potato and tobacco plants' resistance to salt stress (Yan et al., 2016; Badawi et al., 2004). Glutathione peroxidase (GPX) is another important enzyme that scavenges hydrogen peroxide in plants. GPX catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and corresponding alcohols using glutathione (GSH) as a reducing agent (Mittler, 2017). In *Arabidopsis*, seven GPX isoforms have different subcellular localizations and are regulated differently (Rodriguez et al., 2003). Studies have revealed that GPX is essential for plants to respond to a variety of abiotic stressors, including salt, heavy metals, and drought (Miao et al., 2006; Navrot et al., 2006; Passaia et al., 2014). For instance, it has been demonstrated that *Pennisetum glaucum* and soybean plants can tolerate salt stress better when GPX is overexpressed (Islam et al., 2015; Jin et al., 2019). In conclusion, SOD, CAT, APX, and GPX are key enzymes that regulate ROS levels in plants. These enzymes play critical roles in plant responses to various biotic and abiotic stresses. Understanding the regulation and function of these

2.8. Expression regulation of mitochondrial sHSPs in plants

The expression of mitochondrial sHSPs is tightly regulated by various molecular and genetic pathways. One key regulator of mitochondrial sHSPs expression is the heat shock factor (HSF) family of transcription factors. Under heat stress, HSFs attach to heat shock elements (HSEs) located in mitochondrial sHSPs genes' promoter areas and activate their expression. Studies suggest that the Heat shock factor A (HSFA) family protein, HSFA1s is responsible for the transcriptional activation of heat shock genes, including sHSPs, under heat stress conditions (Liu et al., 2011). In *Arabidopsis*, HSFA2 has been shown to play a critical role in the induction of mitochondrial sHSPs under heat stress. HSFA2 binds to the promoters of mitochondrial sHSPs genes and activates their transcription, thereby increasing the production of mitochondrial sHSPs (Liu and Charng, 2012). In addition to HSFA2, other transcription factors have also been shown to regulate mitochondrial sHSPs expression. For example, the basic leucine zipper (bZIP) transcription factor, *AtbZIP60*, has been demonstrated to activate mitochondrial sHSPs expression under endoplasmic reticulum (ER) stress conditions (Iwata et al., 2005). AtbZIP60 is localized to both the cytoplasm and nucleus, and its translocation to the nucleus is regulated by ER stress. Once in the nucleus, AtbZIP60 binds to the promoters of mitochondrial sHSPs genes and induces their expression. Recent studies

have shown that other stress-responsive transcription factors, such as the DREB2A, MYB, WRKY, and NAC have also been shown to regulate mitochondrial sHSPs expression under stress conditions (Schramm et al., 2008). For example, DREB2A binds to the dehydration-responsive element (DRE) present in the promoter region of sHSPs genes and regulates their expression in response to drought stress (Schramm et al., 2008). WRKY and NAC families can also regulate mitochondrial sHSPs expression under various stress conditions, including drought, salt, and oxidative stress, however much investigation is required at this point. In addition to transcription factors, post-transcriptional and post-translational mechanisms also play important roles in regulating mitochondrial sHSPs expression. RNA-binding proteins (RBPs) are known to play a crucial role in the post-transcriptional regulation of sHSPs genes. For example, heterologous expression of *Cucumis sativus L.* glycine-rich RBPs such as *CsGR-RBP3* regulate the expression of *AtHSP20* mRNAs and regulate their translation efficiency (Wang et al., 2018b). Recent studies also suggest that long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) can target mitochondrial sHSPs mRNAs for degradation or translational repression, thereby regulating sHSPs expression (Khuswaha et al., 2021; Song et al., 2021). On the other hand, protein kinases, such as mitogen-activated protein kinases (MAPKs), can phosphorylate and activate HSFs, leading to the induction of sHSPs expression (Mansour et al., 2008; Mo et al., 2021). Furthermore, epigenetic modifications, such as DNA methylation and histone modifications, have been shown to regulate mitochondrial sHSPs expression under stress conditions. For instance, DNA methylation of the promoter region of mitochondrial sHSPs genes can inhibit their expression, while histone modifications, such as acetylation and methylation, can activate or repress mitochondrial sHSPs expression by altering chromatin structure. Studies in *Arabidopsis* suggest that a small HSP protein named IDM2 (Increased DNA methylation 2) acts as a regulator of DNA methylation and gene silencing other than acting as a heat sensor (Qian et al., 2014). Similarly, another report suggests that the histone deacetylase HDA15 represses the expression of sHSPs in *Arabidopsis* under non-stress conditions (Cortijo et al., 2017; Shen et al., 2019). Under stress conditions, the induction of mito-HSP expression is also known to be regulated by the mitochondrial unfolded protein response (UPRmt) in the *C. elegans* pathway (Liu et al., 2019). UPRmt is a conserved stress response pathway that is activated in response to mitochondrial protein misfolding and is known to regulate the expression of mitochondrial sHSPs. In *Arabidopsis*,

UPRmt is activated by the mitochondrial peptidase ClpP3, which cleaves misfolded mitochondrial proteins and activates the UPRmt pathway (Majsec et al., 2017). However, there is not much study on the involvement of small-HSPs in the regulation of the UPRmt pathway which needs more attention. In conclusion, the regulation of mitochondrial sHSPs expression in plants is a complex process that involves multiple molecular and genetic pathways, including transcriptional, post-transcriptional, post-translational, epigenetic, and alternative splicing mechanisms. Understanding these pathways can provide important insights into how plants respond to stress and how their stress tolerance can be improved.

2.9. Future direction: decoding the role of mitochondrial sHSPs in plants and their potential application in the crop improvement program

Despite the significant progress made in understanding the regulation and function of mitochondrial sHSPs in plants, there are still gaps in our understanding that warrant further investigation. One area that requires further attention is the specific roles of individual mitochondrial sHSPs in response to different stress conditions. While several studies have demonstrated the involvement of mitochondrial sHSPs in various stress responses, the exact roles and mechanisms of action of individual mitochondrial sHSPs remain unclear. Future research should aim to identify the specific targets of each mitochondrial sHSPs and the signaling pathways that regulate their expression in response to different stresses. One important area of research could be the development of transgenic or CRISPR/Cas9 mediated gene-edited plants with enhanced sHSPs expression, which could potentially improve crop yield and quality under stress conditions. Several studies have shown that overexpression of sHSPs in transgenic plants can confer enhanced thermotolerance and stress tolerance. For instance, overexpression of cytosolic sHSPs, *OsHSP18.0*, in rice has been shown to improve thermotolerance and increase yield under heat stress (Ju et al., 2017). Similarly, heterologus overexpression of *Camelina sinensis* mitochondrial sHSPs, in *Arabidopsis* has been shown to improve heat stress tolerance and seed germination under stress conditions (Wang et al., 2017). Another area that requires further exploration is the regulatory mechanisms that control the tissue-specific expression of mitochondrial sHSPs. While some mitochondrial sHSPs are expressed predominantly in certain tissues, such as the embryo or seedling, the underlying

molecular mechanisms that drive this tissue-specific expression remain unclear. Identifying the regulatory elements that control the tissue-specific expression of mitochondrial sHSPs could provide insights into their roles in specific developmental stages and stress responses. Furthermore, the potential applications of mitochondrial sHSPs in crop improvement need to be explored. It has been suggested that sHSPs could be used as biomarkers for stress tolerance in crop breeding programs (Chen et al., 2014). This suggests their potential use in breeding programs aimed at developing heat-tolerant crops. However, the potential trade-offs associated with the overexpression of sHSPs, such as reduced growth or yield under non-stress conditions, need to be addressed. Developing transgenic or genome-edited plants with enhanced mitochondrial sHSPs expression could provide a better understanding of the roles and potential applications of these proteins in crop improvement.

In conclusion, the regulation and function of mitochondrial sHSPs in plants are complex and require further investigation. Future research should aim to identify the specific roles and targets of individual mitochondrial sHSPs, the regulatory mechanisms that control their tissue-specific expression, and the potential applications of these proteins in crop improvement. Such studies are essential for developing a comprehensive understanding of the roles and potential applications of mitochondrial sHSPs in plants.

3. Materials and Methods

With an aim at the functional characterization of mitochondrial small heat shock protein (SmsHSP) along with its protective role under abiotic stress conditions, several methodologies were employed. This section presents a brief account of the materials and methods used in the present study.

3.1. Materials and their sources

The names and sources of plant materials, bacterial strains, vectors, enzymes, chemicals, reagents, etc. used for the isolation and cloning of genes, vector construction, protein expression and purification, plant transformation, transgenic and knockout line screening, physiological examination are listed in table (3.1.) alongside their particular sources.

Table 3.1. List of materials and their sources utilized in this research.

Materials	Sources
1. Plant Materials	
a. BARI begun-4 (*Solanum melongena* L.)	Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh.
2. Bacterial Strains	
a. *Escherichia coli* (Top10) b. *Escherichia coli* (BL21, DE3) c. *Agrobacterium tumefaciens* (EHA105)	New England Biolabs Novagen Stratagene
3. Cloning Vector	
a. pCR-TOPO b. pET28a(+) c. pMDC100 (Plant Transformation Vector) d. Entry Vector 1 (pL12R34H-Ap) e. Entry Vector 2 (pL34R12H-CmR-ccdB) f. PCAMBIA1302	Invitrogen Life Technologies, USA Invitrogen Life Technologies, USA Novagen, Germany, ABRC Xue-Chen Wang lab, China Xue-Chen Wang lab, China Invitrogen Life Technologies, USA
4. Markers	
a. 1kb DNA Ladder b. 100bp DNA Ladder c. Protein Markers	Invitrogen Life Technologies, USA Invitrogen Life Technologies, USA Fermantas, GmbH, Germany Biorad, USA
5. Antibiotics	

Kanamycin, Ampicillin, Streptomycin, Rifampicin, Hygromycin, Cefotaxime, Chloramphenicol, Carbenicillin	Sigma Chemical Company, St. Louis, USA
6. Restriction Endonuclease and other Enzymes	
a. Restriction Enzymes b. LR Clonase c. Klenow, T4 DNA Ligase d. RNase A e. Pfu DNA Polymerase	New England Biolabs Inc. MA, USA Invitrogen Life Technologies, USA Roche Applied Science, Germany Promega Fermentas
7. Membrane and Filter Papers	
a. Nylon Membrane (Nitrocellulose) b. 3mm Whatman Sheet	Amersham Biosciences, UK Whatman Co., USA
8. Antibody	
Secondary Antibody (Anti-rabbit IgG)	Sigma Chemical Company, St. Louis, USA
9. Media and Hormones	
a. MS Medium, Hoagland's Medium, b. LB and YEM Medium c. 6-BAP, NAA, Zeatin, 2,4-D, TDZ	Duchefa Biochemie, Netherland Himedia, Bombay Sigma, St. Louis, USA
10. General Chemicals	
Buffers and Solutions	Sigma Chemical Company, St. Louis, USA; USB (Amersham International plc.), Buckinghamshire, UK; Amersham Biosciences, UK; BIO-RAD, USA; Boehringer Manheim, GmbH, Germany; Promega Life Science, Madison, WI, USA
11. Others	
a. Primer b. X-ray Films c. DIG Nonradioactive Nucleic Acid Labelling and Detection System d. Agarose and PAGE Gel Electrophoresis e. DNA Sequencing	IDT, Belgium Kodak, Amersham Biosciences, UK Roche, Switzerland BIO-RAD Macrogen (South Korea)

3.2. Cloning and expression constructs

All the plasmid vectors used in this research program for cloning and protein expression, fluorescence fused constructs, gateway-compatible entry, and binary vectors are enlisted below.

3.2.1. pCR 4.0 TOPO

For the cloning of PCR amplified gene products pCR 4.0 TOPO vector was used which has both kanamycin and ampicillin as its bacterial selection marker.

3.2.2. Gateway cloning vectors

Gateway cloning vectors are efficiently used to easily shuttle insert DNA from one expression plasmid to another. This vector system embraces a destination vector along with two different types of entry vectors.

3.2.2.1. Gateway entry vectors

The Gateway entry vectors can clone the gene of interest into themselves with the help of restriction enzymes. There are two types of entry vectors in this cloning system i.e., entry vector 1 (pL12R34H-Ap) and entry vector 2 (pL34R12H-CmR-ccdB) (Figure 3.1. A, B). Entry vector 1 has the antibiotic selection marker genes for ampicillin and gentamycin whereas entry vector 2 contains chloramphenicol and gentamycin-resistant genes as selectable markers.

3.2.2.2. pET-28a (+)

Mitochondrial small heat shock protein (SmsHSP) genes were expressed under the T7 expression system of the pET-28a (+) vector. It has a bacterial selection marker gene for kanamycin resistance (Figure 3.1. C).

3.2.2 3. Gateway destination vector

The pMD100 Gateway compatible vector was used as the destination vector for this study. This vector contains a kanamycin-resistant gene (NptII) for bacterial and plant selection markers (Figure 3.1. D).

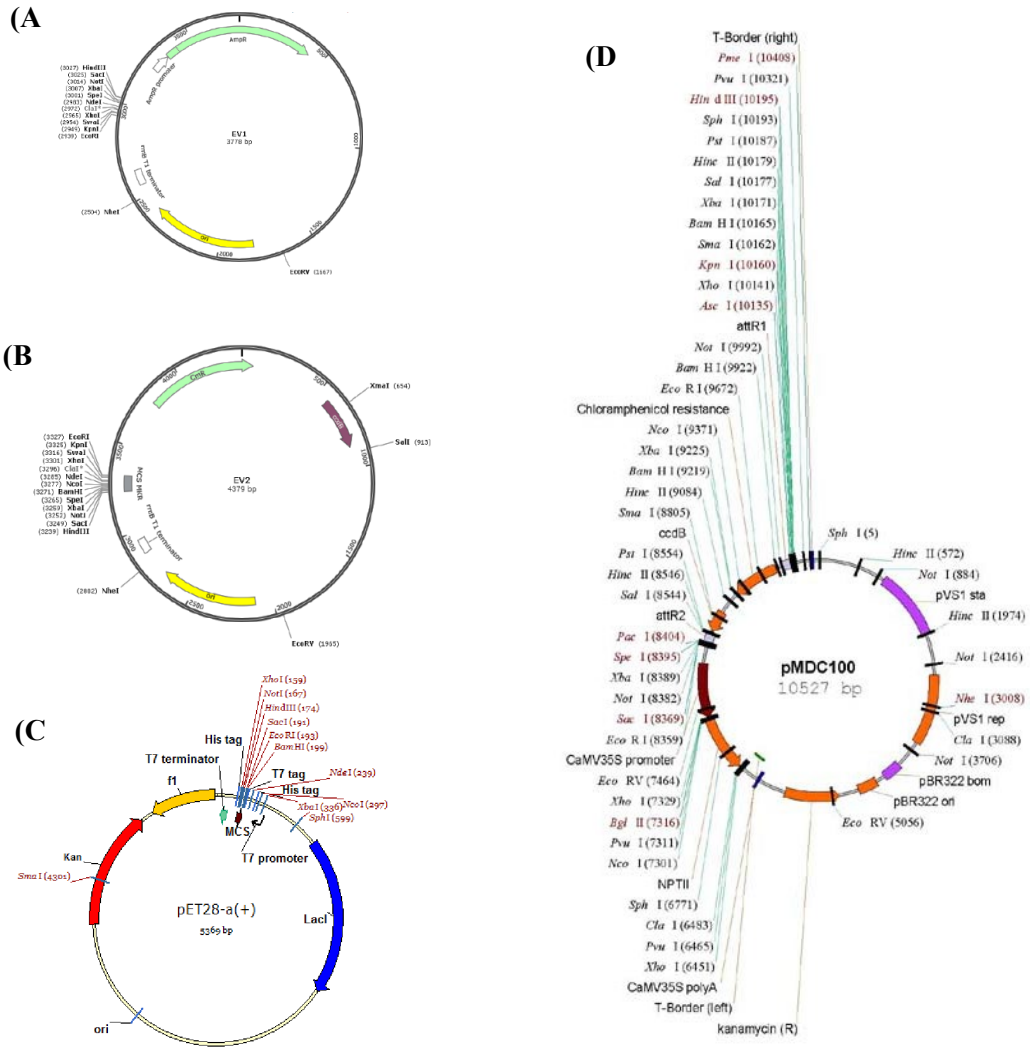


Figure 3.1: The various vectors used for gene cloning. (A) Vector map of entry vector 1 (EV1). (B) Vector map of entry vector 2 (EV2). (C) Vector map of expression vector pET-28a (+). (D) Vector map of destination vector pMDC100.

3.2.3.4. Gateway cloning technique

Gateway cloning is a very easy, flexible, and universal cloning technique. It is constructed on the λ phage site-specific recombination which is used to insert its DNA sequences into *E. coli* genome. This recombination system of phage also controls the decision between lysogenic and lytic pathways (Ptashne, 1992). The recombination is based on the interaction between site-specific attachment (att) sites i.e., attP situated on the lambda

chromosome, and attB, positioned on the E. coli chromosome. These sites serve as a platform for recombination and are recognized by recombination proteins. The recombination protein is a group of many well-characterized proteins/enzymes (Weisberg et al., 1983). These proteins catalyze lambda recombination by bringing together the target sites after binding to them, then cleaving and covalently attaching the DNA. The recombination results in the exchange and ligation of the two fragments of DNA. This multi-round Gateway technique was used to clone or in vitro pyramid the genes with the help of the LR clonase enzyme. The LR reaction performs the recombination of the two attachment sites attL (entry clone) and attR (destination vector) to create a new site attB in the destination vector. At first, the desired DNA fragment is inserted into one of the two entry vector plasmids, with two flanking recombination sequences named attL1 and attL2 to prepare final entry clones with the help of restriction enzymes or BP clonase reactions. Then, the gene cassette in the entry clone is transferred to Gateway compatible destination vector containing the attR recombination sequence by using LR Clonase enzyme mix. The Gateway cloning vector system is composed of a destination vector and two types of entry vectors.

3.3. Bioinformatics analyses of DNA and protein sequences

3.3.1. Sequence identification, phylogenetic analysis, and homology modeling of mitochondrial small heat shock protein (SmsHSP)

Putative eggplant mitochondrial small heat shock protein gene was identified using NCBI expressed sequence tags (ESTs) database. Multiple sequence alignments were performed using the Muscle program (Edgar, 2004), and using MEGAX, a phylogenetic tree was created by the neighbor-joining method, with a bootstrap value of 1000. MODELLER 9v11 (Sali et al., 1995) program was used for homology modeling of mitochondrial small heat shock protein against Arabidopsis mitochondrial small heat shock protein AtHsp21 (Protein databank ID: P31170). The predicted three-dimensional model of mitochondrial small heat shock protein was evaluated using the assessment tools Precheck (Laskowski et al., 1993) and Verify3D (Eisenberg et al., 1997).

3.3.2. Restriction analysis of DNA

The restriction map of a given DNA sequence was analyzed by the NEB cleavage program (http://surl.li/fzroz). This would make it easier to determine the proper restriction enzymes to digest any specific DNA segments for subsequent cloning into various vectors or to make restriction sites in primers.

3.3.3. Domain search

Numerous protein-related databases, including Pfam and NCBI, were used to search for conserved domains among various protein sequences. The fingerprints in the protein sequences were identified by scanning the PRINTS database (Attwood et al., 2003).

3.3.4. Primer designing

Primers were designed using primer blast (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) or Primer-3-input (http://frodo.wi.mit.edu/cgi-bin/primer3/cgi) using customized parameters. The designed set of primers was examined for any likelihood of self or heterodimer formation using the oligo analyzer software. A nucleotide blast was used to confirm the uniqueness of these primers in the NCBI database. IDT and Sigma-Aldrich, both in India and Singapore, produced the vast majority of the primers (Table: 3.2).

3.3.5. In silico promoter analysis

To identify putative conserved plant cis-acting regulatory elements and motifs reported in the literature earlier, the promoter sequences were analyzed using different bioinformatic software i.e., PLACE (Higo et al., 1999) and plantCARE (Lescot et al., 2002).

3.4. Sterilization procedure

All glass wares, bacterial culture media, tissue culture media, and tools were sterilized by autoclaving at 121ºC under 15 lb psi pressure for 20 minutes. Autoclaved cellulose nitrate filter of 0.22 µM pore size (Millipore) was used for filter sterilizing all antibiotics and other heat-labile components.

Table 3.2. The list of the primer pairs used in the study is as follows.

Target sequence	Primer	Sequence (5' - 3')	Amplicon size (bp)
MtsHSP	MtsHSP_F1	TCGGCAAGTTCATACAGCTGCA	750
	MtsHSP_R1	ATCACTGCAGACCAACCTAGCT	
MtsHSP	MtsHSP_F2	GCACATATGGCAACTTCACTTGCTCTC	636
	MtsHSP_R2	ATTGCGGCCGCTCACTCAATTTGAACATTG	
MtsHSP	MtsHSP_qRT_F	CTCACCACCAAGGAGCGTGAGC	172
	MtsHSP_qRT_R	CTTGTCGAGCCCAGGCATATCC	
MtsHSP	MtsHSP-GFP_F	TGCCATGGTGATGGCAACTTCACTTGCTC	636
	MtsHSP-GFP_R	GCACTAGTCTCAATTTGAACATTGAAAAC	
18S rRNA	18S rRNA_qRT_F	CATTGAGAAATGGCCAGACC	172
	18S rRNA_qRT_R	AAGACCGGCAACAGGATTC	
35sP	35sP_F	GGTACCGCGTATTGGCTAGAGCA	1000
	35sP_R	CATATGTGGAGTGTCTCGAACTTCTTCTTCC	
NosT	NosT_F	GCGGCCGCCGATCGTTCAAACATTTGGCA	250
	NosT_R	GAGCTCAATTCCCGATCTAGTAACATAGATG	
NptII	NptII_F	TGGCTGCTATTGGGCGAAGTGC	480
	NptII_R	CCATTCGCCGCCAAGCTCTTCA	
HSP: GFP fused	HSP Fused F	TGCCATGGTGATGGCAACTTCACTTGCTC	1189
	GFP_R1	TGATAATGATCAGCGAGTTGC	
NADH-ubiquinone oxidoreductase	NdhF_qRT_F	GGATATTCGCCAGCTAAAAGCC	172
	NdhF_qRT_R	TGGACCAAAAACAAGCAAGAGG	
PepA	PepA_qPCR_F	TCACACTTGCTGATGCGTTG	170
	PepA_qPCR_R	TTCATCGCTTGCCTCAGAAG	
SOD1	SOD1_qPCR_F	AGCCGTAGTTGTTCATGCTG	172
	SOD1_qPCR_R	AGGACAAACCACGCTTTTGC	
SOD2	SOD2_qPCR_F	CGTGGCTGAAGCAACAATTG	163
	SOD2_qPCR_R	AGCTCGTGAACTACAAGTGC	
SAUR	SAUR _qPCR_F	AACAAGAGGCTGAGTTGTGC	170
	SAUR _qPCR_R	TGTTTTGGGGCAATCGGTTC	
Quinol-cytochrome-c oxidoreductase	Cytc_qPCR_F	ACAAGGACCCAACTTGAACG	107
	Cytc_qPCR_R	TATTCTCTCCCCAGGTAACAGC	
CAT	CAT_qPCR_F	ATTCTGGTGCTCCTGTTTGG	112
	CAT_qPCR_R	ACGTTCCCTGTCAAAGTTGG	
APX	APX_qPCR_F	CCTTTTCCGCACTTCATACC	116
	APX_qPCR_R	TGGATCCAAGGCTACAGTGAG	
HSP_Promoter	HSP_Pro_1F	GGGAGCTCAACGTCATGTCACTGTCAGT	2000
	HSP_Pro_1R	GCCCATGGTTCTGCTATTGCTTACAAGC	
	HSP_Pro_2F	GAGAGCTCACCATGTTTGAAGCCACAAG	1500

	HSP_Pro_2R	GCCCATGGTTCTGCTATTGCTTACAAGC	
	HSP_Pro_3F	GCGAGCTCCTTTTCAAATAAGCTCTTAG	1000
	HSP_Pro_3R	GCCCATGGTTCTGCTATTGCTTACAAGC	
	HSP_Pro_4F	ATGAGCTCCAACTACAACCGAAAGCTGC	500
	HSP_Pro_4R	GCCCATGGTTCTGCTATTGCTTACAAGC	
GUS cloning	Gus_F	CGCCATGGTAGATCTGAGGGTAAATTTC	2350
	NosT_R	GCCTCGAGCCCGATCTAGTAACATAGATG	
spCas9	Cas9_Screen_F	GTGAAGAAGACCGAGGTGCA	750
	Cas9-Screen_R	ACTCGCTGATCTGCTCGATG	

3.5. Plant materials and growth circumstances

The Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, developed the BARI Begun-4 variety of eggplant (Solanum melongena L.), which was used in this study. To investigate the response of SmsHSP expression in various tissues and developmental stages, three-week-old seedlings were cultivated hydroponically in Yoshida Nutrient Solution (YS) (Table 3.3). They were then treated with different stresses including heat (45°C), salt (200 mM NaCl), and drought (150 mM mannitol). Plants that were only treated with water were used as treatment controls (CT). Samples were taken before and after the stress treatment at 0, 1, 2, 4, 6, and 12 hours. These leaf tissues were employed for the extraction of RNA and the subsequent investigation of gene expression. Two-week-old seedlings shifted in soil pots were used for heat and drought stress studies, and seedlings transferred to YS were used for salt stress, to analyze the physiological responses of SmsHSP overexpression lines (OE) under both normal and stressful circumstances. Wild-type plants served as a control (WT). Seedlings had been exposed to heat shock (45°C) for up to 36 hours after one week of acclimatization; YS was supplemented with 200 mM NaCl for salt treatment. Water was denied for 10 continuous days as a drought treatment. To perform RNA extraction, oxidative damage assays, biochemical and enzymatic assays, and stress-related gene expression profiling, eggplant tissues were harvested at different points in time after stress treatments and quickly frozen with liquid nitrogen and stored at 80 °C.

Table 3.3: Composition of modified Yoshida Solution (YS) media

Sl. No	Reagents	gm/l	Preparation
Major nutrients			
1	NH_4NO_3	91.4	1.25 ml of each stock solution was added for 1L media. Before adding the minor elements and iron solution, the pH was corrected to 4.5 with diluted HNO3.
2	K_2SO_4	71.4	
3a	KH_2PO_4	23.1	
3b	K_2HPO_4	4.3	
4	$CaCl_2.6H_2O$	175	
5	$MgSO_4.7H_2O$	324	
Minor nutrients			
a	$MnCl_2.4H_2O$	1.5	
b	$(NH_4)_6$. $Mo_7O_{24}.4H_2O$	0.074	
c	H_3BO_3	0.93	
d	$ZnSO_4.7H_2O$	0.035	
e	$CuSO_4.5H_2O$	0.03	
f	FeNaEDTA	10.5	
g	$FeSO_4$ (made fresh)	2.5	

3.6. Growth conditions of Bacteria

Different isolates of E. coli were incubated and grown either on LB agar at 37ºC or in LB broth with constant shaking at 220 rpm (Table 3.4). *Agrobacterium* cells were grown either in YEM agar at 28ºC or in YEM medium (Table 3.5) with constant shaking at 220 rpm. These bacterial strains were incubated and selected by applying the proper antibiotics. (Table 3.6).

Table 3.4: Composition of LB media.

Component	g/l00 ml	
Tryptone Yeast extract NaCl	1 0.5 1	The above-mentioned ingredients were mixed and dissolved in 80ml of distilled water. The pH was then adjusted to 7.0, and the total volume was raised to 100ml. 1.5% agar was used to prepare the solid medium.

Table 3.5: Composition of YEM media.

Component	g/l00 ml	
Yeast Extract Mannitol NaCl MgSO4.7H2O K2HPO4	0.04 1.00 0.01 0.02 0.05	The above-mentioned ingredients were mixed and dissolved in 80ml of distilled water. The pH was then adjusted to 7.0, and the total volume was raised to 100ml. 1.5% agar was used to prepare the solid medium.

Table 3.6: Concentrations of the antibiotics used in this study.

Antibiotic	Solvent	Concentration
Kanamycin	Water	50 µg/ml
Hygromycin	Water	50 µg/ml
Chloramphenicol	Water	50 µg/ml
Streptomycin	DMSO	15 µg/ml
Rifampicin	DMSO	15 µg/ml
Ampicillin	Water	100 µg/ml
Cefotaxime	Water	250 µg/ml
Carbenicillin	Water	250 µg/ml

3.7. Competent cell preparation and transformation

The *Escherichia coli* and Agrobacterium competent cells were prepared according to a procedure of (Sambrook & Russell, 2006) with trivial modifications of (Höfgen & Willmitzer, 1988) respectively. The competent cells can be used for many standard molecular biology applications.

3.7.1. Preparation of E. coli competent cells (TOP10, BL21 codon plus and DB3.1)

The respective cultures of bacteria were streaked on LB agar plates without antibiotics (for TOP10, DB3.1, and BL21-DE3) or containing the desired antibiotic (chloramphenicol for BL21 Codon Plus strain of E. coli). The plate was maintained at 37^{o}C overnight. In 5 ml

of LB broth, a single, isolated colony was inoculated and grown at 37ºC overnight while being shaken. The next day, a secondary culture was prepared using 1% of the primary culture in 100 ml of LB broth. The secondary culture was transferred to a new falcon tube and centrifuged at 3500 rpm for 15 min at 4oC after reaching OD 0.4–0.6. The pellet was then dissolved in 20 ml of chilled 0.1 M CaCl2 after the supernatant had been removed. After centrifuging the suspension for 15 minutes at 3500 rpm at 4ºC, it was placed on ice for two hours. After discarding the supernatant, the pellet was progressively redissolved in 1.5 ml of CaCl2. The re-suspended pellet was kept on ice and stored at 4ºC overnight. The next day, 1.5 ml of 50% glycerol was added and the suspension was mixed very gently in an icebox at 4ºC. Competent cells were prepared and divided into aliquots holding 100 µl. These aliquots were then transferred to Eppendorf tubes, flash-frozen with liquid nitrogen, and kept at -80ºC until needed. All inoculation and streaking procedures were carried out under sterile conditions in a Laminar Air Flow Hood. The stock solutions of the chemicals used in competent cell preparation are given in below (Table 3.7).

Table 3.7: Stock solutions for competent cell preparation.

Component	Amount	Preparation
0.1 M CaCl2.2H2O	14.7 g/L	The salt was dissolved properly and the volume was made up followed by autoclaving and stored at room temperature.
50 % glycerol	50 ml glycerol/100 ml of water	It was autoclaved and stored at room temperature.

3.7.2. Transformation of *E. coli* cells

A microcentrifuge tube holding competent cells was removed from -80ºC and placed on ice for 10 minutes to defrost the cells before being used to transform DNA into E. coli competent cells. The cells were given the proper quantity of ligated DNA or plasmid (0.2-1.0 g), which was mixed thoroughly with a pipette, and incubated on cold for 30 minutes. The cells were then rapidly cooled on ice for 10 minutes after receiving a 90-second heat pulse at 42ºC. For cell recovery, cells were diluted 10-fold with liquid LB and incubated at 37°C at 200 rpm

for 60 min. The cells were then centrifuged for 10 minutes at 3000 rpm. The pellet was resuspended in 100 µl of LB after the supernatant was decanted, and the mixture was then spread on LB agar plates with the necessary antibiotics (with optimum concentration). The plates were incubated for 12–14 hours at 37°C. Colony PCR was used to check the observed colonies on plates having the cloned insert.

3.7.3. Electro-competent *Agrobacterium* cells preparation

In 5 ml of liquid YEM medium that contained selective antibiotics rifampicin (15 mg/L) and streptomycin (10 mg/ml), where a starter culture of the *A. tumefaciens* strain was grown. Inoculated cells were grown for 48 hours at 28°C on an orbital shaker at 220 rpm. Fresh YEM medium containing 10 mg/ml of streptomycin and 15 mg/L of rifampicin was inoculated with 1 ml of starting culture. The culture was then permitted to reach the log pHase (OD600 0.4 - 0.6). The log phase culture was centrifuged at 5000 rpm for 15 min at 4ºC after being cooled on ice for 30 min. The pellet was centrifuged at 5000 rpm for 5 minutes at 4ºC while being suspended in equal amounts of ice-cold, sterile, double-distilled water. The pellet was centrifuged at 5000 rpm for 5 minutes at 4°C after being resuspended in an equal amount of 0.1 mM HEPES (pH 7.0) and incubated on ice for 1 hour. The obtained bacterial pellet was centrifuged at 7000 rpm for 10 min at 4ºC in an equal amount of 0.1 mM HEPES (pH 7.0) and 10% ice-cold glycerol. The pellet was then placed in 300 µl of 10% glycerol, suspended, and divided into 40 µl aliquots that were kept at -70°C until further use. The stock solutions used in electro-competent cell preparation are given in below (Table 3.8).

Table 3.8: Stock solutions for electro-competent cell preparation.

Component	Amount	Preparation
YEM	1.18 g/100 ml	The readymade powder was dissolved properly and the volume was made up followed by autoclaving and storing at room temperature.
100 mM HEPES buffer	2.383 g/100 ml of water	pH was set to 7.4 and it was filter sterilized and stored at 4 ºC.

3.7.4. *Agrobacterium* electro-transformation

Plasmid DNA (10 ng) was added to the chilled Agrobacterium-competent cells to begin the transformation process. A pre-chilled 2 mm cuvette was used for the bacterial cell suspension transfer process after being cautiously mixed with a pipette tip. Nearly 5 ms of electroporation time was spent at 2.5 KV, 25 µF capacitance, and 201 Ω resistances. The average pulse duration across all subsequent electroporation was 5.25 ms. Following electroporation, 1 ml of new LB medium was added, and the bacterial suspension was incubated for 2–3 hours at 28°C while being shaken. Following incubation, 50 µl of the transformed cells were plated on a YEM plate containing an antibiotic for plasmid selection along with rifampicin (10 mg/l), streptomycin (25 mg/l), and another antibiotic. To allow the transformed Agrobacterium to grow, the plate was incubated at 28°C for 48 hours.

3.8. Isolation of plasmid DNA (mini prep)

The Qiagen Plasmid Miniprep Kit was used to extract plasmid DNA from bacterial cells following the manufacturer's instructions. In a loosely capped 15 ml glass tube filled with 10 ml of LB medium and the necessary antibiotics, one bacterial colony was cultivated at 37°C with frequent shaking. After 12 hours of growth, cells were pelleted by centrifuging at 5,000 rpm for 5 minutes at 4°C. The bacterial pellet was resuspended in 300 µl of the provided resuspension (P1) solution and thoroughly mixed by vertexing after the supernatant was removed. After that 300 µl of lysis solution (P2) was added and gently mixed 4 to 6 times. A neutralization solution (350 µl) was added just after 5 min of lysis and the tube was inverted several times for proper mixing. The suspension was centrifuged for ten minutes at 10,000 rpm, and the supernatant was then run through a QiaSpin column. DNA was washed with wash buffer (500 µl) twice by passing it through the column. Finally, 50 µl of elution buffer was transferred through the column to collect the DNA in a new tube. DNA was quantified by spectrophotometer and stored at 4ºC.

3.9. Isolation of plant genomic DNA

Eggplant (*Solanum melongena* L.) Genomic DNA was extracted from 21days old eggplant seedlings following the cetyl-trimethylammonium bromide (CTAB) protocol

described by (Doyle & Dickson, 1987) with minor modifications. Fresh leaf tissue (0.5 to 1gm) was ground in the presence of liquid nitrogen using mortar, pestle, and preheated CTAB extraction buffer (4% CTAB, 100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA and pH 8.0) plus 2% β-mercapto-ethanol. Approximately 1g of finely ground plant tissue was combined with 10 ml of preheated extraction buffer, and the suspension was incubated at 65°C for 30 min with intermittent mixing every 5 min to guarantee proper mixing of the buffer and plant tissues. After centrifuging the lysate at 12000 rpm for 20 min at room temperature, an equal amount of chloroform: isoamyl alcohol (24:1) solution was added. For 10 to 20 minutes, it was gently but fully mixed. Centrifugation was used to separate the watery pHase for five minutes at room temperature, at 12000 rpm. After transferring the clear supernatant to a new tube, 0.6 volume of ice-cold isopropanol was added, and everything was vigorously mixed for 30 minutes. To get a clear pellet, the combination was centrifuged at 10,000 rpm for 5 minutes. The pellet was washed with 1 ml of cold, 70% ethanol before being centrifuged at 12000 rpm for 2 minutes at 4°C. The ethanol was cautiously removed without damaging the pellet. The centrifugation pellet was air dried before being resuspended in 200 µl of either milli Q water or Tris-EDTA (TE) solution (1 mM EDTA, 10 mM Tris-HCl, and pH 8.0). Stock solutions for genomic DNA isolation are given in below (Table 3.9).

Table 3.9: Stock solutions for genomic DNA isolation.

Component	Amount
2X CTAB extraction buffer: 100 mM Tris (Stock 1 M) 1.4 M NaCl (Stock 5 M) 20 mM EDTA (Stock 0.5 M) 4% (w/v) CTAB Water	 20 ml 56 ml 18 ml 4 g The volume is made up to 200 ml using sterile water. pH was set at 8 then autoclaved and stored at room temperature. 0.2% (v/v) of β-ME was added just before extraction.

3.10. Quality check and quantification of DNA

By measuring spectrophotometric absorption at wavelengths of 260 and 280 nm, the amount of DNA was calculated. The OD260nm formula was used to determine the DNA

concentration (OD260 = 1, which equals 50 ng/ml of dsDNA). The quality of DNA was considered to be good enough if A260/A280 ratio was above 1.8 for DNA. DNA integrity was further examined by passing it through a 0.8% agarose gel.

3.11. Isolation of total plant RNA

Using Trizol reagent (Invitrogen), total RNA was isolated from eggplant tissue samples following the manufacturer's instructions. Approximately 100 mg of plant tissue was ground in liquid nitrogen to make fine powder and 1ml of Trizol reagent was added (Table 3.10). The homogenate was mixed properly. The extracts were put into a 2 ml micro centrifuge tube and 200 µl of chloroform was added for every ml of the initial Trizol after they had fully defrosted. After 15 seconds of vigorous hand shaking, the tubes were incubated at room temperature for an extra 10 minutes. The samples were centrifuged at 12000 X g for 15 min at 4ºC. To prevent contaminating the upper aqueous phase with the interphase or the organic layer, the upper aqueous phase was transferred into a new microcentrifuge tube. To precipitate RNA, 0.6 volume of isopropanol was added to the aqueous phase and kept for 10 min at RT. After centrifuging the RNA particle at 7500 X g for 10 min, it was washed with 75% ethanol (prepared in nuclease-free water). After air drying, pellets were dissolved in 30-40 µl of nuclease-free water and keep it in the freezer (-70ºC) until further use. For long-term storage, precipitated RNA was kept in 75% ethanol and stored at -70ºC. Stock solutions for total RNA isolation are given in below (Table 3.10).

Table 3.10: Stock solutions for total RNA isolation.

Component	Amount
Trizol	A readymade solution from Invitrogen company was used.
DEPC water	1 ml DEPC per L of water was added and stirred overnight by keeping the bottle over a magnetic stirrer and autoclaved.

3.12. Integrity test of total RNA and quantification of RNA

The integrity and quality of the prepared RNA to be used for subsequent cDNA synthesis were confirmed by checking through both spectrophotometric and electrophoresis

methods. Running the isolated RNA through a 1% denaturing formaldehyde agarose gel in 1X MOPS solution ensured its integrity. For this, total RNA (2 µg) was mixed with an appropriate amount of RNA loading buffer (1.5 ml RNA buffer composition: 100 µl Glycerol, 720 µl Formamide, 160 µl10X MOPS, 260 µl 37% Formaldehyde and 260 µl Nuclease-free water) and heated at 65°C for10 min to destroy secondary structures in RNA followed by cooling in ice for 5 min. The gel was run at 5 volts/cm for 45 min in an electrophoresis unit (Bio-Rad, USA). After completion of electrophoresis, the gel was taken in an RNAse-free container and washed thoroughly with DEPC water, and stained with ethidium bromide (10µg/ml). The gel was photographed using a gel documentation system (Alphaimager EP, Innotech). Two distinct bands belonging to 28S and 18S rRNA would appear on a denaturing gel run with intact total RNA (Figure. 3.2). Besides that, in RNA from photosynthetic tissues, two additional bands corresponding to 26S and 16S plastidial rRNAs are often visible. When compared to the 18S rRNA band, the 28S rRNA band should be about two times as strong. The 28S:18S number of 2:1 provides strong evidence that the RNA is intact. The OD260nm method was used to determine the concentration of RNA (OD260=1, or 40 µg /ml of RNA). A ratio of A260/280 should be 2.0, for high-quality pure RNA. Solutions for running RNA in the gel are below (Table 3.11).

Table 3.11. Solutions required for RNA gel running.

Component	Amount
10X MOPS buffer	
200 mM 3-(N-morpHolino) propanesulfonic acid (MOPS)	41.9 g
50 mM sodium acetate	8.2 g
10 mM EDTA	3.72 g
2X RNA loading dye (1.5 ml)	
Deionized formamide	720 µl
10X MOPS	160 µl
DEPC water	200 µl
100% glycerol	80 µl
Formaldehyde	260 µl
Bromophenol blue	80 µl

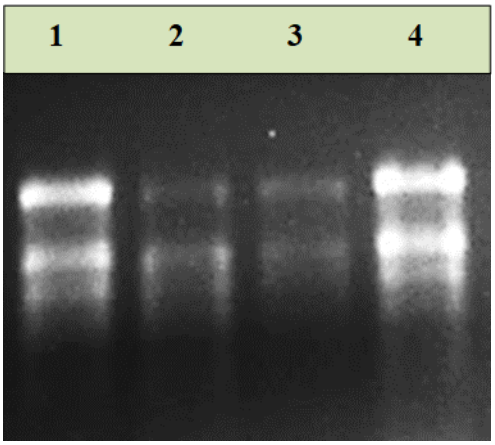


Figure. 3.2: Total RNA from eggplant leaves (4 samples)

3.13. First-strand cDNA synthesis

One microliter of Oligo dT (stock concentration of 500 µg/ ml), 5 µg RNA, and 1 µl of dNTP (Stock concentration of 10 mM) and the required volume of DEPC water was mixed so that the final volume was 12 µl. The combination was heated in a PCR machine for 5 minutes at 65°C before being quickly chilled on ice. It was centrifuged briefly. The 8 µl solution containing 5X first strand synthesis buffer at around 4 µl, 0.1 M DTT at around 2 µl, and 1 µl of RNase was added to the PCR tube containing the RNA sample. It was mixed properly. The mixture was heated at 70°C for 15 minutes to inactivate the reaction after 50 minutes at 50°C in the PCR machine. To keep the produced cDNA for a long time, it was kept at -20°C.

3.14. Quantification of nucleic acids by Nanodrop

Using a Nanodrop machine, the absorbance at 260 and 280 nm was measured to identify the quantity and quality of nucleic acids in the solution. A 50 µg/ml solution of double-stranded DNA, a 40 µg/ml solution of single-stranded RNA, and a 33 µg/ml solution of single-stranded oligonucleotides or primers are all equivalent to one absorbance at 260 nm in a 1-cm quartz cuvette. By examining the A260/A280 ratio, the purity of a DNA or RNA sample was determined.

3.15. Restriction digestion of DNA

1.0 µg of plasmid DNA, 5.0 µl of the proper 10X restriction enzyme buffer, and 10 units of restriction enzyme were used in a 50 µl process to carry out the restriction digestion of the plasmid DNA. The reaction was allowed to sit at 37°C (or at a temperature that is suitable for the particular needs of the restriction enzyme being used) for one hour while the digestion pattern was examined on a 1% agarose gel. The band of interest was cut, and a Qiagen gel purification kit was used to elute the remaining portion.

3.16. Ligation

The general ligation ratio used for the fragment to be cloned and the vector into which it is to be cloned was 3:1. The ligation reaction was conducted in a total reaction volume of 10 µl, which included sterile double-distilled water to make up the volume, 50 ng of restriction digested vector DNA, a known quantity of fragment DNA, 1 µl of 10 X ligase ligation buffer, and 1 µl (400U) of T4 DNA ligase. The entire ligated product was used for transformation after the reaction was allowed to continue overnight at 16°C.

3.17. Gel extraction using a Qiagen gel purification kit

The agarose gel containing the desired band was excised. 500 µl of solubilization buffer (QG buffer) was added to the excised piece of gel and incubated at room temperature till the gel completely melted and dissolved. After adding the solution to the column, it was agitated for 1 minute at 13,000 rpm. Centrifuging at 13,000 rpm for 1 minute while washing was done with 700 µl of PE buffer. At 13,000 rpm for two minutes, an empty column was centrifuged. The DNA was eluted in 15-20 µl of elution buffer by centrifuging at 12,000 rpm. This step was carried out twice to facilitate complete elution.

3.18. PCR purification using a Qiagen gel purification kit

For PCR purification, 5 volumes of PB buffer (supplied with Qiagen PCR purification kit) were added to the PCR product, and the mixture was loaded on to Qiaquick spin column followed by centrifuging at 13,000 rpm for 1 min. After discarding the flow-through, a wash

(PE) solution was used to clean the column. The purified DNA fragment was eluted with 20 µl of elution buffer by centrifuging at 12,000 rpm. This step was carried out twice to facilitate complete elution.

3.19. Polymerase chain reaction (PCR)

Taq DNA polymerase and a combination of forward and reverse converging primers were used to quickly amplify the DNA fragments. The desired quantity of template, 150 ng of forward and reverse primers, 200 µM of each dNTP, and 1.0 U of Taq DNA polymerase were all present in the reaction mixture. In a 50 µl reaction volume, the typical PCR reaction settings were denaturation (94 °C) for 2 min, annealing (58 °C) for 1 min, and extension (72°C) for 1 min/kb of the expected product. To confirm the amplification, an aliquot of the PCR result was run on a 0.8% agarose gel.

3.20. Colony PCR

The putatively transformed clones found growing on the selective media were verified by colony PCR. A sterile pipette tip was used to delicately touch a single colony. The pipette tip was then streaked on a master plate, suspended in 45µl of water, and boiled for 10 minutes. 15µl of the supernatant from the centrifuged mixture was used as a template for PCR to confirm the change after the mixture was centrifuged at 10,000 rpm for one minute.

3.21. Cloning and sequencing of PCR fragments

According to the manufacturer's instructions (Invitrogen, USA), the selected DNA fragment(s) that were produced by the nested PCR-amplified products were gel-purified using columns supplied with a Qiagen gel extraction kit (Cat. No. 28104). Cycle-sequencing was used to sequence the recombinant plasmid and/or the purified PCR products (Macrogen, South Korea).

3.22. Real-Time PCR

With the help of SYBR™ Green PCR Master Mix, real-time qRT-PCR was carried out. Eggplant gene-specific primer and 18S rRNA primer pair were used as internal reference genes. Each well received 15 µl of the master mix to make the finished volume per well 20 µl.

The plate was properly sealed with an optical adhesive cover and then centrifuged briefly (3000 X g for 1 minute) to rotate down the contents and remove any air bubbles. No template controls were also made for each pair of primers. ABI Prism 7500 Sequence Detection System and software were used to conduct the real-time PCRs. (PE Applied Biosystems, USA). The qRT-PCR components and program are mentioned in Table 3.12 and 3.13 respectively.

Table 3.12: The components of the PCR mixture are given below.

Sl. no	Component	Volume (per well)
1	Fast SYBR® Green Master Mix (2✕)	10.0 µl
2	Forward primer (final concentration of 100 nM)	0.2 µl (from 10 µM stock)
3	Reverse primer (final concentration of 100 nM)	0.2 µl (from 10 µM stock)
4	Nuclease free sterile water	4.6 µl
5	Diluted cDNA template	5.0 µl

The specificity of amplification was tested by dissociation curve analysis with the default parameters. For each sample, three technical duplicates were examined. The delta-delta Ct or comparative Ct value technique was used to determine the relative expression ratio for each gene (Livak & Schmittgen, 2001).

Table 3.13: The program of the qRT-PCR.

	Hold	Initial denaturation	PCR		Melt curve	
			40 cycles		0.2°C increment	
			Denaturation	Annealing/Extension	Initial	Final
Time	**2 min**	**10 min**	**15 s**	**60 s**	**5 s per stage**	
Temperature (°C)	50	95	95	58-60 (depending on primer properties)	60	95

3.23. Agarose gel electrophoresis

The concentration of agarose would vary inversely depending on the type of DNA fragments to be loaded. The majority of the time, 1% agarose gels containing EtBr (10 g/ml)

and a 1 kb DNA ladder (Fermentas, Canada) were loaded with a mixture of nucleic acid and PCR products along with 6X DNA loading dye. The electrophoresis of the gel was performed at 5 V/cm for two hours in the electrophoresis machine (Biorad, USA), and the gel was documented using a gel documentation system (AlpHaimager, USA).

3.24. SDS-polyacrylamide gel electrophoresis

According to (Laemmli, 1970), polyacrylamide gel electrophoresis (PAGE) was carried out. Mini-protein instrument of Bio-Rad was used for casting gel and electrophoresis of proteins. Gels were created and operated either without (native) or with SDS (denaturing). The composition of the solutions for the preparation of gel is given in Table 3.14.

Table 3.14: Composition of the solutions required for the preparation of PAGE gel.

1. Acrylamide-bisacrylamide Solution (30%)		
a. Acrylamide	29.2g	The total capacity was increased to 100 ml. Whatman No. 1 filter paper was used to further filter the solution, which was then placed in a dark brown container and kept at 4°C.
b. Bis acrylamide	0.8g	
c. Distilled water	70ml	
2. 1.5 % Ammonium persulfate (APS)		
a. APS	150g	With freshly prepared distilled water, the final volume was made up to 10 ml.
3. N, N, N', N'-tetramethyl ethylene diamine (TEMED)		
a. Undiluted TEMED was stored in a dark, dry, and cool environment.		
4. 4X Resolving Gel Buffer		
a. 1.5 M Tris Base (pH 8.8)	18.2g	The final volume was made up to 100 ml, filtered, and kept at 4°C.
b. Distilled water	80ml	
5. 4X Stacking Gel Buffer		
a. 1.0 M Tris (pH 6.8)	6g	The final volume was made to 100 ml, filtered, and stored at 4 °C.
b. Distilled water	80ml	
6. 2X Stock Sample Buffer		
a.0.5 M Tris–HCl buffer (pH 6.8)	2.4 ml	At room temperature, the final volume was created to be 10 ml.
b. Glycerol	2.0ml	
c. 0.5% bromophenol blue	1.0ml	
7. 1X Electrode Buffer (pH 8.3)		
a. 0.025 M Tris	3g	Made in a total amount of 1000 ml
b. 0.192 M Glycine	14g	

To prepare protein samples for denaturing gel, they were mixed with an equal amount of 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4% 2-mercaptoethanol,

0.01% bromophenol blue). Samples were placed in a boiling water bath for five minutes before being filled either with boiling water for denaturing conditions or without it for native conditions. After the proteins were correctly stacked, gels were run at a constant voltage of 100 V. Initially, gels were run at 50 V. Depending on the further use, gels were either electro blotted onto a nitrocellulose membrane or a Hybond-C+ membrane (both manufactured by Amersham Pharmacia Biotech in England) or dyed with CBB (0.25% Coomassie Brilliant blue R-250, 50% methanol, and 10% acetic acid). The amount of acrylamide solution and buffers required for preparing various percentages of running and stacking polyacrylamide gel is given in Tables 3.14, 3.15, and 3.16.

Table 3.15: Composition of various resolving gels for Tris-Glycine SDS-PAGE.

Sl. No.	Resolving Gel	5ml	10ml	15ml	20ml	25ml
8%						
a.	H_2O	2.3	4.6	6.9	9.3	11.5
b.	30% bis acrylamide	1.3	2.7	4.0	5.3	6.7
c.	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
d.	10% APS	0.05	0.1	0.15	0.2	0.25
e.	10% SDS	0.05	0.1	0.15	0.2	0.25
f.	TEMED	0.003	0.006	0.009	0.012	0.015
10%						
a.	H_2O	1.9	4.0	5.9	7.9	9.9
b.	30% bis acrylamide	1.7	3.3	5.0	6.7	8.3
c.	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
d.	10% APS	0.05	0.1	0.15	0.2	0.25
e.	10% SDS	0.05	0.1	0.15	0.2	0.25
f.	TEMED	0.002	0.004	0.006	0.008	0.01
12%						
a.	H_2O	0.6	3.3	4.9	6.6	8.2
b.	30% bis acrylamide	2.0	4.0	6.0	8.0	10.0
c.	4X Resolving Gel Buffer	1.3	2.5	3.8	5.0	6.3
d.	10% APS	0.05	0.1	0.15	0.2	0.25
e.	10% SDS	0.05	0.1	0.15	0.2	0.25
f.	TEMED	0.02	0.004	0.006	0.008	0.01

Table 3.16: Composition of Stacking Gel for Tris-Glycine SDS-PAGE.

5%	1ml	2ml	3ml	4ml	5ml	6ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1
30% bis acrylamide	0.17	0.33	0.5	0.67	0.83	1.0
4X Resolving Gel	0.13	0.25	0.38	0.5	0.63	0.75
Buffer	0.01	0.02	0.03	0.04	0.05	0.06
10% APS	0.01	0.02	0.03	0.04	0.05	0.06
10% SDS	0.001	0.002	0.003	0.004	0.005	0.006
TEMED						

3.25. Cloning of mitochondrial small heat shock protein (SmsHSP) full-length genes into pET-28a (+) expression vector

For the production of recombinant SmsHSP protein, the coding sequence was amplified and cloned between *Nde*I and *Not*I sites of the pET28a vector. The empty vector and the pET28a:: SmsHSP recombinant plasmid were then transferred into E. coli BL21 (DE3) cells to be used as an expression vector. Growing in liquid LB medium until the OD600 reaches approximately 0.5, E. coli BL21 (DE3) carrying the recombinant pET-28a (+) plasmid was then stimulated with varying concentrations of IPTG (optimized for each protein) for 12 hours at the optimal temperature (37oC/ 18oC). Different IPTG concentrations and incubation temperatures were employed to get the protein expressed in the soluble fraction of cell lysates. SmsHSP was induced by 1 mM of IPTG and expressed at 18º C overnight.

3.25.1. Purification of recombinant proteins using Ni-NTA agarose affinity chromatography

BL21 (DE3) *E. coli* cells culture (1L) was induced and cells were pelleted down after a definite period of incubation. The supernatant was discarded and the cells were washed with water. The recombinant protein was purified from this bacterial culture pellet by lysing the cells. After being dissolved in sonication buffer (sodium phosphate buffer 50 mM with pH 8.0, 10% glycerol, 300 mM NaCl, 1 mg/ml lysozyme, 1 mM PMSF, 0.1% tween 20-, and 10-mM imidazole), the cells were incubated for 30 minutes on ice. Then the solution was sonicated and the clear lysate was collected after centrifugation. This lysate was gently combined with a Ni-NTA agarose slurry for 60 minutes at 4°C and 100 rpm. A bottom outlet capped column

was used to filter the lysate Ni-NTA mixture, and the flow-through (FT) was gathered for a subsequent SDS-PAGE analysis. Wash buffer (50 mM sodium phosphate buffer, pH 8.0, 10% glycerol, 300 mM NaCl, 1 mM PMSF, 0.1% Tween 20-, and 20-mM imidazole) was used to progressively wash the column. Additionally, wash-through (WT) was gathered and kept for SDS-PAGE examination. The protein was eluted using elution buffer, which contained varying concentrations of imidazole (100-250 mM based on the protein), and the eluted fractions were then subjected to SDS-PAGE analysis. Optimum imidazole concentration was again standardized for SmsHSP protein individually. SmsHSP proteins were eluted with 250 mM of imidazole-containing elution buffer.

3.25.2. Confirmation of the expressed recombinant protein

Ni-NTA purified proteins were found to be more than 90% pure by SDS-PAGE analysis. The specific band of the purified protein was confirmed by western blotting using anti-his antibodies (Sigma) that are very specific for the histidine tag present in the recombinantly expressed protein. Western blot was compared with the corresponding CBB-stained gel and purification of the specific protein was confirmed.

3.25.3. Western blotting

Western blot was developed for the SmsHSP. Using a Mini transblot electrophoretic cell, the extracted proteins from BL21 (DE3) E. coli cells were separated on SDS-PAGE and moved to Hybond C+, membrane (GE Healthcare). The electroblotting solution contained 20% methanol (pH 8.0), 20 mM Tris, and 150 mM glycine. To assess the effectiveness of protein transfer, pre-stained molecular weight markers were used. The blot was quickly rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4.7H2O, 1.4 mM KH2PO4, pH 7.3) and then blocked for an hour in PBS with 5% BSA. The blot was then washed three times with PBS containing 0.1% tween-20 (PBST) at a 5-minute interval before being blocked for 1 hour with the primary antibody at 30000 dilutions. The blot was washed with PBST thrice at 5 min intervals. After washing, the blot was further incubated in alkaline-phosphatase conjugated secondary antibody (5000 dilutions in PBS, 1mg of BSA/1 ml of PBS) for 1 h. The blot was then cleaned with PBST three times at a 5-minute delay after being cleaned with PBS containing 0.3% Tween-20 three times. The BCIP/NBT-containing solution (0.1 M Tris-HCl

pH 9.5, 0.1 M NaCl, 5 mM MgCl2 containing 150 g/ml of NBT and 75 g/ml of BCIP) was used to develop the protein-antibody combination onto the blot. Blot was gently kept on shaking and monitored after every 30 s. Finally, the reaction was stopped by replacing the substrate-containing buffer with PBS after the proper development of the band.

3.25.4. Thermal stability and protein solubility assay of mitochondrial small heat shock protein (SmsHSP)

For the thermo-tolerance assay, according to (Zhang et al., 2015), an equal number of E. coli BL21 cells harboring the pET28a::SmsHSP or the control pET21a plasmid were grown at 37°C in LB medium addition with 100 mg/ml ampicillin to an OD600 of 0.3-0.4 before being induced with IPTG at a final concentration of 1 mM for 2-3 hours. In a temperature-controlled water bath, cells were subjected to thermal shock for 30 minutes at various temperatures of 37oC, 45oC, 50oC, and 55oC. Serial cell culture dilutions were created using pre-warmed LB medium, and each dilution (20–30 µl) was then plated in triplicate on LB agar plates having 100 mg/ml of ampicillin and allowed to grow overnight at 37°C. The number of colony-forming units derived from the cells treated at the specified temperatures and those derived from the untreated cells was used to calculate the cell viability. Parallel, Protein solubility was also measured from aliquots of thermos-tolerance experiments. After heat treatment 1 ml culture from each sample was collected and centrifuged at 6000 rpm for 10 minutes to pellet down the cells. With 20 mM Tris-HCl solution (pH 8.0), the cell pellets were washed once. Cell lysates were again centrifuged for 15 minutes at 6000 rpm following sonication in the same solution. By Coomassie blue staining method, the solubility of SmsHSP proteins was analyzed in supernatants and pellet form.

3.25.5. Protein purification and chaperone-like activity assay

The manufacturer's instructions were followed to gather and purify the protein from the supernatant containing mitochondrial small heat shock protein (SmsHSP). Before dialyzing, the eluent containing the SmsHSP protein, all the eluted portions were collected and analyzed by SDS-PAGE. Following the manufacturer's directions, the BCA assay (Pierce) was used to measure the protein concentration. For the chaperone-like activity assay of SmsHSP protein, we followed the report published by (Saito et al., 1999). In the lack and presence of the

SmsHSP protein, we observed and quantified the thermally induced aggregation of alcohol dehydrogenase (ADH) (50oC) and citrate synthase (CS) (45oC). In covered cuvettes in a temperature-controlled water bath for 60 minutes, different amounts of the SmsHSP protein were inoculated with substrate protein with buffer containing 50 mM HEPES-KOH (pH 7.5). In the lack of SmsHSP protein in the mixture, lysozyme was also added at a concentration of 50 µg/ml. The measurement of light absorbance at 360 nm was used to track the aggregation of proteins. The Helios Gamma Spectro-photometer was used to take the readings. (Thermo-Spectronic, Cambridge, UK). Each reaction was performed in three parallel replicate trials.

3.25.6. Electron microscopy

Purified SmsHSP protein and the model substrate citrate synthase (CS) protein were analyzed using electron microscopy before and after thermally induced aggregation. The separate and combined effect of SmsHSP protein with CS protein at the indicated temperature and period was investigated. Before applying 5µl of protein solution, all tested proteins were first adsorbed onto carbon-coated grids that were glow released in the air. The excess protein solution was removed by blotting, and the molecules that had been bound were then stained negatively for the 30s using 5µl of 2% (w/v) uranyl acetate. (pH 4.5). In the lab at ICGEB in New Delhi, electron micrographs were captured using a JEOL 1400 transmission electron microscope operating at 100 kV.

3.25.7. Plasmid protection assay

The antioxidant activity of SmsHSP was evaluated by in vitro plasmid protection assay according to Silva et al (2012). The effectiveness of this experiment was determined by how well it protected plasmid DNA from radicals produced with the presence of 3 mM FeCl3 and 4 mM DTT (oxidant system). The experiment utilized the plasmid DNA pUC19. It was incubated with SmsHSP protein or BSA in 50 mM phosphate buffer at pH 7.0. By examining the stability of the plasmid DNA after electrophoresis in 1% agarose gel and by incubation with ethidium bromide, the protection of the DNA against oxidation was evaluated.

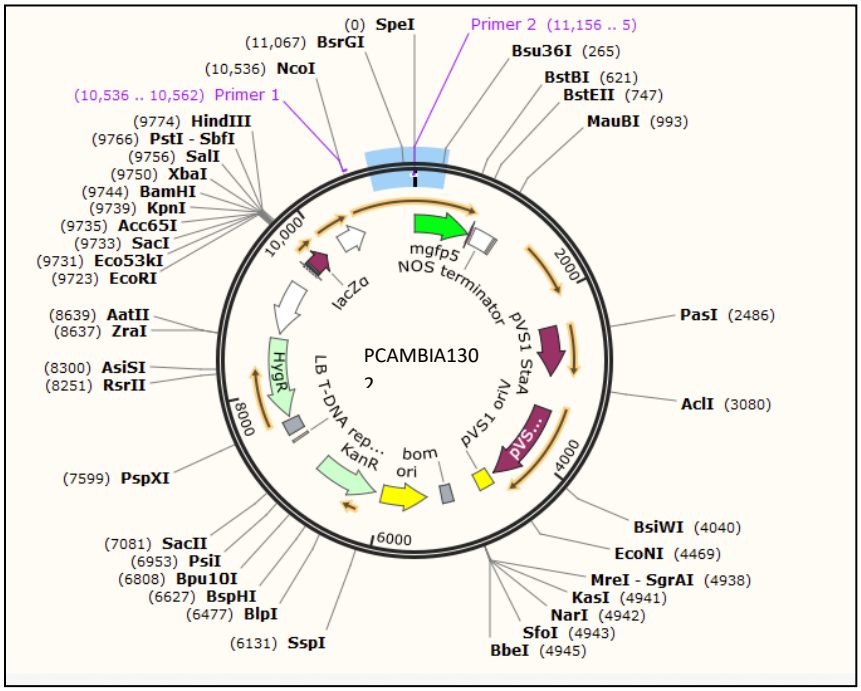


Figure. 3.3: Expression vector PCAMBIA1302 with green fluorescent fusion protein (GFP)

3.26. Fusion protein expression and confocal leisure scanning microscopic observation

For subcellular localization of novel SmsHSP, we used green fluorescent fusion protein (mGFP) based expression vector PCAMBIA1302 with hygromycin selection. The coding sequence of the SmsHSP gene was PCR amplified by removing the stop codon using primer pair F/R with restriction enzyme BglII and SpeI respectively. The amplified product was gel purified and subsequently inserted into PCAMBIA1302 in under 35S promoter and green fluorescent fusion protein (mGFP) with nopaline synthase terminator (NosT). Final expression cassette containing binary vector pCAMBIA1302::CAM35S promoter:: SmsHSP:mGFP::Nos terminator were transferred to Agrobacterium tumefaciens EHA105 by electroporation. The fusion construct was transiently expressed in tobacco leaf epidermal cells using a hypodermal syringe following the method. The leaf sections were examined using a confocal fluorescence scanning microscope after 72 hours of inoculation. (Zeiss LSM510, Germany). Similarly, a stably integrated eggplant cell suspension culture having fluorescent fusion SmsHSP protein was also used to study protein localization following a similar method developed in the crop

improvement lab of M. K. Reddy (data unpublish, ICGEB). Eggplant (Solanum melongena L.) cells suspension culture was prepared by inoculating 2-3 pieces of resistant friable callus (approximately 1gm) into a 250 ml conical flask containing 50 ml autoclaved liquid MS media (Murashige and Skoog 1962). After two weeks when actively growing cells were produced, a 10 ml primary cell suspension culture was transferred to a new conical flask containing 30 ml fresh liquid MS media. Suspension cells are then propagated by regular sub-cultures of an aliquot to fresh liquid MS media. To confirm subcellular localization, mitochondria-specific dyes Mito-tracker TM FM (Invitrogen; https://www.thermofisher.com) were also used. Mito-tracker dyes entered into the cell and passively diffuse across the plasma membrane into the cytosol and bind thiol groups in the mitochondria. Centrifugation was used to break down suspension cells for aspiration of the supernatant for 5 minutes at 3000 rpm. The cells were carefully reconstituted in new, 37°C, liquid MS media with 300 nM of Mito-tracker stained solution added. Cells were incubated in the dark for one hour by shaking at 150 rpm at 28˚C. After staining was completed cells were re-pelleted by centrifugation and suspended in fresh pre-warmed liquid MS media. The sample was prepared by placing 10 µl of suspended cells on a slide, and images were captured using a laser scanning confocal microscope in the dark field. (Zeiss LSM510, Germany). GFP and MitoTracker fluorescence signals were captured using emission filters with band passes of 500 to 535 nm and 488 to 520 nm, respectively. All fluorescence experiments were independently repeated at least three times.

3.27. Cloning of SmsHSP promoter and construction of SmsHSP promoter: GUSNosT expression vector

The genomic DNA was extracted by the CTAB method from 3-week-old eggplant (Solanum melongena) young leaves. The full-length ~2kb upstream sequence of the translation initiation codon (ATG) of small heat shock protein SmsHSP promoter (− 2000 bp to − 1) was amplified by using a pair of PCR primers (HSP_Pro_1F/ HSP_Pro_1R) (Table 2.1) and then cloned into pCR-4-TOPO vector. Three other 5′ deleted fragments of different lengths of SmsHSP promoter (1.5kb,1.0kb,0.5kb) were also amplified by using three pairs of PCR primers (HSP_Pro_2F, 3F, 4F/ HSP_Pro_2R,3R,4R) (Table 3.2). After the amplification of four desired fragments then we sub-cloned this fragment into an entry vector (pL12R34-Ap) using SacI and NcoI restriction enzyme sites. All four 5′ deleted fragments were finally fused

with the uidA (beta-glucuronidase, GUS) reporter gene for the construction of the SmsHSP24.1 promoter+GUSNosT expression vector. Using a high-fidelity DNA polymerase, we amplified the GUS with the Nos terminator gene from the pCAMBIA1301 plasmid. (KOD plus, Toyobo, Japan). The PCR amplicon was cloned into the previously employed entry clone (pL12R34-Ap) using NcoI and XhoI restriction sites and then moved into the pMDC100 vector with the NptII selection marker gene by LR recombinase-mediated Gateway™ (Invitrogen, USA) cloning. Similar to this, to create the CaMV35S promoter: GUS expression vector, the GUS and Nos terminator from pCAMBIA1301 were amplified via PCR, directly cloned into the modified entry clone 1 (pL12R34-Ap), and then transferred into the pMDC100 vector. All expression constructs were finally transferred into Agrobacterium tumefaciens EHA105 strain for eggplant transformation.

3.28. Construction of binary expression vectors and eggplant transformations

With the help of a high-fidelity DNA polymerase (KOD plus, Toyobo, Japan) and a set of primers for the SmsHSP-ORF-F1, F2, and SmsHSP-ORF-R1, R2 regions, the full-length SmsHSP protein-encoding gene was amplified by nested PCR from eggplant (Solanum melongena L.) cDNA. Following sequence confirmation using the universal M13 reverse and forward primers on the macro gen sequencing platform, the SmsHSP coding region was sub-cloned in a Gateway-compatible entry vector (pL12R34-Ap). The promoter was cloned into restriction sites KpnI and NdeI, the SmsHSP gene was cloned into NdeI and NotI restriction sites, and the Nos terminator was cloned into NotI and Sac I sites of entry vector sequentially and confirmed by colony PCR and restriction digestion. A gateway cloning method involving LR recombinase (Invitrogen; https://www.thermofisher.com) was used to transfer a complete gene cassette into the plant transformation vector pMDC100 (Invitrogen, USA), which contains the NptII (kanamycin) gene as a plant selection marker. This final expression cassette pMDC100::CAM35Spromoter:SmsHSP: Nos terminator was transformed into Agrobacterium tumefaciens EHA105 by electroporation and was further used for eggplant transformation. For tissue culture, cotyledonary leaves of 21 days old in vitro grown eggplant seedlings were used. Each cotyledonary leaf was transversely cut into 2-3 pieces and infected with the EHA105 Agrobacterium strain harboring the SmsHSP expression cassette. The transformed cells were selected on MS medium supplemented with kanamycin (100mg/l) and the untransformed Calli

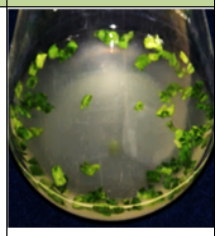
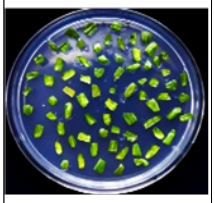
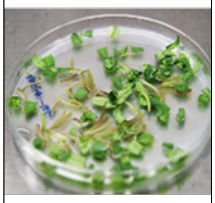
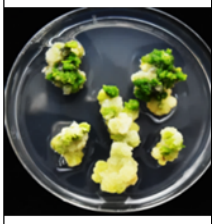
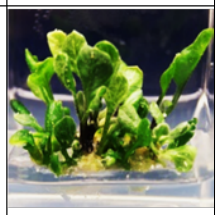
turned black and died. Secondary Calli or micro-Calli developed after three rounds of selection with kanamycin. The micro-Calli was transferred to regeneration media for initiation of shoots. To aid in rooting, the regenerated plantlets were moved to rooting media. Few putative transgenic plants were correctly acclimated and moved to a greenhouse environment. NptII and SmsHSP gene-specific primers were used in PCR to test the potential transgenic lines.

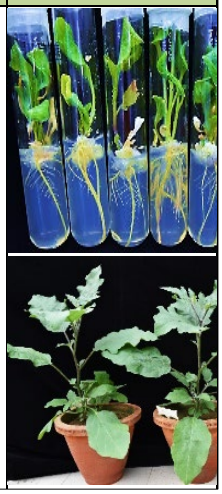
3.28.1. Agrobacterium-mediated transformation approach for developing transgenic eggplant

We have used our optimized Agrobacterium-mediated transformation protocol for developing eggplant transgenic lines. The composition of different media and antibiotics used in eggplant transformation is listed in Table 3.17:

Table 3.17: Outline of Agrobacterium-mediated transformation procedure.

Stage	Media Composition	Duration	Photograph
1.**Seed germination:** In the absence of hormones, sterile eggplant seeds were grown on half strength MS medium at 25 °C for 16 hours.	Half strength MS media (1L: MS 2.2 g + Sucrose (15 g) + P^H (5.7) + Gelrite (4 g)	12-14 days	
2. ***Agrobacterium*** **culture preparation:** *Agrobacterium* cell cultured in YEM medium with constant agitation of 250 rpm at 28 ºC	YEM (100 ml): Yeast extract (0.04 g) + K2HPO4 (0.05 g) + Mannitol (1 g) + NaCl (0.01 g) + MgSO4. 7H2O (0.020 g)	24h	

Stage	Media Composition	Duration	Photograph
3. **Infection with explants:** Re-suspended *Agrobacterium* cell in liquid MS medium, infected with cotyledone, hypocotyl, and root explants in an aseptic environment	Resuspension 1L: MS (2.2 g) + Sucrose (15 g) + Glucose (10 g) + PH (5.2) + Acetosyringone (50 µM)	10 min	
4. **Co-Cultivation with *Agrobacterium*:** Cotyledon, hypocotyl, and root explants were moved to co-cultivation medium after infection and kept in the dark at 28 ºC.	Co-cultivation media (1L): MS (4.4 g) + Glucose (10 g) + Sucrose (30 g) + BAP (2.5 mg/L) + PH (5.2) + Gelrite (4g). After autoclave when temperature below 60°C add 50 µM acetosyringone	48 hours	
5. **Washing:** All explants were properly cleaned with an antibiotic-containing water solution under aseptic conditions after 48 hours of co-cultivation.	Washing component (100 ml): 100 ml of sterile water + antibiotic carbenicillin (250 mg/L) + antibiotic cefatoxime (250 mg/L)	8-10 minute	
6. Selection: Transformed shoots were chosen on an antibiotic-containing selection plate for three to four times, and the selection plates were maintained at 25 °C with 16 hours of light fluorescent illumination.	Selection media (1L): MS (4.4 g) + Glucose (10 g) + Sucrose (30 g) + 6-Benzylaminopurine (2.5 mg/L) + Sorbitol (0.1 mg/L) + Casein hydrolyste (0.02 mg/L) + PH (5.7) + Gelrite (4 g). After autoclave when temperature below 60°C, add selective antibiotic kanamycin (100 mg/L)/ hygromycin (10 mg/L) + cefatoxime (250 mg/L)	10-12 days	
7. Shoot elongation: *In vitro* regenerated Shootlets were moved to an elongation medium and exposed to light fluorescent lighting for 16 hours at 25 °C.	Elongation media (1L): Selection media (1L): MS (4.4 g) + Sucrose (30 g) + 6-Benzylaminopurine (0.25 mg/L) + Sorbitol (0.1 mg/L) + Casein hydrolyste (0.02 mg/L) + PH (5.7) + Gelrite (4 g). +kanamycin (100 mg/L)/ + Cefatoxime (250 mg/L)	12-15 days	

Stage	Media Composition	Duration	Photograph
8. ***In vitro* Rooting:** *In vitro* elongated shoots shifted to root induction medium and maintaining them at 25 °C for 16 hours under fluorescent irradiation	Rooting media (1L): MS (4.4 g) + Sucrose (30 g) + Indolebutyric acid (IBA) (1.0 mg/l) + PH (5.7) + Gelrite (4 g). After autoclave when temperature below 60°C, add selective antibiotic kanamycin (100 mg/L)/ hygromycin (10 mg/L) + Cefatoxime (250 mg/L)	15-18 days	

3.28.1. Detection of genomic integration of transgenes by PCR

To detect the genomic integration of transgene cassette by PCR at T0 generation, we used specific primer pairs of NptII (NptII_F1/NptII_R1) for detection of selectable marker gene and SmsHSP and Nos terminator primer pair (SmsHSP_F2/NosT_R1) for detection of SmsHSP transgene integration. The reaction mixture was set to 50 µl and put into a thermocycler with an initial denaturing period of 5 min at 94oC. This was followed by 35 cycles of denaturation for 1 min at 94oC, annealing for 30 sec at 56oC, extension for 1 min at 72oC, and final extension for 6 min at 72oC. The amplified products were observed in 1.0% agarose gels stained with ethidium bromide (1 µg ml-1).

3.28.2. Selection of seeds for kanamycin resistance

The seeds from wild-type and T1 transgenic lines were germinated in a sterile condition. Around 10 germinating seeds were inoculated in half MS medium with and without kanamycin (150mg/L) in a glass jar. The glass jars were kept in a plant growth house at 28° C under dark conditions till to initiation of germination. When the seeds start to germinate, they are maintained under fluorescent lighting for 16 hours per day at 25 °C to choose seedlings that are kanamycin resistant.

3.28.3. Southern hybridization

Southern hybridization was performed using the DIG nonradioactive nucleic acid labeling and detection system (Roche) following the manufacturer's instructions to prove the presence of the number of copies in transgenic plants. The genomic DNA was extracted from 5.0 g of fresh leaves of transgenic and WT plants by the CTAB method. Using a 0.8% agarose gel to isolate the digested genomic DNA (25 µg, overnight), EtBr staining was used to make it visible. After two rinses in autoclaved MQ for 15 minutes, the gel was removed. To denature the ds of DNA, DNA in agarose gel was treated with 0.2 N HCl for depurination by gentle agitation for 15 mins. The acid solution was decanted and followed by rinsed in autoclaved H2O for 5 mins. The depurination DNA in the gel was treated twice with denaturation buffer (to cleave the DNA at the depurination site) by gentle agitation for 15mins of intervals. This was followed by soaking the gel twice in neutralization buffer for 15 mins of intervals with gentle shaking. Using a 3 mm Whatman paper wick and conventional capillary transfer in the presence of 20X SSC for an overnight period, the denatured DNA was moved from an agarose gel to a nylon membrane (Hybond N+, Amersham Pharmacia). After the transfer was complete, the membrane was washed in 2X SSC for 5 min twice. The DNA was then cross-linked to the membrane by exposure to short wavelength ultraviolet light (Amersham UV-cross linker). Thereafter, the membrane was either stored dry at 4 o C or used immediately to proceed for Pre-hybridization. The Nylon membrane with cross-linked DNA was properly sealed in a hybridization bag and incubated with 10 ml of pre-warmed (65o C) DIG Easy Hyb buffer along with 20 µl of denatured salmon sperm DNA (Sigma) for 2 h at 50oC in a shaking water bath. The 500 bp of SmsHSP gene fragment was labeled with PCR DIG Probe Synthesis Kit (Roche) following the manufacturer's instruction. The probe mixture was denatured by boiling at 100o exactly for 5 min followed by quickly placing it on ice. The denatured probe and 1µl of denatured salmon sperm DNA were mixed with a minimal volume of pre-warmed DIG Easy Hyb (15ml). The membrane was then treated with the hybridization solution, which was then kept on the bottom of a shaking water bath at 50° C overnight. Post-hybridization wash was carried out by washing the membrane twice in 2X SSC with 0.1 % SDS at RT followed by a second wash with 1X SSC with 0.1 % SDS at 65ºC twice for 15 mins of intervals

under constant agitation. After low and high stringency wash to reduce the background, the membrane was

Table 3.18: Composition of the buffers and solutions used for southern hybridization and chemiluminescent detection.

1.	**10X TBE**		
a.	Tris	107.8 g	The complete volume was made up to 1000 ml with distilled water, pH was maintained to 8.2.
b.	EDTA	8.41 g	
c.	Boric acid	559 g	
d.	Distilled water	600 ml	
2.	**Denaturation Solution**		
a.	NaCl (1 M)	29.22g	Total volume was made to 500 ml with distilled water.
b.	NaOH (0.5 N)	10g	
c.	Distilled water	400 ml	
3.	**Neutralisation Solution**		
a.	NaCl	43.83g	The complete volume was made up to 500ml. pH was maintained to 7.5 with concentrated HCl.
b.	Tris	30.28g	
c.	Distilled water	350 ml	
4.	**20X SSC**		
a.	NaCl	175.3g	The complete volume was made up to 1000ml. pH was maintained to 7.0 with concentrated HCl.
b.	Na citrate	88.2 g	
c.	Distilled water	800 ml	
5.	**Maleic acid buffer (10X)**		
a.	1M maleic acid	116g	Final volume was made to 1000 ml, adjust with NaOH (solid) to pH 7.5
b.	1.5 M NaCl	87g	
6.	**Washing Buffer**		
	1X maleic acid buffer with pH 7.5, Tween 20 (0.3% v/v)		
7.	**Detection Buffer**		
a.	0.1M Tris Hcl		Final volume was made to 100 ml with pH 9.5
b.	0.1M Nacl		
8.	**Blocking solution (10X)**		
	Blocking solution 10% (W/V) was dissolved in the maleic acid buffer while being constantly stirred on a heating block. The solution remains cloudy. The solution then autoclaved. 1X working solution freshly prepared by dilution in Maleic acid buffer.		
9.	**Antibody solution**		
	Centrifuge Anti Digoxigenin-AP (vial 3) for 5 min at 10,000 rpm. Dilute the needed amount in blocking solution at a ratio of 1:10,000 (75mU/ml).		
10.	**CSPD solution**		
	CSPD was diluted as 1:100 ratios in detection buffer.		

washed once in 2X SSC for 5 min followed by a brief rinse in washing buffer. After that, the membrane was treated for 30 minutes in a blocking solution and another 30 minutes in an antibody solution. The membrane was rinsed twice with washing buffer at intervals of 15 minutes, then allowed to acclimate for 5 minutes in the detection buffer. Then 2ml of CSPD solution was applied on the upside-facing membrane and the substrate was spread evenly avoiding air bubbles and incubated for 5 mins. Then excess substrate solution was squeezed out, sealed in the bag properly, and kept at 37o C for enhancing the chemiluminescence reaction. Probe-target hybrids were finally visualized chemiluminescent with CSPD. Later the membrane was exposed to X-ray film (Kodak) for 90 mins to 1.5 h at room temperature with an intensifying screen to obtain an image. The composition of the buffers and solutions for southern hybridization are listed in Table 3.18.

3.28.4. DIG labeling of probe DNA

Probe was prepared by labeling the PCR amplified DNA using PCR DIG Probe Synthesis Kit (Roche) with slight modification. A thermostable polymerase includes DIG-dUTP as it amplifies a particular region of the template DNA in PCR probe labeling. A highly labeled, highly focused, and extremely sensitive hybridization probe is the end product. 150 pg of purified plasmid DNA was added with supplier-provided reagents (Roche). The unlabelled probe was amplified as a control. The reaction mixture was set to 50 µl and placed in a thermo-cycler (Applied bioscience) using the following condition (Table 3.19):

Table 3.19: Thermo-cycler condition for preparation of PCR DIG probe.

Steps	Temperature	Time	Cycle number
Primary denaturation	95˚C	2 min	-
2nd denaturation Annealing Elongation	95˚C 60˚C 72˚C	30 s 30 s 1min	Cycles 1 – 10
3rd denaturation Annealing Elongation	95˚C 60˚C 72˚C	30 s 30 s 1min + extra 20 s for each consecutive cycle	Cycles 11 – 30
Final elongation	72˚C	10min	-

The PCR-labeled probe was evaluated by agarose gel electrophoresis. Due to high-density labeling with DIG, the labeled PCR product has a significantly greater molecular weight than the unlabelled probe. 2.0 µl of DIG-labeled probe was used for hybridization.

3.29. Morphological characterization of SmsHSP in transgenic eggplants

3.29. Seed germination assays

Under normal and abiotic stress growth conditions, seed germination tests of WT and transgenic lines were performed. In maximum strength MS growth medium, seeds were plated where 150 mM Mannitol was added for drought stress, and 200 mM NaCl was added for salt stress. Seeds were subjected to heat stress by being put on sterile, empty petri plates and kept in an incubator set at 45°C for 2 and 4 hours before being transferred to full-strength MS agar plates. After that, all dishes were put in a growth chamber with fluorescent lighting set to a 16-hour photoperiod at 25°C. Every day, the germination rates were measured. Representative seedlings were photographed after a 7-day of incubation.

3.29.1. Leaf disc assay

Healthy leaves of plants (both wild-type and transgenic) of the same age were selected for the leaf disc senescence assay. Leaf discs of one-centimeter diameter were cut from each plant and floated on either different stress solution or sterile distilled water (for control). These leaf discs were used for measuring chlorophyll content and experiments were repeated thrice with three replicates each time.

3.29.2. Pigments measurement

According to (Lichtenthaler & Buschmann, 2001), the chlorophyll and carotenoid concentration from the leaf discs were measured spectrophotometrically after extraction in 80% acetone. Three replicates were used in each of the three experiments (n = 3). 100 mg of leaf tissue was completely homogenized in 1 ml of 80% acetone and centrifuged for a couple of minutes at 3000 rpm. A spectrophotometer was used to measure the absorption at 663 nm, 647 nm, and 470 nm while the supernatant was kept in place. Chlorophyll-A (Chl-A), Chlorophyll-B (Chl-B), and total carotenoids (Xanthophylls and carotenoids, x+c) were determined using the following equations-

Chlorophyll-A (µg/ml) = 12.25A663-2.79A647

Chlorophyll-B (µg/ml) = 21.50A647-5.10A663

(X+C) (µg/ml) = (1000A470 -1.82Ca - 85.02Cb)/198

3.29.3. Determination of water loss rates

Ten WT and ten transgenic plants were used to measure the rate of water loss. Three-week-old seedlings were separated from their roots and weighed quickly (fresh weight, FW). The plants were then left on the lab bench at room temperature and weighed at specific intervals (desiccated weights). The proportions of fresh weight loss were calculated relative to the initial weights. Finally, the plants were oven-dried for 24 hours at 50ºC to a constant dry weight (DW). Water contents (WC) were measured according to the formula: WC (%) = (desiccated weight – DW)/ (FW – DW) X100.

3.29.4. Relative water content (RWC) measurement

The relative water content (RWC) of flag leaf was measured according to (Reguera et al., 2013). Flag leaves were taken from wild-type and transgenic plants grown under well-watered and stress conditions and placed in a pre-weighed air-tight vial. The vials were weighed again after the material was added to determine the leaf fresh weight (FW). The samples were then fully moistened for 6 hours, turgid weight was obtained, and the process was repeated. (TW). Following that, samples were dehydrated for 24 hours at 80°C in an oven before being given a dry weight (DW). RWC was determined using the following formula: RWC (%) = (FW-DW)/(TW-DW) X100

3.29.5. Measurement of H_2O_2 content

According to (Velikova et al., 2000) method, the amount of H_2O_2 was measured. The homogenate was centrifuged at 13,000 g for 15 minutes after being extracted from fresh leaf tissues in 5.0 ml of TCA (0.1% w/v) in an ice-cold bath. The 0.5 ml supernatant was mixed with an equal amount of potassium iodide and sodium phosphate buffer (pH 7.5). At 390 nm, the sample's absorbance was determined. The amount of H_2O_2 in plant tissue was calculated using the extinction coefficient (ε=0.28 μM^{-1} cm^{-1}) and represented as nm g^{-1} of H_2O_2 fresh weight.

3.29.6. Estimation of antioxidant enzymes

For tests of the antioxidant enzyme activities, Eggplant leaves from the untreated and stressed state were harvested. 200 mg of fresh leaves were carefully ground and centrifuged at 15000 rpm for 15 min at 4°C in 0.2 M phosphate buffer (pH 8.0). On the ice, the supernatant was retained. As described by (Beyer Jr & Fridovich, 1987), the superoxide dismutase (SOD) activity was carried out. At 560 nm, absorbance was recorded. Following the H_2O_2-dependent oxidation of ascorbic acid (ASC) at 290 nm, ascorbate peroxidase activity (APX) was assessed. The assay for catalase (CAT) activity was carried out following the (Beaumont et al., 1990) procedure, which tracked the dismutation of H_2O_2 at 240 nm. Measurements of electrolyte leakage were made as initially described by (Sairam et al., 2002). Proline measurement was also carried out following the procedure of (Bates et al., 1973).

3.29.7. H_2O_2 and O^{2o-} staining by 3,3'-Diaminobenzidine (DAB) and NBT (Nitro blue tetrazolium chloride) solution

The presence of intercellular H_2O_2 can be stained with a DAB solution. 50 mg DAB was dissolved in 50 ml of water to make 50 ml of DAB staining solution (1 mg/ml). The pH of the mixture was raised to 3.0 by the addition of 0.2 N of HCl. The DAB solution was stirred while 25 µl of Tween-20 (0.05% v/v) and 2.5 ml of 200 mM Na_2HPO4 were added. Plant tissue samples were gently vacuumed for five minutes after being dipped in the appropriate quantity of DAB solution. The serving plates were then wrapped in aluminum foil and gently shaken at 80 to 100 rpm for 4 to 10 hours in the dark. Following the proper color development, tissues were subjected to a series of 90% and 70% ethanol treatments to remove the chlorophyll. On a plain white background, pictures were made, and samples could be kept at 4°C for four days.

Cellular generation of O^{2o-} was visualized histo-chemically as described by (Zhang & Turner, 2008). Fresh leaves were placed in a 50 mM Tris-HCl buffer pH 6.4 containing 0.1% NBT and 0.1% NADH for 10-15 minutes to visualize O^{2o^-} histo-chemically. The leaves were then illuminated with bright white light to produce the blue mono formazan precipitation's signature color. With the help of a Canon DSLR digital EOS 850 D camera in macro-close-up mode, stained leaves were photographed against a white fluorescent light backdrop.

3.30. RNA-seq and transcriptome analysis

Using the RNAeasy Plant Mini Kit (Qiagen: https://www.qiagen.com), total RNA was extracted from 3 weeks old eggplants leave (Solanum melongena L.). Under normal and heat stress circumstances (45°C), RNA was extracted from WT and T_2 generation transgenic plants (OE7). A Bioanalyzer and RNase-free agarose gel electrophoresis were used to validate the quality and quantity of RNA. The Kerala, India-based Agri-Genome Labs performed the RNA sequencing and library building. For the RNA-seq study, only samples with RNA integrity of N7.0 were used. RNA sequencing was done using the Illumina HiSeq 2500 technology (Illumina Inc., CA, USA). Reads that had undergone pre-processing and had their rRNA removed were aligned to the tomato reference genome, and the Sol Genomics Network gene model.
(ftp://ftp.solgenomics.net/tomato_genome/Heinz1706/wgs/assembly/build_3.00/S_lycopersicum_chromosomes.3.00.fa.tar.gz). The reads were used to determine the expression of genes and transcripts using the cufflinks program after being aligned with the reference genome. All control and treated samples underwent differential expression analysis using the Cufflinks software (version 2.2.1). Using the FPKM diagram, gene expression levels were determined. (Fragments per kilobase of transcript per million fragments mapped). Up and down-regulated genes and isoforms were determined independently using the log2 fold change cut-offs of 2 and the p-value cut-offs of 0.01 and 0.05. Genes that were up and down-regulated during the population comparison were grouped into heat maps with a p-value cut-off of 0.05. Using the GOSeq tools, GO enrichment analysis was carried out (default parameters). The importance of each GO category's overrepresentation among differentially expressed (DE) genes is determined by GOseq. Using the tomato reference genome and a bio-conductor tool called path view, the differentially expressed genes that were accessible were subjected to KEGG pathway analysis.

3.31. Validation of selected transcripts using qPCR

Total RNA isolation and cDNA synthesis was done by employing the same methods as described earlier. Few transcripts were chosen for validation using qPCR as explained

earlier. For all experimental validations, three independent biological replicates were used and the primers employed used in this study are listed in the table

3.32. Preparation of CRISPR/Cas9 constructs

Three gateway cloning compatible vectors, referred to as entry vector-1 (EV1), entry vector-2 (EV2), and destination vector (DV), also known as an expression vector, were used in the CRISPR cloning approach. First, the Bsa1 location of the EV1 and EV2 entry vectors was used to clone the gRNA1 and gRNA2, respectively. Then, using gateway cloning and the LR clonase enzyme, both gRNAs from these entry vectors were sequentially cloned into the destination vector, which already contained the Cas9 gene cassette. Through the electroporation technique, the final construct was converted into Agrobacterium EHA105 cells.

3.32.1. Synthesis of gRNA

Since only the exons play a role in gene expression, the target site for knockout was selected to only be in the exonic region. So, the cDNA sequence of the desired gene was used to select the target locations. The process of deletion of a gene will not be affected by intronic gene editing. In CRISPR technology, the promoter, scaffold, and vector backbone are all constant, while the gRNA sequence (complementary to the target spot) is the only variable. Only the scaffold vector needed to be digested with the right restriction enzyme to clone the sgRNA, and the sgRNA could then be altered to meet the needs. The cDNA sequence for the mitochondrial small heat shock protein (SmsHSP) gene was fed into the online CRISPR-direct software to anticipate potential gRNAs. SmsHSP possesses 2 exons and one intron. Scanning sense and antisense strands of both the cDNAs, the CRISPR direct software predicted several prospective target sites which were immediately adjacent to the PAM sequence. Hence the target sequence to be mutated was like 5′-N (20)-NGG-3′ or 5′-CCN-N (20)-3′. We selected two gRNAs for knocking out each gene to increase gene editing frequency. The gRNAs we designed were comprised of 20 nucleotides along with additional four nucleotide overhangs that are 5`CAGG3` in forward oligomer and 5`AAAC3` in reverse oligomer for all the gRNAs. The addition of the four nucleotides overhangs i.e., 5`CAGG3` and 5`AAAC3` on either side

of the gRNA was necessary to facilitate the complementarity-based annealing to the overhangs of digested entry vectors (EV1 or EV2) (Table 3.20).

Table 3.20: Sequences of gRNAs for mitochondrial small heat shock protein (SmsHSP) are as follows.

Gene	gRNA	Orientation (5`→3`)	Oligo sequence with overhangs shown in red colour
SmsHSP	gRNA1	F	CAGGTGGCAACTTCACTTGCTCTC
		R	AAACCCTGAGAGCAAGTGAAGTTG
	gRNA2	F	CAGGCTTTCCCTAGTTTGTTTTCA
		R	AAACGAAAACAAACTAGGGAAAGC

gRNAs were ordered in single-stranded form of 24 nucleotides; which need to be converted to double-stranded form before cloning in the entry vectors. Hence, both the forward and reverse strands of gRNA are annealed together in a thermocycler, and annealing conditions are as follows:

Denaturation	Annealing	Holding
95°C **5 min**	**72°C** **20 min**	**25°C** **10 min**

3.32.2. Preparation of EV1 and EV2 vectors

The vectors used in gateway cloning in this study are Entry vector1 (EV1) or Entry vector2 (EV2). Both the EV1 and EV2 vectors used for cloning gRNA contain either Arabidopsis U3 or U6 promoter along with scaffold (having poly T terminator). The promoter and the scaffold are separated by two Bsa1 restriction enzyme sites. EV1 and EV2 vectors contain gentamycin or gentamycin and chloramphenicol-resistant marker genes respectively, for the selection of transformants. U3 promoter is ~350 bp long while U6 is 390 bp long. The size of the scaffold is ~96 bp. The complete size of EV1 and EV2 vectors with their promoters is 2.8 kb and 4.2 kb respectively. A separate EV1 vector was prepared to clone Cas9 cDNA in the pMDC100 vector. For this, EV1 was double digested with KpnI and BamHI at 27°C for 2

h. The digested vector was run on 1% agarose gel and then column purified. This purified EV1 was ligated with CAM35S promoter (at 16 °C overnight). This EV1-CAM35Spromoter ligation product was transformed into Top10 cells for the selection of transformants on LB media supplemented with gentamycin. The presence of EV1 ligation product was verified by colony PCR. The EV1 ligation product was again double digested with *Bam*HI and *Not*I to ligate the Cas9 gene. Similarly, the Nos terminator was cloned in between Not1 and Sac1 to yield a final construct having a Cas9 expression cassette (EV1 CAM35S promoter: Cas9: NosT). EV1 as well as EV2 have two *Bsa*I cloning sites in between the promoter and scaffold to ligate gRNA in them. Hence, EV1 was digested with *Bsa*I restriction enzyme which is flanked by the U3 promoter and scaffold to ligate gRNA2. In our first attempt, we ligated gRNA2 for the SmsHSP gene in EV1 vectors. On LB culture containing gentamycin, this ligation product was transformed into E Top10 cells and plated. Using a forward primer for the U3 promoter and a reverse primer for gRNA2, the stable colonies created were examined with colony PCR for the presence of gRNA2. Similar to this, the U6 Promoter and scaffold were placed on either end of the BsaI spot where the gRNA1 for the SmsHSP gene was cloned in the EV2 vector. The ligation product of EV2, which contained gRNA1, was transformed into DB3.1 cells and submitted to selection on LB plates supplemented with gentamycin and chloramphenicol. Using a forward primer for the U6 promoter and a reverse primer for gRNA1, the stable colonies created were examined with colony PCR for the presence of gRNA1.

3.32.3. Multi-Round gateway cloning and pyramiding of Cas9 gene and gRNAs into PMDC100 as plant expression vector

Two unique entry vectors, EV1 and EV2, were used for the Multi-Round Gateway cloning process. EV1 (pL12R34H-Ap) contains the recombination attachment sites attL1-attL2 and attR3-attR4, whereas EV2 (pL34R12H-CmR-ccdB) contains the recombination sites attL3-attL4 and attR1-R2. These two entry vectors vary in the recombination sites they contain. For the creation of multi-gene plant transformation constructs, these entry vectors (EV1 and EV2) can be combined with the destination vector (DE) that is gateway compatible (pMDC100-plant transformation vector). The kanamycin resistance gene is a plant selection trait that is included in the pMDC100 vector. For stacking the Cas9 gene and both gRNAs,

three cycles of LR reactions between the pMDC100 vector and the recombinant entry vectors EV1 and EV2 were performed. The EV1 recombinant vector, which contains the CAM35S promoter: Cas9: NosT, and the pMDC100 vector were combined in the first round of LR recombination. The pMDC100 vector and the EV1 vector with the Cas9 expression cassette were combined at a 2:1 ratio (nanomoles) in the presence of the LR clonase enzyme mix and then cultured at 16°C for 19 hours. With the addition of 1 µl of proteinase K, this LR process was stopped. Heat shock was used to transfer a sample of 2 µl of the first round LR reaction product into E. coli K12 cells, which were then chosen on LB agar plates with 50 g/ml kanamycin. Using gene-specific primers and colony PCR, the resistant colonies were examined for the presence of the Cas9 expression cassette. The recombinant 1st LR plasmid (pMDC100:Cas9) was extracted from the positive colonies to be used for the second round of LR cloning. The second round of the LR reaction involved the recombination of the target vector plasmid (pMDC100:Cas9) and the EV2 recombinant vector (having gRNA1 expression cassette: EV2:U6 promoter: gRNA1: scaffold). The pMDC100:Cas9 plasmid and the EV-2 vector carrying the gRNA1 cassette were combined in a 2:1 ratio (nanomoles) in the presence of the LR clonase enzyme mix and then incubated at 16°C for 19 hours. 2 µl of the second round LR reaction product was transformed into E. coli DB3.1 cells and then chosen on LB agar plates supplemented with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol after the LR reaction was terminated using proteinase K. By using particular primers in a colony PCR, the colonies obtained were examined for the presence of Cas9 and gRNA1.The EV1 recombinant vector, which contains the gRNA2 expression cassette: U3promoter: gRNA2: scaffold, and the pMDC100: Cas9: gRNA1 vector were combined in the third round of LR recombination. The pMDC100: Cas9: gRNA1 vector and the EV1 vector carrying the gRNA2 expression cassette were combined in a 2:1 ratio (nanomoles) in the presence of the LR clonase enzyme mix and incubated at 16°C for 19 hours. With the addition of 1 µl of proteinase K, this LR process was stopped. Heat shock was used to transfer a sample of 2 µl of the first round LR reaction product into E. coli K12 cells, which were then chosen on LB agar plates with 50 µg/ml kanamycin. Using gene-specific primers, colony PCR was used to test the resistant colonies for the existence of the gRNA2 expression cassette. To be used for the second round of LR cloning, the positive cells were selected to yield the recombinant third LR plasmid (pMDC100: Cas9: gRNA1: gRNA2). The third round of LR cloning resulted in the selection

of the final recombinant constructs (pMDC100:Cas9:gRNA1:gRNA2), which were electroporated into Agrobacterium (EHA105) and then chosen on a YEM agar dish with 50 µg/ml kanamycin, 50 µg/ml chloramphenicol, and 15 µg/ml rifampicin. The *Npt*II gene, the Cas9 gene, the gRNA1, and the gRNA2 were tested for in the final expression vector construct (pMDC100: Cas9: SmsHSPgRNA1:gRNA2) using particular screening primer sets.

4. Result and Discussion:

Abiotic stressors mostly result in oxidative stress, which disturbs the delicate cellular equilibrium and inhibits the stability and activity of total protein synthesis. Heat stress poses significant risks to agricultural production, particularly the production of eggplants in the Indian subcontinent, as a result of rising global temperatures. According to estimates, South Asian temperatures would rise by about 2.2 °C annually, significantly harming the agricultural productivity for various crops as well as eggplant (Hoegh-Guldberg O. *et al.*, 2018). Therefore, it is crucial to research how high temperatures (HTs) and other abiotic stresses affect crop plant growth and development to maximize agricultural production and food security in the future, particularly for the regions of poor farmers in South Asia, whose subsistence farming is their only source of income. The goal of the current work is to comprehend the crucial function of mitochondrial small heat shock protein under various stress responses and to further exploit this function to engineering crop plants that are highly resilient to environmental stresses.

The results obtained in the course of the investigation have been divided and discussed in the following parts.

Part-I. Cloning, expression profiling, subcellular localization and biochemical characterization of mitochondrial small heat shock protein (SmsHSP24.1)

Part-II. Functional characterization of SmsHSP24.1 through transgenic overexpression

Part-III. Development of SmsHSP24.1 knockout Eggplant lines using CRISPR/Cas9 genome editing system

Part-IV. Understanding the transcriptional regulation of mitochondrial small heat shock protein in Eggplant

Part-I. Cloning, expression profiling, subcellular localization and biochemical characterization of mitochondrial small heat shock protein (SmsHSP24.1)

4.1.1 Sequence identification of novel mitochondrial small heat shock protein gene

By using the tomato mitochondrial small heat shock protein (SlHSP23) (BAA32547.1) full-length cDNA as the sequence, NCBI ESTs blast was able to obtain the full-length putative mitochondrial small heat shock protein gene (1032 bp) from the eggplant (*Solanum melongena* L.) draft genome (Figure 4.1). (Hirakawa et al., 2014), One copy of the mitochondrial small HSP gene with two exons and a 636 bp open reading frame (ORF) was discovered by in silico sequencing analysis using the eggplant draft genome database (Figure. 4.2).

Eggplant mitochondrial small HSP: 1 to 1032

```
                                                                                                    100
atccttatccagcaaaaagaatacaacgtgaagctcagtcaatcggcaagttcatacagctgcatctttctcttcagactcagaagcttgtaagcaatag
                                                                                                    200
cacaaATGGCAACTTCACTTGCTCTCAGGAGGGCTACTGCCTCATCGCTCTTCAATAGGCTCGTCAACCCTGTTCGCTCTGCCTCTGTTTTCCGATCCTT
     M  A  T  S  L  A  L  R  R  A  T  A  S  S  L  F  N  R  L  V  N  P  V  R  S  A  S  V  F  R  S  F
                                                                                                    300
CAATACTAACACTCAGATGACAGCTTATGACCAGGATGATCGTGATGTTGATGTCGAACGCCGGCCCGGTCGCTCCGTCTCTCGCCGCCGGGATGCTTTC
  N  T  N  T  Q  M  T  A  Y  D  Q  D  D  R  D  V  D  V  E  R  R  P  G  R  S  V  S  R  R  R  D  A  F
                                                                                                    400
CCTAGTTTGTTTTCAGATGTATTTGATCCATTCTCACCACCAAGGAGCGTGAGCCAGCTACTAAACATAATGGACCAAATGATGGATTCTCCATTTGTAG
 P  S  L  F  S  D  V  F  D  P  F  S  P  P  R  S  V  S  Q  L  L  N  I  M  D  Q  M  M  D  S  P  F  V
                                                                                                    500
CAGCACCGCGTTCTATGGGCACTGGAGTTGGTGCAAGAAGAGGATGGGACGTGAGAGAGGATGACAATGCTCTGTATATAAAAATGGATATGCCTGGGCT
A  A  P  R  S  M  G  T  G  V  G  A  R  R  G  W  D  V  R  E  D  D  N  A  L  Y  I  K  M  D  M  P  G  L
                                                                                                    600
CGACAAGGAGAACGTGAAGGTTGCGGTGGAGGAGAACACGTTGATCATCAAAGGAGAAGGTGAGAAAGAATCAGAGGATGAAGAGTATAGGAGGAGGTAC
  D  K  E  N  V  K  V  A  V  E  E  N  T  L  I  I  K  G  E  G  E  K  E  S  E  D  E  E  Y  R  R  R  Y
                                                                                                    700
TCGACGAGACTGGAAATTCCTCAAAATATGTACAAATTGGATGGAATTAAGGCGGAGATGAAGAATGGAGTGCTGAAAGTGGCTGTTCCAAAGGTGAAGC
 S  T  R  L  E  I  P  Q  N  M  Y  K  L  D  G  I  K  A  E  M  K  N  G  V  L  K  V  A  V  P  K  V  K
                                                                                                    800
AAGAAGAAAGGAAAGATGTTTTCAATGTTCAAATTGAGTGAgcttctagctgtgctaagtttgtactgtttgggtccgatgttataatttccccccttttt
Q  E  E  R  K  D  V  F  N  V  Q  I  E  *
                                                                                                    900
tcttccccttctccgagctaggttggtctgcagtgatttgtctttgaaaaataaatgaaaatatggttgtaagggtctgttcatgtagctaactgggaag
                                                                                                   1000
ttgttgaatatactgttcttcaagtttttgttactacgagctttggacctgttatagattccaacttctgttgtgtaatggttatatggttctttctatt
tttcgatagaacattcatttttatattagaa
```

Figure 4.1: Eggplant full-length putative mitochondrial small heat shock protein (1032 bp) gene.

Sme2.5_00899.1_g00005.1 contig

```
TGCACATATGGCAACTTCACTTGCTCTCAGGAGGGCTACTGCCTCATCGCTCTTCAATAGGCTCGTCAACCCTGTTCGCTCTGCCTCTGT  90
ACGTGTATACCGTTGAAGTGAACGAGAGTCCTCCCGATGACGGAGTAGCGAGAAGTTATCCGAGCAGTTGGGACAAGCGAGACGGAGACA
  A H  M  A  T  S  L  A  L  R  R  A  T  A  S  S  L  F  N  R  L  V  N  P  V  R  S  A  S  V

TTTCCGATCCTTCAATACTAACACTCAGATGACAGCTTATGACCGGGATGATCGTGATGTTGATGTCGAACGCCGGCCCGGTCGCTCCGC 180
AAAGGCTAGGAAGTTATGATTGTGAGTCTACTGTCGAATACTGGCCCTACTAGCACTACAACTACAGCTTGCGGCCGGGCCAGCGAGGCG
  F  R  S  F  N  T  N  T  Q  M  T  A  Y  D  R  D  D  R  D  V  D  V  E  R  R  P  G  R  S  A

CTCTCGCCGCCGGGATGCTTTCCCTAGTTTGTTTTCAGGTTACTTCTCTATTCTCTGTTTTTACTCGAACTTTGTGATTCAGTAATATGT 270
GAGAGCGGCGGCCCTACGAAAGGGATCAAACAAAAGTCCAATGAAGAGATAAGAGACAAAAATGAGCTTGAAACACTAAGTCATTATACA
 S  R  R  R  D  A  F  P  S  L  F  S

TACAATTAATCGATTCTGTTGCTCTTATACTAGCTGATAATCTGTGTTTTAGACTTATTTGAGCTTCAATTTGATTTTATAAATGTATTT 360
ATGTTAATTAGCTAAGACAACGAGAATATGATCGACTATTAGACACAAAATCTGAATAAACTCGAAGTTAAACTAAAATATTTACATAAA

AACGTCGTCTCAGCTTACTTATTTATCTTTGTTTTTGCTTAGATCTGTGATCTACTGCATGATAATTTCTCAGCTCTGTTACAGATAGAC 450
TTGCAGCAGAGTCGAATGAATAAATAGAAACAAAAACGAATCTAGACACTAGATGACGTACTATTAAAGAGTCGAGACAATGTCTATCTG

GAAATATTACTGTATACTTAAACTCGTTCACTTTCATTCGATTTTACAAATGTATTTCTTCTCAGATATTCTAATTTATCCCTGTTTTCC 540
CTTTATAATGACATATGAATTTGAGCAAGTGAAAGTAAGCTAAAATGTTTACATAAAGAAGAGTCTATAAGATTAAATAGGGACAAAAGG

TCAGATCTGTGATCTAGTATATGATAATTAGTCGGTCCTGTTCAAAGTTTAGCAAACTCATTTGGAGTTTTGGTTGACAGATGTATTTGA 630
AGTCTAGACACTAGATCATATACTATTAATCAGCCAGGACAAGTTTCAAATCGTTTGAGTAAACCTCAAAACCAACTGTCTACATAAACT
                                                                                D  V  F  D

TCCATTCTCACCACCAAGGAGCGTGAGCCAGCTACTAAACATAGTGGACCAAATGATGGATTCTCCATTTGTAGCAGCACCGCGTTCTAT 720
AGGTAAGAGTGGTGGTTCCTCGCACTCGGTCGATGATTTGTATCACCTGGTTTACTACCTAAGAGGTAAACATCGTCGTGGCGCAAGATA
P  F  S  P  P  R  S  V  S  Q  L  L  N  I  V  D  Q  M  M  D  S  P  F  V  A  A  P  R  S  M

GGGCACTGGAGTTGGAGCAAGAAGAGGATGGGACGTGAGAGAGGATGACAATGCTCTGTATATAAAAATGGATATGCCTGGGCTCGACAA  810
CCCGTGACCTCAACCTCGTTCTTCTCCTACCCTGCACTCTCTCCTACTGTTACGAGACATATATTTTTACCTATACGGACCCGAGCTGTT
G  T  G  V  G  A  R  R  G  W  D  V  R  E  D  D  N  A  L  Y  I  K  M  D  M  P  G  L  D  K

GGAGAACGTGAAGGTTGCGGTGGAGGAGAACACGTTGATCATCAAAGGAGAAGGTGAGAAAGAATCAGAGGATGAAGAGTATAGGAGGAG  900
CCTCTTGCACTTCCAACGCCACCTCCTCTTGTGCAACTAGTAGTTTCCTCTTCCACTCTTTCTTAGTCTCCTACTTCTCATATCCTCCTC
E  N  V  K  V  A  V  E  E  N  T  L  I  I  K  G  E  G  E  K  E  S  E  D  E  E  Y  R  R  R

GTACTCGACGAGACTGGAAATTCCTCAAGATATGTACAAATTGGATGGTATTAAGGCGGAGATGAAGAATGGAGTGCTGAAAGTGGCTGT  990
CATGAGCTGCTCTGACCTTTAAGGAGTTCTATACATGTTTAACCTACCATAATTCCGCCTCTACTTCTTACCTCACGACTTTCACCGACA
Y  S  T  R  L  E  I  P  Q  D  M  Y  K  L  D  G  I  K  A  E  M  K  N  G  V  L  K  V  A  V

TCCAAAGGTGAAGCAAGAAGAAAGGAAAGATGTTTTCAATGTTCAAATTGAGTGAGCGGCCGCAAT                      1056
AGGTTTCCACTTCGTTCTTCTTTCCTTTCTACAAAAGTTACAAGTTTAACTCACTCGCCGGCGTTA
P  K  V  K  Q  E  E  R  K  D  V  F  N  V  Q  I  E  *
```

Figure 4.2: Mitochondrial small heat shock protein (Sme2.5_00899.1_g00005.1 contig from Eggplant draft genome database) with an open reading frame (ORF) of 636 bp having two exons.

4.1.2. Cloning and sequence analysis of novel mitochondrial small heat shock protein gene from Eggplant

Using two sets of open reading frame-specific primers ORF- sHSP_F1, F2 and ORF-sHSP_R1, R2 (Primer table. 3.2) from heat-treated Eggplant (*Solanum melongena* L.) leaves, the coding area of the Eggplant mitochondrial small heat shock protein gene was amplified by nested PCR. Utilizing the universal M13 reverse and forward primers, the sequence was

confirmed. The amplified fragment (Figure 4.3) contains a 636 bp open reading frame (ORF) that codes for a protein with an expected molecular mass of 24.1 kDa and an isoelectric value of (pI) 4.84. We, therefore, designated the protein as SmsHSP24.1 and submitted it to NCBI (MF 579857.1) (Figure. 4.4).

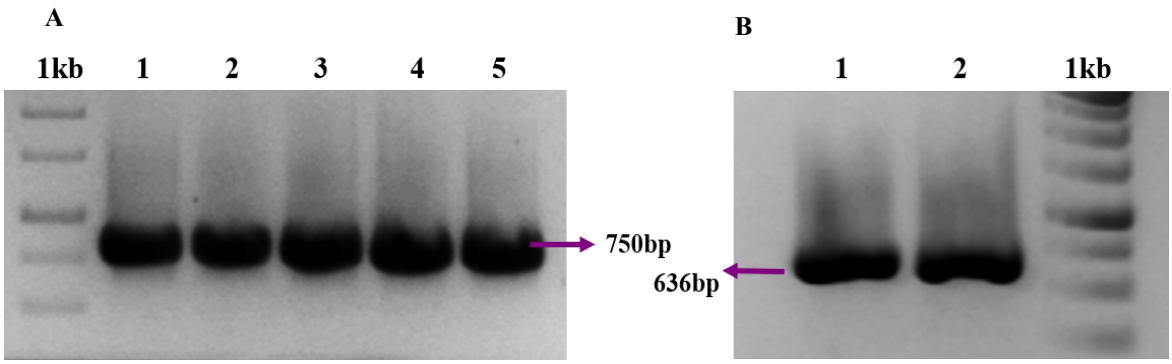


Figure 4.3: Amplification of *SmsHSP24.1* coding region from Eggplant cDNA by nested PCR. (A) First PCR amplification of initial 750bp of *SmsHSP24.1* gene from eggplant cDNA. (B) Second PCR amplification of 636bp of ORF region of *SmsHSP24.1* gene from TOPO vector 1kb: represents 1kb DNA ladder, 1, 2 *SmsHSP24.1* amplified lane

Solanum melongena mitochondrial small heat shock protein 24.1 mRNA, complete cds; nuclear gene for mitochondrial product

GenBank: MF579857.1

GenBank Graphics

```
>MF579857.1 Solanum melongena mitochondrial small heat shock protein 24.1 mRNA, complete
cds; nuclear gene for mitochondrial product
ATGGCAACTTCACTTGCTCTCAGGAGGGCTACTGCCTCATCGCTCTTCAATAGGCTCGTCAACCCTGTTC
GCTCTGCCTCTGTTTTCCGATCCTTCAATACTAACACTCAGATGACAGCTTATGACCAGGATGATCGTGA
TGTTGATGTCGAACGCCGGCCCGGTCGCTCCGTCTCTCGCCGCCGGGATGCTTTCCCTAGTTTGTTTTCA
GATGTATTTGATCCATTCTCACCACCAAGGAGCGTGAGCCAGCTACTAAACATAATGGACCAAATGATGG
ATTCTCCATTTGTAGCAGCACCGCGTTCTATGGGCACTGGAGTTGGTGCAAGAAGAGGATGGGACGTGAG
AGAGGATGACAATGCTCTGTATATAAAAATGGATATGCCTGGGCTCGACAAGGAGAACGTGAAGGTTGCG
GTGGAGGAGAACACGTTGATCATCAAAGGAGAAGGTGAGAAAGAATCAGAGGATGAAGAGTATAGGAGGA
GGTACTCGACGAGACTGGAAATTCCTCAAAATATGTACAAATTGGATGGAATTAAGGCGGAGATGAAGAA
TGGAGTGCTGAAAGTGGCTGTTCCAAAGGTGAAGCAAGAAGAAAGGAAAGATGTTTTCAATGTTCAAATT
GAGTGA
```

Figure 4.4: SmsHSP24.1 protein NCBI gene bank accession no (MF 579857.1).

The deduced amino acid sequence of SmsHSP24.1 showed noticeable homology with other reported mitochondrial small heat shock proteins from diverse species such as *Solanum lycopersicon, Arabidopsis thaliana, Solanum tuberosum, Capsicum annum,*

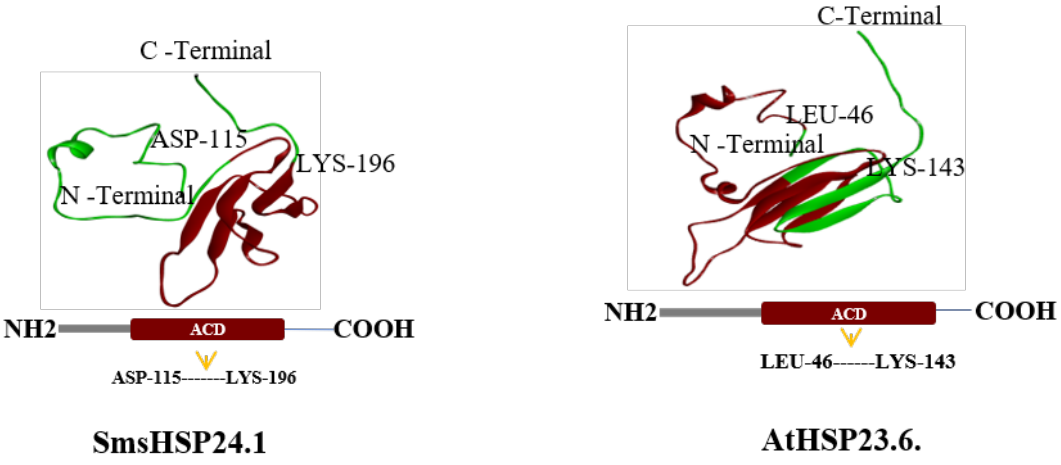


Figure 4.5: Homology modeling of SmsHSP24.1 protein compared to AtHSP23.6 (Protein databank ID P31170). A conserved 81-amino-acid α-crystalline domain (ACD) is present in SmsHSP24.1 at the C-terminus (positions +115 to +196). whereas AtHSP23.6 contains a conserved α-crystalline domain position +46 to +143.

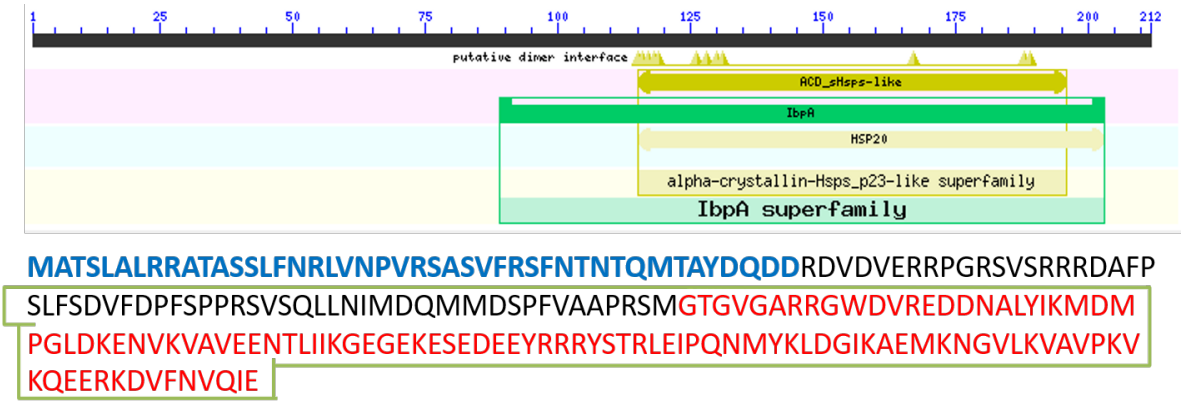


Figure 4.6: SmsHSP24.1 contains a conserved α-crystalline domain (ACD) of 81-amino-acid (positions +115 to +196).

Nicotiana tabacum, Glycine max, etc. According to homology modeling and multiple sequence alignment, SmsHSP24.1 has a conserved 81-amino-acid -crystalline domain (ACD) at the C-terminus (positions +115 to +196) (Figures 4.5, 4.6). This protein is closely related to tomato (SlsHSP23.5) and *Arabidopsis* (AtsHSP26.4), according to a phylogenetic study of deduced amino acid sequences from a wide variety of species (Figure 4.7).

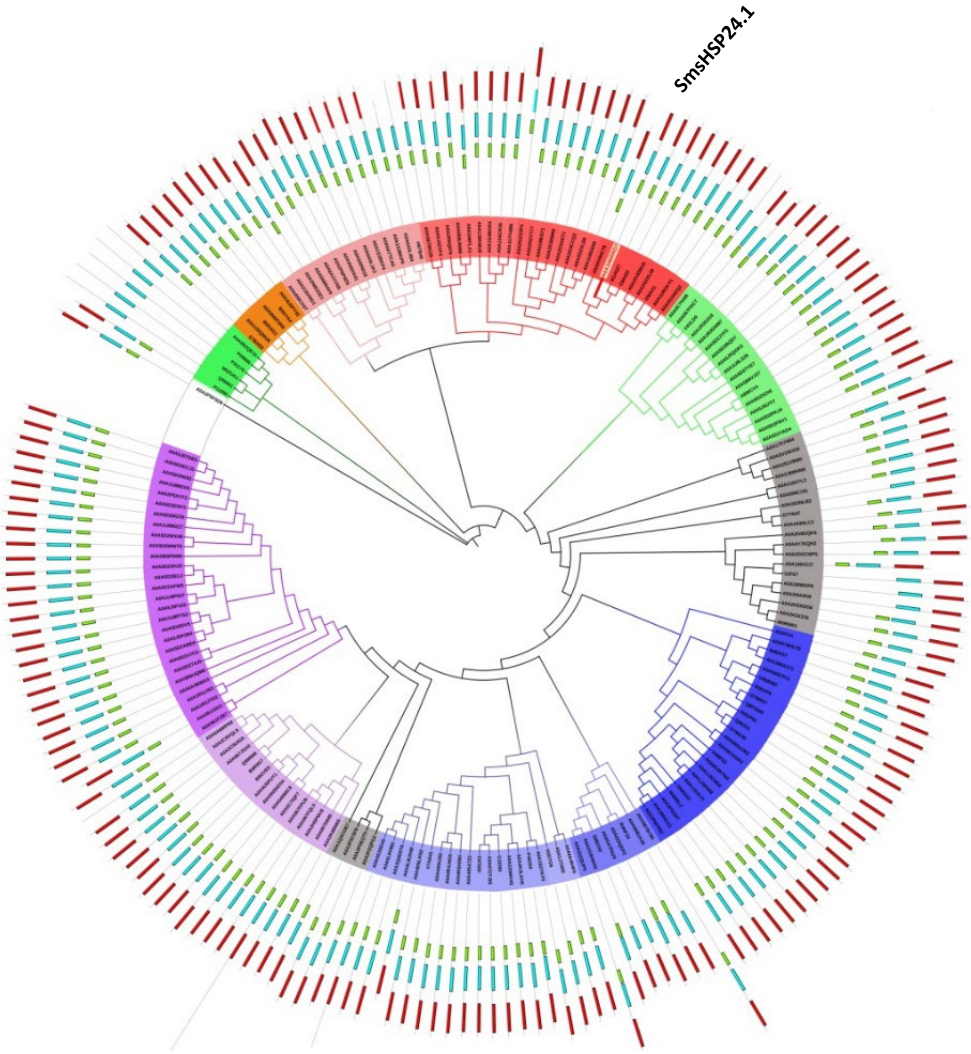


Figure 4.7: Phylogenetic tree depicts the diversity of mitochondria and chloroplast localized small HSPs from diverse plant species

4.1.3. SmsHSP24.1 response to multiple environmental stresses

In this study, we used BARI Begun-4, an Eggplant (*Solanum melongena* L.) variety collected from Bangladesh Agricultural Research Institute (BARI). The collected seeds were first surface

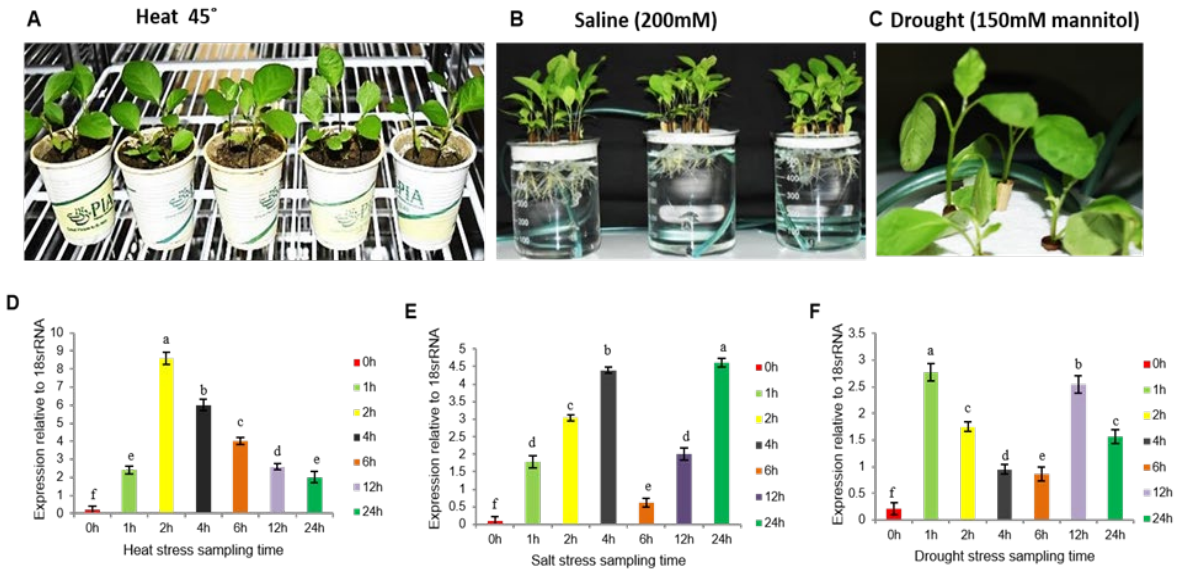


Figure 4.8: Analysis of SmsHSP24.1 expression in response to different abiotic stresses. (A-C) 3-week-old eggplant seedlings treated with 45 °C heat, 200 mM NaCl as salt, and 150 mM mannitol as drought stress were subjected to time-dependent relative quantitative RT-PCR analysis. (D-E) Relative RT-PCR analysis of SmsHSP24.1protein from seedlings under stress (heat, salt, or drought). The findings from RT-qPCR are mean SD (n = 3). Significant differences between samples are denoted by the letters a, b, and c. ($P < 0.05$).

sterilized in the laminar airflow with 1% bavistin and 0.1% mercuric chloride ($HgCl_2$) solution for a time period of 5 minutes. The seeds were then four to five times washed with autoclaved distilled water. After that, the seeds were poured onto the blotting paper to absorb the excess water inside the laminar airflow. Then the properly dried surface sterilized seeds were inoculated into a jam jar containing solidified MS salt (Murashige and Skoog. 1962) with 3% sucrose to support germination and seedlings development. Following two weeks of maintaining under fluorescent lighting on a 16-hour photoperiod at 25.2°C in a growth environment, germinated seedlings were planted hydroponically in Yoshida Solution (YS; Yoshida et al., 1976). Three-week-old seedlings were chosen to be exposed to all stimuli, including heat (45°C), salt (200 mM NaCl), and drought (150 mM mannitol), to better understand how SmsHSP24.1 expression in various tissues (Figure 4.8 A-C). To comprehend

SmsHSP24.1 physiological function under abiotic stress circumstances, we have also investigated the transcript abundance. A time-dependent quantitative RT-PCR analysis was carried out using RNA extracted from the leaves of 3-week-old Eggplant (*Solanum melongena* L.) seedlings treated with 200 mM NaCl as salt, 45˚C heat, and 150 mM mannitol as drought stress. The housekeeping gene 18s rRNA was used to normalize the expression of *SmsHSP24.1*, and the results were presented as the expression fold relative to that of untreated seedlings (Figure. 4.8 D-F). Under heat stress, SmsHSP24.1 showed the largest relative fold change of all the treatments. Within one hour of the heat treatment, there was an increase in transcript levels that peaked at a 9-fold change at two hours and subsequently gradually decreased (Figure 4.8 D). The expression dynamics of the SmsHSP24.1 transcript during salt stress were observed to differ from those under heat stress. During salt stress, the *SmsHSP24.1* transcript was highly upregulated at 4 hr of time point. At 4 hr of salt stress, an approximately 5-fold increase of *SmsHSP24.1* transcript was observed compared to the control. The expression of the *SmsHSP24.1* transcript was gradually reduced up to the 12 hr of salt stress. After that, a noteworthy upregulation was detected again at the 24 hr of salt stress (Figure. 4.8 E). In this case, the expression pattern of the *SmsHSP24.1* transcript was very much similar to 4 hr of salt stress. When Eggplant seedlings were treated with mannitol as drought stress, a quick expression of *SmsHSP24.1* was witnessed. Soon the osmotic agent was added to the plant at 2 hr of time point the *SmsHSP24.1* transcript reaching a peak point. However, the *SmsHSP24.1* transcript started to decline steadily up to 12 hr of drought stress. At 24 hr of drought stress the expression pattern of *SmsHSP24.1* was 1.5-fold lower than the transcript monitored at 1 hr of drought stress (Figure. 4.8 F).

4.1.4. Construction of fluorescent fusion protein-based expression vector for subcellular localization

We used green fluorescent fusion protein (GFP) based expression vector PCAMBIA1302 for subcellular localization of SmsHSP24.1 protein. The coding sequence of *SmsHSP24.1* genes was PCR amplified by removing the stop codon, subsequently inserted into PCAMBIA1302 in the middle of 35S promoter and green fluorescent fusion protein (GFP) with nopaline synthase terminator (NosT) by restriction digestion of enzyme *Bgl*II from

forward and *Spe*I from reverse sites. The final expression cassette containing binary vector was transferred to *Agrobacterium tumefaciens* EHA105 by electroporation (Figure. 4.9).

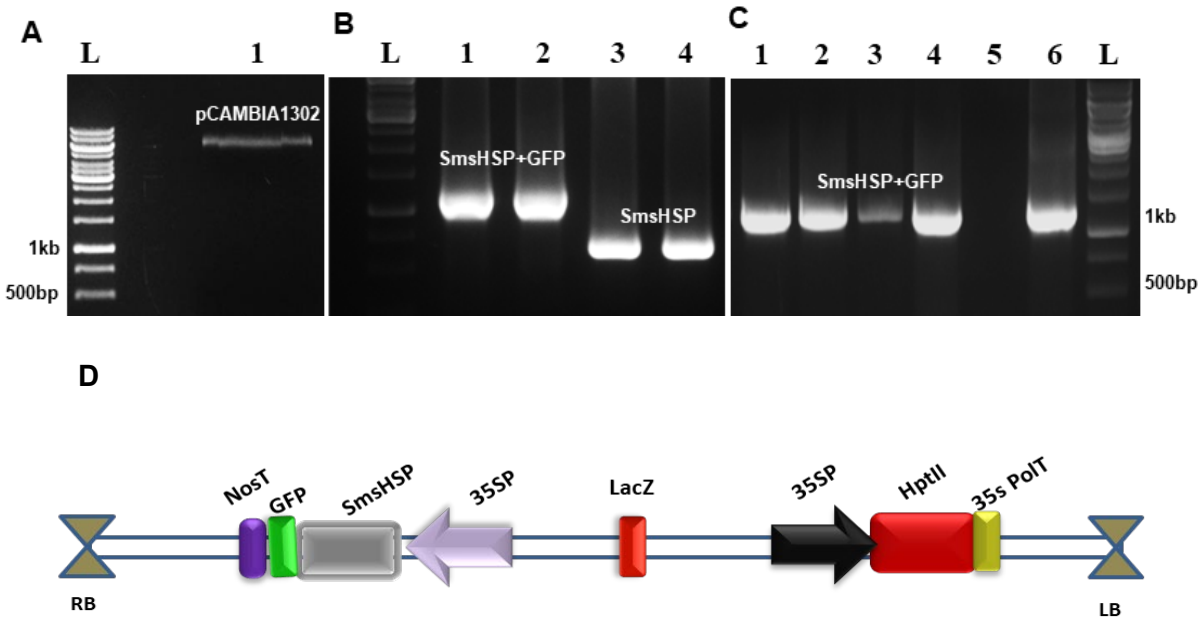
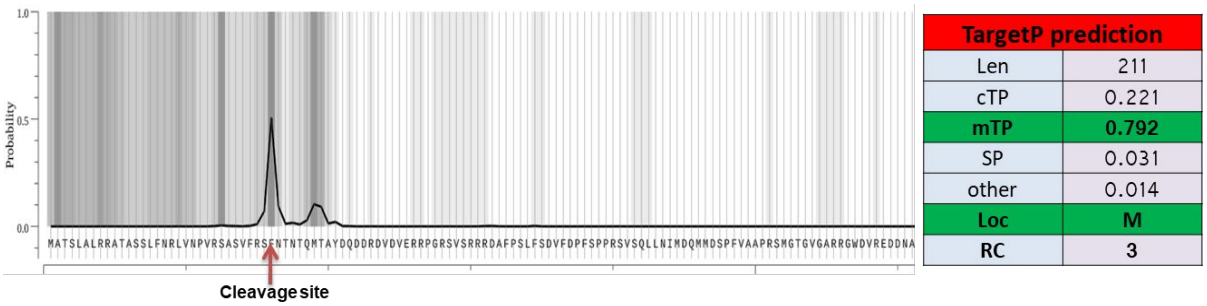


Figure 4.9: Construction of Fluorescent fusion protein-based expression vector. (A) Restriction digestion of pCAMBIA1302 vector with *Bgl*II and *Spe*I. (B) *SmsHSP24.1* and *SmsHSP24.1*-mGFP fused construct conformation in pCAMBIA1302, 1, 2: *SmsHSP24.1*-mGFP fused coding region, 3, 4: *SmsHSP24.1* coding region amplification. (C) PCR confirmation of the *SmsHSP24.1* expression cassette in *Agrobacterium* strain EHA105, 1, 2, 3, 4, 5: *SmsHSP24.1* amplified lane, 6: (+) positive control. (D) Schematic diagram of *SmsHSP24.1*-GFP gene expression cassette in PCAMBIA1302.

4.1.5. In silico signal peptide prediction of SmsHSP24.1 protein

Proteins are synthesized with an organelle-specific signal peptide for proper transportation and performed optimal functions inside the pre-determined organ which determined the fate of the cell. Biochemical reactions are often carried out with complex interactions with different proteins in different organelles and hence, correct transportation and subcellular localization is crucial. Therefore, understanding protein subcellular localization is the first and foremost step in investigating the role of a particular protein. Preliminary we have used bioinformatics-based prediction tools TargetP (http://www.cbs.dtu.dk/services/TargetP/) and SmsHSP24.1 protein targeting signal to be examined using MitoFates. The in-silico tools MitoFates and TargetP both proved that the SmsHSP24.1 protein is localized in mitochondria

with a considerably high score of 0.792 and 0.997, respectively, the in-silico tools MitoFates and TargetP both demonstrated that the SmsHSP24.1 protein is localized in mitochondria. (Figure. 4.10).



TargetP prediction	
Len	211
cTP	0.221
mTP	0.792
SP	0.031
other	0.014
Loc	M
RC	3

Figure 4.10: *In silico* prediction of SmsHSP24.1 protein subcellular localization.

4.1.6. Transient expression of SmsHSP24.1-mGFP protein in tobacco epidermal cell

To evaluate protein subcellular localization, the rapid transformation of tobacco leaf epidermal cells is a comparatively quick, easier, and more effective method. The young tobacco apical leaves (3-week-old seedlings with 5–6 leaves) were infiltrated with *Agrobacterium* strain harboring SmsHSP24.1 protein fused with mGFP. The *Agrobacterium* strain containing blank mGFP (No insert) was also infiltrated as a control. Three plants were Agroinfiltrated at each time. The leaf tissues were examined using a confocal fluorescence scanning microscope 72 hours after inoculation. The transformed leaves were photographed with excitation at 488 nm and emission at 520 nm. SmsHSP24.1-mGFP confocal fluorescence scanning examinations of the leaf epidermal cells revealed punctate structures that appeared to be localized to mitochondria (Figure. 4.11).

4.1.7. Eggplant cell suspension culture confirms SmsHSP24.1 is a nuclear-encoded mitochondria localized protein

Though Agroinfiltration based method is mostly being used to detect the subcellular localization of target proteins in tobacco leaves. However, some drawback of this technique is

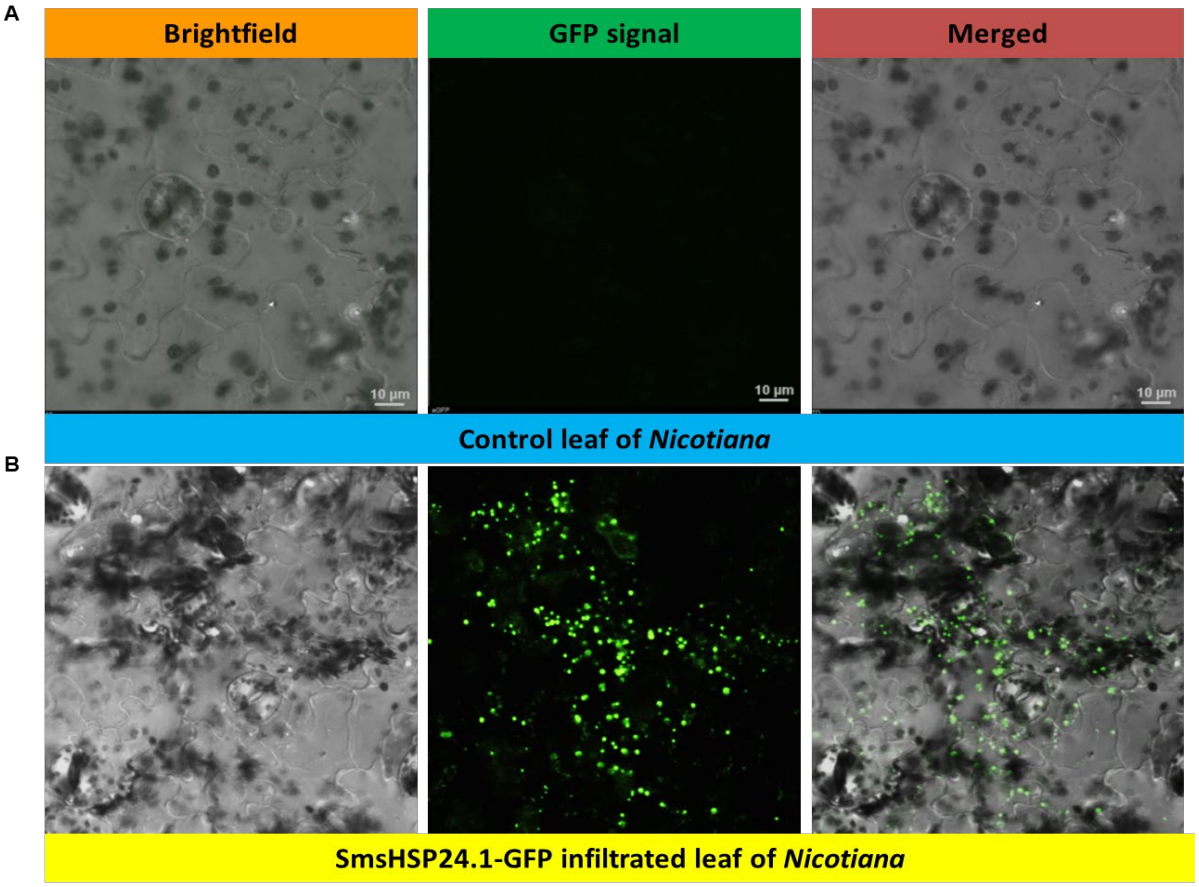


Figure 4.11: Subcellular localization of SmsHSP24.1 protein through agroinfiltration. (A) Fluorescence scanning confocal microscopy of the Agro-infiltrated control tobacco epidermal cells appeared with no GFP fluorescent signal. **(B)** Agro-infiltrated tobacco epidermal cells with pCAMBIA::CAM35SP:*SmsHSP24.1*-mGFP:NosT construct revealed punctate structures in leaf epidermal cells which appeared to be localized in the mitochondria.

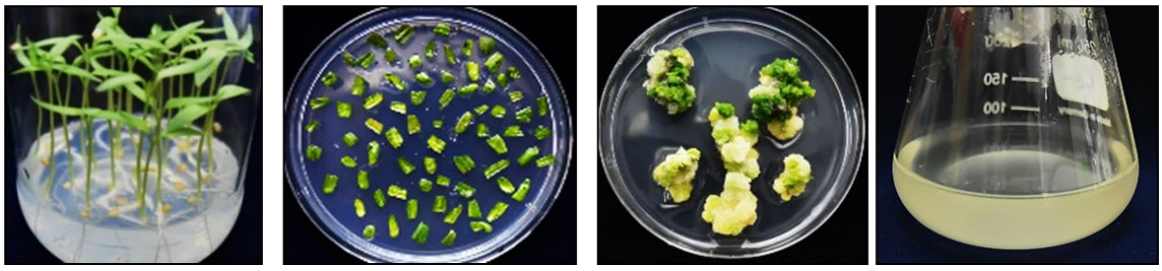


Figure 4.12. Preparation of stably integrated Eggplant cell suspension culture

a high content of chloroplasts and chlorophyll content in green leaves which prevents certain microscopical applications as the strong autofluorescence of chlorophyll can mask the signal of fluorescent tag

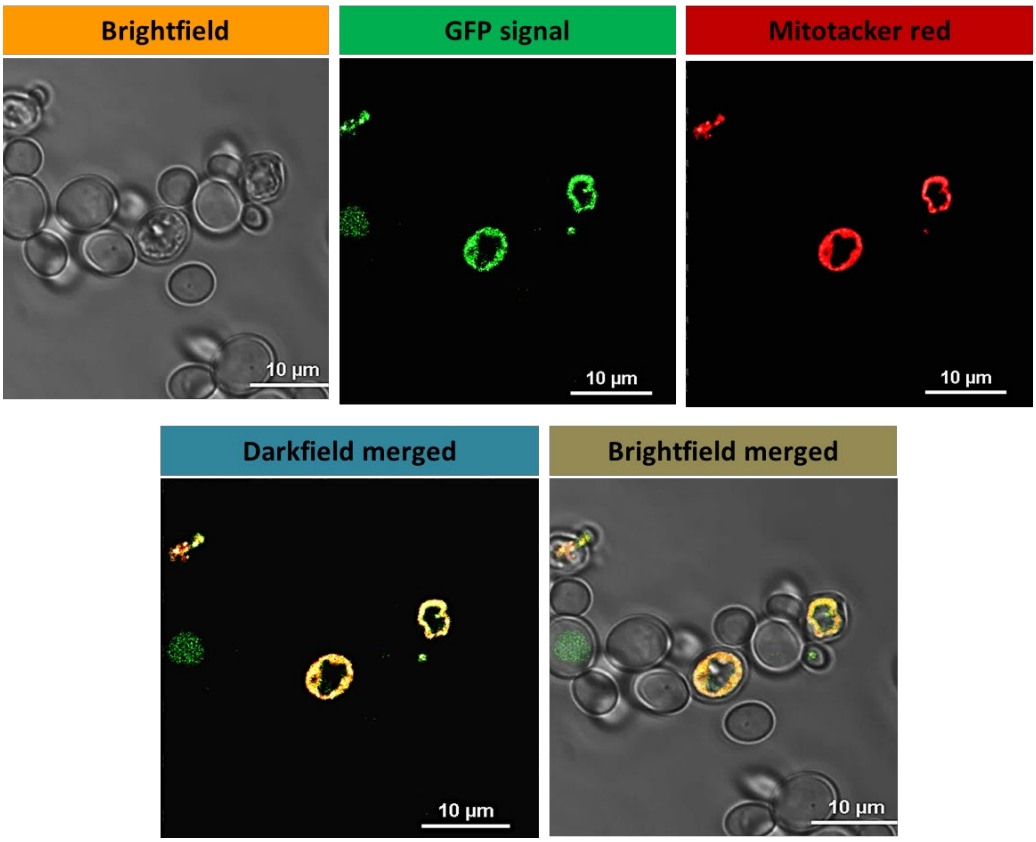


Figure 4.13: Eggplant suspension cells carrying the expression construct (pCAMBIA::CAM35SP:SmsHSP24.1-mGFP:NosT). Co-localization with mitochondria-specific dye MitoTracker Red. The bar is 10 µm.

labeled proteins under UV or confocal microscopy. Considering all the limitations we used a stably transformed Eggplant cell suspension culture for studying organelle-specific protein localization (Figure. 4.12). Laser scanning confocal microscopy of *SmsHSP24.1*-mGFP transformed cells showed GFP signals in small dots that were identified as mitochondria. The mitochondrial localization of SmsHSP24.1-mGFP was further confirmed by co-localization with a mitochondria-selective dye MitoTracker Red. This result clearly showed that Eggplant

mitochondrial small heat shock protein (SmsHSP24.1) is targeted to the mitochondria (Figure. 4.13).

4.1.8. Cloning and heterologous expression of SmsHSP24.1 protein in *E. coli* cells

To obtain large amounts of highly purified SmsHSP24.1 protein for biochemical characterization, we expressed SmsHSP24.1 protein in *E. coli*, a heterologous expression system. For this, the full-length cDNA of *SmsHSP24.1* was cloned into a bacterial expression vector. *SmsHSP24.1* gene was digested with restriction enzyme *Nde*I from the forward side and *Not*I from the reverse side using a previously cloned plasmid. The pET28a vector was also digested with *Nde*I and *Not*I restriction enzyme (Figure. 4.14 A, B). The gel purified digested *SmsHSP24.1* insert and pET28a plasmid both were kept for ligation. The *E. coli* BL21 DE3 cells were used to transformed the ligated product followed by plating on LB agar media that contained kanamycin (50mg/L) as a selective agent. The obtained colonies were screened by colony PCR using gene-specific primers and plasmids were isolated from the positive colonies. The plasmid of pET28a_ *SmsHSP24.1* was confirmed by the restriction digestion by *Nde*I and *Not*I (Figure. 4.14 C, D). Positive pET28a_*SmsHSP24.1* plasmid was sent for sequencing and the construct of pET28a_*SmsHSP24.1* was prepared for recombinant SmsHSP24.1 protein expression. The recombinant plasmid (pET28a_*SmsHSP24.1*) contained a thrombin-cleavable His-tag at the N-terminal end of the protein (Figure. 4.14 E). The pET28a_*SmsHSP24.1* recombinant plasmid and pET28a empty vector plasmid were transformed into *E. coli* BL21 (DE3) cell for recombinant protein production. The BL21 (DE3) cells were grown till OD600 = 0.5 and were induced for the expression of recombinant SmsHSP24.1 protein with different concentrations of IPTG (1, 0.5, 0.3 and 0.1 mM) at various incubation temperatures (18ºC, 28ºC and 37ºC). The expression of SmsHSP24.1 was analyzed on 12% SDS-PAGE, and subsequently stained with Coomassie brilliant blue (CBB). A highly induced band of ~24.1 kDa represented SmsHSP24.1 protein was observed in the induced fraction at both temperatures with all IPTG concentrations (Figure. 4.15 A).

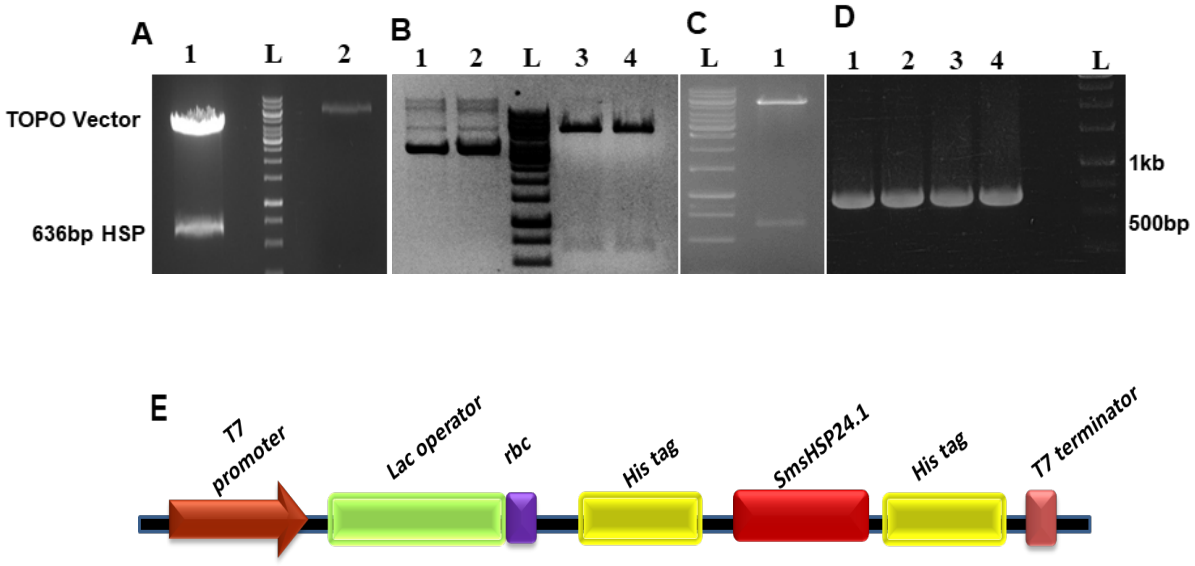


Figure 4.14: Construction of pET28a::*SmsHSP24.1* expression vector for recombinant protein production. (A) Restriction digestion of pET-28a (+) and TOPO vector containing *SmsHSP24.1*, 1: 636 bp *SmsHSP24.1* from TOPO vector, 2: pET-28a (+) vector. (B) *SmsHSP24.1* gene conformation in pET-28a (+) vector, 1, 2: Uncut pET-28a (+) vector, 3,4: Digested pET-28a (+) vector. (C) *SmsHSP24.1* gene confirmation by restriction digestion from plasmid isolated from host *E.coli* BL21 (DE3) cell. (D) PCR confirmation of the *SmsHSP24.1* expression cassette in *E. coli* BL21 (DE3) cell. © Schematic diagram of SmsHSP24.1 protein expression cassette.

To study the function of SmsHSP24.1, recombinant protein was required to be purified in the native state from the soluble fraction of total cell lysates. It was observed that SmsHSP24.1 came into soluble fraction after induction at a low temperature (18^{o}C) with a low concentration of IPTG (0.5 mM) (Figure. 4.15 C). At lower temperatures, bacterial growth as well as protein synthesis rate slows down and thus, recombinant protein gets enough time to refold and come in the soluble fraction. The recombinant SmsHSP24.1 protein was purified by routine affinity chromatography with immobilized Ni-NTA and eluted using 250 mM of imidazole. The flow-through (FT), wash-through (WT) and elution fractions (E-1 to E-5) were run on 12 % SDS-PAGE and stained with CBB. The obtained purified (> 95 %) protein showed only a single band of ~26.4 kDa (including His-tag). The eluted purified protein fractions were pooled and dialyzed against MOPS buffer (10 mM, pH 7.0). The concentration of protein was estimated

to be around 2µg/µl. Then the western blotting techniques were applied to confirm the purified protein using anti-his antibodies. (Figure. 4.15 B).

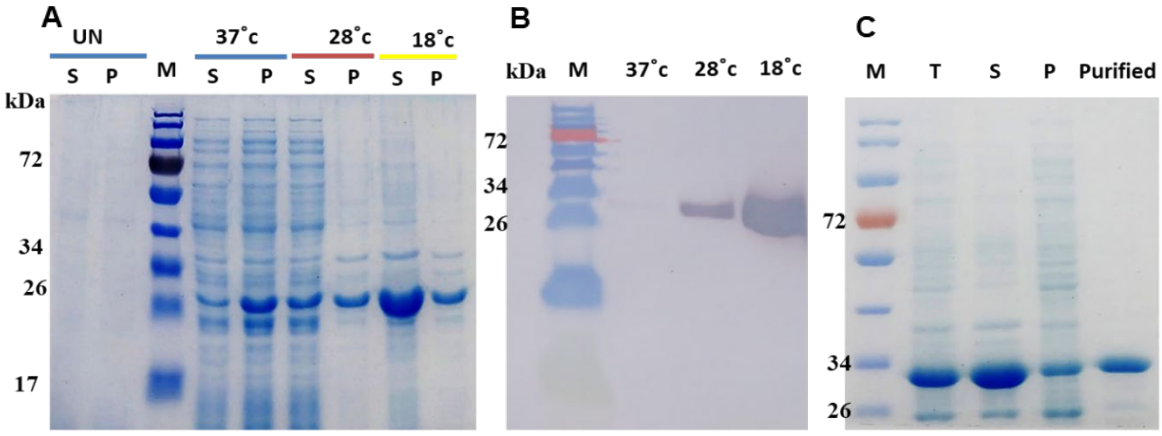


Figure 4.15: Recombinant SmsHSP24.1 protein expression and confirmation by western blotting. **(A)** Heterologous expression of SmsHSP24.1 protein at three different temperatures, (M) indicates protein marker, (UN) indicates uninduced cell, (S) indicates soluble, (P) indicates insoluble pellet, **(B)** Western blot of SmsHSP24.1 protein with Anti-His tag antibody. **(C)** Analysis of the purified SmsHSP24.1 proteins; (T) represents total lysates, (S) represents soluble format, (P) represents insoluble pellet and the last lane is purified SmsHSP24.1 protein.

4.1.9. Heterologous overexpression of SmsHSP24.1 protein enhances thermo-tolerance of *E. coli* cells under severe heat stress

To analyze whether SmsHSP24.1 protein is capable to act as a chaperone by examining the thermotolerance competency of the recombinant *E. coli* cells. The BL21 (DE3) strain of *E. coli* cells having pET28a::*SmsHSP24.1* expression vector was grown. Empty pET28a vectors in BL21 (DE3) strain of *E. coli* cells were also grown as controls. The growth rates of the blank pET28a and pET28a-*SmsHSP24.1* harboring strains showed no noticeable difference, indicating that *E. coli* cells growth did not affect by SmsHSP24.1 protein overexpression under normal growth conditions (Figure. 4.16 A). Remarkably, we noticed under heat stresses, heterologous expression of SmsHSP24.1 protein enabled the *E. coli* cells to efficiently protect the heat shock as high as 55°C for one hour time period, whereas the control *E. coli* cells were found to be completely killed (Figure. 4.16 A). Following one-hour heat treatment at the temperature of 50°C, the viability of *E. coli* cells in absence of SmsHSP24.1 protein was 10% in contrast to 65% of *E. coli* cells expressing SmsHSP24.1 protein. Cell survival was measured

by counting colony-forming units (Figure. 4.16 C). We again enquired whether the survival rate and the prevention of protein aggregation of *E. coli* cells were in part due to the capability of the SmsHSP24.1 protein. The soluble fractions of the SmsHSP24.1 protein expressing *E. coli* cells revealed a significant increase in the level of soluble proteins without affecting heat shock at different temperatures. SmsHSP24.1 protein

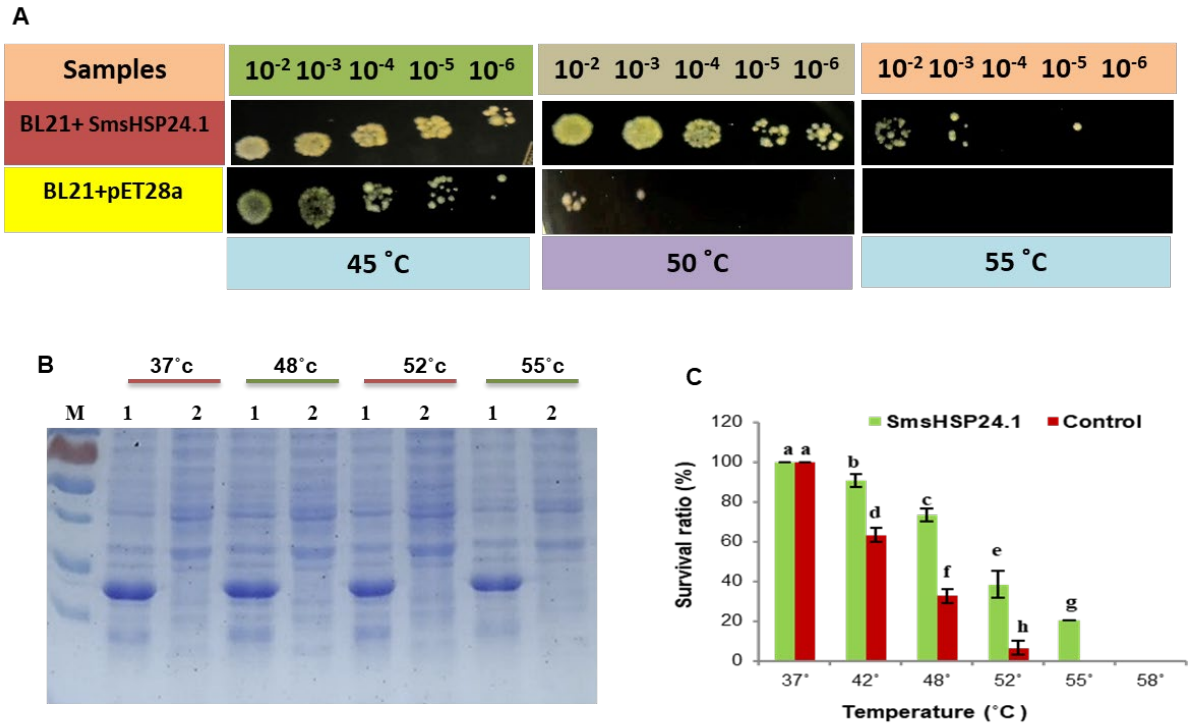


Figure 4.16: SmsHSP24.1 protein enhances thermo-tolerance of *E. coli* cells. (A) Heat-shocked BL21 cells harboring the pET28a::SmsHSP24.1 and control pET21a plasmids were cultured in LB media for an hour at 45°C, 50°C, and 55°C. (B) SDS-PAGE examination of the soluble fractions in the pET28a-SmsHSP24.1 bearing BL21 cells revealed a considerable increase in total soluble proteins when compared to pET28a-blank at various temperatures. (C) Colony-forming units were measured from cells that were heat-treated and those that weren't at various temperatures i.e., 37˚C, 48˚C, 52˚C, 55˚C and 58˚C.

expressed more prominently than other proteins in the *E. coli* cells at all indicated temperatures when compared with the control cells without expressing SmsHSP24.1 protein (Figure. 4.16 B)**.** These observations suggested that the SmsHSP24.1 protein could enhance the viability of *E. coli* transformant cells by acting as a highly efficient molecular chaperon under heat stresses.

4.1.10. SmsHSP24.1 protein acts as a molecular chaperon via polyhedral assembly formation

To determine if the novel SmsHSP24.1 protein can serve as a chaperone and improve survival under stressful circumstances, we carried out several experiments. We performed an electron microscopy examination of the negatively stained protein sample at low (4ºC) and high (45ºC) temperatures which indicated that the SmsHSP24.1 protein formed a wide distribution of oligomeric assemblies (Figure. 4.17 A). All oligomeric assemblies including dimers and a tetramer are probably a building block of the larger oligomers. Notably, when SmsHSP24.1 protein was heated at 45ºC, transmission electron microscopic (TEM) data revealed that the SmsHSP24.1 protein

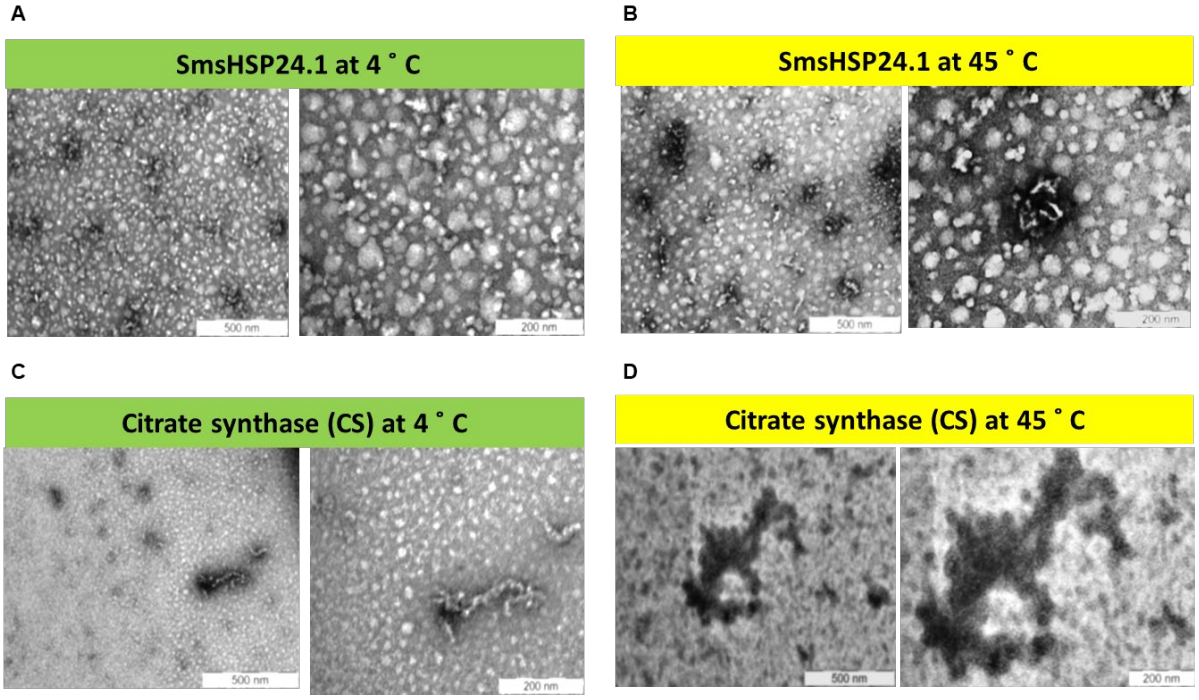


Figure 4.17: Chaperone activity assay of SmsHSP24.1 protein. (A, B) Transmission electron microscopy images of SmsHSP24.1 protein at 4˚C and 45˚C. (C, D) Citrate synthase (CS) protein pictures were taken using transmission electron microscopy at 4˚C and 45˚C. Scale bars indicate 500 nm and 200 nm.

assembled into multiple polyhedral topologies (Figure. 4.17 B). To gain insight, we next demonstrated by electron microscopy to find out the chaperoning activity of the SmsHSP24.1 protein. In this experiment, we used two heat-sensitive model substrate proteins, alcohol dehydrogenase (ADH) and citrate synthase (CS). Normally, citrate synthase (CS) and alcohol dehydrogenase (ADH) are rapidly inactivated and aggregated when heated at 45ºC and 50ºC respectively. Electron microscopy analysis of the model substrates citrate synthase (CS) at low temperature (4ºC) remained in their active forms (Figure. 4.17 C). At 45ºC when checked under an electron microscope, we noticed that CS is completely denatured and aggregated (Figure. 4.17 D). We further investigated the status of the combined effect of SmsHSP24.1 protein with CS at the same temperature of 45ºC for 60 min. It was found that SmsHSP24.1 protein clearly prevented model substrate citrate synthase (CS) denaturation (Figure. 4.18 A, B). Instead of being aggregated or unfolded, model substrate citrate synthase (CS) was completely protected from thermal aggregation with the help of different polyhedron structures of SmsHSP24.1 protein. By using a light scattering spectrophotometer experiment, the thermally-induced aggregation of the enzymes alcohol dehydrogenase (ADH) at 50°C and citrate synthase (CS) at 45°C was also evaluated. Normally, citrate synthase (CS) and alcohol dehydrogenase (ADH) are rapidly inactivated and aggregated when heated to 45ºC and 50ºC respectively. CS and ADH began to form insoluble aggregates that could be detected by light scattering spectrophotometer (Figure. 4.18 C, D). SmsHSP24.1 protein was incubated with substrates CS and ADH at different molar ratios. As determined by the absorption, the increasing trend slowed down at different SmsHSP24.1: CS/ADH ratios after 60 min at 45ºC/50ºC respectively. SmsHSP24.1 prevented CS from aggregating at a 1:1 molar ratio (SmsHSP24.1: CS). In contrast, SmsHSP24.1 completely prevented CS aggregation at a 2:1 molar ratio (Figure 4.18 C). Similar to SmsHSP24.1, ADH was also discovered to be stopped from aggregating at a 2:1 molar ratio. (Figure. 4.18 D).

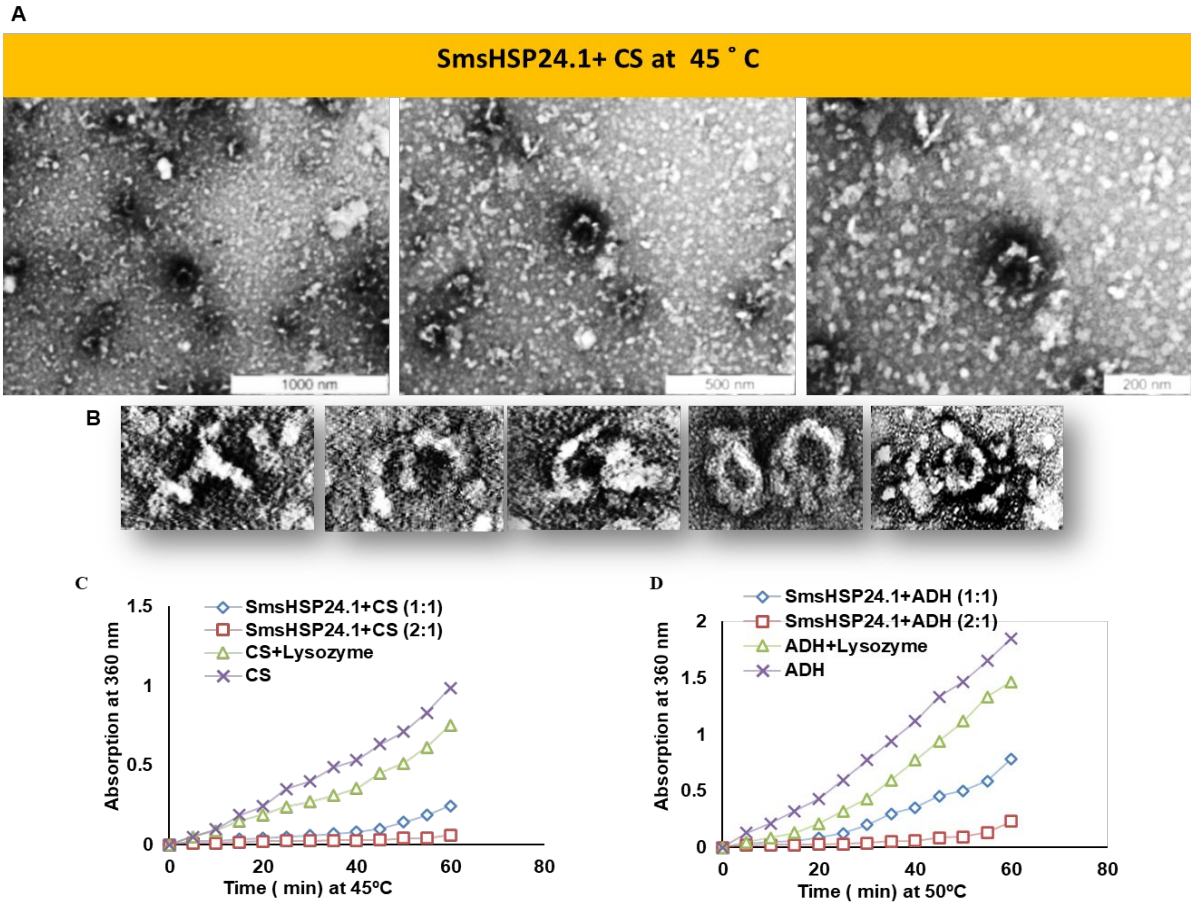


Figure 4.18: SmsHSP24.1 protein protects host client protein via polyhedral assembly formation. (A) Electron microscopy images clearly showed that when incubated at 45 °C for 60 min, SmsHSP24. 1 protein prevents model substrate CS. Scale bars show 1000 nm (Left), 500 nm (Middle) and 200 nm (Right panel). (B) SmsHSP24.1protein forming different polyhedron assemblies. (C, D) Light scattering at 360 nm was used to identify the thermal aggregation of CS and ADH.

Further, we have analyzed the chaperone activity of SmsHSP24.1 protein using a thermo-protection assay of restriction enzyme *Nde*I as a substrate. Restriction enzyme *Nde*I is thermolabile and enzymatic activity could reduce or completely damaged when its pre-incubating at high temperatures. We checked the protection of *Nde*I restriction enzyme against heat inactivation by preincubating with 5 units of restriction enzyme *Nde*I mixed with 5 mg of either acetylated BSA or recombinant purified SmsHSP24.1 protein. Then we kept the mixture at different temperatures ranging from 37°C to 55°C for 60 min followed by assaying the

digestion activity of restriction enzyme *Nde*I on plasmid DNA. We found without SmsHSP24.1 protein the restriction endonuclease *Nde*I completely failed its function after incubation for 60 min at temperatures above 45°C. At this temperature, BSA failed to protect against thermal inactivation of *Nde*I, whereas SmsHSP24.1 protein provided significant protection of *Nde*I restriction enzyme even at 52°C. However, the SmsHSP24.1 protein was ineffective against the thermal inactivation of *Nde*I at 55°C. This experiment demonstrated that at higher temperatures the purified SmsHSP24.1 protein remains the restriction enzyme *Nde*I in a folding competent state and the hexa-histidine tag at N-terminus is not interrupting in this action (Figure. 4.19).

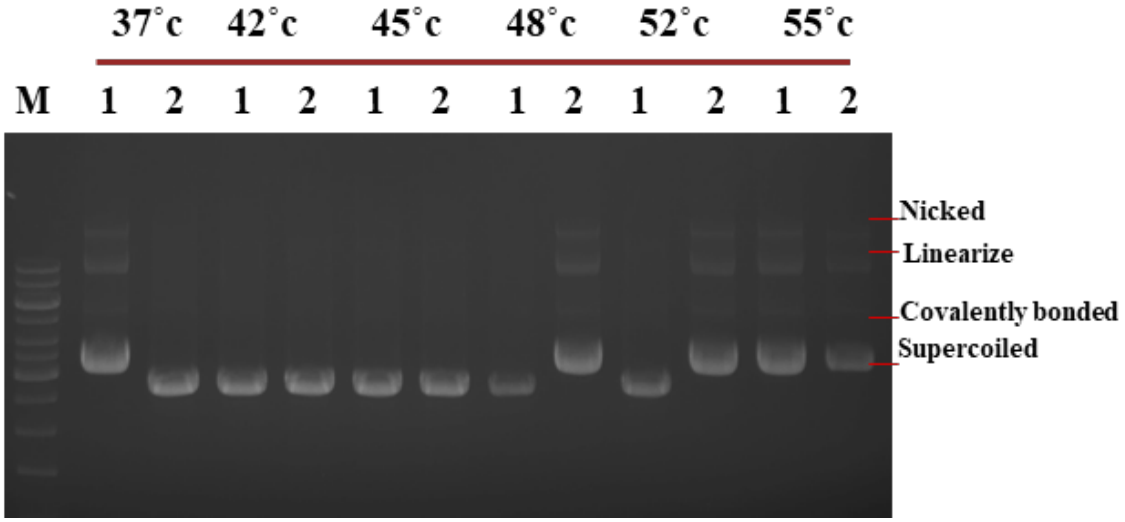


Figure 4.19: Plasmid DNA digestion by *Nde*I restriction enzyme (RE) incubated with or without purified SmsHSP24.1 protein at indicated temperatures. 37 °C used as control where lane 1 indicates undigested plasmid, and lane 2 indicates digested plasmid. From 42 °C to 55 °C lane 1 indicates restriction enzyme (RE) *Nde*I with SmsHSP24.1 protein, lane 2 indicates restriction enzyme (RE) *Nde*I without SmsHSP24.1 protein.

Discussion:

Plants respond to heat and other abiotic stressors through the accumulation of molecular chaperones like heat shock proteins (HSPs). Among the HSP subgroups small heat shock protein (sHSPs) belongs to the most abundant groups with 12 to 42 kDa molecular weight. The sHSPs are universally present in all three kingdoms of life (de Jong et al., 1993; Narberhaus, 2002). Plant sHSPs are nuclear-encoded genes and transported to different subcellular compartments based on amino acid sequence similarity. It can be subdivided into different subfamilies, of which two are localized in the mitochondria (MI and MII), six are localized in the nucleus or cytosol (CI, CII, CIII, CIV, CV, CVI) and the others in the endoplasmic reticulum (ER), plastids (P), and the peroxisomes (Masood Siddique et al., 2008; Sun et al., 2002). sHSPs share a signature alpha-crystallin domain (ACD) at the C-terminal region containing approximately ~90 amino acids long, which has a β-sandwich conserved structure (Basha et al., 2010; Sarkar et al., 2009). Under *in vitro* circumstances, heat shock proteins are frequently found to accumulate into 12–40 subunits forming large oligomers, where single dimers are used as the building block (Fu, 2014). sHSPs can bind substrate proteins without the help of ATP and they have a very high ability to bind stress imposed unfolded or denatured substrates (Fragkostefanakis et al., 2015; Waters, 2013). Under stress conditions plant small sHSPs also hold membranes and proteins and assist in protein refolding (Wang et al., 2004). Under normal environmental growth conditions, most of the sHSPs transcript cannot be detected, but in response to heat stress, they are quickly detected (Joshi, 1994; Vierling, 1991). Also, the accumulation of diverse types of sHSPs during various environmental stresses and developmental phenomena indicates that each sHSP might have a general as well as a specific adaptive role in protecting cells from stresses. Since the discovery of the first small heat shock protein (sHSP) in *Drosophila melanogaster*, various other sHSPs have been detected in numerous plant species (M. Siddique et al., 2008; Wang et al., 2017) There are several reports demonstrated the role of sHSPs, However, the biological functions of sHSPs not completely understood. In our work, we have successfully cloned a full-length cDNA of mitochondrial small heat shock protein (SmsHSP24.1) from heat treated Eggplant (*Solanum melongena* L.) leaves. Based on the multiple sequence alignment and phylogenetic analysis with other plants sHSPs, SmsHSP24.1 showed noticeable homology with mitochondrial small heat shock protein. Particularly phylogenetic analysis revealed

SmsHSP24.1 protein is highly similar to *Solanum lycopersicon* mitochondrial small heat shock protein 23.5 (BAA32547.1). Whereas, homology modelling of SmsHSP24.1 protein indicated that this protein is closely related to *Arabidopsis* mitochondrial small heat shock protein (AtsHSP26.4) this might be the reason for *Solanum lycopersicon* mitochondrial small heat shock protein 23.5 structural information is not available in the protein data bank (PBD). Homology modelling of SmsHSP24.1 protein showed 81-amino-acid long conserved α-crystalline domain (ACD) at the C-terminus region (positions +115 to +196) Likewise, *Arabidopsis* mitochondrial small heat shock protein (AtsHSP26.4) also contain conserved α-crystalline domain (ACD) at the C-terminus region but the position varies from +46 to +143 position. The α-crystallin domain (ACD) is an ancient domain conserved among all kingdoms (Paul et al 2006). sHSPs mostly share a diverse N-terminal domain and a signature α-crystallin domain (ACD) with approximately 90 amino acids long β-sandwich structure at the C-terminus end. The main role of that signature α-crystalline domain is to bind to denatured or unfolded proteins to prevent their nonspecific accumulation. The structural core of all small HSPs is the signature domain ACD forms.

During various stresses condition, it is proposed that plant small sHSPs are up-regulated and act as molecular chaperones by protecting other unfolded or aggregated substrate proteins from stress-induced damages. Numerous reports have previously explained a correlation between the expression of plant small sHSPs and their role in adaptation to several stress conditions. The chloroplast-localized small heat shock protein (sHSP21) in tomatoes is activated by heat shock in the leaf tissue of tomato plants (Neta-Sharir et al., 2005). An *Arabidopsis* small heat shock protein (AtHSP15.7) is actively produced by both heat and oxidative stress (Ma et al., 2006). Similarly, a chloroplast-localized rice small heat shock protein (OsHSP26) is induced by oxidative and heat stress (Lee et al., 2000). Also, the accumulation of *Tamarix hispida* small heat shock protein (ThHSP18.3) is activated by heat, drought, cold, and salt stresses (Gao et al., 2012). On the other hand, RcHSP17.8, is rapidly induced in leaves of *R. chinensis* by heat stress, drought, cold and salt (Jiang et al., 2009). Likewise, *Primula forrestii* small heat shock protein (PfHSP17.1) from *Primula leave* was also induced by various abiotic stress treatments like heat, drought, PEG, cold, salt, and oxidative stresses (Zhang et al., 2013). In addition, LimHSP16.45 a *David Lily* small heat shock protein gene, is induced by heat and cold stresses (Mu et al., 2013). Although, over the years have

immensely broaden our understanding of the diversity and evolution of plant sHSPs, and numerous reports already indicated that they are vital for surviving under different abiotic stress conditions, but still, we are distant from understanding their mood of action (Waters & Vierling, 2020). We performed time-dependent reliant quantitative RT-PCR research to explore the influence of heat, high salinity, and dehydration treatment on new SmsHSP24.1 expression. Even though we found induced expression of SmsHSP24.1 protein under all given abiotic stresses i.e.; heat, salinity and drought nonetheless, the relative expression fold of SmsHSP24.1 under heat stress is much greater than that under salinity or drought treatment. This result of our study also supports the view that like other small sHSPs, the expression of SmsHSP24.1 protein is one of the unique strategies for plants for rapid adaptation under heat stress conditions. Furthermore, our observations revealed the imperative and persistent role of SmsHSP24.1 expression for adaptation of Eggplant under heat, drought and salinity stress condition.

Understanding protein subcellular localization is the first and foremost step in investigating the role of a particular protein and function elucidation of individual proteins and the organization of the cell as a whole. Earlier, an array of approaches has also been demonstrated and successfully used to determine the subcellular localization of a protein in plant molecular biology mostly by performing transient expression of the target protein. Generally, subcellular localization of tasted proteins is studied through transient expression of the desired protein in heterologous plant system such as onion epidermal cells (Hattori et al., 2009; Ma et al., 2018; Nebenführ, 2014), *Arabidopsis* (Jun et al., 2015), rice (Kuijt et al., 2004), tobacco (Dvořák Tomaštíková et al., 2020; Kokkirala et al., 2010; Sasaki et al., 2018) or tomato (Kokkirala et al., 2010). Nevertheless, in different plant species subcellular localization in a heterologous way may not devotedly replicate the native subcellular localization. Ectopic expression of transformed proteins may outcome in mislocalization as heterologous expression system lack of conserved signal sequences (Marion et al., 2008). The transient expression-based Agroinfiltration method is mostly being used to detect the subcellular localization of target proteins in green leaves. However, the drawback of this technique is the high content of chloroplasts and chlorophyll content in green leaves which prevent particular microscopic signal and protein studies. Under UV microscope the bright autofluorescence signal of chlorophyll can cover the signal of fluorescent tag labelled proteins under such circumstance

(Elliott et al., 1999; Mathur & Koncz, 1998) Considering all the limitations associated with detecting subcellular localization of a new protein with the host plant cells, we developed an alternative method where we effectively examined the expression of targeted proteins and to track down its place of function in its own system. Avoiding the need for heterologous expression not only overcomes mislocalization, devoid of green chloroplast also helps to surpass the difficulties of auto-fluorescence of chlorophyll and photosynthesis-related proteins during confocal microscopic imaging. We found plant cell suspension culture-based protein expression system is the easiest and most reliable way to examine the subcellular localization of targeted proteins. MitoTracker™ FM Agroinfiltration on tobacco-leaf and eggplant cell suspension cultures provided unambiguous proof that new SmsHSP24.1 is a protein localized in the mitochondria. A class of extremely conserved molecular chaperones small sHSPs maintain protein homeostasis by averting aggregation or irreparable unfolding of proteins under several stress situations (Horwitz, 1992; Jakob et al., 1993; Yu et al., 2021).). In response to heat shock some small sHSPs can express up to 1% of entire cellular proteins, displaying their essential function in cellular stress response and thermotolerance (Hsieh et al., 1992), The specialty of small sHSPs mostly bind with denaturing or unfolded proteins in an ATP-independent manner, consequently averting their irreparable aggregation. Plant sHSPs typically exist as single oligomers, with the majority studied comprising dodecamers so far. In our experiment, thermal-induced aggregation of substrate protein was measured when mixed with SmsHSP24.1 protein at various molar ratios in the presence or absence of. SmHSP24.1 capability was demonstrated through a thermal survivability test. SmHSP24.1 protein can prevent the aggregation of E. coli cells at temperatures up to 55^{o}C for one hour. Our findings reflect similar findings from earlier research on RcHSP17.8 thermotolerance in *E. coli* (Jiang et al., 2009); CsHSP17.2 in *E. coli* and *P. pastoris* cells (Wang et al., 2017; Zhang et al., 2015). Our investigation provided clear evidence that the heterologous overexpression of SmsHSP24.1 was responsible for E. coli cells' ability to survive under heat stress. Unlike other chaperons, sHSPs have a high affinity for binding with aggregation-prone proteins in an ATP-independent manner (Kuang et al., 2017). Alpha-crystallin domain (ACD) is the basic signature domain of sHSPs (El-Gebali et al., 2018). The ACD is approximately 90 amino acids long-chain seven-stranded β-sandwich structured region which assembled the structural core of every small sHSPs (Waters & Vierling, 2020). The ACD is edged by approximately 85

amino acids long variable length N-terminal domain (NTD), and a small C-terminal extension (CTE). Both CTR and NTR regions of all sHSPs display high flexibility, wide sequence difference and assist to the oligomerization of sHSPs (Yu et al., 2021). Most of the sHSPs gathered into big oligomers contain at least 12 single monomers (Basha et al., 2012; Haslbeck & Vierling, 2015; Waters & Vierling, 2020). Under heat shock conditions the formation of super-molecular assemblies might be a usual property for most small sHSPs (de Jong et al., 1993; Zhang et al., 2015). For example, the mammalian small heat shock protein (sHSP28) was found to shape big assemblies like larger than 2,000 kDa size (Carra et al., 2019). Similarly, the calf alpha-crystallin was found to assemble more than 500,000 kDa size structure protein (Zhang et al., 2015). Our findings, which are in agreement with earlier findings propose a potential mechanism of ATP- independent chaperon activity of sHSPs driven by the development of multimeric topological structures. Our electron microscope research reveals that SmsHSP24.1, which can bind with its taregt protein even at a higher temperature of 50^{o}C, forms polyhedral structures. On the other hand, in heat shock-treated cells sHSPs from animals have been commonly spotted with granules like structure having molecular size larger than 2,000 kDa (Carra et al., 2019; Fu, 2014) . In support with these previous reports, our work strongly associates that the super-molecular assemblies of SmsHSP24.1 protein able to exhibit chaperone-like activity**.** It has also been reported that oligomerisation of small heat shock proteins is crucial for stability of protein, which sequentially boosts protein-protein interactions and chaperone activity of sHSPs (Santhoshkumar & Sharma, 2001). Widespread biochemical investigations and starting work in support of this sHSPs chaperone model mostly involve with CI sHSPs from *Pisum sativum* (Lee et al., 2000; Malik et al., 1999), but the work again broaden to other classes of plant sHSPs (Basha et al., 2012). In *Saccharomyces cerevisiae* and *E. coli* the mechanism of binding stress injured denatured proteins by small sHSPs and refolding it by the large heat shock protein like Hsp70 or Hsp100 have been described earlier (Cashikar et al., 2005; Ma et al., 2006; Mogk et al., 2003) . From plants and other organisms, many studies with sHSPs have been documented the abilities of various sHSPs to keep the denaturing proteins in soluble state which is very much similar with our findings. Soluble fractions analysis of the heat stressed SmsHSP24.1 protein expressing cells revealed a significant increase in the level of soluble proteins without affecting heat shocked. Our data also demonstrated that the novel SmsHSP24.1 provided significant protection of restriction enzyme *Nde*I against thermal

inactivation indicating the ability of SmsHSP24.1 protein to act as chaperone activity further. As the restriction endonuclease *Nde*I without SmsHSP24.1 completely lost its activity at temperatures above 45°C. Nevertheless, thermal inactivation of *Nde*I can be prevented up to 52°C. This may be happened due to the circumstance that the entire denaturation of the of *Nde*I may be happening at 55°C. Earlier it has been reported that small heat shock proteins (sHSPs) protect the thermal aggregation of diverse restriction enzymes and assist in maintaining their biological function (Lee et al., 1995; Lini et al., 2008; Mani et al., 2015; Reddy et al., 2012; Reddy et al., 2011; Santhoshkumar & Sharma, 2001). Our results which are in agreement with previous findings suggest a possible mechanism of ATP- independent chaperon activity of sHSPs.

Part-II. Functional characterization of *SmsHSP24.1* through transgenic overexpression

4.2.1 Cloning of *SmsHSP24.1* in plant transformation vector, pMDC100

As mentioned earlier in material and method, we employed the site-specific homologous recombination method to assemble the *SmsHSP24.1* cassette (CaMV-35S promoter: *SmsHSP24.1* gene: Nos terminator) in pMDC100 plant transformation vector. Initially, the CaMV-35S promoter was cloned into entry vector1 (EV1) within *Kpn*I *and Nde*I restriction sites. The coding region of the mitochondrial small heat shock protein (*SmsHSP24.1*) gene was amplified from eggplant cDNA by nested PCR with N′ terminals *NdeI* and C′ terminals *Not*I restriction sites (Figure. 4.20 A, B). Nos terminator was cloned into entry vector-1 (EV1I) within *Not*I *and Sac*I restriction sites.

Colony-PCR and restriction digestion were performed in sequence to validate the entry vector (Figure 4.20 C). Finally, the SmsHSP24.1 PCR product that had been digested and purified as well as the EV1 carrying the CaMV-35S promoter and Nos terminator were maintained to be ligated using T4 DNA ligase at 16°C. The treated material was transformed into *E. coli* (DH5) competent cells after being incubated for an overnight period, and then it was plated on LB medium with gentamycin as selection pressure. Colony-PCR and plasmid restriction digestion were used to evaluate the viability of the growing colonies and confirm the presence of all three cassette components. Further, the complete *SmsHSP24.1* gene cassette was transferred into the pMDC100 vector containing *Npt*II (kanamycin) gene as a plant selection marker by site-specific homologous recombination method. Colonies with the gene cassette were selected using two antibiotics (one for EV1 and the other for destination vector pMDC100 vector). Plasmids were extracted from positive colonies and electroporated into *Agrobacterium tumefaciens* EHA105 competent cells, which were then used for aubergine transformation (Figure. 4.20 D).

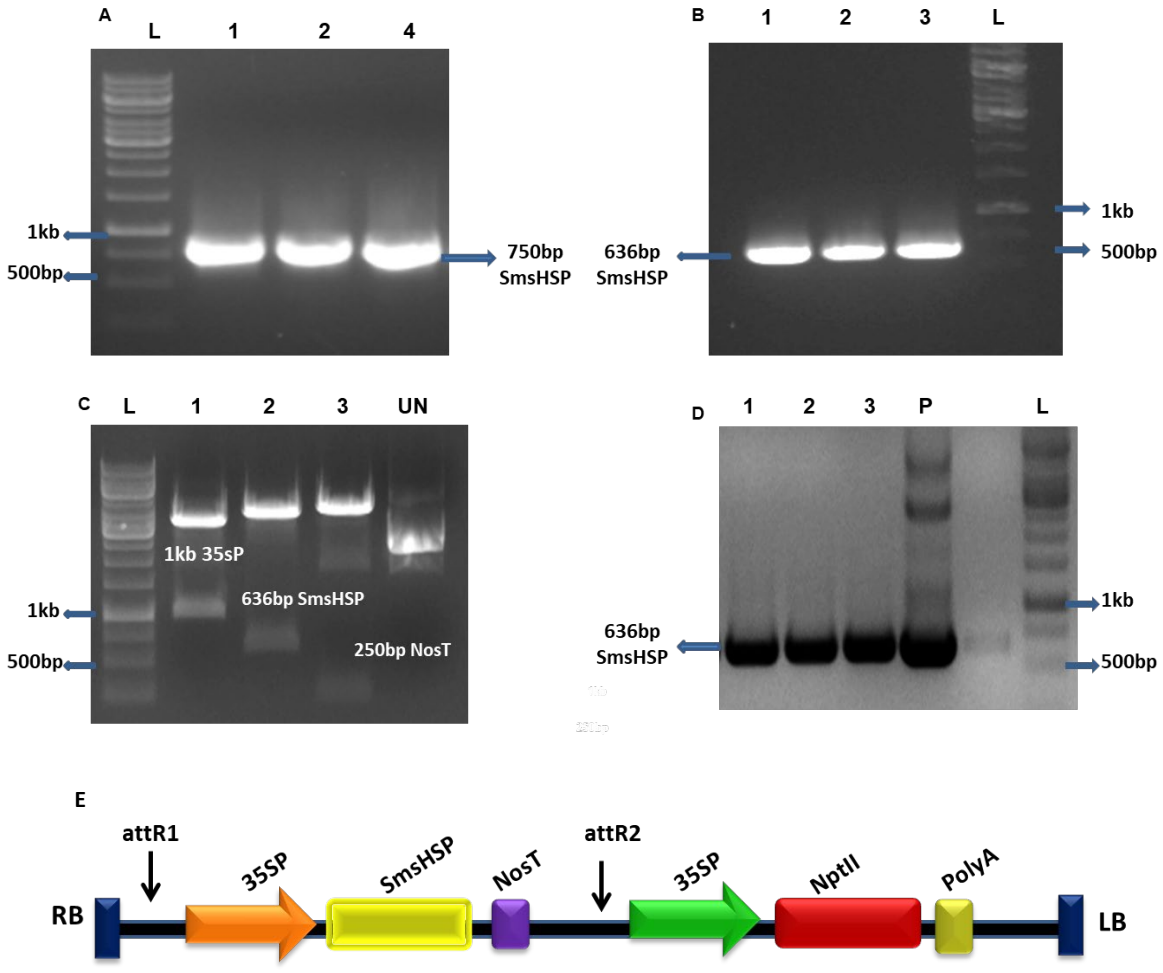


Figure 4.20: Construction of plant transformation vector. (A, B) Amplification of *SmsHSP24.1* coding region from eggplant cDNA by nested PCR, L: represents 1kb DNA ladder, 1, 2, 3: *SmsHSP24.1* amplified lane. (C) The *SmsHSP24.1* gene, CAM35s promoter (35SP) and Nos terminator (NosT) regions sub-cloned into gateway compatible entry vector (pL12R34-Ap) and restriction digestion conformation, 1: Digestion with *Kpn*I and *Nde*I for 1 kb 35s promoter, 2: Digestion with *Nde*I and *Not*I for 636 bp SmsHSP24.1 coding region, 3: Digestion with *Not*I and *Sac*I for 250 bp Nost, 4: EV1 (pL12R34-Ap) undigested plasmid. (D) PCR confirmation of the *SmsHSP24.1* expression cassette in *Agrobacterium* strain EHA105, 1, 2, 3: *SmsHSP24.1* amplified lane, 4: (+) positive control. (E) Schematic diagram of *SmsHSP24.1* gene expression cassette in pMDC100.

4.2.2. Plant material

We utilized the BARI Begun-4 type of eggplant (*Solanum melongena L.*), created by the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

4.2.3. Standardization of eggplant *in vitro* regeneration protocol for efficient gene manipulation

An efficient and reproducible *in vitro* regeneration protocol is a prerequisite for *Agrobacterium*-mediated gene transfer. Initially, it was necessary to determine the composition of the most effective regeneration media for an easy and efficient transformation

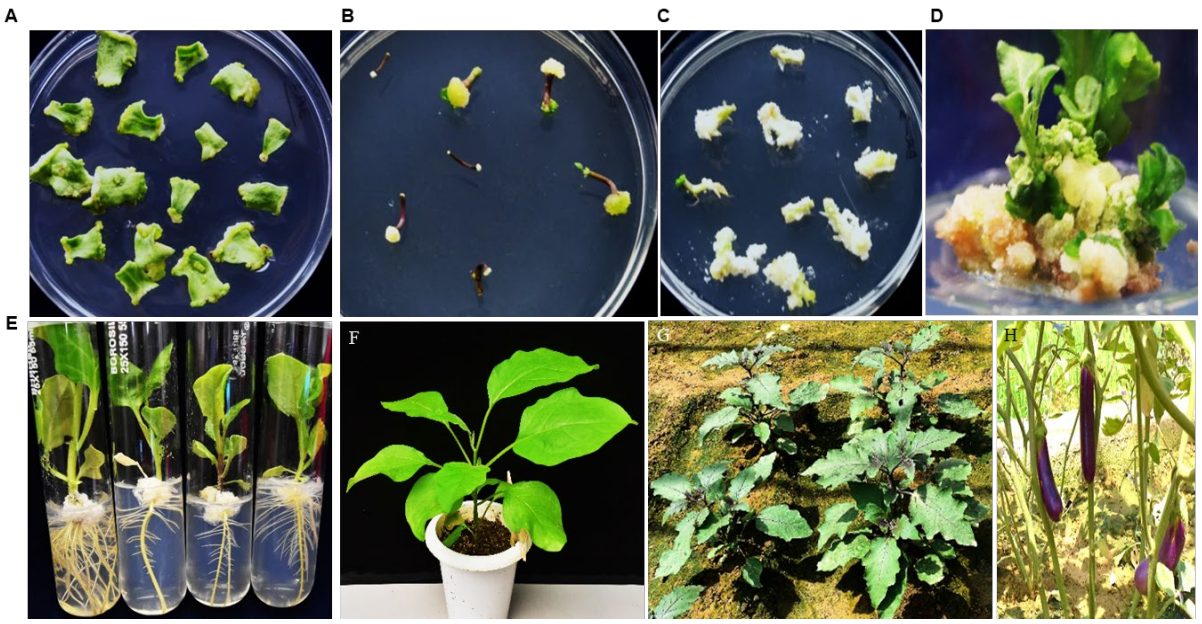


Figure 4.21: *In vitro* regeneration of eggplant from BARI begun 4. (A, B, C) Shoot initiation from cotyledon, hypocotyl, or root explants. (D) The formation of several shoots from all three different explants. (E) Root formation from the regenerated shoot. (F) Acclimatization of plantlets. (G, H) Field establishment and setting of successive flowers and fruits.

for eggplant (*Solanum melongena* L.) variety BARI Begun-4. To assess multiple shoot regeneration, explants were first grown on MS medium supplemented with various amounts of either cytokinin alone or auxin. At four to five weeks of culture, most of the explants began to produce numerous shoots.

Table. 4.1. Effects of different concentrations of cytokinin (BAP and Zeatin) alone or in combination with Auxin (NAA) on *in vitro* shoot regeneration of three different explants of *Solanum melongena* L.

Plant growth regulator(mg/L)	Mean no of shoots/explants			Regeneration frequency (%)		
BARI Begun-4						
	Cotyledon	**Hypocotyl**	**Root**	**Cotyledon**	**Hypocotyl**	**Root**
BAP						
0.50	1.25±0.25p	1.25±0.25q	1.16±0.27j	41.67±0.14f	41.67±0.14f	16.67±0.11e
1.00	3.41±0.14i	3.16±0.16h	1.33±0.22h	50.0±0.15e	50.0±0.15e	25.0±0.13d
1.50	4.41±0.14c	3.16±0.11h	1.41±0.19g	58.33±0.14c	58.33±0.14d	33.33±0.14b
2.00	4.50±0.15b	3.58±0.14d	1.58±0.14e	66.67±0.14b	66.67±0.14c	33.33±0.14b
2.50	**5.08±0.08a**	**4.08±0.14a**	**2.08±0.08a**	**75.0±0.13a**	**83.33±0.13a**	**41.67±0.14a**
3.00	3.91±0.08e	3.83±0.11c	1.50±0.15f	58.33±0.14c	66.67±0.14c	33.33±0.14b
BAP+NAA						
1.5+.2	0.83±0.20s	0.66±0.22w	0.66±0.22l	41.67±0.14f	33.33±0.14g	16.67±0.11e
1.5+.5	1.16±0.24q	1.0±0.21t	1.16±0.24j	50.0±0.15e	50.0±0.15e	25.00±0.13d
2.0+.2	2.08±0.08l	1.83±0.11m	1.33±0.18h	50.0±0.15e	50.0±0.15e	33.33±0.14b
2.0+.5	2.83±0.11j	2.75±0.13j	1.58±0.14e	58.33±0.14c	58.33±0.14d	33.33±0.14b
2.5+.2	3.66±0.14f	3.58±0.14d	1.83±0.16c	50.0±0.14e	66.67±0.14c	25.0±0.14d
2.5+.5	3.91±0.08e	3.91±0.08b	1.91±0.14b	58.33±0.15c	75.0±0.13b	33.33±0.14b
3.0+.2	1.66±0.14n	1.41±0.14p	1.33±0.14h	41.67±0.14f	50.0±0.15e	16.67±0.11e
3.0+.5	1.33±0.14o	1.58±0.14o	1.33±0.14h	50.0±0.15e	58.33±0.14d	16.67±0.11e
Zeatin						
0.5	1.16±0.24q	0.75±0.21v	0.66±0.22l	41.67±0.14f	33.33±0.14g	8.33±0.08f
1.0	2.83±0.11j	1.08±0.25s	1.25±0.21i	50.0±0.15e	41.67±0.14f	16.67±0.11e
1.5	3.58±0.14g	2.08±0.08k	1.33±0.18h	50.0±0.15e	50.0±0.15e	25.0±0.13d
2.0	3.91±0.08e	2.83±0.11i	1.41±0.14g	58.33±0.14c	58.33±0.14d	25.0±0.13d
2.5	4.25±0.13d	3.50±0.15e	1.91±0.14b	58.33±0.14c	66.67±0.14c	33.33±0.14b
3.0	3.58±0.14g	2.75±0.13j	1.41±0.14g	50.0±0.15e	50.0±0.15e	25.0±0.13d
Zeatin+NAA						
1.5+.2	0.66±0.14t	0.58±0.14u	0.58±0.14m	33.33±0.14g	41.67±0.14f	16.67±0.11e
1.5+.5	1.08±0.22r	1.16±0.20r	1.08±0.22k	41.67±0.14f	41.67±0.14f	16.67±0.11e
2.0+.2	2.08±0.08l	1.91±0.08l	1.25±0.17i	41.67±0.14f	50.0±0.15e	25.0±0.13d
2.0+.5	2.75±0.13k	2.83±0.11i	1.50±0.15f	50.0±0.15e	58.33±0.14d	33.33±0.13b
2.5+.2	3.50±0.15h	3.25±0.13g	1.58±0.19e	41.67±0.14f	50.0±0.15e	25.0±0.13d
2.5+.5	3.91±0.08e	3.41±0.14f	1.66±0.14d	50.0±0.15e	66.67±0.14c	33.33±0.13b
3.0+.2	1.66±0.14n	1.75±0.13n	1.25±0.13i	41.67±0.14f	33.33±0.13g	16.67±0.11e
3.0+.5	1.75±0.13m	1.83±0.11m	1.50±0.15f	41.67±0.14f	41.67±0.14f	16.67±0.11e

The findings are expressed as mean±standard error. Each hormone treatment contained 45 explants. At least three independent experiments were performed. Values denoted by various letters at the $P< 0.05$ level are statistically significant. Data were recorded after 60 days of inoculation of explants.

The composition of MS+2.5 mg/L BAP produced the greatest number of regenerated shoots. In BARI Begun 4, the best rate of regeneration (75.00±0.13%) and the largest number of shoots per explant (5.08±0.08%) were both noted (Table. 4.1). We decreased the content of BAP in MS medium to standardize the shoot elongation media so that the regenerated shoot could appropriately lengthen and become suitable for root induction. The combination of MS + BAP (0.2 mg/L), casein hydrolysate (0.2 mg/L), and sorbitol (0.1 mg/L) in the medium produced the best elongation after 12–14 days of culture. Nevertheless, cotyledonary explants of BARI begun 4 had the highest proportion of elongation. Several types and concentrations of auxins were added to full or half-strength MS medium to induce the roots of the elongated shoots. At 9 days of culture, we saw that 80% of the shoots had developed strong roots. With full-strength MS media supplemented with 1.0 mg/L Indolebutyric acid (IBA), each micro-cutting segment generated a 6.66±0.14 mean number of roots. The recently revived shoots were moved to our improved rooting medium. Plantlets were moved to tiny plastic pots with soil and compost after sufficient root growth (2:1). In potted soil, the transplanted plantlets' survival rate was shown to be 80%. (Figure 4.21 A-H).

4.2.4. Optimizing the concentration of antibiotics for the selection of transgenic plants

Eggplant shoots and cotyledonary leaves were inoculated into optimal shoot regeneration medium supplemented with various concentrations of kanamycin (i.e. 50 mg/L, 75 mg/L, 100 mg/L, and 150 mg/L) to determine the concentration of selectable marker for the screening of potential transformed lines. By adding 100 mg/L of kanamycin, shoot regrowth from the control explants was prevented. The explants also became brown after 3 weeks of culture. As a result, it was revealed that 100 mg/L of kanamycin was appropriate for transgenic shoot selection. Control eggplant seeds were germinated on a medium treated with two different doses of kanamycin (i.e., 100 mg/L and 150 mg/L) to find the optimal antibiotic concentration for choosing T_1 transgenic lines. When control seeds failed to germinate, MS media treated with 150 mg/L of kanamycin blocked root development in seedlings that had already germinated. As a result, it was established that 150 mg/L of kanamycin was appropriate for choosing T_1 transgenic eggplant lines (Figure. 4.22). Also, 100, 250, or 350 mg/L of cefotaxime did not significantly impair shoot regeneration. Nevertheless,

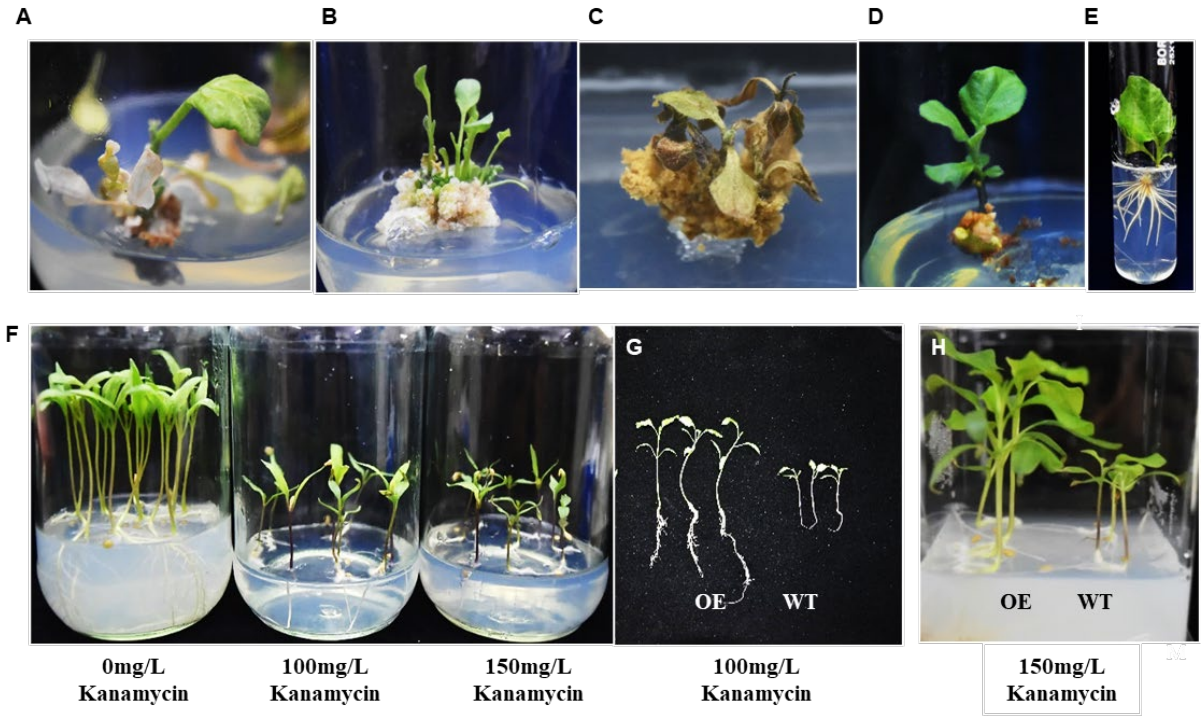


Figure 4.22: Optimizing the concentration of antibiotics for the selection of transgenic plants. (A) Browning of a non-transformed shoot from BARI begun 4 under a kanamycin selection of 50 mg/L. (B) During kanamycin selection at 50 mg/L, the transformed shoot from BARI begun 4 remained green. (C) Under 100 mg/L kanamycin selection, non-transformed shoots from the same variety died. (D) Transformed shoots maintain their green color and thrive when exposed to 100 mg/L of kanamycin. © Well-rooted transformed shoots at a kanamycin concentration of 100 mg/L. (F) To determine the ideal concentration for choosing T_1 transgenic lines, a germination test was performed using control eggplant seeds with various selection pressures in the medium (concentrations ranging from 0 mg/L, 100 mg/L, and 150 mg/L kanamycin). Control seedlings (WT) ability to generate roots was inhibited by MS media supplemented with 100 and 150 mg/L of kanamycin, whereas transgenic lines (OE) exhibit well-developed roots.

following co-cultivation, the growth of *Agrobacterium* was successfully suppressed by 250 mg/L cefotaxime. From that point on, 250 mg/L of cefotaxime was employed to regulate *Agrobacterium* growth. (Figure 4.23 A-E).

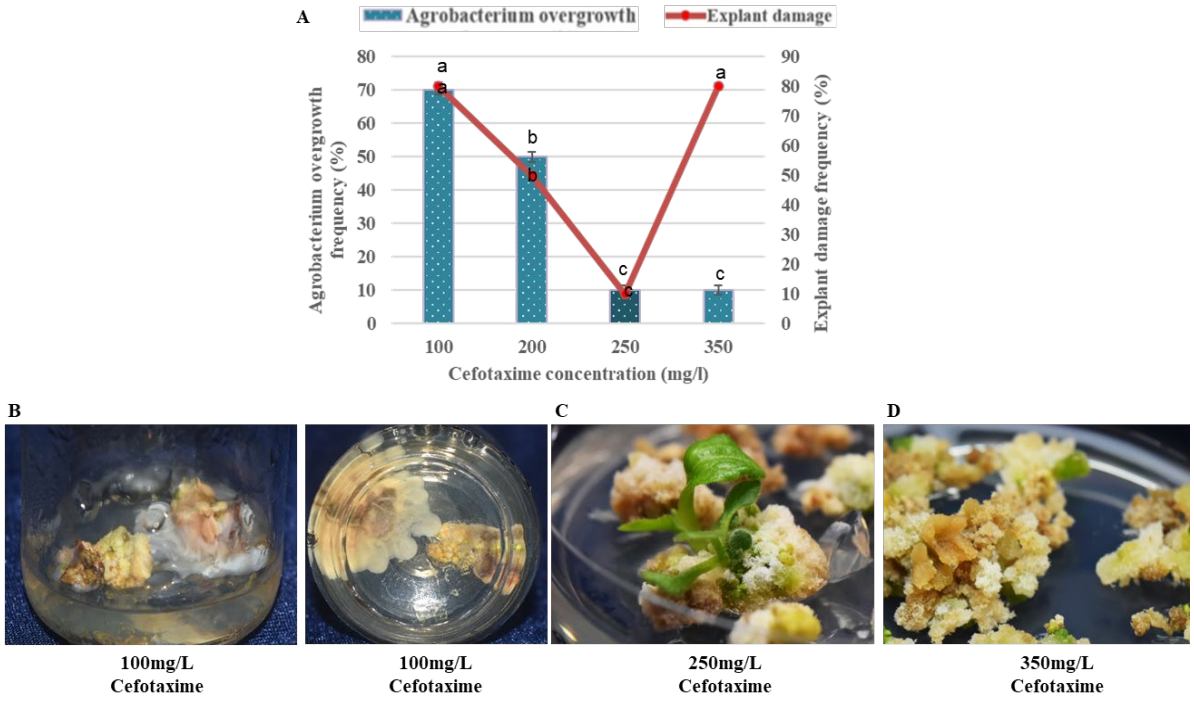


Figure 4.23: Optimization of cefotaxime concentrations to inhibit the growth of *Agrobacterium* after co-cultivation. (A) *Agrobacterium* overgrowth frequency (%) under different concentrations of cefotaxime. Three different experiments' findings are reported as means and standard errors. Different letters indicate statistical significance at $P < 0.05$ level. (B) *Agrobacterium* overgrowth was observed under 100 mg/L cefotaxime selection. (C) *Agrobacterium* was effectively inhibited under 250 mg/L cefotaxime selection. (D) *Agrobacterium* overgrowth was inhibited whereas severe cell damage occurred under 350mg/L cefotaxime selection.

4.2.5. Generation of constitutively overexpressed SmsHSP24.1 Eggplant lines

Cotyledons of 10-day-old eggplant seedlings were utilized as explants, and they were infected with *Agrobacterium* having required gene cassette. The SmsHSP24.1 gene cassette along with the kanamycin gene was transformed into BARI Begun-4. On MS media that had adequate antibiotics added in, the transformed cells were chosen. Green shoots started to emerge on the explants after three rounds of selection. Explants that showed no signs of callus initiation or regeneration even after two or three subsequent transfers on the selection medium were removed. The regenerating shootlets were transferred to rooting media after a suitable amount of elongation. The micro shoots were placed in plastic pots with soil and compost after they have shown good rooting.

4.2.6. Analysis of the putative transgenic plants for the genomic integration of transgene cassette at T_0 generation plant by PCR

Putative T_0 transgenic plants developed from pMDC100:CaMV35S promoter:SmsHSP24.1 gene:Nos terminator construct were PCR screened with primer sets of the selectable marker gene (*Npt*II_F1/*Npt*II_R1) and *SmsHSP24.1* and NosT primer sets (sHSP_F2/NosT_R1). Putative T_0 transgenic plants showed the expected size bands for *SmsHSP24.1* (~0.9 kb) and kanamycin (~0.45 kb) similar to the positive control, whereas the corresponding bands were missing in the WT. This indicated that all the putative T_0 transgenic lines were positive transgenic (Figure. 4.24 C). The T_1 seeds were harvested from the above T_0 transgenic lines. T_1 transgenic plants were used for further molecular analysis.

4.2.7. Molecular detection of genomic integration of transgenes

Under 150 mg/L kanamycin selection and PCR reconfirmation, homozygous T_2 transgenic lines were selected, (Figure. 4.24 B). The SmsHSP24.1 gene-specific probe was employed in a Southern blot assay on the same lines to validate the copy number of transgene integration. From the control and transgenic eggplant lines, we utilized 20 µg of genomic DNA. Restriction enzymes *Nde*I were utilized to digest the genomic DNA of the control and transgenic eggplant lines while kept overnight. On 0.8% agarose gel, fragments of the digested DNA were sorted by electrophoresis. For the capillary transfer of DNA fragments, HybondTM nylon-positive membrane (GE Healthcare Ltd, UK) was utilized. For further hybridization, construct-specific digoxigenin-labeled probes (DIG probes) were utilized. Each DIG probe was created from the cDNA coding regions of SmsHSP24.1 (sHSP-Probe F1/sHSP-Probe R1) using a PCR DIG probe synthesis kit (Roche, Germany). According to the manufacturer's instructions, the blots were identified (DIG High Prime DNA labeling and Detection Starter Kit II, Roche, Germany). Using morphological features identical to those of wild-type eggplants, we were able to produce two singles, two doubles, one triple, and one multi-copy (containing five copies of insertions) transgenic event (Figure. 4.24 D-F). After this, we selected single-copy integrated transgenic lines (OE1 and OE7) from the T_2 generation with strong transgene expression for further physiological study.

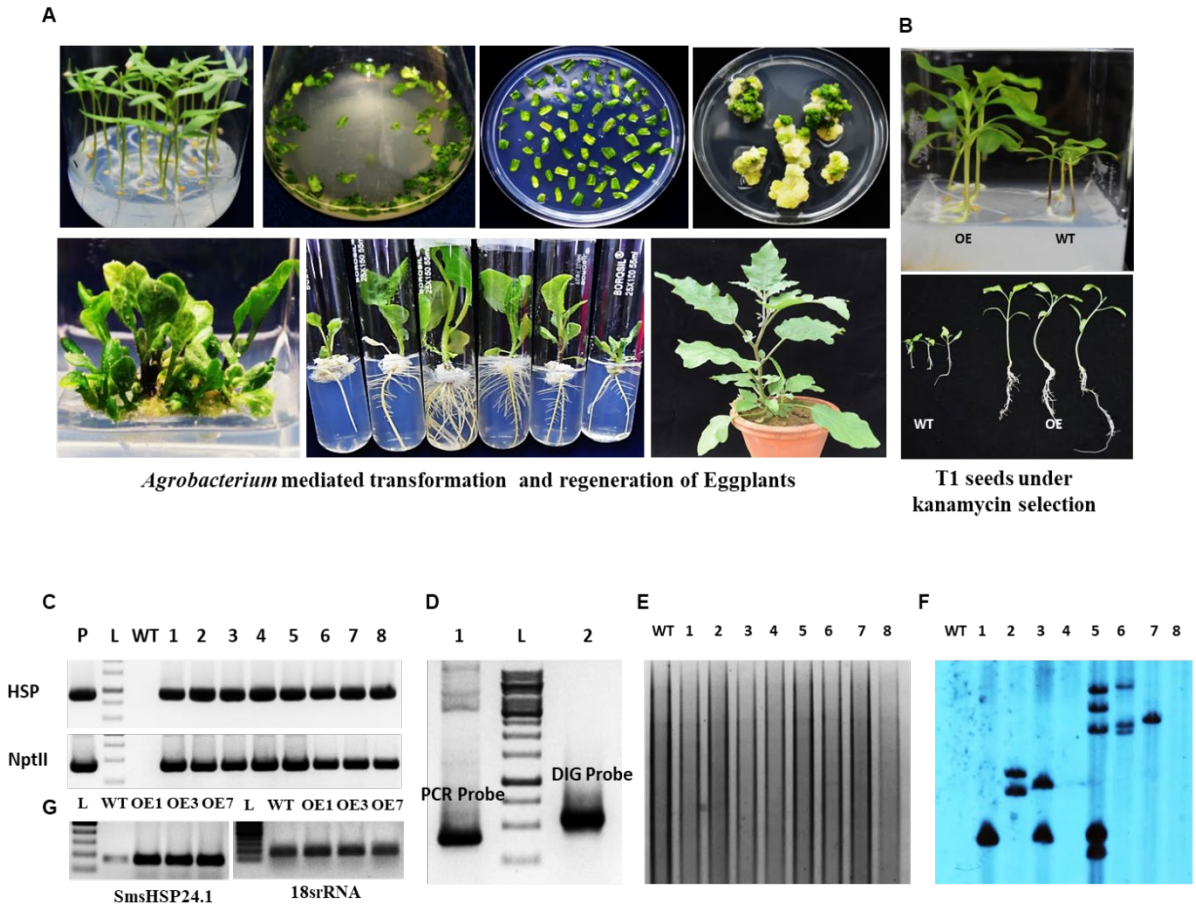


Figure 4.24: *Agrobacterium* mediated transformation and molecular detection of *SmsHSP24.1* gene in eggplant genome. (A) Cotyledonary leaf infected with EHA105 *Agrobacterium* strain harboring *SmsHSP24.1* expression cassette and shoot regeneration from infected explants. (B) Transgenic overexpressed lines were selected under 100 mg/l kanamycin. (C) PCR amplification of *SmsHSP24.1*+NosT terminator and kanamycin (*Npt*II) genes in wild type (WT) and eight OE lines (1-8). L: 1Kb DNA ladder, (P): Positive PCR control. (D-E) Southern blot analysis of WT and eight T_2 transgenic lines using *SmsHSP24.1* cDNA specific probes. (F) Copy number determination by Southern blot analysis, L: 1kb DNA ladder, 1: *Nde*I, 2: *BamH*I. (G) Semi-quantitative RT-PCR of *SmsHSP24.1* transcript in transgenic lines (OE-1, 3, and 7) compared to WT. 18s rRNA, as a housekeeping gene, was used as a reference gene (middle panel).

4.2.8. Analysis *of SmsHSP24.1* transcript in the transgenic rice plants

Semi-quantitative RT-PCR of single-copy T_2 transgenic lines was performed to examine the transcript level and mRNA expression level, respectively. Total RNA was isolated from the transgenic as well as WT counterpart. Semi-quantitative RT-PCR analysis with *SmsHSP24.1* gene-specific primers revealed a higher level of SmsHSP24.1 expression compared to the wild type. 18srRNA (a housekeeping gene) was used as a positive control to check the quality of the synthesized cDNA (Figure. 4.24 G).

4.2.9. SmsHSP24.1 overexpression exerts improved tolerance to abiotic stresses

Transgenic (OE1, OE3 and OE7) and WT plants were examined in normal and stressful conditions to learn more about the function of SmsHSP24.1 overexpression in eggplant. In the untreated condition, both WT and OE lines grew well and produced new leaves. OE lines, in contrast to WT, demonstrated high thermo-tolerance under heat stress (45°C) for 36 hours, and they fully recovered once the stimulus was removed. When water was withheld from WT plants for 10 days, the plants displayed significant withering and some even perished, but OE lines only showed mild indications of dehydration. 80% of the OE lines regenerated after one week of re-watering, compared to 30% of WT plants (Figure. 4.27). At the recovery stage from the effects of drought stress, a more startlingly substantial number of auxiliary shoots were seen. Moreover, compared to OE lines, WT plants either showed considerable growth inhibition or perished after being exposed to 200 mM NaCl for three days. Moreover, it was shown that OE line seedlings were less likely to develop chlorosis than WT plants (Figure 4.26). In addition, under heat, salt, and drought stress, the OE seedlings considerably outperformed the WT plants in terms of fresh weight, dry weight, root length, shoot length, and chlorophyll content (Figure. 4.25 E-G, 4.26 E-G and 4.27 B-C).

4.2.10. Growth performance of the SmsHSP24.1 transgenic plants in response to combine stress under field conditions

In the ICGEB net house facility in New Delhi, India, the combined effects of heat and drought stress were carried out in field conditions. Both OE and WT lines had seedlings that were 2.5 months old and reproductive stage were chosen for this combination stress

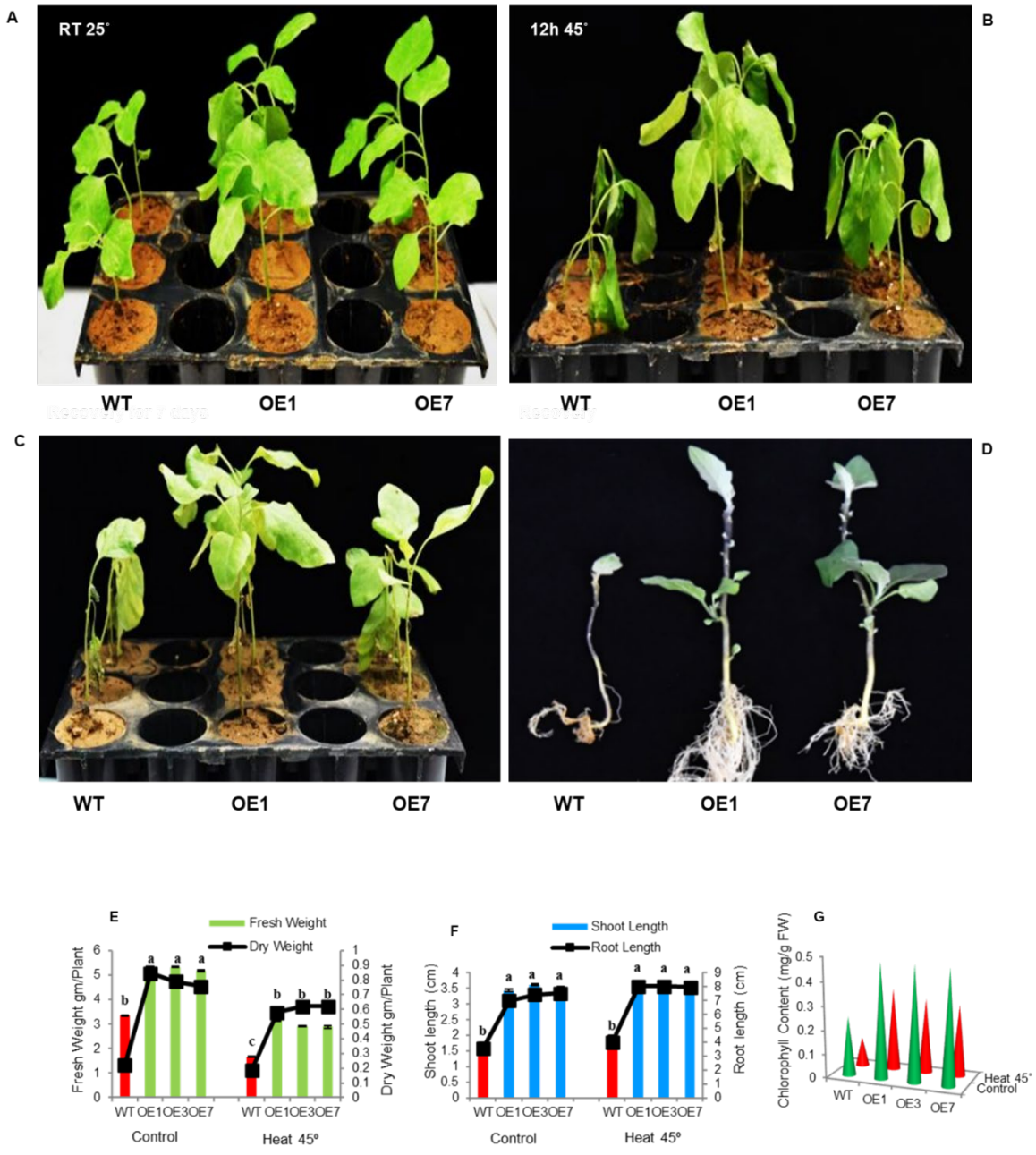


Figure 4.25: Growth characteristics of SmsHSP24.1-overexpressing transgenic eggplant under heat-stressed circumstances. (A-D) Growth characteristics of WT and transgenic (OE) eggplant strains at 45°C. (E-G) All transgenic seedlings had considerably increased total fresh weight, dry weight, root length, shoot length, and chlorophyll contents than the WT line. Data are presented as mean SD (n = 3). Significant changes between several lines are denoted by the letters a, b, c, and others ($P< 0.05$).

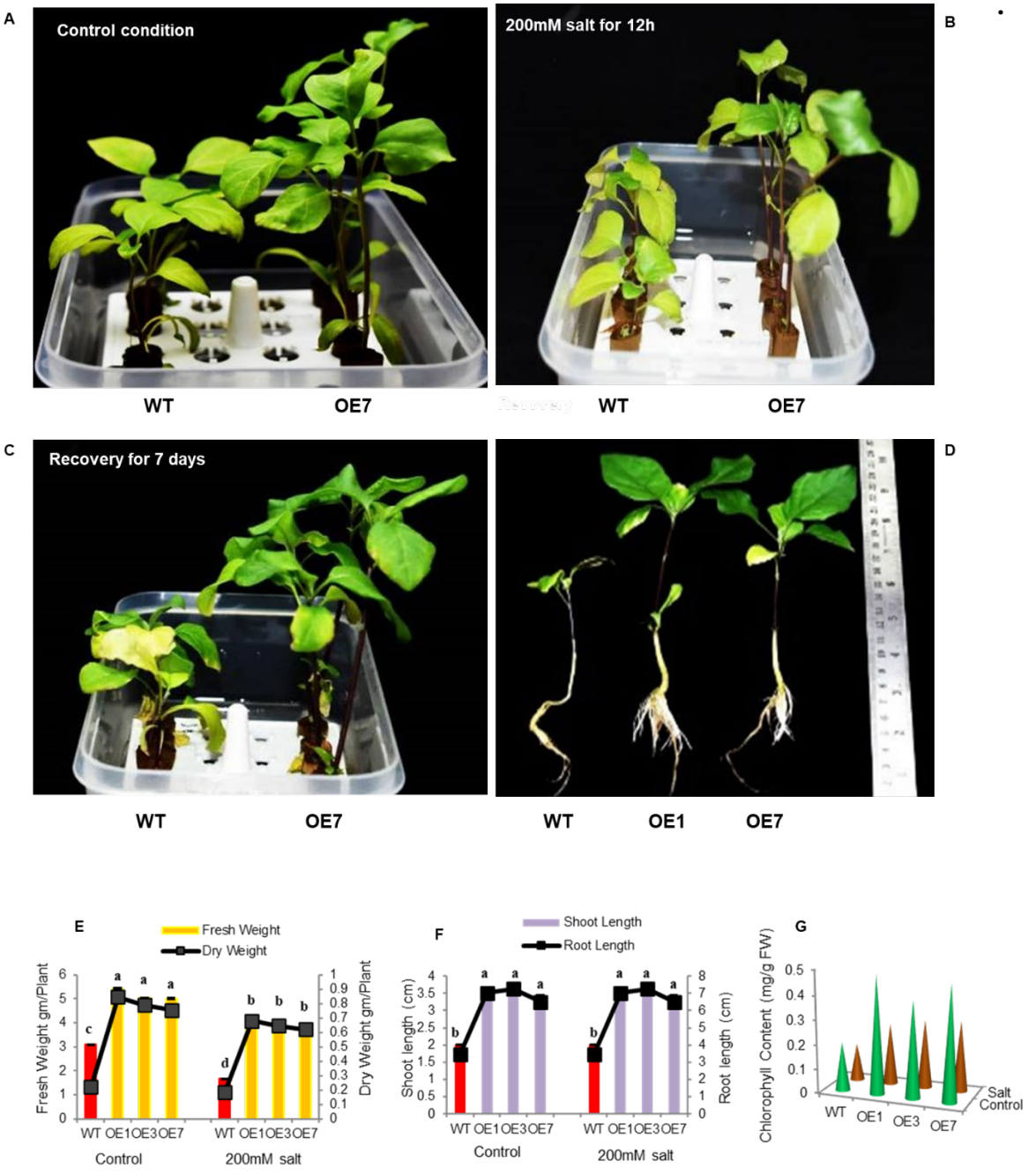


Figure 4.26: Growth characteristics of SmsHSP24.1-overexpressing transgenic eggplant lines grown under salt-stressed circumstances. (A-D) Growth characteristics of the transgenic (OE) and WT Eggplant lines under a 200 mM NaCl salt stress environment. (E-G) All transgenic seedlings had considerably increased total fresh weight, dry weight, root length, shoot length, and chlorophyll contents than the WT line. Data are presented as mean SD (n = 3). Significant changes between several lines are denoted by the letters a, b, c, and others ($P< 0.05$).

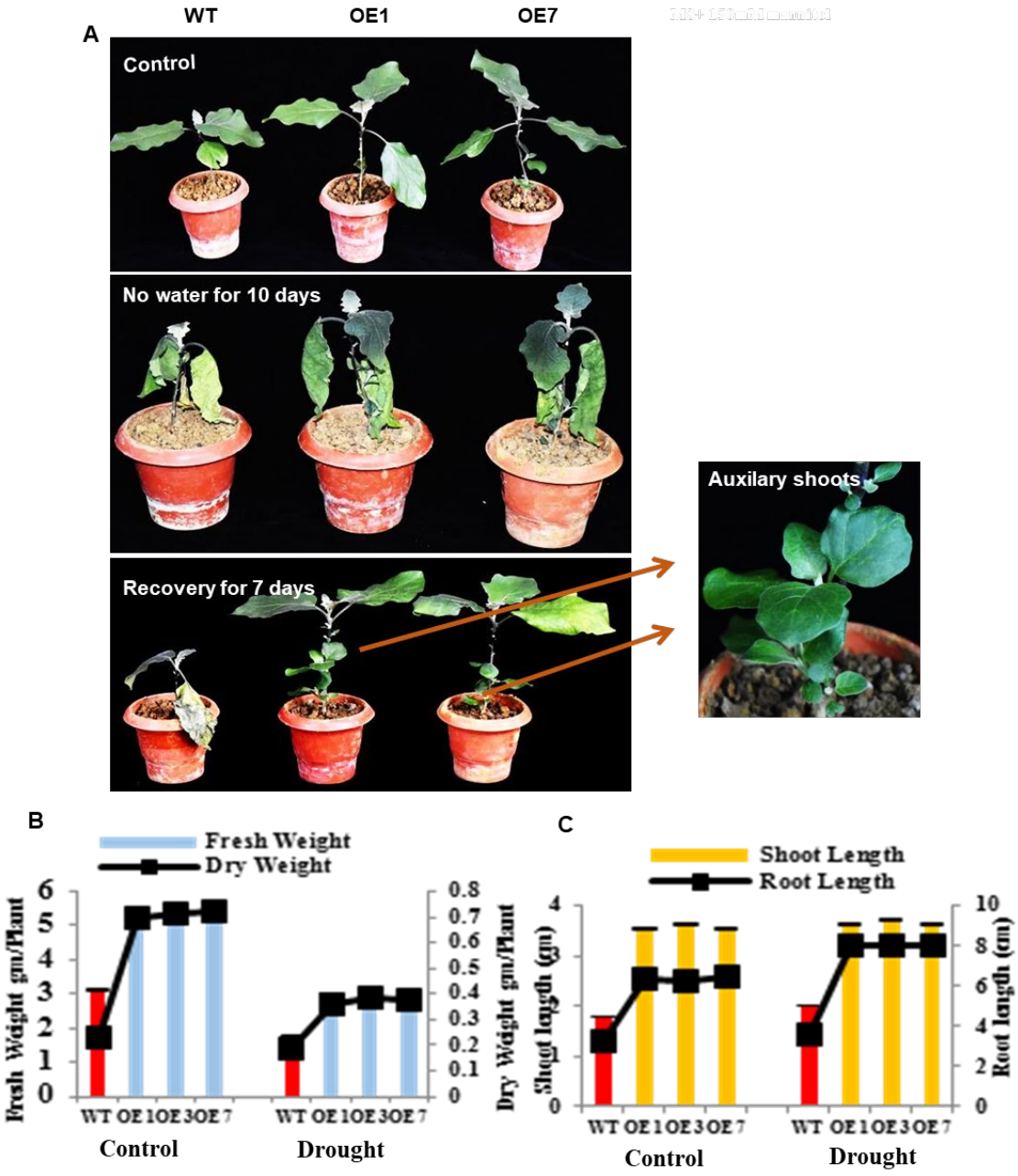


Figure 4.27: The growth physiology of transgenic eggplant lines overexpressing SmsHSP24.1 protein drought conditions. (A) Transgenic and WT eggplant lines' growth parameters over 10 days of water withdrawal followed by a week of re-watering. (B, C) Compared to the WT line, all transgenic seedlings had significantly increased total fresh weight, dry weight, root and shoot length. Data are presented as mean SD (n = 3)

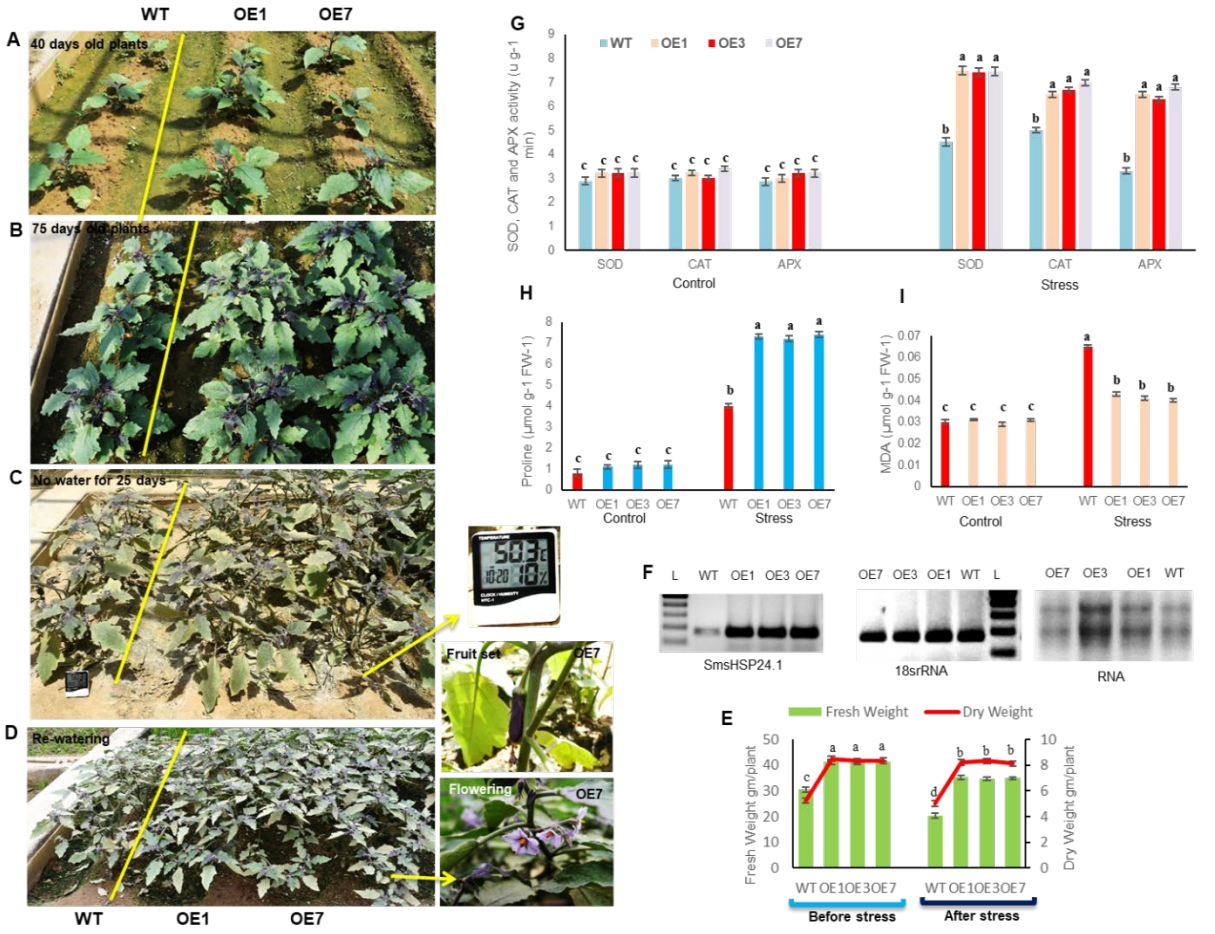


Figure 4.28: Transgenic plants that overexpressed SmsHSP24.1 protein preserved physiologic balance at the field level under combined (heat and drought) conditions. (A, B) Fully grown transgenic and WT plants under usual field conditions. (C) Water withdrawal-induced phenotypes of 75-day-old eggplant seedlings (D) Transgenic and WT seedling phenotypes at the recovery stage (E) All transgenic seedlings had considerably greater total fresh and dry weight and dried weight. (F) Semi-quantitative RT-PCR evaluation of SmsHSP24.1 from transgenic and WT lines that were cultivated in the field. (G-I) In both transgenic and WT lines, many biochemical parameters including proline, MDA, and ROS-scavenging enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were measured. Data are presented as mean SD (n = 3). Significant changes between samples from various transgenic lines are denoted by the letters a, b, c, and others ($P < 0.05$).

experiment. During the experiment most WT seedlings were found to be badly harmed under these circumstances, however OE lines remained healthy and green (Figure. 4.28 A-C). Once stress was removed, both OE lines (OE1 and OE7) recovered fast with no impact on blooming or fruit setting, however the WT plants severely slowed down the growth (Figure. 4.28 D). Also, in all OE lines, all other physiological parameters demonstrated greater fresh and dry weight in comparison to WT under both normal and stress conditions (Figure. 4.28 E). Moreover, semi-quantitative RT-PCR analysis revealed that all OE lines in control and combined stressed plots accumulated more SmsHSP24.1 transcript than WT lines (Figure. 4.28 F). From the combined stress plot, the enzymatic activity of ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) was also seen in both OE and WT lines. When compared to WT plants, it was discovered that OE lines maintained a higher level of all the ROS-scavenging enzymes (Figure. 4.28 G). Similar to proline content, overexpressed lines exhibited a rise, but MDA content had dramatically decreased in transgenic lines compared to wild-type plants following stress treatment (Figure. 4.28 H, I). This information was observed to be consistent with RNA-seq and semi-quantitative RT-PCR study performed on heat stress OE lines that were three weeks old.

4.2.11. In both normal and stressful circumstances, the SmsHSP24.1 overexpressing plant maintains an elevated amount of cellular ROS

We performed a leaf disc test separately for heat (45ºC heat for 36 hr), osmotic stress (150 mM Mannitol), salt (200 mM NaCl), and Methyl viologen (MV) (10 mM) to examine the H_2O_2, O_2 contents, and cell death following stress to assess the role of overexpressed SmsHSP24.1 in reducing ROS damage (Figure. 4.29). After stress treatments, SmsHSP24.1 overexpressing plants showed less and slower chlorosis than WT plants. As shown by nitro blue tetrazolium (NBT), diaminobenzidine (DAB) and Trypan Blue histochemical staining, the transgenic plants overexpressing SmsHSP24.1 protein had a considerably reduced amount of ROS generation and cell death compared to WT plants following MV and heat treatment (Figure. 4.29 A, B, 5 A). We also observed that following treatment with heat, salt, mannitol, and MV, the H_2O_2 levels in WT plants were around three to four times greater than those in overexpressed transgenic lines (Figure. 4.29 D). Yet, it was intriguing to see that under normal circumstances, the transgenic lines also maintained a little greater cellular H_2O_2 level than WT

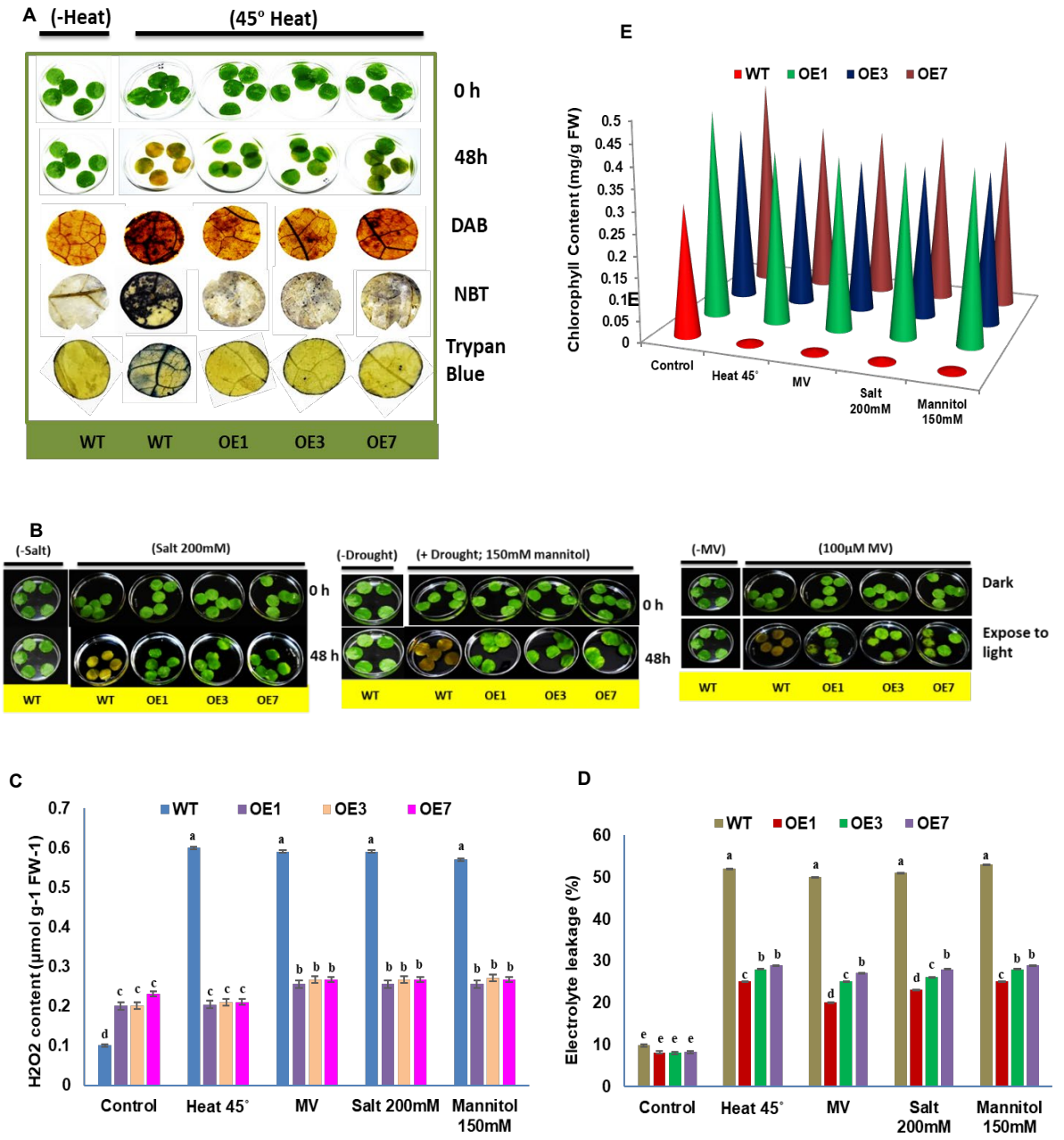


Figure 4.29: Examining how cells are affected by heat, salinity, drought, and other stressors. (A) WT and three transgenic eggplant lines (OE) leaf strips were exposed to a 45°C heat stress for 48 hours. WT leaves that had not been heated or processed were utilized as a control. Histochemical labeling was used to distinguish between O_2 and H_2O_2 build-up and dead cells in the leaf strips of transgenic and WT lines. (B) WT and OE line leaf strips under salt, drought, and MV stress. WT leaves that hadn't been heated or handled served as a control (C-E) Responses to heat, salt, MV (methyl viologen), and osmotic stressors were evaluated using measurements of H_2O_2, chlorophyll content, and electrolytic leakage. Data are presented as mean SD (n = 3). Significant changes between several lines are denoted by the letters a, b, c, and others ($P< 0.05$).

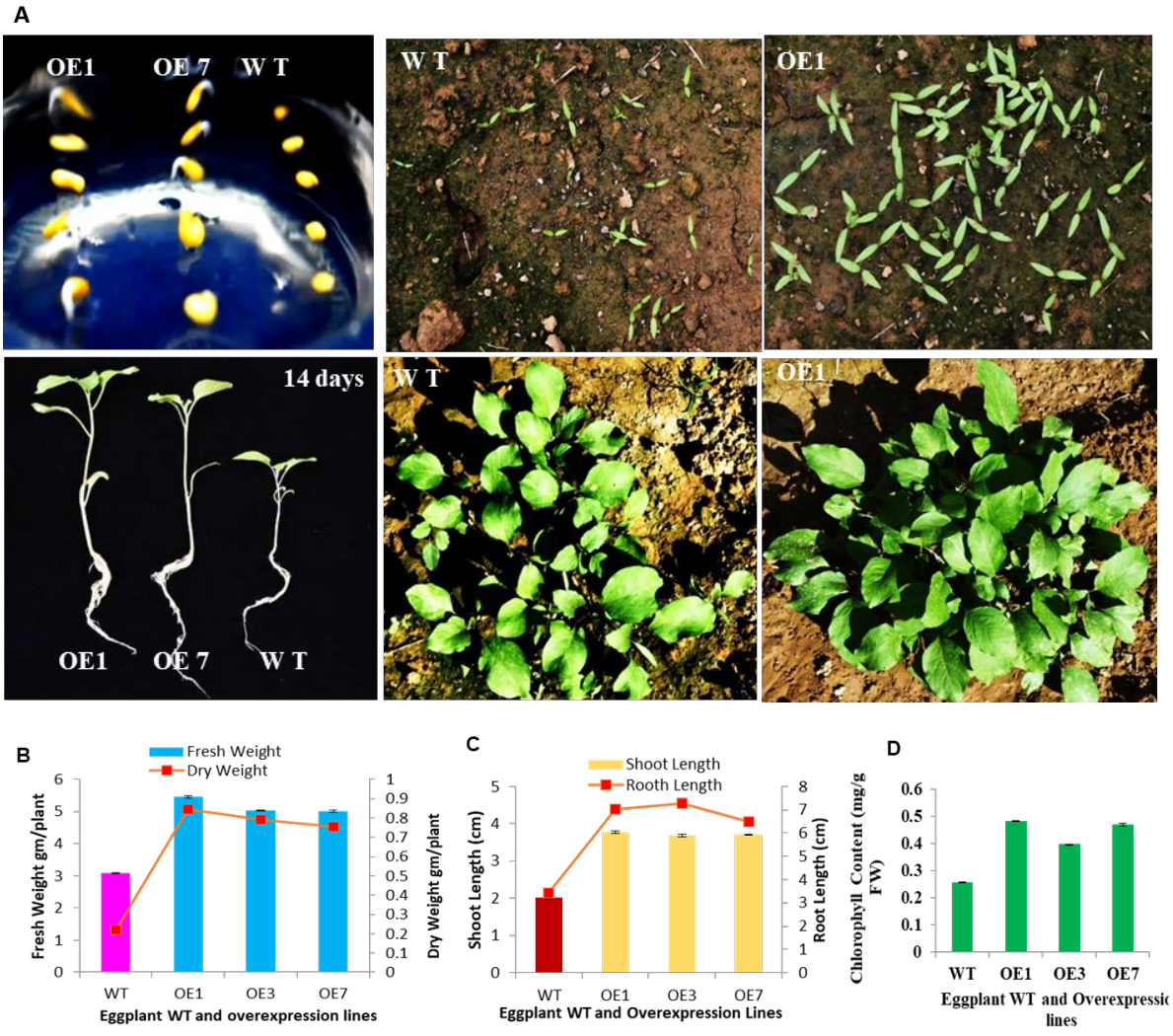


Figure 4.30: Germination rate and growth physiology of WT and transgenic eggplant lines (OE) under normal environmental growth conditions. (A) *In-vitro* and field seed germination and seedling vigor of OE lines (B-D) Chlorophyll content, root length, shoot length, dry weight overall, and total fresh weight in OE and WT lines a. Data are presented as mean SD (n = 3).

plants. Under typical environmental circumstances, it had no detrimental effects on development, and when stressed, there was no deadly increase in H_2O_2 levels compared to WT plants. Also, a comparison of transgenic and WT plants revealed that transgenic plants avoided ion leakage and preserved chlorophyll due to protection from oxidative damage via reduced ROS generation (Figure. 4.29 E).

4.2.12. Overexpressed SmsHSP24.1 lines show early seed germination and vigour in the seedlings

In vitro and in the field, we found that OE lines (OE1 and OE7) had significantly higher rates of early seed germination as well as seedling vigor than WT. In the growing medium, 95% germination rate was seen in OE lines (OE1 and OE7) within 3 to 4 days, but to reach about 80% germination rate in WT it took 6 days (Figure. 4.30 A). When seeds were planted in the field under regular circumstances, a similar outcome was seen. When OE seedlings were compared to WT seedlings using physiological measures taken two weeks after germination, all of the OE seedlings had significantly greater total fresh weight, dry weight, root length, shoot length, and chlorophyll content (Figure. 4.30 B-D).

4.2.13. Construction of RNA-Seq library and high-throughput sequencing

To adapt to unfavorable environmental conditions, plants may regulate their own physiological, biochemical, molecular, and cellular processes, actively modify the transcriptome, activate stress tolerance mechanisms, or regulate their biological processes in response to salinity, drought stress, extreme temperature, metal ion toxicity, and other abiotic stresses. We carried out RNA sequencing and transcript quantification on transgenic and WT lines to elucidate the molecular mechanisms of SmsHSP24.1 overexpression-mediated regulation in control as well as heat stress conditions in eggplant.

When initial sequencing was done, the adaptor sequences and low-quality bases are removed from the raw reads during the preprocessing stage using Adapter Removal-v2 (version 2.2.0, any settings modi ed for the tool used in the analysis: -minlength 30, trimqualities, minquality 30). Ribosomal RNA sequences are eliminated from the preprocessed reads using bowtie2 (version 2.2.9; any settings changed for the analysis tools: -N 1) and following process utilizing custom scripts, the samtools tool (version 1.3.1, used with default settings), the sambamba tool (version 0.6.7, used with default parameters), and the BamUtil utility (version 1.0.13, used with default parameters. Except for the wild-type plant after giving two hours of heat stress (WT2hH), where the value was 87% after the removal of rRNA, more than 95% of all readings were of high quality (HQ) after adaptor trimming and base quality checking. According to

(Fernandez-Pozo et al., 2015), mapping of cleaned reads with the tomato reference genome and gene model retrieved from Sol Genomics Network resulted in the mapping of 65.60% of (OERT), control plant at room temperature (WTRT) 52.80%, overexpressed plant after giving two hours of heat stress (OE2hH) 56.88%, and 57.76% of WT2hH reads (Table. 4.2).

Table 4.2: RNA-seq reads alignment summary

Sample Name	Total Read Count	Read Count after rRNA removal	QC Pass %	Aligned %	Unaligned %
OERT	560,98,118	548,17,600	97.72	65.6	34.4
WTRT	835,59,126	808,99,814	96.82	65.27	34.73
OE2hHeat	735,03,888	721,77,768	98.2	56.88	43.12
WT2hHeat	824,94,878	719,89,620	87.27	57.76	42.24

Assembly data: Table with assembly parameters

4.2.14. Differential gene expression (DEGs) in SmsHSP24.1 overexpressed transgenic (OE) and wild-type (WT) lines

Significant transcription changes were found by using differential gene expression analyses in several of the physiologically critical pathways (Figure. 4.31-33 and Table. 4.3). Important genes' up- and down-regulation were also shown by heat map illustrations of differentially expressed genes (DEGs) in wild type (WT) and transgenic (OE) lines with cluster dendrograms based on FPKM plot and gene ontology (GO) categorization (Figure. 4.34-39). Among the numerous DGEs, Photosystem II reaction center protein M (Solyc09g064580), Chloroplastic matK (Solyc09g061390), and genes involved in the electron transport chain (Solyc09g074540, Solyc11g044636) are significantly up-regulated in OE vs. WT samples at RT (Figure. 4.34, Table. 4.3), whereas SOD (Solyc01g067740), ABC transporter (Figure. 4.36). Similarly, under normal environmental conditions, there was a significant down-regulation of transmembrane transporters such as the F-Box domain-containing protein

(Solyc07g044920), the iron-nicotianamine transporter (Solyc03g082620), the calcium-binding protein CML44, the LOB1 (Solyc06g064540) while the cell number control protein

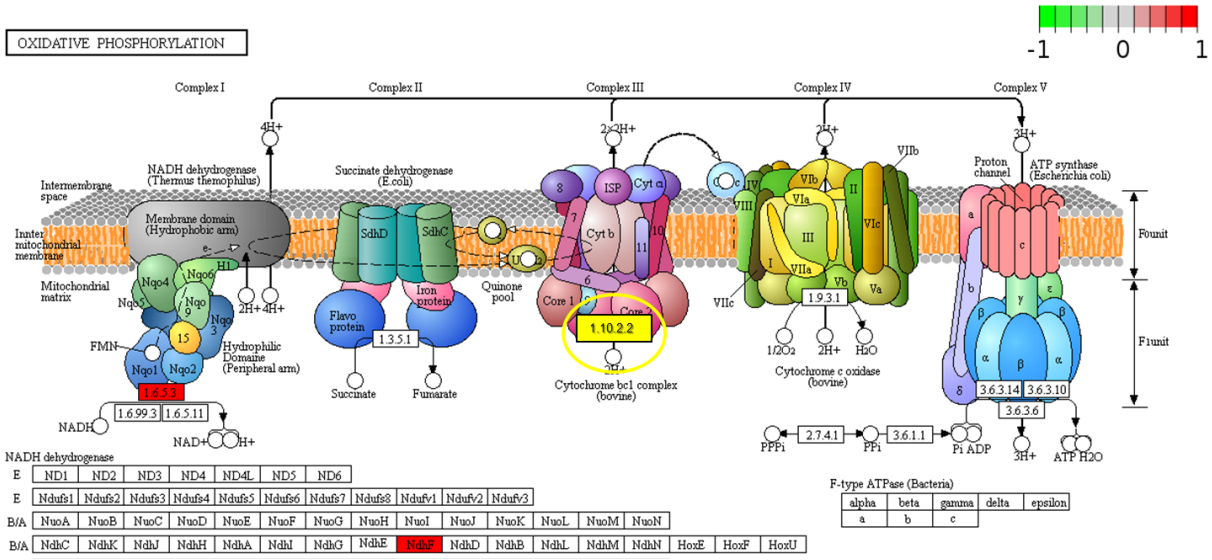


Figure 4.31: The first protein unit NADH-ubiquinone oxidoreductase in electron transport chain (ETC) extensively affected in the leaves sample of SmsHSP24.1 overexpression line (OE) compared to control (WT).

PLAC, and the FA desaturase domain-containing protein were up-regulated in eggplant control lines compared to OE lines upon heat stress (Figure. 4.36, Table. 4.4). According to to GO term-based gene classification, several genes were discovered to be strongly impacted by DEGs in both WT and OE leaves samples. Twelve crucial pathway-related genes were found to express themselves differentially between WT and OE lines (Figure. 4.37 and Table. 4.3). Similar to this, the exact same event resulted in a total down-regulation of 185 genes. BZIP transcription factor (Solyc01g109880.3), ethylene-responsive factor 2; ethylene-binding protein (Solyc09g075420.3), and NAC4 domain protein (Solyc11g017470.2) were found to be down-regulated among transcription factor genes, compared to a total of 18 distinct transcription factor (TFs), most of which were auxin-responsive/ethylene-responsive, DREB, GATA, and TCP family transcripts were found to be up-regulated in OE lines.

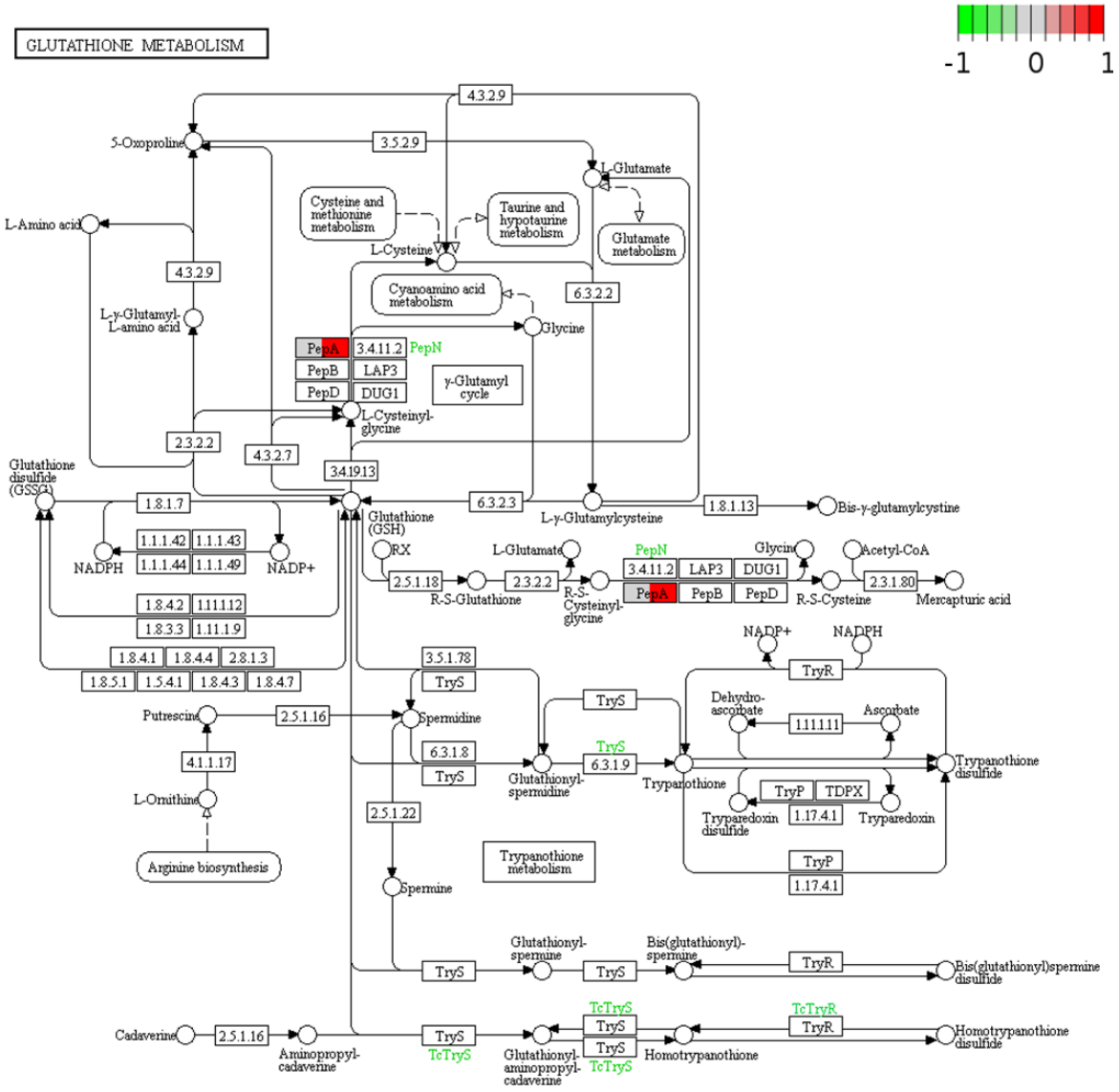


Figure 4.32: Differential gene expression (DEGs) shows PepA (aminopeptidase) positively affected in the Glutathione metabolism pathway in SmsHSP24.1 overexpressed (OE) transgenic plants

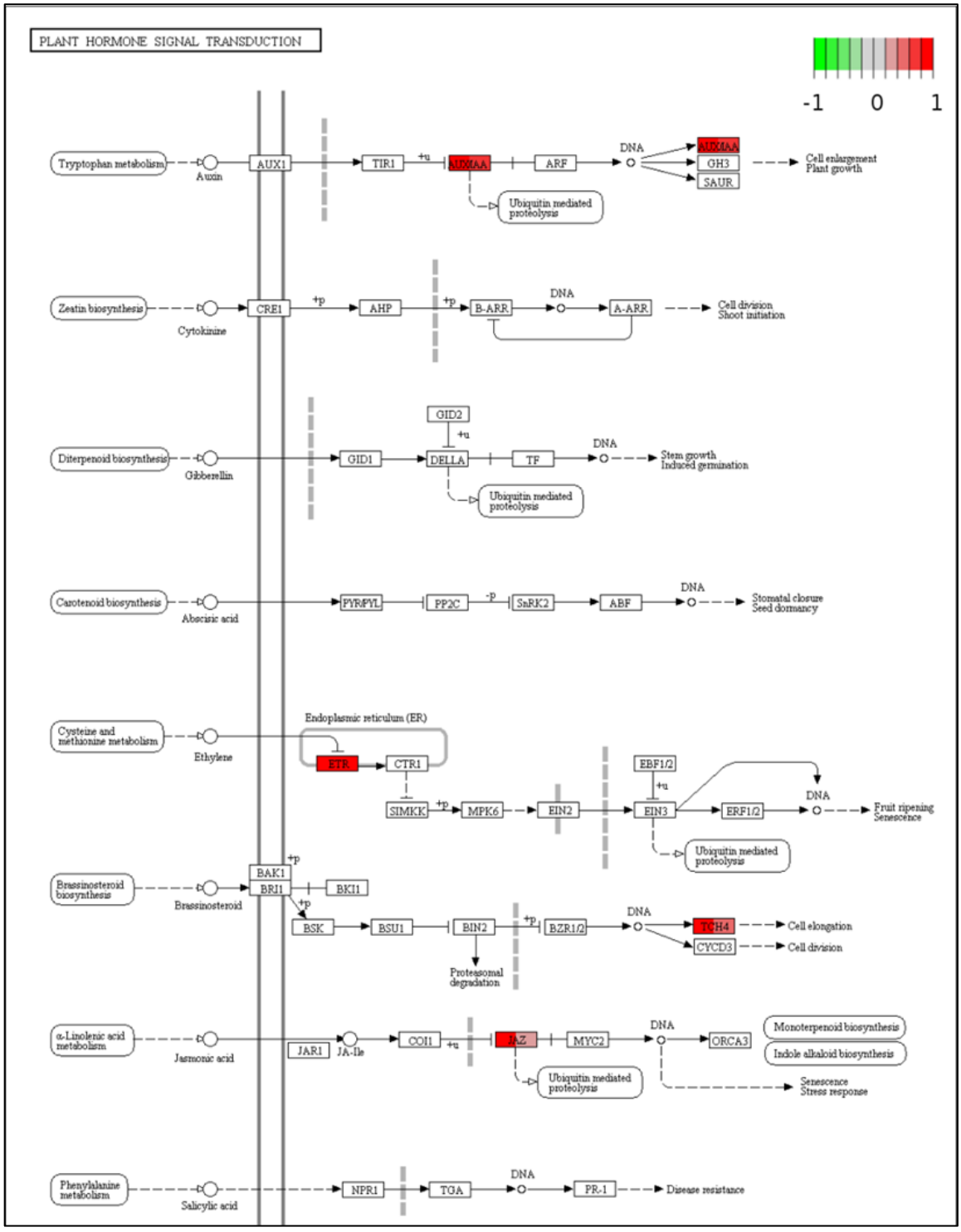


Figure 4.33: Plant hormone biosynthesis/carrier-related transcripts were significantly up-regulated in OE lines compared to WT lines.

Table 4.3: Up-regulated DGEs in Overexpressed OE lines compared to wild-type Eggplant line.

Gene symbol	Gene ID	log2(fold change)	Protein name
1. DGEs related to Auxin-response and transport			
Solyc01g	110900.1	2.22	15A-like auxin induced protein
Solyc01g	110940.3	2.06	15A-like auxin induced protein
Solyc03g	033590.1	2.58	SAUR-like auxin-induced protein
Solyc03g	082510.1	2.20	SAUR32 like auxin responsive protein
Solyc03g	123410.1	4.39	ABP19a like auxin binding protein
Solyc05g	008060.3	2.25	Auxin effluence carrier component
Solyc06g	053840.3	4.58	Auxin induced protein
Solyc07g	041720.1	3.23	ABP19a like auxin-binding protein
Solyc09g	007810.3	3.36	Auxin responsive factor
Solyc09g	083280.3	2.01	Auxin responsive/induced protein
Solyc10g	080880.2	2.39	Auxin efflux component
Solyc11g	069190.2	2.30	Auxin induced/response factor
Solyc11g	011660.2	2.73	Uncharacterized protein
Solyc06g	053260.1	3.18	58 like small auxin-up protein
2. DGEs related to Cell wall reorganization			
Solyc02g	087060.3	2.27	WAT1 related protein
Solyc02g	091920.3	4.03	Xyloglucan hydrolase
Solyc02g	092840.1	3.43	XLT2-like xyloglucan galactosyltransferase
Solyc03g	071570.3	2.73	Pectate lyase
Solyc05g	014000.3	3.48	Pectate lyase
Solyc05g	055840.3	2.64	86A1-like putative UDP galactosyltransferase
Solyc05g	055845.1	2.47	86A1-like putative UDP galactosyltransferase
Solyc06g	009190.3	2.59	Pectate lyase
Solyc06g	062580.3	2.074	Beta galactosidase
Solyc06g	083580.3	2.48	Pectin esterase

Gene symbol	Gene ID	log2(fold change)	Protein name
Solyc07g	017600.3	3.14	Pectate lyase
Solyc07g	049610.1	3.23	XLT2-like xyloglucan galactosyltransferase
Solyc07g	052690.3	2.37	Beta-amylase
Solyc07g	052695.1	2.45	Beta-amylase
Solyc07g	052980.3	2.63	Xyloglucan hydrolase
Solyc08g	075020.3	3.49	Pectin acetyl esterase
Solyc08g	079040.1	2.14	gt19 like putative xyloglucan galactosyltransferase
Solyc09g	008320.3	2.08	Xyloglucan hydrolase
Solyc09g	091430.3	4.28	Pectin esterase
Solyc11g	005770.2	3.38	Pectate lyase
Solyc02g	063140.3	3.92	3 ketoacyl CoA synthase
3. DGEs related to Lipid biosynthesis			
Solyc02g	069490.3	2.51	Sterol reductase
Solyc03g	025320.3	4.43	Alcohol acyl-transferase
Solyc04g	009380.2	2.41	Palmitoyl transferase
Solyc05g	009270.3	3.88	3-ketoacyl-CoA synthase
Solyc05g	012790.3	2.68	S-acyltransferase; Palmitoyltransferase
Solyc05g	013207.1	3.78	3 ketoacyl CoA synthase
Solyc06g	074390.3	2.87	Fatty acyl CoA reductase
Solyc08g	067260.3	2.26	3 ketoacyl CoA synthase
Solyc09g	083050.3	3.07	3 ketoacyl CoA synthase
Solyc10g	011820.3	2.55	Delta (8) fatty acid desaturase
Solyc11g	072990.2	2.49	3 ketoacyl CoA synthase
Solyc12g	006820.2	2.22	3 ketoacyl CoA synthase
4. DGEs related to Cellular redox homeostasis			
Solyc01g	006290.3	3.63	Peroxidase
Solyc03g	093180.1	3.23	Peroxisomal membrane protein
Solyc02g	087850.1	2.30	Putative ovule protein
Solyc05g	053300.3	2.36	Dihydrolipoyl dehydrogenase
Solyc08g	062970.1	2.48	C6 like putative glutaredoxin
Solyc08g	082590.3	2.00	Uncharacterized protein
Solyc10g	007110.3	2.25	Tyrosine aminotransferase1
Solyc11g	066390.2	3.93	Superoxide dismutase (Cu-Zn)

Gene symbol	Gene ID	log2(fold change)	Protein name
5. DGEs related to Histone modification			
Solyc01g	110150.2	2.03	SETD1B like histone lysine N-methyltransferase
Solyc02g	077480.1	2.63	Histone H3.1and H3.2
Solyc06g	005420.1	2.07	Histone H4 and CaH4
Solyc06g	084020.3	3.63	Histone H1
Solyc10g	008910.1	2.80	Histone H3.1and H3.2
Solyc11g	073250.2	2.55	Histone H2A
6. DGEs related to chlorophyll biosynthetic process			
Solyc01g	105030.3	2.18	Chloroplastic; CAB-10A; LHCP CP24 10A like chlorophyll a-b binding protein
Solyc01g	105050.3	2.50	Chloroplastic; CAB-10B; LHCP Chlorophyll a-b binding protein
Solyc06g	063360.3	2.09	Chlorophyll a-b binding protein
Solyc10g	006230.3	2.06	chloroplastic; LHCI type II CAB-7; Chlorophyll a-b binding protein
Solyc10g	018580.1	2.26	Chloroplastic protein TIC 214
Solyc11g	021290.2	2.02	Chloroplastic protein TIC 214
Solyc11g	021300.1	2.40	Translocon at the inner envelope membrane of chloroplasts 214
Solyc12g	035550.1	2.95	Translocon at the inner envelope membrane of chloroplasts 214
Solyc10g	018300.2	2.22	Transketolase
Solyc01g	094750.3	4.14	86A8-like cytochrome P450
Solyc01g	107730.3	3.30	CycD3
Solyc02g	089160.3	2.47	85A1 like cytochrome P450; Dwarf protein
Solyc03g	111950.3	3.27	C6-oxidase; 71A3-like cytochrome P450
Solyc04g	054260.3	2.31	Cytochrome P450
Solyc05g	055400.3	5.04	CYP77A19
Solyc08g	081220.1	2.67	86A22 like cytochrome P450

Gene symbol	Gene ID	log2(fold change)	Protein name
Solyc11g	007540.2	2.67	CYP77A20
Solyc09g	064500.3	2.25	Photosystem II Psb28 protein
Solyc06g	060340.3	2.65	Photosystem II 22 kDa protein,
Solyc01g	109260.3	2.43	PDV2-like plastid division protein
7. DGEs related to transcription factors			
Solyc04g	054910.3	3.06	Ethylene responsive RAP2-13-like transcription factor
Solyc05g	052030.1	2.72	Ethylene response transcription factor ERF4
Solyc08g	008305.1	2.33	ERF061
Solyc01g	110310.3	3.55	GATA transcription factor
Solyc02g	067340.3	2.15	Transcription factor
Solyc02g	077710.1	3.86	GATA zinc finger domain protein
Solyc03g	121240.1	2.49	bHLH87 isoform X2 transcription factor
Solyc06g	070900.3	3.20	Transcription factor 17
Solyc10g	055410.2	3.29	TCP Transcription factor
8. DGEs related to ATP synthesis coupled electron transport			
Solyc01g	017110.1	3.73	Chloroplastic NAD(P)H-quinone oxidoreductase and plastoquinone oxidoreductase subunit 5
Solyc01g	017333.1	4.48	Uncharacterized protein
Solyc01g	065620.1	2.10	Chloroplastic NAD(P)H-quinone oxidoreductase and plastoquinone oxidoreductase chain 4
Solyc03g	043610.2	3.17	ATP synthase subunit a protein 6
Solyc11g	044636.1	2.12	Chloroplastic NAD(P)H-quinone oxidoreductase and plastoquinone oxidoreductase subunit 5

number of severely impacted genes. Twelve important pathway-related genes were discovered to express themselves differently in OE-treated lines compared to WT (Figure. 4. 37-4.39 and Table 4.3). Similarly, the identical event results in the down-regulation of 185 genes in total. Among the genes for transcription factors, BZIP transcription factor (Solyc01g109880.3), ethylene-responsive factor 2; ethylene-binding protein (Solyc09g075420.3) and NAC4 domain protein (Solyc11g017470.2) were found to be down-regulated whereas a total of 18 different transcription factor (TFs), most of which were auxin-responsive/ethylene-responsive, DREB, GATA and TCP family transcripts were found to be up-regulated in OE lines. SOD, Catalase, and Ascorbate-peroxidase, well-known ROS-scavenging genes, were markedly increased in OE lines under untreated and treated circumstances. Several genes involved in the production of fatty acids (Table 4.3), chlorophyll (Table 4.4), the developmental pathway (Table 4.5), and mitochondrial integral membrane proteins have been shown to be elevated. Transcripts linked to auxin biosynthesis/carrier were increased in OE lines among phytohormones (Table. 4.3). When OE lines were compared to WT lines, a total of 14 auxin pathway genes were substantially elevated.

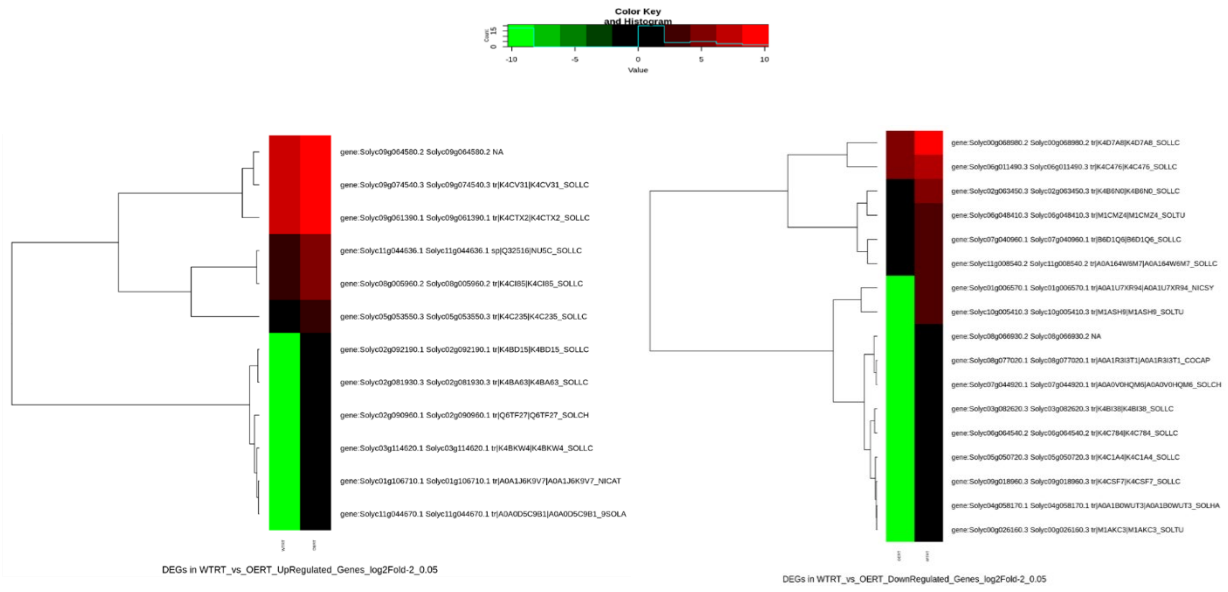


Figure 4.34: Differentially expressed and up and down-regulated genes are shown in a heat map (SmsHSP24.1 Overexpressed transgenic (OE) vs. Wild Type under control conditions)

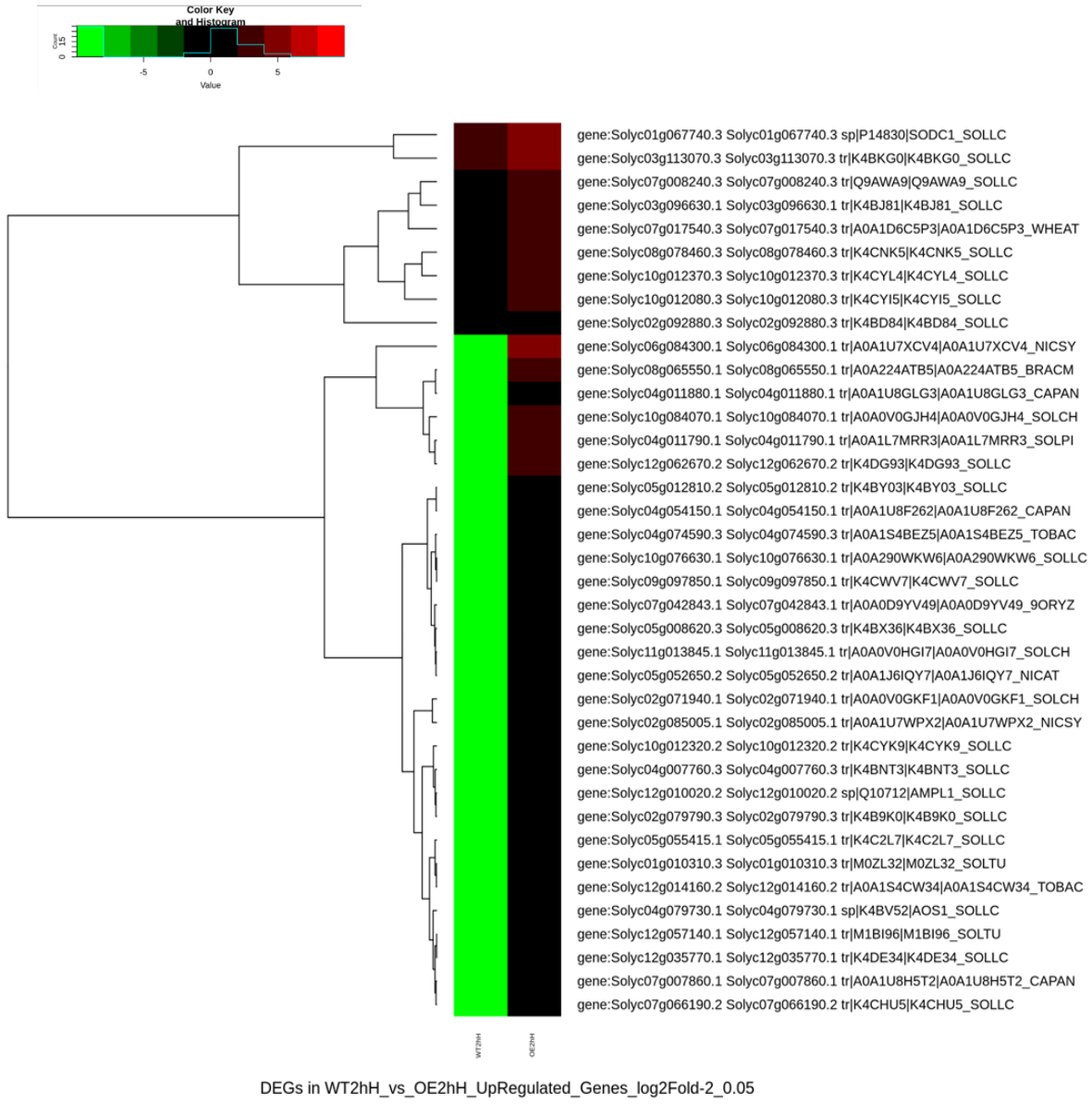


Figure 4.35. Heatmap of differentially expressed up-regulated genes in SmsHSP24.1 overexpressed transgenic line under 2-hour heat stress condition (OE2h) compared to wild type lines under same heat stress conditions (WT2h).

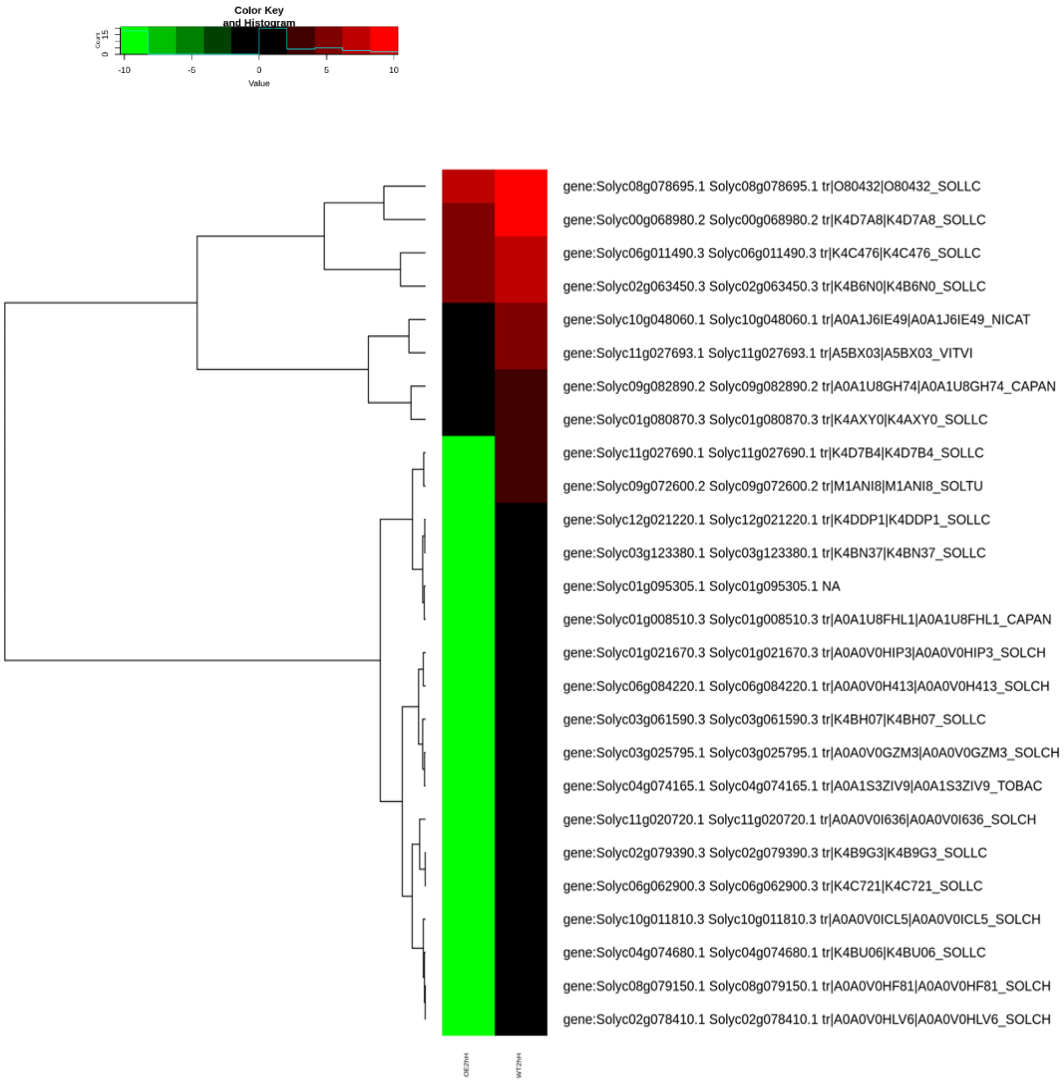


Figure 4.36. Heat map of differentially expressed (DEGs) down-regulated genes in SmsHSP24.1 overexpressed transgenic line under 2-hour heat stress conditions (OE2h) compared to wild type lines under 2-hour heat stress conditions (WT2h).

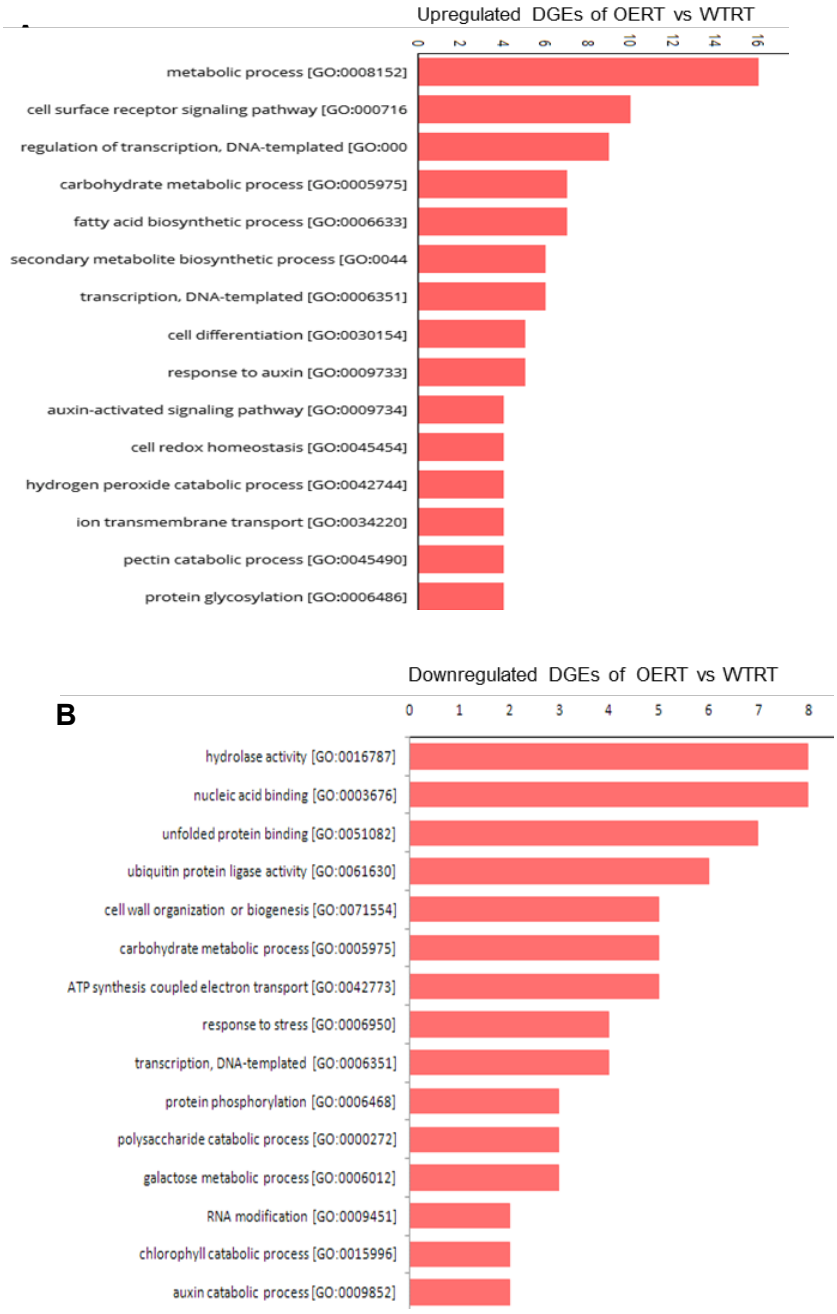


Figure 4.37: Differentially expressed genes (DEGs) according to gene ontology (GO) (SmsHSP24.1 overexpressed transgenic (OE) vs. wild type (WT) lines were grown in both stress-free and regulated environments). (A, B) GO for both up-and down-regulated differentially expressed genes in SmsHSP24.1overexpressed OERT vs. WTRT lines under control environmental growth conditions. OERT: SmsHSP24.1overexpressed line in room temperature, WTRT: Wild type line in room temperature

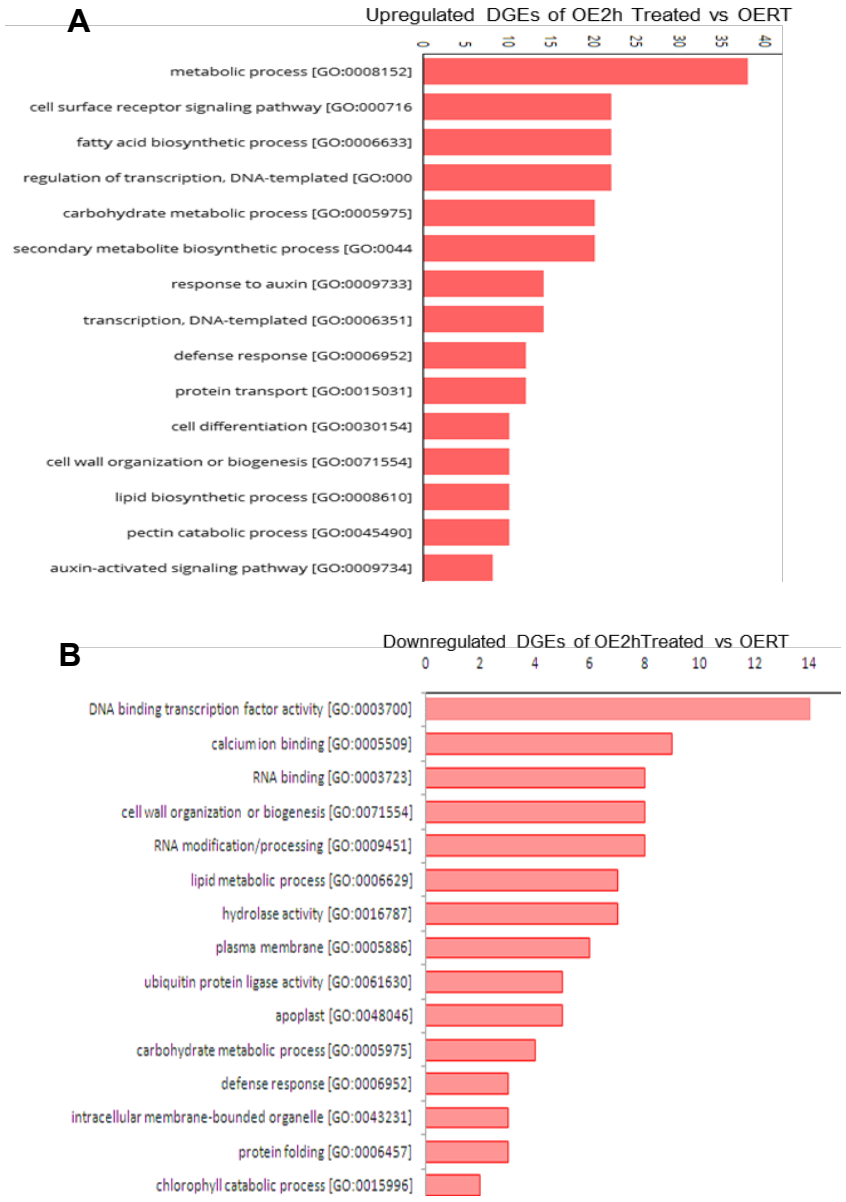


Fig 4.38: Gene ontology (GO) classification of differentially expressed genes (DEGs) in SmsHSP24.1 overexpressed transgenic (OE) and wild type (WT) lines under 2-hour heat stress conditions. (A, B) GO for both up-and down-regulated differentially expressed genes in SmsHSP24.1overexpressed transgenic line under heat stress conditions. The y-axis and x-axis indicate the names of clusters and the gene number of each cluster respectively. Only the biological processes were used for GO term analysis. Wild type line in room temperature, OE2h: SmsHSP24.1overexpressed line under 2hr heat (45˚C) stress condition.

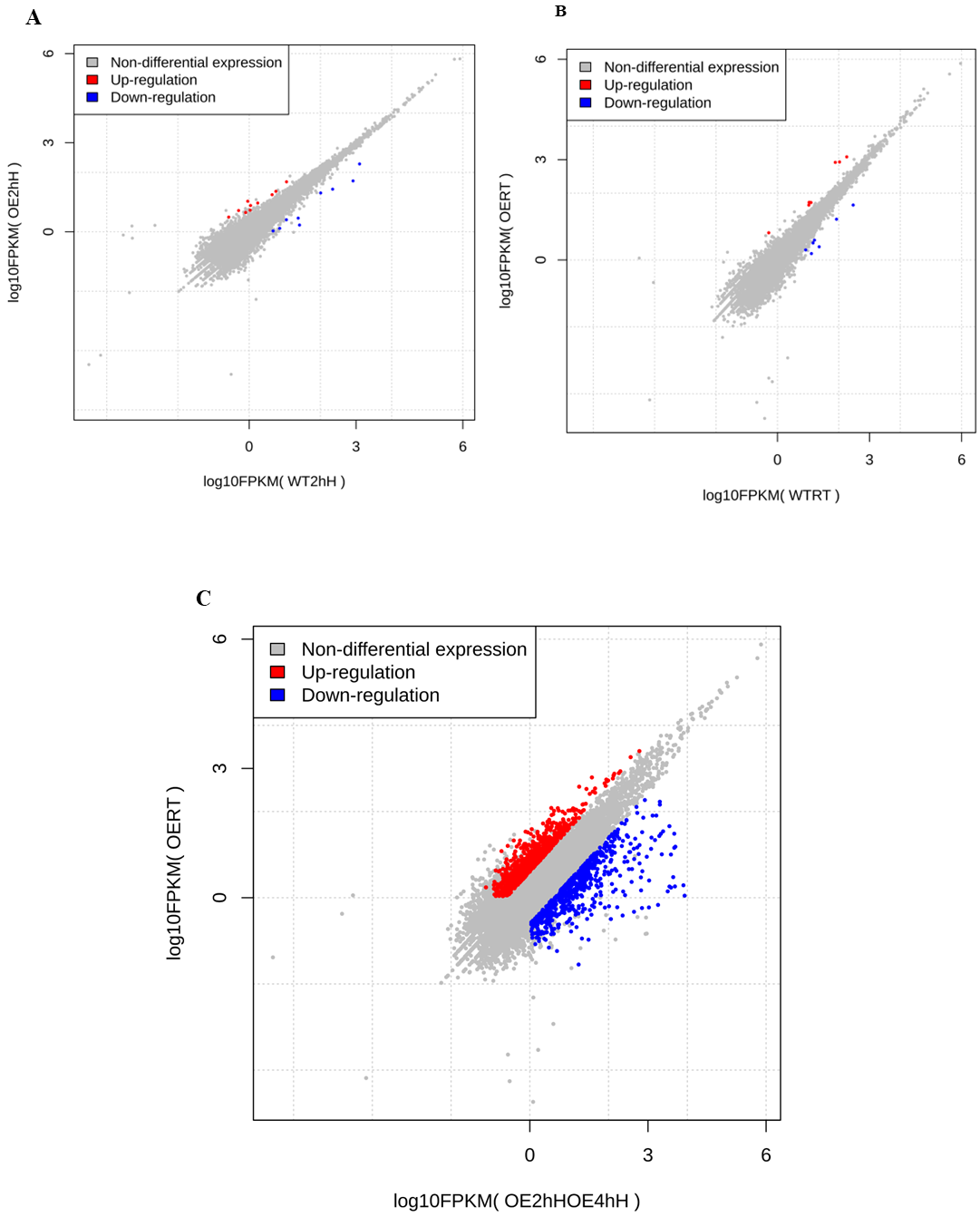


Figure 4.39. (A, B, C) Scatter plot of FPKM in overexpressed (OE) and wild type (WT) lines under control and heat stress.

Table 4.4: Down-regulated DGEs in overexpressed OE lines compared to wild type (WT) eggplant.

Gene symbol	log2(fold change)	Protein name
1. Hydrolase activity [GO:0016787]		
Solyc05g055290.3	-2.52001	Uncharacterized protein
Solyc08g077210.3	-2.0285	Inositol-1,4,5-triphosphate-5-phosphatase
Solyc09g010940.3	-2.19575	Uncharacterized protein
Solyc09g065890.3	-3.9333	Uncharacterized protein
Solyc09g075890.3	-8.22641	Uncharacterized protein
Solyc09g091050.3	-2.70169	Uncharacterized protein
Solyc11g020720.1	-2.34042	Putative hydrolase-like
Solyc12g096660.2	-2.56891	Uncharacterized protein
2. Nucleic acid binding [GO:0003676]		
Solyc01g096750.1	-3.53618	AGO4D
Solyc02g021060.3	-2.58953	Polyprotein, putative
Solyc03g058970.3	-2.19536	Polyprotein from transposon TNT 1-94
Solyc05g056360.2	-2.4012	Uncharacterized protein
Solyc08g065820.2	-5.9472	Uncharacterized protein
Solyc09g013120.3	-2.4022	Uncharacterized protein
Solyc10g055620.2	-2.54615	Putative ovule protein
Solyc11g071690.2	-2.40188	Uncharacterized protein
3. Unfolded protein binding [GO:0051082]		
Solyc01g106210.3	-4.57933	Uncharacterized protein
Solyc01g106260.3	-4.74412	Uncharacterized protein
Solyc03g007890.3	-7.90436	Uncharacterized protein
Solyc04g009770.3	-2.64908	Uncharacterized protein
Solyc04g081570.3	-2.19139	Putative endoplasmin-like
Solyc05g055160.3	-2.11419	Uncharacterized protein
Solyc06g036290.3	-8.68791	Putative heat shock protein 83-like
Solyc07g047790.3	-3.05419	Uncharacterized protein
Solyc11g020040.2	-10.7598	Uncharacterized protein
Solyc11g071830.2	-7.29679	DnaJ protein isogeny ANJ1
Solyc12g015880.2	-2.30258	Molecular chaperone Hsp90-1

4. Ubiquitin protein ligase activity [GO:0061630]		
Solyc01g050040.3	-3.12777	Uncharacterized protein
Solyc02g065540.2	-3.6894	Uncharacterized protein
Solyc03g114160.1	-2.15148	RING-type E3 ubiquitin transferase
Solyc05g005670.1	-2.9996	RING-type E3 ubiquitin transferase
Solyc05g010650.3	-2.08492	RING-type E3 ubiquitin transferase
Solyc06g083150.3	-3.5218	Uncharacterized protein
5. Cell wall organization or biogenesis [GO:0071554]		
Solyc03g007120.3	-3.04653	Uncharacterized protein
Solyc03g093130.3	-6.73563	Xyloglucan endotransglucosylase/hydrolase
Solyc07g006850.2	-3.27768	Xyloglucan endotransglucosylase/hydrolase
Solyc09g092520.3	-2.20259	Xyloglucan endotransglucosylase/hydrolase
Solyc12g017240.2	-6.35822	Xyloglucan endotransglucosylase/hydrolase
6. Carbohydrate metabolic process [GO:0005975]		
Solyc01g058140.3	-2.16348	Putative beta-glycosidase-like
Solyc02g063220.3	-2.59015	Mannose-6-phosphate isomerase
Solyc11g068440.2	-2.03865	Uncharacterized protein
Solyc12g008840.2	-2.57425	Beta-galactosidase
Solyc12g098810.2	-3.62086	Uncharacterized protein

Auxin-induced SAUR-like protein transcript was 4.3-fold upregulated. Moreover, cytokinin oxidoreductase and genes sensitive to cytokines were found to be significantly upregulated in OE lines. Our RNA-Seq findings show significant transcriptional reprogramming in multiple physiologically important pathways of the transgenic lines, suggesting that this major transcriptional change may have profound impacts on OE lines (Table. 4.3). There were also DGEs found to be down-regulated in other critical pathways (Table 4.4), including 8 DGEs involved in hydrolase activity (GO:0016787), 6 DGEs with ubiquitin-protein ligase activity

(GO:0061630), 5 DGEs involved in cell wall organization or biogenesis pathway (GO:0071554), and 2 each of auxin catabolic process (GO:0009852) and chlorophyll catabolic process (GO:00159 (Table. 4.4).

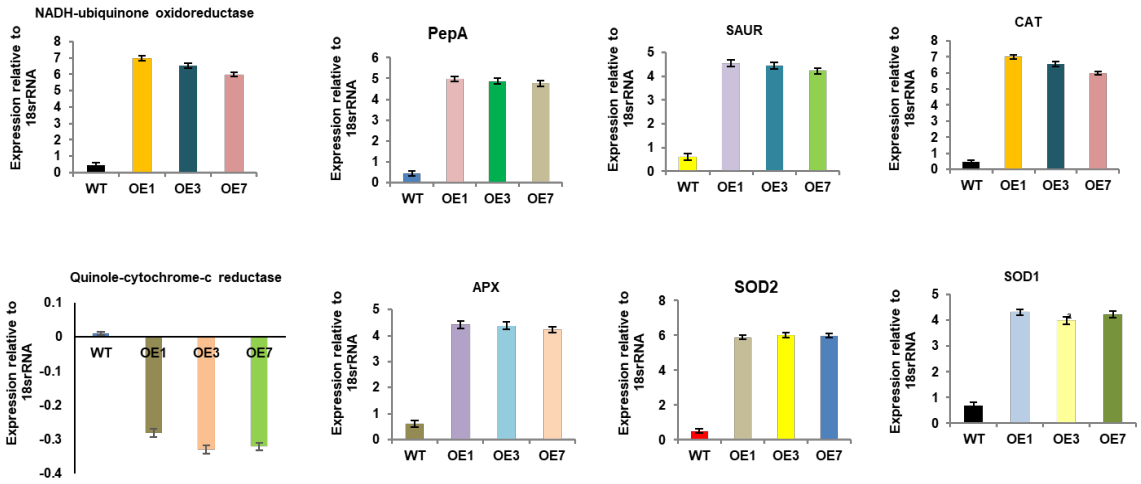


Figure 4.40: Validation of a few severely impacted genes (NADH-ubiquinone oxidoreductase, Quinol-cytochrome c-oxidoreductase, PepA, SOD1, SOD2, CAT, APX, and SAUR) using time-dependent relative quantitative RT-PCR. The housekeeping gene 18srRNA was used to standardize the mRNA level, and the fold change in expression was determined in comparison to the WT sample.

We also carried out a qPCR study on the electron transport chain (ETC), glutathione metabolism (GSH), and auxin production and transport, which are three important regulatory processes. In ETC, it was discovered that under both normal and stressful conditions, the mRNA for NADH-ubiquinone oxidoreductase was dramatically increased, but the transcript for quinol-cytochrome c-oxidoreductase was somewhat downregulated. Similar findings were also obtained via qPCR cross-validation (Figure. 4.40). The GSH pathway's PepA (aminopeptidase) gene was discovered to be significantly elevated in OE lines. Compared to WT, it was almost five times greater in OE lines. Similarly, auxin carrier proteins such as auxin-responsive SAUR-like genes showed substantial overexpression. Similarly, RNA-seq results of SOD1, SOD2, CAT, and APX transcripts that were differently expressed in OE lines were further supported by qPCR (Figure. 4.40).

Discussion:

We utilize the λ-phage site-specific recombination strategy (Papagiannis et al., 2007) for cloning *SmsHSP24.1* gene in pMDC100 plant transformation vector. This method is convenient and excludes the tedious DNA restriction and ligation procedures encountered in standard cloning methods. Preliminary cloning of the desired *SmsHSP24.1* gene cassette (promoter:gene: terminator) is performed in the destination vector (pMDC100 in this case) compatible entry vectors (EV1 and EV2). The three vectors that include destination and entry vectors 1 and 2 have been previously designed in our laboratory. The MCS was taken from the pCAMBIA backbone and the plant selection marker. Once the destination vector compatible plasmid or entry vectors 1 and 2 possess the designed transgene cassette of interest, *SmsHSP24.1* in our case, this can be recombined into a variety of plant transformation vectors (Destination vector) that possess gene combination onto a single T-DNA region by this technology. In this study, *SmsHSP24.1* gene was stacked on a single T-DNA region by a gateway cloning system. The multi-round gateway cloning strategy (as described in Materials and Methods) involves the assembly of many genes through a series of recombination steps while alternating between two different entry vectors, EV1 and EV2. The two entry vectors differ in the attachment (att) sites; EV1 carries the attachment sites attL1-attL2 and attR3-attR4 while EV2 (CmR-ccdB) carries attL3-attL4 and attR1-attR2. Recombination occurs between specific att sites on the interacting DNA molecules. These locations act as recombination proteins' binding sites (Weisberg et al., 1983). Recombination between the attL and attR sites occurs during lambda integration, creating the attB and attP sites (Landy, 1989). Thus, EV1 and EV2 (containing attL sites) can be used in conjunction with the compatible destination vectors (pMDC100, in the present case) carrying the attR sites. As a prerequisite, all the components of the SmsHSP24.1 cassette (promoter: gene: terminator) were cloned in the entry vectors, EV1 and subsequently assembled in the T-DNA region of destination vector pMDC100. Though we have cloned only a single gene, this technology paves the way for further pyramiding of other genes if required.

The delivery of genes of interest for overexpression or gene editing components like Cas9 for targeting agronomically significant traits is critical for precision breeding. Although

Agrobacterium-mediated transformation and regeneration protocols for many agricultural plants have been created, a thorough and repeatable technique for effective eggplant transformation and regeneration has yet to be developed. A few reports (Na et al., 2016; Pratap et al., 2011) have indicated the possibilities of eggplant regeneration and transformation. *In vitro* regeneration procedure is mostly affected by media composition and the supplement of plant growth regulators. According to studies, the majority of in vitro shoot regeneration from eggplants may be distinguished by combining benzylaminopurine with either naphthalene acetic acid (BAP+NAA) or indole acetic acid (BAP+IAA) as well as Kinetine (BAP+Kinetine) (Kaur et al., 2011; Rahman et al., 2006; Scoccianti et al., 2000; Shivaraj & Rao, 2011). Zeatin riboside has recently been shown by (García-Fortea et al., 2020) to be the best hormone for producing shoots in eggplants that operates regardless of genotype. The optimal BAP supplement for cotyledon, hypocotyl, and root explant shoot regeneration is 2.5 mg/L BAP alone. By simply lowering the concentration of BAP (0.2 mg/L) in the same MS medium, maximal shoot elongation was also accomplished, and this hormone concentration was effectively used for all future transformation events. We have extensively studied several key factors that significantly affect eggplant transformation frequency. The selection frequency of stably integrated transformed cells depends on the optimum concentration of the selection agent. At an optimum kanamycin concentration of 100 mg/L (4.16 shoots/explant), we discovered a higher number of shoot induction, however it was still possible to acquire non-transgenic shoots at this dosage. This is not exceptional as comparable observations have been made for several plant species, and it is believed that non-transgenic cells can survive because nearby transformed cells are not fully selected (Khatun et al., 2022). Our discovery that kanamycin is superior to other selection agents for choosing eggplant transformants is supported by other investigations (Franklin et al., 2004; Sagare & Mohanty, 2012). According to (Potrykus, 1991), Southern analysis is crucial for demonstrating the integration of the foreign gene into the host genome and counting the number of distinct transgenic insertions. Because single, undamaged transgene insertions are best for analysis, it's also important to look for additional rearrangements and multicopy tandem insertions at the region in question. In certain loci, the presence of an odd number of bands implies truncation, rearrangement, or multiple copy insertion. It is possible to create an instructive blot by selecting a restriction enzyme with a distinct site between the marker gene and the gene of interest (GOI). Each

independent insertion will have a single band visible after being probed with the marker gene. If the blot is re-probed with the transgene a new banding pattern will emerge (Bhat & Srinivasan, 2002). In our study, two singles, two double, one triple and one multi-copy (containing five copies of insertions) transgenic events were detected, while, no signal was detected in WT counterpart. This may be attributed to the use of a virulent EHA105 strain. We have investigated the role of overexpression of SmsHSP24.1 protein on Eggplant growth and development under stress and normal environmental condition. In addition to chaperon activity, we discovered that SmsHSP24.1-OE lines exhibited faster seed germination rates than WT seeds, as well as rapid seedling development and vigor regardless of the integration location. Previous investigations of sHSPs have also noted altered phenotypes such as altered pollen growth and development, altered seed and root development, altered response to pathogen and UV damage, etc (Murakami et al., 2004; Sun et al., 2002; Zou et al., 2012). Although insertional inactivation of functional genes by *Agrobacterium*-mediated transformation might change phenotypic, all of our lines, which have various copy counts, exhibit the same rate of germination. This appears to be a consequence of SmsHSP24.1 overexpression, providing an opportunity to make use of this trait to promote speedy seed germination and rapid seedling growth and vigour. Those characteristics are crucial for good productivity and effective resource use in commercial farming (Finch-Savage & Bassel, 2016). It has gradually become apparent that maintaining cellular ROS homeostasis is essential for seed germination and that this control has been known as the "oxidative window of seed germination" (Bailly et al., 2008; El-Maarouf-Bouteau & Bailly, 2008). In addition, plant cell signalling pathways supported environmental and developmental processes that governed seed germination, growth, and the transition to blooming (Meng et al., 2019). The primary controllers of ROS generation are mitochondria (Noctor et al., 2007). It is now well acknowledged that the primary enzymes responsible for the generation of superoxide in higher eukaryotes are the enzymes NADH:ubiquinone oxidoreductase (complexes I) and ubiquinol:cytochrome c oxidoreductase (complexes III) (Drose & Brandt, 2008; Larosa & Remacle, 2018). The energy source and essential organelles for plant seed germination are mitochondria (Van Dingenen et al., 2016; Wei et al., 2019). Due to decreased efficiency in photosynthetic redox processes, adverse environmental circumstances frequently cause ROS to develop in mitochondria. Moreover, it has the ability to alter proteins, membrane lipids,

DNA, and other biomacromolecules. ROS scavenging is crucial for defending the cells (Driedonks et al., 2015). Recent research has shown that overexpressing HSP40 in tomatoes conferred thermotolerance together with enhanced APX and superoxide dismutase (SOD) activity (Qiu et al., 2006). Similar effects of our work where SmsHSP24.1 overexpression on ROS-scavenging proteins in response to heat stress have been seen in other investigations as well. Overexpression of ZmHSP16.9 in tobacco increased POD, CAT, and SOD activity, while overexpression of RcHSP17.8 increased SOD activity in *Arabidopsis* (Jiang et al., 2009; Sun et al., 2016). Moreover, LimHSP16.45 overexpression increased SOD and CAT activities in transgenic *A. thaliana* (Mu et al., 2013). Therefore, it is possible to postulate that the tiny HSP proteins have a favourable impact on thermotolerance by preserving the conformation and activity of ROS-scavenging proteins, which lowers the ROS concentration (Kong et al., 2014). Nonetheless, other reports cited OsHSP18.2 overexpression as having significant effects on seed vigor, lifespan, and seedling establishment in transgenic *A. thaliana* lines (Kaur et al., 2015). Similar to this, *A. thaliana* plants overexpressing CsHSP17.2 had faster germination rates and longer hypocotyl elongation lengths than wild-type controls (Wang et al., 2017). In addition, plants overexpressing the OsHsp17.0 and OsHsp23.7 genes had better germination abilities than wild-type plants (Zou et al., 2012). Recent research has also revealed that rice lines with silenced class I small heat shock proteins (RNAiCI-sHsp) exhibit delayed seed germination and thermotolerance. Rice plants with RNAiCI-sHsp had considerably shorter seeds than non-transgenic lines (Sarkar et al., 2019). Almost all investigations that have been published thus far have demonstrated that sHSPs influence plant stress responses in a beneficial way (Sun et al., 2016). Different levels of susceptibility to abiotic stressors were seen at different phases of a plant's life cycle. The reproductive stage is recognized to be the most vulnerable to stimuli, such as temperature, salt, oxidative stresses, etc., among all developmental stages (Ghanem et al., 2009; Ji et al., 2010; Oliver et al., 2005; Oshino et al., 2007). Moreover, reports indicated that minor abiotic stress during the reproductive stage might permanently reduce a plant's production. Dicots and monocots are both equally susceptible to stress-related pollen sterility (Ghanem et al., 2009; Karni & Aloni, 2002; Kim et al., 2001; Pressman et al., 2002). Environmental factors have been discovered to be sensitive to the process of grain filling, which is the building of reserves in the growing and mature grain, and to have a significant impact on both the quantity and quality of the seed (Zhang et

al., 2006). Many stress-regulated genes are activated depending on the conditions that plants are exposed to, and it is believed that the products of these genes act as cellular protectants against stress-related damage (Shinozaki et al., 2003; Thomashow, 1999). It has been demonstrated that the expression of stress-regulated genes plays regulatory roles in stress responses, which eventually contribute to the effective yield production.

Damage from heat stress results in altered metabolic processes, including those involved in starch synthesis, photosynthesis, osmotic regulation, antioxidant system, and membrane thermostability. A variety of genes and signal transduction are involved in the response to heat stress (Kotak et al., 2007). As was already said, abiotic conditions eventually produce oxidative stress in plants as a result of the production of reactive oxygen species, which can seriously harm DNA, proteins, lipids, and carbohydrates. Plants have a very effective system of enzymes that work together to control the cascades of uncontrolled oxidation, scavenge for ROS, and shield cells from oxidative damage as part of their antioxidant apparatus. We carried out RNA sequencing and transcript quantification on transgenic and WT lines to elucidate the molecular mechanisms of SmsHSP24.1 overexpression-mediated regulation in control as well as heat stress conditions in Eggplant. Interesting findings from our transcriptome analysis support the hypothesis that SmsHSP24.1 may play a role in quick seed germination and abiotic stress tolerance.

In comparison to WT, the mRNA level was down-regulated in OE lines under both stressed and non-stressed circumstances for quinol-cytochrome c-oxidoreductase (EC 1.10.2.2) and highly up-regulated for NADH-ubiquinone oxidoreductase (EC 1.6.5.3). The first electron receptor, NADH-ubiquinone oxidoreductase, and the third complex in the electron transport chain, quinol-cytochrome c-oxidoreductase, may have been particularly affected by this differential expression of electron acceptor and donor, which may have caused an imbalance in the electron gradient and, under normal growth conditions, increased levels of cellular ROS. Also, a biochemical examination of ROS generation indicates that OE lines have a somewhat higher ROS level than wild-type plants. This is consistent with our theory that SmsHSP24.1 overexpression enhances germination via changing the cellular ROS gradient. SmsHSP24.1 OE lines showed no adverse growth effects in contrast to the enhanced cellular ROS. The RNA-Seq study showed that numerous essential ROS-scavenging genes were simultaneously elevated in OE lines compared to WT, including superoxide dismutase (Solyc06g048410.3,

Solyc06g048420.2), catalase (CAT) (Solyc04g082460.3, and APX (Solyc09g007270.3). proline dehydrogenase, glutamate synthase, PepA glutaredoxin, and other important cell redox homeostasis transcripts have all been discovered to be increased in OE lines. These results are supported by qPCR expression crosscheck and enzymatic analyses. Our findings suggest that overexpression of SmsHSP24.1 causes an elevation of cellular ROS at normal environmental growth conditions without being fatal to the host cell. Nonetheless, this reprogrammed global transcripts in OE lines. Hypocotyl elongation, which results from cell expansion and is mostly regulated by auxin hormone, determines rapid seedling growth. Recent research has shown that enhanced cell growth brought about by the overexpression of AtSAUR36 (Stamm & Kumar, 2013), AtSAUR41 (Bai et al., 2019) AtSAUR19 (Spartz et al., 2017), and AtSAUR63 (Tian et al., 2022) promotes hypocotyl elongation. Our RNA-seq data indicate overexpression of a number of genes connected to the auxin-biosynthesis and carrier pathway, which is consistent with these results. The OE lines' expression studies of a chosen eggplant-specific auxin-responsive protein SAUR32 gene confirmed its overexpression. Our results point to a complex gene regulation network of SmsHSP24.1 protein on plant growth and development, suggesting that OE lines retain a much higher SAUR32 transcript level and other auxins biosynthetic and carrier transcripts that positively influence hypocotyl growth. The molecular basis for SmsHSP24.1-OE plants' improved heat tolerance is also shown by RNA-seq data. The findings imply that cellular transcript reprogramming modifies multiple important physiological processes in addition to maintaining redox equilibrium. Field research of the OE lines in circumstances of extreme heat and drought shows superior crop production compared to the WT Eggplants. In OE lines, RNA-seq investigations point to altered cell wall biosynthesis (Table 4.3). Previous research has amply demonstrated that altering a cell wall's biophysical characteristics is a critical reaction to external stimuli like heat, essential to sustain the cell's overall function and development (Lima et al., 2013; Wu et al., 2018). In our analysis, we also discovered other important changed transcripts related to lipid metabolism, mitochondrial and chloroplast membrane proteins, histone and histone modification, and transcription factor genes. Many plants have shown significant transcriptional reprogramming under heat stress, which impacts a variety of characteristics (Barah et al., 2013; Zou et al., 2012). Our findings demonstrate a comparable impact on the whole transcriptome in OE lines. During stress signalling and response, the lipid content of the cell membrane must dynamically

alter (Hou et al., 2016). Fatty acyl Co-A reductase (FAR), one of 13 DGEs, functions as an interface between plants and their biotic and abiotic environments, considerably reducing non-stomatal water loss, and acting as the first line of defence (Chai et al., 2018; Rowland et al., 2006). Similar to this, 20 DGEs connected to chloroplasts have been identified in OE lines. Chlorophyll, which is essential for plant growth and development, is one of the main targets of heat stress damage. In both normal and stressful circumstances, a noticeably larger overexpression of these genes is essential for the growth of chlorophyll and photosynthesis. Another method of controlling expression is chromatin remodelling. An important strategy for reversing environmental alterations is DNA methylation pattern modification and histone modification. This frequently coincides with dynamic changes in genes that respond to stress (Kim et al., 2015). Similar to this, TFs play a role in abiotic stress reactions as well as typical growth and development. The presence of TFs such as ethylene-responsive elements has also been noted. Abiotic stress tolerance and enhanced growth and development are conferred by multiple transcriptions reprogramming caused by overexpression of SmsHSP24.1 in environments with typical environmental conditions.

Part-III. Development of SmsHSP24.1 knockout eggplant lines using CRISPR/Cas9 genome editing system

4.3.1. Cloning of gRNAs into EV1 and EV2 with *Bsa*I restriction digestion

The Entry vector1 (EV1) and Entry vector2 (EV2) were used to clone gRNAs targeted to knock out of *SmsHSP24.1* gene. The genetic organization of *SmsHSP24.1* has 2 exons and one intron. We have selected two gRNAs for knocking out *SmsHSP24.1* gene to increase editing frequency (Figure. 4.41).

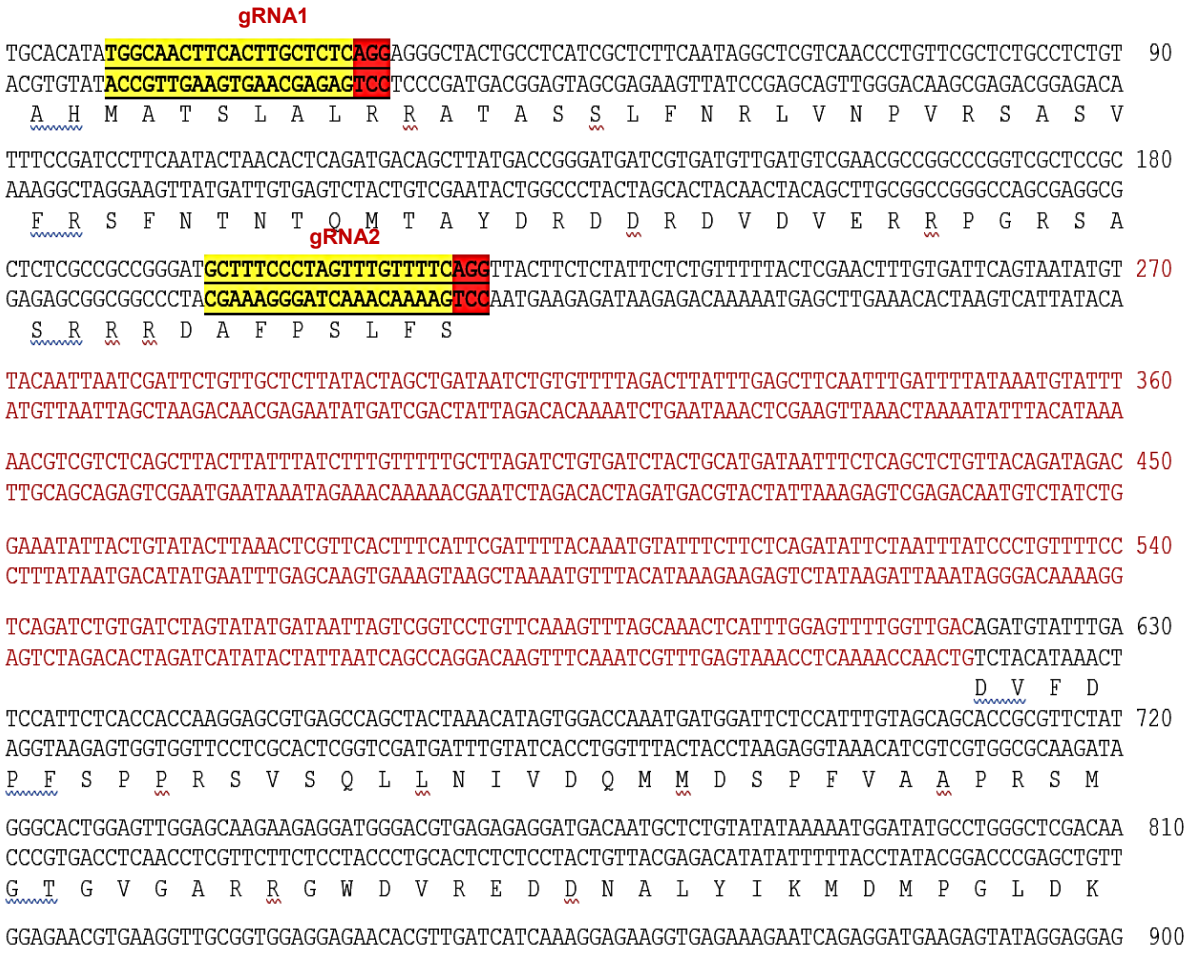


Figure 4.41: Genomic sequence of *SmsHSP24.1* possess 2 exons and one intron

Both EV1 and EV2 have *Bsa*I cloning sites in between the promoter (AtU3P for EV1 and AtU6P for EV2) and scaffold to ligate gRNA in them. Hence, EV1 was digested with *Bsa*I restriction enzyme which is flanked by the U3 promoter and scaffold to ligate gRNA2 (Figure. 4.43 A). We ligated gRNA2 for the SmsHSP24.1 gene in EV1 vectors in our initial effort. In LB medium containing gentamycin, this ligation product was converted into *E. coli* Top10 cells and plated. Using a forward primer for the U3 promoter and a reverse primer for gRNA2, the resistant colonies created were examined by colony PCR for the presence of gRNA2. The PCR results for gRNA2 confirmation in EV1 produced a band with a size of about 370 base pairs. Colony PCR of EV1 produced a band of the desired size, indicating that the gRNA2 cassette was correctly assembled in the EV1 (Figure. 4.43 B).

This is similar to how we cloned gRNA1 for SmsHSP24.1 gene in EV2 vectors at *Bsa*I digested site, flanked on each end by U6 Promoter and scaffold (Figure. 4.42 A, B). In LB plates supplemented with gentamycin and chloramphenicol, the ligation product of EV2, which contained gRNA1, was transformed into DB3.1 cells and exposed to selection. The resistant colonies produced were tested by colony PCR for the presence of gRNA1 using a forward primer for the U6 promoter and a reverse primer for gRNA1. The gRNA1 confirmation in EV2 PCR produced a band with a size of around 670 bp (Figure. 4.42 C). A band of the necessary size was also obtained by EV2 colony PCR, demonstrating that the gRNA1 cassette was properly assembled in the EV2.

4.3.2. Cloning of Cas9 into Entry vector1 (EV1)

Entry vector1 (EV1) was prepared to clone Cas9 cDNA. First, EV1 was double digested with *Kpn*I and *BamH*I restriction sites. Cas9 cDNA was digested with the same restriction sites. Both digested EV1 and Cas9 genes were ligated and transferred into E-Top10 cells and plated on LB media containing gentamycin. The presence of EV1 ligation product was verified by colony PCR (Figure. 4.42 D). The PCR products for Cas9 validation in EV1 produced a band of approximately 750 bp in size. Colony PCR of EV1 produced a band of the desired size, confirming that the Cas9 cassette was correctly assembled in the EV1.

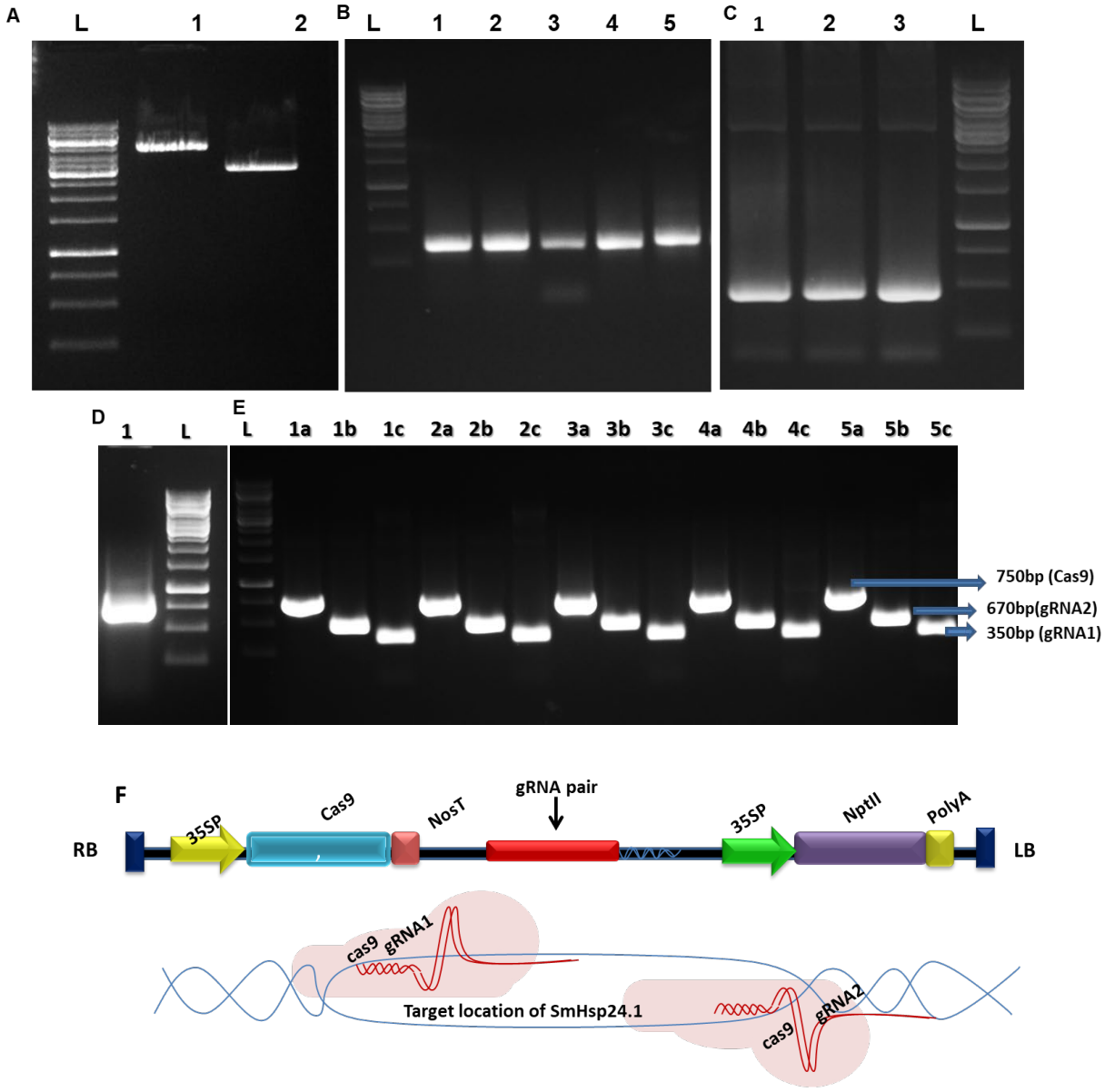


Figure 4.42: Construction of CRISPR/Cas9 expression vector. (A) Restriction digestion of Entry vector (EV1 and EV2) with BsaI; Lane L represents 1kb DNA ladder, and lane 1 represents digested EV1 vector. Lane 2 represents digested EV2 vector. (B) PCR amplification of EV1 vector with AtU3 promoter+gRNA2 in ETOP10; Lane L represents 1kb DNA ladder, lanes 1-5 represent PCR amplification of AtU3 promoter+gRNA2. (C) PCR amplification of EV2 vector with AtU6 promoter+gRNA1 in DB3.1; Lane L represents 1kb DNA ladder, lanes 1-3 represents PCR amplification AtU6 promoter+gRNA1. (D) PCR amplification of Cas9 in PMDC100. (E) PCR amplification of full CRISPR/Cas9 expression cassette (Cas9+ At U6-gRNA1+AtU3–gRNA2 region in PMDc100). Lane L represents 1kb DNA ladder, lanes 1-5 represent PCR amplified region. (F) Schematic diagram of CRISPR/Cas9 expression cassette.

4.3.3. Pyramiding Cas9 gene and gRNAs into pMDC100 as plant expression vector

The Multi-Round Gateway™ technology was successfully used to pyramid the Cas9 gene and the gRNAs into pMDC100 plant expression vector. The first round of LR recombination contains the CAM35Spromoter:Cas9:NosT and the pMDC100 vector. The second round of LR reaction constituted the recombination between the expression cassette: EV2:AtU6promoter:gRNA1:scaffold and the destination vector pMDC100:Cas9. The pMDC100:Cas9:gRNA1 vector was combined with the gRNA2 expression cassette, AtU3promoter, and scaffold during the third stage of LR recombination. The Cas9 gene, gRNA1, and gRNA2 were verified to be present in the finished expression vector construct using specific sets of screening primers (pMDC100:Cas9:AtU6gRNA1:AtU3gRNA2). By using PCR to detect the presence of a complete expression cassette, the predicted size bands for Cas9 (750 bp), gRNA1 (370 bp), and gRNA2 (670 bp) were confirmed (Figure. 4.43 E).

4.3.4. Transformation of Eggplant (*Solanum melongena* L.) and generation of SmsHSP24.1 knockout lines

Our previously optimized *Agrobacterium*-mediated transformation protocol was used for the transformation of pMDC100:Cas9:AtU6gRNA1:AtU3gRNA2 expression cassette into BARI Begun-4 Eggplant variety. The. Cotyledons explants were used from 10-day-old Eggplant seedlings. Frist explants were infected with *Agrobacterium* containing the pMDC100:Cas9:AtU6gRNA1:AtU3gRNA2 expression cassette. The transformed cells were selected on MS medium supplemented with 100 mg/L kanamycin. Despite using our previously optimized *Agrobacterium*-mediated transformation protocol to develop transformed Eggplant shoots but surprisingly regeneration frequency was severely retarded. Hardly, we have generated 8-10 transformed shoots from all the batches. After three rounds of selection, the regenerated shootlets were transferred to rooting media. The micro shoots were placed in plastic pots with soil and compost after they have shown strong roots (2:1). The potential knockout lines were accustomed to the environment and moved to a greenhouse (Figure. 4.43 A-D).

4.3.5. Molecular confirmation of T0 Eggplant lines

Ten (10) T_0 putative transgenic plants along with WT plants were screened by PCR using the *Npt*II (kanamycin) and Cas9 gene-specific primers to check their integration into the eggplant genome. All the 10 plants were positive for kanamycin while one plant showed no Cas9 band. From the 10 plants nine plants were positive for Cas9 (~0.75 bp band of Cas9 gene) and all 10 plants were positive for kanamycin (~0.45 kb band of *Npt*II gene), showing 100% transformation efficiency for kanamycin and 90% for Cas9 (Figure. 4.43 E, F).

To determine gene editing frequency, we sent six (6) Cas9-positive plants (L2, L3, L5, L7, L8, and L10) for DNA sequencing. The partial sequence of *SmsHSP24.1* gene from Cas9 positive plants was amplified having the target sites (comprising both the gRNAs target sites) such that the amplicon was of ~300 bp. This amplicon was sent for DNA sequencing to analyse the mutation frequency. DNA sequencing results of T_0 plants showed no desired mutations in the expected region which is upstream of PAM sequence. Then these cas9-positive T_0 transgenic Eggplant plants were grown under optimal conditions in the greenhouse until maturity to progress the lines to the T1 generation (Figure 4.44).

4.3.6. Analysis of mutation inheritance in T_1 generation

T_1 seeds were collected from putative T_0 lines. After germination of ten (10) T_1 plants from each T_0 line (L2, L3, L5) were selected for sequencing. We did PCR of thirty plants (T_1 generation) of *SmsHSP24.1* genes along with wild type (WT) using Cas9 and the corresponding gene-specific primer set. PCR product from Cas9 primer set showed an expected 750 bp fragment. The partial amplicon of *SmsHSP24.1* genes using gene-specific primer sets showed expected bands of ~600 bp. Then these amplicons were sent for DNA sequencing to analyse the inheritance pattern of these CRISPR-mediated mutations. When all the sequence (30) results came again we found no desired mutations in the expected region of the PAM sequence (Figure. 4.45). To find out the probable reason behind this, first, we decided to check the copy number of *SmsHSP24.1* genes in the Eggplant genome. We assumed if a single copy of *SmsHSP24.1* genes is actually present in the Eggplant genome and if this gene is essential for Eggplant, then it might difficult for Eggplant cells to survive after knocking out

and making non-functional of this gene. Further, we conducted the copy number analysis of this gene in wild-type Eggplant.

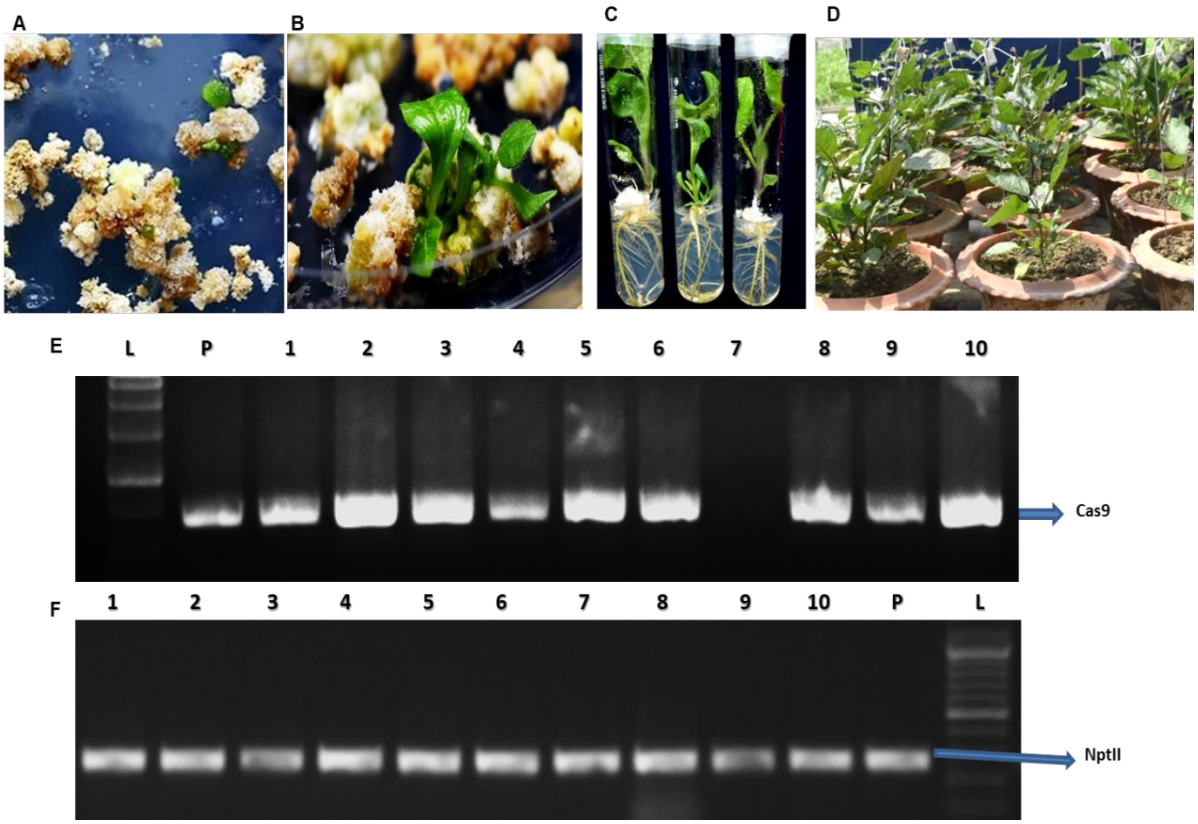


Figure 4.43: *Agrobacterium* mediated transformation of Eggplant (*Solanum melongena* L.) and generation of SmsHSP24.1 knockout lines. (A) Cotyledonary leaf infected with EHA105 *Agrobacterium* strain harboring CRISPR/Cas9 expression construct. (B) Initiation of shoot regeneration. (C) *In vitro* root formation of the regenerated shoots. (D) Acclimatization and establishment of putative knockout lines. (E) PCR amplification of Cas9 genes using specific primers (1-10): PCR screen for putative Cas9 lines, L: 1Kb DNA ladder, (P): Positive PCR control. (E) PCR amplification of *Npt*II genes using specific primers (1-10): PCR screen for *Npt*II lines, L: 1Kb DNA ladder, (P): Positive PCR control.

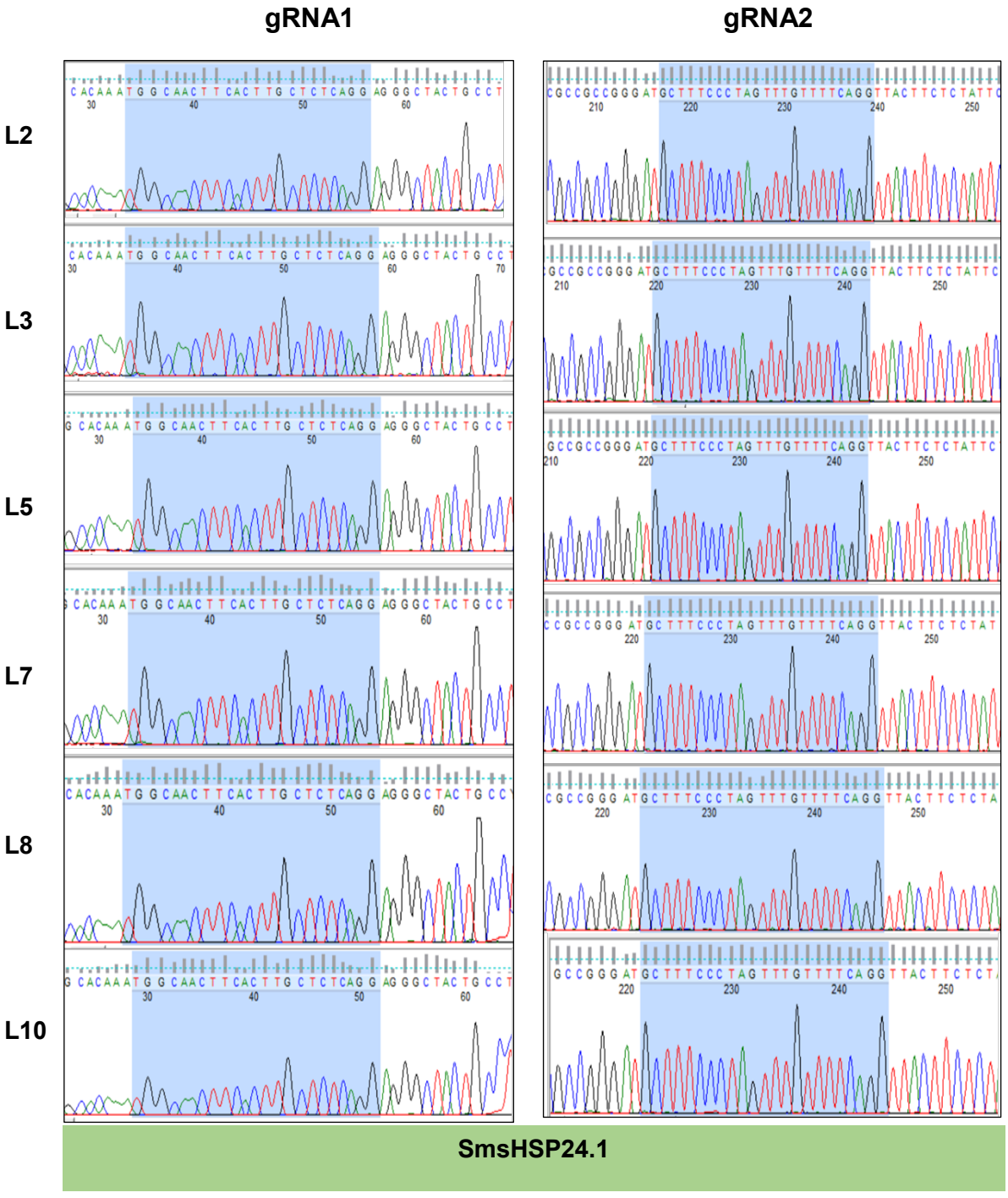


Figure 4.44: DNA sequencing result of six (6) T_0 lines (L2, L3, L5, L7, L8, L10). T_0 lines showed no desired mutations in 3-6 bp upstream of PAM sequence**.**

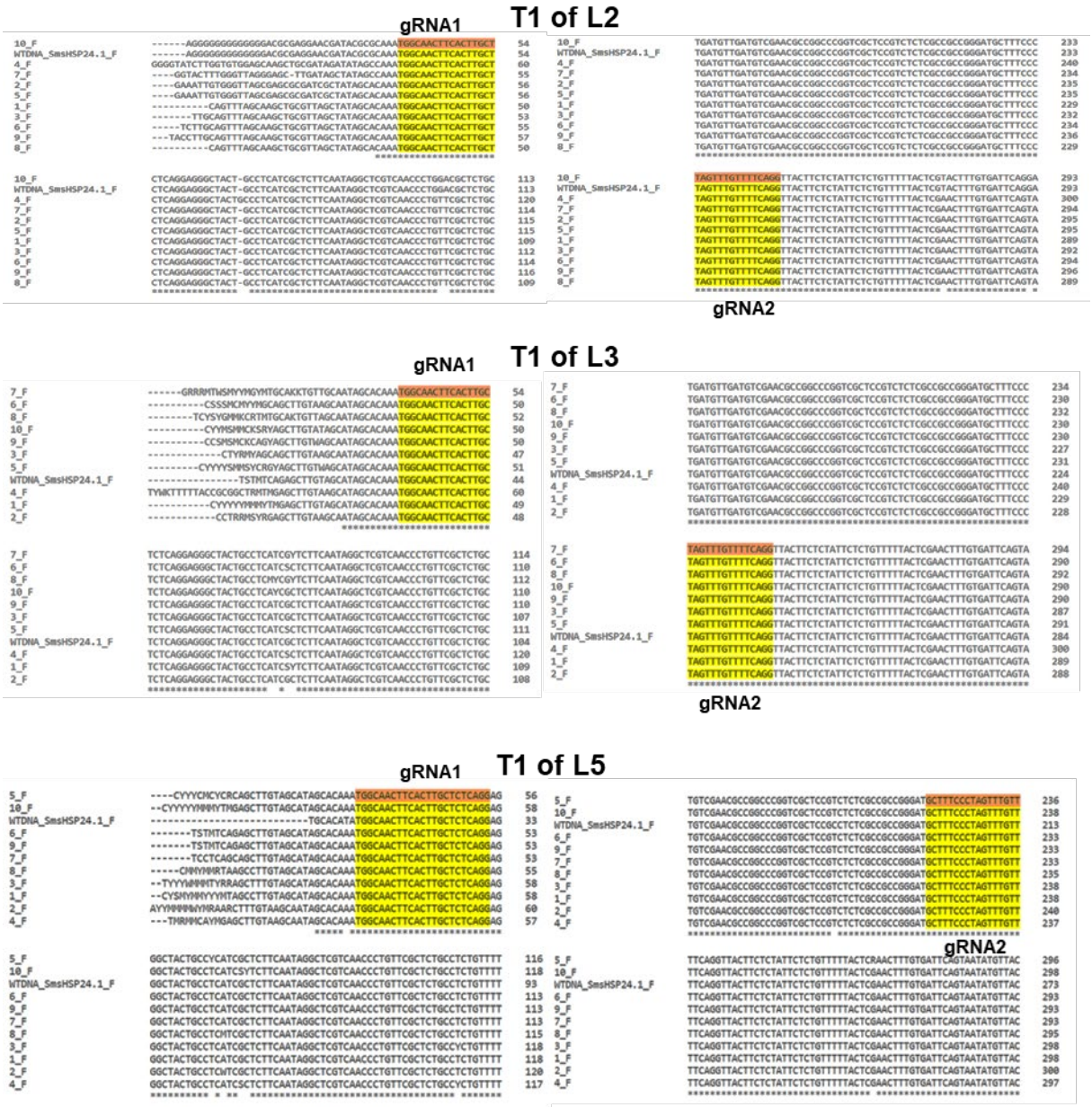


Figure 4.45: DNA sequencing result of T_1 generation of L2, L3, L5 T0 lines. Ten (10) T_1 plants from each T_0 lines showed no desired mutations is 3-6 bp upstream of PAM sequence.

4.3.7. Copy Number Analysis

To confirm the copy number of *SmsHSP24.1* gene in the eggplant genome we used *SmsHSP24.1* gene-specific probe. Genomic DNA (approximately 20 µg) was isolated from the leaf tissue of wild-type eggplant. *Nde*I and *Bam*HI were two of the restriction enzymes (REs) employed to overnight digest the genomic DNA of the eggplant. On 0.8% agarose gel, fragments of the digested DNA were sorted by electrophoresis. For the capillary transfer of DNA fragments, HybondTM nylon-positive membrane (GE Healthcare Ltd, UK) was utilized. For the following hybridization, gene-specific digoxigenin-labelled probes (DIG probes) were utilized. Each DIG probe was created from the cDNA coding regions of *SmsHSP24.1* (sHSP-Probe F1/sHSP-Probe R1) (Table. 3.2) using a PCR DIG probe synthesis kit (Roche, Germany). According to the manufacturer's instructions, the blots were detected (DIG High Prime DNA Labelling and Detection Starter Kit II, Roche, Germany). We discovered that the *SmsHSP24.1* gene is only found in one copy in the eggplant genome (Figure. 4.46).

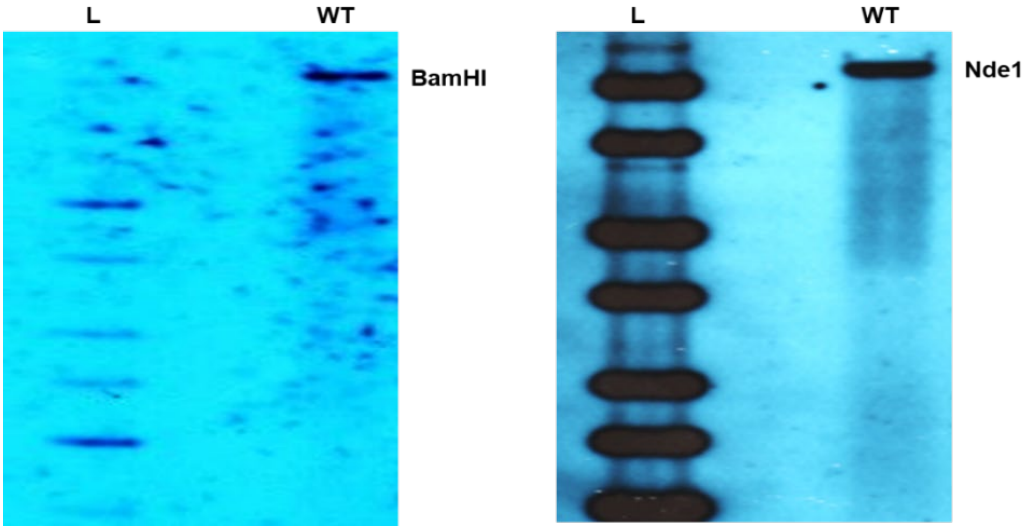


Figure 4.46: Copy number analysis of *SmsHSP24.1* gene in Eggplant genome. Restriction enzyme digestion results showed only a single copy of *SmsHSP24.1* gene present in the Eggplant genome.

Discussion:

Using a CRISPR/Cas-based genome editing method makes it simple to explore functional genomics. Characterization of natural mutants has uncovered several significant biological pathways and gene functions during the past few decades. Alternately, targeted genome editing is a powerful technique that has been utilized to swiftly modify genes in organisms. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases were earlier genome editing tools (TALENs). Over the past few years, there has been incredible progress in CRISPR-based genome editing technologies. Since 2013, several successful applications of CRISPR/Cas9-mediated knockout have recently been documented. For e. g., in *Arabidopsis* Eif(iso)4E (Pyott et al., 2016), UGT79B2 and UGT79B3 (Zhao et al., 2016), OST1 (Osakabe et al., 2016); in rice OsERF922 (Wang et al., 2016), OsSWEET13 (Diana et al., 2022), OsMPK2, OsBADH2 (Shan et al., 2013), OsMPK5 (Xie & Yang, 2013), OsDEP1 (Shan et al., 2014), Gn1a, DEP1, GS3, and IPA1 (Li et al., 2016), OsDERF1, OsPMS3, OsMSH1, OsMYB5 (Zhang et al., 2014), OsAOX1a, OsAOX1b, OsAOX1c, OsBEL (Xu et al., 2015), OsHAK-1 (Nieves-Cordones et al., 2017), OsPRX2 (Mao et al., 2018); in tomato PSY1, MYB12, and SGR1 (Yang et al., 2023), SlMAPK3 (Wang et al., 2017) and Rin (Ito et al., 2015), in grape (Wang et al., 2018); in soybean GmPDS11 and GmPDS18 (Du et al., 2016) and in cassava (Odipio et al., 2017) phytoene desaturase (OsPDS) gene in rice has been recently knocked out which proved to be important for nutrient enhancement in rice (Shan et al., 2013). In hexaploid wheat, three mildew resistance locus (MLO) homoeoallele has been successfully mutated which showed the possibilities of multiplex genome editing through a gene knock-out approach (Wang et al., 2004). Many researchers have also applied CRISPR/Cas tools to gene functional analysis (Lino et al., 2018). Occurrences of several abiotic stresses are the main causes behind the lessening of eggplant yield in Bangladesh. As implied by earlier reports (Lund et al., 1998; Vierling, 1991); it might be speculated that mitochondrial small heat shock protein plays a crucial role in protecting eggplants from various stresses. Consequently, understanding the function of Eggplant mitochondrial small heat shock protein (SmsHSP) would help to develop stress tolerant plant using transgenic approach. Therefore, we applied CRISPR/Cas9 system to knockout the SmsHSP genes for better understanding of the actual role of this gene for eggplant function and survivability. In our

experiment we have successfully developed several Cas9 positive plants through *Agrobacterium* mediated transformation. We have sequenced number of plants from T_0 and T_1 generation. Surprisingly, we haven't found any desire mutations in the expected region of *SmsHSP24.1* gene. Further we have conducted the copy number analysis to find out the exact copy of *SmsHSP24.1* gene present in Eggplant genome. We have done Southern blot analysis with *SmsHSP24.1* gene-specific probe. Our Southern blot analysis confirmed that only single copy of *SmsHSP24.1* gene present in Eggplant genome. At that point, from our experimental data we assumed that the single copy of *SmsHSP24.1* gene might be essential for eggplant survivality. We also assumed that if the single copy of *SmsHSP24.1* gene is mutated or already been knocked out that mutated cell won't survive during transformation process. At the same time non-edited but Cas9 positive plant could grow during transformation. We thought whatever Cas9 positive plant we got after transformation that was non-mutated but Cas9 positive. Our findings harmonised with the experiment of (Morrow et al., 2004), they also found that Mito-sHSP22 in Drosophila is a key player in the aging process and absence of this protein shortens Drosophila's lifespan by 40%. Similarly, (Hsu et al., 2003) also observed sHSP RNAi in *Caenorhabditis elegans*, shortens lifespan of wild-type animals by less than 25%. Plant mitochondria are crucial for plant metabolism and are closely unified into cellular homeostasis (Van Aken, 2021). Disturbance of normal mitochondrial function could be detrimental to cell viability. Our findings also indicated the same that knocked out the function of single copy mitochondrial small heat shock protein (*SmsHSP24.1*) gene made the Eggplant cell unable to regenerate during transformation process.

Part-IV. Understanding the transcriptional regulation of *SmsHSP24.1* gene in Eggplant

4.4.1. Cloning of *SmsHSP24.1* promoter from Eggplant (*Solanum melongena* L.)

Understanding the promoter is essential for developing a vector system for efficient genetic transformation and discovering the function of any unknown gene. Primarily, this objective was aimed at the functional characterization of a small heat shock protein (*SmsHSP24.1*) promoter which is supposed to be used as an inducible way for crop genetic improvement. In this study first, we amplified the ~2kb upstream sequence (predicted as a small heat shock protein promoter) of the translation initiation codon (ATG) of small heat shock protein promoter (− 2000 bp to − 1 bp from ORF of *SmsHSP24.1* gene) by using a primer sets (HSP_Pro_1F/ HSP_Pro_1R) from genomic DNA of eggplant (*Solanum melongena* L.). After the successful amplification of the full-length *SmsHSP24.1* promoter sequence we cloned the amplified product into the pCR-4-TOPO vector. Sequence confirmation was subsequently done by universal M13 reverse and forward primers. The function of the cis-acting elements and the stress-specific core functional regions associated with this promoter was identified by deletion experiments.

4.4.2. Construction of SmsHSP24.1 promoter:GUSNosT expression vector

After successfully cloning and confirming the pCR-4-TOPO vector having 2kb upstream sequence predicted as a small heat shock protein promoter, then 5′ three other deleted fragments of different lengths (1.5kb,1.0kb,0.5kb) were also amplified by using a pair of PCR primers (HSP_Pro_2F, 3F, 4F/ HSP_Pro_2R,3R,4R) (Table. 2.1). All four 2.0kb fragments of SmsHSP24.1 promoter (− 2000 bp to − 1 bp from ORF of *SmsHSP24.1* gene), 1.5kb SmsHSP24.1 promoter (− 1500 bp to − 1 bp from ORF of *SmsHSP24.1* gene), 1.0kb SmsHSP24.1 promoter (− 1000bp to − 1 bp from ORF of *SmsHSP24.1* gene) and 0.5kb SmsHSP24.1 promoter (− 500 bp to − 1 bp from ORF of *SmsHSP24.1* gene) then sub-cloned into entry vector (pL12R34-Ap) using *Sac*I and *Nco*I restriction sites. All four 5′ deleted fragments were then fused with the uidA (beta-glucuronidase, GUS) reporter gene for the

construction of *SmsHSP24.1* promoter+GUSNosT expression vector; we again amplified GUS and Nos terminator from pCAMBIA1301 plasmid with primer pair,

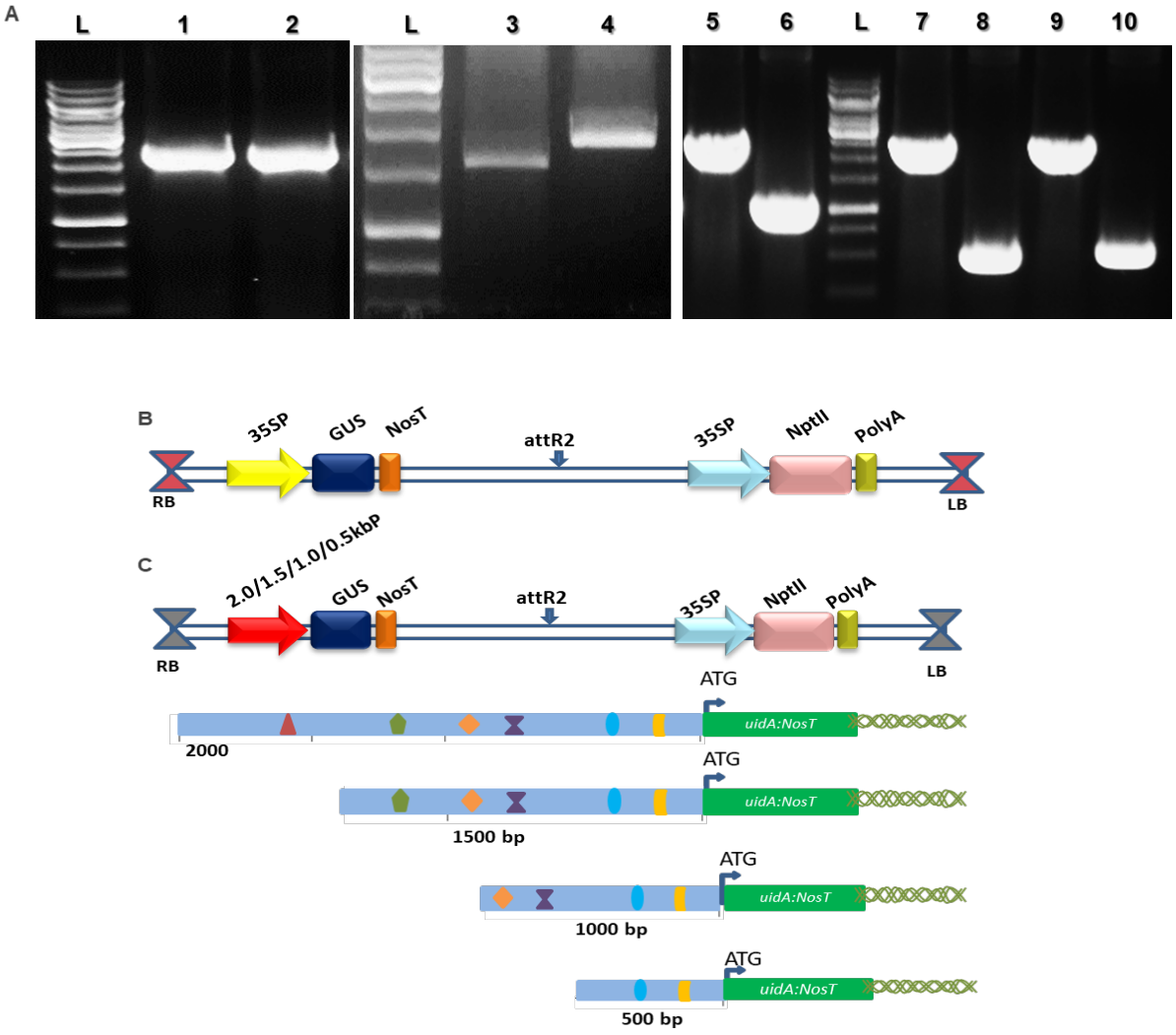


Figure 4.47: Construction of SmsHSP24.1 promoter:GUSNosT and CaMV35S promoter: GUSNosT plant expression vector. (A) PCR confirmation of the SmsHSP24.1 promoter:GUS NosT expression cassette in *Agrobacterium* strain EHA105 strain, Lane L represent 1kb ladder, Lane 1 represent 2.0kb amplified SmsHSP24.1 promoter region, Lane 2 represents amplified 2.0kb GUS portion, Lane 3 represent 1.5kb amplified SmsHSP24.1 promoter region, Lane 4 represent amplified 2.0kb GUS portion, Lane 5 represent 1.0kb amplified SmsHSP24.1 promoter region, Lane 6 represent amplified 2.0kb GUS portion, Lane 7 represent 0.5kb amplified SmsHSP24.1 promoter region, Lane 8 represent amplified 2.0kbGUS portion, Lane 9 represent amplified CaMV 35Spromoter region, Lane 10 represent amplified 2.0kbGUS portion. (B) Schematic diagram of CaMV35spromoter: GUSNosT expression cassette. (C) Schematic diagram of four 5′ deleted SmsHSP24.1 promoter:GUSNosT gene expression cassette in pMDC100.

GUS_F/NosT_R using a high-fidelity DNA polymerase (KOD plus, Toyobo, Japan). The PCR amplified GUS+Nos terminator portion was then cloned into previously used entry vector (pL12R34-Ap) already having all four 5′ deleted SmsHSP24.1 promoter regions. Using LR recombinase-mediated Gateway™ (Invitrogen, USA) cloning, the GUS F/NosT R amplified region was cloned between *Nco*I and *Xho*I restriction sites and subsequently placed into the plant expression vector pMDC100 having the *Npt*II selection marker gene in its backbone. Finally, the plant expression vector was introduced into the *Agrobacterium tumefaciens* EHA105 strain for transformation to the eggplant genome (Figure. 4.47 A). Using LR recombinase-mediated GatewayTM (Invitrogen, USA) cloning, the PCR-amplified GUS and Nos terminator from pCAMBIA1301 were directly cloned into the modified Entry clone 1 (pL12R34-Ap) under the CaMV35S promoter and Nos terminator between NcoI and XhoI restriction sites. This GUS expression vector was transferred into eggplant using the *Agrobacterium tumefaciens* EHA105 strain as the host cell (Figure. 4.47). For the construction of CaMV35S promoter:GUS expression vector, PCR amplified GUS and Nos terminator from pCAMBIA1301 was directly cloned into the entry vector 1 (pL12R34-Ap). Entry vector 1 (pL12R34-Ap) already had CaMV35S promoter thus we just placed our amplified product into it using the restriction enzymes *Nco*I and *Xho*I and then subsequently transferred it into pMDC100 vector. The backbone of pMDC100 vector already has plant selection marker gene kanamycin thus making it suitable for eggplant transformation. LR recombinase-mediated Gateway™ (Invitrogen, USA) cloning strategy was used to make all expression vectors. Finally, *Agrobacterium tumefaciens* EHA105 strain was used as a host cell for the transfer of this GUS expression vector into eggplant (Figure. 4.47).

4.4.3. Generation of SmsHSP24.1 promoter+GusNosT stable transgenic eggplant lines

For *Agrobacterium* mediated stable transformation, we used BARI Begun-4 Eggplant variety. *A. tumefaciens* has been employed to alter the genetic makeup of many plants due to its innate ability to do so. The SmsHSP24.1 promoter+Gus+NosT gene cassette harbouring vector was transformed into BARI Begun-4 through *Agrobacterium*-mediated transformation method. Our previously optimized protocol was extended for stable transformation and

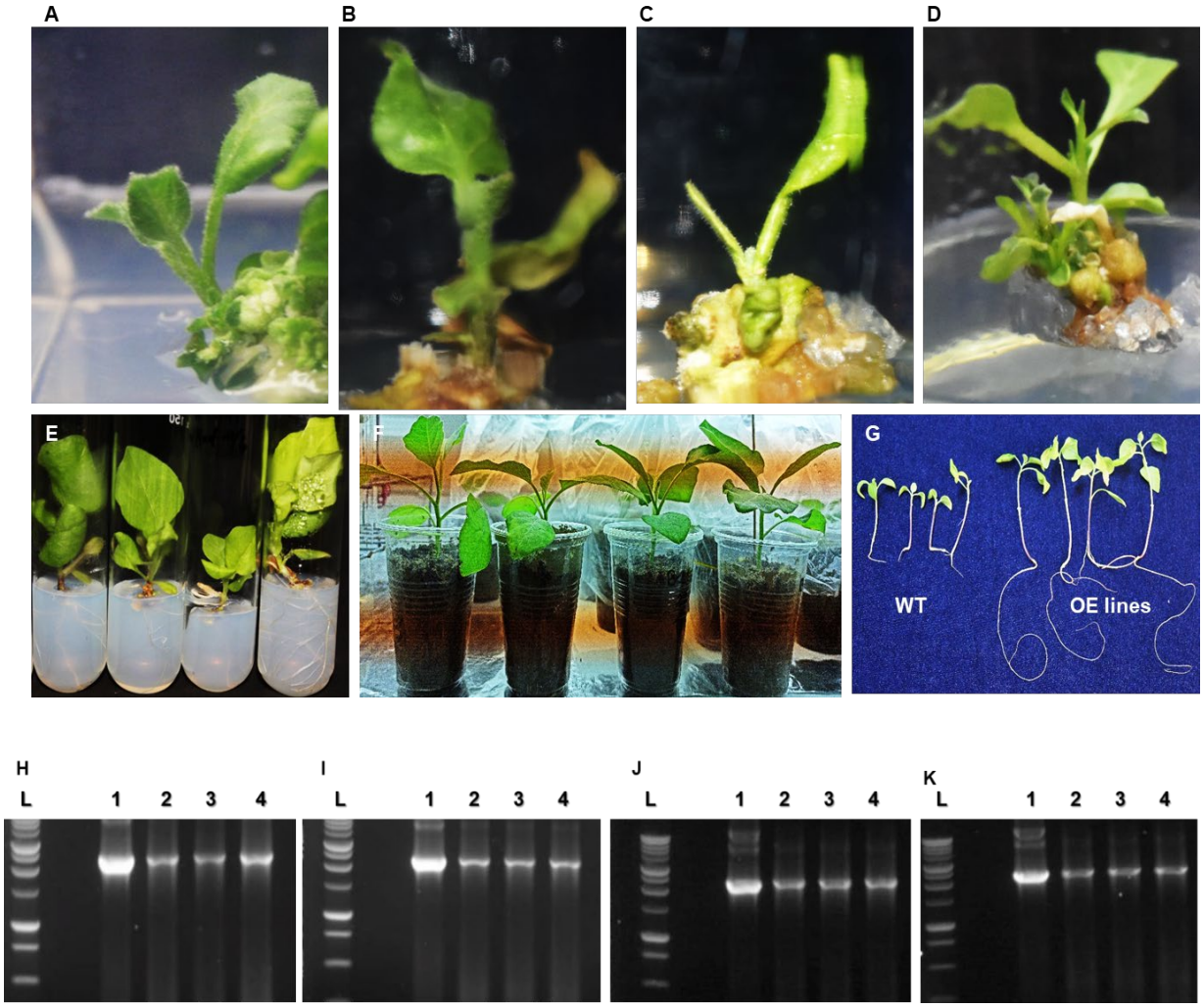
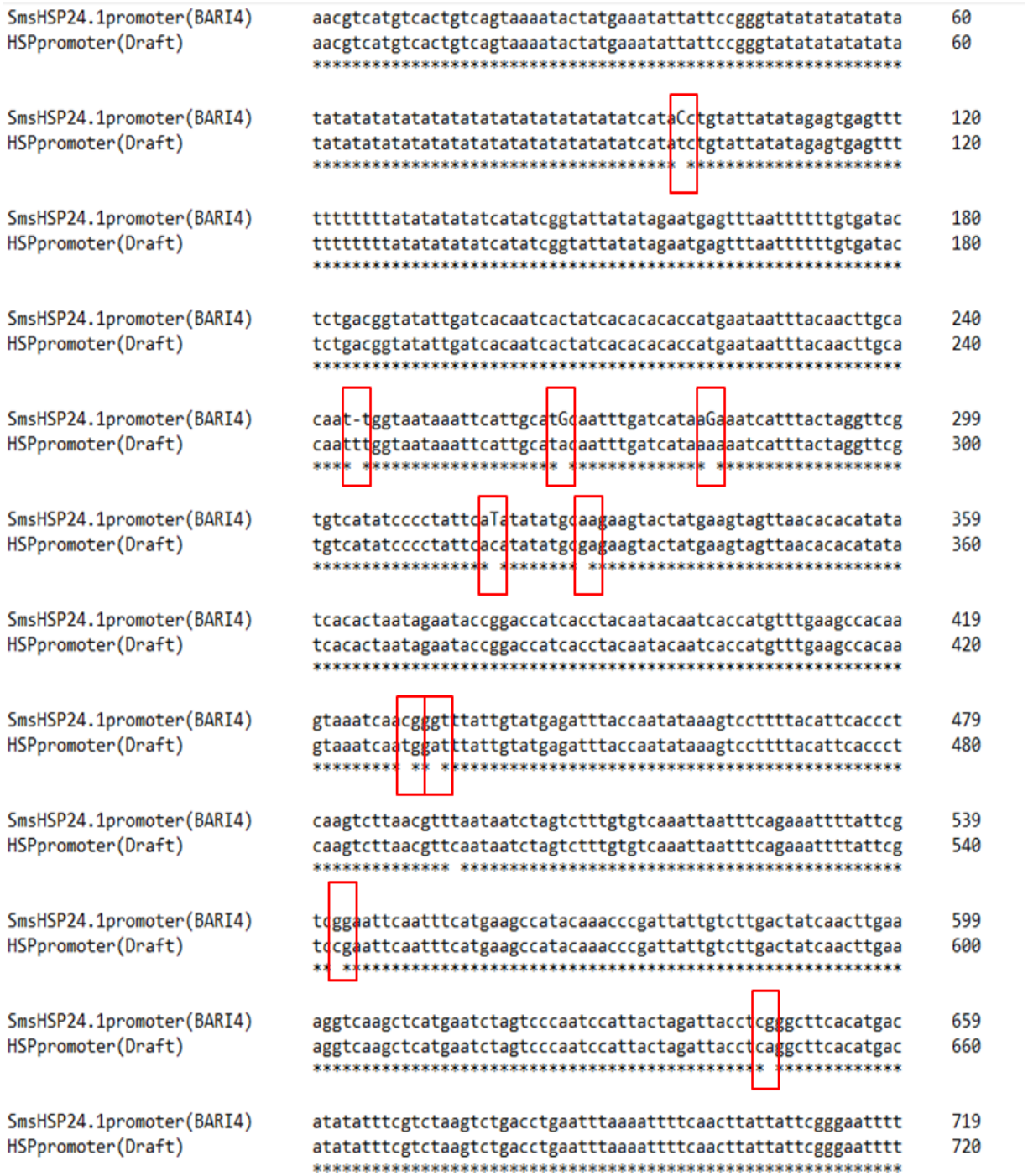


Figure 4.48: *Agrobacterium*-mediated transformation of SmsHSP24.1 promoter:GUSNosT expression vector into BARI Begun-4 Eggplant variety. (A) Shoot regeneration from construct having 2.0kb SmsHSP24.1 promoter:GUSNosT region. (B) Shoot regeneration from 1.5kb SmsHSP24.1 promoter:GUSNosT construct. (C) Shoot regeneration from 1.0kb construct. (D) Regenerated shoots from 0.5kb construct. (E) *In vitro* rooting of four 5′ deleted SmsHSP24.1 promoter:GUSNosT lines. (F) Acclimatization of four different putative transgenic lines. (G) Transgenic lines were selected under 150 mg/l kanamycin. (H-K) PCR amplification of Gus+NosT terminator from 5′ deleted *SmsHSP24.1* promoter::GUS fused transgenic eggplants lines, Lane L represents 1kb ladder, Lane 1-3 represents Gus+NosT amplified region from 2.0kb,1.5kb, 1.0kb,0.5kb SmsHSP24.1 promoter respectively.

regeneration. After successful transformation, we generated four different 5′ deleted SmsHSP24.1 promoter::GUS fused transgenic eggplants lines. PCR confirmation with two pairs of primers (kanamycin primer sets produced ~0.45 kb band length and Gus+NosT primer pair produced ~2.0 kb band length) confirmed the T_0 positive plants. Homozygous T_2 generation transgenic lines were selected under 150 mg/l kanamycin selection pressure and reconfirmed by PCR screening was selected for giving all abiotic stresses (Figure. 4.48 A-K).

4.4.4. Prediction of stress-responsive element in *SmsHSP24.1* promoter

Homology between the full-length promoter sequence (~2kb upstream sequence of SmsHSP24.1 protein) from genomic DNA of BARI begun 4 eggplant variety and draft mitochondrial small heat shock protein promoter sequence from database showed 22 mismatches and 3 gaps. This mismatch could be due to different inbred lines (BARI begun 4 Bangladeshi Eggplant variety) used in this study (Figure. 4.49). The full-length *SmsHSP24.1* promoter sequence was analyzed by New PLACE, Plantpan 2.0, and PlantCARE software and the result was shown in Figure. 4.50. It was found that TATA box was present in 2 different positions as box2, 3 different locations as box4, 7 different positions as box 5 and one box3. CAAT box were found at 37 different positions scattered in whole promoter. Many Cis-regulatory elements are predicted to be stress-responsive elements in this promoter. These include LTR (cis-regulatory element responsible for low-temperature interactiveness), TCA (cis-regulatory element associated with salicylic acid responsiveness), ABRE (ABA-responsive element), G-BOX (cis-regulatory element in light receptiveness), DRE core (dehydration responsive element binding), TGACG Motif (connected with MeJA responsiveness). The SmsHSP24.1 also contains motifs that are involved in stress-related regulation. These include CCAAT Box (drought response), HSF (Heat stress response), MYB recognition site (transcriptional regulation, drought response), STRE (stress regulator, Msn2p/4p binding domain), MADF:trihelix (stress response, pathogen response), MYB (drought response), MYCCONSENSUSAT (CBF/DREB1 transcription regulation in clod).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HSPpromoter(Draft) aacgtcatgtcactgtcagtaaaatactatgaaatattattccgggtatatatatatata 60
SmsHSP24.1promoter(BARI4) tatatatatatatatatatatatatatatatatcataCctgtattatatagagtgagttt 120
HSPpromoter(Draft) tatatatatatatatatatatatatatatatatcatatctgtattatatagagtgagttt 120
SmsHSP24.1promoter(BARI4) ttttttttatatatatatcatatcggtattatatagaatgagtttaatttttttgtgatac 180
HSPpromoter(Draft) ttttttttatatatatatcatatcggtattatatagaatgagtttaatttttttgtgatac 180
SmsHSP24.1promoter(BARI4) tctgacggtatattgatcacaatcactatcacacacaccatgaataatttacaacttgca 240
HSPpromoter(Draft) tctgacggtatattgatcacaatcactatcacacacaccatgaataatttacaacttgca 240
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HSPpromoter(Draft) tgtcatatcccctattcacatatatgcgagaagtactatgaagtagttaacacacatata 360
SmsHSP24.1promoter(BARI4) tcacactaatagaataccggaccatcacctacaatacaatcaccatgtttgaagccacaa 419
HSPpromoter(Draft) tcacactaatagaataccggaccatcacctacaatacaatcaccatgtttgaagccacaa 420
SmsHSP24.1promoter(BARI4) gtaaatcaacgggtttattgtatgagatttaccaatataaagtccttttacattcaccct 479
HSPpromoter(Draft) gtaaatcaatggatttattgtatgagatttaccaatataaagtccttttacattcaccct 480
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HSPpromoter(Draft) tccgaattcaatttcatgaagccatacaaacccgattattgtcttgactatcaacttgaa 600
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HSPpromoter(Draft) aggtcaagctcatgaatctagtcccaatccattactagattacctcaggcttcacatgac 660
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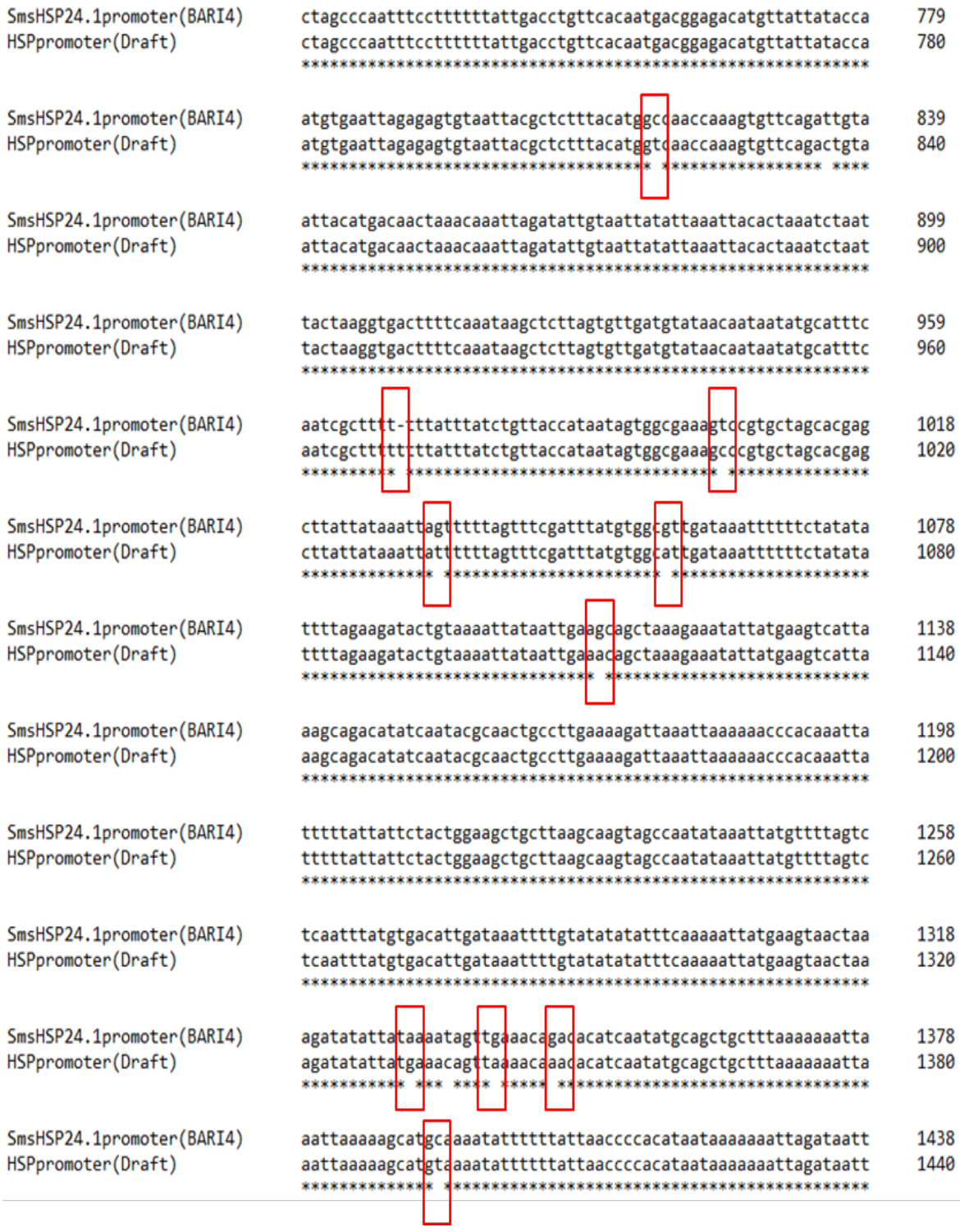
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HSPpromoter(Draft) ctagcccaatttccttttttattgacctgttcacaatgacggagacatgttattataccа 780
SmsHSP24.1promoter(BARI4) atgtgaattagagagtgtaattacgctctttacatggccaaccaaagtgttcagattgta 839
HSPpromoter(Draft) atgtgaattagagagtgtaattacgctctttacatggtcaaccaaagtgttcagactgta 840
SmsHSP24.1promoter(BARI4) attacatgacaactaaacaaattagatattgtaattatattaaattacactaaatctaat 899
HSPpromoter(Draft) attacatgacaactaaacaaattagatattgtaattatattaaattacactaaatctaat 900
SmsHSP24.1promoter(BARI4) tactaaggtgacttttcaaataagctcttagtgttgatgtataacaataatatgcatttc 959
HSPpromoter(Draft) tactaaggtgacttttcaaataagctcttagtgttgatgtataacaataatatgcatttc 960
SmsHSP24.1promoter(BARI4) aatcgctttt-tttatttatctgttaccataatagtggcgaaagtccgtgctagcacgag 1018
HSPpromoter(Draft) aatcgctttttttttatttatctgttaccataatagtggcgaaagcccgtgctagcacgag 1020
SmsHSP24.1promoter(BARI4) cttattataaattagttttagtttcgatttatgtggcgttgataaatttttttctatata 1078
HSPpromoter(Draft) cttattataaattatttttagtttcgatttatgtggcattgataaatttttttctatata 1080
SmsHSP24.1promoter(BARI4) ttttagaagatactgtaaaattataattgaagcagctaaagaaatattatgaagtcatta 1138
HSPpromoter(Draft) ttttagaagatactgtaaaattataattgaaacagctaaagaaatattatgaagtcatta 1140
SmsHSP24.1promoter(BARI4) aagcagacatatcaatacgcaactgccttgaaaagattaaattaaaaaacccacaaatta 1198
HSPpromoter(Draft) aagcagacatatcaatacgcaactgccttgaaaagattaaattaaaaaacccacaaatta 1200
SmsHSP24.1promoter(BARI4) tttttattattctactggaagctgcttaagcaagtagccaatataaattatgttttagtc 1258
HSPpromoter(Draft) tttttattattctactggaagctgcttaagcaagtagccaatataaattatgttttagtc 1260
SmsHSP24.1promoter(BARI4) tcaatttatgtgacattgataaattttgtatatatatttcaaaaattatgaagtaactaa 1318
HSPpromoter(Draft) tcaatttatgtgacattgataaattttgtatatatatttcaaaaattatgaagtaactaa 1320
SmsHSP24.1promoter(BARI4) agatatattataaaatagttgaaacagacacatcaatatgcagctgctttaaaaaaatta 1378
HSPpromoter(Draft) agatatattatgaaacagttaaaacaaacacatcaatatgcagctgctttaaaaaaatta 1380
SmsHSP24.1promoter(BARI4) aattaaaaagcatgcaaaatatttttttattaaccccacataataaaaaaattagataatt 1438
HSPpromoter(Draft) aattaaaaagcatgtaaaatatttttttattaaccccacataataaaaaaattagataatt 1440

Figure 4.49: Sequence alignment between the full length (2kb upstream of gene) SmsHSP24.1 promoter sequence from genomic DNA of BARI begun 4 eggplant variety and draft mitochondrial small heat shock protein promoter from the database.

In addition to the above-mentioned motifs, this promoter also has motifs that bind to stress-related transcription factors and enhances HSP protein production as well as growth promotion. For example, AT-Hook (flowering initiation, developmental process and stress response) is present in 154 different positions in this promoter. AP2; ERF (hormone and abiotic stress

regulator). NAM, NAC is a major transcription factor that regulated salt stress tolerance in plants are also present in SmHSP24.1. MBS is an MYB binding site that senses drought. WRKY proteins bind in W-Box motifs that regulate plant development and biotic and abiotic stress response. The deletion fragment of *SmsHSP24.1* promoter was constructed to identify the impact of the motifs under different abiotic stress. Four different deletion fragments were constructed denoted as 0.5 kb contains MYCCONSENSUSAT, G-Box, ABRE, DRE, AP2: ERF, MADF; Trihelix, MYB, STRE, HSF, CCAAT Box, AT-Hook, LTR, TCA-Element, 0.1 kb contains NAM; NAC, MBS motifs in addition to 0.5 kb's motifs, 1.5 kb contains Dof, TGACG motif, W-box motif in addition to 1.0 kb's motifs, 2.0 kb contains MYB Recognition site, MYC, MADF motifs in addition to 1.5kb's motifs. Therefore, this data revealed that *SmsHSP24.1* promoter contains a number of motifs that regulate HSP protein expression during heat, cold, salt and drought stress as well as under biotic stress with growth promotion activity. Among four different deletion fragments of *SmsHSP24.1* promoter, 0.5 kb portion has dense stress-related motifs to regulate HSP gene expression while 2.0 kb portion has all the necessary motifs to functionalize HSP gene when it is required to express (Table 4.5).

4.4.5. Histochemical staining of the transgenic Eggplant harbouring the 5′ deleted SmsHSP24.1 promoter sequence under different abiotic stress condition

We performed GUS histochemical staining assays on the transgenic eggplants harboring the 5′ deleted SmsHSP24.1 promoter:GUSNosT and CaMV35spromoter: GUSNosT expression constructs. Homozygous T_2 lines from each construct were selected under 150 mg/l kanamycin and reconfirmed by PCR screening was selected for giving various abiotic stresses to find out the functional regulatory motif present within the fragments of the SmsHSP24.1 promoter region. 3-week-old eggplant (*Solanum melongena* L.) seedlings treated with 45˚C heat, 150 mM mannitol as drought or osmotic stress, 200 mM Sodium chloride (NaCl) as salt stress (Figure. 4.51-52). The Histochemical staining of leaf tissues

***Solanum melongena* mitochondrial small heat shock protein (SmsHSP 24.1) promoter**

-1957 AACGTCATGTCACTGTCAGTAAAATACTATGAAATATTATTCCGGGTATATATATATATATATATATATATATATA

-1879 TATATATATATATATCATACCTGTATTATATAGAGTGAGTTTTTTTTTTTATATATATATCATATCGGTATTATATAGA

TGACG Motif, **MADF**

-1800 ATGAGTTTAATTTTTTGTGATACTCTGACG**GTATA**TTGATCACAATCACTATCACACACACCATGAATAATTTACAA C

MYC STRE

-1722 TTGCACAATTGGTAATAAATTCATTGCATGCAATTTGATCATAAGAAATCATTTACTAGGTTCGTGTCATATCCCCTA

-1644 TTCATATATATGCAAGAAGTACTATGAAGTAGTTAACACACATATATCACACTAATAGAATACCGGACCATCACCTA

CCAAT Box, MYB Recognition Site

-1567 CAATACAATCACCATGTTTGAAGCCACAAGTAAATCAACGGGTTTATTGTATGAGATTTACCAATATAAAGTCCTTT

AT-Hook HSF

-1490 TACATTCACCCTCAAGTCTTAACGTTTAATAATCTAGTCTTTGTGTCAAATTAATTTCAGAAATTTTATTCGTCGGAA

W-Box

-1412 TTCAATTTCATGAAGCCATACAAACCCGATTATTGTCTTGACTATCAACTTGAAAGGTCAAGCTCATGAATCTAGTC

MYB

-1335 CCAATCCATTACTAGATTACCTCGGGCTTCACATGACATATATTTCGTCTAAGTCTGACCTGAATTTAAAATTTTCAA

W-Box TGACG Motif

-1257 CTTATTATTCGGGAATTTTCTAGCCCAATTTCCTTTTTTATTGACCTGTTCACAATGACGGAGACATGTTATTATACC

MYB

-1179 AATGTGAATTAGAGAGTGTAATTACGCTCTTTACATGGCCAACCAAAGTGTTCAGATTGTAATTACATGACAACTA

Dof

-1103 AACAAATTAGATATTGTAATTATATTAAATTACACTAAATCTAATTACTAAGGTGACTTTTCAAATAAGCTCTTAGTG

-1025 TTGATGTATAACAATAATATGCATTTCAATCGCTTTTTTTATTTATCTGTTACCATAATAGTGGCGAAAGTCCGTGCT

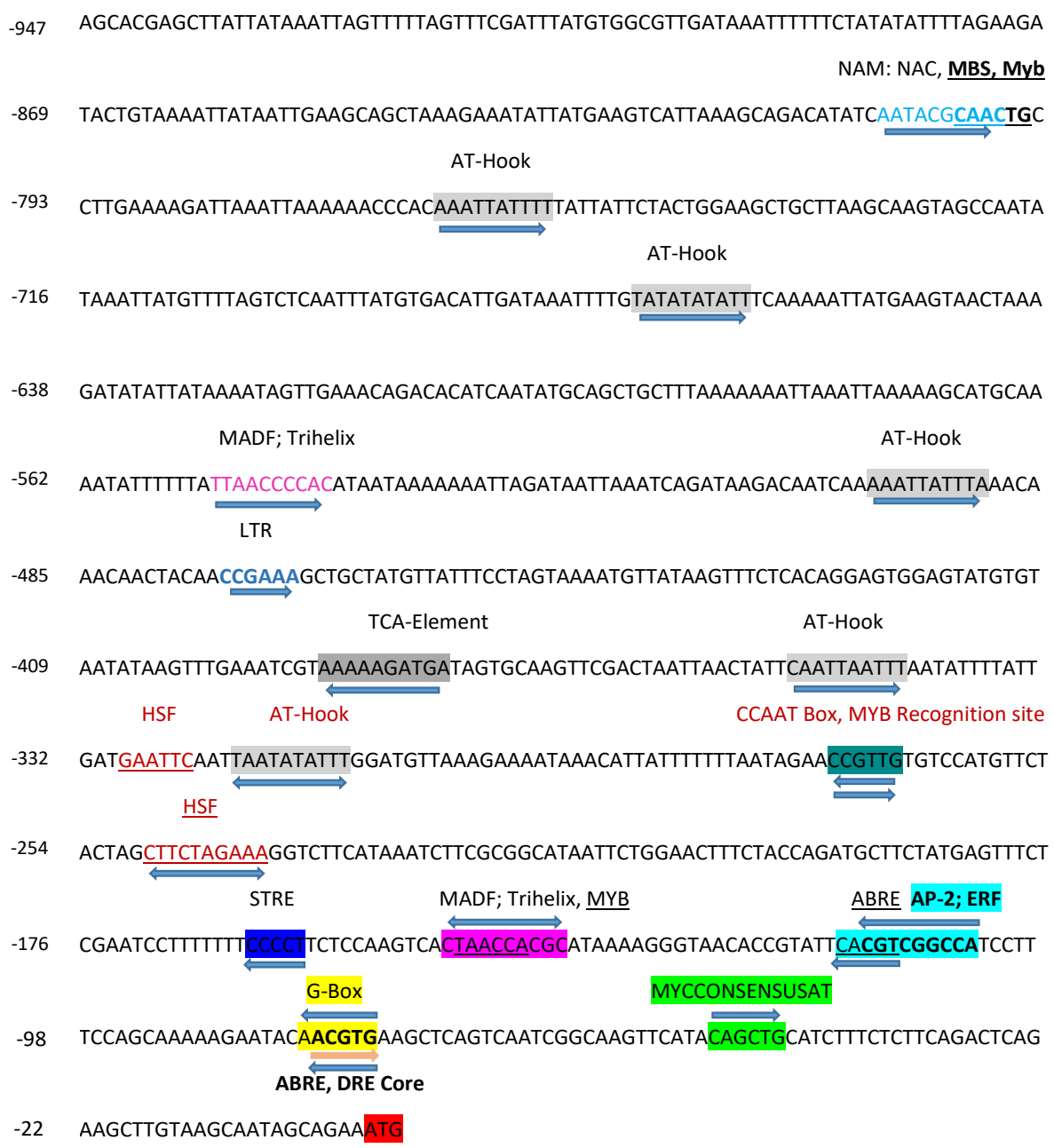


Figure 4.50: cis-regulatory motifs on the promoter of SmsHSP24.1 are shown in a picture. Various cis-regulatory motifs are highlighted in color and placed upstream of the translation start point on the promoter sequence.

Table 4.5: Prediction of stress-responsive element in SmsHSP24.1 promoter.

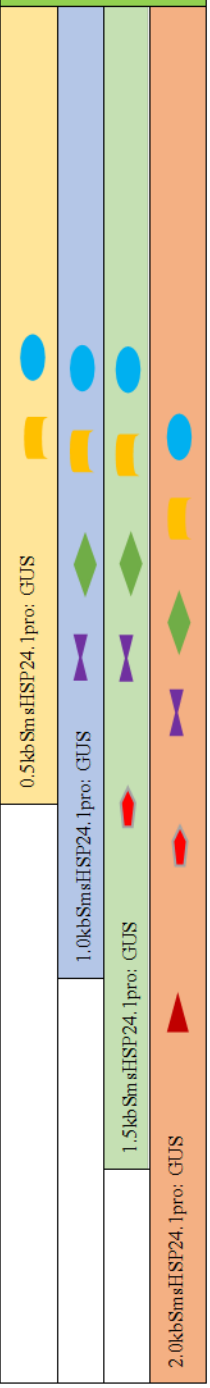
Fragment	Motif Sequence	Motif Name	Function Annotation	Function Overlap
0.5 kb	aaattattta	AT-Hook	Flowering initiation, developmental processes and stress responses	
	ccgaaa	LTR	cis-acting element involved in low-temperature responsiveness	
	gaattc	HSF	Heat stress response	
	aaaaagatga	TCA Element	cis-acting element involved in salicylic acid responsiveness	
	ccgttg	CCAAT Box	MYBHv1 binding site	
	ccgttg	MYB Recognition Site	Transcription regulator, Drought response	
	cttctagaaa	HSF	Heat Stress Transcription Factor	
	cccct	STRE	Stress Regulator, Msn2p/4p binding domain	
	ctaaccacgc	MADF; trihelix	Stress Response, Pathogen Response	
	taacca	MYB	Transcription regulator, Drought response	
	cacgt	ABRE	cis-acting element involved in the abscisic acid responsiveness	
	aacgtg	G-BOX	cis-acting regulatory element involved in light responsiveness	
	acgtg	DRE Core	Dehydration Responsive Element Binding	
	cgtcggcca	AP-2; ERF	Hormone and abiotic stress regulator	
	cagctg	MYCCONSENSUSAT	Regulates the transcription of CBF/DREB1 genes in the cold in Arabidopsis	
1.0 kb	aatacgcaac	NAM, NAC	Abiotic Stress regulation (salt)	
	caactg	Myb	Transcription Regulator, drought-inducibility	
	ttaaccccac	MADF; trihelix	Stress Response, Pathogen Response	
	caactg	MBS	MYB binding site involved in drought-inducibility	
1.5 kb	caactg	DOF	Plant Development, Biotic and Abiotic Stress Response	
	tgacg	TGACG Motif	cis-acting regulatory element involved in the MeJA-responsiveness	
	ggtcaa	W-Box	Plant Development, Biotic and Abiotic Stress Response	
2.0 kb	gaattc	HSF	Heat Stress Transcription Factor	
	caacgg	CCAAT Box	MYBHv1 binding site, drought-inducibility	
	cccct	STRE	Stress Regulator, Msn2p/4p binding domain	
	acggtata	MADF	Stress Response, Pathogen Response	

Heat, Drought and Cold Stress Response

Salt and Drought + Heat, Drought and Cold Stress Response

Biotic and Abiotic Stress Response + Salt and Drought + Heat, Drought and Cold Stress Response

Regulation, Heat stress + Biotic and Abiotic Stress Response + Salt and Drought + Heat, Drought and Cold Stress Response



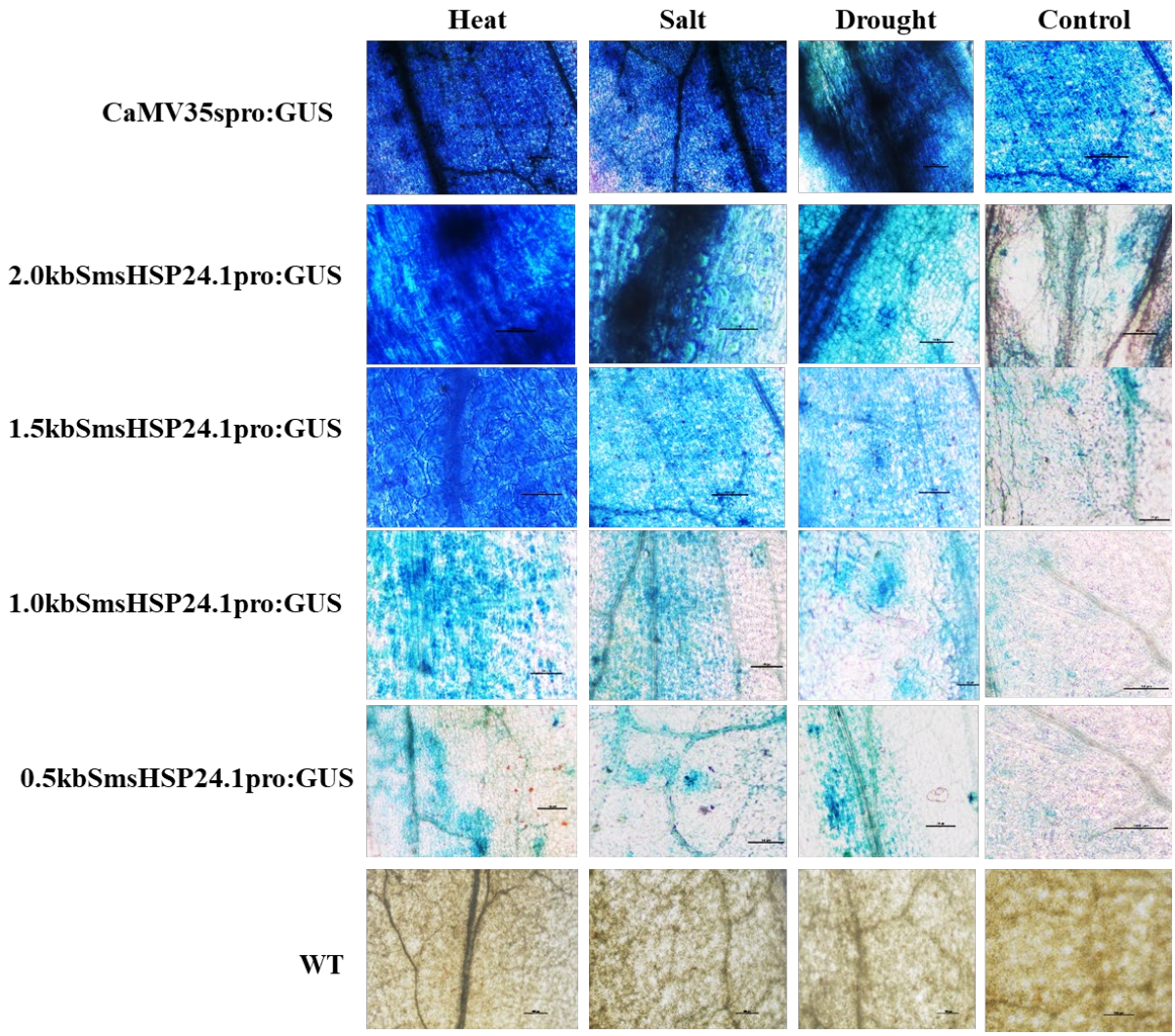


Figure 4.51: Histochemical staining on the transgenic eggplants under different abiotic stress conditions. Leaf tissues of transgenic plant harboring full length 2.0kb SmsHSP24.1 promoter:GUSNosT and CaMV35spromoter+GUSNosT expression cassette showed strong GUS expression under heat, salt and drought stress conditions. GUS expression was slightly seen in all the transgenic lines when kept in at controlled environment. While GUS activity was not detected in leaf tissues of the WT plants in control and stress conditions. The highest GUS expression was detected in 2.0kb SmsHSP24.1 promoter under heat stress.

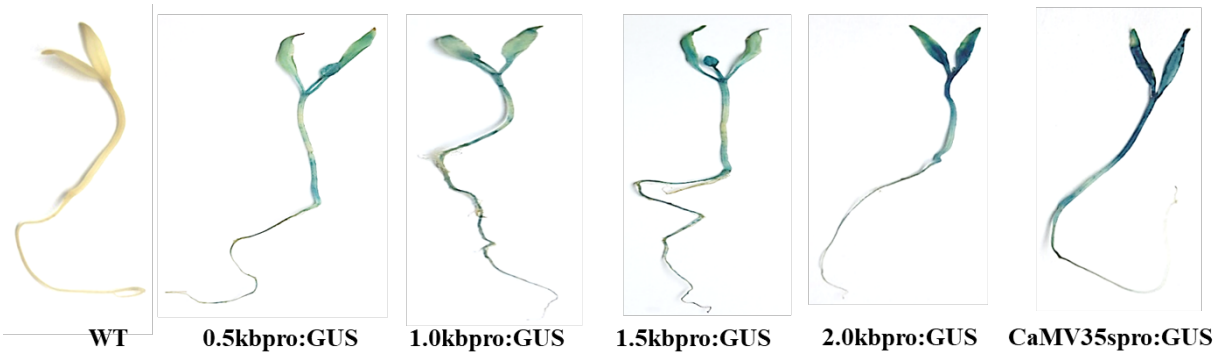


Figure 4.52: Histochemical staining on the 21 days old transgenic eggplant seedlings under heat stress conditions. Full-length SmsHSP24.1 promoter (~2kb upstream sequence of SmsHSP24.1 protein) is more responsive against abiotic stress compared to other deletion fragments.

and 21 days old transgenic eggplant seedlings harboring full-length 2.0kb SmsHSP24.1 promoter:GUSNosT expression cassette showed strong GUS expression under heat stress and exposed the function of this promoter in an inducible way (Figure 4.51-52). Whereas GUS expression was gradually declined in the 5′ deleted fragment of SmsHSP24.1 promoter harboring transgenic plants under heat, salt or even drought stress condition. The histochemical staining of leaf tissues of CaMV35spromoter+GUSNosT expression cassette harboring transgenic plants showed strong GUS expression under all heat, salt and drought stress conditions while GUS activity was not detected in leaf tissues of the WT plants.

Discussion:

How a plant grows, develops, and adapts is governed by factors such as gene expression, promoters, the cis-acting elements that start transcription, and others. A recent development in agricultural genetic improvement is transgenic technology. The usage of well-characterized promoters is essential for developing a vector system for efficient genetic transformation and for understanding the function of the gene. Studies on gene function in transgenic plants require strategies to accurately control the expression of candidate genes. Experimental control of transgene expression is normally achieved by constructing a gene cassette linking the coding sequence to a promoter that shows a predetermined pattern and level of expression in the animal to be transformed. The 35S and 34S promoters from the cauliflower mosaic virus and the figwort mosaic virus, respectively, as well as promoters of plant genes that are constitutively expressed, like actin and ubiquitin, are frequently used promoters that give relatively strong expression in most tissues throughout development (Potenza et al., 2004). Moreover, several promoters from genes whose regulation is typically tissue-specific have been used in transformation research. These promoters provide transgenic expression patterns that are repeatable in designated cell types, but they often cannot be modified afterwards. Strong tissue-specific promoters that are expressed only in vascular tissues (Liu & Jia, 2003), anthers (Kato et al., 2010), or seed tissues are a few examples of those that have been utilized to drive plant transgenes (Furtado et al., 2008; Jones et al., 2005; Qu & Takaiwa, 2004). Moreover, it may one day be possible to create synthetic, customized tissue-specific promoters (Venter, 2007). A helpful research tool is a capacity to stimulate de novo transgene expression at particular phases of plant growth or in chosen tissues in the same transgenic plant. When constitutive up-or down-regulation of the target gene has negative effects on plant growth and development, when constitutive expression requires excessive amounts of energy or nutrients, or when the effects of the transgene will be compared across various tissues or growth stages of the same plant, inducible promoters are especially crucial. In transgenic applications, a number of inducible promoters have been described, including those that are triggered by the administration of certain chemicals or stimuli including heat, drought, mechanical injury, and light (Argüello-Astorga & Herrera-Estrella, 1998; Corrado & Karali, 2009; Moore et al., 2006; Padidam et al., 2003). Constitutive promoters are frequently employed to functionally describe plant genes in transgenic plants; however, they might have

significant drawbacks due to their lack of specificity and poor control over protein production. Yet, by directing overexpression or knockdown of the target genes to certain tissues and/or during particular developmental phases, promoters that offer precise regulation of temporal or spatial transgenic expression help such research.

Our previous studies on the expression analysis of the SmsHSP24.1 protein unambiguously showed that this protein is sensitive to a variety of environmental conditions, including those caused by heat, salt, and mannitol. Its transcript level has rapidly increased, which indicates that the SmsHSP24.1 protein has begun to react to stressful situations. Overexpression of SmsHSP24.1 in BARI Begun 4 also displayed significant abiotic stress tolerance in both seedling and reproductive stages. To deeply understand the signalling and regulation of SmsHSP24.1 protein, we constructed four deletion constructs of the *SmsHSP24.1* promoter fused with GUS and overexpressed it again in BARI begun 4. Indicators of a promoter's role in stress and developmental processes may be found in the patterns of gene expression. In BARI begun 4, GUS gene expression was driven by *SmsHSP24.1*_promoter and observed in leaf tissue as maximum as well as other tissue. CaMV35spromoter+GUSNosT expression cassette was included to characterize the maximum level of GUS expression in transgenic plants compared to the control. A gradual decrease of GUS activity was observed for 2.0kbSmsHSP24.1 promoter:GUSNosT to 0.5kbSmsHSP24.1 promoter:GUSNosT compared to CaMV35spromoter+GUSNosT. Under heat stress, 2.0kbSmsHSP24.1 promoter:GUSNosT showed maximum GUS expression as region of 2kb promoter contains three HSF (Heat Stress Transcription Factor) binding motifs. 1.5kb, 1.0 kb and 0.5kb promoters have two HSF but still decrease of GUS expression was observed due to less amount to other abiotic responsive element presence in 1.5kb to 0.5kb promoter and there is a possible networking interplay. The same notion can be drawn for the salt, cold and drought stress response in terms of GUS expression pattern as 2.0kb to 1.0kb have major salt responsive elements (NAM, NAC, Dof, W-box and STRE) with a drop of GUS expression in 0.5 kb because of their absence. 2.0 kb has CCAAT box responsible for drought inducibility is absent in 1.5kb and 1.0 kb has Myb (responsible for drought inducibility) missing in 0.5kb. GUS expression is significantly decreased from 2.0kb to 0.5kb because of the loss of motifs that are required for promoter activation during stress. In this study, we also predict the presence of biotic responsible

element in the SmsHSP24.1 making this promoter more significant. Presence of MADF; trihelix, DOF. TGACG Motif, W-Box, MADF motifs make it a pathogen-responsive promoter. TGACG motif is a cis-acting regulatory element involved in the MeJA-responsiveness and Methyl Jasmonate has been reported to be a powerful elicitor of numerous defence mechanisms in response to pathogen-mediated wounding. Our experiment data found that full-length *SmsHSP24.1* promoter (~2kb upstream sequence of SmsHSP24.1 protein) is more responsive against abiotic stress compared to other deletion fragments. As it is mentioned in (Table 4.5), 2.0kb promoter has a maximum number of motifs that are responsible for binding to regulatory molecules during stress to drive the SmsHSP24.1 gene expression. GUS expression analysis also confirmed that despite of hovering all necessary regulatory elements in 0.5kb promoter for stress response, overexpressing any gene under 2.0 kb SmsHSP24.1 promoter is the best choice as it can maximize the gene response. All of the motifs present in *SmsHSP24.1* promoter strongly justify its expression profile observed in GUS assay and signify its inducible expression in the transgenic line. Thus, this promoter could be used for crop improvement programs and discover the function of any unidentified gene.

5. Summary:

Despite being widely present and closely related to protein homeostasis and survival under stress, small HSPs (sHSPs) are one of the HSP subgroups that have remained elusive and understudied. In three areas of life, there are a total of 12 diverse subfamilies of sHSP. All organisms have sHSPs, yet just the plant lineage has evolved mitochondrial localized subfamilies (Waters and Vierling, 2020) except in the instance of Drosophila HSP22 (Morrow et al., 2000). Furthermore, heat-stressed plants accumulate significantly more mitochondrial sHSPs than HSP60 and HSP70, and this accumulation coincides with an improvement in plant heat tolerance, suggesting that mitochondrial sHSPs play a significant role in plant heat tolerance. However, not much is known regarding the function of mitochondria-localized sHSPs and their potential impact on plant growth and development. In this study, we investigated the physiological functions of a small HSP (SmsHSP24.1) that were deliberately targeted to mitochondria as well as its protective function in stressful situations from a unique source, the Eggplant. We have created transgenic SmsHSP24.1 overexpression and SmsHSP24.1 knockdown Eggplant lines to better understand the effects of the SmsHSP24.1 protein in both normal and stressful environments. Under both normal and stressful circumstances, its impact on growth and development has been thoroughly explored. In particular, early germination and seedling vigour in transgenic lines show considerable modification due to global reprogramming in critical biological pathways. We have cloned SmsHSP24.1 promoter fused with GUS reporter gene to understand the signalling and regulation of SmsHSP24.1 protein. Various stress-related *cis*-regulatory elements present in the full-length SmsHSP24.1 promoter (~2kb upstream sequence of SmsHSP24.1 protein) strongly justify its expression profile observed in GUS assay and signify its inducible expression in the transgenic line. CRISPR/Cas9 mediated knocked out of SmsHSP24.1 protein also indicated the essential role of SmsHSP24.1 in Eggplant. All of our findings indicated an entirely novel source of the mitochondria-localized small heat shock protein (SmsHSP24.1), which could potentially be useful for developing crops that are climate-resilient in the future. Our results are summarized below.

1. The full-length cDNA sequence for *SmsHSP24.1* was amplified by nested PCR from heat-treated Eggplant (*Solanum melongena* L.) leaves. The amplified fragment comprised an open reading frame (ORF) with a length of 636 base pairs

(bp) that encodes a protein with an apparent molecular mass of 24.1 kDa. As a consequence, we designated our protein name SmsHSP24.1 and uploaded it to the NCBI gene bank (MF 579857.1). Multiple sequence alignment, homology modelling, and blastp homology search demonstrated that SmsHSP24.1 contained a conserved 81-amino acid α-crystalline domain (ACD) near the C-terminus (positions +115 to +196).

2. A time-dependent quantitative RT-PCR analysis of SmsHSP24.1 protein during heat stress was higher than it was under salinity or drought treatment. Expression profiling of SmsHSP24.1 clearly revealed the imperative and persistent role of SmsHSP24.1 towards abiotic stresses adaptation in Eggplant.
3. Transient expression assay of *Agrobacterium* harbouring *SmsHSP24.1* fused with mGFP on tobacco leaf (3-week-old seedlings) indicated discreet localization other than the nucleus. Furthermore, co-localization analysis with mitochondria-specific dye in Eggplant cell suspension culture conclusively confirmed that this novel SmsHSP24.1 protein targeted to the mitochondria.
4. Heterologous expression and biochemical characterization of SmsHSP24.1 protein proved that the SmsHSP24.1 protein enhances the thermo-tolerance of *E. coli* cells under severe heat stress (55°C for one hour). Electron microscopy study suggests the formation of the polyhedral structure of SmsHSP24.1 acts as a molecular chaperon by binding with its client protein at a higher temperature of 50 ºC. SmsHSP24.1 protein prevents the thermal aggregation of restriction enzyme *Nde*I and helps in retaining their biological function.
5. The stable integration of transgene in T_0 and T_1 generation plants was confirmed by PCR and Southern blot analysis. We obtained 2 single-copy, 2 double-copy, one triple copy and one multi-copy (containing 5 copy insertions) transgenic events. Transgene integration was further validated by expression at the transcript level by RT-PCR.
6. SmsHSP24.1 overexpressed Eggplant lines exhibit early seed germination, seedling vigour and improved tolerance to heat, salt and drought stresses. It was also observed that the SmsHSP24.1 over-expressing lines possess improved photosynthetic capacity, higher fresh weight, dry weight, root length, shoot length and chlorophyll content.

7. Overexpressing lines keeps cellular ROS levels stable under both normal and stressful circumstances. Leaf disc assay showed slow chlorosis in SmsHSP24.1 overexpressing lines compared to WT plants after salt (200 mM NaCl), heat (45 ºC for 36 hr), drought (150 mM mannitol), and MV (10µM) stress treatments.
8. Growth performance of the SmsHSP24.1 transgenic plants in response to combine stress under field conditions revealed that transgenic lines showed better growth in terms of shoot and root length, and total biomass. Transgenic lines recovered quickly without any effect on flowering and fruit setting after withdrawal of stress whereas the WT plants were completely retarded.
9. CRISPR/Cas9 expression vector was constructed successfully which targeted to knock out a native copy of *SmsHSP24.1* gene in Eggplant genome. CRISPR/Cas9 expression cassette successfully transferred to Eggplant genome through *Agrobacterium*-mediated transformation. Transformation efficiency was severely retarded during the transformation process. Again, copy number analysis proved that only a single copy of *SmsHSP24.1* gene is present in Eggplant genome which might be essential for Eggplant growth and survivability.
10. 5′ promoter regions were deleted of different lengths (2.0kb, 1.5kb,1.0kb,0.5kb) and fused with the uidA (beta-glucuronidase, GUS) reporter gene to produce the promoter:GUS vector. Various stress-related putative cis-regulatory elements were also identified in the promoter region of *SmsHSP24.1* gene. GUS fused SmsHSP24.1 promoter region successfully transferred to Eggplant genome through *Agrobacterium*-mediated transformation. Histochemical staining on leaf tissues of 21 days old transgenic Eggplant seedlings harbouring full-length 2.0kb SmsHSP24.1 promoter:GUSNosT expression cassette showed strong GUS expression under heat stress and exposed the function of this promoter in an inducible way. This promoter could be used for crop improvement programs and discover the function of any unidentified gene.

5.1. Conclusion:

Finally, in Eggplant, we discovered a novel SmsHSP24.1 protein that is localized to the mitochondria and characterized this protein under both normal and stressful circumstances. Rapid germination and seedling vigor were also noted whereas overexpression of SmsHSP24.1 improved abiotic stress resistance, particularly in temperatures up to 45°C in the field conditions.

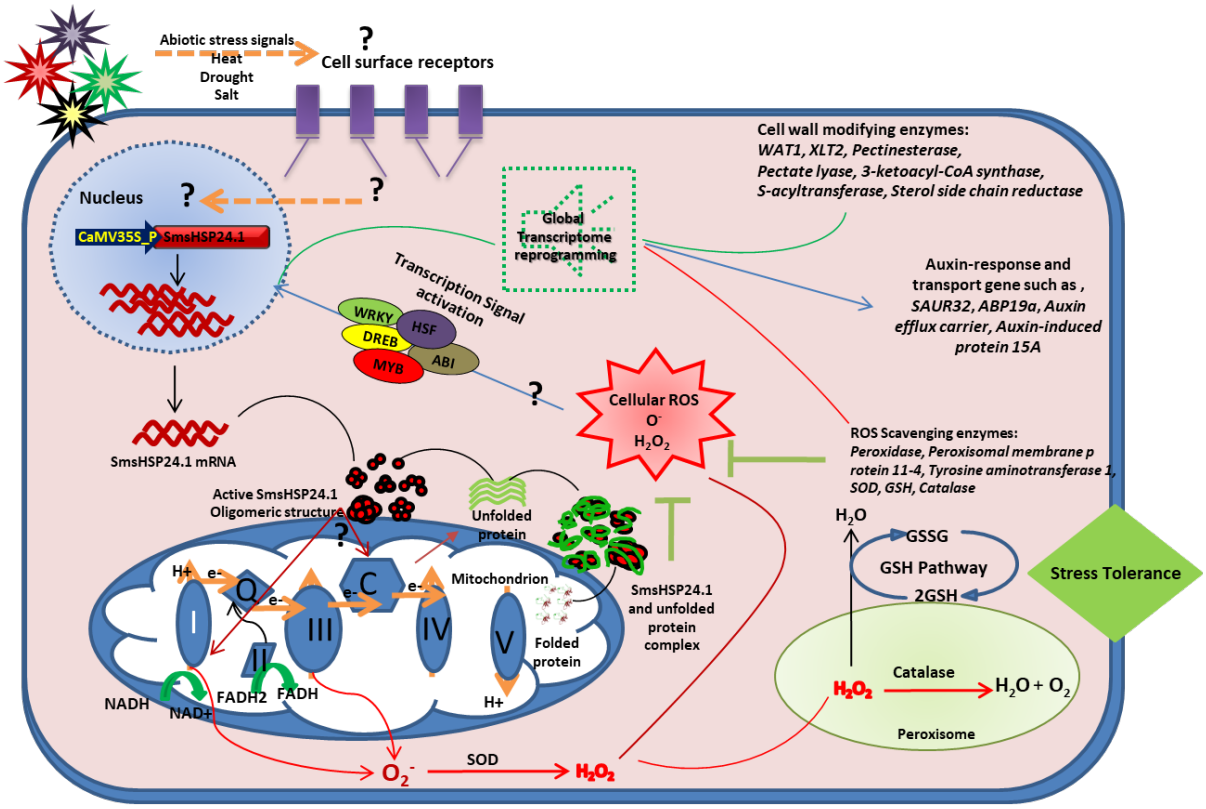


Figure 4.53: A hypothetical paradigm for conceptualizing the SmsHSP24.1-mediated plant response to abiotic stress. Increased ROS may be caused by interactions between SmsHSP24.1 overexpression and the mitochondrial electron transport chain (ETC). The glutathione (GHS) pathway contains a number of reactive oxygen species (ROS) scavenging enzymes that are upregulated and keeps ROS levels below the lethal dose, which may act as a transcriptional activation signal for a number of stress-induced transcription factors and ultimately change global transcription.

Overexpression also led to an altered ROS gradient and transcriptional reprogramming of critical biological pathways. Copy number analysis proved that only a single copy of *SmsHSP24.1* gene is present in Eggplant genome. Full-length small heat shock protein promoter analysis revealed the existence of several stress-responsive cis-regulatory regions, demonstrating the promoter's ability to act in an induced manner. So, based on our research, we put forth a theory regarding the potential mechanism of SmsHSP24.1 overexpression and its effects on ROS regulation and global transcriptome reprogramming in transgenic Eggplant lines compared to WT (Figure 4.53). Further research into targeted cytosolic or mitochondrial protein profiling under both normal and stressful circumstances is interesting. (Escobar et al., 2021) recent research has demonstrated that mitochondria-localized sHSPs control cellular homeostasis by coordinating between several subcellular compartments, particularly between plastids, cytosol, and mitochondria in *Arabidopsis*. In order to comprehend the regulatory mechanism of mitochondria-localized small HSPs in plant growth and development, comparative studies of protein profiling in OE lines against WT plants will be important in subsequent lines of research. The results of the current study also revealed that mitochondrial sHSPs gave plants an evolutionary advantage that may be used to create crop plants that are resistant to different abiotic stresses.

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7. Publications:

i. **Mst. Muslima Khatun**, Bhabesh Borphukan, Iftekhar Alam, C. A. Keya, Haseena Khan, M K Reddy and Md Salimullah (2022). An Improved Agrobacterium Mediated Transformation and Regeneration Protocol for Successful Genetic Engineering and CRISPR/Cas9 Based Genome Editing in Eggplant. Scientia Horticulturae 293. doi.org/10.1016/j.scienta.2021.110716

ii. **Mst. Muslima Khatun,** Bhabesh Borphukan, Iftekhar Alam, C. A. Keya, Saaimatul Huq, V. Panditi, Haseena Khan, M K Reddy and Md Salimullah (2021). Mitochondria-targeted novel SmsHSP24.1 overexpression stimulates early seedling vigour and stress tolerance by multi-pathway transcriptome-reprogramming. Front. Plant Sci. 12:741898.

iii. **Mst. Muslima Khatun**, Bhabesh Borphukan, Imran Khan, Mohammad Riazul Islam and Md Salimullah (2023). Beyond the Dark Side: Revisiting the Significance of Reactive Oxygen Species (ROS) in Plant Growth and Development. (Manuscript under preparation)

iv. **Mst. Muslima Khatun**, Imran Khan, Bhabesh Borphukan, Md Salimullah and Mohammad Riazul Islam (2023). Functional characterization of SmsHSP24.1 promoter in response to abiotic stresses by deletion analysis in transgenic Eggplant. (Manuscript under preparation).

v. **Mst. Muslima Khatun**, Bhabesh Borphukan, Imran Khan, Md Salimullah and Mohammad Riazul Islam (2023). CRISPR/Cas9 mediated knockout reveals the indispensable role of SmsHSP24.1 protein for Eggplant growth and survivability. (Manuscript under preparation)

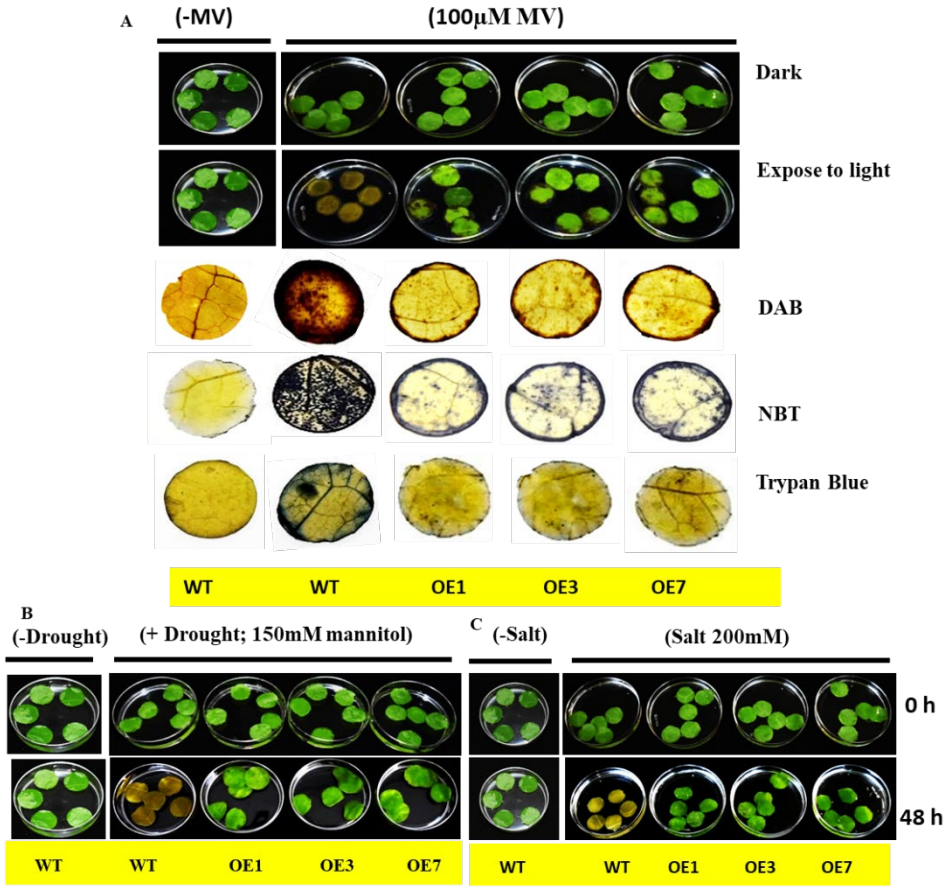
Abstract/Poster presentation

i. **Mst. Muslima Khatun**, Bhabesh Borphukan, Iftekhar Alam, Haseena Khan, Md Salimullah, M K Reddy, (2019). Understanding the role of SmsHSP24.1, a novel mitochondrial small heat shock protein and engineering multi-stress resistance crop plants. Sensing and signaling in pant stress response, India-EMBO symposium, 15th-17th April 2019 New Delhi, India.

ii. **Mst. Muslima Khatun**, Bhabesh Borphukan, Iftekhar Alam, Haseena Khan, Md Salimullah, M K Reddy, (2019). Engineering novel SmsHSP24.1 gene for multi-stress resistance crop plant. International Conference on Plant stress biology and food security, 18-20th April, 2019 2019, ICGEB, New Delhi, India.

iii. **Mst. Muslima Khatun,** Bhabesh Borphukan, Iftekhar Alam, C. A. Keya, Saaimatul Huq, V. Panditi, Haseena Khan, M K Reddy and Md Salimullah. (2021). Mitochondria-targeted SmsHSP24.1 overexpression stimulates early seedling vigour and stress tolerance by multi-pathway transcriptome-reprogramming. 16th FAOBMB congress Christchurch, New Zealand.

Appendices



Fingure: Assessment of cellular damage of transgenic eggplants lines overexpressing SmsHSP24.1 protein in response to methyl viologen (MV), 200 mM NaCl and 150 mM mannitol induced oxidative stress.

(A) Leaf disc were pre-incubated with 10 µM methyl viologen (MV; paraquat) and then exposed to sunlight subsequently relative H_2O_2 and O^{2-} accumulation and death cells were assessed by histochemical staining. (B,C) Leaf disc were incubated for 48 hours in 200 mM NaCl as salt and 150 mM Mannitol as drought stress.

- **Table: Effects of different concentration of IBA, IAA and NAA on root induction from regenerated shoots of *Solanum melongena* L.**

Plant growth regulator (mg/L)	Days require for root induction	Number of roots per shoot	Average root lengths (cm)
BARI begun 4			
IBA			
0.2	12.08±0.19^{d}	3.16±0.11^{g}	4.91±0.19^{f}
0.5	11.16±0.16^{h}	4.41±0.14^{b}	6.25±0.17^{b}
1.0	9.08±0.08^{j}	6.66±0.14^{a}	6.66±0.14^{a}
1.5	11.75±0.17^{f}	4.08±0.08^{d}	5.08±0.08^{e}
IAA			
0.2	14.16±0.11^{a}	2.58±0.14^{j}	3.58±0.14^{j}
0.5	13.91±0.08^{b}	3.08±0.19^{h}	4.75±0.21^{g}
1.0	11.75±0.13^{f}	3.41±0.14^{f}	5.08±0.22^{e}
1.5	12.08±0.19^{d}	2.83±0.11^{i}	4.33±0.22^{h}
NAA			
0.2	12.41±0.14^{c}	3.08±0.08^{h}	3.91±0.08^{i}
0.5	11.58±0.14^{g}	3.41±0.14^{f}	5.66±0.14^{c}
1.0	10.08±0.08^{i}	4.16±0.16^{c}	5.91±0.08^{d}
1.5	11.83±0.16^{e}	3.75±0.13^{e}	4.91±0.08^{f}

The results are expressed as mean±standard error. Each hormone treatment contained 12 shoots. At least three independent experiments were performed. Values with the different letters indicate statistical significance at $P < 0.05$ level. Data were recorded after 30days of inoculation of shoots.

- **Bioinformatics analysis pipeline for Eggplant samples**

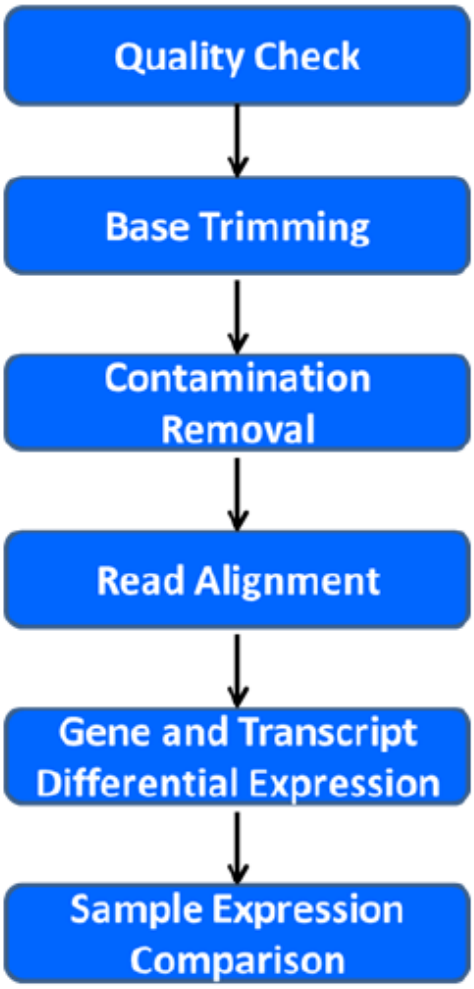


Figure 1: Transcriptome Bioinformatics Pipeline

- **The average base composition and quality**

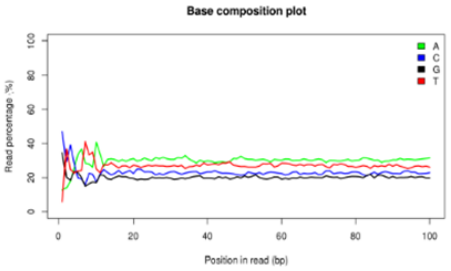


Figure 2: Average base composition of R1 reads of OERT

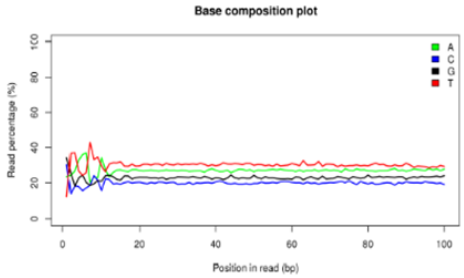


Figure 3: Average base composition of R2 reads of OERT

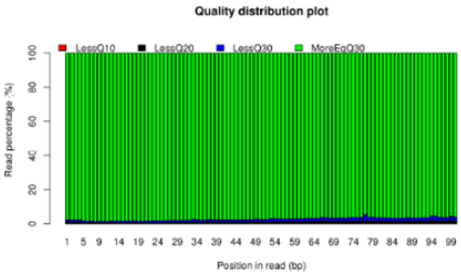


Figure 4: Average base quality of R1 reads of OERT

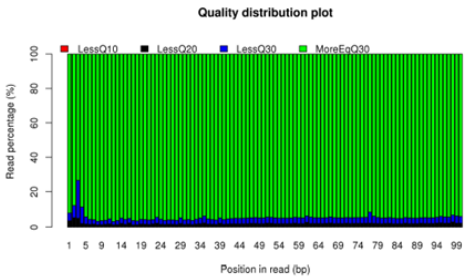


Figure 5: Average base quality of R2 reads of OERT

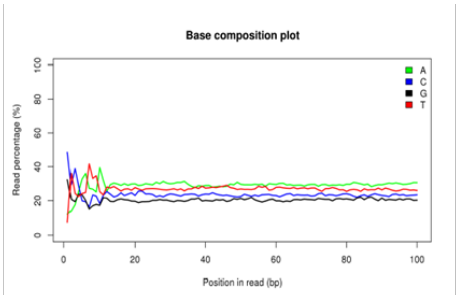


Figure 10: Average base composition of R1 reads of WTRT

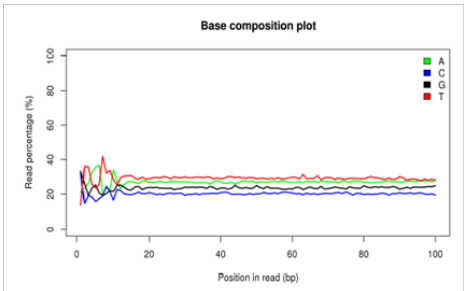
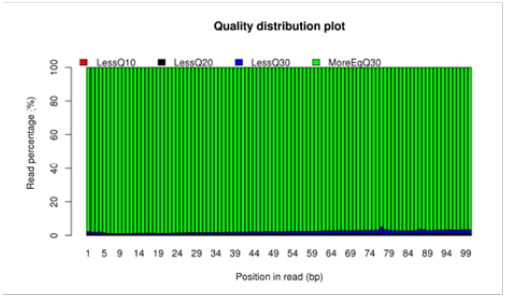


Figure 11: Average base composition of R2 reads of WTRT



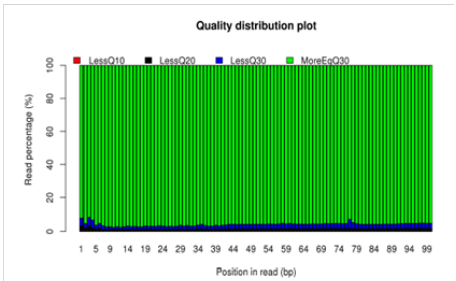


Figure 13: Average base quality of R2 reads of WTRT

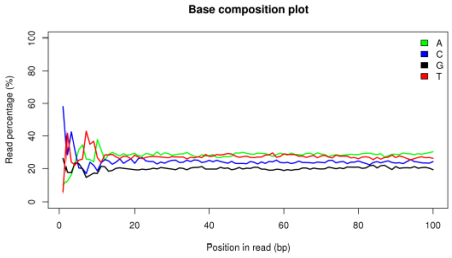


Figure 14: Average base composition of R1 reads of WT2hH

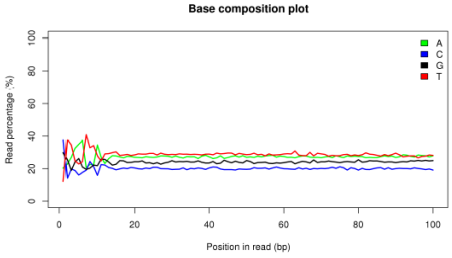


Figure 15: Average base composition of R2 reads of WT2hH

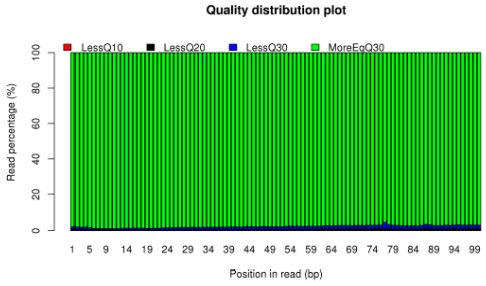


Figure 16: Average base quality of R1 reads of WT2hH

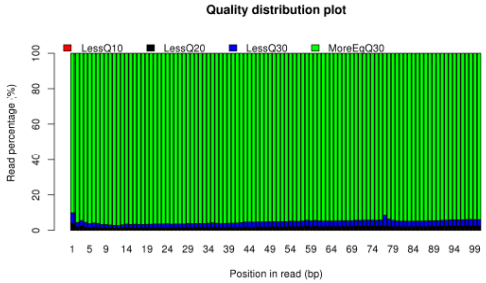


Figure 17: Average base quality of R2 reads of WT2hH

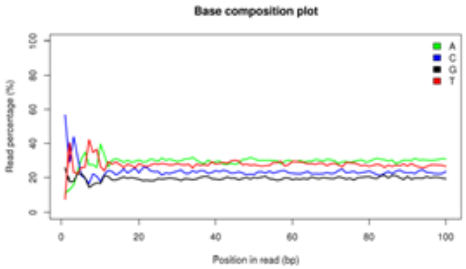


Figure 18: Average base composition of R1 reads of OE2hH

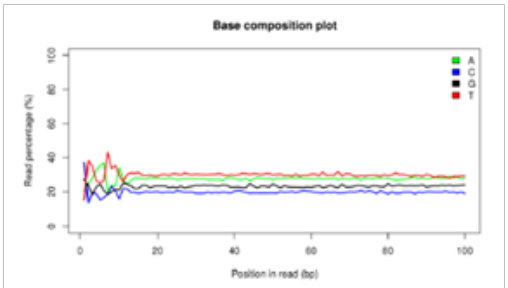


Figure 19: Average base composition of R2 reads of OE2hH

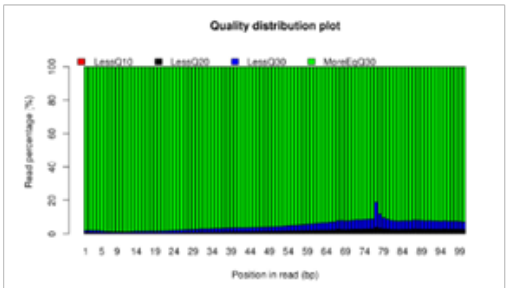


Figure 20: Average base quality of R1 reads of OE2hH

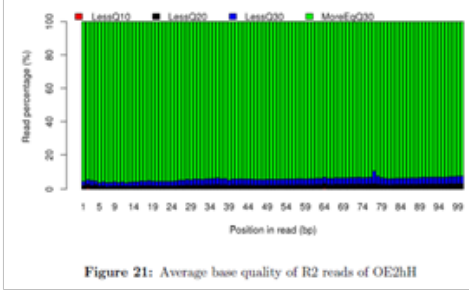


Figure 21: Average base quality of R2 reads of OE2hH

- Chromosomal distribution of differentially expressed transcript between control eggplant sample (OERT vs WTRT)

Chromosome	Differentially expressed transcript count
SL3.0ch00	14
SL3.0ch01	191
SL3.0ch02	184
SL3.0ch03	159
SL3.0ch04	116
SL3.0ch05	111
SL3.0ch06	137
SL3.0ch07	110
SL3.0ch08	134
SL3.0ch09	119
SL3.0ch10	105
SL3.0ch11	105
SL3.0ch12	85

- Chromosomal distribution of differentially expressed transcript between heat stressed eggplant sample (OE2h vs WT2h)

Chromosome	Differentially expressed transcript count
SL3.0ch00	1
SL3.0ch01	6
SL3.0ch02	7
SL3.0ch03	5
SL3.0ch04	8
SL3.0ch05	5
SL3.0ch06	4
SL3.0ch07	5
SL3.0ch08	4
SL3.0ch09	3
SL3.0ch10	7
SL3.0ch11	4
SL3.0ch12	7