DECIPHERING THE DIFFERENTIALLY EXPRESSED HEAT STRESS RESPONSIVE CANDIDATE GENES IN RICE

(Oryza sativa)

M.Sc. (Ag.) Thesis

by

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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

Poola Lohitha

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REQUIREMENTS FOR THE DEGREE OF

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CERTIFICATE - I

This is to certify that the thesis entitled **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)*"** submitted in partial fulfillment of the requirements for the degree of **"Master of Science in Agriculture"** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Poola Lohitha** under my/our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or certificate course. All the assistance and help received during the course of the investigations have been duly acknowledged.

Chairman

Date: 16/7/19

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

Chairman (Dr. Girish Chandel) 16/7/19

Member (Dr. Zenu Jha)

Member (Dr. Sunil Nair)

Member (Dr. R R. Saxena)

CERTIFICATE – II

This is to certify that the thesis entitled **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice** ***(Oryza sativa)"*** submitted by **Poola Lohitha** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur, in partial fulfilment of the requirements for the degree of **Master of Science in Agriculture** in the Department of **Plant Molecular Biology and Biotechnology** has been approved by the external examiner and Student's Advisory Committee after oral examination.

23/7/19

Signature External Examiner

(Name Dr. Suneeta Patra)

Date: 23/7/19

Major Advisor ____________________

Head of the Department ____________________

Faculty Dean ____________________

Approved/Not approved

Director of Instructions ____________________

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Date:

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Symbol/ notation	Indicates
%	Percent
µ	Micro
&	And
ºC	Degree Celsius
@	at the rate of
Bp	Base pair
et al.	And others
gm	Gram
i.e	That is
µg	Microgram
Ml	Millilitre
M	Molar
mg	Milligram
Ng	Nanogram
ppm	Parts per million
Tm	Melting temperature
Viz	As follows

LIST OF ABBREVIATIONS

Abbreviations	Details
ABA	Abscicic acid
cDNA	Complementary DNA
CSI	Chlorophyll stability index
dATP	Deoxy adenosine 5’ triphosphate
dCTPs	Deoxy cytidine 5’ triphosphates
DEPC	Di ethyl pyrocarbonate
dGTPs	Deoxy guanosine 5’ triphosphate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
dTTPs	Deoxy thymidine 5’ triphosphates
EDTA	Ethylene diamine tetra acetic acid
ESTs	Expressed sequence tags
EtBr	Ethidium bromide
HSFs	Heat stress transcription factors
HSG	Heat shock gene
Hsp	Heat shock protein
IAA	Indole acetic acid
IPCC	Intergovernmental panel on climate change
LEA	Late embryogenesis abundant
MDA	Malondialdehyde
MSI	Membrane stability index
MW	Molecular weight
PCR	Polymerase Chain Reaction
pH	Log 1/(H+)
PMC	Pollen mother cells
QTLs	Qunatitative trait loci
RIL	Recombinant inbred line

RNA	Ribonucleic acid
Abbreviations	**Details**
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Real time Polymerase chain reaction
smHSP	Small heat shock proteins
SNP	Single nucleotide polymorphisms
SSR	Simple sequence repeats

THESIS ABSTRACT

a) Title of the Thesis: “Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)”*

b) Full Name of Student: Poola Lohitha

c) Major Subject: Plant Molecular Biology and Biotechnology

d) Name and Address of the Major Advisor: Dr. Girish Chandel (Professor) Dept. of Plant Molecular Biology and Biotechnology, College of Agriculture, IGKV, Raipur.

e) Degree to be Awarded: M.Sc.(Ag.) Plant Molecular Biology and Biotechnology

Signature of the Student

Signature of Major Advisor

Date: ______________

Signature of Head of the Department

Global warming is expected to increase the earth’s surface mean temperatures by 1.5 to 4.8 °C by 2100 (IPCC 2014) and it is reported to reduce the yield of major crop plants including rice by 41% by the end of 21st century. Rice is the most important staple food crop across the world and feeds more than 3 billion people in Asia and Africa. Heat stress affects the growth of rice crop at all stages of growth like vegetative, reproductive and ripening phases but the effect is severe during flowering stage, as it leads to spikelet sterility that causes reduced yields. Improved varieties of rice with heat tolerance are required to meet the complex challenges like increasing population, decreasing arable land and increasing temperatures. Expression analysis of genes responsive to heat stress in different crops will be of great importance in today’s agriculture.

Transcriptome sequencing of rice after exposure to high temperature treatments could provide information on genes, that are differentially regulated on exposure to heat stress and its dissection is necessary to identify and characterize key genes responsible for heat tolerance. High temperatures produces the new group of special proteins under stress called as ‘Heat Shock Proteins (HSPs)’, which are categorized into five families based on their molecular weights: 1) small heat-shock proteins (sHSPs), 2) Hsp60, 3) Hsp70, 4) Hsp90 and 5) Hsp100. Studies reported plants synthesize large amounts of sHSPs under heat stress functioning as molecular chaperons, suggesting that they play a major role for enduring thermo-tolerance in plants. Higher plants posses at least 20 sHSPs. Transcription of HSPs is guarded by some regulatory proteins, called heat stress transcription factors (Hsfs). At least 21 Hsfs are seen in plants, with its specific role in regulation. Heat shock proteins perform a major role in signalling, translation, host-defence mechanisms, carbohydrate metabolism and amino acid metabolism by maintaining the functional conformation of proteins, preventing accumulation of non-native proteins and refolding. sHSPs facilitate stabilization of denatured proteins under heat stress. Based on this, the current study was planned to identify some key genes that are responsible for heat tolerance in fourteen rice genotypes (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mashuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) at late vegetative stage before panicle initiation. Genotypes belonging to diverse genetic background were grown in two sets under control and stress conditions (42˚C) for identifying heat tolerant traits. Phenological and biochemical characterization of rice genotypes was done under heat stress and in parallel a set of known heat related candidate genes were selected for expression analysis studies using semi-quantitative RT-PCR analysis. Under phenological and biochemical characterization six parameters are taken into consideration. They are membrane stability index (MSI), pollen fertility, spikelet fertility, chlorophyll content, proline and MDA levels.

Phenological and biochemical studies on rice resulted in identifying some of the tolerant and susceptible genotypes for heat stress. Genotypes RRF-127 , Nagina-22, Karma mashuri, CGZR-1 and Annada had recorded lower electrolyte leakage of 19.8%, 21.1%, 27.5%, 51.7% and 69.8% respectively under stress when compared to control. Lowest percentage of decrease in pollen fertility under stress conditions was observed in the genotypes RRF-127, Nagina-22, GP-145-103, Annada and CGZR-1 as 14.4%, 18.1%, 24.9%, 28.3% and 37.9% respectively. Percentage of Spikelet fertility decrease under stress was lowest in the genotypes RRF-127, Nagina-22, Annada, Karma mashuri and CGZR-1 as 14%, 19%, 25%, 31% and 42% respectively. When comparing the fold increase in proline content for stress over control, Nagina-22 showed highest fold increase of 20.6 folds followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mashuri (6.0 fold). In chlorophyll content, Annada has the lowest fold decrease of 1.0 folds for all chla, chlb and total chlorophyll content followed by Karma mashuri (1.0 fold) under stress when compared with control. When comparing the fold increase in MDA content for stress over control. The lowest fold increase in MDA content was shown by RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mashuri (2.5 fold).

Among the five genes (OsHSP26.7, OsHSP16.9, OsHSP DnaJ, OsHSP18 and 60Kda chaperon), selected for the study, OsHSP DnaJ has shown consistent expression under both control and stress with no significant change. The remaining four genes has shown up-regulation in all the genotypes under stress. OsHSP 26.7 gene has shown strong up-regulation in rice genotype RRF-127 (14.3 fold) followed by Annada (13.9 fold), Karma mashuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold). OsHSP 16.9 gene has shown up-regulation in RRF-127 (10.0 fold) followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mashuri (3.1 fold). OsHSP 18 gene showed up-regulation in GP-145-103 (17.7 fold) followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and poornima (8.9 fold). 60Kda chaperon gene has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. Among all the fourteen genotypes selected for the study, RRF-127, Annada, Karma mashuri, CGZR-1 and GP-145-103 showed a positive regulation towards heat stress and genotypes like MTU-1010, ARB-6-11 and GP-145-55 showed negative regulation towards heat stress. These findings were observed to be in correlation with the phenological and biochemical characterization and expression analysis studies carried out with five different heat responsive genes belonging to small heat shock proteins family, which had differential expression under heat stress when compared to control conditions. However positive induction of these genes leads to a key role in identification of different transcription factors, that have been responsible for different heat tolerant mechanisms or in cross-linking of different signalling pathways to activate plant defence mechanisms in rice under stress. This can be taken as a base for heat tolerance response of rice crop, which may be useful for further validation studies of the candidate genes responsive for heat stress in rice as well as other crop plants.

सारांश

(a).थीसिस का शीर्षक	"चावल (ओरिज़ा सैटिवा) में विभेदित रूप से व्यक्त गर्मी तनाव उत्तरदायी उम्मीदवार जीन का निर्णय लेना"
(b).छात्र का पुरा नाम	पूला लोहिथा
(c).प्रमुख विषय	पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
(d).प्रमुख सलहाकार का नाम और पता	डॉ॰ गिरीश चंदेल पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी विभाग, कृषि महाविद्यालय, आई जी के वी, रायपुर
(e).अवार्ड होने वाली उपाधि	एमएससी(कृषि) पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
	छात्र के हस्ताक्षर
प्रमुख सलहाकार के हस्ताक्षर	
दिनांक	विभागाध्यक्ष के हस्ताक्षर

ग्लोबल वार्मिंग से 2100 (IPCC 2014) तक पृथ्वी की सतह के औसत तापमान में 1.5 से 4.8 21˚C की वृद्धि होने की उम्मीद है और यह 21 वीं सदी के अंत तक चावल सहित प्रमुख फसल पौधों की उपज को 41% तक कम करने की सूचना है। चावल दुनिया भर में सबसे महत्वपूर्ण प्रधान खाद्य फसल है और एशिया और अफ्रीका में 3 बिलियन से अधिक लोगों को खिलाती है। गर्मी का तनाव वनस्पति, प्रजनन और पकने वाले चरणों की तरह सभी चरणों में चावल की फसल के विकास को प्रभावित करता है, लेकिन फूल के चरण के दौरान प्रभाव गंभीर होता है, क्योंकि यह स्पाइकलेट बाँझपन की ओर जाता है जो कम पैदावार का कारण बनता है। बढ़ती आबादी, घटती कृषि योग्य भूमि और बढ़ते तापमान जैसी जटिल चुनौतियों का सामना करने के लिए गर्मी सहिष्णुता के साथ चावल की उन्नत किस्मों की आवश्यकता होती है। विभिन्न फसलों में गर्मी के तनाव के प्रति उत्तरदायी जीन का अभिव्यक्ति विश्लेषण आज के कृषि में बहुत महत्व रखेगा।

उच्च तापमान उपचार के संपर्क में आने के बाद चावल की ट्रांसक्रिप्शनल अनुक्रमण जीन पर जानकारी प्रदान कर सकता है, जो कि गर्मी के तनाव के संपर्क में अंतर पर विनियमित होते हैं और गर्मी के सहिष्णुता के लिए जिम्मेदार प्रमुख जीन की पहचान करना और उनका विच्छेदन करना आवश्यक

है। उच्च तापमान तनाव के तहत विशेष प्रोटीन के नए समूह का निर्माण करता है जिसे 'हीट शॉक प्रोटीन (एचएसपी)' कहा जाता है, जिसे उनके आणविक भार के आधार पर पांच परिवारों में वर्गीकृत किया गया है: 1) छोटे हीट-शॉक प्रोटीन (sHSPs), 2) H660, 3) Hsp70, 4) Hsp90 और 5) Hsp100। अध्ययन में बताया गया है कि पौधे आणविक चैपरों के रूप में हीट स्ट्रेस फंक्शनिंग के तहत बड़ी मात्रा में एसएचएसपी का संश्लेषण करते हैं, यह सुझाव देते हैं कि वे पौधों में थर्मो-टॉलरेंस को समाप्त करने के लिए एक प्रमुख भूमिका निभाते हैं। उच्चतर पौधे कम से कम 20 sHSPs रखते हैं। एचएसपी के ट्रांसक्रिप्शन को कुछ नियामक प्रोटीनों द्वारा संरक्षित किया जाता है, जिसे हीट स्ट्रेस ट्रांसक्रिप्शन कारक (Hsfs) कहा जाता है। विनियमन में इसकी विशिष्ट भूमिका के साथ, पौधों में कम से कम 21 Hsfs देखे जाते हैं। हीट शॉक प्रोटीन प्रोटीन, कार्यात्मक गैर-देशी प्रोटीन के संचय को रोकने और refolding को रोकने के द्वारा सिग्नलिंग, अनुवाद, मेजबान-रक्षा तंत्र, कार्बोहाइड्रेट चयापचय और अमीनो एसिड चयापचय में एक प्रमुख भूमिका निभाते हैं। sHSPs गर्मी तनाव के तहत विकृत प्रोटीन के स्थिरीकरण की सुविधा देता है। इसके आधार पर, वर्तमान अध्ययन में कुछ प्रमुख जीनों की पहचान करने की योजना बनाई गई थी जो चौदह चावल के जीनोटाइप (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR) में गर्मी सहनशीलता के लिए जिम्मेदार हैं 1, अन्नदा, पूर्णिमा, कर्म मशूरी, ARB-6-11, GP-145-40, MTU-1010, RRF-127) पैतृक दीक्षा से पहले देर से वानस्पतिक अवस्था में। गर्मी सहन करने वाले लक्षणों की पहचान के लिए नियंत्रण और तनाव की स्थिति (42)C) के तहत विविध आनुवंशिक पृष्ठभूमि से संबंधित जीनोटाइप दो सेटों में उगाए गए थे। चावल के जीनोटाइप के फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन गर्मी के तनाव के तहत किया गया था और समानांतर में ज्ञात गर्मी से संबंधित उम्मीदवार जीन का एक सेट अर्ध-मात्रात्मक आरटी-पीसीआर विश्लेषण का उपयोग करके अभिव्यक्ति विश्लेषण अध्ययन के लिए चुना गया था। फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन के तहत छह मापदंडों को ध्यान में रखा जाता है। वे झिल्ली स्थिरता सूचकांक (एमएसआई), पराग प्रजनन, स्पाइकलेट प्रजनन क्षमता, क्लोरोफिल सामग्री, प्रोलाइन और एमडीआर स्तर हैं।

चावल पर जैविक और जैव रासायनिक अध्ययन ने गर्मी के तनाव के लिए कुछ सहिष्णु और अतिसंवेदनशील जीनोटाइप की पहचान की। नियंत्रण की तुलना में तनाव के दौरान जीनोटाइप आरआरएफ -127, नगीना -22, कर्म मशूरी, सीजीजेडआर -1 और अन्नदा में क्रमशः 19.8%, 21.1%, 27.5%, 51.7% और 69.8% का इलेक्ट्रोलाइट रिसाव दर्ज किया गया था। तनाव की स्थिति के तहत पराग की उर्वरता में कमी का सबसे कम प्रतिशत क्रमश: RRF-127, नगीना -22, GP-145-103, अन्नदा और CGZR-1 में 14.4%, 18.1%, 24.9%, 28.3% और 37.9% के रूप में देखा गया। । तनाव के तहत

स्पाइकलेट प्रजनन क्षमता का प्रतिशत जीनोटाइप आरआरएफ -127, नगीना -22, अन्नदा, कर्म मशूरी और सीजीजेडआर -1 में क्रमशः 14%, 19%, 25%, 31% और 42% के रूप में सबसे कम था। नियंत्रण पर तनाव के लिए प्रोलाइन सामग्री में गुना वृद्धि की तुलना करते समय, नगीना -22 ने CGZR-1 (11.4 गुना), RRF-127 (11.2 गुना), अन्नदा (10.5 गुना), GP-145 के बाद 20.6 सिलवटों की उच्चतम वृद्धि दिखाई। -103 (7.0 गुना) और कर्मा मशूरी (6.0 गुना)। क्लोरोफिल सामग्री में, अन्नदा में नियंत्रण के साथ तुलना में तनाव के तहत कर्मा मशूरी (1.0 गुना) द्वारा पीछा किया जाने वाला सभी क्लो, च्लब और कुल क्लोरोफिल सामग्री के लिए 1.0 गुना की कमी है। नियंत्रण पर तनाव के लिए एमडीए सामग्री में गुना वृद्धि की तुलना करते समय। MDA सामग्री में सबसे कम गुना वृद्धि RRF-127 द्वारा 1.1 गुना वृद्धि के साथ दिखाई गई जिसके बाद नगीना -22 (1.4 गुना), CGZR-1 (1.5 गुना), अन्नदा (1.8 गुना), GP-145-103 (2.1 गुना) है। और कर्मा मशूरी (2.5 गुना)।

अध्ययन के लिए चुने गए पांच जीनों (OsHSP26.7, OsHSP16.9, OsHSP DJJ, OsHSP18 और 60Kda चैपरॉन) के बीच, OsHSP DnaJ ने बिना किसी महत्वपूर्ण बदलाव के नियंत्रण और तनाव दोनों के तहत लगातार अभिव्यक्ति दिखाई है। शेष चार जीनों ने तनाव के तहत सभी जीनोटाइप में अप-विनियमन दिखाया है। ओएनएचएसपी 26.7 जीन ने अन्नदा (13.9 गुना), कर्मा मशूरी (11.5 गुना), जीपी-145-103 (8.6 गुना) और सीजीजेडआर -1 (3.7 गुना) के बाद चावल जीनोटाइप आरआरएफ-127 (14.3 गुना) में मजबूत विनियमन दिखाया है।) । OsHSP 16.9 जीन ने RRF-127 (10.0 गुना) और अन्नदा (3.7 गुना), CGZR-1 (3.4 गुना), GP-145-103 (3.2 गुना) और कर्मा मशूरी (3.1 गुना) के बाद अप-विनियमन दिखाया है। ओजीएचएसपी 18 जीन ने जीपी-145-103 (17.7 गुना) में सीजीजेडआर -1 (14.1 गुना), अन्नदा (13.8 गुना), जीपी 145-55 (11.9 गुना) और पूर्णिमा (8.9 गुना) के बाद विनियमन दिखाया। 60Kda चैपरोन जीन ने गर्मी तनाव के तहत लगभग सभी जीनोटाइप में अप-विनियमन दिखाया है, लेकिन अप-विनियमन न्यूनतम स्तर तक था। अध्ययन के लिए चुने गए सभी चौदह जीनोटाइप्स में से, RRF-127, अन्नदा, कर्म मशूरी, CGZR-1 और GP-145-103 में MTU-1010, ARB-6-11 और GP- जैसे हीट तनाव और जीनोटाइप के लिए एक सकारात्मक विनियमन दिखाया गया है। 145-55 ने ऊष्मा तनाव के प्रति नकारात्मक नियमन दिखाया। इन निष्कर्षों को फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन और अभिव्यक्ति विश्लेषण अध्ययन के साथ सहसंबंध में देखा गया था, जो छोटे ऊष्मा शॉक प्रोटीन परिवार से संबंधित पांच अलग-अलग गर्मी उत्तरदायी जीन के साथ किया गया था, जिसमें नियंत्रण की स्थिति की तुलना में गर्मी के तनाव के तहत अंतर अभिव्यक्ति थी। हालाँकि, इन जीनों का सकारात्मक समावेश विभिन्न प्रतिलेखन कारकों की पहचान करने में महत्वपूर्ण भूमिका निभाता है, जो विभिन्न ताप सहिष्णु तंत्रों के लिए या तनाव के तहत चावल में पादप रक्षा तंत्र को सक्रिय

करने के लिए विभिन्न संकेतन पथों के क्रॉस-लिंकिंग के लिए जिम्मेदार रहे हैं। यह चावल की फसल की गर्मी सहिष्णुता प्रतिक्रिया के लिए एक आधार के रूप में लिया जा सकता है, जो चावल और साथ ही अन्य फसल पौधों में गर्मी के तनाव के लिए उत्तरदायी उम्मीदवार जीन के आगे सत्यापन अध्ययन के लिए उपयोगी हो सकता है।

CHAPTER – I

INTRODUCTION

Rice is the most important staple food crop in the world, directly feeding more than 3 billion people across Asia, Africa, and Latin America. It is cultivated in more than 159 million hectares every year by households more than 100 millions in at least 114 countries across Asia and Africa (Tonini and Cabrera, 2011). "In the developing world, rice is the source of 27% dietary energy and 20% of dietary protein (Mottaleb *et al.*, 2012)". Rice is produced in wide range of locations and climatic conditions. Despite, many abiotic stresses such as drought, salinity and heat adversely affect plant growth and productivity. In the past three decades, surface temperatures of the earth have become more warmer than it was during any preceding decade since 1850, due to rapid increase in green house gas concentrations, Global mean surface temperatures increases by 1.5 to 4.8 ºC by 2100 (IPCC 2014). Plant growth and performance was damaged irreversibly due to increasing temperatures, majorly affecting the yield of crop and also quality of the yield (Wahid *et al.*, 2007). "Rice yields have been estimated to be reduced by 41% by the end of the 21st century (Ceccarelli *et al.*, 2010 and Shah *et al.*, 2011)". It's been reported that rice yields were decreased with high night temperatures, as per the yield data from field experiments. "With increasing concerns about global warming, the effect of temperatures stress on rice production has become a major focus in many countries in tropical, subtropical and temperate regions that produce rice (Horie *et al.*, 1996; Peng *et al.*, 2004; Wassmann *et al.*, 2009 and Nagarajan *et al.*, 2010)."

Heat stress affects the growth of rice plants at all stages of their growth like vegetative, reproductive and/or ripening phases (Wassmann *et al.*, 2009) by causing reduction in the photosynthesis rate and stomatal conductance. "In particular, spikelet sterility induced by high temperatures during flowering (Satake and Yoshida, 1978) is a serious problem, because it directly reduces yield (Prasad *et al.*, 2006 and Mohammed and Tarpley, 2011)". High temperatures during grain filling stage reduces the grain quality.

“Most importantly, Rice is immensely sensitive to heat stress episodes of short duration (>35◦C for ≥1 h) which coincides with the reproductive stage, especially anthesis (Prasad *et al.*, 2006; Jagadish *et al.*, 2007, Jagadish *et al.*, 2008, Jagadish *et al.*, 2010 and Sathishraj *et al.*, 2015)”. Additionally, nominal effect on spikelet fertility is seen due to temperatures higher than 38˚C which occurs an hour after anthesis (Yoshida *et al.*, 1981; Jagadish *et al.*, 2007). “Climate models have, with greater certainty indicated increased frequency of hotter days with temperatures above known critical threshold(>33◦C; Jagadish *et al.*, 2007) to coincide with the flowering stage”. Anther dehiscence was inhibited by high temperatures above 35 ºC at the flowering stage, resulting in low amount of pollen shed on the stigma, which leads to incomplete fertilization (Jagadish *et al.*, 2010). “Even if a sufficient number of pollen grains are shed on the stigma, in some cases, the pollen germination and pollen tube growth are poor under heat stress (Satake and Yoshida, 1978)”. Thus, primarily disturbed development of pollen because of aberrant anther dehiscence and secondarily shedding were considered as the causes for spikelet sterility at the flowering stage, induced by heat.

Some adaptation strategies to heat in rice have been recently described. 1) Escape strategy, ability of the plants to adjust the time of the day of anthesis based on their sensitivity to climate conditions during the 7-days period preceding anthesis and is advanced earlier in the morning, if conditions during the seven preceding days are hot and humid, which allows the plants to escape the hottest time of the day. 2) Avoidance strategy, in dry air environments, panicle temperature is lowered by up to 10 ºC when compared to air temperature. This is because of the transpirational cooling ability of the plant and so stabilizes the spikelet fertility by maintaining panicle temperature below the critical threshold. Reported genetic variability was seen in the cooling capacity of panicles through its transpiration and the leaves surrounding it. 3) “True tolerance, involves different physiological mechanisms which were induced under heat stress (Kondamudi *et al.*, 2012; Bahuguna *et al.*, 2015; Bahuguna and Jagadish 2015)”. Short exposure of rice seedlings to high temperature affects the ultra-structure of cells, with major changes in chloroplast and mitochondria, results in reduced metabolism and thus reducing plant growth (Pareek *et al.*, 1997).

In response to heat stress, plants adjust themselves to the metabolism and morphology suitable to sustain in the stress conditions. Generally high temperature induces expression of heat shock proteins (HSPs) and normal cellular protein production, at least in part, is suppressed (Shah *et al*., 2011). “HSPs can improve or stabilise photosynthesis, partitioning of assimilates, nutrient and water use efficiency and the thermal stability of cellular membranes (Wahid *et al*., 2007)”. “Damaged protein restoration is also aided by some of the HSPs and molecular chaperones (Kumari *et al*., 2013)”. Investigation of these mechanisms in further agricultural production system is needed, to exploit them in the development of heat stress tolerant rice cultivars. Despite the occurance of different strategies (escape, avoidance), sterility has still remained as a major concern in the areas cultivating rice. As large percent of the rice growing areas are characterized by humid conditions, limiting the transpirational cooling incidence, there by reducing panicle temperature. In addition, the occurrence of combinations of different abiotic stresses such as heat, drought and salinity in farmers' fields increases, lowering the transpiration cooling because of partial stomatal closure. Under future climates, this immensely increases the vulnerability of rice in the most productive regions. To cope with future climate scenarios, it is vitally important to develope rice varieties with heat tolerance under varying humidity conditions.

“Heat tolerance genetics was poorly understood as it is complex and controlled by multiple genes (Wahid *et al.,* 2007; Xue *et al.*, 2012; Driedonks *et al.,* 2016)”. “Fairly more heritability has seen in rice heat tolerance and most of the genetic variation is additive (Yoshida 1981)”. Many HSPs have been reported and their genetics (controlling genes, location of genes, dominance/recessive) are known. Even then combination of certain genes are very critical for successful breeding of the cultivar, e.g. cultivars should posses the optimal genes/alleles for flowering time, height, etc., and the effectiveness of HSP genes in an elite genetic background is not known. As per the recent genetic studies, its been reported that probably, plant heat-tolerance is a polygenic trait. “In wheat different components of tolerance, controlled by different sets of genes, are critical for heat tolerance at different stages of development or in different tissues (Barakat *et al.,* 2011)”. “Shah *et al.,* (2011) emphasized that indica rice is generally more heat tolerant than japonica rice, however there is a genotypic variation in spikelet fertility at high temperature in both species". Understanding the tolerance genetic base and increasing the level of breeding for heat tolerance still continues.

The present study could provide clues for identification of candidate genes responsive to heat stress, for further functional analysis and to explain their precise role in heat stress response. As key genes are identified, it increases the efficiency and realizes the opportunities for genetic engineering. This is a fundamental aspect of research into heat stress responsive genes, which is explored in the present study. The present study entitled **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)*"** is planned with following objectives:

1. Phenological and biochemical characterization of rice genotypes under heat stress.

2. Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.

CHAPTER – II

REVIEW OF LITERATURE

Rice *(Oryza sativa)* is one of the major staple foods among three most important food crops in the world, feeding more than 2.7 billion people. In India rice cultivation occupies an area of 44.6m ha with total yield output of 80 million tonnes (paddy) and average productivity of 1855 Kg/ha.

2.1. Taxonomic classification of Rice:

Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Cyperales
Family: Gramineae
Genus: Oryza
Species: Sativa
Subspecies: Indica, japonica, javanica.

2.2. Morphology of rice plant:

Generally cultivated rice is treated as semi-aquatic annual grass. Rice plant at maturity has main stem and tillers, with each productive tiller having a flowering head called panicle. There is a great variation in plant height according to varieties but average height ranges from 0.4m – more than 5m, in some. The morphology of rice is divided into two phases;

Vegetative phase – germination, seedling and tillering stages

Reproductive phase – panicle initiation and heading stages.

Rice plant has has fibrous root system with seminal, nodal and lateral roots. The seminal root is formed by radical, nodal roots develop from basal nodes as lants grow, nodal roots developing from higher nodes and helping plant in absorbing food are called lateral roots. The stem of rice plant is known popularly as culm or haulm, made up of large number of series of nodes and internodes. Node is the solid part of culm bearing leaf. Early tillers are emerged in an alternate manner from main culm, lower most nodes gives rise to primary tillers and they give rise to secondary and tertiary tillers. Each tiller is considered as an independent plant. Leaves are formed on the culm near a node in alternative manner and the top most leaf beneath panicle is called flag leaf. Mostly leaves contain small paired ear like lobes on both sides of blade base called as auricles. The terminal part of rice tiller is an inflorescence called as panicle, bearing rice spikelets that develop into grain. Each spikelet in rice is considered as a single flower enclosed by lemma and palea. It has six stamens, 3 in each whorl. The pistil has stigma, style and ovary. Self pollination occurs in rice when pollen is dusted on the stigma after dehiscence of anthers. After fertilization ovary is developed into seed and it is called as grain. The morphology of rice plant and spikelet is shown in figure 2.1 below

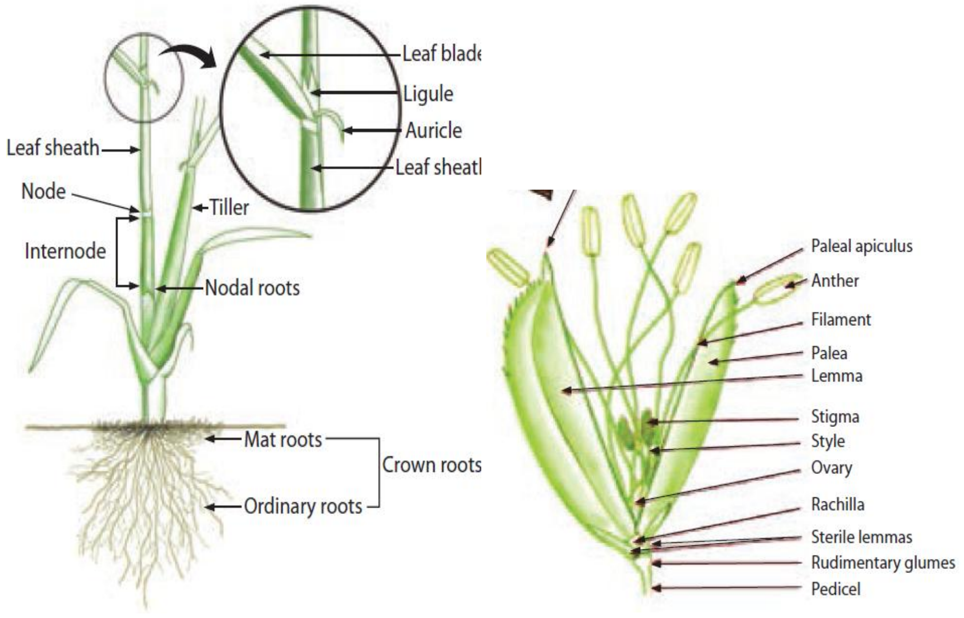


Fig 2.1: Parts of rice stem, tillers and spikelet. [Maclean *et al.*, (2013), *Rice Almanac*]

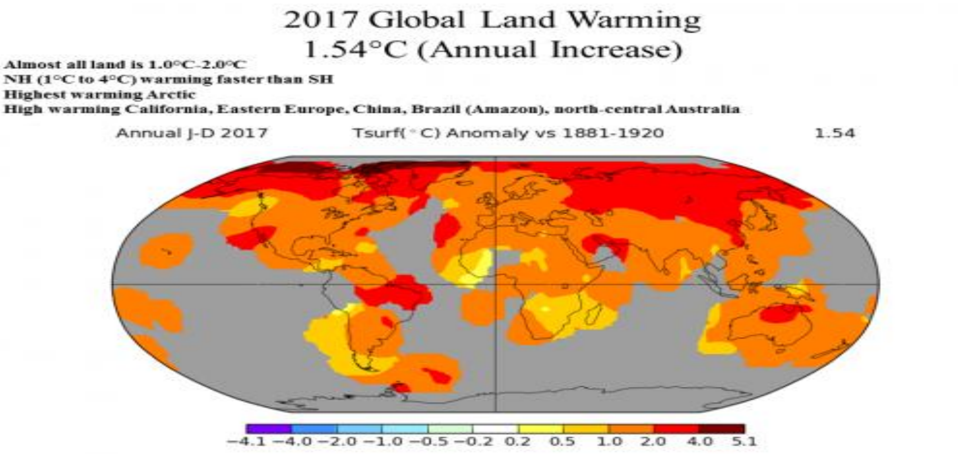
Rice is a plant of tropical climate and a normal variety grown in tropical region has a life period of 120 days i.e it spends about 60 days in vegetative phase, 30 in reproductive phase and the remaining 30 in ripening phase. As the plant is a tropical plant is requires a temperature range of 20-35˚C during its developmental stages. The following table 2.1 shows the stage and optimum temperature required.

Table 2.1: Optimum temperature required for different stages of rice plant. [Maclean *et al.*, (2013), *Rice Almanac*]

Stage	optimum temperature (˚C)
Germination	20-35
Seedling emergence	25-30
Rooting	25-28
Leaf elongation	31
Tillering	25-31
Panicle initiation	15-30
Anthesis	30-33
Ripening	20-25

2.3. Climate change and raising temperatures :

Earth’s surface temperature is increasing in logistic fashion and in the past three decades it became more warmer than it was during any preceeding decade, since 1850. This drastic increase in temperature is due to rise in green house gas concentration and global warming. As per the report given by IPCC 2014, Intergovernmental Panel on Climate Change, the global mean surface temperatures increases by 1.5 to 4.8 ºC by 2100.



Source: NASA GISS, Surface Temperature Surface Temperature Analysis

Fig 2.2: Global land warming 2017. [NASA GISS, surface temperature analysis.]

2.4. Impact of high temperature on rice and its adaptations:

Heat stress affects the rice crop during its all stages of development like vegetative, reproductive and ripening stage. Particularly, rice plants are sensitive to high temperatures during anthesis and leads to improper or reduced pollination and results in spikelet sterility. This reduces the rice yields. Hence it is estimated that increasing global mean surface temperature reduces the rice yields by 41% by the end of 21st century (Ceccarelli *et al*., 2010; Shah *et al*., 2011).

One of the important agricultural goals now a days as per the increasing global temperatures is elucidating the approaches for sustaining high yields of crop plants under heat stress. The physiological, biochemical and molecular responses that take place in plants in accordance with heat stress are shown below in the figure 2.3

Figure 2.3: Major effects of high temperature on plants. [Hasanuzzaman *et al.*, (2013)]

Heat stress, like any other abiotic stress might uncouple enzymes and metabolic pathways that causes accumulation of unwanted and harmful ROS (reactive oxygen species) like singlet oxygen (1O2), superoxide radical (O2•−), hydrogen peroxide (H2O2) responsible for oxidative stress. Though chloroplasts are major sites of ROS generation, they are also generated in peroxisomes and mitochondria. This oxidative stress induced by thermal stress is because of peroxidation of membrane lipids, cell membrane stability disruption by protein denaturation and increased malondialdehyde (MDA) content in leaves by reduced antioxidant enzyme activities. Even though ROS have a lot of destructive effects on metabolic process of plants, they have also hypothesized to trigger heat shock responses by having signaling behaviour towards development of heat tolerance in plants.

2.4.1. Plant Adaptation to Heat Stress:

Considering the preferred temperature for the growth of living organisms, they are classified into following types.

a) Psychrophiles: grows at low temperature ranging between 0 and 10 °C

b) Mesophyles: growing well between 10 and 30˚C;favours moderate temperature

c) Thermophyles: grows wellin temperature ranging from 30 to 65˚C and even in higher temperatures.

Larcher, classified all plant species on the basis of thermotolerance into three groups as shown in fig.2.4

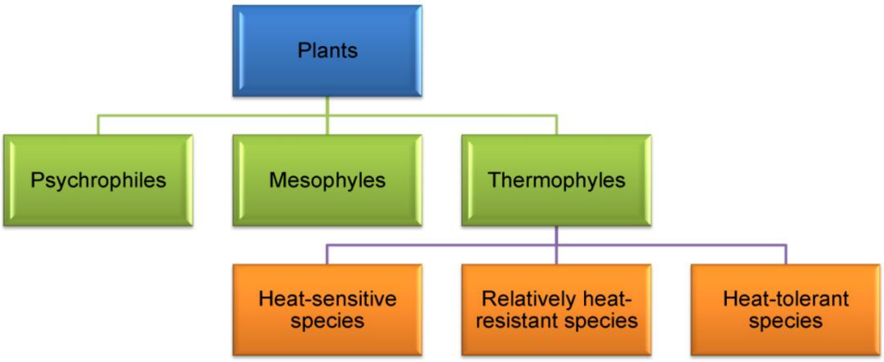


Fig 2.4: Classification of plants based on their heat tolerance. [Larcher, 1995]

Fitter *et al.,* (2002) reported that plant adaptation to heat stress involves various mechanisms like avoidance and tolerance. Plant survival in hot environment is achieved by various ways that include combinations of avoidance and tolerance strategies (fig 2.5)

Avoidance strategies that operate under high temperatures include changing leaf blade orientation parallel to sun rays (paraheliotropism), membrane lipid composition alterations, reduced absorption of solar radiation due to presence of small hairs (tomentose). An escape mechanism like early maturation is also seen in hot environments which is closely correlated for low yields.

Tolerance strategies that appear in heat stress plants count ion-transporters, late embryogenesis abundance (LEA) proteins, osmoprotectants, antioxidant defense, factors involving transcriptional control and signalling cascades.

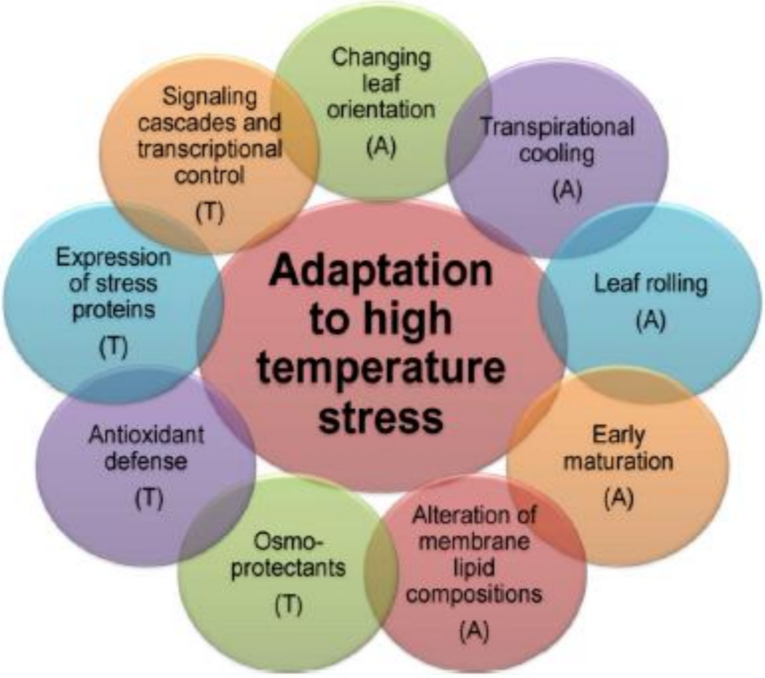


Fig. 2.5: Different adaptation mechanisms of plants to high temperature.
A: avoidance T: tolerance [Fitter *et al.*, (2002).]

Reis *et al.,* (2012) studies stated that, In general, protein denaturation takes place under HT as the reduced cellular volume increases the chances of degradative molecular interactions. So, to compensate this under heat stress conditions heat shock proteins (HSP's) are activated. HSP's are good at maintaining and repairing companion protein structure. They target the incorrectly aggregated and non-native proteins, help in degrading and removing them from cells. Their primary function is to control proper folding and conformation of structural (i.e., cell membrane) and functional (i.e., enzyme) proteins. They ensure the proper functioning of many cellular proteins under elevated temperature conditions.

The schematic diagram shown in figure 2.6, represents the molecular regulatory mechanism of heat shock proteins as per a hypothetical cellular model. The steps involved are as follows:

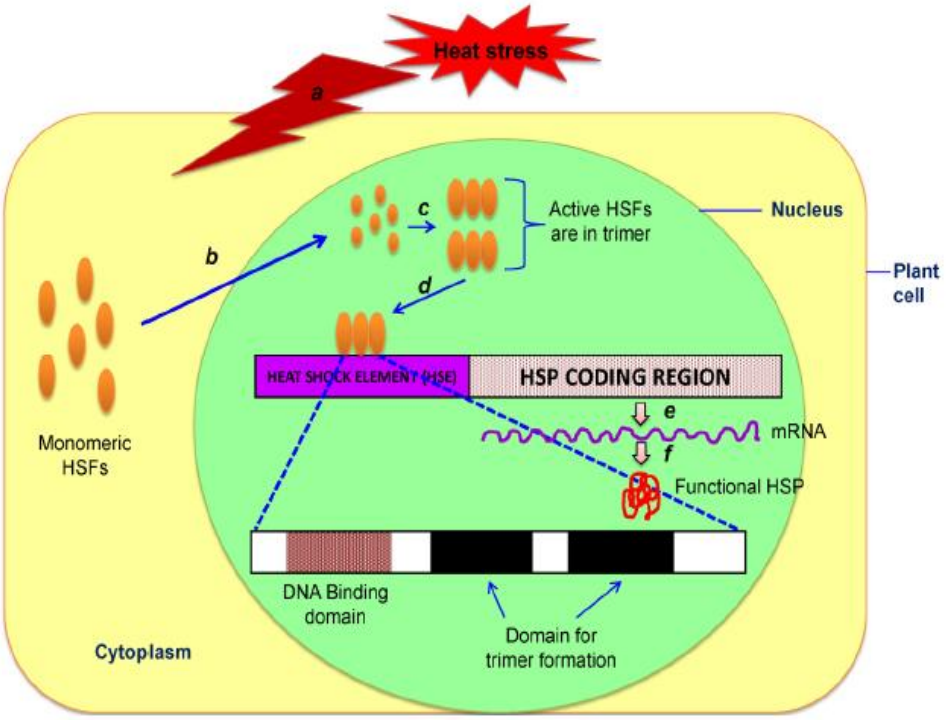


Fig 2.6: Schematic representation showing molecular regulatory mechanism of HSP's. a) plant cell receives the heat stress, **b)** monomeric heat shock factors (HSFs) enters into the nucleus from cytoplasm, **c)** In nucleus, HSF monomers form active trimers, **d)** These trimers bind to the specific genomic region (promoter or heat shock element, HSE) of the respective heatshock gene (HSG), **e)** (Molecular dissection of the HSF binding region of HSE showing that it consists of one DNA binding domain and two domains for trimerization of HSFs) Successful transcription occurs, **f)** Translation and post-translational modification takes place leading to production of functional HSP responsible for heat stress tolerance. **[Reis *et al.*, (2012).]**

Zafar *et al.*, (2018) studied several approaches enveloping genetic, biochemical and physiological mechanisms for mitigating heat stress impact on rice. They utilized the knowledge of genetic information for increasing the conventional breeding approaches for developing heat tolerant germplasm in rice. The figure 2.7, Given below shows all the mechanisms for heat tolerance.

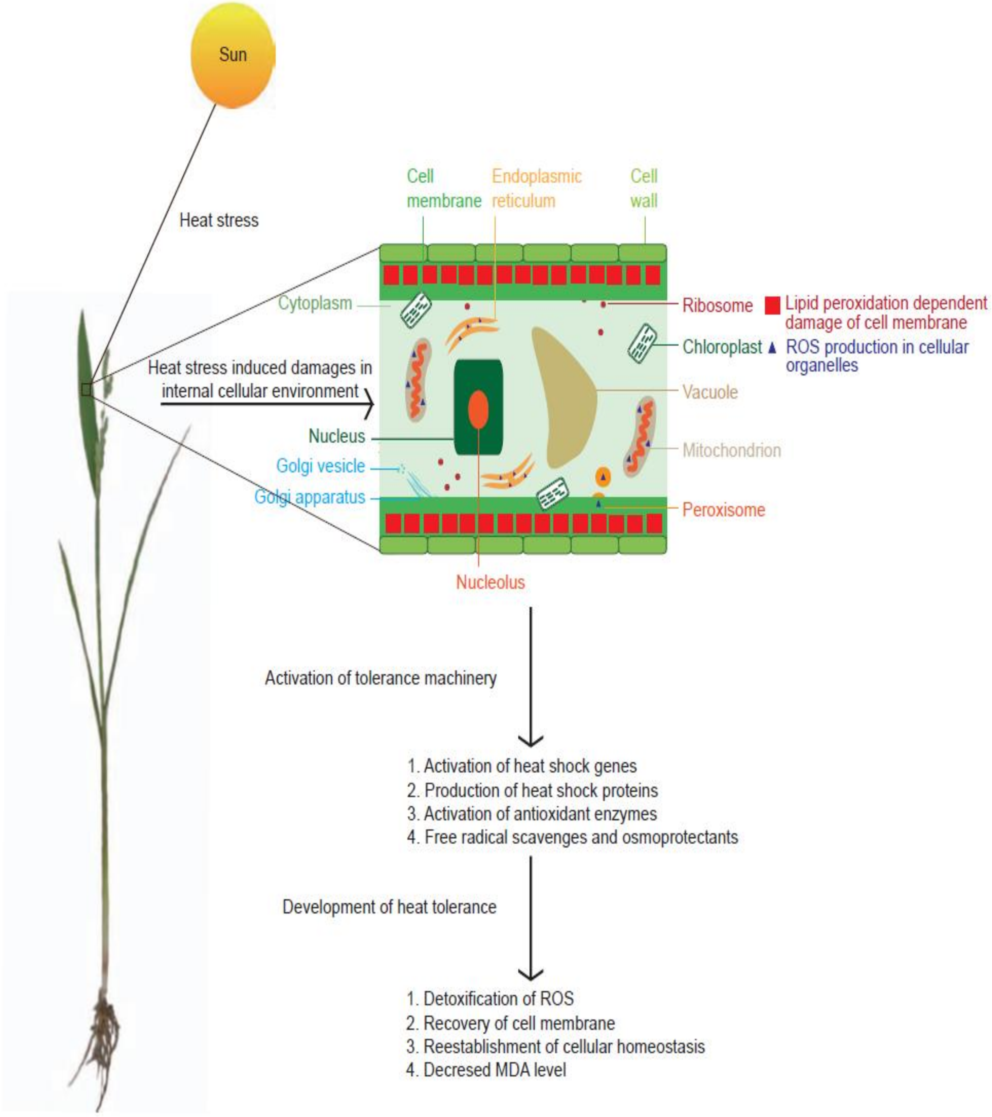


Fig 2.7: Number of mechanisms and compounds produced after exposing rice plant to heat stress for its rescue. [Zafar *et al.*, (2018)].

2.5. Phenological and biochemical characterization of rice and other crops under heat stress.

Phenological characters that are considered in this study, under heat stress includes:

- Membrane Stability Index-[MSI],
- Pollen fertility,
- Spikelet fertility.

Biochemical characters acknowledged in this study, under heat stress cover:

- Chlorophyll,
- Proline,
- Malondialdehyde (MDA).

2.5.1. Membrane stability or cell membrane thermo-stability (CMT):

Dexter *et al.,* (1932) investigated hardiness of plants by measuring electrical conductivity. The principle of increasing permeability and electrical conductivity in an injured tissue is more intimate in plant physiology . Electrical conductivity is measured by changing electrolyte concentration. In this study, they estimated the hardiness of three varieties of alfalfa by determining the electrical conductivity in which exosmosis of electrolytes from frozen tissues takes place. A progressive hardening of Grimm alfalfa, a hardy variety, was found as the winter season arrived, and little or no hardening in Hairy Peruvian, a very tender strain. Common alfalfa, Utah was intermediate in response.

Martineau *et al.,* (1979) evaluated a simple rapid technique of assessing genotypic differences for heat stress by measuring membrane thermo-stability, which was previously used in sorghum. In this technique, electrical conductance of amount of electrolytes leaked from heat damaged leaf cells is measured. A sigmoidal response was observed between the degree of injury and the temperature causing that injury. Genotypic differences for heat tolerance are determined by difference in relative positions of response curve with respect to temperature treatment. While detecting genotypic differences greatest sensitivity occurred at about 50% injury. As the genotypic differences were found to be greatest in newly formed tissues, it concluded that most recently developed leaves should be used for the assay. As per this study, the cultivars showing the low injury readings constantly, are those having the relatively greater membrane thermo-stability.

Blum *et al.,* (1981) used cell membrane stability as a measure of heat and drought tolerance in wheat. 66 cultivars were tested for heat tolerance using 44˚C temperature as standard heat treatment. Among them, there were most significant differences in the percent injury ranging from 14.8% (in 'H895') to 77.0% (in 'CD 31/75' durum wheat).

Agarie *et al.,* (1995) explored the potentiality of measuring the cell membrane stability adaptations for evaluating drought and heat tolerance in rice. They exposed the leafs to deccisation by polyethylene glycol (PEG) and to heat stress by temperature treatment of 42˚C. Electrolyte leakage (EL) was increased linearly from both the treatments like PEG and heat stress, reflecting that this technique is used for assessing the injury probably at the membrane level due to environmental stress in rice.

Hasan *et al.,* (2007) reported that to evaluate heat tolerance, most widely used and accepted method is membrane thermostability while yield parameters are evaluated by heat susceptibility index. Wheat cultivars under normal and heat stress at post anthesis conditions differed in terms of CMT measured by relative electrolyte leakage at 55˚C elated temperature. Heat killing time is the time required to reach 50% membrane leakage in a cultivar. Among the cultivars, Aghrani recorded the longest heat killing time (150min) closely followed by Kanchan (120 min) and CB-30 (120 min). shortest heat killing time irrespective of growing condition was recorded in Sonora (30min). Based on this criteria of heat killing time Aghrani, Kanchan and CB-30 were regarded as the heat tolerant cultivars and Sonara as the heat sensitive. High killing time indicates that cultivars showed lower membrane leakage at elevated temperatures, due to tendency of the genotype to acclimatize against stress.

Hemantaranjan *et al.,* (2014) studied physiological responses of plants to high temperature and mechanisms of heat tolerance to contribute for traditional breeding. For the processes such as photosynthesis and respiration, continued function of cellular membranes under heat stress is vital. So to increase selection efficiency physiological and biochemical screening techniques are used as a complement to empirical breeding methods. Heat tolerance securing genes may be lost in breeding programs, hence membrane thermal stability (MTS) is considered as significant selection criteria for heat stress tolerance. As the purity and functions of biological membranes are sensitive to heat stress, tertiary and quaternary structures of membrane proteins gets transformed boosting up the permeability of membranes, which is obvious from enhanced electrolyte leakage. This increased the electrical conductivity under heat stress, resulting the decreased membrane thermal stability.

Usman *et al.*, (2015) carried out experiments in chilli pepper to study mechanisms for heat tolerance. They evaluated six genotypes for cellular membrane thermostability (CMT) and for HSP70 gene expression. The genotypes were classified as follows: heat-tolerant (greater than 60%), moderately tolerant (30% to 60%), and susceptible (less than 30%), based on mean values of CMT. The highest CMTs of 89.27%, 88.03%, and 85.10% were recorded for heat tolerant plants AVPP0702, AVPP0116, and AVPP9905, respectively. Under heat stress, heat shock protein 70kDa was identified to be differentially expressed. Changed CMT values were observed and correlated with differential heat shock protein HSP70 expression. Genotypes that have high CMT were observed to have high expression of HSP70, indicating thermo-tolerance. Hence the degree of differences detected in HSP70 and membrane stability were used to improve thermotolerance in studied genotypes.

Tiwari *et al.*, (2017) conducted an experiment to evaluate heat stress traits in wheat to develop high yielding heat tolerant wheat varieties, with 4 varieties namely Halna, HD2733, Raj3765 and K9006. Under field conditions, delayed sowing was used to give the heat stress treatment. To screen and evaluate different wheat varieties for heat tolerance they selected cell membrane stability as one of the parameters. Cell membrane stability is the measure of electrolyte diffusion from the cells and during heat stress electrical conductivity increases as the cell membrane damage occurs, resulting in solute leakage. The maximum membrane stability index values were recorded in Halna (69) and Raj 3765 (63).

Sharma *et al.,* (2017) examined alterations in the cell membrane stabitility (CMT) due to heat stress in different genotypes of bread wheat. They exposed different varieties (HDR 77, HD 2864, HD 2967, PBW 373, HS 365, Raj 4037, JAUW 584, PBW 175, RSP 561, HW 2045, HD2385, HD2687) of single genotype to varying temperatures of 45˚C and 100˚C. Changes in cell membrane stability of leaf samples was calculated and the highest percentage of conductivity was seen in HDR77, predicting the highest susceptibility of this variety for heat stress. Lowest percentage of conductivity was observed in HD2687, which predicts that this is the highly resistant variety for heat stress in bread wheat among the selected genotypes.

ElBasyoni *et al.,* (2017) demonstrated identification of potentially new genomic regions associated with cell membrane stability (CMS), using genome-wide association mapping. They exposed leaf tissues to heat treatment and screened a global spring wheat panel for CMS, potential quantitative trait loci (QTL)/genes linked with CMS were identified using genome wide association-mapping and relationship between the field performance and measured CMS was estimated. Highly significant differences were indicated among the 2111 spring wheat accessions with respect to CMS. Several SNPs were found to be undoubtedly linked to CMS and their annotations indicated that these SNPs are linked with important functional genes that control solute transport through cell membrane and other biochemical activities related to abiotic stress tolerance.

2.5.2. Pollen Fertility:

Herrero *et al.,* (1980) determined in vitro pollen germination of different maize genotypes during anthesis , affected by high temperature stress. At the beginning of anthesis, tassels from field grown plants were cut out and placed in water. Later they were transferred to growth chambers with the day temperatures maintained at 27˚C, 32˚C, 38˚C and night 6˚C cooler temperatures were maintained. After 24 and 48hrs, in vitro germination was measured both in growth chamber as well as on field collected pollen. As the temperature increased pollen germination steadily decreased in some genotypes. All genotypes witnessed lower germination at 38˚C, when compared with 27 and 32˚C. After 48hrs at 38˚C, several genotypes showed no germination. This study suggested that pollen germination of many genotypes reduces on prolonged exposure to temperature above 32˚C.

Pressman *et al.,* (2002) continuously exposed tomato ‘Trust’ to high temperatures (day/night temperatures of 32/26 °C) and observed the reduction in number of pollen grains per flower and the viability. Heat stress effect on pollen viability was correlated with changes in carbohydrate metabolism in different parts of anther during its development. In favourable temperature conditions (28/22 °C), accumulation of starch in pollen grains reached maximum value of 3d before anthesis and then reduced towards anthesis. During anther development, total soluble sugars concentration progressively increased in anther walls and in pollen grains (but not in the locular fluid), meeting maximum at anthesis. Heat stress interrupted the transient increase in starch concentration, leading to reduced concentrations of soluble sugars in anther walls and pollen grains. Their results suggested the dominant effect of heat stress on pollen development is decrease in starch concentration 3d before anthesis leading to reduced sugar concentration in mature pollen grains, which contributes to the decreased pollen viability in tomato.

Sakata *et al.,* (2008) reported that male reproductive development is dominantly affected by abiotic stresses. In particular, male sterility is is caused by high temperature in many plant species. They used double-rowed barley (*Hordeum vulgare* L. cv. ‘Haruna-nijyo’) as a model For studying male reproductive development and high-temperature injury correlation in plants. High temperature injury leads to premature advancement of early developmental programs in anthers, including proliferation arrest, anther wall cell degradation and progression to meiosis in pollen mother cells (PMCs). All these require broad amendments in transcription. These findings suggested that male reproductive development is more sensitive when compared with female reproductive development under heat stress leading to anther sterility.

Rang *et al.,* (2011) studied five rice genotypes under high temperature, water stress and combination of both during flowering, which is most sensitive developmental stage and their response is quantified through spikelet fertility. Significant differences in anther dehiscence between treatments and genotypes, with a moderately enhanced partnership with the number of germinated pollen grains on the stigma were revealed using microscopic analysis. Strong relationship was found between the spikelet fertility and the number of pollen germinated on stigmas. All the 3 treatments lead to spikelet stertility but highest spikelet sterility was found in high temperature stress in all 5 genotypes. N22 showed better anther dehiscence , higher in vivo pollen germination and higher spikelet fertility when compared with IR-64, Apo and Moroberekan under high temperature, water stress and combined stress, suggesting its ability to resist multiple abiotic stresses.

Shah *et al.*, (2011) reported the impact of temperature increment on rice production. They stated that compared to mean daily temperatures or day-time temperature, high temperatures at night are more devastating to rice production. Sometimes this high night temperature may lead to complete sterility, as booting and flowering are most sensitive stages to temperature. At increased temperatures, humidity also plays an important role in imparting spikelet sterility. Some phenotypic markers for high-temperature tolerance includes Flowering at cooler times of day, more pollen viability, larger anthers, longer basal dehiscence and presence of long basal pores. Some of the adaptive measures to mitigate the yield loss during high temperatures are adjustment of sowing time, choosing varieties that allow avoidance of peak stress periods and exogenous application of some plant hormones.

Harsant *et al.*, (2013) investigated The effect of high temperatures on harvest index (HI) and morphological components that contribute to HI in *Brachypodium distachyon.* It is a C3 grass, identified as an amenable plant for addressing critical issues related with enhancing cereal crop yields under global climate change. The results explained that due to high temperatures more than 32˚C, HI was eliminated. Primary reasons for the reducing yields at 32˚C were declined pollen viability, retention of pollen in anthers, and pollen germination. Microspore abortion at uni-nucleate stage in correlation with abnormal tapetal development are reasons for yield failure at 36˚C. In *B. distachyon*, the The response of male reproductive development and tiller branching patterns to increasing temperatures provides support for the use of this C3 grass in estimating molecular control of HI in global warming.

Das *et al.,* (2014) studied pollen in two groups of genotypes belonging to lowland and upland conditions. These two group of plants were subjected to six different day/night temperature regimes (25/15, 30/20, 35/25, 40/30, 45/35 and 50/40 ◦C) during panicle initiation for three days. Various morphological, physiological and biochemical aspects of pollen were determined during anthesis in each regime. panicle extrusion, flowering period, and number of anthesing (opened) spikelets were sceptically altered by High temperatures above 35/25 ◦C. Pollen viability, tube length, anther dehiscence and number of pollen on stigma decreased significantly. It was noticed that the pollen of low land genotypes group was more adversely effected compared to the upland genotypes groups under high temperature stress. Hence the viability of pollens was precisely more in upland genotypes. Among all the genotypes examined, loss of pollen viability was highest in Swarna.

Kumar *et al.,* (2015) conducted a study indicating the rise in temperature with respect to pollen viability. The threshold temperature for pollination of rice is 35◦C and temperature above this causes pollen sterility and fertilization is severely affected. They studied 11 rice genotypes in indigenous field conditions during kharif 2010 and 2011. The spikelet attributes of the crop were severely affected due to increased mean temperature of about 12◦C during full flowering stage. Scanning electron microscopy studies showed that under high temperature pollen size was clearly reduced. Spikelet sterility of about 90% was observed in all genotypes.

Jagadish *et al.,* (2015) addressed the relevance of vapour pressure scantiness under fully flooded paddies and enhanced susceptibility of rice production to heat stress or infrequent occurrence of combined heat and drought stress under egressing water-saving rice technologies and the major disconnect with high night temperature response between field and controlled environments in terms of spikelet sterility. Expression of transpiration cooling, an efficient heat-avoiding mechanism, will decline with the shift from fully flooded paddies to water-saving technologies, such as direct-seeded and aerobic rice cultivation, provoking stress damage. This change can possibly introduce higher sensitivity to previously unaffected developmental stages such as floral meristem (panicle) initiation and spikelet differentiation, and further enhance vulnerability at the known sensitive gametogenesis and flowering stages. More than the mean temperature rise, increased variability and a more rapid increase in nighttime temperature compared with the daytime maximum leads to sterility.

Fahad *et al.,* (2018) interpreted that in rice, vegetative growth continues upto 40˚C day temperature but floret development is extremely sensitive to temperatures exceeding 35˚C. High night temperatures adversely effect the yield than the day temperatures. Heat stress results in floret sterility due to deprived anther dehiscence, defective pollination and aberrant pollen germination. Imbalance in protein expression, abandoned biosynthesis, partitioning and translocation of soluble sugars, imbalance in phytohormones release, and loss of pollen water content leads to decreased pollen viability. Response of rice to heat stress is through adjustment of various physiochemical mechanisms like growth inhibition, leaf senescence. Antioxidant enzymes, calcium and iron also plays a vital role in handling heat stress.

2.5.3. Spikelet Fertility:

Cheng *et al.,* (2009) conducted a pot experiment with four different treatments that include two levels of CO2 (ambient, 380 ppm; and elevated, 680 ppm) and two levels of night temperatures (22 and 32 ˚C) for understanding the impact of both high night temperature and increased CO2 concentration at the time of reproductive growth on rice growth and spikelet fertility. Results indicated increased plant and stem dry weight in both the conditions, while elevated CO2 concentrations increased and high night temperature decreased ear dry weight. Only the high night temperatures lead to the decrease in fertilized spikelet number. Elevated concentrations of CO2 increased individual grain weight.

Mohammed *et al.,* (2010) explained the multiple rice yield determining parameters affected by night temperatures and spikelet position. These multiple yield parameters included productive tillers number, spikelet sterility, grain length, width and weight. Rice plants were grown in green house under normal (27 ◦C) and high night temperature (32 ◦C) conditions, using infrared heaters. A decreased yield of about 90% was observed under high night temperature, which was affected by spikelet sterility (61%), and grain length (2%), width (2%), and weight. The resulted spikelet sterility in rice under high night temperature was due to decreased pollen germination and increased respiration rates. Along with high night temperatures, spikelet position on the panicle is also considered as a factor for determining yield in rice as spikelets at tip weighed more than basal spikelets on panicle.

Van Oort *et al.,* (2014) reviewed the available fertility functions i.e fertility as a function of panicle temperature and proposed a simple and new model integrating insights on diurnal temperature patterns and relative humidity. Their model also includes recent studies on relationship between panicle temperature and sterility. Their study included the impact of environmental conditions on flowering time. Model is emphasized for typical arid and typical humid conditions. Differences in simulation fertility up to 59% was observed due to the presence of large differences between existing fertility functions and simulated fertility. As transpirational cooling was ignored, it led to the overestimation of spikelet sterility to 14-73%. A reduction in sterility of about 7-35% was observed by shifting the flowering times from 12:00 to 9:00. They concluded that any impact of climate change simulation is highly dependent on fertility function choices.

Nguyen *et al.,* (2014) constructed, calibrated and validated a simulation model for predicting spikelet sterility in rice caused under heat stress conditions. The model consists of equations for estimating the probability distributions of panicle heading dates in field, spikelet flowering date on panicle and spikelet flowering time during day, and two sterility response functions to temperature at the time of meiosis and spikelet flowering time. Peak heading of panicle was observed after 4-6 days from initial heading and lasted for 12 days after heading. Date of panicle heading distribution was fitted into Poisson’s equation. The spikelet flowering peak on panicle was quite good fitted into normal distribution function, opening time of flower followed normal distribution with standard deviation around 1 hour, the fertility responses to high temperature during meiosis and anthesis were fitted into logistic functions of heating degree day above 31˚C on 12 days before anthesis of spikelet and temperature of air at time of spikelet flowering, respectively. Hence the model consisting of above equations is used to predict the response of spikelet fertility / sterility to air temperature with precision.

Wu *et al.,* (2016) investigated the phytohormonal changes in rice under heat stress and their impact on yield and other attributes, by growing four rice varieties (Nagina22, Huanghuazhan, Liangyoupeijiu, and Shanyou 63) in pots and treating them with 3 high temperatures under greenhouse conditions for 15 days at early reproductive growth phase. Nagina22, huanghuazhan, and liangyoupeijiu showed decreased yields due reduced spikelet fertility, spikelets per panicle, and grain weight. Active cytokinins, gibberellin A1 (GA1), and indole-3-acetic acid (IAA) were reduced as a result of heat stress and abscisic acid (ABA) and bound cytokinins in young panicles were increased. Correlation analysis suggested that yield is closely associated with changes in phytohormones, under heat stress.

Ishimaru *et al.,* (2016) facilitated breeding towards heat-resilient rice development in terms of spikelet sterility and quality of grain. In recent years identification of rice cultivars with heat tolerance at different growth stages was done. In these cultivars a trait named as early morning flowering (EMF) trait, effectively escapes heat as it shifts the time of day flowering to cooler early mornings. Some genetic resources for EMF were found in wild rice accessions. Heat-tolerant japonica type rice cultivars were found in which white-back type of chalky grains (WBCG) formation was mitigated. Mapping of QTLs related to heat tolerance at flowering, EMF and for WBCG in grainquality was done on rice chromosomes.

Fu *et al.,* (2016) reported that inferior spikelets of rice has lower ferlity and kernel weight compared to the superior spikelets under heat stress. Two rice plants having different heat tolerance were exposed to 40˚C during anthesis, to analyse the mechanisms responsible for the above mentioned problem. The results determined that fertility and kernel weight greatly decreased in superior spikelets as compared with inferior onesand this was due to their different organ temperatures. The superior spikelets had more organ temperatures. Under heat stress, both the superior and inferior spikelets fertility and kernel weight decreased due to reduced panicle number per plant, which in turn increases canopy temperature. Thus a rice plant with upright growth habit and loose panicles would be more heat stress susceptible because of higher canopy and spikelet temperatures.

Sehgal *et al.*, (2018) investigated on the effect of drought and heat stress on seed filling in food crops. They stated that even if these abiotic stresses affect the crop growth at all stages but the effect is critical at the seed filling phase of the reproductive growth. Seed yields are considerably affected by reduced seed size and number, ultimately affecting '100 seed weight' which is taken as the commercial trait. Various processes like production and translocation of photoassimilates , biosynthesis of seed reserves and minerals by importing precursors, occurring in leaves influence seed filling. Due to the involvement of different enzymes and transporters present in leaves and seeds, in all the processes of seed filling it is severely affected by heat stress.

Zhang *et al.,* (2018) studied the role of auxins for elongation of pollen tube in pistils, leading to successful pollination in plants. Heat stress treatment of 40 °C for 2 hrs was given to two rice genotypes with differeing heat tolerance, after flowering. Under heat stress, nipponbare (NPB) and high temperature susceptible (HTS), its mutant showed sharply reduced spikelet fertility. In mutant HTS, 1-naphthaleneacetic acid (NAA) reversed the spikelet sterility induced by stress. In NPB, pollentubes were visible in ovule and that of HTS were not visible. When the application of auxin NAA was done, we found pollen tubes of HTS in ovule. Hence it was concluded that heat tolerance enhancement by NAA in plants was accomplished by increasing the auxin levels, promoting elongation of pollen tube in pistil.

Lawas *et al.*, (2018) explained the association of spikelet sterility and grain yield with failure of exertion of panicle or sheathed panicle (SP) phenotype in rice. They identified a unique phenotype with sheathed panicle (cv. Sathi) with potential of maintaining normal seed set and grain weight under stress (38°C for 20 d). Inspite of the high temperature of the spikelet tissues (38˚C - 40˚C) during anthesis, a higher number of pollen count and the germinated pollen on the stigma was observed which was more than the required threshold for successful seed set. They recorded the lipid peroxidation and carbohydrate metabolism enzymes during grain filling. Under heat stress they were decreased, suggesting that the minimum impact of heat stress on carbohydrate enzymes leads to normal sucrose unloading and starch synthesis in grain. This results in normal grain size under prolonged exposure of sheathed panicle (SP) phenotype to heat stress.

2.5.4. Chlorophyll content:

Cui *et al.,* (2006) evaluated the process of photosynthesis and functionality of PS-II in response to heat stress in two tall fescue (*Festuca arundinacea*) cultivars namely Jaguar 3 brand (heat-tolerant) and TF 66 (heat-sensitive). Reduction in net-photosynthesis was caused in two cultivars under heat stress because of both stomatal ans non-stomatal limitations, increase in photo-inhibition and reduction in Rubisco activity. Functionality of PS-II was modified in two plant leaves under stress which was manifested by lower variable chlorophyll fluorescence yield (Fv), maximal PS-II photo-chemical efficiency (Fv/Fm), open reaction center efficiency in light (*Φ*PSIIopen). Their results reported that high temperature stress caused decrease in chlorophyll a+b and chlorophyll/carotenoid ratios.

Veerasamy *et al.,* (2007) investigated the exogenous application of zeatin riboside (ZR), a synthetic cytokinin on leaves of creeping bent grass (Agrostis stolonifera L.) under heat stress and their effects. The plants were grown in growth chamber with control (20/15 8C, day/night) and heat (35/30 8C) treatments. Before heat stress treatment, 10 µmol ZR was sprayed on foliage for 3 days and then during 35 days of heat stress treatment, it is sprayed for once in a week. In normal conditions of heat stress without ZR, there is decline in Leaf chlorophyll content, photochemical efficiency (Fv/Fm). In heat stress conditions with ZR application, high chlorophyll content and Fv/Fm. Hence, the exogenous ZR application mitigated the negative heat stress effects like chlorophyll degradation and lower photochemical efficiency.

Song *et al.,* (2013) explained how photosynthesis was effected by the application of nitric oxide exogenously in rice seedlings under heat stress. There was an indicative decrease in net photosynthetic rate (PN) because of non-stomatal components, under heat stress. This high temperature induced reduction of PN was alleviated by the application of nitric oxide donors like sodium nitroprusside

(SNP) or S-nitrosoglutathione (GSNO). Measurement of fluorescence of chlorophyll under high temperature reported that there is indicative increase in initial fluorescence (Fo) and non-photochemical quenching (NPQ), while maximal fluorescence (Fm), maximal PS-II photochemistry efficiency (Fv/Fm), efficiency of actual PS-II (ӨPSII), and photochemical quenching (qp) decreased. SNP or GSNO pre-treatment led to the mitigation of increased Fo and decreased Fm, Fv/Fm, UPSII, qp. It also reported the elevation of NPQ values. Their results suggested that application of NO can protect the process of photosynthesis from damage caused due to heat stress.

Sailaja *et al.*, (2015) deciphered the response of elevated temperature in 11 mega rice cultivars that are popularly grown in India by multiple organisational analysis considering physiological, biochemical and molecular levels. Many traits along with photosynthesis were studied at both vegetative and reproductive stages, which has an established correlation with the yield under heat stress. Their results, evaluated nagina22 among all the 11 genotypes, as best performer in consideration with yield as well as all other physiological, biochemical and molecular traits. Nagina22 exhibited lesser decrease in chlorophyll content, lesser decrease in net photosynthetic rate and other parameters like photosynthetic / fluorescence was also reduced, leading to least reduce of spikelet fertility and grain yield under stress.

Wang *et al.*, (2018) reported chloroplasts as metabolic centers and their role in developing physiological adaptiveness to heat stress. During this adaptive physiological process occurring in chloroplasts, metabolic reprogramming occurs and heat shock proteins protecting other proteins at heat stress are expressed. Heat stress induces inhibition of photosynthetic activity in plants by damaging different key components functioning in metabolic processes with collateral biomass production reduction leading to low crop yield. They focused on the events like chlorophyll breakdown, reactive oxygen species generation, protein turnover etc through extensive and metabolic reprogramming under heat stress .This kind of diverse metabolic reprogramming in chloroplasts is a requisite for systemic acquired adjustment to heat stress.

Sangwan *et al.,* (2018) conducted experiment in wheat fields of CCSHAU, Hisar and reported the drastic effect of high temperature on chlorophyll, which can also be reflected by normalized difference vegetative index (NDVI). Their study evaluated the content of chlorophyll with the use of SPAD and NDVI with the Green Seeker™ Handheld Optical Sensor Unit on bread wheat recombinant inbred lines (RILs) that were derived from WH1021 (heat tolerant) and WH711 (heat sensitive) genotypes of wheat. Assessment of RILs was done under different sowing dates i.e normal and late sowing and the measurements were taken at anthesis and 10days after anthesis. Indicative negative effect is seen in leaf chlorophyll content due to heat stress. As the temperature raises, a decrease in NDVI and average chlorophyll content was observed.

2.5.5. Proline content:

Ahmed *et al.,* (2011) investigated the proline content of wheat seedlings as a screening criteria for heat stress. For measuring seedling proline content, 20 different genotypes of wheat were grown at 25℃ and 35℃ in phytotron. Membrane stability was also calculated in addition with proline for screening them into tolerant and susceptible varieties. The wheat genotypes recording <50% membrane injury were considered under heat tolerant (HT) group and the genotypes recording >50% membrane injury were considered as heat sensitive (HS). The HT group genotypes, at high temperature produced almost double the proline content (>200%) than normal temperature while the proline content of HS genotypes was less at 35℃ when compared with HT genotypes. Membrane injury % and proline content of seedlings at 35℃ maintained an indicative negative correlation among all genotypes of wheat tested.

Han *et al.,* (2013) reported the impact of heat stress on proline, total soluble sugars and MDA in different lettuce varities at seedling stage. The seedlings at 4-5 leaf stage are subjected to heat treatments of 25/20℃ (d/n), 30/25℃ (d/n), 38/33℃ (d/n), 42/37℃ (d/n) by placing them in artificial climate boxes. After 3 days of heat stress, leaf samples were collected for various analysis. The results showed the progressive increase of proline and total soluble sugars in lettuce seedlings at high temperatures of 38/33℃ (d/n). Proline has a kind of osmotic adjustments and increase of proline content under stress leads to plant resistance by augmenting the organisms adaptability to environmental stress. Results depicted that non-heat resistant varieties proline content increased rapidly when compared with heat-resistant varieties.

Gosavi *et al.,* (2014) studied the heat stress effect on different physiological and biochemical parameters of drought susceptible, tolerant and wild sorghum genotypes at seedling stage. Under heat stress, proline accumulation was significantly increased in seedlings when compared with control. As heat stress produces oxidative damage as secondary stress, sorghum seedlings can cope up with that by higher anti-oxidant enzyme activities under stress. Wild sorghum genotypes showed higher accumulation of proline and anti-oxidant enzymes in stressed conditions and used for transferring heat stress tolerance trait by breeding programmes. They are also used for mining of genes responsible for heat tolerance. In above studies mean fold increase of proline accumulation was recorded higher in drought tolerant (30%), wild genotypes (23%) and lower in drought susceptible (18%) genotypes.

Harsh *et al.,* (2016) conducted an experiment for finding out the impact of short term heat stress in moth bean. As the heat stress causes an array of changes at physiological, biochemical and molecular levels, in this experiment the effect of short term heat stress on antioxidants and osmoprotectants was found in 37 genotypes (32 mutants and 5 varieties) of moth bean. Short term heat stress was imposed by placing the 7 day old seedlings in hot air oven at 42˚C for one hour. After 3 days of heat exposure, analysis of different parameters was done. Results showed the indicative over-accumulation of proline under heat stress conditions.

Sarkar *et al* (2016) aimed at studying the elevated temperatures effect on wheat seedlings of four different cultivars namely gayetri (GY), gandhari (GN), kedar (KD), PBW343 in consideration with tolerance / susceptibility to stress, biochemical and anti-oxidative responses. They exposed the cultivars seedlings to various temperatures from 25°C to 40°C for 6 h. GN was found as heat tolerant, GY and KD were moderately tolerant and PBW343 was heat sensitive, as per the calculated heat susceptibility index. Upto 35 °C, there was an increase in the compatible osmolytes like proline and total sugar content in cellular compartments. These results suggested that wheat can tolerate oxidative stress induced by heat up to certain period, which was illustrated by increase of osmoprotectants like proline and non-enzymatic oxidants under stress.

Mishra *et al.,* (2018) deciphered the over-expression of *OsSIZ1* rice gene in Arabidopsis improving all the drough, heat and salt stress tolerance. A post translational modification named sumoylation, affects plants cellular processes through conjugation of small ubiquitin like modifier (SUMO) to substrate target protein. One of the important cellular functions that SUMO conjugation regulates is responding to different abiotic stresses due to environment. A crucial step in sumoylation pathway is facilitated by SIZ1, a SUMO E3 ligase. In this report it has been demonstrated that over expression of *OsSIZ1* rice gene in Arabidopsis led to increased multiple abiotic stress tolerance. Further analysis confessed that plants over-expressing *OsSIZ1* showed higher transcript levels of *P5CS*, proline biosynthesis gene under environmental abiotic stresses. Here proline acts as osmoprotectant to mitigate damage due to heat and other stresses.

2.5.6. Malondialdehyde (MDA) content:

Kazim *et al.,* (2013) carried out an experiment for screening 8 local Pakistan rice accessions during their early growth stages like germination and seedling stages for heat stress by exposing them to heat shock temperature of 42±3°C for various time periods (24, 48, 72 h). Thermo-tolerance at germinating stage was explored using promptness index (P.I) and germination stress index (G.S.I), while relative membrane permeability assessed by electrolyte leakage, content of melondialdehyde (MDA) and hydrogen peroxide were used at seedling stage. It’s been reported that during germination stage, heat stress resulted in delayed germination and lower germination percentage. Under stressed conditions there was more leakage of electrolyte when compared with normal, as it is associated with lipid peroxidation level and hydrogen peroxide that increases under stress. Thus MDA content increases relatively more in heat susceptible varieties than heat tolerant varieties. Among the 8 Pakistan rice varieties, Kanwal-95 showed more thermo-tolerance.

Wilson *et al.*, (2014) evaluated fifty *Brassica juncea* genotypes at four day old seedling stage for heat stress considering biochemical components. These four day old seedlings were given heat treatment of 45 ºC for about 4.5 hrs and estimated survival percentage, leakage of electrolytes and chlorophyll content. In these genotypes lipid peroxidation was determined by MDA content. In stressed conditions, tolerant genotypes recorded MDA of 4.66 (MDA g-1 f. wt. of tissue) while susceptible genotypes recorded 7.44 (MDA g-1 f. wt. of tissue). During heat stress, ROS are produced as secondary stress which react with unsaturated fatty acids in membranes and results in lipid peroxidation, which leads to MDA accumulation.

Zafar *et al.*, (2017) conducted an experiment for exploring the heat tolerance mechanisms in rice during early seedling stage. Heat stress treatment of 45±2°C was given for 12 hrs in 46 rice genotypes that include 39 super basmati mutants (M5generation) and 7 varieties. The heat stress is followed by recovery of 3 days under normal temperature (28±2°C). The heat tolerance of genotypes was determined using various parameters including morphological changes, physiological and cell membrane thermostability, photosynthetic pigments and malondialdehyde content. They identified 15 rice genotypes (11 mutants and 4 varieties) as heat tolerant based on relatively lower MDA content. The increased levels of MDA under stress conditions when compared with normal conditions determined higher peroxidation of lipid membrane due to stress. Basmati-385 recorded lowest MDA content (31.74 µM/g f. wt.) under high temperature indicating least peroxidation of lipid membranes under stress.

Borriboon *et al.*, (2018) reported that decline in rice productivity is a major concern due to this high temperature in thailand. They investigated the content of hydrogen peroxide, malondialdehyde (MDA) and membrane stability from seedlings of rice, grown from seeds which are exposed to various temperature treatments (25°C, 35°C and 40°C) for short (1 week) and long (2 weeks) duration before germination. They designed the experiment in RCBD containing 6 replications with 3 rice cultivars Dular, KDML105 and Riceberry. Among these riceberry produced large numder of normal seedlings under both short and long term treatment of heat, while KDML105 produced more percentage of abnormal seedlings at high temperature. Both electrolyte leakage and MDA content determine the membrane stability under heat stress. Abnormal seedlings of riceberry recorded the highest MDA content after exposing to heat stress for two weeks.

Sharma *et al.,* (2018) aimed at deciphering the heat stress effect on wheat varieties and their different mechanisms adopted to encounter the stress. Different wheat varieties were grown on MS media and they are subjected to continuous high temperatures for 5 days after plantlets were appeared. Antioxidative enzyme activity was increased significantly under stress in wheat varities. After stress application, the high content of MDA and proline in these varieties suggested that they are suited better to overcome the injury due to oxidative stress.

2.6. Expression analysis of candidate genes responsible for heat stress:

Agarwal *et al.,* (2003) narrated isolation and characterization of HSP101 gene from rice. Southern analysis determined that there seems to be a single gene per haploid genome coding for HSP101 protein in rice. Northern analysis showed that HSP101 transcript expression is induced by heat and the induction is transcient. Western analysis reported that this protein is rapidly expressed during stress period and retained for longer duration in japonica rice types compared to indica rice types during post-stress recovery period. OsHSP101 gene constituted 5 exons and 4 introns as per the comparative analysis of nucleotide sequences of HSP101 cDNA and the gene. Analogous to rice, Athsp101 has 4 introns in the genomic clone. They were the first one to report that plant HSP100 protein expression correlates disappearance of protein granules in yeast cells and also reported distinct rice-type dependent protein expression pattern.

Hu *et al.,* (2009) examined, heat stressed rice seedlings global expression profiling and compared their results with previous data available under other abiotic stresses. These comparision led to the conclusion that, while most HSFs and HSPs had highly similar and overlapped response and regulation patterns under different stresses, some of those genes exhibit significantly specific response to different stress. Distribution of 99 different heat stress genes were marked on 12 chromosomes of rice. 5 different heat shock gene families were indicated by different colours on chromosomes of rice in figure 2.8 below.

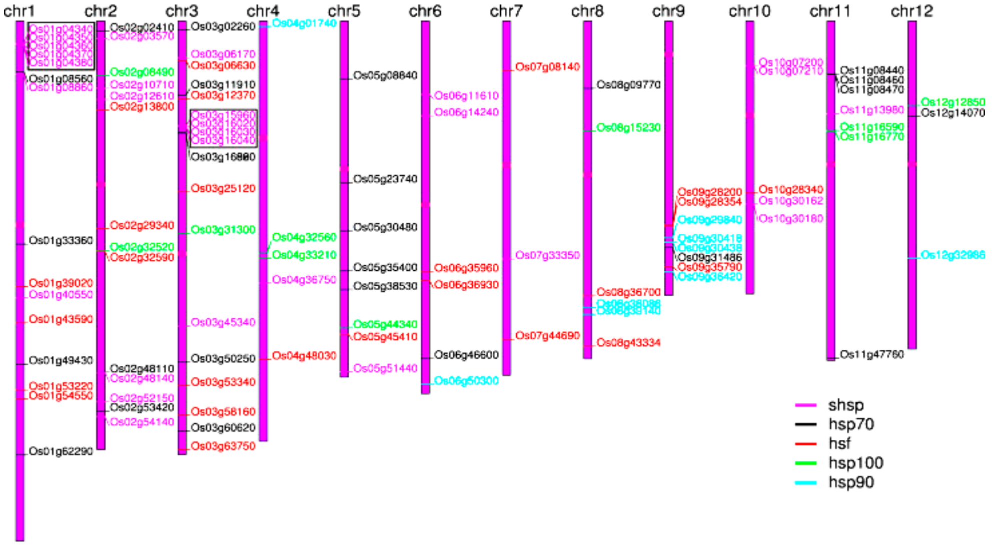


Fig. 2.8 Chromosome distribution of heat stress transcription factors and heat shock proteins. Two tandem clustered genes of sHSPs are indicated by black boxes at chromosomes 1 and 3. [Hu *et al.*, (2009)]

Zou *et al.*, (2011) reported that heat stress response in rice is complicated, involving up- or down-regulation of numerous proteins related to different metabolic pathways. Protection proteins, proteins involved in protein biosynthesis, protein degradation, energy and carbohydrate metabolism, and redox homeostasis come under this list. In addition, enhanced thermotolerance in transgenic rice was obtained by over expression of rice genes and genes from other plants. On other side, heterologous expression of some rice proteins led to high thermotolerance in bacteria and other easily transformed plants. They gave the proteomic characterization of rice in response to heat stress. The table 2.2 below shows some transgenic rice with increased tolerance to heat.

Table 2.2: Transgenic rice with increased heat tolerance.

Gene	Protein	Source	Comments	References
AtHsp101	HSP101	Arabidopsis thaliana	Pusa basmati 1; CaMV 35S promoter;Transgenic plants showed increased tolerance to high temperature.	Katiyar-Agarwal *et al.,* (2003
mtHsp70	HSP70	Oryza sativa	Nipponbare; CaMV 35S promoter; mtHsp70 over-expression suppresses PCD by maintaining 4wm and preventing ROS signal amplification	Qi *et al.,* (2011)
sHSP 17.7	HSP17.7	Oryza sativa	Hoshinoyume; CaMV 35S promoter;Transgenic plants exhibited significantly increased thermotolerance	Murakami *et al.,* (2004)

Jung *et al.*, (2012) performed genome wide transcriptome analysis of rice to identify strongly induced immediate early genes due to high temperature stress. Using the NSF45K array they compared the effects of high temperature (37˚C) treatment (for 1 hr) of seedlings relative to control (28˚C) and identified 710 genes that show at least 2 fold up-regulation at both time points. Among them they defined 244 genes as early heat stress responsive groups and 238 genes as prolonged heat stress responsive groups. As per Gene Ontology (GO) enrichment analysis, chaperon-mediated protein folding cofactor was the most over-represented GO term in association with prolonged heat stress, while enrichment of processes involving cell redox homeostasis, intracellular protein transport and regulation of transcription (DNA dependent) in association with early heat stress response. In addition, they also observed strongest correlation between heat and drought. Amongst the genes upregulated in both heat and drought, *OsNAC6/SNAC2 (Os03g60080)* is induced by cold, drought and high salinity. Hence this gene was suggested as good target gene for developing crop with increased tolerance to multiple abiotic stress conditions. HSP10/CPN10 family developed specialized functions to get over prolonged heat stress only, while HSP20 family genes evolved to combat with multiple abiotic stress. Figure 2.9

below, shows the expression pattern of early and prolonged heat stress genes responsive to other abiotic stresses.

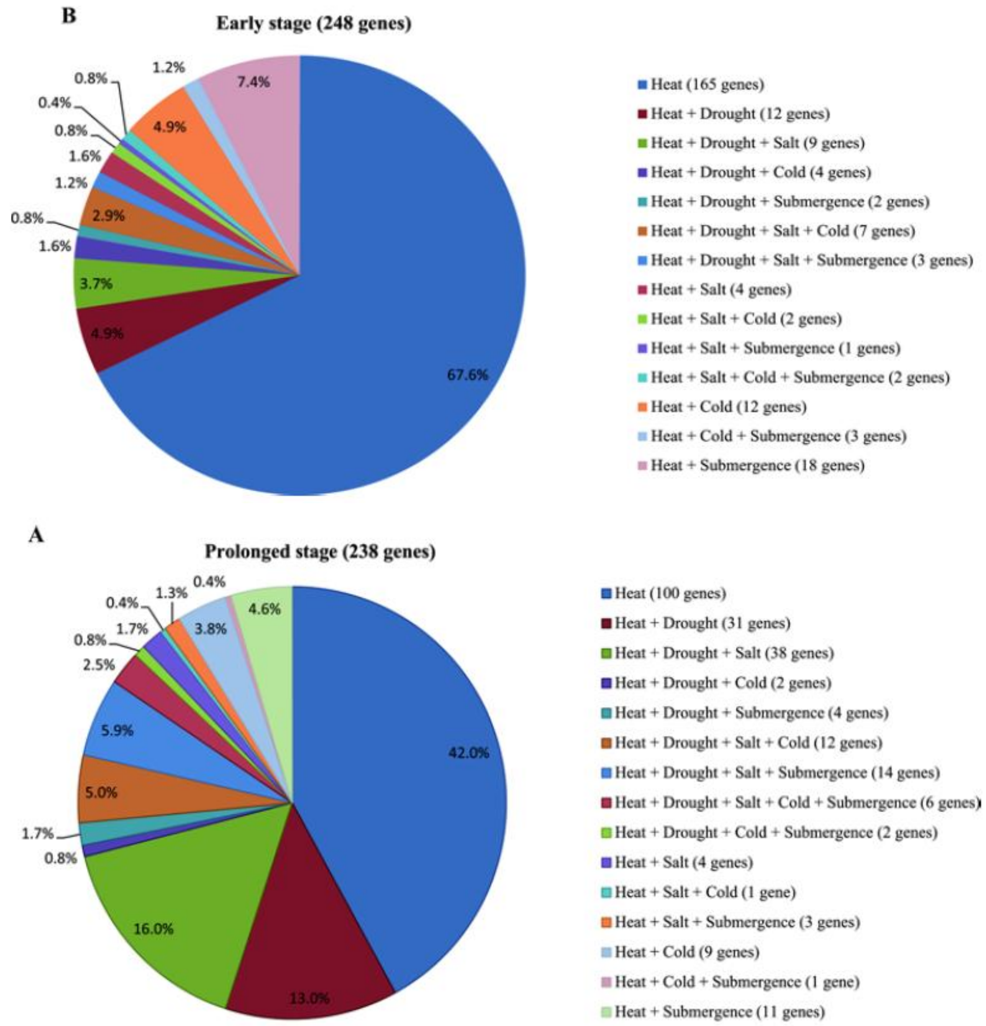


Fig 2.9 Expression patterns of early and prolonged heat stress responsive genes in response to other abiotic stress. [Jung *et al.*, (2012)]

Cao *et al.*, (2013) investigated the differential gene expression analyses in rice leaves of 21 days old seedlings subjected to different heat stress by performing cDNA-AFLP, to identify transcripts induced by heat stress. They were further verified by real-time RT-PCR. In total, more than 3000 different fragments were identified. Among them 49 fragments have been selected for sequencing and those differentially expressed genes are classified into different groups. 6 out of these 49 fragments were measured using real time RT-PCR. In addition they also conducted high performance liquid chromatography (HPLC) to analyse variations in 3 different polyamines like Putrescine, Spermidine, Sperimine at heat stress conditions. The transcripts of 6 gene fragments responded to heat stress, that are measured using real time RT-PCR are *Os04g0429600*, *Os06g0124900*, *SRP14*, *Os02g0611200*, *Os02g0788800*, and *Os07g0620200*. Among them *Os06g0124900* gene was observed to show the pronounced effect, it increased more than twice both in heat sensitive "Shuanggui 1" and heat tolerant "Huanghuazhan".

Hasanuzzaman *et al.*, (2013) studied that the ability of a plant to perceive the high temperature stimulus, generate and transmit the signal and to initiate the respective biochemical , physiological changes determines the plant survival under heat stress. At molecular level, alterations in the gene expression responsible for osmoprotectants, detoxifying enzymes, regulatory proteins and transporters is involved. The below figure 2.10 explains schematic flow of heat induced signal transduction.

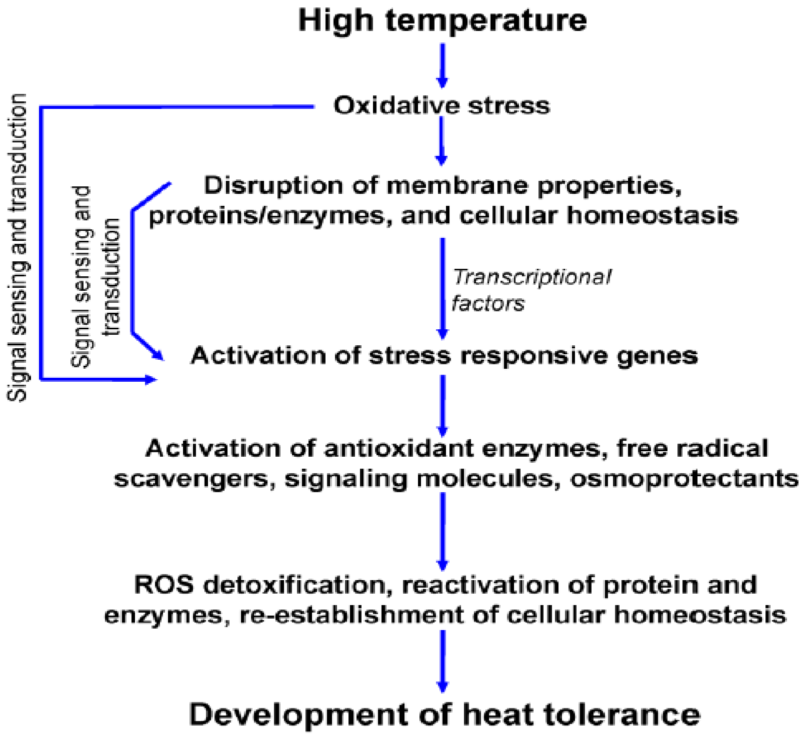


Fig 2.10 Schematic flow of heat induced signal transduction mechanism and heat tolerance development in plants. [Hasanuzzaman *et al.*, (2013).]

Qu *et al.*, (2013) elaborated on the heat stress related response networks in plants, in conjunction with Hsf and Hsp response pathways, ROS response and hormones network. Heat stress response elements production during particular physiological periods of plants is also interpreted. Heat stress leads to osmotic and oxidative secondary stresses. So to resist heat stress, production of many enzymes, non-enzymes, antioxidants and hormones occurs through heat stress response networks. Figure 2.11

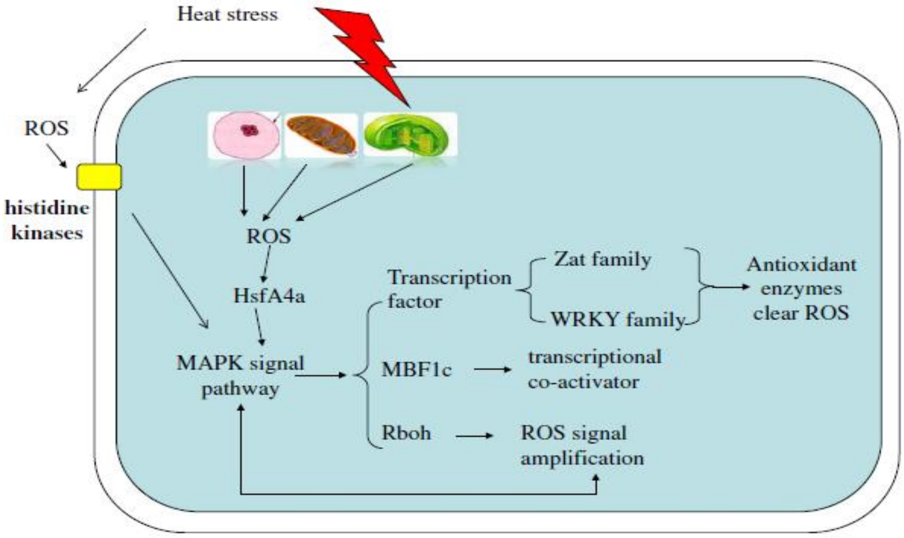


Fig. 2.11 The response of ROS. During heat stress, endogenous and exogenous ROS accumulates. Endogenous ROS are sensed through histidine kinases, and HsfA4a act as the sensors of exogenous ROS. Subsequently, the MAPK signal pathway is activated and induces the expression of transcription factors, MBF1c and Rboh. The transcription factors, including the Zat and WRKY families, induce the expression of antioxidant enzymes, and MBF1c acts as a transcriptional co-activator. Rboh enhances the production and maintenance of ROS signaling. **[Qu *et al.*, (2013)].**

Li *et al*., (2013) worked on switch grass (*Panicum virgatum L*.) which is considered as a model herbaceous bio-energy crop, for its rapid growth rate, reliable biomass yield, water and nutrients minimal requirement, adaptability of growing on marginal lands and widespread distribution throughout North America. They reported on switch grass physiology, cell wall composition and biomass yield under high temperature. Switch grass cultivar Alamo was subjected to long-term heat stress treatment (38°/30°C, day/night, for 50 days) and observed significant decrease in plant height and total biomass in the heat stressed plants when compared to controls. Transcriptome analysis with switchgrass Affymetrix gene chips was done using total RNA from control and heat stress plants followed by normalization and pre-processing, leading to the identification of 5365 probesets as differentially expressed using a 2-fold cutoff. Among them, no.of probesets up-regulated were 2233 (2000 switchgrass unigenes) and 3132 probesets (2809 unigenes) were down-regulated. From this list, using RT-PCR, differential expression of 42 randomly selected genes were validated. Rice orthologs were restored for 78.7% of the switchgrass probesets that are heat stress responsive. As per Gene ontology (GOs) enrichment analysis using AgriGO program genes related to ATPase regulator, chaperone binding, and protein folding were significantly up-regulated while those related to protein modification, transcription, phosphorus and nitrogen metabolic processes, were significantly down-regulated by heat stress. This study identified conceivable connections between the identified GOs, physiological responses and heat response phenotype in switch grass plants. Comparative transcriptome analysis in response to heat stress among four monocots – switch grass, rice, wheat and maize was done and it resulted in the identification of 16 common genes, most of which are related to the process of protein refolding in stress conditions. The figure 2.12 Shown below describes the comparison of heat stress transcriptomes among 4 monocots.

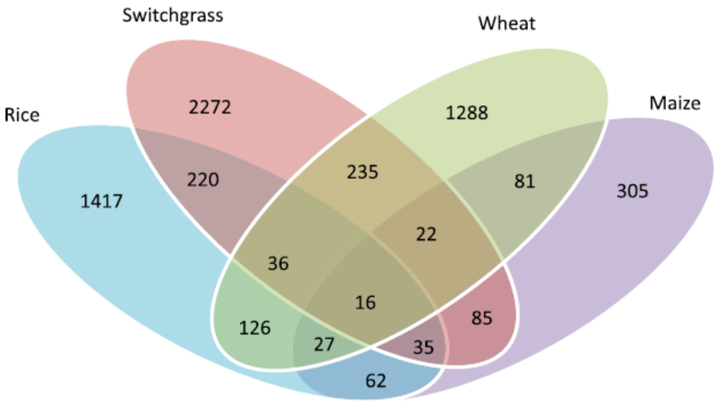


Fig 2.12 Comparision of heat stress transcriptome of switchgrass, rice, wheat and maize showing 16 common genes, most of which are responsible for protein refolding. [Li *et al.*, (2013)]

Rajaram *et al.*, (2014) studies reported the heat shock responses in cyanobacteria, role and regulation of molecular chaperons. The key to stress adaptability of cyanobacteria is its ability to maintain the protein conformational homeostasis (folding – misfolding – refolding or aggregation – degradation). Unlike in other bacteria, cyanobacteria posses several genes that encode DnaK and

DnaJ family proteins. Hsp60 family proteins and some small Hsps are more prevalent during heat stress. In unicellular cyanobacteria like Synechocystis and Synechococcus, the HSP100 (ClpB) family proteins and some small Hsps contribution towards heat stress has been elucidated. Their study also documented the regulation of chaperon genes by many cis-elements and trans-acting factors. Some heat shock protein genes and their role in cyanobacteria is given in the table 2.3, below.

Table 2.3. HSP genes and their role in cyanobacteria.

Family	Gene	Cyanobacteria	Function	Reference(s)
Hsp100	clpBI, clpBII	Synechococcus	Acquired thermotolerance, cold tolerance	Eriksson & Clarke (1996), xPorankiewicz & Clarke (1997)
Hsp90	htpG	Synechococcus	Innate and acquired thermotolerance, protection of photosynthetic apparatus	Tanaka & Nakamoto (1999), sato et.al (2010)
Hsp60 Hsp10	groEL-1, groEL-2 (cpn60)	Synechocystis, Anabaena	Thermotolerance, GroEL: nitrogenfixing conditions; Cpn60: nitrogen replete conditions.	Chaurasia & Apte (2009),Rajaram & Apte (2008)
sHsp	hsp16.6 /hsp17/ hspA	Synechocystis, Synechococcus	Protects membrane fluidity, thermotolerance	Nakamoto *et al.,* (2000),Horva´th *et al.,* (1998),Lee *et al.,* (2000)

Usman *et al.,* (2015) conducted experiments to study the mechanisms for heat tolerance in chilli pepper. To determine these mechanisms, they evaluated six genotypes for cellular membrane thermo-stability(CMT) and HSP70 gene expression. In most of the genotypes membrane integrity was not damaged by high temperature treatment (50˚C), which was indicated by mean value of CMT. As per this, genotypes are classified as heat-tolerant (greater than 60%), moderately tolerant (30% to 60%), and susceptible (less than 30%). The heat-tolerant plants, AVPP0702, AVPP0116, and AVPP9905, recorded the highest CMTs at 89.27%, 88.03%, and 85.10% respectively. Under heat stress, they observed a significant increase in levels of HSP70 genes after 2h of 42˚C treatment indicating that this is quickly and sharply promoted by heat shock. Among all the six genotypes considered for the study, ‘ kulai’ showed the down-regulation of HSP70 gene and was found to be heat-sensitive genotypes. In eukaryotes, HSP70 is one of the most abundant HSPs. It binds to hydrophobic patches of partially unfolded proteins, in an ATP-dependent manner preventing the protein aggregation. Even if accumulation of HSP70 takes place during heat shock, they need their constitutively expressed cognates(HSC70) for general cellular functions, as they control protein homeostasis. They aid in the folding of nascent polypeptides releasing from ribosome and sorting of proteins to cellular organelles by interacting with mitochondrial and chloroplast protein import complexes and also form a link with ubiquitin-mediated proteasomal degradation pathway.

Chen *et al.,* (2016) elucidated molecular pathways contributing to heat sensitivity of critical developemental stages like transitions from syncytium to the cellularization stage during endosperm formation in young seeds. A set of putative imprinted genes, showing down-regulation under severe heat stress were identified using transcriptomic comparison of seeds exposed to moderate(35˚C) and severe heat stress (39˚C) with control (28˚C). Under moderate and severe heat stress, several type I MADS box genes specifically expressed during syncytial stage were differentially regulated. Precocious and delayed cellularization under moderate and severe stress are associated with suppression and over accumulation of these genes. They showed that rice seed size is regulated by modulating the expression of OsMADS87, one of the heat sensitive, imprinted genes affiliated with syncytial stage endosperm. Transgenic seeds lacking OsMADS87 show increased endosperm cellularization and also have decreased sensitivity to moderate heat stress concerned with seed size reduction when compared with seeds from wild type plants and plants over-expressing OsMADS87. The figure 2.13 Given below shows transitions in endosperm formation.

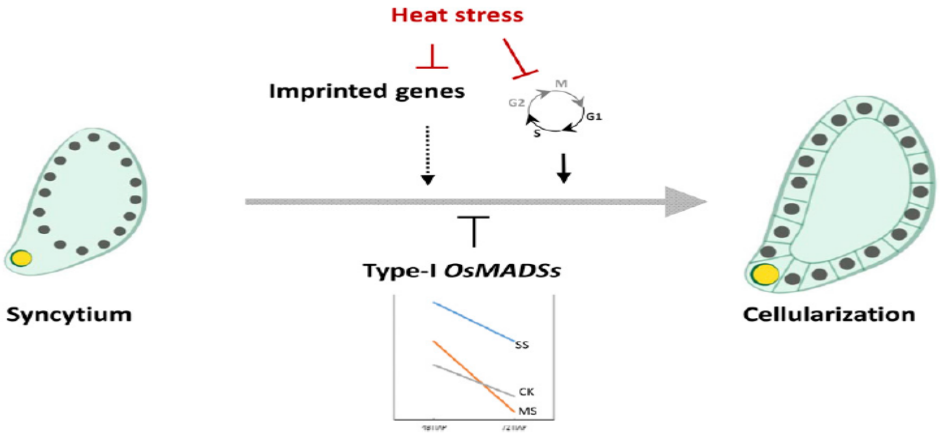


Fig 2.13 Proposed model incorporating main genes and pathways that are impacted when young rice seeds are exposed to heat stress. Arrows and T-bars indicate signalling promotion and inhibition, respectively. CK, MS, and SS indicate nonstressed, moderatestressed, and severe-stressed conditions, respectively. **[Chen *et al.*, (2016)].**

Kumar *et al.*, (2017) conducted proteomic analysis of heat sensitive rice genotype IET 21405 and revealed a total of 73 protein spots in rice leaf using two dimensional electrophoresis (2-DE) and MALDI-TOF/MS based proteomic approaches. Functional assessment of these proteins stated that, among these 73, few proteins are expected to involve in photosynthesis, tricarboxylic acid (TCA) cycle, and for energy production. The major role in acquiring heat stress response and thermotolerance is accumulation of HSPs under control of HSFs. These HSPs functionally act as molecular chaperons, repair and aid in renaturation of stress-damaged proteins. Distribution pattern of identified 73 proteins under high temperature, according to their biological function is shown in the figure 2.14, below. Mainly proteins found are functionally related to defense followed by energy metabolism, trafficking and signal transduction.

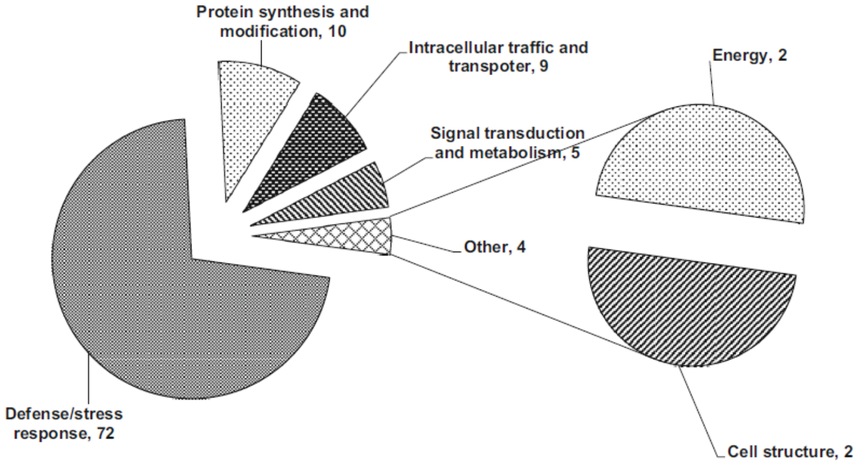


Fig 2.14 Distribution pattern of proteins under stress according to their biological function. [Kumar *et al.*, (2017).]

Su *et al.*, (2018) reported in vivo heat-regulated RNA structuromes. They obtained structural data on >14,000 mRNAs by applying structure-seq chemical [dimethylsulfate (DMS)] structure probing to rice (Oryza sativa L.) seedlings with and without 42 °C heat shock for 10 minutes. They have shown that RNA secondary structure broadly regulates gene expression in response to heat stress. Their results revealed RNA unfolding over biological temperature range, by significant heat induced ascent of DMS reactivity in the global transcriptome. They have found that increase in heat-induced DMS reactivity correlates with significant decrease in transcript abundance, as quantified from RNA-seq time course. This finally indicated that mRNA unfolding promotes transcript degradation and the basic mechanism for this outcome appears to be mRNA unfolding at both 5′ and 3′-UTRs facilitating access to RNA degradation machinery.

Vishwakarma *et al.*, (2018) constructed suppression subtractive hybradization (SSH) library in heat and drought stress tolerant Indian bread wheat cultivar C306, to generate a genetic resource of heat stress responsive genes/ESTs. Two subtractive cDNA libraries were prepared from RNA isolated from 93 days old plants at grian filling stage treated with 37˚C and 42˚C. Obtained ESTs were reconfirmed by using reverse northern dot blot hybridisation. A total of 175 contigs and 403 singlets were obtained from 1728 ESTs by gene ontology analysis. A transcript showing homology to *Hsp90* was observed to be up-regulated(7.6 fold) under heat stress by validating with qRT-PCR. *TaHsp90* heterologous expression was validated in *E. coli* BL21 and was confirmed by protein gel blot and MALDI-TOF analysis. The developed heat stress responsive SSH library led to single out a number of heat responsive genes/ESTs used for untangling the heat tolerance mechanism in wheat.

Nevame *et al.,* (2018) expressed that chalkiness in rice is occurred due to genetic and environmental factors, notably high temperature(HT). Many grain qualities are compromised due to heat stress, transparency being the most important among them. In rice, chalkiness is commonly studied in combination with other quality traits like amylose content, gel consistency and protein storage. They presented some of the relatively stable QTLs related to chalkiness, amylose content and gel consistency. Stable QTL distribution and availability of potential material resource is regarded as a expediency to find out novel stable QTLs related to chalkiness under heat stress. Hence to alleviate the high temperature effect , pyramiding of relatively stable and non-allelic QTLs regulating low chalkiness endosperm is done into adaptable rice varieties.

Kilasi *et al.,* (2018) aimed at identifying QTLs for tolerance of vegetative stage under heat stress and corresponding candidate genes in rice, as heat stress tolerance is a multigenic trait and candidate genes are known poorly. They generated single nucleotide polymorphic (SNP) markers by using genotyping-by-sequencing and genotype 150 F8 recombinant inbred lines (RILs) gathered by crossing heat tolerant "N22" and heat susceptible "IR64" varieties. 4,074 high quality SNP markers corresponding to 1,638 recombinationally unique events from mapping population were used to construct a linkage map. They identified 6 QTLs for root length and 2 for shoot length under control conditions with 2.1-12% effect. One QTL *rlht5.1* was determined for "root length under heat stress," with 20.4% effect. Four QTL were diagnosed for "root length under heat stress as percent of control" that illustrated the total phenotypic variation from 5.2 to 8.6%. Three QTL with 5.3–10.2% effect were classified for "shoot length under heat stress," and seven QTL with 6.6–19% effect were described for "shoot length under heat stress expressed as percentage of control." Amidst the QTL identified six were overlapping between those selected using shoot traits and root traits: two were overlapping between QTL identified for “shoot length under heat stress” and “root length expressed as percentage of control” and two QTL for “shoot length as percentage of control” were overlapping a QTL each for “root length as percentage of control” and “shoot length under heat stress.” Genes coding 1,037 possible transcripts were identified based on their location in 10 QTL regions for vegetative stage heat stress tolerance. Among these, according to previous research in the literature, 213 transcript annotations were reported to be connected to stress tolerance. transcription factors, chaperone proteins (e.g., alpha-crystallin family heat shock protein 20 and DNAJ homolog heat shock protein), proteases, protein kinases, phospholipases, and proteins related to disease resistance and defense and several novel proteins are included in the putative candidate genes currently annotated as expressed and hypothetical proteins.

Soda *et al.,* (2018) reported that cytoskeleton plays a crucial role in stress tolerance; however, entanglement of intermediate filaments (IFs) in stress tolerance response remains ambiguous in crop plants. Clear evidence related to the unique involvement of Ifs in cellular protection against abiotic stresses in rice was provided in their study. Under different abiotic stress, abundance of transcript with genes encoding *Oryza sativa* intermediate filament (*OsIF)* showed 2-10 fold up-regulation. In transgenic rice, over expression of *OsIF* increased tolerance to salinity and heat stress, where as its knock-down (KD) effected plants towards more sensitivity. This indicates IFs role in promoting survival under stress. In the presence of high salt and heat stress, seeds of *OsIF* over expressed rice showed normal germination, better growth, ultra-structure of chloroplast was maintained and favourable K+/Na+ ratio when compared with wild type and KD plants. In the *OsIF* over expressed rice, better performance of both photosystem I and II was suggested based on photosynthesis analysis and chlorophyll a florescence data. Higher accumulation of proline and trehalose was revealed by metabolite profiling showing 2-4 fold increase, in over expressing rice than wild types. Thus under stress conditions, IFs overexpressing plants could maintain significantly high yield. In over expressed IFs seedlings, higher accumulation of compatible solutes takes place resulting in maintenance of osmotic homeostasis, organelle ultra-structure which corresponds to better performance of photosynthetic machinery. In future, better insight into the re-orientation of intermediate filaments under stress and their involvement in cellular machinery protection is provided by live cell imaging studies of OsIF:GFP (green fluorescent protein) fusion protein. Alisoltani *et al.*, (2018) reported that it is essential to have a targeted selection scheme to identify functional biomarkers than the selection of SSR's randomly. They aimed at developing a new method for identifying functional SSR markers using the available rice and wheat RNA-seq data under heat SSR stress and obtained unique SSR patterns. They revealed that GC/GC and GCC/GGC repeats were significantly more common under heat stress conditions on comparison with controls of both wheat and rice samples. Interestingly, genes containing these motifs participated in abiotic stress responses and included various heat shock proteins (HSPs) and DREB/CBF proteins. Their study suggested the possibility of using genes with altered SSRs as functional markers. Work flow of plant RNA-seq SSR analysis is shown in the figure 2.15 Below.

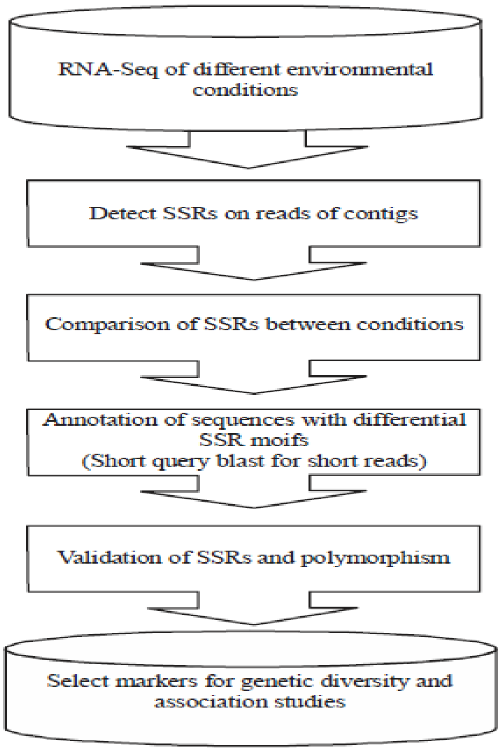


Fig 2.15 Work flow of plant RNA-seq SSR analysis. [Alisoltani *et al.*, (2018).]

CHAPTER-III

MATERIALS AND METHODS

The present investigation on **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)*"** was performed during the year 2018-2019 at Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, India. This chapter elaborates the experimental material and methods adopted during the course of the analysis. The detail of the materials used, methods adopted were presented as under the individual headings and sub headings.

3.1. Experimental site

The experiment was conducted in the green house facility and different laboratories of Department of Plant Molecular Biology & Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur.

3.2. Experimental material

The experimental material for the present investigation includes fourteen diverse genotypes of rice belonging to landraces, established varieties, and susceptible varieties from different research stations of Chhattisgarh. The details of these rice genotypes used in this study are presented in Table 3.1

Table 3.1: Different rice genotypes used in this experiment.

SN	Genotype	SN	Genotype
1	GP-145-103	8	Annada
2	SL – 62	9	Poornima
3	Dagad Deshi	10	Karma mahsuri
4	Nagina -22 (Check)	11	ARB – 6-11
5	Swarna	12	GP -145 -40
6	GP – 145-55	13	MTU-1010
7	CGZR -1	14	RRF-127

3.3. Experimental Design

The experiment consisting of fourteen rice genotypes was laid out in completely randomized design (CRD) with two replications for each experimental unit. The mean value work out from the measurements recorded on three randomly selected plants for different characteristics used for statistical analysis. The data collected was analyzed using analysis of variance (ANOVA) technique. OPSTAT statistical software package was used for this purpose (Sheoran *et al* 1998).

3.4. Experimental details

The present study was aimed to investigate the differential expression of various candidate genes that are responsive under heat stress conditions in rice. Semi-quantitative method of expression analysis was conducted for understanding the gene response in heat stress conditions. This was also followed by phenotypic and biochemical characterization of rice under heat stress. The whole work involves comparison of various biochemical and phenotypic characters with gene expression under heat stress and concluding that particular changes in biochemical and phenotypic characters under heat stress are due to up-regulation of few genes.

3.4.1 Phenological and Biochemical characterization of rice genotypes under heat stress.

3.4.1.1 Methods

Stress treatment:

Heat stress is given at the end of vegetative stage, before the panicle initiation of plants. Summer field conditions were mimicked by flooding the trays with water and increasing the green house temperatures. The green house temperature is increased gradually from 30 ºC at 6:00 am to 42 ºC at 11:00 am. Constant temperature of 42 ºC was maintained for 6 hrs continuously. The rice plants were treated with heat stress until 17:00 pm, with gradual adjustments to the greenhouse’s temperature down to 28 ºC–30 ºC at night (18:00 to 6:00). This stress is given continuously for 6 days from the beginning of the late vegetative stage. Leaf samples were collected after stress treatment from control and stress plants for further studies.

3.4.1 a) Membrane Stability Index-[MSI] :

Membrane stability index is measured by electrolyte leakage, at high stress stage when temperature becomes high enough to cause heat hardening of leaves.

1. Leaf samples were collected from control and stress conditions of all the fourteen genotypes.
2. Weigh 1g of leaf samples and wash it with deionized water to remove the residues present on them.
3. Cut the washed leaf samples into small pieces of 1cm and place in testtubes containing 15ml deionozed water and incubate at 24˚C for 12h.
4. Electrical conductivity (EC1) of the solution was measured using conductivity meter.
5. Subsequently, the samples were autoclaved at 120˚C for 20min and then cooled to room temperature.
6. Now the final Electrical conductivity (EC2) of the solution was measured.

Electrolyte leakage from the leaf samples is calculated by the formulae

$$\text{Electrolyte leakage (EL \%)} = \frac{EC1}{EC2} \text{ X } 100$$

3.4.1 b) Pollen fertility :

Pollen fertility is calculated using staining methods with the help of stains like aectocarmine (2%) or tetrazolium.

1. Pollen is collected from the 6 anthers of all the genotypes from control and stress conditions, and they are stored in ethanol (70%) to arrest the stage.
2. Then the anthers are punched using needle and pollen is distributed on the microscope slide and place a drop of acetocarmine (2%) dye.
3. Cover the slide with cover slip and observe under microscope at 40x just after preparation of slide.
4. The deeply stained / normal looking pollen grains are counted as the viable pollen and the colourless / shrivelled pollen are counted as the non-viable pollen.

The pollen fertility is calculated using the formulae

$$\text{Pollen fertility (\%)} = \frac{no.of\ viable\ pollen}{total\ no.of\ pollen\ in\ the\ microscopic\ field} \text{ X } 100$$

3.4.1 c) Spikelet fertility :

spikelet fertility at maturity was used to screen heat tolerance. It is calculated by counting the empty and filled grains.

1. Three randomly selected matured panicles were harvested from all the fourteen genotypes in stress and control conditions.
2. The harvested panicles were manually threshed and the number of filled, unfilled and total no.of grains per panicle are recorded.
3. These readings were taken by pressing each floret between forefinger and thumb to determine if the grain is filled or not. Both partially and fully filled spikelets were categorized as filled spikelets.

Spikelet fertility is calculated as shown below

$$\text{Spikelet fertility (\%)} = \frac{no.of\ filled\ grains}{total\ no.of\ grains\ formed\ (florets)} \text{ X } 100$$

Spikelet fertility is calculated in control and stress conditions. The negative effect (% decrease from control) was determined in every genotype.

3.4.1 d) Determination of chlorophyll by Acetone method :

The chlorophyll (Chl) content of leaves was estimated both in control and stress plants of all the fourteen rice genotypes following the method of Arnon (1949).

Reagents:

80% Acetone – The reagent is prepared by adding 80ml Acetone and 20ml distilled water.

Procedure:

1. 0.1 g of leaf tissue was cut into small pieces and homogenized with mortar and pestle in 2 ml of 80 % acetone.

2. The homogenate was centrifuged at 5000 rpm for 5 minute and the supernatant is collected in a volumetric flask.

3. The residue is re-extracted with 2 ml of 80% Acetone and centrifuged. Supernatant is collected in the same volumetric flask.

4. The procedure was repeated until the residues turned colourless. The final volume is made up to 10 ml.

5. The absorption of solution was measured at 663 nm, 646 nm, and 470 nm against the solvent (80% Acetone) blank using spectrophotometer (Biomate 5, Thermo Electron Corporation).

The amount of the chlorophyll present in extract was estimated as mg chlorophyll per gram of tissue using following formula-

$$\text{mg chlorophyll a/g leaf tissue} = 12.7\,(A663) - 2.69\,(A645)\ \text{x}\ \frac{V}{1000\ X\ W}$$

$$\text{mg chlorophyll b/g leaf tissue} = 22.9\,(A645) - 4.68\,(A663)\ \text{x}\ \frac{V}{1000\ X\ W}$$

$$\text{mg total chlorophyll /g leaf tissue} = 20.2\,(A645) + 8.02\,(A663)\ \text{x}\ \frac{V}{1000\ X\ W}$$

Where,

A = absorbance at specific wave length

V = final volume of chlorophyll extract in 80 % acetone

W = fresh weight of tissue extracted

Estimation of Chlorophyll Stability Index

The chlorophyll stability index (CSI) of fourteen rice genotypes in both control and stress conditions was determined according to Sairam *et al.* (1997) and calculated as follows:

$$\text{CSI} = \frac{total\ \ chlorop\,hyll\ \ under\ \ stress}{total\ \ chlorop\,hyll\ \ under\ \ control}\ \text{X}\ 100$$

3.4.1 e) Determination of Proline by Acid Ninhydrin method :

The free proline content in the leaf tissues of fourteen rice genotypes in both stress and control conditions were estimated as per the method given by Bates *et al*., (1990).

Reagents:

1. 6M Orthophosphoric acid – Take 38.1ml of orthophosphoric acid in 100ml DW

2. 3% Aqueous sulphosalicylic acid – Add 3gm of sulphosalicylic acid in 100ml DW
3. Glacial acetic acid
4. Toluene
5. Proline
6. Acid ninhydrin – heat 1.25gm of ninhydrin in 30ml glacial acetic acid and 20ml 6M phosphoric acid on hot top magnetic stirrer until dissolved.store at 4˚C and use within 24hrs.
7. Standards – dissolve 0.1gm proline in 100ml DW. Prepare 10 concentrations by taking 1-10ml from proline solution and make up volume to 100ml by DW. Now take 1ml each from above concentrations separately and add 1ml sulphosalicylic acid. To this add 2ml glacial acetic acid and then add 2ml acid ninhydrin.

Procedure:

1. 0.5 gm of fresh leaf tissue was ground and homogenized in a mortar and pestle with 10ml of 3% (w/v) aqueous sulphosalicylic acid.
2. The homogenate was filtered through Whattman no.2 filter paper.
3. 2 ml of filtered extract was taken for analysis in test tube and 2ml of glacial acetic acid and 2 ml of ninhydrin were added.
4. The reaction mixture was incubated in boiling water bath for 1 hour.
5. Terminate the reaction by placing the test tube in ice bath.
6. 4 ml of toluene was added to the reaction mixture and it was vortexed for 1-2 minute.
7. A pinkish red coloured toluene layer was formed on top. The layer was separated carefully in another tube and warm to room temperature.
8. Optical density was measured at 520 nm using toluene as blank by UV-visible spectrophotometer (Biomate 5, Thermo Electron Corporation).
9. A series of standard was run with pure proline in a similar way, using DW as blank and standard curve was prepared.

10. The amount of free proline in test sample was calculated from the standard curve using following formula and expressed in fresh-weight basis (µ mol/g f.wt.).

$$\mu \text{ moles per g tissue} = \frac{\mu\text{g proline } /\text{ml X ml toluene}}{115.5} \text{ X } \frac{5}{g\ sample}$$

where, 115.5 is the molecular weight of proline.

3.4.1 f) Determination of MDA enzyme :

MDA content (an indicator of lipid peroxidation) was calculated from all fourteen genotypes of rice under both stress and control conditions using method given by Heath and Packer (1986).

Reagents:

1. 0.1% W/V TCA – Take 0.1ml of TCA in 100ml DW.
2. 0.5% TBA in 20% TCA – Add 20ml TCA to 100ml DW and then dilute 0.5gm TBA in this 20% TCA.

Procedure:

1. 1gm of leaf tissue is homogenized by adding 5ml, 0.1% (w/v) trichloro acetic acid (TCA).
2. Centrifuge the homogenate for 10mins at 15000 rpm at 4˚C.
3. Collect supernatant and mix 1ml of supernatant with 4ml of 0.5% thiobarbuteric acid (TBA) diluted in 20% TCA. Now incubate in waterbath at 95˚C for 30mins.
4. End reaction by incubation the tubes on ice. Incase, the solution is not clear then centrifuge for a further 5mins at 15000 rpm at 4˚C.
5. Measure the absorbance at 532 and 600nm.

OD 600 values are substracted from the MDA-TBA complex values at 532nm and MDA content is calculated using the Lambert – Beer Law with an extinction coefficient $\mathcal{E}^{M}$ = 155 m$M^{-1}Cm^{-1}$.

$$\text{nmoles per 1ml of reaction mix} = \frac{A532-A600}{155} \text{ x } 1000,$$

as we took reaction mix of 5ml, multiply the values by 5

Results are represented as nmoles MDA g^{-1}FW. Where, FW = fresh weight.

Statistical analysis:

The experiment consisting 14 rice genotypes was laid out in completely randomised design (CRD) with three replications for each experimental unit. The mean value work out from all the measurements recorded on three randomly selected plants for different characteristics were used for statistical analysis. The data collected was analyzed using analysis of variance (ANOVA) technique. OPSTAT statistical software package has been used for this purpose.

3.4.2 Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.

3.4.2.1 Collection of leaf tissue and isolation of RNA:

Leaf samples of fourteen rice genotypes were collected from both control and stress induced plants.Leaves of control and stress plants harvested after 6th day of stress implosion. After collection, samples were snap frozen in liquid nitrogen and kept in a -80°C freezer for total RNA extraction.

3.4.2.2 RNA extraction:

RNA was extracted from the collected plant tissues by following combined procedure that employed both Trizol reagent and spin Columns (Trizol + spin protocol). The protocol was standardized in the laboratory to obtain quality RNA from leaves of rice. In this protocol, tissues were homogenized initially in Trizol reagent followed by precipitation and purification.

The RNA precipitate was purified by a column based method using Qiagen’s Plant RNAeasy Mini kit. Detailed stepwise procedure is described below. The extracted RNA was checked on denaturing formaldehyde agarose gel (FA gel) for checking its quality and integrity. Quantitative assessment was done on Nano drop spectrophotometer (Thermo scientific, ND1000).

Materials used:

- Liquid nitrogen along with container and dispenser
- Mortar and pestle
- Trizol reagent
- Chloroform Isoamyl mixture (*i.e. CIA* 24:1)
- Isopropanol
- Ethanol (Absolute ethanol and 70% ethanol)
- RNA isolation kit (Qiagen)
- DEPC treated water
- Refrigerated centrifuge
- Vortex mixture
- Eppendorf tubes
- Filter tips, pippette *etc.*

Preparation of utensils for RNA Extraction:

1. Mortar pestles and other glassware used for RNA extraction were soaked in 30% Hydrogen Peroxide for 15 minutes. Further, they were rinsed with DEPC water and autoclaved at 121˚C for 15mins. Then they were baked overnight in hot air oven.
2. R*NAS*e free 1.5 ml microcentrifuge tubes and R*NAS*e free filter tips were used every time.

Preparation of platform for RNA Extraction:

1. Clean the work area by spraying RnaZap over the platform and spread blotting paper.

2. Wipe the autoclaved motar and pestles using RnaZap. Before starting the procedure RnaZap is sprayed on hands covered with glouses and rubbed against each other.

RNA Extraction Procedure:

1. The collected samples were cut into fine pieces.
2. Tissue samples were crushed to fine powder by using liquid nitrogen.
3. The homogenate up to 0.5ml was transferred to RNASe free eppendorf tubes.
4. Add 700 µl of Trizol reagent to the sample and vortexed well.
5. Add 700 µl of chloroform: isoamyl alcohol mixture and samples were vortexed again. Samples were then allowed to sit at room temperature for 10 minutes.
6. Samples were centrifuged at 13000 rpm for 10 minutes under 40C.
7. Transfer 600 µl of top aqueous phase carefully into clean 1.5 ml eppendorf tubes.
8. Add 600 µl of isopropanol in to the samples and mix by inverting twice to precipitate RNA.
9. The entire precipitate was transferred to RNeasy mini spin column sitting in a 2 ml collection tube.
10. The Rneasy column was centrifuged at 10,000 rpm for 15 sec.
11. The flow through was discarded and RNeasy mini spin column (retaining RNA) was reused by placing it in fresh 2ml collection tube.
12. The column was then washed with 700 µl RW1 buffer by centrifuging at 10,000 rpm for 15 sec.
13. 500 µl of RPE buffer was added to RNeasy mini spin column followed by centrifugation at 10,000 rpm for 5 sec.

14. The flow through was discarded and Rneasy spin column was then placed in a new 1.5 ml collection tube.

15. About 30-50 µl (depending on expected yield) of RNASe free water was added to the Rneasy spin column and centrifuged at 10,000 for 1min.

16. The isolated RNA was stored immediately at -20 ºC.

- **NOTE**: All centrifugation steps involving spin column must be followed at room temperature.

3.4.2.3 cDNA synthesis:

RNA isolated and quantified was used for cDNA synthesis using Bio-RAD iScriptTM cDNA Synthesis kit as per manufacturer’s instructions. Reaction mix and temperature profile for cDNA synthesis are presented in Table 3.2 and 3.3. The kit consisted of following components and is suitable for 1pg to 1µg of RNA. Kit components include following items:

1. 5X iScript Reaction Mix
2. iScript Reverse Transcriptase
3. Nuclease free water

Procedure for cDNA synthesis:

1. Thaw the template RNA samples on ice. Workout the amount/ volume of RNA required reaching 1 µg concentration and adjusting the volume of water accordingly. Dispense the required quantities of RNA and water in each tube (0.2 ml capacity).

2. Prepare the master mix on ice by mixing the mentioned amounts of 5x iScript reaction mix, iScript Reverse transcriptase, nuclease free water.

3. Mix by pipetting.

4. Dispense 10 µl of master mix into each tube containing RNA samples and water. Mix by gentle tapping and spin down the contents.

5. Incubate the tubes in PCR machine on reverse transcription cycling program described in table 3.3.

Table 3.2: cDNA synthesis (reverse-transcription) reaction components.

Component	Volume per reaction	Final concentration
5x iScript reaction mix	4 µl	1x
iScriptReverseTranscriptase	1 µl	-
Nuclease free water	Variable	-
RNA template	1-51 µl	1 µg
Total volume	20 µl	

Table 3.3: Reverse transcription cycling program

Step	Temperature	Time	No.of cycles
Priming	25˚C	5min	1
Reverse transcription	46˚C	20min	1
RT inactivation	95˚C	1min	1
Optional step	4˚C	Hold	

Quantification and dilution of cDNA:

Quality and quantity of cDNA were estimated by measuring the absorbance using Nanodrop spectrophotometer (ND1000). Two microlitre of DNA was placed over tip of Nanodrop to record absorbance at 260nm. The absorption ration (A260/ A280) and (A260/ A280) was recorded for each sample to estimate quantity and purity of cDNA. cDNA was diluted to uniform concentration of about 50 ηg/µl using Milli Q distal water.

3.4.2.4 Semi quantitative RT- PCR based gene expression analysis:

Semi quantitative reverse transcriptase PCR was carried out to study the expression of heat stress responsive transcription factor genes in rice genotypes under control and heat stress conditions. The cDNA generated from the total RNA isolated from leaf tissues of fourteen rice samples in total (i.e control and stress) were subjected to semi-quantitative expression profiling in 20 µl reaction using gene specific primers. The resultant PCR product was then resolved on 1.5 % Agarose gel at 100V. The presence of amplicons and their respective intensity were recorded under gel documentation system. The expression was analyzed by comparing the relative fluorescent intensities of cDNA amplicons under gel documentation system. *OsActin* (primer designed from rice)was used as an internal control for normalization of RNA concentration. Details of PCR components and temperature profile are depicted below in table 3.4 and table 3.5.

Table 3.4. PCR components their quantity used for semi-quantitative PCR.

Components	Concentration	Quantity
cDNA	1000 ng/µl	2 µl
PCR Buffer	1X(1.5mM MgCl2)	2 µl
dNTP mix	200 µM each	2 µl
Primer Forward	10 µM	1 µl
Primer Reverse	10 µM	1 µl
Taq Polymerase	1U/ µl	0.5 µl
Nanopure water	-	11.5 µl
Total		20 µl

Table 3.5. Temperature profiles used for semi quantitative RT-PCR

Activity	Temperature	Duration	Cycles
Initial Denaturation	94 ˚C	1 min	1
Denaturation	94 ˚C	30 sec	
Annealing	Variable(56-64˚C)	45 sec	35
Extension	72˚C	30 sec	
Final Extension	72˚C	5 min	1
Store	4˚C	∞	1

3.4.2.5 Digitalization of expression data:

In order to evaluate gene expression pattern based on numerical values, the semi quantitative RT-PCR generated fluorescence data were digitalized using GelQuant.NET Analyzer software (*www.biochemlabssolutiond.com*). The relative expression of genes was expressed in terms of fold change for the samples under heat stress with respect to control conditions. The genes used in this study are given below in table 3.6 along with their specific forward and reverse primers, melting temperatures.

Table 3.6. Forward and reverse gene specific primers used for semi-quantitative RT-PCR amplifications:

S No.	Gene	Locus ID	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Tm (˚C)	Product size (bp)
1	OsHsp 16	LOC_Os06g14240	GTCTGCGTTAGCTCCTGAAG	ACCACATCCTTCTCCCTCTC	62	290
2	OsHsp 16.9	LOC_Os01g04270	GTGATGGCCAGTCAAGTAGA	CTGCATCTCTGTTGGATCAC	61	219
3	OsHsp DnaJ	LOC_Os01g01160	GAAGACAAGTCTGGCTGGAG	CACAGCACACCCTTCTAACC	62	246
4	17.9 kDa CI heat shock protein	LOC_Os03g15960	AGAAGGAGGAGGTCAAGGTG	AGATCTGGATGGACTTGACG	62	255
5	OsHsp 18	LOC_Os01g08860	AAGAAGAAGAGGCGATCGAG	CTTGATGTCGGAGGACTTGA	61	259
6	18.9 kDa heat shock protein	LOC_Os02g03570	AGCATGGACTGGAAGGAGAC	CTTCCTGTCGTTGTCCTTGG	63	291
7	OsHsp 26.7	LOC_Os03g14180	CGTGAGGGTTTAAGCAGTGT	AGCTCAGTGTCTCAGCCTTG	62	250
8	60 kDa chaperonin	LOC_Os10g32550	AGTTCGAGCCTCAGATGTTG	ACCAACTTCAGCTTCACTGG	62	239
9	OsHsf A4a	LOC_Os01g54550	AAGGATGCGTACTTCGTCAG	GGTAAGAAACTGCTGCCAGA	62	257
10	OsHsf A7	LOC_Os01g39020	GTGATGATGTCTGGGAAGAG	TCTCATGCCTCTAGTGTTCC	59	177

3.5 Details of commercial kits, laboratory tools and equipments used in this study

Table 3.7 List and details of laboratory tools and equipments used in the study

SN	Product	Make/ Manufacture
1	-86℃ Ultra low temperature frezzer	Haier Biomedical
2	-20℃ Deep freezer	Vestfrost
3	4℃ Cabinet	Cellfrost
4	Ice maker	SCOTSMAN FRIMONT AF100 AS-E 230/50/1
5	Magnetic stirrer	Remi
6	Refrigerated centrifuge	Sorvall, Thermo Fisher Scientific
7	Bench top microcentrifuge	1-14K Microfuge, Sigma
8	Vortex Mixture	Tarsons
9	Electronic weighing balance	Elico
10	Water bath	Yarco
12	pH meter	Tarsons
13	Pipetteman Set (All volume range)	Finnpipette, Thermo Fisher Scientific
14	UV Transulliminator	Macroview UV transilluminator, Hoefer
15	Tissue lyzer	Molbio
16	96 well thermal cycler	Veriti 96 well thermal cycler, Applied Biosystems
17	Gel electrophoresis system	Midi Submarine electrophoresis system, Tarsons
18	Gel documentation system	Chemi doc MP Gel imaging system, Biorad laboratories,
19	Spectrophotometer for DNA/ RNA quantification	Nanodrop ND1000 spectrophotometer, Thermo

Table 3.8 List and details of molecular biology reagents, commercial kits and enzymes used in the study

SN	Product	Make/ Manufacture
1	Phire hot start taq DNA Polymerase	Cat# F122S, Finnzyme, Thermo Life Science
2	PCR components (dNTPs, buffer, DMSO, MgCl2 *etc*)	Finnzyme, Thermo Life Science
3	RNA stabilization reagent,RNAlater	Cat# R0901, Sigma
4	Trizol reagent	Cat# 15596018, Invitrogen
5	DEPC Treated water	Cat# AM9906, Ambion
6	UltraPure Distilled water	Ref 10977-015, Invitrogen
7	RNA isolation kit	Cat# 74904, Rneasy plant mini kit, Qiagen
8	cDNA synthesis kit	Cat# 1708891, iScript^{TM} cDNA synthesis kit, BIO-RAD
9	1 Kb DNA ladder	New England Biolabs & Merck Bioscience (Genei)
10	6X Organge G gel loading dye	Cat# 612601580061730, Merck Bioscience (Genei)
11	Oligos/ primers, (OD- 3,scale-25 nM) with HPSF purification	Eurofins Genomics India Pvt Ltd.

CHAPTER – IV
RESULTS AND DISCUSSION

The present investigation under study entitled as **“Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)”*** was undertaken to understand the expression of known heat related genes using diverse rice genotypes. Further the correlation of genes expression levels with phenological and biochemical traits associated with the heat response in rice was also assessed.

4.1. Quantification of heat stress considering physiological and biochemical parameters.

Fourteen rice genotypes namely GP-145-103, SL-62, Dagad Deshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127, belonging to landraces, established varieties, susceptible varieties and advanced breeding lines were taken as the study material. All these genotypes were grown in trays under two different conditions i.e. control and heat stress by altering temperatures in green house. High temperature stress was given by mimicking the summer field conditions based on weather data of previous years with flooding water in trays.

The plant morphology of fourteen rice genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) at vegetative stage before panicle initiation under both stress and control conditions including the complete view of experimental set up of rice genotypes at vegetative stage under controlled conditions prior to induction of heat stress are shown in figure 4.1 and 4.2.

All the plants were induced stress treatment at vegetative stage before panicle initiation. High temperature treatment is given to the stress trays by adjusting the green house temperature to 42˚C at daytime, continuously for 6 days. After 6 days of high temperature treatment, three sets of leaf tissues were collected

For phenotypic, biochemical and expression studies. Likewise, three sets of leaves from control plants were also collected at same time.

Fig 4.1: Overall view of the experimental set up of 14 rice genotypes at vegetative stage before stress induction.

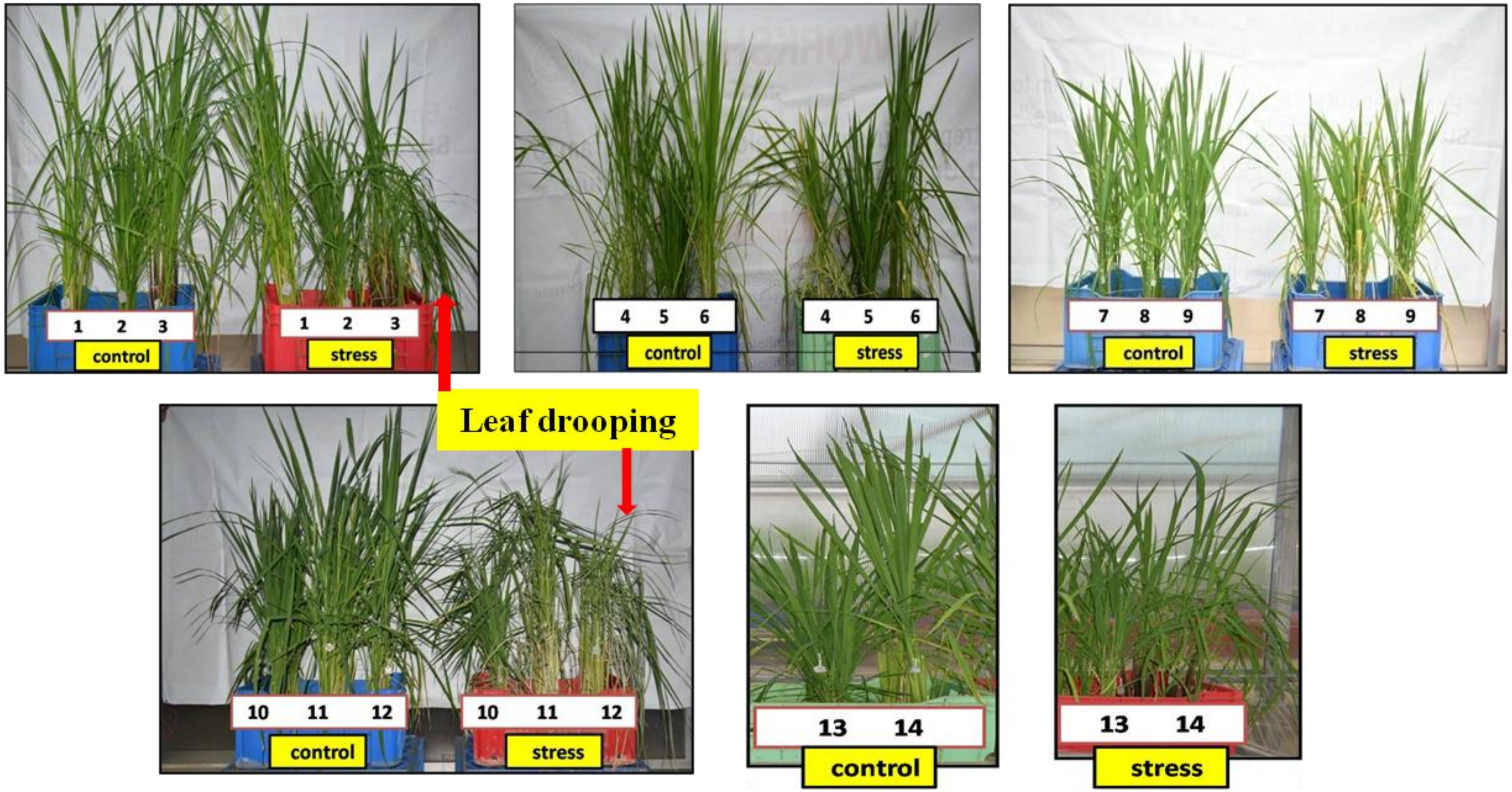


Fig 4.2: Plant morphology of 14 rice genotypes under control and stress conditions at vegetative stage before panicle initiation.1) DagadDeshi, 2) SL-62, 3) GP-145-103, 4) Nagina-22, 5) Swarna, 6) GP-145-55, 7) Poornima, 8) Annada, 9) CGZR-1, 10) Karma mahsuri, 11) ARB-6-11, 12) GP-145-40, 13) RRF-127, 14) MTU-1010.

4.1.1. Phenotyping of rice genotypes for heat stress related traits

4.1.1.1. Membrane stability index (MSI):

Uninterrupted function of cellular membranes is very important under stress conditions, for photosynthesis and respiration processes to occur precisely. Hence, cell membrane stability was reported to play critical role under conditions of high temperature, as a major component of heat tolerance. Membrane stability index is calculated by measuring the relative electrolyte leakage from cells due to injury occurred under stress. Electrolyte leakage is the measure of electrical conductivity of the tissues. Wide variation in the fold increase of membrane stability among the tested genotypes was recorded which ranged from 19.8% fold increase in RRF-127 to 292.9% in MTU-1010, given in the below table 4.1.

Table 4.1: Membrane stability index of fourteen rice genotypes under control and stress conditions

		MSI (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% increase**
1	GP-145-103	23.21	53.57	130.8
2	SL-62	23.52	55.86	137.4
3	DagadDeshi	33.92	83.49	146.1
4	Nagina-22	47.39	57.36	21.1
5	Swarna	15.08	23.66	56.9
6	GP-145-55	31.01	95.96	209.5
7	CGZR-1	26.36	40.00	51.7
8	Annada	40.64	69.00	69.8
9	Pornima	17.51	62.39	256.2
10	Karma mahsuri	28.28	36.07	27.5
11	ARB-6-11	22.73	67.97	199.1
12	GP-145-40	23.50	59.73	154.2
13	MTU-1010	21.00	82.50	292.9
14	RRF-127	25.00	29.95	19.8

Previous studies reported that heat tolerant genotypes show low levels of electrolyte leakage, indicating less injury to the membrane [Sarsu *et al.*, (2018); Sharma *et al.*, (2017); Hemantaranjan *et al.*, (2014)]. In the present study, the lowest levels of electrolyte leakage was observed in RRF-127 with 19.8% increase under stress when compared with control and reported as heat tolerant genotype followed by Nagina-22 (21.1%), Karma mahsuri (27.5%), CGZR-1 (51.7%) and Annada (69.8%) as shown graphically in figure 4.3.

4.1.1.2. Pollen fertility

High temperatures generally reduce the growth and production at all stages of the crop, but the effect is more pronounced on the yield reduction, when heat stress occurs at flowering stage (Prasad *et al.,* 2006; Mohammed and Tarpley 2011). Heat stress effects pollen development by decreasing the starch concentration before anthesis, which leads to decrease in sugar concentration in mature pollens, contributing to sterile pollen (Pressman *et al.,* 2002). Pollen fertility is calculated by using Acetocaramine dye. The fertile pollen are stained red and the non-fertile pollen stay transparent or unstained as shown in figure 4.4. Wide variation is observed in the pollen fertility of the tested genotypes under stress. The fold decrease in pollen fertility ranged from 14.4% in RRF-127 to 66.5% in MTU-1010as given below in table 4.2.

Table 4,2: Pollen fertility of fourteen rice genotypes under control and heat stress conditions

		Fertility (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% decrease**
1	GP-145-103	78	58	24.9
2	SL-62	57	28	50.2
3	DagadDeshi	51	20	60.5
4	Nagina-22	49	40	18.1
5	Swarna	71	37	47.9
6	GP-145-55	67	25	62.8
7	CGZR-1	53	33	37.9
8	Annada	50	36	28.3
9	Pornima	69	37	46.2
10	Karma mahsuri	79	54	31.4
11	ARB-6-11	83	30	63.3
12	GP-145-40	56	22	61.8
13	MTU-1010	54	18	66.5
14	RRF-127	85	73	14.4

Many studies reported that pollen fertility decreases under stress conditions and the genotypes showing lowest decrease in pollen fertility are considered as the tolerant ones for heat stress (Fahad *et al.*, 2018; Jagadish *et al.*, 2015; Kumar *et al.*, 2015). In the present study lowest decrease in the pollen fertility, under stress conditions was observed in RRF-127 (14.4%) followed by Nagina-22 (18.1%), GP-145-103 (24.9%), Annada (28.3%) and CGZR-1 (37.9%) shown graphically in figure 4.5.

4.1.1.3. Spikelet fertility

Heat stress affects spikelet fertility by decreasing pollen germination and increasing respiration rates, which subsequently reduces yield (Mohammed *et al.*, 2010). High temperatures reduce seed size and number, which ultimately affects '100 seed weight'. This occurs because high temperatures decrease translocation of photo assimilates, seed reserves biosynthesis that takes place in leaves during seed filling (Sehgal *et al.*, 2018). Wide variation is observed in the spikelet fertility of the tested genotypes under stress. The fold decrease in spikelet fertility ranged from 14% in RRF-127 to 71% in ARB-6-11 as given below in table 4.3.

Table 4.3: Spikelet fertility of fourteen rice genotypes under control and stress conditions

		Fertility (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% decrease**
1	GP-145-103	49	31	37
2	SL-62	54	28	48
3	DagadDeshi	49	19	61
4	Nagina-22	48	39	19
5	Swarna	47	35	26
6	GP-145-55	65	25	62
7	CGZR-1	36	22	42
8	Annada	76	56	25
9	Pornima	69	36	48
10	Karma mahsuri	77	53	31
11	ARB-6-11	79	22	71
12	GP-145-40	69	36	48
13	MTU-1010	52	13	56
14	RRF-127	83	71	14

Earlier studies reported the decreased spikelet fertility under heat stress conditions and the genotype having the lowest change in spikelet fertility under stress conditions when compared to control is considered as the tolerant genotype (Wu *et al.*, 2016; Fu *et al.*, 2016; Zhang *et al.*, 2018). In the present study, lowest decrease in spikelet fertility under stress conditions was observed in RRF-127 (14%) followed by Nagina-22 (19%), Annada (25%), Karma mahsuri (31%) and CGZR-1 (42%) shown graphically in figure 4.6.

4.1.2. Quantification of heat stress considering biochemical parameters

4.1.2.1. Chlorophyll content

Under heat stress conditions, for photosynthesis to take place at normal level, it requires maintenance of chlorophyll content. Studies by Ashraf *et al.*, (1994) revealed that degradation of chlorophyll (a, b, total) under abiotic stress is induced due to osmotic stress that results as secondary stress because of the occurrence of more active OH hydroxyl radical. There was a wide variation in the recorded chlorophyll content of heat stress leaves of all the fourteen genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) as mentioned in the table 4.4.

The mean chlorophyll a content under control conditions was recorded as 2.529 mg/g leaf tissue and it ranged from 1.610 mg/g in MTU-1010 to 3.410 mg/g in CGZR-1 (Table 4.7). While mean chlorophyll a content under stress conditions was recorded as 1.874mg/g leaf tissue and it ranged from 0.523mg/g in swarna to 2.743 mg/g in CGZR-1 (Table 4.8). Lowest fold decrease in chlorophyll a content was recorded in the genotype Nagina-22 (1.0 fold) followed by Karma mahsuri (1.0 fold), Annada (1.0 fold), GP-145-103 (1.2 fold) and CGZR-1 (1.2 fold) (Fig. 4.7a).

The mean chlorophyll b content under control conditions was recorded as 1.303mg/g leaf tissue and it ranged from 0.370 mg/g in GP-145-40 to 2.820 mg/g in GP-145-55 (Table 4.7). While mean chlorophyll b content under stress condition was recorded as 0.873mg/g leaf tissue and it ranged from 0.203 mg/g in RRF-127 to 2.300 mg/g in GP-145-103 (Table 4.8). Minimum fold decrease in chlorophyll b content was recorded in the genotype Annada (1.0 fold) followed by ARB-6-11 (1.0 fold), GP-145-103 (1.0 fold), Karma mahsuri (1.1 fold) and GP-145-40 (1.1 fold) (Fig. 4.7b).

The mean total chlorophyll content under control conditions was recorded as 3.831mg/g leaf tissue and it ranged from 2.023 mg/g in MTU-1010 to 5.820 mg/g in GP-145-55 (Table 4.7). While mean total chlorophyll content under stress conditions was recorded as 2.747 mg/g leaf tissue and it ranged from 0.853 mg/g in Swarna to 4.970 mg/g in GP-145-103 (Table 4.8). Lowest fold decrease in total chlorophyll content was recorded in genotype Annada (1.0 fold) followed by GP-145-103 (1.0 fold), Karma mahsuri (1.0 fold), GP-145-40 (1.1 fold) and CGZR-1 (1.2 fold) (Fig. 4.7c).

Table 4.4: Chlorophyll a, chlorophyll b and total chlorophyll content of fourteen rice genotypes under stress and control conditions.

	chlorophyll content	**chl a (mg/g)**			**chl b (mg/g)**			**total chl. (mg/g)**		
S. No	**Genotype**	**control**	**stress**	**fold decrease**	**control**	**stress**	**fold decrease**	**Control**	**stress**	**fold decrease**
1	GP-145-103	2.71	2.17	1.2	2.31	2.30	1.0	5.02	4.97	1.0
2	SL-62	2.99	2.34	1.3	2.15	1.29	1.7	4.83	3.63	1.3
3	DagadDeshi	2.40	1.72	1.4	1.29	0.84	1.5	3.69	2.56	1.4
4	Nagina 22	2.55	2.55	1.0	1.82	1.24	1.5	4.36	3.79	1.2
5	Swarna	1.72	0.52	3.3	1.05	0.32	3.2	2.76	0.85	3.2
6	GP-145-55	3.00	1.14	2.6	2.82	0.62	4.5	5.82	1.76	3.3
7	CGZR-1	3.41	2.74	1.2	1.44	1.11	1.3	4.85	3.89	1.2
8	Annada	2.69	2.68	1.0	0.62	0.93	0.7	3.32	3.21	1.0
9	Pornima	3.09	1.76	1.8	1.06	0.41	2.6	4.15	2.16	1.9
10	Karma mahsuri	2.54	2.55	1.0	1.93	1.80	1.1	4.47	4.34	1.0
11	ARB-6-11	2.70	2.20	1.2	0.49	0.48	1.0	3.19	2.69	1.2
12	GP-145-40	1.90	1.29	1.5	0.37	0.34	1.1	2.07	1.93	1.1
13	MTU 1010	1.61	1.54	1.0	0.41	0.34	1.2	2.02	1.62	1.2
14	RRF-127	2.60	0.93	2.8	0.48	0.20	2.4	3.08	1.13	2.7

4.1.2.2. Proline content

Proline is a cyclic amino acid synthesized from glutamate. Proline acts as a compatible solute, i.e. it can accumulate to high concentrations in the cell cytoplasm without interrupting cellular structure and metabolism (Yancey *et al.*,1982). The proline content of leaf tissues is estimated according to the method of Bates *et al.*, (1973). The result of the proline estimation is given in the table 4.5. There was a wide variation in the recorded proline content of all the fourteen genotypes.

The mean proline content under control conditions was recorded as 0.407 µ mol/g fresh weight and it ranged from 0.064 µ mol/g in GP-145-103 to 1.65 µ mol/g in GP-145-40 (Table 4.7), while the mean proline content under stress conditions increased to 1.213 µ mol/g fresh weight and ranged from 0.095 µ mol/g in SL-62 to 2.044 µ mol/g in GP-145-55 (Table 4.8). When comparing the fold increase of proline content in stress over control, the highest fold increase in proline content was recorded in the genotype Nagina-22 (20.6 fold) followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mahsuri (6.0 fold) as shown in figure 4.8.

Table 4.5: Proline content of fourteen rice genotypes under control and stress conditions

		Proline(µmole/g)		
S.No	**Genotypes**	**control**	**stress**	**fold increase**
1	GP-145-103	0.06	0.45	7.0
2	SL-62	0.06	0.10	1.5
3	DagadDeshi	0.09	0.38	4.1
4	Nagina22	0.07	1.40	20.6
5	Swarna	0.27	1.57	5.9
6	GP-145-55	1.00	2.04	2.0
7	CGZR-1	0.17	1.98	11.4
8	Annada	0.18	1.90	10.5
9	Pornima	0.37	1.91	5.1
10	Karma mahsuri	0.07	0.40	6.0
11	ARB-6-11	0.98	1.94	2.0
12	GP-145-40	1.65	1.67	1.0
13	MTU 1010	0.52	0.92	1.8
14	RRF-127	0.11	1.25	11.2

An elevated level of proline during stress conditions reduce the osmotic potential and help in diffusion of water into the cells and maintain high turgor potential in the cell (Basu *et al.,* 2007). Other functions of proline accumulation were also proposed, including stabilization of macromolecules (Schobert and Tschesche, 1978), sink for carbon and nitrogen after stress recovery (Farooq *et al.,* 2009) and as a scavenger against reactive oxygen species (Joseph *et al.,* 2015). The concentrations of increased proline under stress conditions are directly proportional to the intensity of heat stress.

4.1.2.3 Malondialdehyde (MDA) content

Lipid peroxidation in the cell membranes is the destructive effect of oxidative damage caused due to heat stress. MDA content is the ultimate result of lipid peroxidation, which changes cells membrane stability. MDA content has been widely used as a criteria for assessing abiotic stress in various plants (Jain *et al.*, 2001; Moller *et al.,* 2007). MDA content of leaf tissues is estimated according to the method given by Heath and Paker (1968). The result of the MDA estimation is given in the table 4.6. Significant variation was recorded in the MDA content of fourteen rice genotypes.

The mean MDA content under control conditions was recorded as 0.372 µ mol/g fresh weight and it ranged from 0.097 µ mol/g in GP-145-55 to 0.785 µ mol/g in RRF-127 (Table 4.7), while the mean MDA content under stress conditions increased to 0.922 µ mol/g fresh weight and ranged from 0.265 µ mol/g in Annada to 1.684 µ mol/g in GP-145-40 (Table 4.8). When comparing the fold increase of MDA content in stress over control, the highest fold increase in MDA content was recorded in the genotype DagadDeshi (6.6 fold) followed by Annada (5.9 fold), Nagina-22 (3.9 fold), CGZR-1 (3.4 fold) and GP-145-103 (3.2 fold) as shown in figure 4.9.

Table 4.6: MDA content of fourteen rice genotypes under control and stress conditions.

		MDA (nmole / g)		
S.No	**Rice Genotype**	**control**	**stress**	**fold increase**
1	GP-145-103	0.59	1.23	2.1
2	SL-62	0.22	0.59	2.7
3	DagadDeshi	0.24	1.56	6.6
4	Nagina 22	0.37	0.51	1.4
5	Swarna	0.42	1.62	3.9
6	GP-145-55	0.10	0.33	3.4
7	CGZR-1	0.28	0.43	1.5
8	Annada	0.15	0.27	1.8
9	Pornima	0.29	0.95	3.2
10	Karma mahsuri	0.30	0.75	2.5
11	ARB-6-11	0.41	1.27	3.1
12	GP-145-40	0.64	1.68	2.6
13	MTU 1010	0.24	1.43	5.9
14	RRF-127	0.79	0.90	1.1

Increased levels of MDA content was observed in heat stress conditions. MDA content in cells is said to be inversely related to anti-oxidant enzymes because, as the anti-oxidant activity increases, oxidative stress causing lipid peroxidation decreases, and results in decreased MDA content. Studies reported that MDA content is relatively more in heat susceptible varieties under stress conditions due to increased lipid peroxidation (Kazim 2013; Wilson *et al.*, 2014; Zafar *et al.*, 2017). The heat tolerant genotypes recorded relatively low MDA content under stress than susceptible ones. In this present study the lowest amount MDA content under stress was recorded in RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mahsuri (2.5 fold) shown graphically in figure 4.9.

The comparison between all the phenotypic and biochemical characterizations of fourteen rice genotypes under stress conditions when compared with control i.e fold increase and fold decrease are shown in the figure 4.10.

Table 4.7: Mean and Range for all biochemical traits of fourteen rice genotypes unde control conditions.

	Chlorophyll a Control (mg/g)	Chlorophyll b Control (mg/g)	Total Chlorophyll Control (mg/g)	Proline Control (μ mol/g f.wt)	MDA control (μmols / g f.wt)
Genotype	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**
GP-145-103	2.710±0.000	2.310±0.006	5.023±0.004	0.064±0.000	0.59±0.008
SL-62	2.993±0.003	2.150±0.000	4.833±0.003	0.064±0.000	0.219±0.008
DagadDeshi	2.400±0.000	1.290±0.000	3.690±0.001	0.093±0.000	0.237±0.015
Nagina 22	2.547±0.003	1.817±0.003	4.360±0.000	0.068±0.001	0.366±0.016
Swarna	1.720±0.000	1.047±0.003	2.763±0.003	0.265±0.004	0.419±0.006
GP-145-55	3.000±0.000	2.820±0.000	5.820±0.000	1.001±0.000	0.097±0.000
CGZR-1	3.410±0.000	1.440±0.010	4.847±0.009	0.173±0.004	0.284±0.008
Annada	2.693±0.003	0.520±0.000	3.317±0.003	0.182±0.002	0.150±0.009
Pornima	3.090±0.000	1.060±0.000	4.147±0.003	0.374±0.000	0.293±0.004
Karma mahsuri	2.540±0.000	1.930±0.000	4.470±0.000	0.067±0.001	0.301±0.012
ARB-6-11	2.700±0.000	0.493±0.003	3.190±0.001	0.977±0.001	0.411±0.006
GP-145-40	1.900±0.000	0.370±0.000	2.070±0.000	1.650±0.006	0.639±0.006
MTU 1010	1.610±0.000	0.413±0.003	2.023±0.003	0.519±0.001	0.243±0.008
RRF-127	2.600±0.000	0.480±0.000	3.077±0.003	0.111±0.000	0.785±0.004
Mean	2.529	1.303	3.831	0.407	0.372
Minimum	1.610	0.370	2.023	0.064	0.097
Maximum	3.410	2.820	5.820	1.65	0.785
CD (*p*=0.05)	0.004	0.01	0.01	0.007	0.026
SE(m)±	0.002	0.004	0.003	0.002	0.009
SE(d)±	0.002	0.005	0.005	0.003	0.013
C.V.	0.106	0.473	0.151	0.995	4.134

Table 4.8: Mean and Range for all biochemical traits of fourteen rice genotypes under stress conditions.

Genotype	Chlorophyll a Stress (mg/g) Mean±S.E.	Chlorophyll b Stress (mg/g) Mean±S.E.	Total Chlorophyll Stress (mg/g) Mean±S.E.	Proline Stress (µ mol/g f.wt) Mean±S.E.	MDA stress (µmols / g f.wt) Mean±S.E.
GP-145-103	2.173±0.003	2.300±0.000	4.970±0.000	0.447±0.008	1.128±0.004
SL-62	2.340±0.000	1.290±0.000	3.630±0.000	0.095±0.003	0.585±0.027
DagadDeshi	1.720±0.000	0.837±0.015	2.560±0.012	0.380±0.003	1.563±0.006
Nagina 22	2.550±0.000	1.240±0.000	3.790±0.000	1.402±0.000	0.512±0.009
Swarna	0.523±0.003	0.323±0.003	0.853±0.003	1.567±0.000	1.617±0.002
GP-145-55	1.140±0.000	0.620±0.000	1.760±0.000	2.044±0.001	0.326±0.000
CGZR-1	2.743±0.003	1.107±0.003	3.890±0.000	1.975±0.001	0.432±0.004
Annada	2.680±0.000	0.051±0.000	3.210±0.000	1.903±0.000	0.265±0.002
Pornima	1.760±0.000	0.410±0.000	2.163±0.003	1.906±0.001	0.945±0.006
Karma mahsuri	2.547±0.003	1.800±0.000	4.343±0.003	0.404±0.000	0.748±0.004
ARB-6-11	2.203±0.003	0.480±0.000	2.687±0.003	1.941±0.001	1.271±0.006
GP-145-40	1.290±0.000	0.340±0.000	1.930±0.000	1.667±0.006	1.684±0.004
MTU 1010	1.540±0.000	0.340±0.000	1.623±0.003	0.921±0.000	1.430±0.007
RRF-127	0.933±0.003	0.203±0.003	1.133±0.003	0.246±0.001	0.901±0.004
Mean	1.874	0.873	2.747	1.213	0.922
Minimum	0.523	0.203	0.853	0.095	0.265
Maximum	2.743	2.300	4.970	2.044	1.684
CD (*p*=0.05)	0.006	0.012	0.011	0.009	0.025
SE(m)±	0.002	0.004	0.004	0.003	0.009
SE(d)±	0.003	0.006	0.005	0.004	0.012
C.V.	0.202	0.83	0.238	0.435	1.634

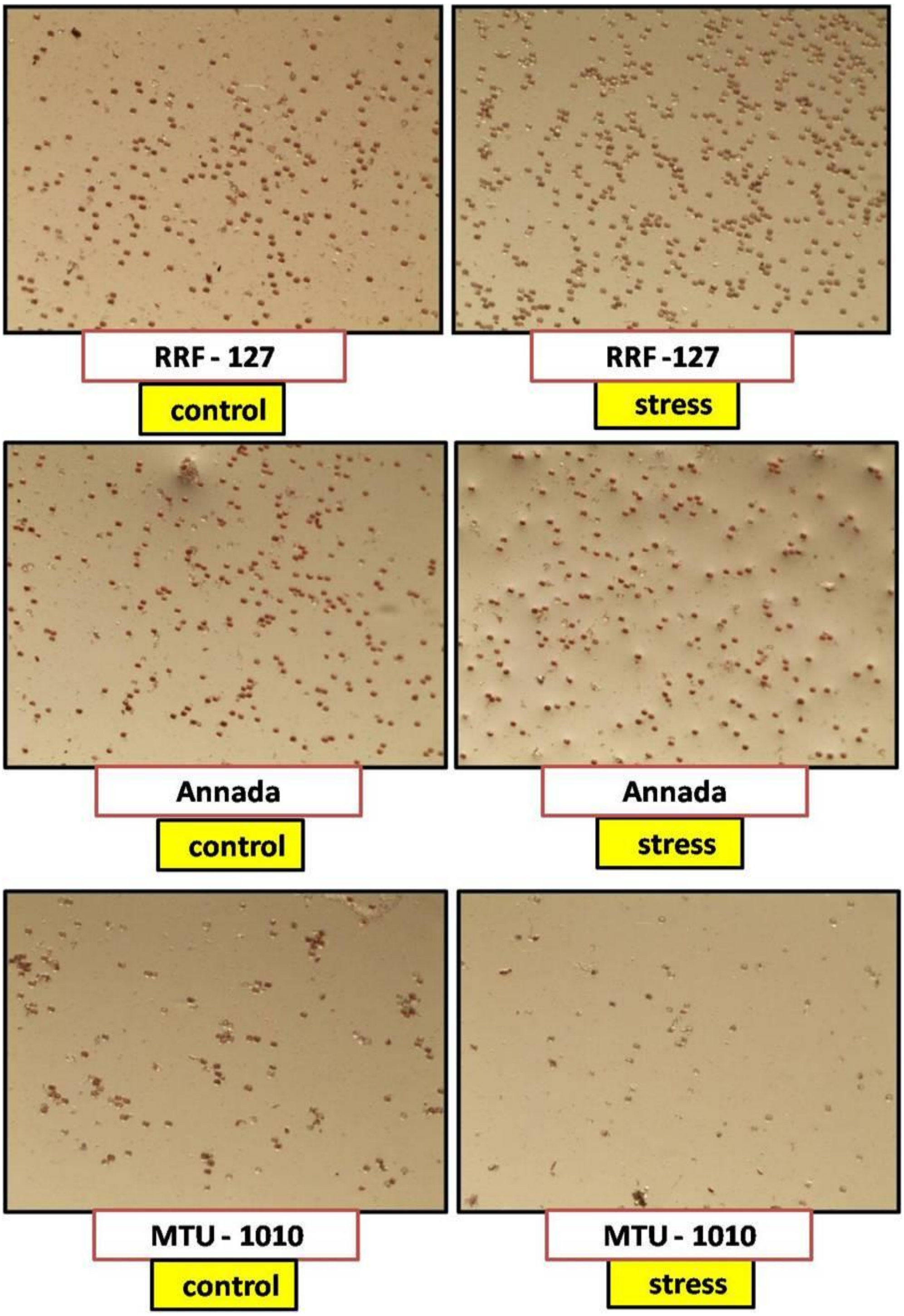


Fig 4.4: Fertile (red/stained) pollen and sterile (transparent/unstained) pollen in rice genotypes under control and stress conditions.

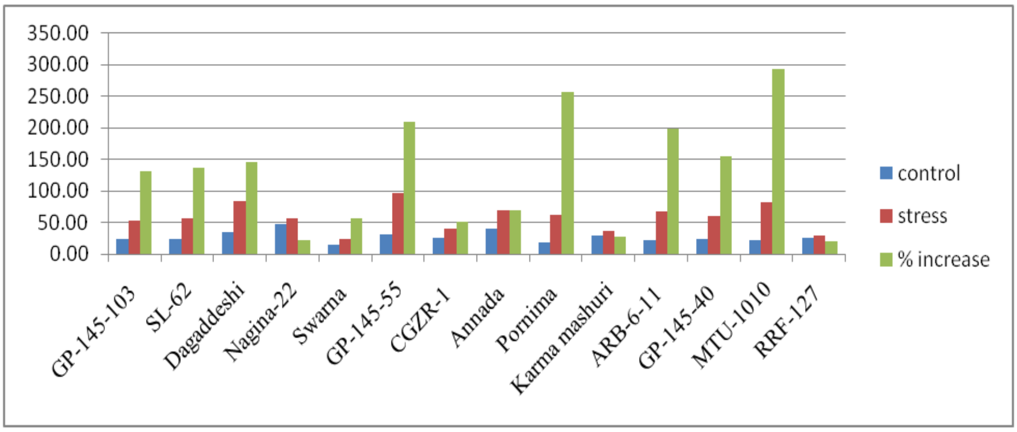


Fig 4.3: Membrane Stability Index (MSI) of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

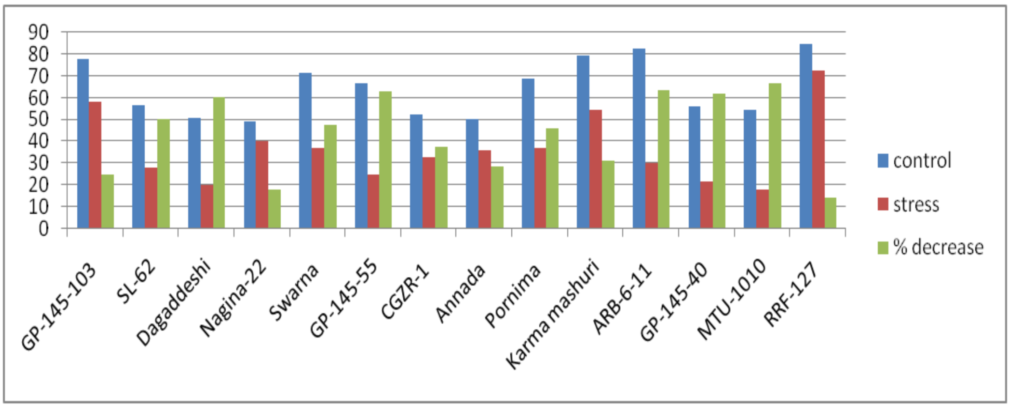


Fig 4.5: Pollen fertility of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

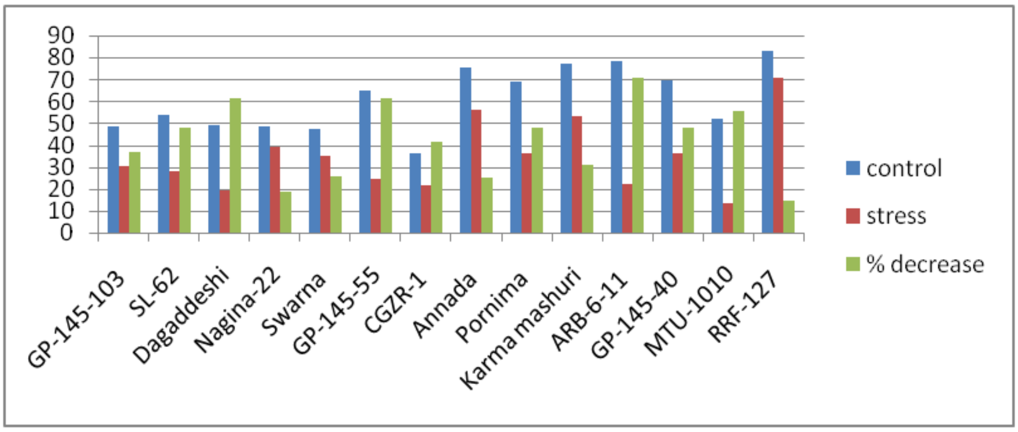


Fig 4.6: Spikelet fertility of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

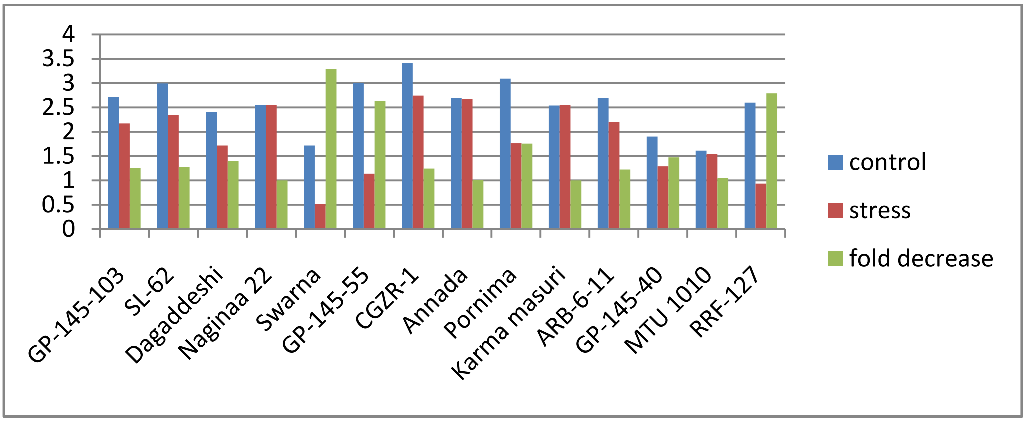


Fig 4.7a: Chlorophyll –a content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

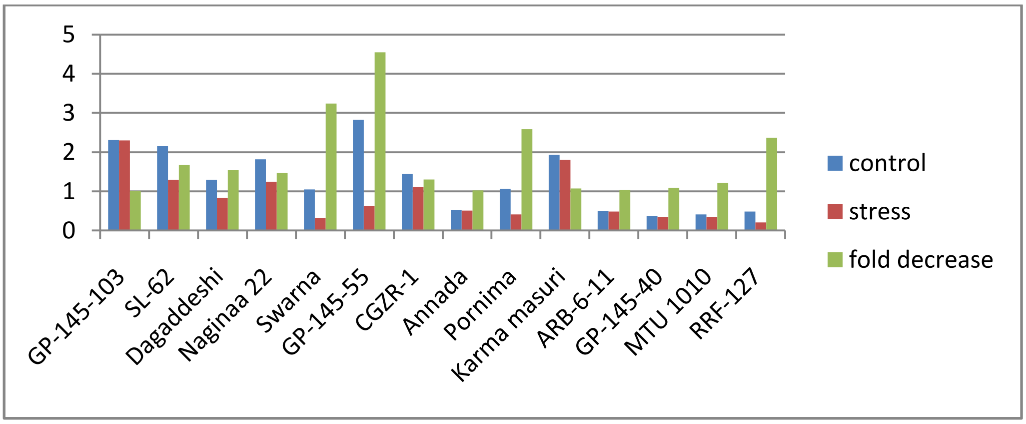


Fig 4.7b: Chlorophyll –b content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

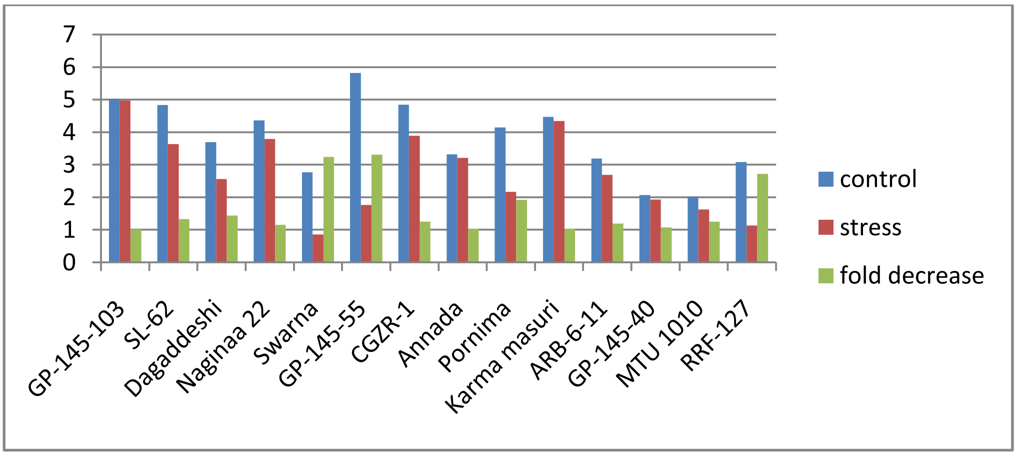


Fig 4.7c: Total Chlorophyll content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

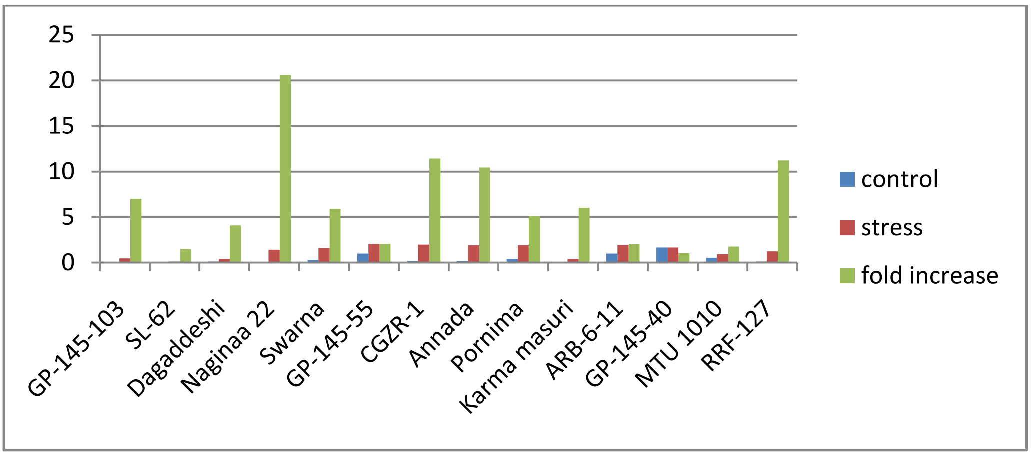


Fig 4.8: Proline content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

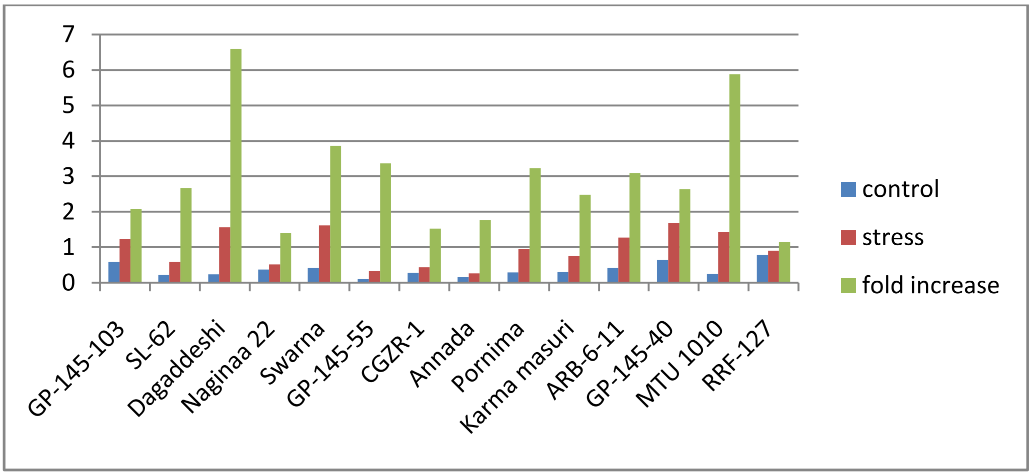


Fig 4.9: MDA content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

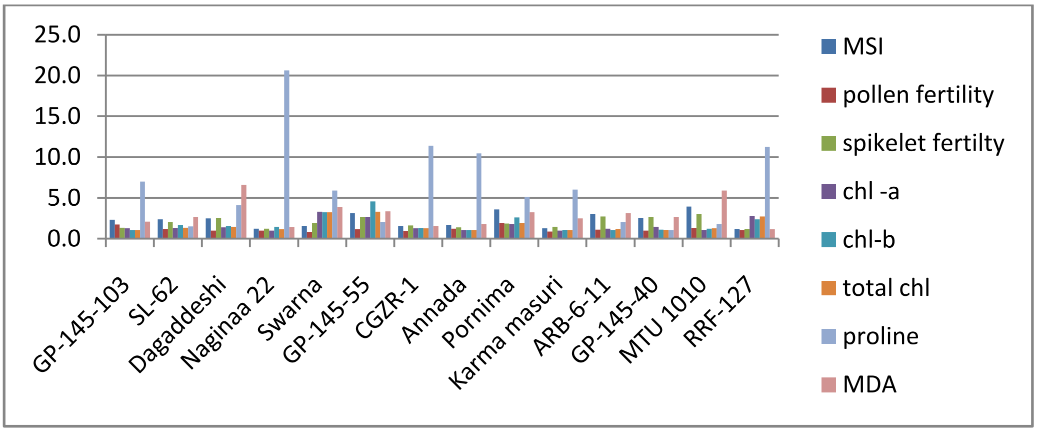


Fig 4.10: Overall Fold Increase or Decrease in all phenotypic and biochemical characters of fourteen rice genotypes under stress conditions at vegetative stage before panicle initiation.

4.2. Expression analysis of heat responsive genes using semi-quantitative RT-PCR

As mentioned earlier, the fourteen rice genotypes were grown both under control and stress conditions. The stress treatment of 42°C was given at day time continuously for 6 days by maintaining the temperatures in green house, at vegetative stage before panicle initiation. After 6 days of stress treatment, the leaf samples were harvested from both control and stress plants in liquid nitrogen and the they are preserved in 80 °C for RNA isolation. RNA should be extracted immediately after the sample collection as RNA degrades very fast. Isolation of total RNA was done using TRIzol (Invitrogen, USA) manufacturer's protocol including some minor modifications. The concentration of the isolated RNA was quantified using Nanodrop spectrophotometer ND-1000 ® (NanoDrop Technologies, USA). From this isolated RNA , synthesis of cDNA was done using BIORAD iScriptTM cDNA Synthesis Kit as per manufacturer's instruction. Then the expression pattern of five different genes was analysed using Semi quantitative RT-PCR. These genes include OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon (Table 3.6).

4.2.1. Standardization of RT-PCR parameters for expression profiling of heat responsive genes

Considering the concentration of RNA, assessed by Nanodrop ND1000 spectrophotometer, initially samples were diluted to 1500ng/µl for normalization. After cDNA synthesis, normalization was checked by using Os-ACTIN gene (house-keeping gene) as internal control. Further relative quantification of selected 5 genes was done by conducting semi-quantitative RT-PCR analysis by using gene specific primers on by comparing the florescence intensities of individual bands observed in each well. NRCPB, New Delhi provides the primer sequences. The melting temperature, template DNA and other parameters related to RT-PCR, of selected genes were standardized for expression analysis. The first-strand cDNA was obtained from 1 µg of total RNA in a 20 µl reaction mixture, and 20 ng/µl of synthesized cDNA was used as template for PCR reaction (94°C for 2 min, 15 s at 94°C, 30 s at 60-64°C, and 45 s at 72°C, then 72°C for 7 min followed by 4°C).

4.2.2. Semi-quantitative expression profiling of heat stress responsive genes in rice genotypes under heat stress

Gene expression analysis by semi-quantitative RT-PCR was undertaken to calculate the transcript abundance differences among both control and stress (high temperatures) conditions in genotypes. In the present study, the semi-quantitative RT-PCR analysis of five heat stress responsive genes was performed for all the fourteen genotypes (-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) under stress and control conditions at vegetative stage before panicle initiation. The differential expression of five heat stress responsive genes (OsHSP 26.7, OsHSP 16.9, OsHSP 18, OsHSP DnaJ and 60kDa chaperon) in all the fourteen rice genotypes under both control and stress conditions was indicated in the figure 4.16. The genotype specific fold increase and decrease of heat stress responsive genes in rice genotypes under stress as compared with control conditions are given in table 4.9. The results are discussed below in detail.

Note: The increase or decrease in the Fold value was calculated by measuring the band intensity and size using GelQuantNET software.

In this study, semi quantitative RT-PCR of OsHSP 26.7 gene has shown up-regulation under heat stress conditions in all the rice genotypes. Table 4.9 shows the fold increase in all the fourteen rice genotypes. The rice genotype RRF-127 showed the highest up-regulation of 14.3 fold increase followed by Annada (13.9 fold), Karma mahsuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold) as shown in figure 4.11. Previous studies reported that OsHSP 26.7 gene belongs to sHSP (small heat shock proteins) class of genes and showed strong up-regulation under heat stress conditions in comparison to control (Chandel *et al.*, 2013).

Semi quantitative RT-PCR of OsHSP 16.9 gene, in the present investigation has shown up-regulation in almost all rice genotypes under heat stress conditions. Table 4.9 shows fold increase in all genotypes except for DagadDeshi. The rice genotype RRF-127 showed the highest up-regulation of 10.0

fold increase followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mahsuri (3.1 fold) as shown in figure 4.12. Its been reported that OsHSP 16.9 gene belongs to sHSP (small heat shock proteins) class of genes and showed slight up-regulation under heat stress conditions when compared with control (Chandel *et al.,* 2013).

In this study, semi quantitative RT-PCR of OsHSP DnaJ gene, has shown almost the same expression under control and stress conditions and no convincing difference was observed in heat stress. Table 4.10 shows the quantified expression of the gene under both and stress conditions. Consistent expression was seen in the genotypes like GP-145-103, DagadDeshi, GP-145-55, CGZR-1, and Annada as shown in figure 4.13. As per the reports of previous work done, the gene OsHSP DnaJ also showed no significant change in the expression under control and stress (Chandel *et al.,* 2013) and this gene also belongs to sHSP (small heat shock proteins) family.

OsHSP 18 gene expression was also studied in the present investigation using semi quantitative RT-PCR and it showed up-regulation in almost all genotypes of rice under heat stress. Table 4.9 shows fold increase in all genotypes of rice. The rice genotype GP-145-103 has shown highest up-regulation of 17.7 fold increase followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and Poornima (8.9 fold) as shown in the figure 4.14. This gene also belongs to the family sHSP (small heat shock proteins) class of genes and showed up-regulation under heat stress conditions (Chandel *et al.,* 2013).

In the present study, semi quantitative RT-PCR of 60Kda chaperon gene was studied and it has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. Table 4.9 shows fold increase in all the genotypes of rice. The rice genotype Annada has shown highest up-regulation of 1.9 fold increase followed by CGZR-1 (1.5 fold), Nagina -22 (1.4 fold), GP-145-103 (1.3 fold) and Karma mahsuri (1.3 fold) as shown in figure 4.15. 60Kda chaperon was also reported to be up-regulated by minimal levels under heat stress but it is lately induced under stress (Chandel *et al.,* 2013).

Heat stress triggers few defence mechanisms like obvious gene expression, which results in the increased synthesis of particular protein groups called "heat shock proteins (HSPs)". Generally HSPs are classified into 5 evolutionarily conserved families based on their molecular weight, which include HSP 60, 70, 90, 100 and small HSPs (Krishna 2003). As the present investigation studied the expression of five different genes mentioned above, most of them belong to the small HSPs family. Brief explanation about the small HSPs and their mechanism is explained under 4.2.3.

Table 4.9: Fold increase of all up-regulated genes in rice genotypes under high temperature stress compared with control conditions

		Fold increase				
S.No	**Rice Genotypes**	**OsHSP 26.7**	**OsHSP 16.9**	**OsHSP 18**	**OsHSP DnaJ**	**60kDa chaperon**
1	GP-145-103	8.6	3.2	17.7	1.0	1.3
2	SL – 62	1.7	2.3	8.4	0.9	1.0
3	DagaDeshi	2.4	1.5	5.9	1.0	1.1
4	Nagina -22 (Check)	3.0	2.9	4.8	0.8	1.4
5	Swarna	3.6	1.4	3.3	0.9	1.2
6	GP – 145-55	3.3	1.3	11.9	1.0	1.2
7	CGZR -1	3.7	3.4	14.1	1.0	1.5
8	Annada	13.9	3.7	13.8	1.0	1.9
9	Poornima	1.6	1.3	8.9	0.9	1.2
10	Karma mahsuri	11.5	3.1	6.1	1.1	1.3
11	ARB – 6-11	3.0	1.7	1.2	0.9	1.2
12	GP -145 -40	2.4	1.3	4.8	0.7	1.2
13	MTU-1010	2.3	1.4	2.0	1.1	1.1
14	RRF-127	14.3	10.0	5.2	1.1	1.1

4.2.3 Small heat shock protein family (smHSPs)

Small heat shock proteins (sHSPs) are the largest ubiquitous HSP subgroup whose molecular weight ranges from 12 t0 42 KDa. "Small HSPs sequence analysis indicates that members belonging to this family includes evolutionarily divergent N-terminal part, which is followed by a conserved α-crystallin domain and a short C-terminal tail (de Jong *et al.*, 1993)". Along the evolutionary scale, sHSPs genes number increases where as bacteria had only one or two sHSPs and multi cellular organisms have large no.of sHSPs in their genome. Its been recognized that ten separate sHSPs families have been conserved in both monocots and dicot plants, which indicates the diversity for sHSPs mechanisms (Siddique *et al.*, 2008; Scharf *et al.*, 2001). Among the ten, sHSPs encoded by 4 families are localized to cytoplasm and other 6 families are localized to different cellular organelles like nucleus, chloroplasts, endoplasmic reticulum, mitochondria and peroxisomes (Basha *et al.*, 2010). In vivo studies reported that sHSPs function as molecular chaperons. Plants synthesize large amounts of sHSPs when they are exposed to high temperature suggesting that they play a major role for enduring thermo-tolerance in plants (Charng *et al.*, 2006). Higher plants have at least 20 sHSPs and chances of presence of 40 kinds of these sHSPs in one plant species has been reported (Vierling 1991). Some regulatory proteins called heat stress transcription factors (Hsfs) control the transcription of heat shock protein genes. Plants have at least 21 Hsfs, each one having its own specific role in regulation. They also cooperate in all phases of responses due to periodical stress like triggering, maintenance and recovery. "The smHsps cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, preventing irreversible unfolding or wrong protein aggregation (Mogk, *et al.*, 2003)". "Recent findings showed that the smHSPs 18.1 isolated from *Pisum sativum*, as well as the sHsps 16.6 from *Synechocystis* sp. PCC6803 under *in vitro* conditions, binds to unfolded proteins and allows further refolding by HSP70/HSP100 complexes (Mogk *et al.*, 2003)". A very strong positive qualitative relation was noticed between the thermo-tolerance and accumulation of sHSPs in plastids.

"The current model for sHSP chaperone activity was defined based on studies of a cytosolic sHSP family named as Class I sHSPs (sHSP-CI), which represent the most abundant sHSP in plants (Guan *et al.*, 2004)". According to this model, a large homo-oligomer is formed by the assembly of sHSPs, which is binded to denatured proteins by ATP-independent fashion, and keeps them in folding competent state. Then this complex cooperates with molecular chaperons like HSP70 and HSP90, which are ATP-dependent to refold those denatured proteins. “Notably, sHSP has a much larger binding stoichiometry than other molecular chaperones, which has led to the speculation that sHSP functions as a reservoir to stabilize the flood of denatured proteins in response to stress (Lee *et al.,* 2000; Ehrnsperger *et al.,* 1997)”. “Its been proposed that major mechanism through which sHSPs expose normally inaccessible, hydrophobic client binding surfaces by heat-induced oligomer dissociation, Nevertheless, the details about the interactions between sHSP and non-native proteins and how these non-native proteins are subsequently refolded are still lacking. This is partially due to limited knowledge on the molecular structure of sHSPs (Haslbeck *et al.,* 2005)”. “Among the few solved crystallographic structures of sHSPs is a wheat TaHsp16.9- CI, the basic building block of wHSP16.9 is a dimer, which further assembles as a 12-mer consisting of two trimers of dimmers, in solution to this wHSP16.9 can dissociate into smaller oligomeric states in a temperature dependent manner (van Montfort *et al.,* 2001)”. Based on this mechanism it is observed that heat-induced sHSPs oligomers dissociation may expose the hydrophobic patches that were buried in the oligomeric interface, which leads to binding and stabilization of denatured proteins under heat stress and imparts thermo-tolerance.

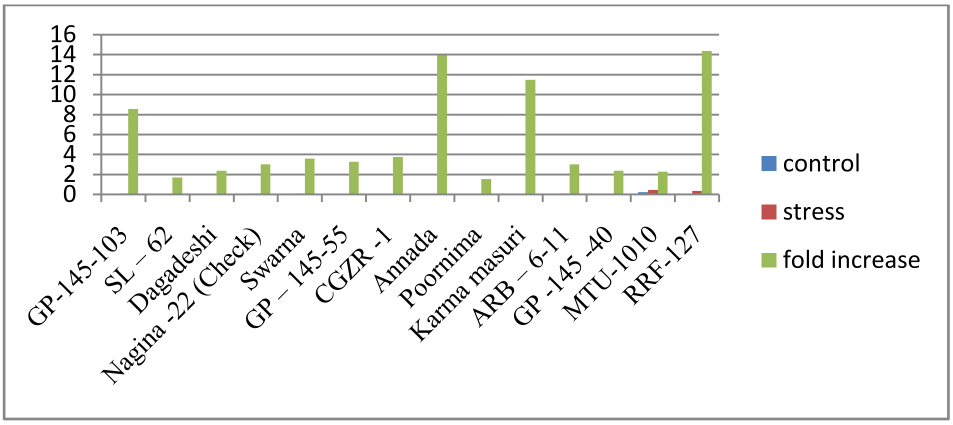


Fig 4.11: **Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP26.7 gene (strongly up-regulated)**

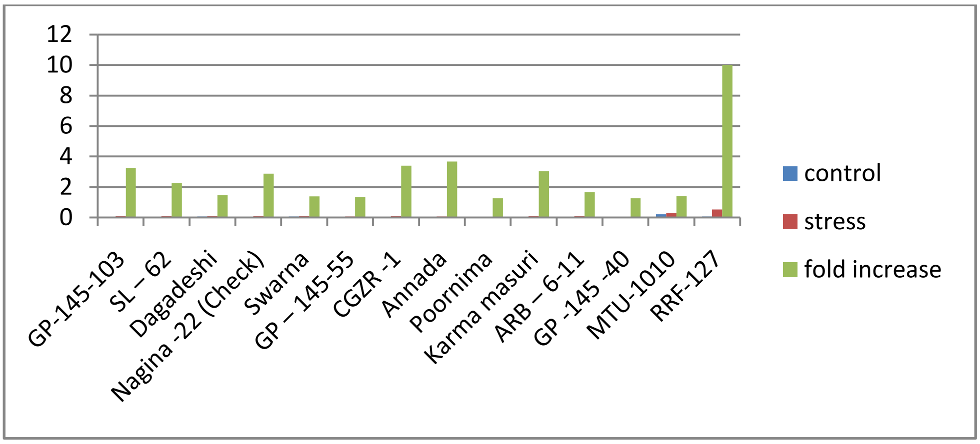


Fig 4.12: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP16.9 gene (up-regulated)

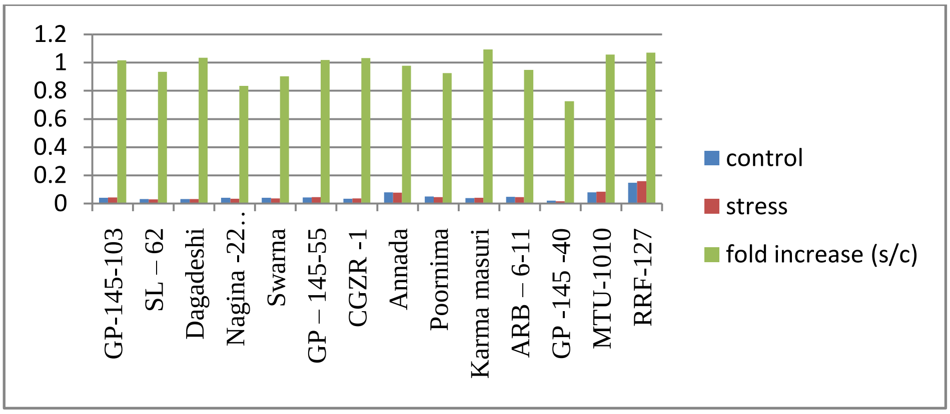


Fig 4.13: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP DnaJ gene (consistent expression)

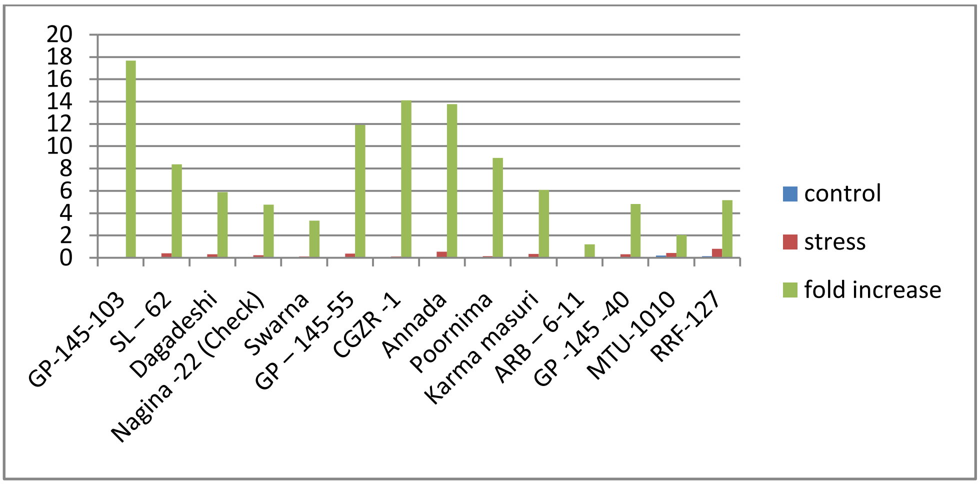


Fig 4.14: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP18 gene (up-regulation

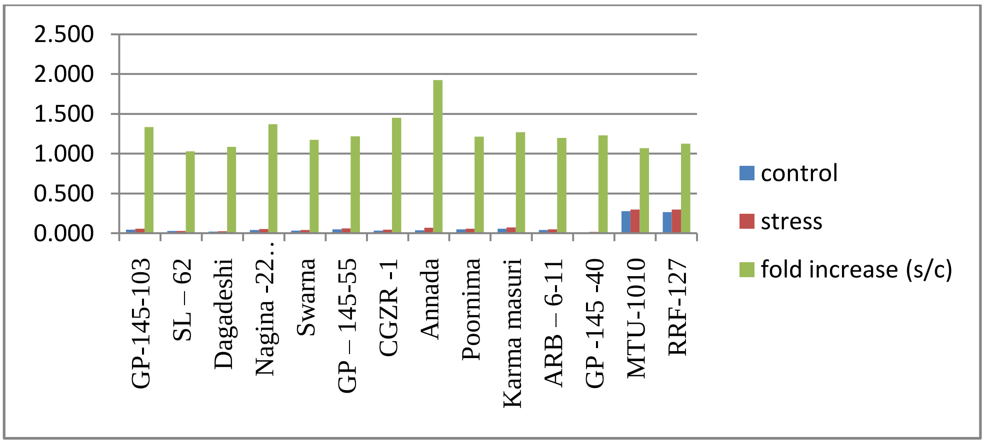


Fig 4.15: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on 60Kda chaperon gene (slight up-regulation)

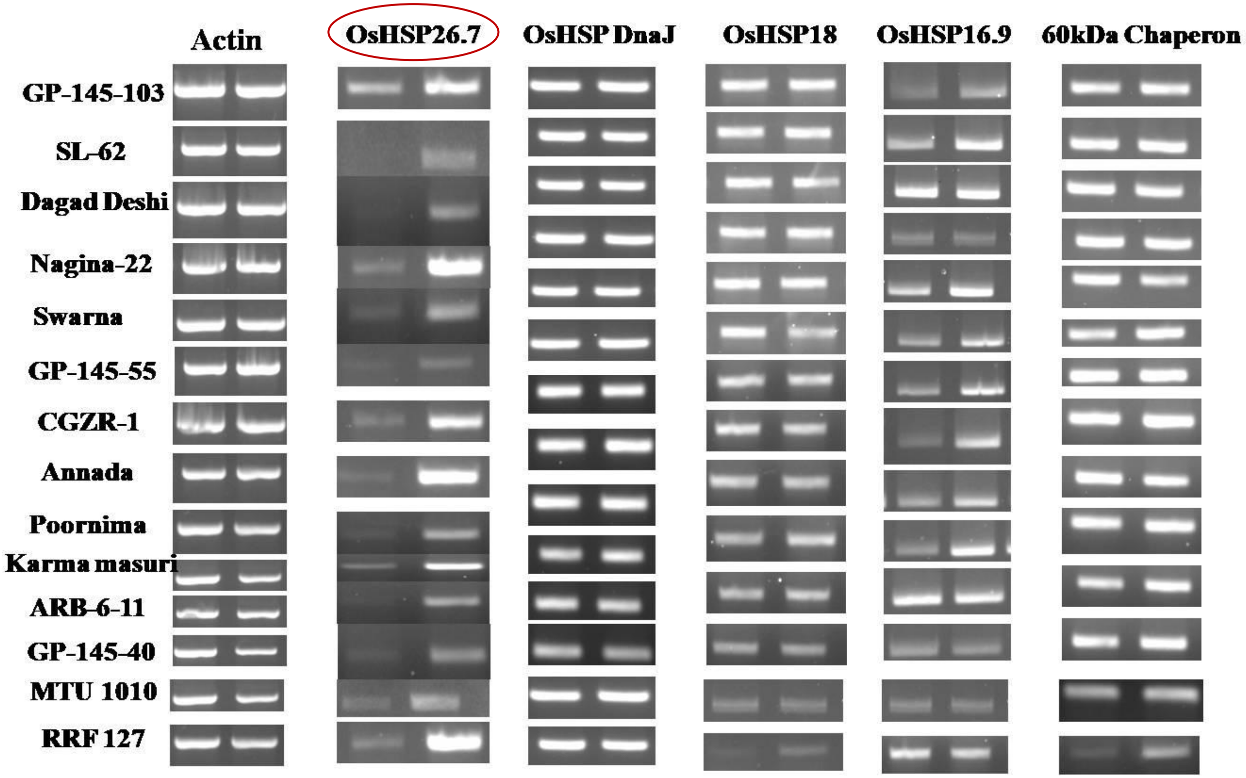


Fig 4.16: Semi quantitative RT-PCR Analysis of five heat responsive genes for fourteen rice genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) under control and stress condition

4.3 Correlation between Gene expression pattern and various phenological, biochemical traits

High temperature is a severe environmental stress and the major constraint on plant productivity with an obvious effect on plant growth. The aim of this work was to study the response of rice genotypes under high temperature stress at the phenological, biochemical and molecular levels. Characterization of rice genotypes contrasting in their response to high temperature stress was performed at the phenological and biochemical level by determination of MSI, Pollen fertility, Spikelet fertility, chlorophyll content, free proline content and MDA content under temperature stress condition. Pollen fertility and Spikelet fertility were positively correlated and MSI was negatively correlated with the yield per plant which was significant. The remaining traits showed non-significant correlation with yield per plant. In this study rice genotypes RRF-127 and Nagina-22 showed minimum decrease in MSI, pollen fertility and spikelet fertility. Chlorophyll-a degradation was positively correlated with chlorophyll-b and total chlorophyll content and it was negatively correlated with MDA levels and was significant, which correctly indicated the increase in MDA levels under high temperatures with decreased chlorophyll content. Chlorophyll-b degradation was positively correlated with chlorophyll-a, total chlorophyll content and it was negatively correlated with proline content and was significant, which indicated the increase in proline accumulation under high temperature with decreased chlorophyll content.

The expression level of five high temperature responsive genes (OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon) was also analyzed by reverse transcription–polymerase chain reaction (RT-PCR). The result revealed that the all genes are up regulated under high temperature stress treatment in rice genotypes expect OsHSP DnaJ which showed consistent expression both under control and stress conditions. Very prominent result was observed for OsHSP26.7 gene in all rice genotype under high temperature, and the genotypes RRF-127 and Annada which were screened as heat stress tolerance genotype on the basis of biochemical and phonological studies carried out in this work showed the highest fold increase. Positive correlation was found among the expression of OsHSP26.7, OsHSP16.9, 60kDa chaperon and it was significant. Rice genotype RRF-127 showed a higher level of up regulation for gene OsHSP26.7 (14.3 fold), followed by OsHSP16.9 (10.0 fold). Annada showed higher level of up-regulation for gene OsHSP26.7 (13.9 fold), followed by OsHSP16.9 (3.7 fold) and 60kDa chaperon (1.9 fold). Based on the expression pattern all five genes (OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon) under study were identified as positively up regulated under high temperature in rice genotypes. These genes are not expressed in the control plants, but only in the stressed plants. This result was used as a basis to select for the gene OsHSP26.7 as the most responsive gene under high temperature and genotypes RRF-127 and Annada as the heat-tolerant genotypes on the basis of study at phenological, biochemical and molecular levels. The table 4.10 given below shows the correlation among all the phenological, biochemical traits and the gene expression

Table 4.10: Correlation between various phenological and biochemical traits.

	MSI	Pollen fertility	spikelet fertility	chl a	chl b	chl total	proline	MDA	Yield/plant
MSI	1								
Pollen fertility	-0.503NS	1							
spikelet fertility	-0.499NS	0.999**	1						
chl a	0.054NS	0.076NS	0.048NS	1					
chl b	-0.142NS	0.173NS	0.157NS	0.653*	1				
chl total	-0.079NS	0.130NS	0.108NS	0.867**	0.931**	1			
proline	0.095NS	0.104NS	0.099NS	-0.147NS	-0.552*	-0.394NS	1		
MDA	-0.054NS	-0.344NS	-0.340NS	-0.543*	-0.305NS	-0.397NS	-0.215NS	1	
Yield/plant	-0.640*	0.642*	0.643*	0.202NS	0.016NS	0.101NS	0.072NS	-0.396NS	1

* (significance at .05), ** (significance at .01) NS (non-significant)

CHAPTER – V

SUMMARY AND CONCLUSIONS

All abiotic stress conditions like drought, salinity, cold and high temperatures effects the agricultural production as they hinder the expression of genes to their full potential. Among them heat stress is the major constraint in the present days, due to increasing earth's surface temperature as a result of global warming. Hence its necessary to produce crops with higher heat stress tolerance to combat yield loss. The first step in generating the tolerant crops is to understand the complete mechanisms involved in response to high temperatures in plants. Research done at the level of genes (Genomics), proteins (Proteomics) and metabolites (Metabolomics) is fundamental understanding the plant responses to high temperatures. Identification of key regulators through gene expression patterns related to stress interactions is the important aspect of using functional genomics tools. So to study the effect of heat stress on the expression of genes that respond to high temperatures, that lead to heat-tolerance, the present investigation was conducted with two major objectives:

1. **Phenological and Biochemical characterization of rice genotypes under heat stress.**
    - Genotypes RRF-127 , Nagina-22, Karma mashuri, CGZR-1 and Annada had recorded lower electrolyte leakage i.e membrane stability index under stress when compared with control as 19.8%, 21.1%, 27.5%, 51.7% and 69.8% respectively.
    - Lowest decrease in pollen fertility under stress conditions when compared to control was observed in the genotypes RRF-127, Nagina-22, GP-145-103, Annada and CGZR-1 as 14.4%, 18.1%, 24.9%, 28.3% and 37.9% respectively.
    - Spikelet fertility decrease under stress conditions when compared to control was found to be lowest in the genotypes RRF-127, Nagina-22, Annada, Karma mashuri and CGZR-1 as 14%, 19%, 25%, 31% and 42% respectively.

- When comparing the fold increase in proline content for stress over control among all the fourteen genotypes, Nagina-22 showed highest fold increase of 20.6 folds followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mashuri (6.0 fold).
- In chlorophyll content, Annada has the lowest fold decrease of 1.0 folds for all chla, chlb and total chlorophyll content followed by Karma mashuri (1.0 fold) under stress when compared with control
- When comparing the fold increase in MDA content for stress over control among all fourteen genotypes, the lowest fold increase in MDA content was shown by RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mashuri (2.5 fold).

1. **Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.**

Wet lab conformation using RT-PCR analysis for the expression pattern of five selected genes showed a genotype specific banding pattern under control and heat stress conditions. A significant level of up-regulation was observed in all the five genotypes under stress as these belong to small HSPs family, whose genes become responsive in all abiotic stress conditions.

- OsHSP 26.7 gene has shown strong up-regulation under heat stress conditions in all the rice genotypes. The rice genotype RRF-127 showed the highest up-regulation of 14.3 fold increase followed by Annada (13.9 fold), Karma mashuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold).
- OsHSP 16.9 gene has shown up-regulation in almost all rice genotypes under heat stress conditions. The rice genotype RRF-127 showed the highest up-regulation of 10.0 fold increase followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mashuri (3.1 fold).

- OsHSP DnaJ gene, has shown almost the same expression under control and stress conditions and no convincing difference was observed in heat stress.
- OsHSP 18 gene showed up-regulation in almost all genotypes of rice under heat stress. The rice genotype GP-145-103 has shown highest up-regulation of 17.7 fold increase followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and poornima (8.9 fold).
- 60Kda chaperon gene has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. The rice genotype Annada has shown highest up-regulation of 1.9 fold increase followed by CGZR-1 (1.5 fold), Nagina -22 (1.4 fold), GP-145-103 (1.3 fold) and Karma mashuri (1.3 fold).

CONCLUSIONS

- one of the effective biochemical changes that takes place during heat stress condition is the over accumulation of proline, which acts as osmo-protectant. The membrane stability index (MSI), chlorophyll content (chla-a, chl-b and total chl) of leaves in all the genotypes of rice decreased remarkably when subjected to high temperatures. A significant increase in the levels of proline and MDA content was observed under high temperatures, revealing that accumulation of proline and MDA content takes place during stress conditions.
- High temperature stress imposed at late vegetative stage before panicle initiation, reduced the pollen fertility and spikelet fertility in all rice genotypes revealing the impaired pollen development and fertilization under stress.
- A positive correlation was recorded for yield per plant and pollen fertility and spikelet fertility under stress and it was significant, where as yield is negatively correlated with Membrane stability index (MSI).
- Phenological and biochemical studies (MSI, pollen fertility, spikelet fertility, chlorophyll content, proline and MDA content) on rice genotypes resulted in identifying some of the tolerant and susceptible genotypes for

heat stress. Among all the fourteen genotypes selected for the study, RRF-127, Annada, Karma mashuri, CGZR-1 and GP-145-103 showed a positive regulation towards heat stress and genotypes like MTU-1010, ARB-6-11 and GP-145-55 showed negative regulation towards heat stress.

- There are a number of genes involved in heat stress mechanisms that regulate the expression of genome in response to environment and physiological signals under high temperature conditions. In wet lab conformation using RT-PCR analysis of 5 genes belonging to the family of small HSPs (OsHSP 26.7, OsHSP 16.9, OsHSP DnaJ, OsHSP 18 and 60Kda chaperon), there was a correlation in between phonological and biochemical traits with the genotypes (RRF-127, Annada, Karma mashuri and CGZR-1) which had shown a significant level of up-regulation among all the genes under stress conditions.
- OsHSP26.7 gene is strongly up-regulated and it is recorded as one of the most responsive genes under high temperature stress.
- Genotypes RRF-127 and Annada showed a positive regulation for almost all the phonological and biochemical traits which are positively correlated along with significant up-regulation in the expression analysis of all the five genes. By this we can say that, these genotypes are tolerant towards high temperatures.

SUGGESTIONS FOR FUTURE RESEARCH WORK

- The study delineates and employs the strategy for discovering useful genes responsive to heat stress in rice genotypes. Same can be carried out for the identification of other key genes responsible for heat tolerance in rice as well as other food crops. The same strategy can also be employed for other agricultural important crops.
- High temperature these days are turning out to be one of the serious challenges for crop production. There are many stress related genes that are expressed under high temperatures and today there is an immense need for examining and analyzing stress related genes, their products and mechanisms under multiple environmental conditions.

- There is a great need to understand the signal transduction pathways role along with post-transcriptional and translational regulatory mechanisms in both biotic and abiotic stress responses to examine and understand the function of various stress related genes in order to improve stress tolerance towards heat in rice as well as other crops.
- Identification of genes and mechanisms of general stress processes as well as stress-specific responses and identifying the function of genes whose functions are currently unknown will help in determining their role in stress tolerance.
- Hundreds of heat specific transcription factors respond to heat stress, understanding the function of these Hsfs will help to unravel the mechanisms of heat stress tolerance.
- More attempts should be made for the validation of genes for abiotic stresses (salinity, drought, cold, nutrients) with genotypes of diverse genetic background in order to identify the key genes responsible for different abiotic traits.
- Genes that are significantly up and down regulated in heat tolerant cultivars when compared to a susceptible cultivar can serve as a candidate genes for heat stress tolerance.
- Genes which are screened from the expression analysis studies of rice could be used for narrow down process and transcriptome sequencing to measure the transcript abundance at varying heat stress levels.

REFERENCES

Agarie, S., Hanaoka, N., Kubota, F., Agata, W. and Kaufman, P.B., 1995. Measurement of cell membrane stability evaluated by electrolyte leakage as a drought and heat tolerance test in rice (Oryza sativa L.). *Journal of the Faculty of Agriculture, Kyushu University*, *40*(1), pp.233-240.

Agarwal, M., Sahi, C., Katiyar-Agarwal, S., Agarwal, S., Young, T., Gallie, D.R., Sharma, V.M., Ganesan, K. and Grover, A., 2003. Molecular characterization of rice hsp101: complementation of yeast hsp104 mutation by disaggregation of protein granules and differential expression in indica and japonica rice types. *Plant molecular biology*, *51*(4), pp.543-553.

Ahmed, J.U. and Hassan, M.A., 2011. Evaluation of seedling proline content of wheat genotypes in relation to heat tolerance. *Bangladesh Journal of Botany*, *40*(1), pp.17-22.

Alisoltani, A., Shiran, B., Sarvestani, N.R., Fallahi, H., Feto, N.A. and Ebrahimie, E., 2018. Changes in Microsatellite Motifs in Response to Abiotic Stresses: a Case Study Using Wheat and Rice RNA-sequencing Data. *Asian Journal of Scientific Research*, *11*, pp.12-21.

Arnon, D.I., 1949. Copper enzyme polyphenoloxides in isolated chloroplast in Beta Vulgaris Plant physiology, 24: 1–15.

Ashraf, M.Y., Azmi, A.R., Khan, A.H. and Ala, S.A., 1994. Effect of water stress on total phenols, peroxidase activity and chlorophyll content in wheat [Triticum aestivum L.]. *Acta physiologiae plantarum*, *16*(3).

Bahuguna RN, Jagadish KS (2015) Temperature regulation of plant phenological development. Environ Exp Bot 111:83–90

Bahuguna RN, Pal JJ, Shah D, Lawas LM, Khetarpal S, Jagadish KS (2015) Physiological and biochemical characterization of NERICA rice: a novel source of heat tolerance at the vegetative and reproductive stages in rice. Physiol Plant 154(4):543–559

Barakat MM, Al-Doss AA, Elshafei AA, Moustafa KA (2011) Identification of new microsatellite marker linked to the grain filling rate as indicator for heat tolerance genes in F2 wheat population. AJCS 5(2):104–110. ISSN:1835-2707

Basha, E., Jones, C., Wysocki, V. and Vierling, E., 2010. Mechanistic differences between two conserved classes of small heat shock proteins found in the plant cytosol. *Journal of Biological Chemistry*, *285*(15), pp.11489-11497.

Bates, L., Waldren, R.P. and Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. Plant and Soil, 39: 205–207.

Blum, A. and Ebercon, A., 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat 1. *Crop Science*, *21*(1), pp.43-47

Borriboon, W., Lontom, W., Pongdontri, P., Theerakulpisut, P. and Dongsansuk, A., 2018. Effects of Short-and Long-Term Temperature on Seed Germination, Oxidative Stress and Membrane Stability of Three Rice Cultivars (Dular, KDML105 and Riceberry). *Pertanika Journal of Tropical Agricultural Science*, *41*(1).

Cao, Y., Zhang, Q., Chen, Y., Zhao, H., Lang, Y., Yu, C. and Yang, J., 2013. Identification of differential expression genes in leaves of rice (Oryza sativa L.) in response to heat stress by cDNA-AFLP analysis. *BioMed research international*, *2013*.

Ceccarelli, S., Grando, S., Maatougui, M., Michael, M., Slash, M., Haghparast, R., Rahmanian, M., Taheri, A., Al-Yassin, A., Benbelkacem, A. and Labdi, M., 2010. Plant breeding and climate changes. *The Journal of Agricultural Science*, *148*(6), pp.627-637.

Chandel, G., Dubey, M. and Meena, R., 2013. Differential expression of heat shock proteins and heat stress transcription factor genes in rice exposed to different levels of heat stress. *Journal of plant biochemistry and biotechnology*, *22*(3), pp.277-285.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Hsu, F.C. and Ko, S.S., 2006. Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. *Plant Physiology*, *140*(4), pp.1297-1305.

Chen, C., Begcy, K., Liu, K., Folsom, J.J., Wang, Z., Zhang, C. and Walia, H., 2016. Heat stress yields a unique MADS box transcription factor in determining seed size and thermal sensitivity. *Plant physiology*, *171*(1), pp.606-622.

Cheng, W., Sakai, H., Yagi, K. and Hasegawa, T., 2009. Interactions of elevated [CO2] and night temperature on rice growth and yield. *Agricultural and forest meteorology*, *149*(1), pp.51-58.

Cui, L., Li, J., Fan, Y., Xu, S. and Zhang, Z., 2006. High temperature effects on photosynthesis, PSII functionality and antioxidant activity of two Festuca arundinacea cultivars with different heat susceptibility. *Botanical Studies*, *47*(1), pp.61-69.

Das, S., Krishnan, P., Nayak, M. and Ramakrishnan, B., 2014. High temperature stress effects on pollens of rice (Oryza sativa L.) genotypes. *Environmental and Experimental Botany*, *101*, pp.36-46.

de Jong, W.W., Leunissen, J.A. and Voorter, C.E., 1993. Evolution of the alpha-crystallin/small heat-shock protein family. *Molecular biology and evolution*, *10*(1), pp.103-126.

Dexter, S.T., Tottingham, W.E. and Graber, L.F., 1932. Investigations of the hardiness of plants by measurement of electrical conductivity. *Plant Physiology*, *7*(1), p.63.

Driedonks N, Rieu I, Vrizen WH (2016) Breeding for plant heat tolerance at vegetative and

Ehrnsperger, M., Gräber, S., Gaestel, M. and Buchner, J., 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The EMBO journal*, *16*(2), pp.221-229.

ElBasyoni, I., Saadalla, M., Baenziger, S., Bockelman, H. and Morsy, S., 2017. Cell membrane stability and association mapping for drought and heat tolerance in a worldwide wheat collection. *Sustainability*, *9*(9), p.1606.

El-Esawi, M.A. and Alayafi, A.A., 2019. Overexpression of rice Rab7 gene improves drought and heat tolerance and increases grain yield in rice (Oryza sativa L.). *Genes*, *10*(1), p.56.

Fahad, S., Ihsan, M.Z., Khaliq, A., Daur, I., Saud, S., Alzamanan, S., Nasim, W., Abdullah, M., Khan, I.A., Wu, C. and Wang, D., 2018. Consequences of high temperature under changing climate optima for rice pollen characteristics-concepts and perspectives. *Archives of Agronomy and Soil Science*, *64*(11), pp.1473-1488.

Fitter, A.H.; Hay, R.K.M. *Environmental Physiology of Plants*, 3rd ed.; Academic Press:London, UK, 2002.

Fu, G., Feng, B., Zhang, C., Yang, Y., Yang, X., Chen, T., Zhao, X., Zhang, X., Jin, Q. and Tao, L., 2016. Heat stress is more damaging to superior spikelets than inferiors of rice (Oryza sativa L.) due to their different organ temperatures. *Frontiers in plant science*, *7*, p.1637.

Gosavi, G.U., Jadhav, A.S., Kale, A.A., Gadakh, S.R., Pawar, B.D. and Chimote, V.P., 2014. Effect of heat stress on proline, chlorophyll content, heat shock proteins and antioxidant enzyme activity in sorghum (Sorghum bicolor) at seedlings stage.

Guan, J.C., Jinn, T.L., Yeh, C.H., Feng, S.P., Chen, Y.M. and Lin, C.Y., 2004. Characterization of the genomic structures and selective expression profiles of nine class I small heat shock protein genes clustered on two chromosomes in rice (Oryza sativa L.). *Plant molecular biology*, *56*(5), pp.795-809.

Han, Y., Fan, S., Zhang, Q. and Wang, Y., 2013. Effect of heat stress on the MDA, proline and soluble sugar content in leaf lettuce seedlings. *Agricultural Sciences*, *4*(05), p.112.

Harsant, J., Pavlovic, L., Chiu, G., Sultmanis, S. and Sage, T.L., 2013. High temperature stress and its effect on pollen development and morphological components of harvest index in the C3 model grass Brachypodium distachyon. *Journal of experimental botany*, *64*(10), pp.2971-2983.

Harsh, A., Sharma, Y.K., Joshi, U., Rampuria, S., Singh, G., Kumar, S. and Sharma, R., 2016. Effect of short-term heat stress on total sugars, proline and some antioxidant enzymes in moth bean (Vigna aconitifolia). *Annals of Agricultural Sciences*, *61*(1), pp.57-64.

Hasan, M.A., Ahmed, J.U., Bahadur, M.M., Haque, M.M. and Sikder, S., 2007. Effect of late planting heat stress on membrane thermostability, proline content and heat susceptibility index of different wheat cultivars. *Journal of the National Science Foundation of Sri Lanka*, *35*(2).

Hasanuzzaman, M., Nahar, K., Alam, M., Roychowdhury, R. and Fujita, M., 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International journal of molecular sciences*, *14*(5), pp.9643-9684.

Haslbeck, M., Franzmann, T., Weinfurtner, D. and Buchner, J., 2005. Some like it hot: the structure and function of small heat-shock proteins. *Nature structural & molecular biology*, *12*(10), p.842.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives in Biochemistry and Biophysics* 125**,** 189–198.

Hemantaranjan, A., Bhanu, A.N., Singh, M.N., Yadav, D.K., Patel, P.K., Singh, R. and Katiyar, D., 2014. Heat stress responses and thermotolerance. *Adv. Plants Agric. Res*, *1*(12), pp.10-15406.

Herrero, M.P. and Johnson, R.R., 1980. High Temperature Stress and Pollen Viability of Maize 1. *Crop science*, *20*(6), pp.796-800.

HorieT, Matsui T, Nakagawa H, Omasa K.1996. Effect of elevated CO_2 and global climate change on rice yield in Japan. In: Omasa K, Kai K, Toda H, Uchijima Z, Yoshino M, eds. *Climate change and plants in East Asia*. Tokyo: Springer-Verlag, 39–56.

Hu, W., Hu, G. and Han, B., 2009. Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. *Plant Science*, *176*(4), pp.583-590.

IPCC, 2014. Intergovernmental Panel on Climate Change. Fifth Assessment Report of the Intergovernmental Panel on Climate Change: the Impacts, Adaptation and Vulnerability. Cambridge University Press, United Kingdom and New York, NY, USA.

Ishimaru, T., Hirabayashi, H., Sasaki, K., Ye, C. and Kobayashi, A., 2016. Breeding efforts to mitigate damage by heat stress to spikelet sterility and grain quality. *Plant Production Science*, *19*(1), pp.12-21.

Jagadish, S.V.K., Craufurd, P.Q. and Wheeler, T.R., 2007. High temperature stress and spikelet fertility in rice (Oryza sativa L.). *Journal of experimental botany*, *58*(7), pp.1627-1635.

Jagadish, S.V.K., Cairns, J., Lafitte, R., Wheeler, T.R., Price, A.H. and Craufurd, P.Q., 2010. Genetic analysis of heat tolerance at anthesis in rice. *Crop Science*, *50*(5), pp.1633-1641.

Jagadish, S.V.K., Murty, M.V.R. and Quick, W.P., 2015. Rice responses to rising temperatures–challenges, perspectives and future directions. *Plant, Cell & Environment*, *38*(9), pp.1686-1698.

Jung, K.H., Ko, H.J., Nguyen, M.X., Kim, S.R., Ronald, P. and An, G., 2012. Genome-wide identification and analysis of early heat stress responsive genes in rice. *Journal of Plant Biology*, *55*(6), pp.458-468.

Kazim, A.M., Abid, A. and Saddia, G., 2013. Response of rice (Oryza sativa L.) under elevated temperature at early growth stage: Physiological markers. *Russian Journal of Agricultural and Socio-Economic Sciences*, *20*(8).

Kilasi, N.L., Singh, J., Vallejos, C.E., Ye, C., Jagadish, S.K., Kusolwa, P. and Rathinasabapathi, B., 2018. Heat stress tolerance in rice (Oryza sativa L.): Identification of quantitative trait loci and candidate genes for seedling growth under heat stress. *Frontiers in plant science*, *9*.

Kondamudi R, Swamy KN, Chakravarthy DVN, Vishnuprasanth V, Rao YV, Rao PR, Sarla N,Subrahmanyam D, Voleti SR (2012) Heat stress in rice – physiological mechanisms and adaptation strategies page under the book crop stress and its management perspective and strategies. In: Venkateswarlu B, Shanker AK, Maheswari M (eds) Crop stress and its management: perspectives and strategies. Springer, Dordrecht, pp 193–223

Krishna, P., 2003. Plant responses to heat stress. In *Plant responses to abiotic stress* (pp. 73-101). Springer, Berlin, Heidelberg.

Kumar, N., Kumar, N., Shukla, A., Shankhdhar, S.C. and Shankhdhar, D., 2015. Impact of terminal heat stress on pollen viability and yield attributes of rice (Oryza sativa L.). *Cereal research communications*, *43*(4), pp.616-626.

Kumar, N., Suyal, D.C., Sharma, I.P., Verma, A. and Singh, H., 2017. Elucidating stress proteins in rice (Oryza sativa L.) genotype under elevated temperature: a proteomic approach to understand heat stress response. *3 Biotech*, *7*(3), p.205.

Kumari S, Roy S, Singh P, Singla-Pareek SL, Pareek A (2013) Cyclophilins: proteins in search of function. Plant Signal Behav 8:e22734

Larcher, W. *Physiological Plant Ecology*; Springer: Berlin, Germany, 1995.

Lawas, L.M., Bheemanahalli, R., Solis, C.A. and Krishna Jagadish, S.V., 2018. Sheathed Panicle Phenotype (cv. Sathi) Maintains Normal Spikelet Fertility and Grain Filling under Prolonged Heat Stress in Rice. *Crop Science*, *58*(4), pp.1693-1705.

Lee, G.J. and Vierling, E., 2000. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiology*, *122*(1), pp.189-198.

Li, Y.F., Wang, Y., Tang, Y., Kakani, V.G. and Mahalingam, R., 2013. Transcriptome analysis of heat stress response in switchgrass (Panicum virgatum L.). *BMC plant biology*, *13*(1), p.153.

Maclean, J., Hardy, B. and Hettel, G., 2013. *Rice Almanac: Source book for one of the most important economic activities on earth*. IRRI.

Martineau, J.R., Specht, J.E., Williams, J.H. and Sullivan, C.Y., 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability 1. *Crop Science*, *19*(1), pp.75-78.

Mishra, N., Srivastava, A.P., Esmaeili, N., Hu, W. and Shen, G., 2018. Overexpression of the rice gene OsSIZ1 in Arabidopsis improves drought-, heat-, and salt-tolerance simultaneously. *PloS one*, *13*(8), p.e0201716.

Mogk, A., Schlieker, C., Friedrich, K.L., Schönfeld, H.J., Vierling, E. and Bukau, B., 2003. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *Journal of Biological Chemistry*, *278*(33), pp.31033-31042.

Mohammed, A.R. and Tarpley, L., 2010. Effects of high night temperature and spikelet position on yield-related parameters of rice (Oryza sativa L.) plants. *European journal of agronomy*, *33*(2), pp.117-123.

Mohammed, A.R. and Tarpley, L., 2011. Morphological and physiological responses of nine southern US rice cultivars differing in their tolerance to enhanced ultraviolet-B radiation. *Environmental and experimental botany*, *70*(2-3), pp.174-184.

Mottaleb, K.A., Rejesus, R.M., Mohanty, S., Murty, M.V.R., Li, T., Valera, H.G. and Gumma, M.K., 2012. *Ex ante impact assessment of a drought tolerant rice variety in the presence of climate change* (No. 323-2016-11905).

Nagarajan, S., Jagadish, S.V.K., Prasad, A.H., Thomar, A.K., Anand, A., Pal, M. and Agarwal, P.K., 2010. Local climate affects growth, yield and grain quality of aromatic and non-aromatic rice in northwestern India. *Agriculture, Ecosystems & Environment*, *138*(3-4), pp.274-281.

Nevame, A.Y.M., Emon, R.M., Malek, M.A., Hasan, M.M., Alam, M., Muharam, F.M., Aslani, F., Rafii, M.Y. and Ismail, M.R., 2018. Relationship between High Temperature and Formation of Chalkiness and Their Effects on Quality of Rice. *BioMed research international*, *2018*.

Nguyen, D.N., Lee, K.J., Kim, D.I., Anh, N.T. and Lee, B.W., 2014. Modeling and validation of high-temperature induced spikelet sterility in rice. *Field crops research*, *156*, pp.293-302.

Pareek A, Singla SL, Grover A (1997) Short-term salinity and high temperature stress-associated ultrastructural alterations in young leaf cells of Oryza sativa L. Ann Bot 80:629–639

Prasad, P.V.V., Boote, K.J., Allen Jr, L.H., Sheehy, J.E. and Thomas, J.M.G., 2006. Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field crops research*, *95*(2-3), pp.398-411.

Pressman, E., Peet, M.M. and Pharr, D.M., 2002. The effect of heat stress on tomato pollen characteristics is associated with changes in carbohydrate concentration in the developing anthers. *Annals of Botany*, *90*(5), pp.631-636.

Qu, A.L., Ding, Y.F., Jiang, Q. and Zhu, C., 2013. Molecular mechanisms of the plant heat stress response. *Biochemical and biophysical research communications*, *432*(2), pp.203-207.

Rajaram, H., Chaurasia, A.K. and Apte, S.K., 2014. Cyanobacterial heat-shock response: role and regulation of molecular chaperones. *Microbiology*, *160*(4), pp.647-658.

Rang, Z.W., Jagadish, S.V.K., Zhou, Q.M., Craufurd, P.Q. and Heuer, S., 2011. Effect of high temperature and water stress on pollen germination and spikelet fertility in rice. *Environmental and Experimental Botany*, *70*(1), pp.58-65.

Reis, S.P., Lima, A.M. and de Souza, C.R.B., 2012. Recent molecular advances on downstream plant responses to abiotic stress. *International Journal of Molecular Sciences*, *13*(7), pp.8628-8647.

Sailaja, B., Subrahmanyam, D., Neelamraju, S., Vishnukiran, T., Rao, Y.V., Vijayalakshmi, P., Voleti, S.R., Bhadana, V.P. and Mangrauthia, S.K., 2015. Integrated physiological, biochemical, and molecular analysis identifies important traits and mechanisms associated with differential response of rice genotypes to elevated temperature. *Frontiers in plant science*, *6*, p.1044.

Sairam, R.K., Deshmukh, P.S., and Shukla, D.S., 1997. Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. Journal of Agronomy and Crop Science, 178: 171–178.

Sakata, T. and Higashitani, A., 2008. Male sterility accompanied with abnormal anther development in plants–genes and environmental stresses with special reference to high temperature injury. *Int. J. Plant Dev. Biol*, *2*(4).

Sangwan, S., Ram, K., Rani, P., Munjal, R., 2018. Effect of Terminal High Temperature on Chlorophyll Content and Normalized Difference Vegetation Index in Recombinant Inbred Lines of Bread Wheat. *International Journal of Current Microbiology and Applied Sciences, 7(6),* PP. 1174-1183.

Sarkar, J., Chakraborty, B. and Chakraborty, U., 2016. Temperature stress induced antioxidative and biochemical changes in wheat (Triticum aestivum L.) cultivars. *Journal of Plant Stress Physiology*, pp.22-30.

Sarsu, F., Ghanim, A.M., Das, P., Bahuguna, R.N., Kusolwa, P.M., Ashraf, M., Singla-Pareek, S.L., Pareek, A., Forster, B.P. and Ingelbrecht, I., 2018. *Pre-Field Screening Protocols for Heat-Tolerant Mutants in Rice*. Springer.

Satake, T. and Yoshida, S., 1978. High temperature-induced sterility in indica rices at flowering. *Japanese Journal of Crop Science*, *47*(1), pp.6-17.

Scharf, K.D., Siddique, M. and Vierling, E., 2001. The expanding family of Arabidopsis thaliana small heat stress proteins and a new family of proteins containing α-crystallin domains (Acd proteins). *Cell stress & chaperones*, *6*(3), p.225.

Sehgal, A., Sita, K., Siddique, K.H., Kumar, R., Bhogireddy, S., Varshney, R.K., HanumanthaRao, B., Nair, R.M., Prasad, P.V. and Nayyar, H., 2018. Drought or/and Heat-Stress Effects on Seed Filling in Food Crops: Impacts on Functional Biochemistry, Seed Yields, and Nutritional Quality. *Frontiers in plant science, 9*.

Shah F, Huang J, Kul K, Nie L, Shah T, Chen C (2011) Impact of high-temperature stress on rice plant and its traits related to tolerance. J Agric Sci 149:545–556

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C. and Wang, K., 2011. Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, *149*(5), pp.545-556.

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C. and Wang, K., 2011. Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, *149*(5), pp.545-556.

Sharma, D., Rachhoya, H.K., Sharma, M. and Agarwal, R., 2018. Effect of Rising Temperature Stress on Growth and Physiology of Domestic Crops of

Rajasthan, India. *International Journal of Current Microbiology and Applied Sciences*, 7(7), p. 1426-1440.

Sharma, R., Sharma, R., Sudan, J., Salgotra, R.K. and Singh, R., 2017. Research Article Alterations in cellular membrane stability due to heat stress in different genotypes of bread wheat. *Electronic Journal of Plant Breeding*, *8*(4), pp.1022-1027.

Siddique, M., Gernhard, S., von Koskull-Döring, P., Vierling, E. and Scharf, K.D., 2008. The plant sHSP superfamily: five new members in Arabidopsis thaliana with unexpected properties. *Cell Stress and Chaperones*, *13*(2), pp.183-197.

Soda, N., Gupta, B.K., Anwar, K., Sharan, A., Singla-Pareek, S.L. and Pareek, A., 2018. Rice intermediate filament, OsIF, stabilizes photosynthetic machinery and yield under salinity and heat stress. *Scientific reports*, *8*(1), p.4072.

Song, L., Yue, L., Zhao, H. and Hou, M., 2013. Protection effect of nitric oxide on photosynthesis in rice under heat stress. *Acta physiologiae plantarum*, *35*(12), pp.3323-3333.

Tiwari, A., Prasad, S., Jaiswal, B., Gyanendra, K., Singh, S., Singh, K.N., 2017. Effect of Heat Stress on Yield Attributing Traits in Wheat (Triticum aestivum L.). *International Journal of Current Microbiology and Applied Sciences* , 6(12), p. 2738-2744.

Tonini, A. and Cabrera, E., 2011. *Opportunities for global rice research in a changing world* (No. 2215-2019-1630).

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A. and Latif, M.A., 2015. Expression of target gene Hsp70 and membrane stability determine heat tolerance in chili pepper. *Journal of the American Society for Horticultural Science*, *140*(2), pp.144-150.

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A. and Latif, M.A., 2015. Expression of target gene Hsp70 and membrane stability determine heat tolerance in chili pepper. *Journal of the American Society for Horticultural Science*, *140*(2), pp.144-150.

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A., Latif, M.A. and Oladosu, Y., 2014. Heat shock proteins: functions and response against heat stress in plants. *Int. J. Sci. Technol. Res*, *3*(11), pp.204-218.

van Montfort, R.L., Basha, E., Friedrich, K.L., Slingsby, C. and Vierling, E., 2001. Crystal structure and assembly of a eukaryotic small heat shock protein. *Nature Structural & Molecular Biology*, *8*(12), p.1025.

Van Oort, P.A.J., Saito, K., Zwart, S.J. and Shrestha, S., 2014. A simple model for simulating heat induced sterility in rice as a function of flowering time and transpirational cooling. *Field Crops Research*, *156*, pp.303-312.

Veerasamy, M., He, Y. and Huang, B., 2007. Leaf senescence and protein metabolism in creeping bentgrass exposed to heat stress and treated with cytokinins. *Journal of the American Society for Horticultural Science*, *132*(4), pp.467-472.

Vierling, E., 1991. The roles of heat shock proteins in plants. *Annual review of plant biology*, *42*(1), pp.579-620.

Vishwakarma, H., Junaid, A., Manjhi, J., Singh, G.P., Gaikwad, K. and Padaria, J.C., 2018. Heat stress transcripts, differential expression, and profiling of heat stress tolerant gene TaHsp90 in Indian wheat (Triticum aestivum L.) cv C306. *PloS one*, *13*(6), p.e0198293.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–233

Wahid, A., Gelani, S., Ashraf, M. and Foolad, M.R., 2007. Heat tolerance in plants: an overview. *Environmental and experimental botany*, *61*(3), pp.199-223.

Wang, Q.L., Chen, J.H., He, N.Y. and Guo, F.Q., 2018. Metabolic reprogramming in chloroplasts under heat stress in plants. *International journal of molecular sciences*, *19*(3), p.849.

Wang, Q.L., Chen, J.H., He, N.Y. and Guo, F.Q., 2018. Metabolic reprogramming in chloroplasts under heat stress in plants. *International journal of molecular sciences*, *19*(3), p.849.

Wilson, R.A., Sangha, M.K., Banga, S.S., Atwal, A.K. and Gupta, S., 2014. Heat stress tolerance in relation to oxidative stress and antioxidants in Brassica juncea. *Journal of environmental biology*, *35*(2), p.383.

Wu, C., Cui, K., Wang, W., Li, Q., Fahad, S., Hu, Q., Huang, J., Nie, L. and Peng, S., 2016. Heat-induced phytohormone changes are associated with disrupted early reproductive development and reduced yield in rice. *Scientific reports*, *6*, p.34978.

Xue DW, Jiang H, Hu J, Zhang XQ, Guo LB, Zeng DL, Dong GJ, Sun GC, Qian Q, 2012. Characterization of physiological response and identification of associated genes under heat stress in rice seedlings. Plant Physiol Biochem 61:46–53

Yoshida S (1981) Fundamentals of rice crops science. International Rice Research Institute, Los Banos, The Philippines

Yoshida, S., Satake, T. and Mackill, D.S., 1981. High-temperature stress in rice [study conducted at IRRI, Philippines]. *IRRI Research Paper Series (Philippines)*.

Zafar, S.A., Hameed, A., Khan, A.S. and Ashraf, M., 2017. Heat shock induced morpho-physiological response in indica rice (Oryza sativa L.) at early seedling stage. *Pakistan Journal of Botany*, *49*(2), pp.453-463.

Zafar, S.A., Hameed, A., Nawaz, M.A., Wei, M.A., Noor, M.A. and Hussain, M., 2018. Mechanisms and molecular approaches for heat tolerance in rice (Oryza sativa L.) under climate change scenario. *Journal of Integrative Agriculture*, *17*(4), pp.726-738.

Zhang, C., Li, G., Chen, T., Feng, B., Fu, W., Yan, J., Islam, M.R., Jin, Q., Tao, L. and Fu, G., 2018. Heat stress induces spikelet sterility in rice at anthesis through inhibition of pollen tube elongation interfering with auxin homeostasis in pollinated pistils. *Rice*, *11*(1), p.14.

Zou, J., Liu, C. and Chen, X., 2011. Proteomics of rice in response to heat stress and advances in genetic engineering for heat tolerance in rice. *Plant Cell Reports*, *30*(12), pp.2155-2165.

APPENDIX–A:

Department of Agricultural Meteorology
Indira Gandhi Agricultural University, Raipur (C.G.) - 492 006

Station: Labhandi **Monthly Meteorological Data: 2018**

Month	**Max. Temp. (°C)**	**Min. Temp. (°C)**	**RainFall (mm)**	**Rainy days**	**Relative Humidity (%)**		**Vapour pressure (mm)**		**Wind Velocity (Kmph)**	**Evaporation (mm)**	**Sun Shine (hours)**
					I	**II**	**I**	**II**			
Jan.	28.5	10.1	0	0	83	23	8.1	6.4	2.2	103.2	8.7
Feb.	31.5	15	16.4	3	81	30	10.9	9.4	2.8	112.6	8.3
Mar	36.3	19.6	1.2	0	64	21	11.8	8.8	3.2	186.7	11.1
Apr.	39.3	23.8	41.4	4	61	23	15.2	11.3	4.7	235.7	8.7
May	41.8	27.4	15.4	3	62	31	19.4	17.4	6.7	312.4	8.9
Jun.	36.9	26.3	141.5	9	78	46	21.8	19.6	6.7	226.9	6.4
Jul.	31.2	25.4	328	11	90	74	22.7	22.7	7.5	100.5	1.4
Aug.	29.8	24.8	560.2	15	93	81	22.8	23.3	5.6	92.2	1.5
Sep.	31.3	24.5	84.8	7	92	65	22.3	20.7	3.7	96.9	4.9
Oct.	32.9	21.4	0	0	88	45	18.4	16.3	1.5	116.8	8.1
Nov.	31.3	16	0	0	87	34	12.9	11.2	1.3	100.7	8.5
Dec.	25.9	12.4	47.2	2	88	42	10.2	9.8	1.5	70.6	4.7
Total			**1236.1**							**1755.2**	
Average	**33.1**	**20.6**			**81**	**43**	**16.4**	**14.7**	**4**		**6.8**

Source: http://igau.edu.in/agromet.htm

RESUME

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2 messages

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Dear Mr Editor,

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सारांश

(a).थीसिस का शीर्षक	"चावल (ओरिज़ा सैटिवा) में विभेदित रूप से व्यक्त गर्मी तनाव उत्तरदायी उम्मीदवार जीन का निर्णय लेना"
(b).छात्र का पुरा नाम	पूला लोहिथा
(c).प्रमुख विषय	पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
(d).प्रमुख सलहाकार का नाम और पता	डॉ॰ गिरीश चंदेल पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी विभाग, कृषि महाविद्यालय, आई जी के वी, रायपुर
(e).अवार्ड होने वाली उपाधि	एमएससी(कृषि) पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
	छात्र के हस्ताक्षर
प्रमुख सलहाकार के हस्ताक्षर	
दिनांक	विभागाध्यक्ष के हस्ताक्षर

ग्लोबल वार्मिंग से 2100 (IPCC 2014) तक पृथ्वी की सतह के औसत तापमान में 1.5 से 4.8 21°C की वृद्धि होने की उम्मीद है और यह 21 वीं सदी के अंत तक चावल सहित प्रमुख फसल पौधों की उपज को 41% तक कम करने की सूचना है। चावल दुनिया भर में सबसे महत्वपूर्ण प्रधान खाद्य फसल है और एशिया और अफ्रीका में 3 बिलियन से अधिक लोगों को खिलाती है। गर्मी का तनाव वनस्पति, प्रजनन और पकने वाले चरणों की तरह सभी चरणों में चावल की फसल के विकास को प्रभावित करता है, लेकिन फूल के चरण के दौरान प्रभाव गंभीर होता है, क्योंकि यह स्पाइकलेट बाँझपन की ओर जाता है जो कम पैदावार का कारण बनता है। बढ़ती आबादी, घटती कृषि योग्य भूमि और बढ़ते तापमान जैसी जटिल चुनौतियों का सामना करने के लिए गर्मी सहिष्णुता के साथ चावल की उन्नत किस्मों की आवश्यकता होती है। विभिन्न फसलों में गर्मी के तनाव के प्रति उत्तरदायी जीन का अभिव्यक्ति विश्लेषण आज के कृषि में बहुत महत्व रखेगा।

उच्च तापमान उपचार के संपर्क में आने के बाद चावल की ट्रांसक्रिप्शनल अनुक्रमण जीन पर जानकारी प्रदान कर सकता है, जो कि गर्मी के तनाव के संपर्क में अंतर पर विनियमित होते हैं और गर्मी के सहिष्णुता के लिए जिम्मेदार प्रमुख जीन की पहचान करना और उनका विच्छेदन करना आवश्यक

है। उच्च तापमान तनाव के तहत विशेष प्रोटीन के नए समूह का निर्माण करता है जिसे 'हीट शॉक प्रोटीन (एचएसपी)' कहा जाता है, जिसे उनके आणविक भार के आधार पर पांच परिवारों में वर्गीकृत किया गया है: 1) छोटे हीट-शॉक प्रोटीन (sHSPs), 2) H660, 3) Hsp70, 4) Hsp90 और 5) Hsp100। अध्ययन में बताया गया है कि पौधे आणविक चैपरों के रूप में हीट स्ट्रेस फंक्शनिंग के तहत बड़ी मात्रा में एसएचएसपी का संश्लेषण करते हैं, यह सुझाव देते हैं कि वे पौधों में थर्मो-टॉलरेंस को समाप्त करने के लिए एक प्रमुख भूमिका निभाते हैं। उच्चतर पौधे कम से कम 20 sHSPs रखते हैं। एचएसपी के ट्रांसक्रिप्शन को कुछ नियामक प्रोटीनों द्वारा संरक्षित किया जाता है, जिसे हीट स्ट्रेस ट्रांसक्रिप्शन कारक (Hsfs) कहा जाता है। विनियमन में इसकी विशिष्ट भूमिका के साथ, पौधों में कम से कम 21 Hsfs देखे जाते हैं। हीट शॉक प्रोटीन प्रोटीन, कार्यात्मक गैर-देशी प्रोटीन के संचय को रोकने और refolding को रोकने के द्वारा सिग्नलिंग, अनुवाद, मेजबान-रक्षा तंत्र, कार्बोहाइड्रेट चयापचय और अमीनो एसिड चयापचय में एक प्रमुख भूमिका निभाते हैं। sHSPs गर्मी तनाव के तहत विकृत प्रोटीन के स्थिरीकरण की सुविधा देता है। इसके आधार पर, वर्तमान अध्ययन में कुछ प्रमुख जीनों की पहचान करने की योजना बनाई गई थी जो चौदह चावल के जीनोटाइप (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR) में गर्मी सहनशीलता के लिए जिम्मेदार हैं 1, अन्नदा, पूर्णिमा, कर्म मशूरी, ARB-6-11, GP-145-40, MTU-1010, RRF-127) पैतृक दीक्षा से पहले देर से वानस्पतिक अवस्था में। गर्मी सहन करने वाले लक्षणों की पहचान के लिए नियंत्रण और तनाव की स्थिति (42)C) के तहत विविध आनुवंशिक पृष्ठभूमि से संबंधित जीनोटाइप दो सेटों में उगाए गए थे। चावल के जीनोटाइप के फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन गर्मी के तनाव के तहत किया गया था और समानांतर में ज्ञात गर्मी से संबंधित उम्मीदवार जीन का एक सेट अर्ध-मात्रात्मक आरटी-पीसीआर विश्लेषण का उपयोग करके अभिव्यक्ति विश्लेषण अध्ययन के लिए चुना गया था। फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन के तहत छह मापदंडों को ध्यान में रखा जाता है। वे झिल्ली स्थिरता सूचकांक (एमएसआई), पराग प्रजनन, स्पाइकलेट प्रजनन क्षमता, क्लोरोफिल सामग्री, प्रोलाइन और एमडीआर स्तर हैं।

चावल पर जैविक और जैव रासायनिक अध्ययन ने गर्मी के तनाव के लिए कुछ सहिष्णु और अतिसंवेदनशील जीनोटाइप की पहचान की। नियंत्रण की तुलना में तनाव के दौरान जीनोटाइप आरआरएफ -127, नगीना -22, कर्म मशूरी, सीजीजेडआर -1 और अन्नदा में क्रमशः 19.8%, 21.1%, 27.5%, 51.7% और 69.8% का इलेक्ट्रोलाइट रिसाव दर्ज किया गया था। तनाव की स्थिति के तहत पराग की उर्वरता में कमी का सबसे कम प्रतिशत क्रमश: RRF-127, नगीना -22, GP-145-103, अन्नदा और CGZR-1 में 14.4%, 18.1%, 24.9%, 28.3% और 37.9% के रूप में देखा गया। । तनाव के तहत

स्पाइकलेट प्रजनन क्षमता का प्रतिशत जीनोटाइप आरआरएफ -127, नगीना -22, अन्नदा, कर्म मशूरी और सीजीजेडआर -1 में क्रमशः 14%, 19%, 25%, 31% और 42% के रूप में सबसे कम था। नियंत्रण पर तनाव के लिए प्रोलाइन सामग्री में गुना वृद्धि की तुलना करते समय, नगीना -22 ने CGZR-1 (11.4 गुना), RRF-127 (11.2 गुना), अन्नदा (10.5 गुना), GP-145 के बाद 20.6 सिलवटों की उच्चतम वृद्धि दिखाई। -103 (7.0 गुना) और कर्मा मशूरी (6.0 गुना)। क्लोरोफिल सामग्री में, अन्नदा में नियंत्रण के साथ तुलना में तनाव के तहत कर्मा मशूरी (1.0 गुना) द्वारा पीछा किया जाने वाला सभी क्लो, च्लब और कुल क्लोरोफिल सामग्री के लिए 1.0 गुना की कमी है। नियंत्रण पर तनाव के लिए एमडीए सामग्री में गुना वृद्धि की तुलना करते समय। MDA सामग्री में सबसे कम गुना वृद्धि RRF-127 द्वारा 1.1 गुना वृद्धि के साथ दिखाई गई जिसके बाद नगीना -22 (1.4 गुना), CGZR-1 (1.5 गुना), अन्नदा (1.8 गुना), GP-145-103 (2.1 गुना) है। और कर्मा मशूरी (2.5 गुना)।

अध्ययन के लिए चुने गए पांच जीनों (OsHSP26.7, OsHSP16.9, OsHSP DJJ, OsHSP18 और 60Kda चैपरॉन) के बीच, OsHSP DnaJ ने बिना किसी महत्वपूर्ण बदलाव के नियंत्रण और तनाव दोनों के तहत लगातार अभिव्यक्ति दिखाई है। शेष चार जीनों ने तनाव के तहत सभी जीनोटाइप में अप-विनियमन दिखाया है। ओएनएचएसपी 26.7 जीन ने अन्नदा (13.9 गुना), कर्मा मशूरी (11.5 गुना), जीपी-145-103 (8.6 गुना) और सीजीजेडआर -1 (3.7 गुना) के बाद चावल जीनोटाइप आरआरएफ-127 (14.3 गुना) में मजबूत विनियमन दिखाया है।) । OsHSP 16.9 जीन ने RRF-127 (10.0 गुना) और अन्नदा (3.7 गुना), CGZR-1 (3.4 गुना), GP-145-103 (3.2 गुना) और कर्मा मशूरी (3.1 गुना) के बाद अप-विनियमन दिखाया है। ओजीएचएसपी 18 जीन ने जीपी-145-103 (17.7 गुना) में सीजीजेडआर -1 (14.1 गुना), अन्नदा (13.8 गुना), जीपी 145-55 (11.9 गुना) और पूर्णिमा (8.9 गुना) के बाद विनियमन दिखाया। 60Kda चैपरोन जीन ने गर्मी तनाव के तहत लगभग सभी जीनोटाइप में अप-विनियमन दिखाया है, लेकिन अप-विनियमन न्यूनतम स्तर तक था। अध्ययन के लिए चुने गए सभी चौदह जीनोटाइप्स में से, RRF-127, अन्नदा, कर्म मशूरी, CGZR-1 और GP-145-103 में MTU-1010, ARB-6-11 और GP- जैसे हीट तनाव और जीनोटाइप के लिए एक सकारात्मक विनियमन दिखाया गया है। 145-55 ने ऊष्मा तनाव के प्रति नकारात्मक नियमन दिखाया। इन निष्कर्षों को फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन और अभिव्यक्ति विश्लेषण अध्ययन के साथ सहसंबंध में देखा गया था, जो छोटे ऊष्मा शॉक प्रोटीन परिवार से संबंधित पांच अलग-अलग गर्मी उत्तरदायी जीन के साथ किया गया था, जिसमें नियंत्रण की स्थिति की तुलना में गर्मी के तनाव के तहत अंतर अभिव्यक्ति थी। हालाँकि, इन जीनों का सकारात्मक समावेश विभिन्न प्रतिलेखन कारकों की पहचान करने में महत्वपूर्ण भूमिका निभाता है, जो विभिन्न ताप सहिष्णु तंत्रों के लिए या तनाव के तहत चावल में पादप रक्षा तंत्र को सक्रिय

करने के लिए विभिन्न संकेतन पथों के क्रॉस-लिंकिंग के लिए जिम्मेदार रहे हैं। यह चावल की फसल की गर्मी सहिष्णुता प्रतिक्रिया के लिए एक आधार के रूप में लिया जा सकता है, जो चावल और साथ ही अन्य फसल पौधों में गर्मी के तनाव के लिए उत्तरदायी उम्मीदवार जीन के आगे सत्यापन अध्ययन के लिए उपयोगी हो सकता है।

DECIPHERING THE DIFFERENTIALLY EXPRESSED HEAT STRESS RESPONSIVE CANDIDATE GENES IN RICE

(Oryza sativa)

M.Sc. (Ag.) Thesis

by

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INDIRA GANDHI KRISHI VISHWAVIDYALAYA,
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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

Poola Lohitha

IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

Master of Science

in

Agriculture

(Plant Molecular Biology and Biotechnology)

UE ID: 20171827408 **College ID: 120117181**

June, 2018

CERTIFICATE - I

This is to certify that the thesis entitled **“Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)”*** submitted in partial fulfillment of the requirements for the degree of **“Master of Science in Agriculture”** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Poola Lohitha** under my/our guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or certificate course. All the assistance and help received during the course of the investigations have been duly acknowledged.

Chairman

Date:

THESIS APPROVED BY THE STUDENT’S ADVISORY COMMITTEE

Chairman (Dr. Girish Chandel) ____________________

Member (Dr. Zenu Jha) ____________________

Member (Dr. Sunil Nair) ____________________

Member (Dr. R R. Saxena) ____________________

CERTIFICATE – II

This is to certify that the thesis entitled **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)*"** submitted by **Poola Lohitha** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur, in partial fulfilment of the requirements for the degree of **Master of Science in Agriculture** in the Department of **Plant Molecular Biology and Biotechnology** has been approved by the external examiner and Student's Advisory Committee after oral examination.

Signature External Examiner

(Name ____________________)

Date:

Major Advisor ____________________

Head of the Department ____________________

Faculty Dean ____________________

Approved/Not approved

Director of Instructions ____________________

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Symbol/ notation	Indicates
%	Percent
µ	Micro
&	And
ºC	Degree Celsius
@	at the rate of
Bp	Base pair
et al.	And others
gm	Gram
i.e	That is
µg	Microgram
Ml	Millilitre
M	Molar
mg	Milligram
Ng	Nanogram
ppm	Parts per million
Tm	Melting temperature
Viz	As follows

LIST OF ABBREVIATIONS

Abbreviations	Details
ABA	Abscicic acid
cDNA	Complementary DNA
CSI	Chlorophyll stability index
dATP	Deoxy adenosine 5’ triphosphate
dCTPs	Deoxy cytidine 5’ triphosphates
DEPC	Di ethyl pyrocarbonate
dGTPs	Deoxy guanosine 5’ triphosphate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
dTTPs	Deoxy thymidine 5’ triphosphates
EDTA	Ethylene diamine tetra acetic acid
ESTs	Expressed sequence tags
EtBr	Ethidium bromide
HSFs	Heat stress transcription factors
HSG	Heat shock gene
Hsp	Heat shock protein
IAA	Indole acetic acid
IPCC	Intergovernmental panel on climate change
LEA	Late embryogenesis abundant
MDA	Malondialdehyde
MSI	Membrane stability index
MW	Molecular weight
PCR	Polymerase Chain Reaction
pH	Log 1/(H+)
PMC	Pollen mother cells
QTLs	Qunatitative trait loci
RIL	Recombinant inbred line

RNA	Ribonucleic acid

Abbreviations	Details
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Real time Polymerase chain reaction
smHSP	Small heat shock proteins
SNP	Single nucleotide polymorphisms
SSR	Simple sequence repeats

THESIS ABSTRACT

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a) Title of the Thesis: “Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)”*

b) Full Name of Student: Poola Lohitha

c) Major Subject: Plant Molecular Biology and Biotechnology

d) Name and Address of the Major Advisor: Dr. Girish Chandel (Professor) Dept. of Plant Molecular Biology and Biotechnology, College of Agriculture, IGKV, Raipur.

e) Degree to be Awarded: M.Sc.(Ag.) Plant Molecular Biology and Biotechnology

Signature of the Student

Signature of Major Advisor

Date: _____________

Signature of Head of the Department

Global warming is expected to increase the earth’s surface mean temperatures by 1.5 to 4.8 ºC by 2100 (IPCC 2014) and it is reported to reduce the yield of major crop plants including rice by 41% by the end of 21st century. Rice is the most important staple food crop across the world and feeds more than 3 billion people in Asia and Africa. Heat stress affects the growth of rice crop at all stages of growth like vegetative, reproductive and ripening phases but the effect is severe during flowering stage, as it leads to spikelet sterility that causes reduced yields. Improved varieties of rice with heat tolerance are required to meet the complex challenges like increasing population, decreasing arable land and increasing temperatures. Expression analysis of genes responsive to heat stress in different crops will be of great importance in today’s agriculture.

Transcriptome sequencing of rice after exposure to high temperature treatments could provide information on genes, that are differentially regulated on exposure to heat stress and its dissection is necessary to identify and characterize key genes responsible for heat tolerance. High temperatures produces the new group of special proteins under stress called as ‘Heat Shock Proteins (HSPs)’, which are categorized into five families based on their molecular weights: 1) small heat-shock proteins (sHSPs), 2) Hsp60, 3) Hsp70, 4) Hsp90 and 5) Hsp100. Studies reported plants synthesize large amounts of sHSPs under heat stress functioning as molecular chaperons, suggesting that they play a major role for enduring thermo-tolerance in plants. Higher plants posses at least 20 sHSPs. Transcription of HSPs is guarded by some regulatory proteins, called heat stress transcription factors (Hsfs). At least 21 Hsfs are seen in plants, with its specific role in regulation. Heat shock proteins perform a major role in signalling, translation, host-defence mechanisms, carbohydrate metabolism and amino acid metabolism by maintaining the functional conformation of proteins, preventing accumulation of non-native proteins and refolding. sHSPs facilitate stabilization of denatured proteins under heat stress. Based on this, the current study was planned to identify some key genes that are responsible for heat tolerance in fourteen rice genotypes (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mashuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) at late vegetative stage before panicle initiation. Genotypes belonging to diverse genetic background were grown in two sets under control and stress conditions (42˚C) for identifying heat tolerant traits. Phenological and biochemical characterization of rice genotypes was done under heat stress and in parallel a set of known heat related candidate genes were selected for expression analysis studies using semi-quantitative RT-PCR analysis. Under phenological and biochemical characterization six parameters are taken into consideration. They are membrane stability index (MSI), pollen fertility, spikelet fertility, chlorophyll content, proline and MDA levels.

Phenological and biochemical studies on rice resulted in identifying some of the tolerant and susceptible genotypes for heat stress. Genotypes RRF-127 , Nagina-22, Karma mashuri, CGZR-1 and Annada had recorded lower electrolyte leakage of 19.8%, 21.1%, 27.5%, 51.7% and 69.8% respectively under stress when compared to control. Lowest percentage of decrease in pollen fertility under stress conditions was observed in the genotypes RRF-127, Nagina-22, GP-145-103, Annada and CGZR-1 as 14.4%, 18.1%, 24.9%, 28.3% and 37.9% respectively. Percentage of Spikelet fertility decrease under stress was lowest in the genotypes RRF-127, Nagina-22, Annada, Karma mashuri and CGZR-1 as 14%, 19%, 25%, 31% and 42% respectively. When comparing the fold increase in proline content for stress over control, Nagina-22 showed highest fold increase of 20.6 folds followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mashuri (6.0 fold). In chlorophyll content, Annada has the lowest fold decrease of 1.0 folds for all chla, chlb and total chlorophyll content followed by Karma mashuri (1.0 fold) under stress when compared with control. When comparing the fold increase in MDA content for stress over control. The lowest fold increase in MDA content was shown by RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mashuri (2.5 fold).

Among the five genes (OsHSP26.7, OsHSP16.9, OsHSP DnaJ, OsHSP18 and 60Kda chaperon), selected for the study, OsHSP DnaJ has shown consistent expression under both control and stress with no significant change. The remaining four genes has shown up-regulation in all the genotypes under stress. OsHSP 26.7 gene has shown strong up-regulation in rice genotype RRF-127 (14.3 fold) followed by Annada (13.9 fold), Karma mashuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold). OsHSP 16.9 gene has shown up-regulation in RRF-127 (10.0 fold) followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mashuri (3.1 fold). OsHSP 18 gene showed up-regulation in GP-145-103 (17.7 fold) followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and poornima (8.9 fold). 60Kda chaperon gene has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. Among all the fourteen genotypes selected for the study, RRF-127, Annada, Karma mashuri, CGZR-1 and GP-145-103 showed a positive regulation towards heat stress and genotypes like MTU-1010, ARB-6-11 and GP-145-55 showed negative regulation towards heat stress. These findings were observed to be in correlation with the phenological and biochemical characterization and expression analysis studies carried out with five different heat responsive genes belonging to small heat shock proteins family, which had differential expression under heat stress when compared to control conditions. However positive induction of these genes leads to a key role in identification of different transcription factors, that have been responsible for different heat tolerant mechanisms or in cross-linking of different signalling pathways to activate plant defence mechanisms in rice under stress. This can be taken as a base for heat tolerance response of rice crop, which may be useful for further validation studies of the candidate genes responsive for heat stress in rice as well as other crop plants.

सारांश

(a).थीसिस का शीर्षक	"चावल (ओरिज़ा सैटिवा) में विभेदित रूप से व्यक्त गर्मी तनाव उत्तरदायी उम्मीदवार जीन का निर्णय लेना"
(b).छात्र का पुरा नाम	पूला लोहिथा
(c).प्रमुख विषय	पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
(d).प्रमुख सलहाकार का नाम और पता	डॉ॰ गिरीश चंदेल पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी विभाग, कृषि महाविद्यालय, आई जी के वी, रायपुर
(e).अवार्ड होने वाली उपाधि	एमएससी(कृषि) पादप आणविक जीवविज्ञान एव जैव प्रौदयौगिकी
	छात्र के हस्ताक्षर
प्रमुख सलहाकार के हस्ताक्षर	
दिनांक	विभागाध्यक्ष के हस्ताक्षर

ग्लोबल वार्मिंग से 2100 (IPCC 2014) तक पृथ्वी की सतह के औसत तापमान में 1.5 से 4.8 21˚C की वृद्धि होने की उम्मीद है और यह 21 वीं सदी के अंत तक चावल सहित प्रमुख फसल पौधों की उपज को 41% तक कम करने की सूचना है। चावल दुनिया भर में सबसे महत्वपूर्ण प्रधान खाद्य फसल है और एशिया और अफ्रीका में 3 बिलियन से अधिक लोगों को खिलाती है। गर्मी का तनाव वनस्पति, प्रजनन और पकने वाले चरणों की तरह सभी चरणों में चावल की फसल के विकास को प्रभावित करता है, लेकिन फूल के चरण के दौरान प्रभाव गंभीर होता है, क्योंकि यह स्पाइकलेट बाँझपन की ओर जाता है जो कम पैदावार का कारण बनता है। बढ़ती आबादी, घटती कृषि योग्य भूमि और बढ़ते तापमान जैसी जटिल चुनौतियों का सामना करने के लिए गर्मी सहिष्णुता के साथ चावल की उन्नत किस्मों की आवश्यकता होती है। विभिन्न फसलों में गर्मी के तनाव के प्रति उत्तरदायी जीन का अभिव्यक्ति विश्लेषण आज के कृषि में बहुत महत्व रखेगा।

उच्च तापमान उपचार के संपर्क में आने के बाद चावल की ट्रांसक्रिप्शनल अनुक्रमण जीन पर जानकारी प्रदान कर सकता है, जो कि गर्मी के तनाव के संपर्क में अंतर पर विनियमित होते हैं और गर्मी के सहिष्णुता के लिए जिम्मेदार प्रमुख जीन की पहचान करना और उनका विच्छेदन करना आवश्यक

है। उच्च तापमान तनाव के तहत विशेष प्रोटीन के नए समूह का निर्माण करता है जिसे 'हीट शॉक प्रोटीन (एचएसपी)' कहा जाता है, जिसे उनके आणविक भार के आधार पर पांच परिवारों में वर्गीकृत किया गया है: 1) छोटे हीट-शॉक प्रोटीन (sHSPs), 2) H660, 3) Hsp70, 4) Hsp90 और 5) Hsp100। अध्ययन में बताया गया है कि पौधे आणविक चैपरों के रूप में हीट स्ट्रेस फंक्शनिंग के तहत बड़ी मात्रा में एसएचएसपी का संश्लेषण करते हैं, यह सुझाव देते हैं कि वे पौधों में थर्मो-टॉलरेंस को समाप्त करने के लिए एक प्रमुख भूमिका निभाते हैं। उच्चतर पौधे कम से कम 20 sHSPs रखते हैं। एचएसपी के ट्रांसक्रिप्शन को कुछ नियामक प्रोटीनों द्वारा संरक्षित किया जाता है, जिसे हीट स्ट्रेस ट्रांसक्रिप्शन कारक (Hsfs) कहा जाता है। विनियमन में इसकी विशिष्ट भूमिका के साथ, पौधों में कम से कम 21 Hsfs देखे जाते हैं। हीट शॉक प्रोटीन प्रोटीन, कार्यात्मक गैर-देशी प्रोटीन के संचय को रोकने और refolding को रोकने के द्वारा सिग्नलिंग, अनुवाद, मेजबान-रक्षा तंत्र, कार्बोहाइड्रेट चयापचय और अमीनो एसिड चयापचय में एक प्रमुख भूमिका निभाते हैं। sHSPs गर्मी तनाव के तहत विकृत प्रोटीन के स्थिरीकरण की सुविधा देता है। इसके आधार पर, वर्तमान अध्ययन में कुछ प्रमुख जीनों की पहचान करने की योजना बनाई गई थी जो चौदह चावल के जीनोटाइप (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR) में गर्मी सहनशीलता के लिए जिम्मेदार हैं 1, अन्नदा, पूर्णिमा, कर्म मशूरी, ARB-6-11, GP-145-40, MTU-1010, RRF-127) पैतृक दीक्षा से पहले देर से वानस्पतिक अवस्था में। गर्मी सहन करने वाले लक्षणों की पहचान के लिए नियंत्रण और तनाव की स्थिति (42)C) के तहत विविध आनुवंशिक पृष्ठभूमि से संबंधित जीनोटाइप दो सेटों में उगाए गए थे। चावल के जीनोटाइप के फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन गर्मी के तनाव के तहत किया गया था और समानांतर में ज्ञात गर्मी से संबंधित उम्मीदवार जीन का एक सेट अर्ध-मात्रात्मक आरटी-पीसीआर विश्लेषण का उपयोग करके अभिव्यक्ति विश्लेषण अध्ययन के लिए चुना गया था। फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन के तहत छह मापदंडों को ध्यान में रखा जाता है। वे झिल्ली स्थिरता सूचकांक (एमएसआई), पराग प्रजनन, स्पाइकलेट प्रजनन क्षमता, क्लोरोफिल सामग्री, प्रोलाइन और एमडीआर स्तर हैं।

चावल पर जैविक और जैव रासायनिक अध्ययन ने गर्मी के तनाव के लिए कुछ सहिष्णु और अतिसंवेदनशील जीनोटाइप की पहचान की। नियंत्रण की तुलना में तनाव के दौरान जीनोटाइप आरआरएफ -127, नगीना -22, कर्म मशूरी, सीजीजेडआर -1 और अन्नदा में क्रमशः 19.8%, 21.1%, 27.5%, 51.7% और 69.8% का इलेक्ट्रोलाइट रिसाव दर्ज किया गया था। तनाव की स्थिति के तहत पराग की उर्वरता में कमी का सबसे कम प्रतिशत क्रमश: RRF-127, नगीना -22, GP-145-103, अन्नदा और CGZR-1 में 14.4%, 18.1%, 24.9%, 28.3% और 37.9% के रूप में देखा गया। । तनाव के तहत

स्पाइकलेट प्रजनन क्षमता का प्रतिशत जीनोटाइप आरआरएफ -127, नगीना -22, अन्नदा, कर्म मशूरी और सीजीजेडआर -1 में क्रमशः 14%, 19%, 25%, 31% और 42% के रूप में सबसे कम था। नियंत्रण पर तनाव के लिए प्रोलाइन सामग्री में गुना वृद्धि की तुलना करते समय, नगीना -22 ने CGZR-1 (11.4 गुना), RRF-127 (11.2 गुना), अन्नदा (10.5 गुना), GP-145 के बाद 20.6 सिलवटों की उच्चतम वृद्धि दिखाई। -103 (7.0 गुना) और कर्मा मशूरी (6.0 गुना)। क्लोरोफिल सामग्री में, अन्नदा में नियंत्रण के साथ तुलना में तनाव के तहत कर्मा मशूरी (1.0 गुना) द्वारा पीछा किया जाने वाला सभी क्लो, च्लब और कुल क्लोरोफिल सामग्री के लिए 1.0 गुना की कमी है। नियंत्रण पर तनाव के लिए एमडीए सामग्री में गुना वृद्धि की तुलना करते समय। MDA सामग्री में सबसे कम गुना वृद्धि RRF-127 द्वारा 1.1 गुना वृद्धि के साथ दिखाई गई जिसके बाद नगीना -22 (1.4 गुना), CGZR-1 (1.5 गुना), अन्नदा (1.8 गुना), GP-145-103 (2.1 गुना) है। और कर्मा मशूरी (2.5 गुना)।

अध्ययन के लिए चुने गए पांच जीनों (OsHSP26.7, OsHSP16.9, OsHSP DJJ, OsHSP18 और 60Kda चैपरॉन) के बीच, OsHSP DnaJ ने बिना किसी महत्वपूर्ण बदलाव के नियंत्रण और तनाव दोनों के तहत लगातार अभिव्यक्ति दिखाई है। शेष चार जीनों ने तनाव के तहत सभी जीनोटाइप में अप-विनियमन दिखाया है। ओएनएचएसपी 26.7 जीन ने अन्नदा (13.9 गुना), कर्मा मशूरी (11.5 गुना), जीपी-145-103 (8.6 गुना) और सीजीजेडआर -1 (3.7 गुना) के बाद चावल जीनोटाइप आरआरएफ-127 (14.3 गुना) में मजबूत विनियमन दिखाया है।) । OsHSP 16.9 जीन ने RRF-127 (10.0 गुना) और अन्नदा (3.7 गुना), CGZR-1 (3.4 गुना), GP-145-103 (3.2 गुना) और कर्मा मशूरी (3.1 गुना) के बाद अप-विनियमन दिखाया है। ओजीएचएसपी 18 जीन ने जीपी-145-103 (17.7 गुना) में सीजीजेडआर -1 (14.1 गुना), अन्नदा (13.8 गुना), जीपी 145-55 (11.9 गुना) और पूर्णिमा (8.9 गुना) के बाद विनियमन दिखाया। 60Kda चैपरोन जीन ने गर्मी तनाव के तहत लगभग सभी जीनोटाइप में अप-विनियमन दिखाया है, लेकिन अप-विनियमन न्यूनतम स्तर तक था। अध्ययन के लिए चुने गए सभी चौदह जीनोटाइप्स में से, RRF-127, अन्नदा, कर्म मशूरी, CGZR-1 और GP-145-103 में MTU-1010, ARB-6-11 और GP- जैसे हीट तनाव और जीनोटाइप के लिए एक सकारात्मक विनियमन दिखाया गया है। 145-55 ने ऊष्मा तनाव के प्रति नकारात्मक नियमन दिखाया। इन निष्कर्षों को फेनोलॉजिकल और जैव रासायनिक लक्षण वर्णन और अभिव्यक्ति विश्लेषण अध्ययन के साथ सहसंबंध में देखा गया था, जो छोटे ऊष्मा शॉक प्रोटीन परिवार से संबंधित पांच अलग-अलग गर्मी उत्तरदायी जीन के साथ किया गया था, जिसमें नियंत्रण की स्थिति की तुलना में गर्मी के तनाव के तहत अंतर अभिव्यक्ति थी। हालाँकि, इन जीनों का सकारात्मक समावेश विभिन्न प्रतिलेखन कारकों की पहचान करने में महत्वपूर्ण भूमिका निभाता है, जो विभिन्न ताप सहिष्णु तंत्रों के लिए या तनाव के तहत चावल में पादप रक्षा तंत्र को सक्रिय

करने के लिए विभिन्न संकेतन पथों के क्रॉस-लिंकिंग के लिए जिम्मेदार रहे हैं। यह चावल की फसल की गर्मी सहिष्णुता प्रतिक्रिया के लिए एक आधार के रूप में लिया जा सकता है, जो चावल और साथ ही अन्य फसल पौधों में गर्मी के तनाव के लिए उत्तरदायी उम्मीदवार जीन के आगे सत्यापन अध्ययन के लिए उपयोगी हो सकता है।

CHAPTER – I
INTRODUCTION

Rice is the most important staple food crop in the world, directly feeding more than 3 billion people across Asia, Africa, and Latin America. It is cultivated in more than 159 million hectares every year by households more than 100 millions in at least 114 countries across Asia and Africa (Tonini and Cabrera, 2011). “In the developing world, rice is the source of 27% dietary energy and 20% of dietary protein (Mottaleb *et al.*, 2012)”. Rice is produced in wide range of locations and climatic conditions. Despite, many abiotic stresses such as drought, salinity and heat adversely affect plant growth and productivity. In the past three decades, surface temperatures of the earth have become more warmer than it was during any preceding decade since 1850, due to rapid increase in green house gas concentrations, Global mean surface temperatures increases by 1.5 to 4.8 ºC by 2100 (IPCC 2014). Plant growth and performance was damaged irreversibly due to increasing temperatures, majorly affecting the yield of crop and also quality of the yield (Wahid *et al.*, 2007). “Rice yields have been estimated to be reduced by 41% by the end of the 21st century (Ceccarelli *et al.*, 2010 and Shah *et al.*, 2011)”. It’s been reported that rice yields were decreased with high night temperatures, as per the yield data from field experiments. “With increasing concerns about global warming, the effect of temperatures stress on rice production has become a major focus in many countries in tropical, subtropical and temperate regions that produce rice (Horie *et al.*, 1996; Peng *et al.*, 2004; Wassmann *et al.*, 2009 and Nagarajan *et al.*, 2010).”

Heat stress affects the growth of rice plants at all stages of their growth like vegetative, reproductive and/or ripening phases (Wassmann *et al.*, 2009) by causing reduction in the photosynthesis rate and stomatal conductance. “In particular, spikelet sterility induced by high temperatures during flowering (Satake and Yoshida, 1978) is a serious problem, because it directly reduces yield (Prasad *et al.*, 2006 and Mohammed and Tarpley, 2011)”. High temperatures during grain filling stage reduces the grain quality.

“Most importantly, Rice is immensely sensitive to heat stress episodes of short duration (>35◦C for ≥1 h) which coincides with the reproductive stage, especially anthesis (Prasad *et al.*, 2006; Jagadish *et al.*, 2007, Jagadish *et al.*, 2008, Jagadish *et al.*, 2010 and Sathishraj *et al.*, 2015)”. Additionally, nominal effect on spikelet fertility is seen due to temperatures higher than 38˚C which occurs an hour after anthesis (Yoshida *et al.*, 1981; Jagadish *et al.*, 2007). “Climate models have, with greater certainty indicated increased frequency of hotter days with temperatures above known critical threshold(>33◦C; Jagadish *et al.*, 2007) to coincide with the flowering stage”. Anther dehiscence was inhibited by high temperatures above 35 ºC at the flowering stage, resulting in low amount of pollen shed on the stigma, which leads to incomplete fertilization (Jagadish *et al.*, 2010). “Even if a sufficient number of pollen grains are shed on the stigma, in some cases, the pollen germination and pollen tube growth are poor under heat stress (Satake and Yoshida, 1978)”. Thus, primarily disturbed development of pollen because of aberrant anther dehiscence and secondarily shedding were considered as the causes for spikelet sterility at the flowering stage, induced by heat.

Some adaptation strategies to heat in rice have been recently described. 1) Escape strategy, ability of the plants to adjust the time of the day of anthesis based on their sensitivity to climate conditions during the 7-days period preceding anthesis and is advanced earlier in the morning, if conditions during the seven preceding days are hot and humid, which allows the plants to escape the hottest time of the day. 2) Avoidance strategy, in dry air environments, panicle temperature is lowered by up to 10 ºC when compared to air temperature. This is because of the transpirational cooling ability of the plant and so stabilizes the spikelet fertility by maintaining panicle temperature below the critical threshold. Reported genetic variability was seen in the cooling capacity of panicles through its transpiration and the leaves surrounding it. 3) “True tolerance, involves different physiological mechanisms which were induced under heat stress (Kondamudi *et al.*, 2012; Bahuguna *et al.*, 2015; Bahuguna and Jagadish 2015)”. Short exposure of rice seedlings to high temperature affects the ultra-structure of cells, with major changes in chloroplast and mitochondria, results in reduced metabolism and thus reducing plant growth (Pareek *et al.*, 1997).

In response to heat stress, plants adjust themselves to the metabolism and morphology suitable to sustain in the stress conditions. Generally high temperature induces expression of heat shock proteins (HSPs) and normal cellular protein production, at least in part, is suppressed (Shah *et al*., 2011). “HSPs can improve or stabilise photosynthesis, partitioning of assimilates, nutrient and water use efficiency and the thermal stability of cellular membranes (Wahid *et al*., 2007)”. “Damaged protein restoration is also aided by some of the HSPs and molecular chaperones (Kumari *et al*., 2013)”. Investigation of these mechanisms in further agricultural production system is needed, to exploit them in the development of heat stress tolerant rice cultivars. Despite the occurance of different strategies (escape, avoidance), sterility has still remained as a major concern in the areas cultivating rice. As large percent of the rice growing areas are characterized by humid conditions, limiting the transpirational cooling incidence, there by reducing panicle temperature. In addition, the occurrence of combinations of different abiotic stresses such as heat, drought and salinity in farmers' fields increases, lowering the transpiration cooling because of partial stomatal closure. Under future climates, this immensely increases the vulnerability of rice in the most productive regions. To cope with future climate scenarios, it is vitally important to develope rice varieties with heat tolerance under varying humidity conditions.

“Heat tolerance genetics was poorly understood as it is complex and controlled by multiple genes (Wahid *et al.,* 2007; Xue *et al.,* 2012; Driedonks *et al.,* 2016)”. “Fairly more heritability has seen in rice heat tolerance and most of the genetic variation is additive (Yoshida 1981)”. Many HSPs have been reported and their genetics (controlling genes, location of genes, dominance/recessive) are known. Even then combination of certain genes are very critical for successful breeding of the cultivar, e.g. cultivars should posses the optimal genes/alleles for flowering time, height, etc., and the effectiveness of HSP genes in an elite genetic background is not known. As per the recent genetic studies, its been reported that probably, plant heat-tolerance is a polygenic trait. “In wheat different components of tolerance, controlled by different sets of genes, are critical for heat tolerance at different stages of development or in different tissues (Barakat *et al.,* 2011)”. “Shah *et al.,* (2011) emphasized that indica rice is generally more heat tolerant than japonica rice, however there is a genotypic variation in spikelet fertility at high temperature in both species”. Understanding the tolerance genetic base and increasing the level of breeding for heat tolerance still continues.

The present study could provide clues for identification of candidate genes responsive to heat stress, for further functional analysis and to explain their precise role in heat stress response. As key genes are identified, it increases the efficiency and realizes the opportunities for genetic engineering. This is a fundamental aspect of research into heat stress responsive genes, which is explored in the present study. The present study entitled **“Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)”*** is planned with following objectives:

1. Phenological and biochemical characterization of rice genotypes under heat stress.

2. Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.

CHAPTER – II
REVIEW OF LITERATURE

Rice *(Oryza sativa)* is one of the major staple foods among three most important food crops in the world, feeding more than 2.7 billion people. In India rice cultivation occupies an area of 44.6m ha with total yield output of 80 million tonnes (paddy) and average productivity of 1855 Kg/ha.

2.1. Taxonomic classification of Rice:

Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Cyperales
Family: Gramineae
Genus: Oryza
Species: Sativa
Subspecies: Indica, japonica, javanica.

2.2. Morphology of rice plant:

Generally cultivated rice is treated as semi-aquatic annual grass. Rice plant at maturity has main stem and tillers, with each productive tiller having a flowering head called panicle. There is a great variation in plant height according to varieties but average height ranges from 0.4m – more than 5m, in some. The morphology of rice is divided into two phases;

Vegetative phase – germination, seedling and tillering stages

Reproductive phase – panicle initiation and heading stages.

Rice plant has has fibrous root system with seminal, nodal and lateral roots. The seminal root is formed by radical, nodal roots develop from basal nodes as lants grow, nodal roots developing from higher nodes and helping plant in absorbing food are called lateral roots. The stem of rice plant is known popularly as culm or haulm, made up of large number of series of nodes and internodes. Node is the solid part of culm bearing leaf. Early tillers are emerged in an alternate manner from main culm, lower most nodes gives rise to primary tillers and they give rise to secondary and tertiary tillers. Each tiller is considered as an independent plant. Leaves are formed on the culm near a node in alternative manner and the top most leaf beneath panicle is called flag leaf. Mostly leaves contain small paired ear like lobes on both sides of blade base called as auricles. The terminal part of rice tiller is an inflorescence called as panicle, bearing rice spikelets that develop into grain. Each spikelet in rice is considered as a single flower enclosed by lemma and palea. It has six stamens, 3 in each whorl. The pistil has stigma, style and ovary. Self pollination occurs in rice when pollen is dusted on the stigma after dehiscence of anthers. After fertilization ovary is developed into seed and it is called as grain. The morphology of rice plant and spikelet is shown in figure 2.1 below

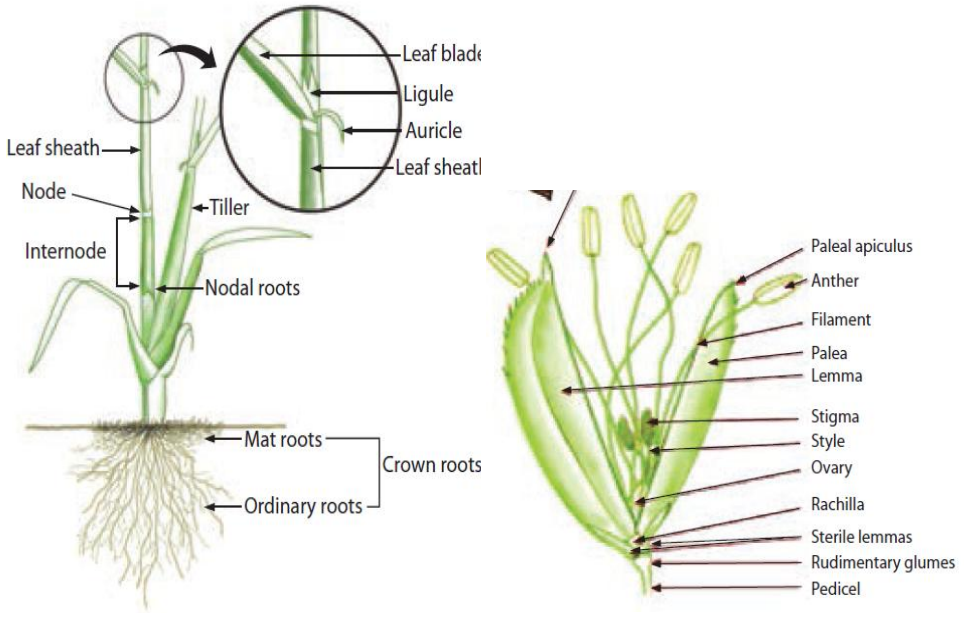


Fig 2.1: Parts of rice stem, tillers and spikelet. [Maclean *et al.*, (2013), *Rice Almanac*]

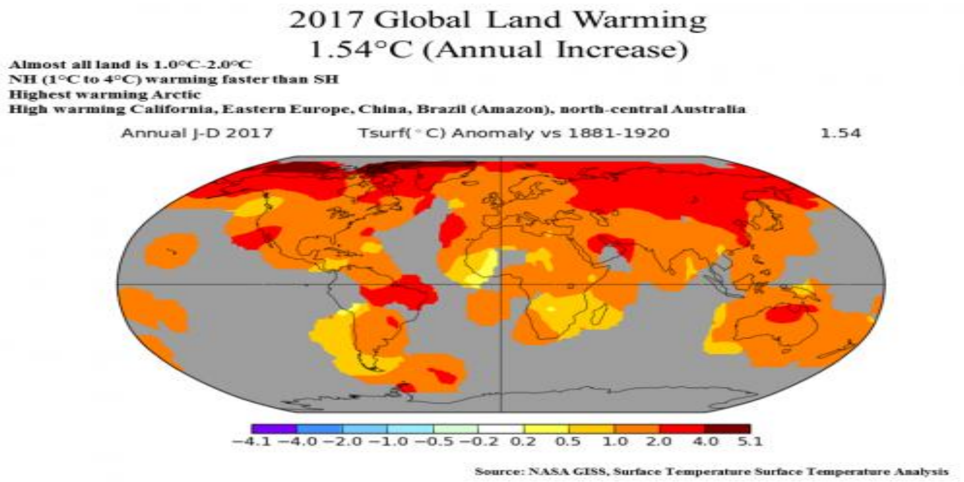
Rice is a plant of tropical climate and a normal variety grown in tropical region has a life period of 120 days i.e it spends about 60 days in vegetative phase, 30 in reproductive phase and the remaining 30 in ripening phase. As the plant is a tropical plant is requires a temperature range of 20-35˚C during its developmental stages. The following table 2.1 shows the stage and optimum temperature required.

Table 2.1: Optimum temperature required for different stages of rice plant. [Maclean *et al.*, (2013), *Rice Almanac*]

Stage	optimum temperature (˚C)
Germination	20-35
Seedling emergence	25-30
Rooting	25-28
Leaf elongation	31
Tillering	25-31
Panicle initiation	15-30
Anthesis	30-33
Ripening	20-25

2.3. Climate change and raising temperatures :

Earth's surface temperature is increasing in logistic fashion and in the past three decades it became more warmer than it was during any preceeding decade, since 1850. This drastic increase in temperature is due to rise in green house gas concentration and global warming. As per the report given by IPCC 2014, Intergovernmental Panel on Climate Change, the global mean surface temperatures increases by 1.5 to 4.8 ºC by 2100.



Source: NASA GISS, Surface Temperature Surface Temperature Analysis

Fig 2.2: Global land warming 2017. [NASA GISS, surface temperature analysis.]

2.4. Impact of high temperature on rice and its adaptations:

Heat stress affects the rice crop during its all stages of development like vegetative, reproductive and ripening stage. Particularly, rice plants are sensitive to high temperatures during anthesis and leads to improper or reduced pollination and results in spikelet sterility. This reduces the rice yields. Hence it is estimated that increasing global mean surface temperature reduces the rice yields by 41% by the end of 21st century (Ceccarelli *et al.*, 2010; Shah *et al.*, 2011).

One of the important agricultural goals now a days as per the increasing global temperatures is elucidating the approaches for sustaining high yields of crop plants under heat stress. The physiological, biochemical and molecular responses that take place in plants in accordance with heat stress are shown below in the figure 2.3

Figure 2.3: Major effects of high temperature on plants. [Hasanuzzaman *et al.*, (2013)]

Heat stress, like any other abiotic stress might uncouple enzymes and metabolic pathways that causes accumulation of unwanted and harmful ROS (reactive oxygen species) like singlet oxygen (1O2), superoxide radical (O2•−), hydrogen peroxide (H2O2) responsible for oxidative stress. Though chloroplasts are major sites of ROS generation, they are also generated in peroxisomes and mitochondria. This oxidative stress induced by thermal stress is because of peroxidation of membrane lipids, cell membrane stability disruption by protein denaturation and increased malondialdehyde (MDA) content in leaves by reduced antioxidant enzyme activities. Even though ROS have a lot of destructive effects on metabolic process of plants, they have also hypothesized to trigger heat shock responses by having signaling behaviour towards development of heat tolerance in plants.

2.4.1. Plant Adaptation to Heat Stress:

Considering the preferred temperature for the growth of living organisms, they are classified into following types.

a) Psychrophiles: grows at low temperature ranging between 0 and 10 °C

b) Mesophyles: growing well between 10 and 30˚C;favours moderate temperature

c) Thermophyles: grows wellin temperature ranging from 30 to 65˚C and even in higher temperatures.

Larcher, classified all plant species on the basis of thermotolerance into three groups as shown in fig.2.4

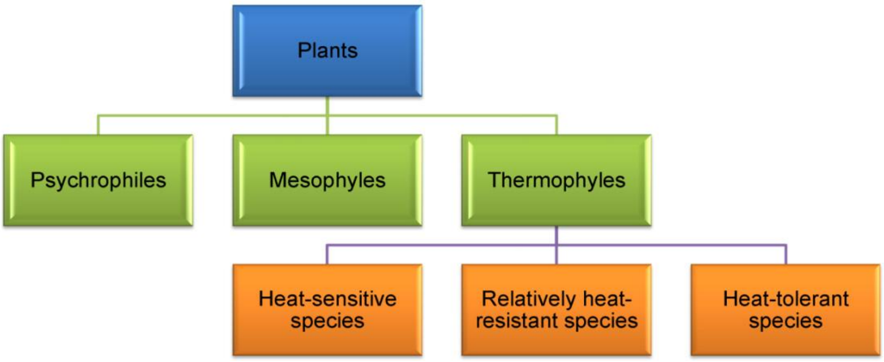


Fig 2.4: Classification of plants based on their heat tolerance. [Larcher, 1995]

Fitter *et al.*, (2002) reported that plant adaptation to heat stress involves various mechanisms like avoidance and tolerance. Plant survival in hot environment is achieved by various ways that include combinations of avoidance and tolerance strategies (fig 2.5)

Avoidance strategies that operate under high temperatures include changing leaf blade orientation parallel to sun rays (paraheliotropism), membrane lipid composition alterations, reduced absorption of solar radiation due to presence of small hairs (tomentose). An escape mechanism like early maturation is also seen in hot environments which is closely correlated for low yields.

Tolerance strategies that appear in heat stress plants count ion-transporters, late embryogenesis abundance (LEA) proteins, osmoprotectants, antioxidant defense, factors involving transcriptional control and signalling cascades.

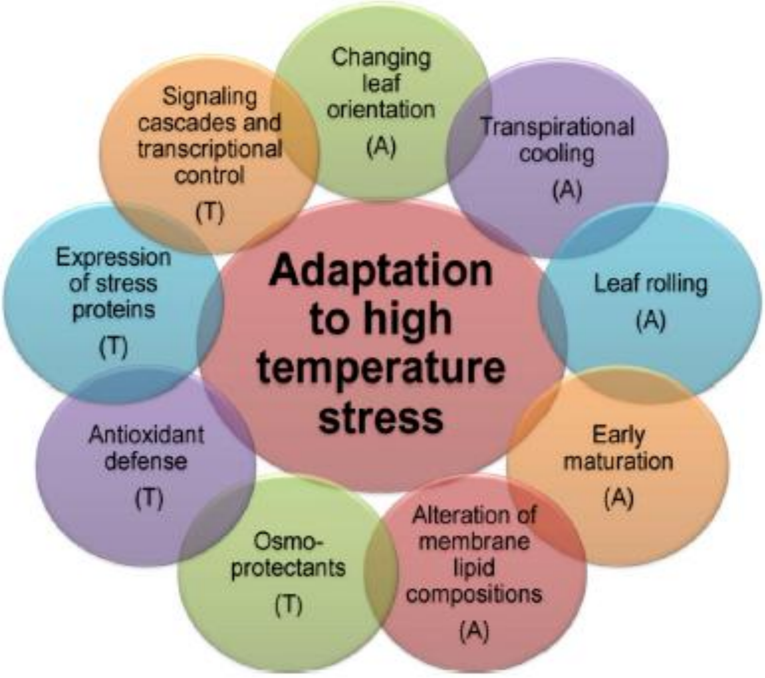


Fig. 2.5: Different adaptation mechanisms of plants to high temperature.
A: avoidance T: tolerance [Fitter *et al.*, (2002).]

Reis *et al.*, (2012) studies stated that, In general, protein denaturation takes place under HT as the reduced cellular volume increases the chances of degradative molecular interactions. So, to compensate this under heat stress conditions heat shock proteins (HSP's) are activated. HSP's are good at maintaining and repairing companion protein structure. They target the incorrectly aggregated and non-native proteins, help in degrading and removing them from cells. Their primary function is to control proper folding and conformation of structural (i.e., cell membrane) and functional (i.e., enzyme) proteins. They ensure the proper functioning of many cellular proteins under elevated temperature conditions.

The schematic diagram shown in figure 2.6, represents the molecular regulatory mechanism of heat shock proteins as per a hypothetical cellular model. The steps involved are as follows:

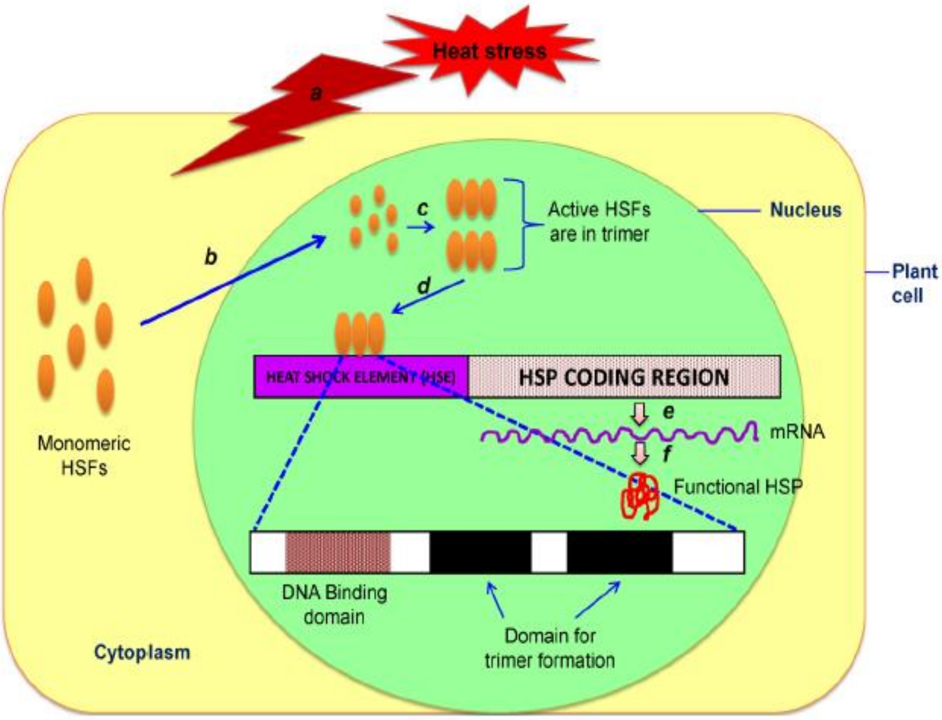


Fig 2.6: Schematic representation showing molecular regulatory mechanism of HSP's. a) plant cell receives the heat stress, **b)** monomeric heat shock factors (HSFs) enters into the nucleus from cytoplasm, **c)** In nucleus, HSF monomers form active trimers, **d)** These trimers bind to the specific genomic region (promoter or heat shock element, HSE) of the respective heatshock gene (HSG), **e)** (Molecular dissection of the HSF binding region of HSE showing that it consists of one DNA binding domain and two domains for trimerization of HSFs) Successful transcription occurs, **f)** Translation and post-translational modification takes place leading to production of functional HSP responsible for heat stress tolerance. **[Reis *et al.*, (2012).]**

Zafar *et al.*, (2018) studied several approaches enveloping genetic, biochemical and physiological mechanisms for mitigating heat stress impact on rice. They utilized the knowledge of genetic information for increasing the conventional breeding approaches for developing heat tolerant germplasm in rice. The figure 2.7, Given below shows all the mechanisms for heat tolerance.

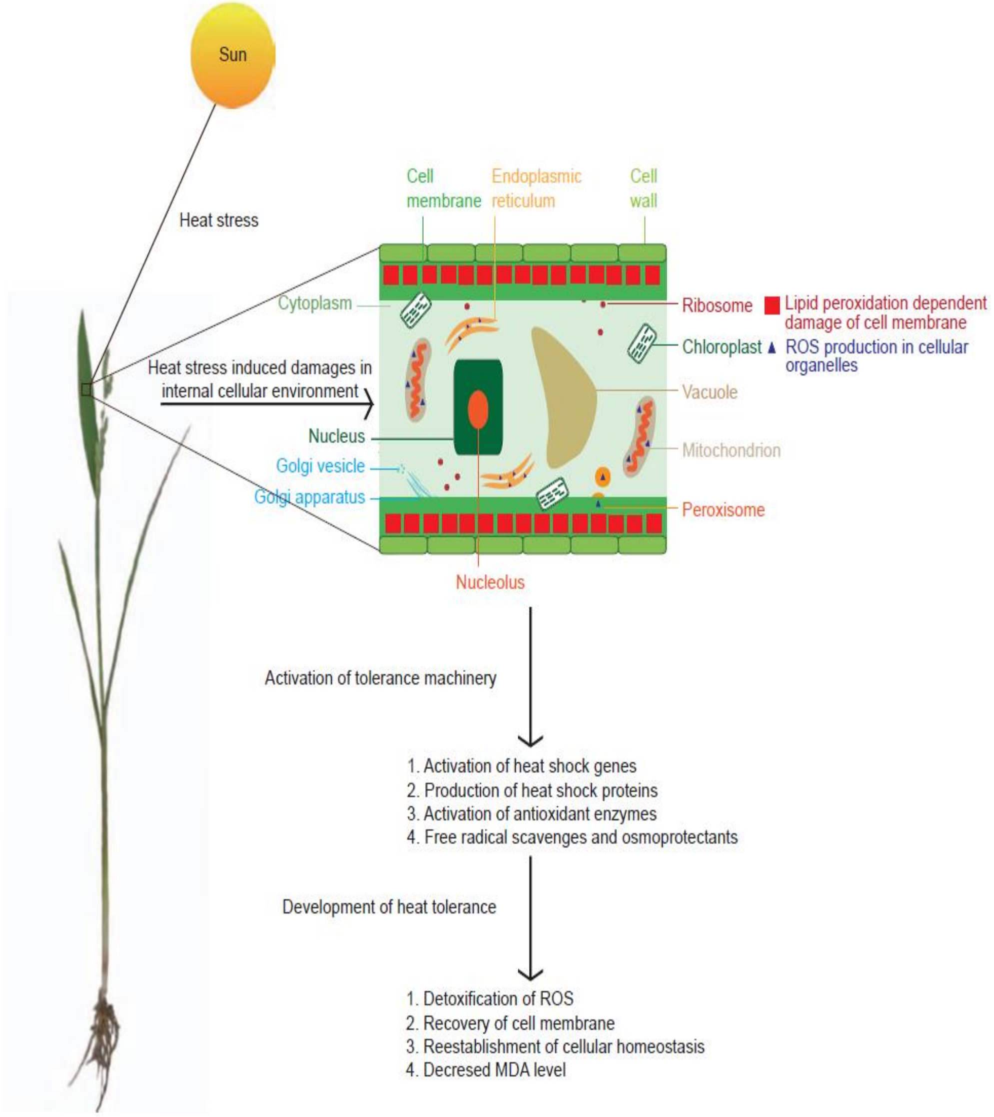


Fig 2.7: Number of mechanisms and compounds produced after exposing rice plant to heat stress for its rescue. [Zafar *et al.*, (2018)].

2.5. Phenological and biochemical characterization of rice and other crops under heat stress.

Phenological characters that are considered in this study, under heat stress includes:

- Membrane Stability Index-[MSI],
- Pollen fertility,
- Spikelet fertility.

Biochemical characters acknowledged in this study, under heat stress cover:

- Chlorophyll,
- Proline,
- Malondialdehyde (MDA).

2.5.1. Membrane stability or cell membrane thermo-stability (CMT):

Dexter *et al.,* (1932) investigated hardiness of plants by measuring electrical conductivity. The principle of increasing permeability and electrical conductivity in an injured tissue is more intimate in plant physiology . Electrical conductivity is measured by changing electrolyte concentration. In this study, they estimated the hardiness of three varieties of alfalfa by determining the electrical conductivity in which exosmosis of electrolytes from frozen tissues takes place. A progressive hardening of Grimm alfalfa, a hardy variety, was found as the winter season arrived, and little or no hardening in Hairy Peruvian, a very tender strain. Common alfalfa, Utah was intermediate in response.

Martineau *et al.,* (1979) evaluated a simple rapid technique of assessing genotypic differences for heat stress by measuring membrane thermo-stability, which was previously used in sorghum. In this technique, electrical conductance of amount of electrolytes leaked from heat damaged leaf cells is measured. A sigmoidal response was observed between the degree of injury and the temperature causing that injury. Genotypic differences for heat tolerance are determined by difference in relative positions of response curve with respect to temperature treatment. While detecting genotypic differences greatest sensitivity occurred at about 50% injury. As the genotypic differences were found to be greatest in newly formed tissues, it concluded that most recently developed leaves should be used for the assay. As per this study, the cultivars showing the low injury readings constantly, are those having the relatively greater membrane thermo-stability.

Blum *et al.,* (1981) used cell membrane stability as a measure of heat and drought tolerance in wheat. 66 cultivars were tested for heat tolerance using 44˚C temperature as standard heat treatment. Among them, there were most significant differences in the percent injury ranging from 14.8% (in 'H895') to 77.0% (in 'CD 31/75' durum wheat).

Agarie *et al.,* (1995) explored the potentiality of measuring the cell membrane stability adaptations for evaluating drought and heat tolerance in rice. They exposed the leafs to deccisation by polyethylene glycol (PEG) and to heat stress by temperature treatment of 42˚C. Electrolyte leakage (EL) was increased linearly from both the treatments like PEG and heat stress, reflecting that this technique is used for assessing the injury probably at the membrane level due to environmental stress in rice.

Hasan *et al.,* (2007) reported that to evaluate heat tolerance, most widely used and accepted method is membrane thermostability while yield parameters are evaluated by heat susceptibility index. Wheat cultivars under normal and heat stress at post anthesis conditions differed in terms of CMT measured by relative electrolyte leakage at 55˚C elated temperature. Heat killing time is the time required to reach 50% membrane leakage in a cultivar. Among the cultivars, Aghrani recorded the longest heat killing time (150min) closely followed by Kanchan (120 min) and CB-30 (120 min). shortest heat killing time irrespective of growing condition was recorded in Sonora (30min). Based on this criteria of heat killing time Aghrani, Kanchan and CB-30 were regarded as the heat tolerant cultivars and Sonara as the heat sensitive. High killing time indicates that cultivars showed lower membrane leakage at elevated temperatures, due to tendency of the genotype to acclimatize against stress.

Hemantaranjan *et al.,* (2014) studied physiological responses of plants to high temperature and mechanisms of heat tolerance to contribute for traditional breeding. For the processes such as photosynthesis and respiration, continued function of cellular membranes under heat stress is vital. So to increase selection efficiency physiological and biochemical screening techniques are used as a complement to empirical breeding methods. Heat tolerance securing genes may be lost in breeding programs, hence membrane thermal stability (MTS) is considered as significant selection criteria for heat stress tolerance. As the purity and functions of biological membranes are sensitive to heat stress, tertiary and quaternary structures of membrane proteins gets transformed boosting up the permeability of membranes, which is obvious from enhanced electrolyte leakage. This increased the electrical conductivity under heat stress, resulting the decreased membrane thermal stability.

Usman *et al.*, (2015) carried out experiments in chilli pepper to study mechanisms for heat tolerance. They evaluated six genotypes for cellular membrane thermostability (CMT) and for HSP70 gene expression. The genotypes were classified as follows: heat-tolerant (greater than 60%), moderately tolerant (30% to 60%), and susceptible (less than 30%), based on mean values of CMT. The highest CMTs of 89.27%, 88.03%, and 85.10% were recorded for heat tolerant plants AVPP0702, AVPP0116, and AVPP9905, respectively. Under heat stress, heat shock protein 70kDa was identified to be differentially expressed. Changed CMT values were observed and correlated with differential heat shock protein HSP70 expression. Genotypes that have high CMT were observed to have high expression of HSP70, indicating thermo-tolerance. Hence the degree of differences detected in HSP70 and membrane stability were used to improve thermotolerance in studied genotypes.

Tiwari *et al.*, (2017) conducted an experiment to evaluate heat stress traits in wheat to develop high yielding heat tolerant wheat varieties, with 4 varieties namely Halna, HD2733, Raj3765 and K9006. Under field conditions, delayed sowing was used to give the heat stress treatment. To screen and evaluate different wheat varieties for heat tolerance they selected cell membrane stability as one of the parameters. Cell membrane stability is the measure of electrolyte diffusion from the cells and during heat stress electrical conductivity increases as the cell membrane damage occurs, resulting in solute leakage. The maximum membrane stability index values were recorded in Halna (69) and Raj 3765 (63).

Sharma *et al.,* (2017) examined alterations in the cell membrane stabitility (CMT) due to heat stress in different genotypes of bread wheat. They exposed different varieties (HDR 77, HD 2864, HD 2967, PBW 373, HS 365, Raj 4037, JAUW 584, PBW 175, RSP 561, HW 2045, HD2385, HD2687) of single genotype to varying temperatures of 45˚C and 100˚C. Changes in cell membrane stability of leaf samples was calculated and the highest percentage of conductivity was seen in HDR77, predicting the highest susceptibility of this variety for heat stress. Lowest percentage of conductivity was observed in HD2687, which predicts that this is the highly resistant variety for heat stress in bread wheat among the selected genotypes.

ElBasyoni *et al.,* (2017) demonstrated identification of potentially new genomic regions associated with cell membrane stability (CMS), using genome-wide association mapping. They exposed leaf tissues to heat treatment and screened a global spring wheat panel for CMS, potential quantitative trait loci (QTL)/genes linked with CMS were identified using genome wide association-mapping and relationship between the field performance and measured CMS was estimated. Highly significant differences were indicated among the 2111 spring wheat accessions with respect to CMS. Several SNPs were found to be undoubtedly linked to CMS and their annotations indicated that these SNPs are linked with important functional genes that control solute transport through cell membrane and other biochemical activities related to abiotic stress tolerance.

2.5.2. Pollen Fertility:

Herrero *et al.,* (1980) determined in vitro pollen germination of different maize genotypes during anthesis , affected by high temperature stress. At the beginning of anthesis, tassels from field grown plants were cut out and placed in water. Later they were transferred to growth chambers with the day temperatures maintained at 27˚C, 32˚C, 38˚C and night 6˚C cooler temperatures were maintained. After 24 and 48hrs, in vitro germination was measured both in growth chamber as well as on field collected pollen. As the temperature increased pollen germination steadily decreased in some genotypes. All genotypes witnessed lower germination at 38˚C, when compared with 27 and 32˚C. After 48hrs at 38˚C, several genotypes showed no germination. This study suggested that pollen germination of many genotypes reduces on prolonged exposure to temperature above 32˚C.

Pressman *et al.,* (2002) continuously exposed tomato ‘Trust’ to high temperatures (day/night temperatures of 32/26 °C) and observed the reduction in number of pollen grains per flower and the viability. Heat stress effect on pollen viability was correlated with changes in carbohydrate metabolism in different parts of anther during its development. In favourable temperature conditions (28/22 °C), accumulation of starch in pollen grains reached maximum value of 3d before anthesis and then reduced towards anthesis. During anther development, total soluble sugars concentration progressively increased in anther walls and in pollen grains (but not in the locular fluid), meeting maximum at anthesis. Heat stress interrupted the transient increase in starch concentration, leading to reduced concentrations of soluble sugars in anther walls and pollen grains. Their results suggested the dominant effect of heat stress on pollen development is decrease in starch concentration 3d before anthesis leading to reduced sugar concentration in mature pollen grains, which contributes to the decreased pollen viability in tomato.

Sakata *et al.,* (2008) reported that male reproductive development is dominantly affected by abiotic stresses. In particular, male sterility is is caused by high temperature in many plant species. They used double-rowed barley (*Hordeum vulgare* L. cv. ‘Haruna-nijyo’) as a model For studying male reproductive development and high-temperature injury correlation in plants. High temperature injury leads to premature advancement of early developmental programs in anthers, including proliferation arrest, anther wall cell degradation and progression to meiosis in pollen mother cells (PMCs). All these require broad amendments in transcription. These findings suggested that male reproductive development is more sensitive when compared with female reproductive development under heat stress leading to anther sterility.

Rang *et al.,* (2011) studied five rice genotypes under high temperature, water stress and combination of both during flowering, which is most sensitive developmental stage and their response is quantified through spikelet fertility. Significant differences in anther dehiscence between treatments and genotypes, with a moderately enhanced partnership with the number of germinated pollen grains on the stigma were revealed using microscopic analysis. Strong relationship was found between the spikelet fertility and the number of pollen germinated on stigmas. All the 3 treatments lead to spikelet stertility but highest spikelet sterility was found in high temperature stress in all 5 genotypes. N22 showed better anther dehiscence , higher in vivo pollen germination and higher spikelet fertility when compared with IR-64, Apo and Moroberekan under high temperature, water stress and combined stress, suggesting its ability to resist multiple abiotic stresses.

Shah *et al.*, (2011) reported the impact of temperature increment on rice production. They stated that compared to mean daily temperatures or day-time temperature, high temperatures at night are more devastating to rice production. Sometimes this high night temperature may lead to complete sterility, as booting and flowering are most sensitive stages to temperature. At increased temperatures, humidity also plays an important role in imparting spikelet sterility. Some phenotypic markers for high-temperature tolerance includes Flowering at cooler times of day, more pollen viability, larger anthers, longer basal dehiscence and presence of long basal pores. Some of the adaptive measures to mitigate the yield loss during high temperatures are adjustment of sowing time, choosing varieties that allow avoidance of peak stress periods and exogenous application of some plant hormones.

Harsant *et al.*, (2013) investigated The effect of high temperatures on harvest index (HI) and morphological components that contribute to HI in *Brachypodium distachyon.* It is a C3 grass, identified as an amenable plant for addressing critical issues related with enhancing cereal crop yields under global climate change. The results explained that due to high temperatures more than 32˚C, HI was eliminated. Primary reasons for the reducing yields at 32˚C were declined pollen viability, retention of pollen in anthers, and pollen germination. Microspore abortion at uni-nucleate stage in correlation with abnormal tapetal development are reasons for yield failure at 36˚C. In *B. distachyon*, the The response of male reproductive development and tiller branching patterns to increasing temperatures provides support for the use of this C3 grass in estimating molecular control of HI in global warming.

Das *et al.,* (2014) studied pollen in two groups of genotypes belonging to lowland and upland conditions. These two group of plants were subjected to six different day/night temperature regimes (25/15, 30/20, 35/25, 40/30, 45/35 and 50/40 ◦C) during panicle initiation for three days. Various morphological, physiological and biochemical aspects of pollen were determined during anthesis in each regime. panicle extrusion, flowering period, and number of anthesing (opened) spikelets were sceptically altered by High temperatures above 35/25 ◦C. Pollen viability, tube length, anther dehiscence and number of pollen on stigma decreased significantly. It was noticed that the pollen of low land genotypes group was more adversely effected compared to the upland genotypes groups under high temperature stress. Hence the viability of pollens was precisely more in upland genotypes. Among all the genotypes examined, loss of pollen viability was highest in Swarna.

Kumar *et al.,* (2015) conducted a study indicating the rise in temperature with respect to pollen viability. The threshold temperature for pollination of rice is 35◦C and temperature above this causes pollen sterility and fertilization is severely affected. They studied 11 rice genotypes in indigenous field conditions during kharif 2010 and 2011. The spikelet attributes of the crop were severely affected due to increased mean temperature of about 12◦C during full flowering stage. Scanning electron microscopy studies showed that under high temperature pollen size was clearly reduced. Spikelet sterility of about 90% was observed in all genotypes.

Jagadish *et al.,* (2015) addressed the relevance of vapour pressure scantiness under fully flooded paddies and enhanced susceptibility of rice production to heat stress or infrequent occurrence of combined heat and drought stress under egressing water-saving rice technologies and the major disconnect with high night temperature response between field and controlled environments in terms of spikelet sterility. Expression of transpiration cooling, an efficient heat-avoiding mechanism, will decline with the shift from fully flooded paddies to water-saving technologies, such as direct-seeded and aerobic rice cultivation, provoking stress damage. This change can possibly introduce higher sensitivity to previously unaffected developmental stages such as floral meristem (panicle) initiation and spikelet differentiation, and further enhance vulnerability at the known sensitive gametogenesis and flowering stages. More than the mean temperature rise, increased variability and a more rapid increase in nighttime temperature compared with the daytime maximum leads to sterility.

Fahad *et al.,* (2018) interpreted that in rice, vegetative growth continues upto 40˚C day temperature but floret development is extremely sensitive to temperatures exceeding 35˚C. High night temperatures adversely effect the yield than the day temperatures. Heat stress results in floret sterility due to deprived anther dehiscence, defective pollination and aberrant pollen germination. Imbalance in protein expression, abandoned biosynthesis, partitioning and translocation of soluble sugars, imbalance in phytohormones release, and loss of pollen water content leads to decreased pollen viability. Response of rice to heat stress is through adjustment of various physiochemical mechanisms like growth inhibition, leaf senescence. Antioxidant enzymes, calcium and iron also plays a vital role in handling heat stress.

2.5.3. Spikelet Fertility:

Cheng *et al.,* (2009) conducted a pot experiment with four different treatments that include two levels of CO2 (ambient, 380 ppm; and elevated, 680 ppm) and two levels of night temperatures (22 and 32 ˚C) for understanding the impact of both high night temperature and increased CO2 concentration at the time of reproductive growth on rice growth and spikelet fertility. Results indicated increased plant and stem dry weight in both the conditions, while elevated CO2 concentrations increased and high night temperature decreased ear dry weight. Only the high night temperatures lead to the decrease in fertilized spikelet number. Elevated concentrations of CO2 increased individual grain weight.

Mohammed *et al.,* (2010) explained the multiple rice yield determining parameters affected by night temperatures and spikelet position. These multiple yield parameters included productive tillers number, spikelet sterility, grain length, width and weight. Rice plants were grown in green house under normal (27 ◦C) and high night temperature (32 ◦C) conditions, using infrared heaters. A decreased yield of about 90% was observed under high night temperature, which was affected by spikelet sterility (61%), and grain length (2%), width (2%), and weight. The resulted spikelet sterility in rice under high night temperature was due to decreased pollen germination and increased respiration rates. Along with high night temperatures, spikelet position on the panicle is also considered as a factor for determining yield in rice as spikelets at tip weighed more than basal spikelets on panicle.

Van Oort *et al.,* (2014) reviewed the available fertility functions i.e fertility as a function of panicle temperature and proposed a simple and new model integrating insights on diurnal temperature patterns and relative humidity. Their model also includes recent studies on relationship between panicle temperature and sterility. Their study included the impact of environmental conditions on flowering time. Model is emphasized for typical arid and typical humid conditions. Differences in simulation fertility up to 59% was observed due to the presence of large differences between existing fertility functions and simulated fertility. As transpirational cooling was ignored, it led to the overestimation of spikelet sterility to 14-73%. A reduction in sterility of about 7-35% was observed by shifting the flowering times from 12:00 to 9:00. They concluded that any impact of climate change simulation is highly dependent on fertility function choices.

Nguyen *et al.,* (2014) constructed, calibrated and validated a simulation model for predicting spikelet sterility in rice caused under heat stress conditions. The model consists of equations for estimating the probability distributions of panicle heading dates in field, spikelet flowering date on panicle and spikelet flowering time during day, and two sterility response functions to temperature at the time of meiosis and spikelet flowering time. Peak heading of panicle was observed after 4-6 days from initial heading and lasted for 12 days after heading. Date of panicle heading distribution was fitted into Poisson’s equation. The spikelet flowering peak on panicle was quite good fitted into normal distribution function, opening time of flower followed normal distribution with standard deviation around 1 hour, the fertility responses to high temperature during meiosis and anthesis were fitted into logistic functions of heating degree day above 31˚C on 12 days before anthesis of spikelet and temperature of air at time of spikelet flowering, respectively. Hence the model consisting of above equations is used to predict the response of spikelet fertility / sterility to air temperature with precision.

Wu *et al.,* (2016) investigated the phytohormonal changes in rice under heat stress and their impact on yield and other attributes, by growing four rice varieties (Nagina22, Huanghuazhan, Liangyoupeijiu, and Shanyou 63) in pots and treating them with 3 high temperatures under greenhouse conditions for 15 days at early reproductive growth phase. Nagina22, huanghuazhan, and liangyoupeijiu showed decreased yields due reduced spikelet fertility, spikelets per panicle, and grain weight. Active cytokinins, gibberellin A1 (GA1), and indole-3-acetic acid (IAA) were reduced as a result of heat stress and abscisic acid (ABA) and bound cytokinins in young panicles were increased. Correlation analysis suggested that yield is closely associated with changes in phytohormones, under heat stress.

Ishimaru *et al.,* (2016) facilitated breeding towards heat-resilient rice development in terms of spikelet sterility and quality of grain. In recent years identification of rice cultivars with heat tolerance at different growth stages was done. In these cultivars a trait named as early morning flowering (EMF) trait, effectively escapes heat as it shifts the time of day flowering to cooler early mornings. Some genetic resources for EMF were found in wild rice accessions. Heat-tolerant japonica type rice cultivars were found in which white-back type of chalky grains (WBCG) formation was mitigated. Mapping of QTLs related to heat tolerance at flowering, EMF and for WBCG in grainquality was done on rice chromosomes.

Fu *et al.,* (2016) reported that inferior spikelets of rice has lower ferlity and kernel weight compared to the superior spikelets under heat stress. Two rice plants having different heat tolerance were exposed to 40˚C during anthesis, to analyse the mechanisms responsible for the above mentioned problem. The results determined that fertility and kernel weight greatly decreased in superior spikelets as compared with inferior onesand this was due to their different organ temperatures. The superior spikelets had more organ temperatures. Under heat stress, both the superior and inferior spikelets fertility and kernel weight decreased due to reduced panicle number per plant, which in turn increases canopy temperature. Thus a rice plant with upright growth habit and loose panicles would be more heat stress susceptible because of higher canopy and spikelet temperatures.

Sehgal *et al.*, (2018) investigated on the effect of drought and heat stress on seed filling in food crops. They stated that even if these abiotic stresses affect the crop growth at all stages but the effect is critical at the seed filling phase of the reproductive growth. Seed yields are considerably affected by reduced seed size and number, ultimately affecting ‘100 seed weight’ which is taken as the commercial trait. Various processes like production and translocation of photoassimilates , biosynthesis of seed reserves and minerals by importing precursors, occurring in leaves influence seed filling. Due to the involvement of different enzymes and transporters present in leaves and seeds, in all the processes of seed filling it is severely affected by heat stress.

Zhang *et al.,* (2018) studied the role of auxins for elongation of pollen tube in pistils, leading to successful pollination in plants. Heat stress treatment of 40 °C for 2 hrs was given to two rice genotypes with differeing heat tolerance, after flowering. Under heat stress, nipponbare (NPB) and high temperature susceptible (HTS), its mutant showed sharply reduced spikelet fertility. In mutant HTS, 1-naphthaleneacetic acid (NAA) reversed the spikelet sterility induced by stress. In NPB, pollentubes were visible in ovule and that of HTS were not visible. When the application of auxin NAA was done, we found pollen tubes of HTS in ovule. Hence it was concluded that heat tolerance enhancement by NAA in plants was accomplished by increasing the auxin levels, promoting elongation of pollen tube in pistil.

Lawas *et al.*, (2018) explained the association of spikelet sterility and grain yield with failure of exertion of panicle or sheathed panicle (SP) phenotype in rice. They identified a unique phenotype with sheathed panicle (cv. Sathi) with potential of maintaining normal seed set and grain weight under stress (38°C for 20 d). Inspite of the high temperature of the spikelet tissues (38˚C - 40˚C) during anthesis, a higher number of pollen count and the germinated pollen on the stigma was observed which was more than the required threshold for successful seed set. They recorded the lipid peroxidation and carbohydrate metabolism enzymes during grain filling. Under heat stress they were decreased, suggesting that the minimum impact of heat stress on carbohydrate enzymes leads to normal sucrose unloading and starch synthesis in grain. This results in normal grain size under prolonged exposure of sheathed panicle (SP) phenotype to heat stress.

2.5.4. Chlorophyll content:

Cui *et al.,* (2006) evaluated the process of photosynthesis and functionality of PS-II in response to heat stress in two tall fescue (*Festuca arundinacea*) cultivars namely Jaguar 3 brand (heat-tolerant) and TF 66 (heat-sensitive). Reduction in net-photosynthesis was caused in two cultivars under heat stress because of both stomatal ans non-stomatal limitations, increase in photo-inhibition and reduction in Rubisco activity. Functionality of PS-II was modified in two plant leaves under stress which was manifested by lower variable chlorophyll fluorescence yield (Fv), maximal PS-II photo-chemical efficiency (Fv/Fm), open reaction center efficiency in light (*Φ*PSIIopen). Their results reported that high temperature stress caused decrease in chlorophyll a+b and chlorophyll/carotenoid ratios.

Veerasamy *et al.,* (2007) investigated the exogenous application of zeatin riboside (ZR), a synthetic cytokinin on leaves of creeping bent grass (Agrostis stolonifera L.) under heat stress and their effects. The plants were grown in growth chamber with control (20/15 8C, day/night) and heat (35/30 8C) treatments. Before heat stress treatment, 10 µmol ZR was sprayed on foliage for 3 days and then during 35 days of heat stress treatment, it is sprayed for once in a week. In normal conditions of heat stress without ZR, there is decline in Leaf chlorophyll content, photochemical efficiency (Fv/Fm). In heat stress conditions with ZR application, high chlorophyll content and Fv/Fm. Hence, the exogenous ZR application mitigated the negative heat stress effects like chlorophyll degradation and lower photochemical efficiency.

Song *et al.,* (2013) explained how photosynthesis was effected by the application of nitric oxide exogenously in rice seedlings under heat stress. There was an indicative decrease in net photosynthetic rate (PN) because of non-stomatal components, under heat stress. This high temperature induced reduction of PN was alleviated by the application of nitric oxide donors like sodium nitroprusside

(SNP) or S-nitrosoglutathione (GSNO). Measurement of fluorescence of chlorophyll under high temperature reported that there is indicative increase in initial fluorescence (Fo) and non-photochemical quenching (NPQ), while maximal fluorescence (Fm), maximal PS-II photochemistry efficiency (Fv/Fm), efficiency of actual PS-II (ϴPSII), and photochemical quenching (qp) decreased. SNP or GSNO pre-treatment led to the mitigation of increased Fo and decreased Fm, Fv/Fm, UPSII, qp. It also reported the elevation of NPQ values. Their results suggested that application of NO can protect the process of photosynthesis from damage caused due to heat stress.

Sailaja *et al.*, (2015) deciphered the response of elevated temperature in 11 mega rice cultivars that are popularly grown in India by multiple organisational analysis considering physiological, biochemical and molecular levels. Many traits along with photosynthesis were studied at both vegetative and reproductive stages, which has an established correlation with the yield under heat stress. Their results, evaluated nagina22 among all the 11 genotypes, as best performer in consideration with yield as well as all other physiological, biochemical and molecular traits. Nagina22 exhibited lesser decrease in chlorophyll content, lesser decrease in net photosynthetic rate and other parameters like photosynthetic / fluorescence was also reduced, leading to least reduce of spikelet fertility and grain yield under stress.

Wang *et al.*, (2018) reported chloroplasts as metabolic centers and their role in developing physiological adaptiveness to heat stress. During this adaptive physiological process occurring in chloroplasts, metabolic reprogramming occurs and heat shock proteins protecting other proteins at heat stress are expressed. Heat stress induces inhibition of photosynthetic activity in plants by damaging different key components functioning in metabolic processes with collateral biomass production reduction leading to low crop yield. They focused on the events like chlorophyll breakdown, reactive oxygen species generation, protein turnover etc through extensive and metabolic reprogramming under heat stress .This kind of diverse metabolic reprogramming in chloroplasts is a requisite for systemic acquired adjustment to heat stress.

Sangwan *et al.,* (2018) conducted experiment in wheat fields of CCSHAU, Hisar and reported the drastic effect of high temperature on chlorophyll, which can also be reflected by normalized difference vegetative index (NDVI). Their study evaluated the content of chlorophyll with the use of SPAD and NDVI with the Green Seeker™ Handheld Optical Sensor Unit on bread wheat recombinant inbred lines (RILs) that were derived from WH1021 (heat tolerant) and WH711 (heat sensitive) genotypes of wheat. Assessment of RILs was done under different sowing dates i.e normal and late sowing and the measurements were taken at anthesis and 10days after anthesis. Indicative negative effect is seen in leaf chlorophyll content due to heat stress. As the temperature raises, a decrease in NDVI and average chlorophyll content was observed.

2.5.5. Proline content:

Ahmed *et al.,* (2011) investigated the proline content of wheat seedlings as a screening criteria for heat stress. For measuring seedling proline content, 20 different genotypes of wheat were grown at 25℃ and 35℃ in phytotron. Membrane stability was also calculated in addition with proline for screening them into tolerant and susceptible varieties. The wheat genotypes recording <50% membrane injury were considered under heat tolerant (HT) group and the genotypes recording >50% membrane injury were considered as heat sensitive (HS). The HT group genotypes, at high temperature produced almost double the proline content (>200%) than normal temperature while the proline content of HS genotypes was less at 35℃ when compared with HT genotypes. Membrane injury % and proline content of seedlings at 35℃ maintained an indicative negative correlation among all genotypes of wheat tested.

Han *et al.,* (2013) reported the impact of heat stress on proline, total soluble sugars and MDA in different lettuce varities at seedling stage. The seedlings at 4-5 leaf stage are subjected to heat treatments of 25/20℃ (d/n), 30/25℃ (d/n), 38/33℃ (d/n), 42/37℃ (d/n) by placing them in artificial climate boxes. After 3 days of heat stress, leaf samples were collected for various analysis. The results showed the progressive increase of proline and total soluble sugars in lettuce seedlings at high temperatures of 38/33℃ (d/n). Proline has a kind of osmotic adjustments and increase of proline content under stress leads to plant resistance by augmenting the organisms adaptability to environmental stress. Results depicted that non-heat resistant varieties proline content increased rapidly when compared with heat-resistant varieties.

Gosavi *et al.,* (2014) studied the heat stress effect on different physiological and biochemical parameters of drought susceptible, tolerant and wild sorghum genotypes at seedling stage. Under heat stress, proline accumulation was significantly increased in seedlings when compared with control. As heat stress produces oxidative damage as secondary stress, sorghum seedlings can cope up with that by higher anti-oxidant enzyme activities under stress. Wild sorghum genotypes showed higher accumulation of proline and anti-oxidant enzymes in stressed conditions and used for transferring heat stress tolerance trait by breeding programmes. They are also used for mining of genes responsible for heat tolerance. In above studies mean fold increase of proline accumulation was recorded higher in drought tolerant (30%), wild genotypes (23%) and lower in drought susceptible (18%) genotypes.

Harsh *et al.,* (2016) conducted an experiment for finding out the impact of short term heat stress in moth bean. As the heat stress causes an array of changes at physiological, biochemical and molecular levels, in this experiment the effect of short term heat stress on antioxidants and osmoprotectants was found in 37 genotypes (32 mutants and 5 varieties) of moth bean. Short term heat stress was imposed by placing the 7 day old seedlings in hot air oven at 42˚C for one hour. After 3 days of heat exposure, analysis of different parameters was done. Results showed the indicative over-accumulation of proline under heat stress conditions.

Sarkar *et al* (2016) aimed at studying the elevated temperatures effect on wheat seedlings of four different cultivars namely gayetri (GY), gandhari (GN), kedar (KD), PBW343 in consideration with tolerance / susceptibility to stress, biochemical and anti-oxidative responses. They exposed the cultivars seedlings to various temperatures from 25°C to 40°C for 6 h. GN was found as heat tolerant, GY and KD were moderately tolerant and PBW343 was heat sensitive, as per the calculated heat susceptibility index. Upto 35 °C, there was an increase in the compatible osmolytes like proline and total sugar content in cellular compartments. These results suggested that wheat can tolerate oxidative stress induced by heat up to certain period, which was illustrated by increase of osmoprotectants like proline and non-enzymatic oxidants under stress.

Mishra *et al.,* (2018) deciphered the over-expression of *OsSIZ1* rice gene in Arabidopsis improving all the drough, heat and salt stress tolerance. A post translational modification named sumoylation, affects plants cellular processes through conjugation of small ubiquitin like modifier (SUMO) to substrate target protein. One of the important cellular functions that SUMO conjugation regulates is responding to different abiotic stresses due to environment. A crucial step in sumoylation pathway is facilitated by SIZ1, a SUMO E3 ligase. In this report it has been demonstrated that over expression of *OsSIZ1* rice gene in Arabidopsis led to increased multiple abiotic stress tolerance. Further analysis confessed that plants over-expressing *OsSIZ1* showed higher transcript levels of *P5CS*, proline biosynthesis gene under environmental abiotic stresses. Here proline acts as osmoprotectant to mitigate damage due to heat and other stresses.

2.5.6. Malondialdehyde (MDA) content:

Kazim *et al.,* (2013) carried out an experiment for screening 8 local Pakistan rice accessions during their early growth stages like germination and seedling stages for heat stress by exposing them to heat shock temperature of 42±3°C for various time periods (24, 48, 72 h). Thermo-tolerance at germinating stage was explored using promptness index (P.I) and germination stress index (G.S.I), while relative membrane permeability assessed by electrolyte leakage, content of melondialdehyde (MDA) and hydrogen peroxide were used at seedling stage. It’s been reported that during germination stage, heat stress resulted in delayed germination and lower germination percentage. Under stressed conditions there was more leakage of electrolyte when compared with normal, as it is associated with lipid peroxidation level and hydrogen peroxide that increases under stress. Thus MDA content increases relatively more in heat susceptible varieties than heat tolerant varieties. Among the 8 Pakistan rice varieties, Kanwal-95 showed more thermo-tolerance.

Wilson *et al.,* (2014) evaluated fifty *Brassica juncea* genotypes at four day old seedling stage for heat stress considering biochemical components. These four day old seedlings were given heat treatment of 45 ºC for about 4.5 hrs and estimated survival percentage, leakage of electrolytes and chlorophyll content. In these genotypes lipid peroxidation was determined by MDA content. In stressed conditions, tolerant genotypes recorded MDA of 4.66 (MDA g-1 f. wt. of tissue) while susceptible genotypes recorded 7.44 (MDA g-1 f. wt. of tissue). During heat stress, ROS are produced as secondary stress which react with unsaturated fatty acids in membranes and results in lipid peroxidation, which leads to MDA accumulation.

Zafar *et al.,* (2017) conducted an experiment for exploring the heat tolerance mechanisms in rice during early seedling stage. Heat stress treatment of 45±2°C was given for 12 hrs in 46 rice genotypes that include 39 super basmati mutants (M5generation) and 7 varieties. The heat stress is followed by recovery of 3 days under normal temperature (28±2°C). The heat tolerance of genotypes was determined using various parameters including morphological changes, physiological and cell membrane thermostability, photosynthetic pigments and malondialdehyde content. They identified 15 rice genotypes (11 mutants and 4 varieties) as heat tolerant based on relatively lower MDA content. The increased levels of MDA under stress conditions when compared with normal conditions determined higher peroxidation of lipid membrane due to stress. Basmati-385 recorded lowest MDA content (31.74 µM/g f. wt.) under high temperature indicating least peroxidation of lipid membranes under stress.

Borriboon *et al.,* (2018) reported that decline in rice productivity is a major concern due to this high temperature in thailand. They investigated the content of hydrogen peroxide, malondialdehyde (MDA) and membrane stability from seedlings of rice, grown from seeds which are exposed to various temperature treatments (25°C, 35°C and 40°C) for short (1 week) and long (2 weeks) duration before germination. They designed the experiment in RCBD containing 6 replications with 3 rice cultivars Dular, KDML105 and Riceberry. Among these riceberry produced large numder of normal seedlings under both short and long term treatment of heat, while KDML105 produced more percentage of abnormal seedlings at high temperature. Both electrolyte leakage and MDA content determine the membrane stability under heat stress. Abnormal seedlings of riceberry recorded the highest MDA content after exposing to heat stress for two weeks.

Sharma *et al.,* (2018) aimed at deciphering the heat stress effect on wheat varieties and their different mechanisms adopted to encounter the stress. Different wheat varieties were grown on MS media and they are subjected to continuous high temperatures for 5 days after plantlets were appeared. Antioxidative enzyme activity was increased significantly under stress in wheat varities. After stress application, the high content of MDA and proline in these varieties suggested that they are suited better to overcome the injury due to oxidative stress.

2.6. Expression analysis of candidate genes responsible for heat stress:

Agarwal *et al.,* (2003) narrated isolation and characterization of HSP101 gene from rice. Southern analysis determined that there seems to be a single gene per haploid genome coding for HSP101 protein in rice. Northern analysis showed that HSP101 transcript expression is induced by heat and the induction is transcient. Western analysis reported that this protein is rapidly expressed during stress period and retained for longer duration in japonica rice types compared to indica rice types during post-stress recovery period. OsHSP101 gene constituted 5 exons and 4 introns as per the comparative analysis of nucleotide sequences of HSP101 cDNA and the gene. Analogous to rice, Athsp101 has 4 introns in the genomic clone. They were the first one to report that plant HSP100 protein expression correlates disappearance of protein granules in yeast cells and also reported distinct rice-type dependent protein expression pattern.

Hu *et al.,* (2009) examined, heat stressed rice seedlings global expression profiling and compared their results with previous data available under other abiotic stresses. These comparision led to the conclusion that, while most HSFs and HSPs had highly similar and overlapped response and regulation patterns under different stresses, some of those genes exhibit significantly specific response to different stress. Distribution of 99 different heat stress genes were marked on 12 chromosomes of rice. 5 different heat shock gene families were indicated by different colours on chromosomes of rice in figure 2.8 below.

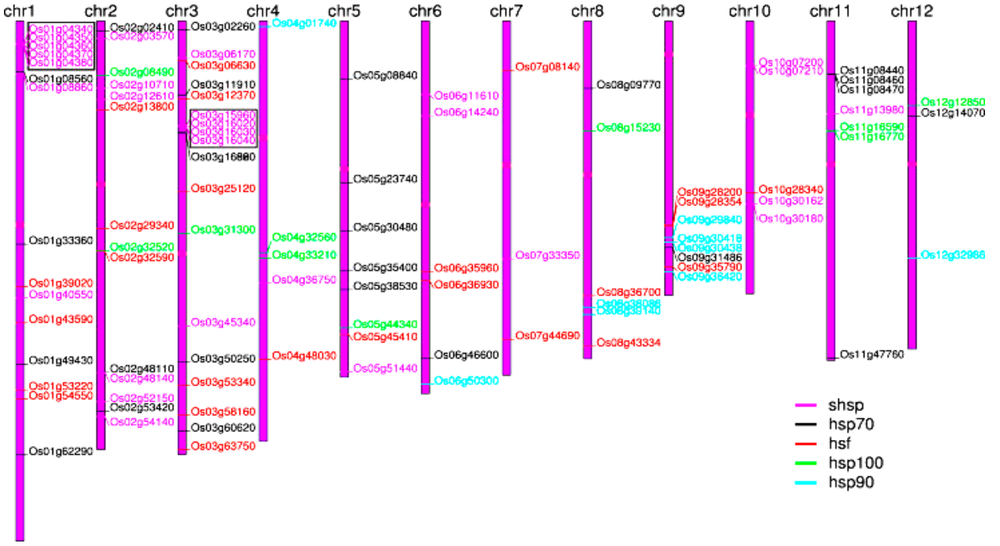


Fig. 2.8 Chromosome distribution of heat stress transcription factors and heat shock proteins. Two tandem clustered genes of sHSPs are indicated by black boxes at chromosomes 1 and 3. [Hu *et al.*, (2009)]

Zou *et al.*, (2011) reported that heat stress response in rice is complicated, involving up- or down-regulation of numerous proteins related to different metabolic pathways. Protection proteins, proteins involved in protein biosynthesis, protein degradation, energy and carbohydrate metabolism, and redox homeostasis come under this list. In addition, enhanced thermotolerance in transgenic rice was obtained by over expression of rice genes and genes from other plants. On other side, heterologous expression of some rice proteins led to high thermotolerance in bacteria and other easily transformed plants. They gave the proteomic characterization of rice in response to heat stress. The table 2.2 below shows some transgenic rice with increased tolerance to heat.

Table 2.2: Transgenic rice with increased heat tolerance.

Gene	Protein	Source	Comments	References
AtHsp101	HSP101	Arabidopsis thaliana	Pusa basmati 1; CaMV 35S promoter;Transgenic plants showed increased tolerance to high temperature.	Katiyar-Agarwal *et al.,* (2003
mtHsp70	HSP70	Oryza sativa	Nipponbare; CaMV 35S promoter; mtHsp70 over-expression suppresses PCD by maintaining 4wm and preventing ROS signal amplification	Qi *et al.,* (2011)
sHSP 17.7	HSP17.7	Oryza sativa	Hoshinoyume; CaMV 35S promoter;Transgenic plants exhibited significantly increased thermotolerance	Murakami *et al.,* (2004)

Jung *et al.*, (2012) performed genome wide transcriptome analysis of rice to identify strongly induced immediate early genes due to high temperature stress. Using the NSF45K array they compared the effects of high temperature (37˚C) treatment (for 1 hr) of seedlings relative to control (28˚C) and identified 710 genes that show at least 2 fold up-regulation at both time points. Among them they defined 244 genes as early heat stress responsive groups and 238 genes as prolonged heat stress responsive groups. As per Gene Ontology (GO) enrichment analysis, chaperon-mediated protein folding cofactor was the most over-represented GO term in association with prolonged heat stress, while enrichment of processes involving cell redox homeostasis, intracellular protein transport and regulation of transcription (DNA dependent) in association with early heat stress response. In addition, they also observed strongest correlation between heat and drought. Amongst the genes upregulated in both heat and drought, *OsNAC6/SNAC2 (Os03g60080)* is induced by cold, drought and high salinity. Hence this gene was suggested as good target gene for developing crop with increased tolerance to multiple abiotic stress conditions. HSP10/CPN10 family developed specialized functions to get over prolonged heat stress only, while HSP20 family genes evolved to combat with multiple abiotic stress. Figure 2.9

below, shows the expression pattern of early and prolonged heat stress genes responsive to other abiotic stresses.

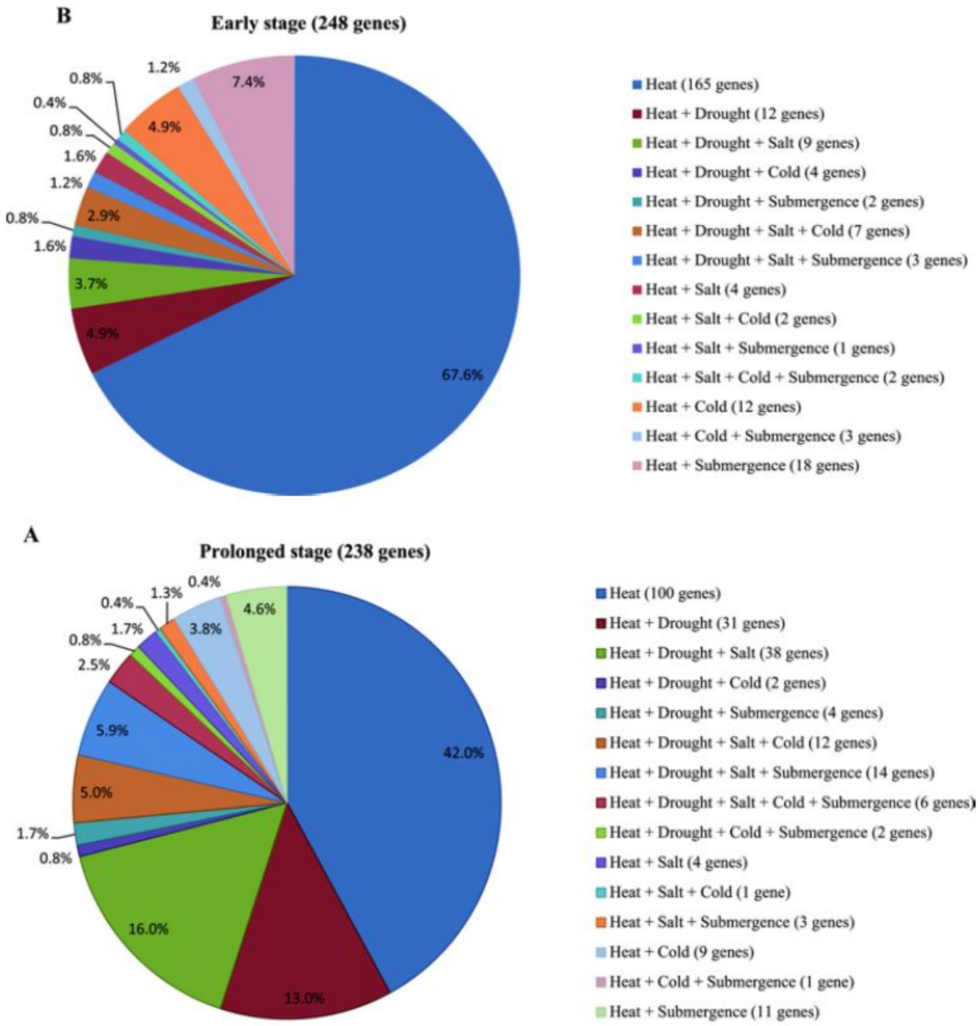


Fig 2.9 Expression patterns of early and prolonged heat stress responsive genes in response to other abiotic stress. [Jung *et al.*, (2012)]

Cao *et al.*, (2013) investigated the differential gene expression analyses in rice leaves of 21 days old seedlings subjected to different heat stress by performing cDNA-AFLP, to identify transcripts induced by heat stress. They were further verified by real-time RT-PCR. In total, more than 3000 different fragments were identified. Among them 49 fragments have been selected for sequencing and those differentially expressed genes are classified into different groups. 6 out of these 49 fragments were measured using real time RT-PCR. In addition they also conducted high performance liquid chromatography (HPLC) to analyse variations in 3 different polyamines like Putrescine, Spermidine, Sperimine at heat stress conditions. The transcripts of 6 gene fragments responded to heat stress, that are measured using real time RT-PCR are *Os04g0429600*, *Os06g0124900*, *SRP14*, *Os02g0611200*, *Os02g0788800*, and *Os07g0620200*. Among them *Os06g0124900* gene was observed to show the pronounced effect, it increased more than twice both in heat sensitive “Shuanggui 1” and heat tolerant “Huanghuazhan”.

Hasanuzzaman *et al.*, (2013) studied that the ability of a plant to perceive the high temperature stimulus, generate and transmit the signal and to initiate the respective biochemical , physiological changes determines the plant survival under heat stress. At molecular level, alterations in the gene expression responsible for osmoprotectants, detoxifying enzymes, regulatory proteins and transporters is involved. The below figure 2.10 explains schematic flow of heat induced signal transduction.

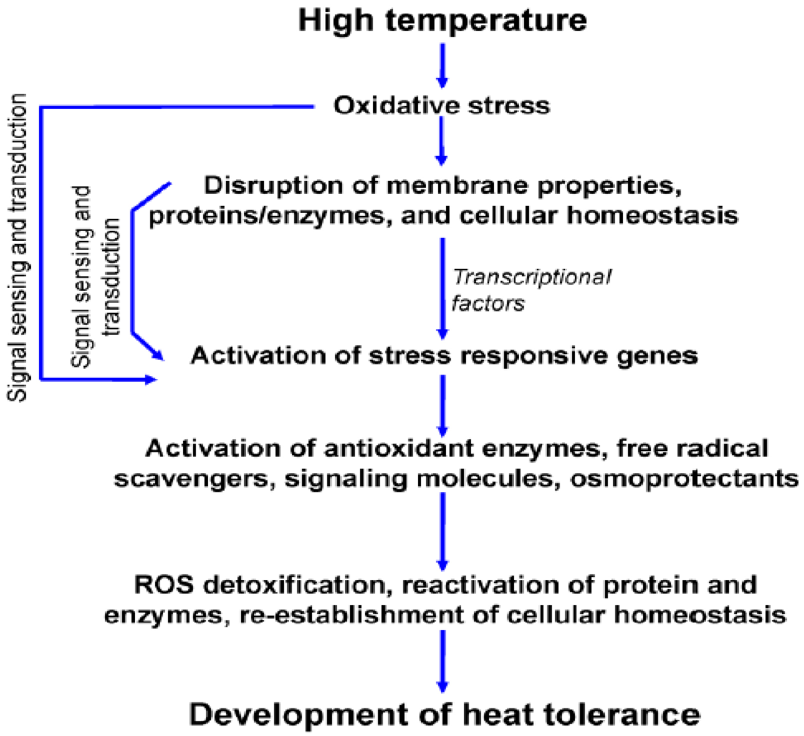


Fig 2.10 Schematic flow of heat induced signal transduction mechanism and heat tolerance development in plants. [Hasanuzzaman *et al.*, (2013).]

Qu *et al.*, (2013) elaborated on the heat stress related response networks in plants, in conjunction with Hsf and Hsp response pathways, ROS response and hormones network. Heat stress response elements production during particular physiological periods of plants is also interpreted. Heat stress leads to osmotic and oxidative secondary stresses. So to resist heat stress, production of many enzymes, non-enzymes, antioxidants and hormones occurs through heat stress response networks. Figure 2.11

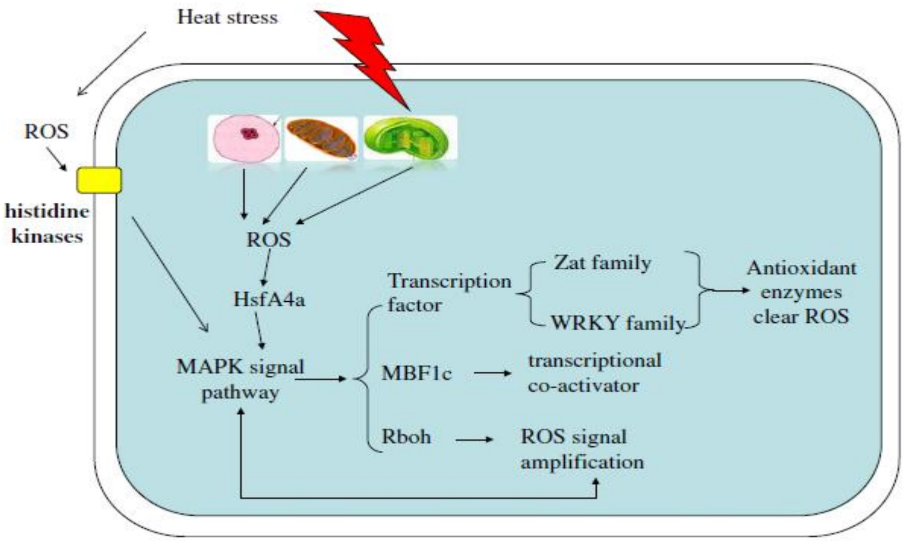


Fig. 2.11 The response of ROS. During heat stress, endogenous and exogenous ROS accumulates. Endogenous ROS are sensed through histidine kinases, and HsfA4a act as the sensors of exogenous ROS. Subsequently, the MAPK signal pathway is activated and induces the expression of transcription factors, MBF1c and Rboh. The transcription factors, including the Zat and WRKY families, induce the expression of antioxidant enzymes, and MBF1c acts as a transcriptional co-activator. Rboh enhances the production and maintenance of ROS signaling. **[Qu *et al.*, (2013)].**

Li *et al.*, (2013) worked on switch grass (*Panicum virgatum L.*) which is considered as a model herbaceous bio-energy crop, for its rapid growth rate, reliable biomass yield, water and nutrients minimal requirement, adaptability of growing on marginal lands and widespread distribution throughout North America. They reported on switch grass physiology, cell wall composition and biomass yield under high temperature. Switch grass cultivar Alamo was subjected to long-term heat stress treatment (38°/30°C, day/night, for 50 days) and observed significant decrease in plant height and total biomass in the heat stressed plants when compared to controls. Transcriptome analysis with switchgrass Affymetrix gene chips was done using total RNA from control and heat stress plants followed by normalization and pre-processing, leading to the identification of 5365 probesets as differentially expressed using a 2-fold cutoff. Among them, no.of probesets up-regulated were 2233 (2000 switchgrass unigenes) and 3132 probesets (2809 unigenes) were down-regulated. From this list, using RT-PCR, differential expression of 42 randomly selected genes were validated. Rice orthologs were restored for 78.7% of the switchgrass probesets that are heat stress responsive. As per Gene ontology (GOs) enrichment analysis using AgriGO program genes related to ATPase regulator, chaperone binding, and protein folding were significantly up-regulated while those related to protein modification, transcription, phosphorus and nitrogen metabolic processes, were significantly down-regulated by heat stress. This study identified conceivable connections between the identified GOs, physiological responses and heat response phenotype in switch grass plants. Comparative transcriptome analysis in response to heat stress among four monocots – switch grass, rice, wheat and maize was done and it resulted in the identification of 16 common genes, most of which are related to the process of protein refolding in stress conditions. The figure 2.12 Shown below describes the comparison of heat stress transcriptomes among 4 monocots.

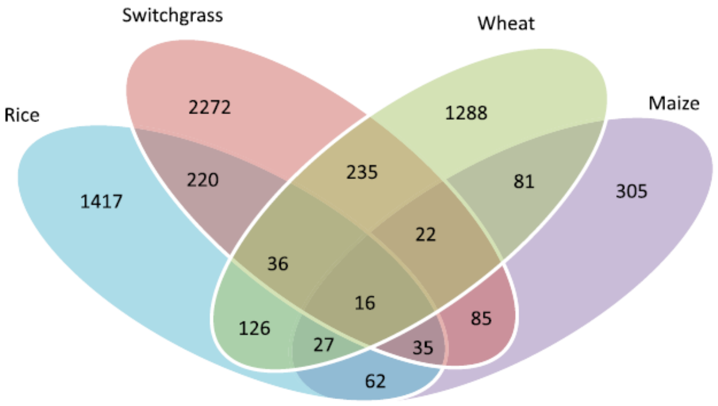


Fig 2.12 Comparision of heat stress transcriptome of switchgrass, rice, wheat and maize showing 16 common genes, most of which are responsible for protein refolding. [Li *et al.*, (2013)]

Rajaram *et al.*, (2014) studies reported the heat shock responses in cyanobacteria, role and regulation of molecular chaperons. The key to stress adaptability of cyanobacteria is its ability to maintain the protein conformational homeostasis (folding – misfolding – refolding or aggregation – degradation). Unlike in other bacteria, cyanobacteria posses several genes that encode DnaK and

DnaJ family proteins. Hsp60 family proteins and some small Hsps are more prevalent during heat stress. In unicellular cyanobacteria like Synechocystis and Synechococcus, the HSP100 (ClpB) family proteins and some small Hsps contribution towards heat stress has been elucidated. Their study also documented the regulation of chaperon genes by many cis-elements and trans-acting factors. Some heat shock protein genes and their role in cyanobacteria is given in the table 2.3, below.

Table 2.3. HSP genes and their role in cyanobacteria.

Family	Gene	Cyanobacteria	Function	Reference(s)
Hsp100	clpBI, clpBII	Synechococcus	Acquired thermotolerance, cold tolerance	Eriksson & Clarke (1996), xPorankiewicz & Clarke (1997)
Hsp90	htpG	Synechococcus	Innate and acquired thermotolerance, protection of photosynthetic apparatus	Tanaka & Nakamoto (1999), sato et.al (2010)
Hsp60 Hsp10	groEL-1, groEL-2 (cpn60)	Synechocystis, Anabaena	Thermotolerance, GroEL: nitrogenfixing conditions; Cpn60: nitrogen replete conditions.	Chaurasia & Apte (2009),Rajaram & Apte (2008)
sHsp	hsp16.6 /hsp17/ hspA	Synechocystis, Synechococcus	Protects membrane fluidity, thermotolerance	Nakamoto *et al.*, (2000),Horva´th *et al.*, (1998),Lee *et al.*, (2000)

Usman *et al.*, (2015) conducted experiments to study the mechanisms for heat tolerance in chilli pepper. To determine these mechanisms, they evaluated six genotypes for cellular membrane thermo-stability(CMT) and HSP70 gene expression. In most of the genotypes membrane integrity was not damaged by high temperature treatment (50˚C), which was indicated by mean value of CMT. As per this, genotypes are classified as heat-tolerant (greater than 60%), moderately tolerant (30% to 60%), and susceptible (less than 30%). The heat-tolerant plants, AVPP0702, AVPP0116, and AVPP9905, recorded the highest CMTs at 89.27%, 88.03%, and 85.10% respectively. Under heat stress, they observed a significant increase in levels of HSP70 genes after 2h of 42˚C treatment indicating that this is quickly and sharply promoted by heat shock. Among all the six genotypes considered for the study, ‘ kulai’ showed the down-regulation of HSP70 gene and was found to be heat-sensitive genotypes. In eukaryotes, HSP70 is one of the most abundant HSPs. It binds to hydrophobic patches of partially unfolded proteins, in an ATP-dependent manner preventing the protein aggregation. Even if accumulation of HSP70 takes place during heat shock, they need their constitutively expressed cognates(HSC70) for general cellular functions, as they control protein homeostasis. They aid in the folding of nascent polypeptides releasing from ribosome and sorting of proteins to cellular organelles by interacting with mitochondrial and chloroplast protein import complexes and also form a link with ubiquitin-mediated proteasomal degradation pathway.

Chen *et al.,* (2016) elucidated molecular pathways contributing to heat sensitivity of critical developemental stages like transitions from syncytium to the cellularization stage during endosperm formation in young seeds. A set of putative imprinted genes, showing down-regulation under severe heat stress were identified using transcriptomic comparison of seeds exposed to moderate(35˚C) and severe heat stress (39˚C) with control (28˚C). Under moderate and severe heat stress, several type I MADS box genes specifically expressed during syncytial stage were differentially regulated. Precocious and delayed cellularization under moderate and severe stress are associated with suppression and over accumulation of these genes. They showed that rice seed size is regulated by modulating the expression of OsMADS87, one of the heat sensitive, imprinted genes affiliated with syncytial stage endosperm. Transgenic seeds lacking OsMADS87 show increased endosperm cellularization and also have decreased sensitivity to moderate heat stress concerned with seed size reduction when compared with seeds from wild type plants and plants over-expressing OsMADS87. The figure 2.13 Given below shows transitions in endosperm formation.

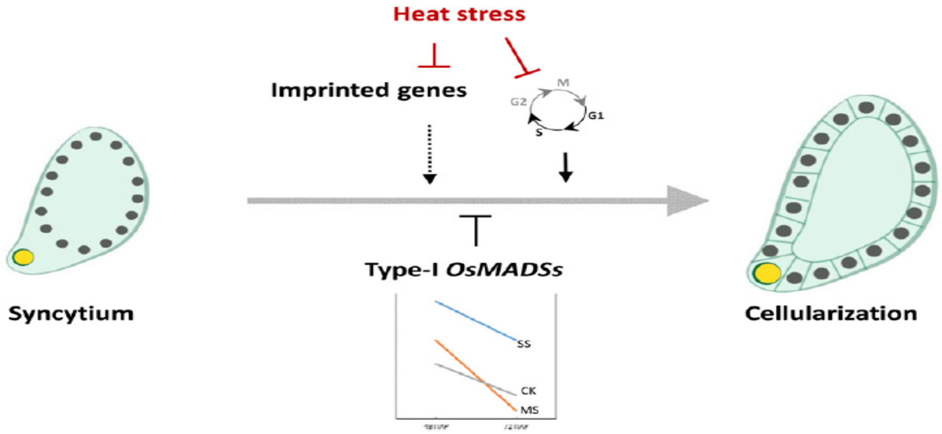


Fig 2.13 Proposed model incorporating main genes and pathways that are impacted when young rice seeds are exposed to heat stress. Arrows and T-bars indicate signalling promotion and inhibition, respectively. CK, MS, and SS indicate nonstressed, moderatestressed, and severe-stressed conditions, respectively. **[Chen *et al.*, (2016)].**

Kumar *et al.*, (2017) conducted proteomic analysis of heat sensitive rice genotype IET 21405 and revealed a total of 73 protein spots in rice leaf using two dimensional electrophoresis (2-DE) and MALDI-TOF/MS based proteomic approaches. Functional assessment of these proteins stated that, among these 73, few proteins are expected to involve in photosynthesis, tricarboxylic acid (TCA) cycle, and for energy production. The major role in acquiring heat stress response and thermotolerance is accumulation of HSPs under control of HSFs. These HSPs functionally act as molecular chaperons, repair and aid in renaturation of stress-damaged proteins. Distribution pattern of identified 73 proteins under high temperature, according to their biological function is shown in the figure 2.14, below. Mainly proteins found are functionally related to defense followed by energy metabolism, trafficking and signal transduction.

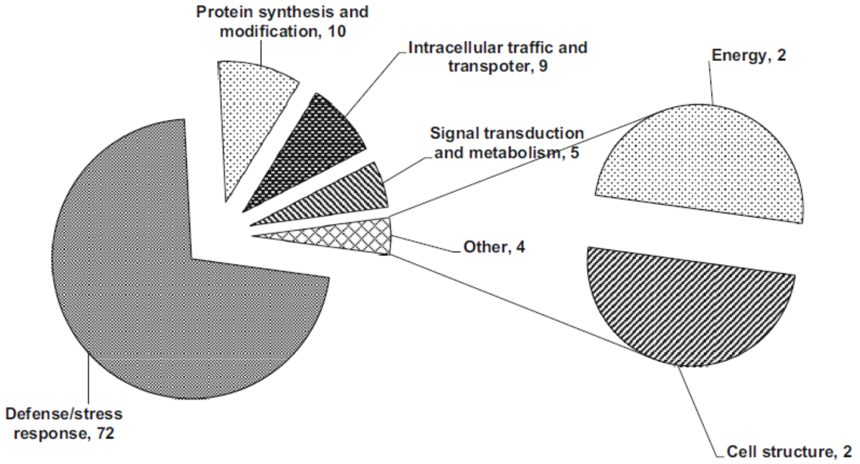


Fig 2.14 Distribution pattern of proteins under stress according to their biological function. [Kumar *et al.*, (2017).]

Su *et al.*, (2018) reported in vivo heat-regulated RNA structuromes. They obtained structural data on >14,000 mRNAs by applying structure-seq chemical [dimethylsulfate (DMS)] structure probing to rice (Oryza sativa L.) seedlings with and without 42 °C heat shock for 10 minutes. They have shown that RNA secondary structure broadly regulates gene expression in response to heat stress. Their results revealed RNA unfolding over biological temperature range, by significant heat induced ascent of DMS reactivity in the global transcriptome. They have found that increase in heat-induced DMS reactivity correlates with significant decrease in transcript abundance, as quantified from RNA-seq time course. This finally indicated that mRNA unfolding promotes transcript degradation and the basic mechanism for this outcome appears to be mRNA unfolding at both 5′ and 3′-UTRs facilitating access to RNA degradation machinery.

Vishwakarma *et al.*, (2018) constructed suppression subtractive hybradization (SSH) library in heat and drought stress tolerant Indian bread wheat cultivar C306, to generate a genetic resource of heat stress responsive genes/ESTs. Two subtractive cDNA libraries were prepared from RNA isolated from 93 days old plants at grian filling stage treated with 37˚C and 42˚C. Obtained ESTs were reconfirmed by using reverse northern dot blot hybridisation. A total of 175 contigs and 403 singlets were obtained from 1728 ESTs by gene ontology analysis. A transcript showing homology to *Hsp90* was observed to be up-regulated(7.6 fold) under heat stress by validating with qRT-PCR. *TaHsp90* heterologous expression was validated in *E. coli* BL21 and was confirmed by protein gel blot and MALDI-TOF analysis. The developed heat stress responsive SSH library led to single out a number of heat responsive genes/ESTs used for untangling the heat tolerance mechanism in wheat.

Nevame *et al.,* (2018) expressed that chalkiness in rice is occurred due to genetic and environmental factors, notably high temperature(HT). Many grain qualities are compromised due to heat stress, transparency being the most important among them. In rice, chalkiness is commonly studied in combination with other quality traits like amylose content, gel consistency and protein storage. They presented some of the relatively stable QTLs related to chalkiness, amylose content and gel consistency. Stable QTL distribution and availability of potential material resource is regarded as a expediency to find out novel stable QTLs related to chalkiness under heat stress. Hence to alleviate the high temperature effect , pyramiding of relatively stable and non-allelic QTLs regulating low chalkiness endosperm is done into adaptable rice varieties.

Kilasi *et al.,* (2018) aimed at identifying QTLs for tolerance of vegetative stage under heat stress and corresponding candidate genes in rice, as heat stress tolerance is a multigenic trait and candidate genes are known poorly. They generated single nucleotide polymorphic (SNP) markers by using genotyping-by-sequencing and genotype 150 F8 recombinant inbred lines (RILs) gathered by crossing heat tolerant “N22” and heat susceptible “IR64” varieties. 4,074 high quality SNP markers corresponding to 1,638 recombinationally unique events from mapping population were used to construct a linkage map. They identified 6 QTLs for root length and 2 for shoot length under control conditions with 2.1-12% effect. One QTL *rlht5.1* was determined for “root length under heat stress,” with 20.4% effect. Four QTL were diagnosed for “root length under heat stress as percent of control” that illustrated the total phenotypic variation from 5.2 to 8.6%. Three QTL with 5.3–10.2% effect were classified for “shoot length under heat stress,” and seven QTL with 6.6–19% effect were described for “shoot length under heat stress expressed as percentage of control.” Amidst the QTL identified six were overlapping between those selected using shoot traits and root traits: two were overlapping between QTL identified for “shoot length under heat stress” and “root length expressed as percentage of control” and two QTL for “shoot length as percentage of control” were overlapping a QTL each for “root length as percentage of control” and “shoot length under heat stress.” Genes coding 1,037 possible transcripts were identified based on their location in 10 QTL regions for vegetative stage heat stress tolerance. Among these, according to previous research in the literature, 213 transcript annotations were reported to be connected to stress tolerance. transcription factors, chaperone proteins (e.g., alpha-crystallin family heat shock protein 20 and DNAJ homolog heat shock protein), proteases, protein kinases, phospholipases, and proteins related to disease resistance and defense and several novel proteins are included in the putative candidate genes currently annotated as expressed and hypothetical proteins.

Soda *et al.,* (2018) reported that cytoskeleton plays a crucial role in stress tolerance; however, entanglement of intermediate filaments (IFs) in stress tolerance response remains ambiguous in crop plants. Clear evidence related to the unique involvement of Ifs in cellular protection against abiotic stresses in rice was provided in their study. Under different abiotic stress, abundance of transcript with genes encoding *Oryza sativa* intermediate filament (*OsIF)* showed 2-10 fold up-regulation. In transgenic rice, over expression of *OsIF* increased tolerance to salinity and heat stress, where as its knock-down (KD) effected plants towards more sensitivity. This indicates IFs role in promoting survival under stress. In the presence of high salt and heat stress, seeds of *OsIF* over expressed rice showed normal germination, better growth, ultra-structure of chloroplast was maintained and favourable K+/Na+ ratio when compared with wild type and KD plants. In the *OsIF* over expressed rice, better performance of both photosystem I and II was suggested based on photosynthesis analysis and chlorophyll a florescence data. Higher accumulation of proline and trehalose was revealed by metabolite profiling showing 2-4 fold increase, in over expressing rice than wild types. Thus under stress conditions, IFs overexpressing plants could maintain significantly high yield. In over expressed IFs seedlings, higher accumulation of compatible solutes takes place resulting in maintenance of osmotic homeostasis, organelle ultra-structure which corresponds to better performance of photosynthetic machinery. In future, better insight into the re-orientation of intermediate filaments under stress and their involvement in cellular machinery protection is provided by live cell imaging studies of OsIF:GFP (green fluorescent protein) fusion protein. Alisoltani *et al.*, (2018) reported that it is essential to have a targeted selection scheme to identify functional biomarkers than the selection of SSR's randomly. They aimed at developing a new method for identifying functional SSR markers using the available rice and wheat RNA-seq data under heat SSR stress and obtained unique SSR patterns. They revealed that GC/GC and GCC/GGC repeats were significantly more common under heat stress conditions on comparison with controls of both wheat and rice samples. Interestingly, genes containing these motifs participated in abiotic stress responses and included various heat shock proteins (HSPs) and DREB/CBF proteins. Their study suggested the possibility of using genes with altered SSRs as functional markers. Work flow of plant RNA-seq SSR analysis is shown in the figure 2.15 Below.

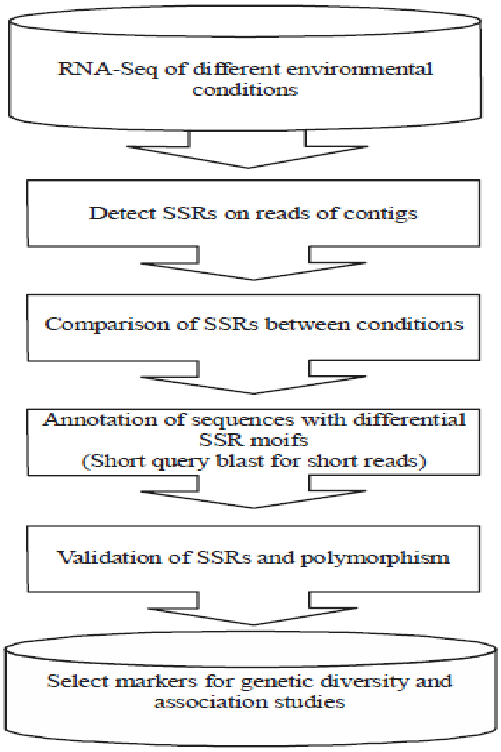


Fig 2.15 Work flow of plant RNA-seq SSR analysis. [Alisoltani *et al.*, (2018).]

CHAPTER-III
MATERIALS AND METHODS

The present investigation on **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)*"** was performed during the year 2018-2019 at Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, India. This chapter elaborates the experimental material and methods adopted during the course of the analysis. The detail of the materials used, methods adopted were presented as under the individual headings and sub headings.

3.1. Experimental site

The experiment was conducted in the green house facility and different laboratories of Department of Plant Molecular Biology & Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur.

3.2. Experimental material

The experimental material for the present investigation includes fourteen diverse genotypes of rice belonging to landraces, established varieties, and susceptible varieties from different research stations of Chhattisgarh. The details of these rice genotypes used in this study are presented in Table 3.1

Table 3.1: Different rice genotypes used in this experiment.

SN	Genotype	SN	Genotype
1	GP-145-103	8	Annada
2	SL – 62	9	Poornima
3	Dagad Deshi	10	Karma mahsuri
4	Nagina -22 (Check)	11	ARB – 6-11
5	Swarna	12	GP -145 -40
6	GP – 145-55	13	MTU-1010
7	CGZR -1	14	RRF-127

3.3. Experimental Design

The experiment consisting of fourteen rice genotypes was laid out in completely randomized design (CRD) with two replications for each experimental unit. The mean value work out from the measurements recorded on three randomly selected plants for different characteristics used for statistical analysis. The data collected was analyzed using analysis of variance (ANOVA) technique. OPSTAT statistical software package was used for this purpose (Sheoran *et al* 1998).

3.4. Experimental details

The present study was aimed to investigate the differential expression of various candidate genes that are responsive under heat stress conditions in rice. Semi-quantitative method of expression analysis was conducted for understanding the gene response in heat stress conditions. This was also followed by phenotypic and biochemical characterization of rice under heat stress. The whole work involves comparison of various biochemical and phenotypic characters with gene expression under heat stress and concluding that particular changes in biochemical and phenotypic characters under heat stress are due to up-regulation of few genes.

3.4.1 Phenological and Biochemical characterization of rice genotypes under heat stress.

3.4.1.1 Methods

Stress treatment:

Heat stress is given at the end of vegetative stage, before the panicle initiation of plants. Summer field conditions were mimicked by flooding the trays with water and increasing the green house temperatures. The green house temperature is increased gradually from 30 ºC at 6:00 am to 42 ºC at 11:00 am. Constant temperature of 42 ºC was maintained for 6 hrs continuously. The rice plants were treated with heat stress until 17:00 pm, with gradual adjustments to the greenhouse’s temperature down to 28 ºC–30 ºC at night (18:00 to 6:00). This stress is given continuously for 6 days from the beginning of the late vegetative stage. Leaf samples were collected after stress treatment from control and stress plants for further studies.

3.4.1 a) Membrane Stability Index-[MSI] :

Membrane stability index is measured by electrolyte leakage, at high stress stage when temperature becomes high enough to cause heat hardening of leaves.

1. Leaf samples were collected from control and stress conditions of all the fourteen genotypes.
2. Weigh 1g of leaf samples and wash it with deionized water to remove the residues present on them.
3. Cut the washed leaf samples into small pieces of 1cm and place in testtubes containing 15ml deionozed water and incubate at 24˚C for 12h.
4. Electrical conductivity (EC1) of the solution was measured using conductivity meter.
5. Subsequently, the samples were autoclaved at 120˚C for 20min and then cooled to room temperature.
6. Now the final Electrical conductivity (EC2) of the solution was measured.

Electrolyte leakage from the leaf samples is calculated by the formulae

$$\text{Electrolyte leakage (EL \%)} = \frac{EC1}{EC2} \text{ X } 100$$

3.4.1 b) Pollen fertility :

Pollen fertility is calculated using staining methods with the help of stains like aectocarmine (2%) or tetrazolium.

1. Pollen is collected from the 6 anthers of all the genotypes from control and stress conditions, and they are stored in ethanol (70%) to arrest the stage.
2. Then the anthers are punched using needle and pollen is distributed on the microscope slide and place a drop of acetocarmine (2%) dye.
3. Cover the slide with cover slip and observe under microscope at 40x just after preparation of slide.
4. The deeply stained / normal looking pollen grains are counted as the viable pollen and the colourless / shrivelled pollen are counted as the non-viable pollen.

The pollen fertility is calculated using the formulae

$$\text{Pollen fertility (\%)} = \frac{no.of\ viable\ pollen}{total\ no.of\ pollen\ in\ the\ microscopic\ field} \text{ X } 100$$

3.4.1 c) Spikelet fertility :

spikelet fertility at maturity was used to screen heat tolerance. It is calculated by counting the empty and filled grains.

1. Three randomly selected matured panicles were harvested from all the fourteen genotypes in stress and control conditions.
2. The harvested panicles were manually threshed and the number of filled, unfilled and total no.of grains per panicle are recorded.
3. These readings were taken by pressing each floret between forefinger and thumb to determine if the grain is filled or not. Both partially and fully filled spikelets were categorized as filled spikelets.

Spikelet fertility is calculated as shown below

$$\text{Spikelet fertility (\%)} = \frac{no.of\ filled\ grains}{total\ no.of\ grains\ formed\ (florets)} \text{ X } 100$$

Spikelet fertility is calculated in control and stress conditions. The negative effect (% decrease from control) was determined in every genotype.

3.4.1 d) Determination of chlorophyll by Acetone method :

The chlorophyll (Chl) content of leaves was estimated both in control and stress plants of all the fourteen rice genotypes following the method of Arnon (1949).

Reagents:

80% Acetone – The reagent is prepared by adding 80ml Acetone and 20ml distilled water.

Procedure:

1. 0.1 g of leaf tissue was cut into small pieces and homogenized with mortar and pestle in 2 ml of 80 % acetone.

2. The homogenate was centrifuged at 5000 rpm for 5 minute and the supernatant is collected in a volumetric flask.

3. The residue is re-extracted with 2 ml of 80% Acetone and centrifuged. Supernatant is collected in the same volumetric flask.

4. The procedure was repeated until the residues turned colourless. The final volume is made up to 10 ml.

5. The absorption of solution was measured at 663 nm, 646 nm, and 470 nm against the solvent (80% Acetone) blank using spectrophotometer (Biomate 5, Thermo Electron Corporation).

The amount of the chlorophyll present in extract was estimated as mg chlorophyll per gram of tissue using following formula-

$$\text{mg chlorophyll a/g leaf tissue} = 12.7\ (A663) - 2.69\ (A645)\ \text{x}\ \frac{V}{1000\ X\ W}$$

$$\text{mg chlorophyll b/g leaf tissue} = 22.9\ (A645) - 4.68\ (A663)\ \text{x}\ \frac{V}{1000\ X\ W}$$

$$\text{mg total chlorophyll /g leaf tissue} = 20.2\ (A645) + 8.02\ (A663)\ \text{x}\ \frac{V}{1000\ X\ W}$$

Where,

A = absorbance at specific wave length

V = final volume of chlorophyll extract in 80 % acetone

W = fresh weight of tissue extracted

Estimation of Chlorophyll Stability Index

The chlorophyll stability index (CSI) of fourteen rice genotypes in both control and stress conditions was determined according to Sairam *et al.* (1997) and calculated as follows:

$$\text{CSI} = \frac{total\ \ chlorop\,hyll\ \ under\ \ stress}{total\ \ chlorop\,hyll\ \ under\ \ control}\ \text{X}\ 100$$

3.4.1 e) Determination of Proline by Acid Ninhydrin method :

The free proline content in the leaf tissues of fourteen rice genotypes in both stress and control conditions were estimated as per the method given by Bates *et al*., (1990).

Reagents:

1. 6M Orthophosphoric acid – Take 38.1ml of orthophosphoric acid in 100ml DW

2. 3% Aqueous sulphosalicylic acid – Add 3gm of sulphosalicylic acid in 100ml DW
3. Glacial acetic acid
4. Toluene
5. Proline
6. Acid ninhydrin – heat 1.25gm of ninhydrin in 30ml glacial acetic acid and 20ml 6M phosphoric acid on hot top magnetic stirrer until dissolved.store at 4˚C and use within 24hrs.
7. Standards – dissolve 0.1gm proline in 100ml DW. Prepare 10 concentrations by taking 1-10ml from proline solution and make up volume to 100ml by DW. Now take 1ml each from above concentrations separately and add 1ml sulphosalicylic acid. To this add 2ml glacial acetic acid and then add 2ml acid ninhydrin.

Procedure:

1. 0.5 gm of fresh leaf tissue was ground and homogenized in a mortar and pestle with 10ml of 3% (w/v) aqueous sulphosalicylic acid.
2. The homogenate was filtered through Whattman no.2 filter paper.

3. 2 ml of filtered extract was taken for analysis in test tube and 2ml of glacial acetic acid and 2 ml of ninhydrin were added.

4. The reaction mixture was incubated in boiling water bath for 1 hour.

5. Terminate the reaction by placing the test tube in ice bath.

6. 4 ml of toluene was added to the reaction mixture and it was vortexed for 1-2 minute.

7. A pinkish red coloured toluene layer was formed on top. The layer was separated carefully in another tube and warm to room temperature.

8. Optical density was measured at 520 nm using toluene as blank by UV-visible spectrophotometer (Biomate 5, Thermo Electron Corporation).

9. A series of standard was run with pure proline in a similar way, using DW as blank and standard curve was prepared.

10. The amount of free proline in test sample was calculated from the standard curve using following formula and expressed in fresh-weight basis (µ mol/g f.wt.).

$$\mu \text{ moles per g tissue} = \frac{\mu\text{g proline /ml X ml toluene}}{115.5} \text{ X } \frac{5}{g\ sample}$$

where, 115.5 is the molecular weight of proline.

3.4.1 f) Determination of MDA enzyme :

MDA content (an indicator of lipid peroxidation) was calculated from all fourteen genotypes of rice under both stress and control conditions using method given by Heath and Packer (1986).

Reagents:

1. 0.1% W/V TCA – Take 0.1ml of TCA in 100ml DW.
2. 0.5% TBA in 20% TCA – Add 20ml TCA to 100ml DW and then dilute 0.5gm TBA in this 20% TCA.

Procedure:

1. 1gm of leaf tissue is homogenized by adding 5ml, 0.1% (w/v) trichloro acetic acid (TCA).
2. Centrifuge the homogenate for 10mins at 15000 rpm at 4˚C.
3. Collect supernatant and mix 1ml of supernatant with 4ml of 0.5% thiobarbuteric acid (TBA) diluted in 20% TCA. Now incubate in waterbath at 95˚C for 30mins.
4. End reaction by incubation the tubes on ice. Incase, the solution is not clear then centrifuge for a further 5mins at 15000 rpm at 4˚C.
5. Measure the absorbance at 532 and 600nm.

OD 600 values are substracted from the MDA-TBA complex values at 532nm and MDA content is calculated using the Lambert – Beer Law with an extinction coefficient $\mathcal{E}^{M}$ = 155 m$M^{-1}Cm^{-1}$.

$$\text{nmoles per 1ml of reaction mix} = \frac{A532-A600}{155} \text{ x } 1000,$$

as we took reaction mix of 5ml, multiply the values by 5

Results are represented as nmoles MDA g^{-1}FW. Where, FW = fresh weight.

Statistical analysis:

The experiment consisting 14 rice genotypes was laid out in completely randomised design (CRD) with three replications for each experimental unit. The mean value work out from all the measurements recorded on three randomly selected plants for different characteristics were used for statistical analysis. The data collected was analyzed using analysis of variance (ANOVA) technique. OPSTAT statistical software package has been used for this purpose.

3.4.2 Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.

3.4.2.1 Collection of leaf tissue and isolation of RNA:

Leaf samples of fourteen rice genotypes were collected from both control and stress induced plants.Leaves of control and stress plants harvested after 6th day of stress implosion. After collection, samples were snap frozen in liquid nitrogen and kept in a -80°C freezer for total RNA extraction.

3.4.2.2 RNA extraction:

RNA was extracted from the collected plant tissues by following combined procedure that employed both Trizol reagent and spin Columns (Trizol + spin protocol). The protocol was standardized in the laboratory to obtain quality RNA from leaves of rice. In this protocol, tissues were homogenized initially in Trizol reagent followed by precipitation and purification.

The RNA precipitate was purified by a column based method using Qiagen’s Plant RNAeasy Mini kit. Detailed stepwise procedure is described below. The extracted RNA was checked on denaturing formaldehyde agarose gel (FA gel) for checking its quality and integrity. Quantitative assessment was done on Nano drop spectrophotometer (Thermo scientific, ND1000).

Materials used:

- Liquid nitrogen along with container and dispenser
- Mortar and pestle
- Trizol reagent
- Chloroform Isoamyl mixture (*i.e. CIA* 24:1)
- Isopropanol
- Ethanol (Absolute ethanol and 70% ethanol)
- RNA isolation kit (Qiagen)
- DEPC treated water
- Refrigerated centrifuge
- Vortex mixture
- Eppendorf tubes
- Filter tips, pippette *etc*.

Preparation of utensils for RNA Extraction:

1. Mortar pestles and other glassware used for RNA extraction were soaked in 30% Hydrogen Peroxide for 15 minutes. Further, they were rinsed with DEPC water and autoclaved at 121˚C for 15mins. Then they were baked overnight in hot air oven.
2. R*NAS*e free 1.5 ml microcentrifuge tubes and R*NAS*e free filter tips were used every time.

Preparation of platform for RNA Extraction:

1. Clean the work area by spraying RnaZap over the platform and spread blotting paper.

2. Wipe the autoclaved motar and pestles using RnaZap. Before starting the procedure RnaZap is sprayed on hands covered with glouses and rubbed against each other.

RNA Extraction Procedure:

1. The collected samples were cut into fine pieces.
2. Tissue samples were crushed to fine powder by using liquid nitrogen.
3. The homogenate up to 0.5ml was transferred to RNASe free eppendorf tubes.
4. Add 700 μl of Trizol reagent to the sample and vortexed well.
5. Add 700 μl of chloroform: isoamyl alcohol mixture and samples were vortexed again. Samples were then allowed to sit at room temperature for 10 minutes.
6. Samples were centrifuged at 13000 rpm for 10 minutes under 40C.
7. Transfer 600 μl of top aqueous phase carefully into clean 1.5 ml eppendorf tubes.
8. Add 600 μl of isopropanol in to the samples and mix by inverting twice to precipitate RNA.
9. The entire precipitate was transferred to RNeasy mini spin column sitting in a 2 ml collection tube.
10. The Rneasy column was centrifuged at 10,000 rpm for 15 sec.
11. The flow through was discarded and RNeasy mini spin column (retaining RNA) was reused by placing it in fresh 2ml collection tube.
12. The column was then washed with 700 μl RW1 buffer by centrifuging at 10,000 rpm for 15 sec.
13. 500 μl of RPE buffer was added to RNeasy mini spin column followed by centrifugation at 10,000 rpm for 5 sec.

14. The flow through was discarded and Rneasy spin column was then placed in a new 1.5 ml collection tube.
15. About 30-50 µl (depending on expected yield) of RNASe free water was added to the Rneasy spin column and centrifuged at 10,000 for 1min.
16. The isolated RNA was stored immediately at -20 ºC.

- **NOTE**: All centrifugation steps involving spin column must be followed at room temperature.

3.4.2.3 cDNA synthesis:

RNA isolated and quantified was used for cDNA synthesis using Bio-RAD iScriptTM cDNA Synthesis kit as per manufacturer's instructions. Reaction mix and temperature profile for cDNA synthesis are presented in Table 3.2 and 3.3. The kit consisted of following components and is suitable for 1pg to 1µg of RNA. Kit components include following items:

1. 5X iScript Reaction Mix
2. iScript Reverse Transcriptase
3. Nuclease free water

Procedure for cDNA synthesis:

1. Thaw the template RNA samples on ice. Workout the amount/ volume of RNA required reaching 1 µg concentration and adjusting the volume of water accordingly. Dispense the required quantities of RNA and water in each tube (0.2 ml capacity).
2. Prepare the master mix on ice by mixing the mentioned amounts of 5x iScript reaction mix, iScript Reverse transcriptase, nuclease free water.
3. Mix by pipetting.
4. Dispense 10 µl of master mix into each tube containing RNA samples and water. Mix by gentle tapping and spin down the contents.
5. Incubate the tubes in PCR machine on reverse transcription cycling program described in table 3.3.

Table 3.2: cDNA synthesis (reverse-transcription) reaction components.

Component	Volume per reaction	Final concentration
5x iScript reaction mix	4 µl	1x
iScriptReverseTranscriptase	1 µl	-
Nuclease free water	Variable	-
RNA template	1-51 µl	1 µg
Total volume	20 µl	

Table 3.3: Reverse transcription cycling program

Step	Temperature	Time	No.of cycles
Priming	25˚C	5min	1
Reverse transcription	46˚C	20min	1
RT inactivation	95˚C	1min	1
Optional step	4˚C	Hold	

Quantification and dilution of cDNA:

Quality and quantity of cDNA were estimated by measuring the absorbance using Nanodrop spectrophotometer (ND1000). Two microlitre of DNA was placed over tip of Nanodrop to record absorbance at 260nm. The absorption ration (A260/ A280) and (A260/ A280) was recorded for each sample to estimate quantity and purity of cDNA. cDNA was diluted to uniform concentration of about 50 ηg/µl using Milli Q distal water.

3.4.2.4 Semi quantitative RT- PCR based gene expression analysis:

Semi quantitative reverse transcriptase PCR was carried out to study the expression of heat stress responsive transcription factor genes in rice genotypes under control and heat stress conditions. The cDNA generated from the total RNA isolated from leaf tissues of fourteen rice samples in total (i.e control and stress) were subjected to semi-quantitative expression profiling in 20 µl reaction using gene specific primers. The resultant PCR product was then resolved on 1.5 % Agarose gel at 100V. The presence of amplicons and their respective intensity were recorded under gel documentation system. The expression was analyzed by comparing the relative fluorescent intensities of cDNA amplicons under gel documentation system. *OsActin* (primer designed from rice)was used as an internal control for normalization of RNA concentration. Details of PCR components and temperature profile are depicted below in table 3.4 and table 3.5.

Table 3.4. PCR components their quantity used for semi-quantitative PCR.

Components	Concentration	Quantity
cDNA	1000 ng/µl	2 µl
PCR Buffer	1X(1.5mM MgCl2)	2 µl
dNTP mix	200 µM each	2 µl
Primer Forward	10 µM	1 µl
Primer Reverse	10 µM	1 µl
Taq Polymerase	1U/ µl	0.5 µl
Nanopure water	-	11.5 µl
Total		20 µl

Table 3.5. Temperature profiles used for semi quantitative RT-PCR

Activity	Temperature	Duration	Cycles
Initial Denaturation	94 ˚C	1 min	1
Denaturation	94 ˚C	30 sec	
Annealing	Variable(56-64˚C)	45 sec	35
Extension	72˚C	30 sec	
Final Extension	72˚C	5 min	1
Store	4˚C	∞	1

3.4.2.5 Digitalization of expression data:

In order to evaluate gene expression pattern based on numerical values, the semi quantitative RT-PCR generated fluorescence data were digitalized using GelQuant.NET Analyzer software (*www.biochemlabssolutiond.com*). The relative expression of genes was expressed in terms of fold change for the samples under heat stress with respect to control conditions. The genes used in this study are given below in table 3.6 along with their specific forward and reverse primers, melting temperatures.

Table 3.6. Forward and reverse gene specific primers used for semi-quantitative RT-PCR amplifications:

S No.	**Gene**	**Locus ID**	**Forward primer (5′ to 3′)**	**Reverse primer (5′ to 3′)**	**Tm (˚C)**	**Product size (bp)**
1	OsHsp 16	LOC_Os06g14240	GTCTGCGTTAGCTCCTGAAG	ACCACATCCTTCTCCCTCTC	62	290
2	OsHsp 16.9	LOC_Os01g04270	GTGATGGCCAGTCAAGTAGA	CTGCATCTCTGTTGGATCAC	61	219
3	OsHsp DnaJ	LOC_Os01g01160	GAAGACAAGTCTGGCTGGAG	CACAGCACACCCTTCTAACC	62	246
4	17.9 kDa CI heat shock protein	LOC_Os03g15960	AGAAGGAGGAGGTCAAGGTG	AGATCTGGATGGACTTGACG	62	255
5	OsHsp 18	LOC_Os01g08860	AAGAAGAAGAGGCGATCGAG	CTTGATGTCGGAGGACTTGA	61	259
6	18.9 kDa heat shock protein	LOC_Os02g03570	AGCATGGACTGGAAGGAGAC	CTTCCTGTCGTTGTCCTTGG	63	291
7	OsHsp 26.7	LOC_Os03g14180	CGTGAGGGTTTAAGCAGTGT	AGCTCAGTGTCTCAGCCTTG	62	250
8	60 kDa chaperonin	LOC_Os10g32550	AGTTCGAGCCTCAGATGTTG	ACCAACTTCAGCTTCACTGG	62	239
9	OsHsf A4a	LOC_Os01g54550	AAGGATGCGTACTTCGTCAG	GGTAAGAAACTGCTGCCAGA	62	257
10	OsHsf A7	LOC_Os01g39020	GTGATGATGTCTGGGAAGAG	TCTCATGCCTCTAGTGTTCC	59	177

3.5 Details of commercial kits, laboratory tools and equipments used in this study

Table 3.7 List and details of laboratory tools and equipments used in the study

SN	Product	Make/ Manufacture
1	-86℃ Ultra low temperature frezzer	Haier Biomedical
2	-20℃ Deep freezer	Vestfrost
3	4℃ Cabinet	Cellfrost
4	Ice maker	SCOTSMAN FRIMONT AF100 AS-E 230/50/1
5	Magnetic stirrer	Remi
6	Refrigerated centrifuge	Sorvall, Thermo Fisher Scientific
7	Bench top microcentrifuge	1-14K Microfuge, Sigma
8	Vortex Mixture	Tarsons
9	Electronic weighing balance	Elico
10	Water bath	Yarco
12	pH meter	Tarsons
13	Pipetteman Set (All volume range)	Finnpipette, Thermo Fisher Scientific
14	UV Transulliminator	Macroview UV transilluminator, Hoefer
15	Tissue lyzer	Molbio
16	96 well thermal cycler	Veriti 96 well thermal cycler, Applied Biosystems
17	Gel electrophoresis system	Midi Submarine electrophoresis system, Tarsons
18	Gel documentation system	Chemi doc MP Gel imaging system, Biorad laboratories,
19	Spectrophotometer for DNA/ RNA quantification	Nanodrop ND1000 spectrophotometer, Thermo

Table 3.8 List and details of molecular biology reagents, commercial kits and enzymes used in the study

SN	Product	Make/ Manufacture
1	Phire hot start taq DNA Polymerase	Cat# F122S, Finnzyme, Thermo Life Science
2	PCR components (dNTPs, buffer, DMSO, MgCl2 *etc*)	Finnzyme, Thermo Life Science
3	RNA stabilization reagent,RNAlater	Cat# R0901, Sigma
4	Trizol reagent	Cat# 15596018, Invitrogen
5	DEPC Treated water	Cat# AM9906, Ambion
6	UltraPure Distilled water	Ref 10977-015, Invitrogen
7	RNA isolation kit	Cat# 74904, Rneasy plant mini kit, Qiagen
8	cDNA synthesis kit	Cat# 1708891, iScript^{TM} cDNA synthesis kit, BIO-RAD
9	1 Kb DNA ladder	New England Biolabs & Merck Bioscience (Genei)
10	6X Organge G gel loading dye	Cat# 612601580061730, Merck Bioscience (Genei)
11	Oligos/ primers, (OD- 3,scale-25 nM) with HPSF purification	Eurofins Genomics India Pvt Ltd.

CHAPTER – IV

RESULTS AND DISCUSSION

The present investigation under study entitled as **"Deciphering the differentially expressed heat stress responsive candidate genes in Rice *(Oryza sativa)"*** was undertaken to understand the expression of known heat related genes using diverse rice genotypes. Further the correlation of genes expression levels with phenological and biochemical traits associated with the heat response in rice was also assessed.

4.1. Quantification of heat stress considering physiological and biochemical parameters.

Fourteen rice genotypes namely GP-145-103, SL-62, Dagad Deshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127, belonging to landraces, established varieties, susceptible varieties and advanced breeding lines were taken as the study material. All these genotypes were grown in trays under two different conditions i.e. control and heat stress by altering temperatures in green house. High temperature stress was given by mimicking the summer field conditions based on weather data of previous years with flooding water in trays.

The plant morphology of fourteen rice genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) at vegetative stage before panicle initiation under both stress and control conditions including the complete view of experimental set up of rice genotypes at vegetative stage under controlled conditions prior to induction of heat stress are shown in figure 4.1 and 4.2.

All the plants were induced stress treatment at vegetative stage before panicle initiation. High temperature treatment is given to the stress trays by adjusting the green house temperature to 42˚C at daytime, continuously for 6 days. After 6 days of high temperature treatment, three sets of leaf tissues were collected

For phenotypic, biochemical and expression studies. Likewise, three sets of leaves from control plants were also collected at same time.

Fig 4.1: Overall view of the experimental set up of 14 rice genotypes at vegetative stage before stress induction.

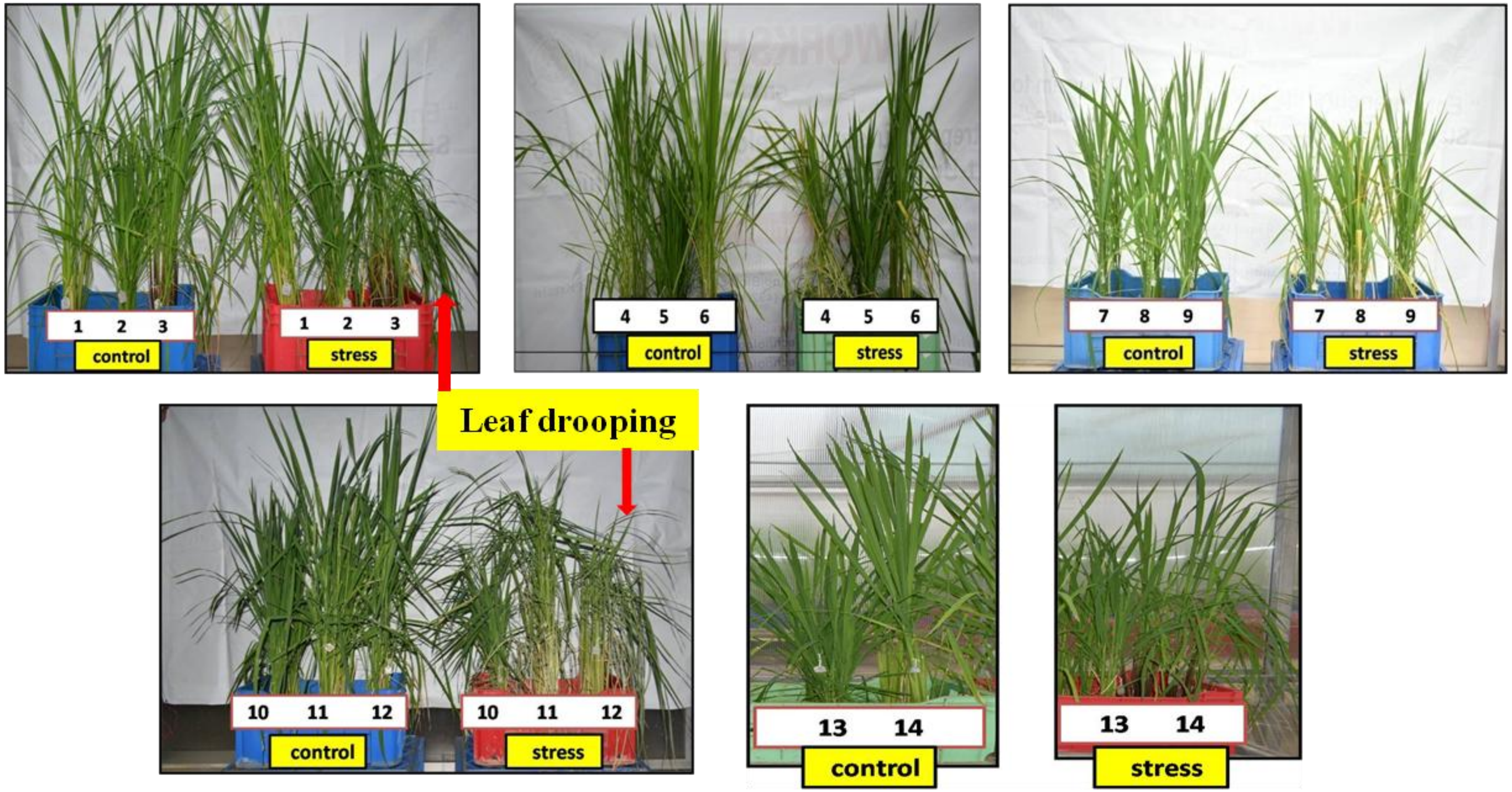


Fig 4.2: Plant morphology of 14 rice genotypes under control and stress conditions at vegetative stage before panicle initiation.1) DagadDeshi, 2) SL-62, 3) GP-145-103, 4) Nagina-22, 5) Swarna, 6) GP-145-55, 7) Poornima, 8) Annada, 9) CGZR-1, 10) Karma mahsuri, 11) ARB-6-11, 12) GP-145-40, 13) RRF-127, 14) MTU-1010.

4.1.1. Phenotyping of rice genotypes for heat stress related traits

4.1.1.1. Membrane stability index (MSI):

Uninterrupted function of cellular membranes is very important under stress conditions, for photosynthesis and respiration processes to occur precisely. Hence, cell membrane stability was reported to play critical role under conditions of high temperature, as a major component of heat tolerance. Membrane stability index is calculated by measuring the relative electrolyte leakage from cells due to injury occurred under stress. Electrolyte leakage is the measure of electrical conductivity of the tissues. Wide variation in the fold increase of membrane stability among the tested genotypes was recorded which ranged from 19.8% fold increase in RRF-127 to 292.9% in MTU-1010, given in the below table 4.1.

Table 4.1: Membrane stability index of fourteen rice genotypes under control and stress conditions

		MSI (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% increase**
1	GP-145-103	23.21	53.57	130.8
2	SL-62	23.52	55.86	137.4
3	DagadDeshi	33.92	83.49	146.1
4	Nagina-22	47.39	57.36	21.1
5	Swarna	15.08	23.66	56.9
6	GP-145-55	31.01	95.96	209.5
7	CGZR-1	26.36	40.00	51.7
8	Annada	40.64	69.00	69.8
9	Pornima	17.51	62.39	256.2
10	Karma mahsuri	28.28	36.07	27.5
11	ARB-6-11	22.73	67.97	199.1
12	GP-145-40	23.50	59.73	154.2
13	MTU-1010	21.00	82.50	292.9
14	RRF-127	25.00	29.95	19.8

Previous studies reported that heat tolerant genotypes show low levels of electrolyte leakage, indicating less injury to the membrane [Sarsu *et al.*, (2018); Sharma *et al.*, (2017); Hemantaranjan *et al.*, (2014)]. In the present study, the lowest levels of electrolyte leakage was observed in RRF-127 with 19.8% increase under stress when compared with control and reported as heat tolerant genotype followed by Nagina-22 (21.1%), Karma mahsuri (27.5%), CGZR-1 (51.7%) and Annada (69.8%) as shown graphically in figure 4.3.

4.1.1.2. Pollen fertility

High temperatures generally reduce the growth and production at all stages of the crop, but the effect is more pronounced on the yield reduction, when heat stress occurs at flowering stage (Prasad *et al.,* 2006; Mohammed and Tarpley 2011). Heat stress effects pollen development by decreasing the starch concentration before anthesis, which leads to decrease in sugar concentration in mature pollens, contributing to sterile pollen (Pressman *et al.,* 2002). Pollen fertility is calculated by using Acetocaramine dye. The fertile pollen are stained red and the non-fertile pollen stay transparent or unstained as shown in figure 4.4. Wide variation is observed in the pollen fertility of the tested genotypes under stress. The fold decrease in pollen fertility ranged from 14.4% in RRF-127 to 66.5% in MTU-1010as given below in table 4.2.

Table 4,2: Pollen fertility of fourteen rice genotypes under control and heat stress conditions

		Fertility (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% decrease**
1	GP-145-103	78	58	24.9
2	SL-62	57	28	50.2
3	DagadDeshi	51	20	60.5
4	Nagina-22	49	40	18.1
5	Swarna	71	37	47.9
6	GP-145-55	67	25	62.8
7	CGZR-1	53	33	37.9
8	Annada	50	36	28.3
9	Pornima	69	37	46.2
10	Karma mahsuri	79	54	31.4
11	ARB-6-11	83	30	63.3
12	GP-145-40	56	22	61.8
13	MTU-1010	54	18	66.5
14	RRF-127	85	73	14.4

Many studies reported that pollen fertility decreases under stress conditions and the genotypes showing lowest decrease in pollen fertility are considered as the tolerant ones for heat stress (Fahad *et al.*, 2018; Jagadish *et al.*, 2015; Kumar *et al.*, 2015). In the present study lowest decrease in the pollen fertility, under stress conditions was observed in RRF-127 (14.4%) followed by Nagina-22 (18.1%), GP-145-103 (24.9%), Annada (28.3%) and CGZR-1 (37.9%) shown graphically in figure 4.5.

4.1.1.3. Spikelet fertility

Heat stress affects spikelet fertility by decreasing pollen germination and increasing respiration rates, which subsequently reduces yield (Mohammed *et al.*, 2010). High temperatures reduce seed size and number, which ultimately affects '100 seed weight'. This occurs because high temperatures decrease translocation of photo assimilates, seed reserves biosynthesis that takes place in leaves during seed filling (Sehgal *et al.*, 2018). Wide variation is observed in the spikelet fertility of the tested genotypes under stress. The fold decrease in spikelet fertility ranged from 14% in RRF-127 to 71% in ARB-6-11 as given below in table 4.3.

Table 4.3: Spikelet fertility of fourteen rice genotypes under control and stress conditions

		Fertility (%)		
S.No	**Rice Genotype**	**control**	**stress**	**% decrease**
1	GP-145-103	49	31	37
2	SL-62	54	28	48
3	DagadDeshi	49	19	61
4	Nagina-22	48	39	19
5	Swarna	47	35	26
6	GP-145-55	65	25	62
7	CGZR-1	36	22	42
8	Annada	76	56	25
9	Pornima	69	36	48
10	Karma mahsuri	77	53	31
11	ARB-6-11	79	22	71
12	GP-145-40	69	36	48
13	MTU-1010	52	13	56
14	RRF-127	83	71	14

Earlier studies reported the decreased spikelet fertility under heat stress conditions and the genotype having the lowest change in spikelet fertility under stress conditions when compared to control is considered as the tolerant genotype (Wu *et al.,* 2016; Fu *et al.,* 2016; Zhang *et al.,* 2018). In the present study, lowest decrease in spikelet fertility under stress conditions was observed in RRF-127 (14%) followed by Nagina-22 (19%), Annada (25%), Karma mahsuri (31%) and CGZR-1 (42%) shown graphically in figure 4.6.

4.1.2. Quantification of heat stress considering biochemical parameters

4.1.2.1. Chlorophyll content

Under heat stress conditions, for photosynthesis to take place at normal level, it requires maintenance of chlorophyll content. Studies by Ashraf *et al.,* (1994) revealed that degradation of chlorophyll (a, b, total) under abiotic stress is induced due to osmotic stress that results as secondary stress because of the occurrence of more active OH hydroxyl radical. There was a wide variation in the recorded chlorophyll content of heat stress leaves of all the fourteen genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) as mentioned in the table 4.4.

The mean chlorophyll a content under control conditions was recorded as 2.529 mg/g leaf tissue and it ranged from 1.610 mg/g in MTU-1010 to 3.410 mg/g in CGZR-1 (Table 4.7). While mean chlorophyll a content under stress conditions was recorded as 1.874mg/g leaf tissue and it ranged from 0.523mg/g in swarna to 2.743 mg/g in CGZR-1 (Table 4.8). Lowest fold decrease in chlorophyll a content was recorded in the genotype Nagina-22 (1.0 fold) followed by Karma mahsuri (1.0 fold), Annada (1.0 fold), GP-145-103 (1.2 fold) and CGZR-1 (1.2 fold) (Fig. 4.7a).

The mean chlorophyll b content under control conditions was recorded as 1.303mg/g leaf tissue and it ranged from 0.370 mg/g in GP-145-40 to 2.820 mg/g in GP-145-55 (Table 4.7). While mean chlorophyll b content under stress condition was recorded as 0.873mg/g leaf tissue and it ranged from 0.203 mg/g in RRF-127 to 2.300 mg/g in GP-145-103 (Table 4.8). Minimum fold decrease in chlorophyll b content was recorded in the genotype Annada (1.0 fold) followed by ARB-6-11 (1.0 fold), GP-145-103 (1.0 fold), Karma mahsuri (1.1 fold) and GP-145-40 (1.1 fold) (Fig. 4.7b).

The mean total chlorophyll content under control conditions was recorded as 3.831mg/g leaf tissue and it ranged from 2.023 mg/g in MTU-1010 to 5.820 mg/g in GP-145-55 (Table 4.7). While mean total chlorophyll content under stress conditions was recorded as 2.747 mg/g leaf tissue and it ranged from 0.853 mg/g in Swarna to 4.970 mg/g in GP-145-103 (Table 4.8). Lowest fold decrease in total chlorophyll content was recorded in genotype Annada (1.0 fold) followed by GP-145-103 (1.0 fold), Karma mahsuri (1.0 fold), GP-145-40 (1.1 fold) and CGZR-1 (1.2 fold) (Fig. 4.7c).

Table 4.4: Chlorophyll a, chlorophyll b and total chlorophyll content of fourteen rice genotypes under stress and control conditions.

	chlorophyll content	**chl a (mg/g)**			**chl b (mg/g)**			**total chl. (mg/g)**		
S. No	**Genotype**	**control**	**stress**	**fold decrease**	**control**	**stress**	**fold decrease**	**Control**	**stress**	**fold decrease**
1	GP-145-103	2.71	2.17	1.2	2.31	2.30	1.0	5.02	4.97	1.0
2	SL-62	2.99	2.34	1.3	2.15	1.29	1.7	4.83	3.63	1.3
3	DagadDeshi	2.40	1.72	1.4	1.29	0.84	1.5	3.69	2.56	1.4
4	Nagina 22	2.55	2.55	1.0	1.82	1.24	1.5	4.36	3.79	1.2
5	Swarna	1.72	0.52	3.3	1.05	0.32	3.2	2.76	0.85	3.2
6	GP-145-55	3.00	1.14	2.6	2.82	0.62	4.5	5.82	1.76	3.3
7	CGZR-1	3.41	2.74	1.2	1.44	1.11	1.3	4.85	3.89	1.2
8	Annada	2.69	2.68	1.0	0.62	0.93	0.7	3.32	3.21	1.0
9	Pornima	3.09	1.76	1.8	1.06	0.41	2.6	4.15	2.16	1.9
10	Karma mahsuri	2.54	2.55	1.0	1.93	1.80	1.1	4.47	4.34	1.0
11	ARB-6-11	2.70	2.20	1.2	0.49	0.48	1.0	3.19	2.69	1.2
12	GP-145-40	1.90	1.29	1.5	0.37	0.34	1.1	2.07	1.93	1.1
13	MTU 1010	1.61	1.54	1.0	0.41	0.34	1.2	2.02	1.62	1.2
14	RRF-127	2.60	0.93	2.8	0.48	0.20	2.4	3.08	1.13	2.7

4.1.2.2. Proline content

Proline is a cyclic amino acid synthesized from glutamate. Proline acts as a compatible solute, i.e. it can accumulate to high concentrations in the cell cytoplasm without interrupting cellular structure and metabolism (Yancey *et al.*,1982). The proline content of leaf tissues is estimated according to the method of Bates *et al.*, (1973). The result of the proline estimation is given in the table 4.5. There was a wide variation in the recorded proline content of all the fourteen genotypes.

The mean proline content under control conditions was recorded as 0.407 µ mol/g fresh weight and it ranged from 0.064 µ mol/g in GP-145-103 to 1.65 µ mol/g in GP-145-40 (Table 4.7), while the mean proline content under stress conditions increased to 1.213 µ mol/g fresh weight and ranged from 0.095 µ mol/g in SL-62 to 2.044 µ mol/g in GP-145-55 (Table 4.8). When comparing the fold increase of proline content in stress over control, the highest fold increase in proline content was recorded in the genotype Nagina-22 (20.6 fold) followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mahsuri (6.0 fold) as shown in figure 4.8.

Table 4.5: Proline content of fourteen rice genotypes under control and stress conditions

		Proline(µmole/g)		
S.No	**Genotypes**	**control**	**stress**	**fold increase**
1	GP-145-103	0.06	0.45	7.0
2	SL-62	0.06	0.10	1.5
3	DagadDeshi	0.09	0.38	4.1
4	Nagina22	0.07	1.40	20.6
5	Swarna	0.27	1.57	5.9
6	GP-145-55	1.00	2.04	2.0
7	CGZR-1	0.17	1.98	11.4
8	Annada	0.18	1.90	10.5
9	Pornima	0.37	1.91	5.1
10	Karma mahsuri	0.07	0.40	6.0
11	ARB-6-11	0.98	1.94	2.0
12	GP-145-40	1.65	1.67	1.0
13	MTU 1010	0.52	0.92	1.8
14	RRF-127	0.11	1.25	11.2

An elevated level of proline during stress conditions reduce the osmotic potential and help in diffusion of water into the cells and maintain high turgor potential in the cell (Basu *et al.,* 2007). Other functions of proline accumulation were also proposed, including stabilization of macromolecules (Schobert and Tschesche, 1978), sink for carbon and nitrogen after stress recovery (Farooq *et al.,* 2009) and as a scavenger against reactive oxygen species (Joseph *et al.,* 2015). The concentrations of increased proline under stress conditions are directly proportional to the intensity of heat stress.

4.1.2.3 Malondialdehyde (MDA) content

Lipid peroxidation in the cell membranes is the destructive effect of oxidative damage caused due to heat stress. MDA content is the ultimate result of lipid peroxidation, which changes cells membrane stability. MDA content has been widely used as a criteria for assessing abiotic stress in various plants (Jain *et al.,* 2001; Moller *et al.,* 2007). MDA content of leaf tissues is estimated according to the method given by Heath and Paker (1968). The result of the MDA estimation is given in the table 4.6. Significant variation was recorded in the MDA content of fourteen rice genotypes.

The mean MDA content under control conditions was recorded as 0.372 µ mol/g fresh weight and it ranged from 0.097 µ mol/g in GP-145-55 to 0.785 µ mol/g in RRF-127 (Table 4.7), while the mean MDA content under stress conditions increased to 0.922 µ mol/g fresh weight and ranged from 0.265 µ mol/g in Annada to 1.684 µ mol/g in GP-145-40 (Table 4.8). When comparing the fold increase of MDA content in stress over control, the highest fold increase in MDA content was recorded in the genotype DagadDeshi (6.6 fold) followed by Annada (5.9 fold), Nagina-22 (3.9 fold), CGZR-1 (3.4 fold) and GP-145-103 (3.2 fold) as shown in figure 4.9.

Table 4.6: MDA content of fourteen rice genotypes under control and stress conditions.

S.No	Rice Genotype	MDA (nmole / g) control	MDA (nmole / g) stress	fold increase
1	GP-145-103	0.59	1.23	2.1
2	SL-62	0.22	0.59	2.7
3	DagadDeshi	0.24	1.56	6.6
4	Nagina 22	0.37	0.51	1.4
5	Swarna	0.42	1.62	3.9
6	GP-145-55	0.10	0.33	3.4
7	CGZR-1	0.28	0.43	1.5
8	Annada	0.15	0.27	1.8
9	Pornima	0.29	0.95	3.2
10	Karma mahsuri	0.30	0.75	2.5
11	ARB-6-11	0.41	1.27	3.1
12	GP-145-40	0.64	1.68	2.6
13	MTU 1010	0.24	1.43	5.9
14	RRF-127	0.79	0.90	1.1

Increased levels of MDA content was observed in heat stress conditions. MDA content in cells is said to be inversely related to anti-oxidant enzymes because, as the anti-oxidant activity increases, oxidative stress causing lipid peroxidation decreases, and results in decreased MDA content. Studies reported that MDA content is relatively more in heat susceptible varieties under stress conditions due to increased lipid peroxidation (Kazim 2013; Wilson *et al.*, 2014; Zafar *et al.*, 2017). The heat tolerant genotypes recorded relatively low MDA content under stress than susceptible ones. In this present study the lowest amount MDA content under stress was recorded in RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mahsuri (2.5 fold) shown graphically in figure 4.9.

The comparison between all the phenotypic and biochemical characterizations of fourteen rice genotypes under stress conditions when compared with control i.e fold increase and fold decrease are shown in the figure 4.10.

Table 4.7: Mean and Range for all biochemical traits of fourteen rice genotypes unde control conditions.

	Chlorophyll a Control (mg/g)	Chlorophyll b Control (mg/g)	Total Chlorophyll Control (mg/g)	Proline Control (µ mol/g f.wt)	MDA control (µmols / g f.wt)
Genotype	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**	**Mean±S.E.**
GP-145-103	2.710±0.000	2.310±0.006	5.023±0.004	0.064±0.000	0.59±0.008
SL-62	2.993±0.003	2.150±0.000	4.833±0.003	0.064±0.000	0.219±0.008
DagadDeshi	2.400±0.000	1.290±0.000	3.690±0.001	0.093±0.000	0.237±0.015
Nagina 22	2.547±0.003	1.817±0.003	4.360±0.000	0.068±0.001	0.366±0.016
Swarna	1.720±0.000	1.047±0.003	2.763±0.003	0.265±0.004	0.419±0.006
GP-145-55	3.000±0.000	2.820±0.000	5.820±0.000	1.001±0.000	0.097±0.000
CGZR-1	3.410±0.000	1.440±0.010	4.847±0.009	0.173±0.004	0.284±0.008
Annada	2.693±0.003	0.520±0.000	3.317±0.003	0.182±0.002	0.150±0.009
Pornima	3.090±0.000	1.060±0.000	4.147±0.003	0.374±0.000	0.293±0.004
Karma mahsuri	2.540±0.000	1.930±0.000	4.470±0.000	0.067±0.001	0.301±0.012
ARB-6-11	2.700±0.000	0.493±0.003	3.190±0.001	0.977±0.001	0.411±0.006
GP-145-40	1.900±0.000	0.370±0.000	2.070±0.000	1.650±0.006	0.639±0.006
MTU 1010	1.610±0.000	0.413±0.003	2.023±0.003	0.519±0.001	0.243±0.008
RRF-127	2.600±0.000	0.480±0.000	3.077±0.003	0.111±0.000	0.785±0.004
Mean	2.529	1.303	3.831	0.407	0.372
Minimum	1.610	0.370	2.023	0.064	0.097
Maximum	3.410	2.820	5.820	1.65	0.785
CD (*p*=0.05)	0.004	0.01	0.01	0.007	0.026
SE(m)±	0.002	0.004	0.003	0.002	0.009
SE(d)±	0.002	0.005	0.005	0.003	0.013
C.V.	0.106	0.473	0.151	0.995	4.134

Table 4.8: Mean and Range for all biochemical traits of fourteen rice genotypes under stress conditions.

Genotype	Chlorophyll a Stress (mg/g) Mean±S.E.	Chlorophyll b Stress (mg/g) Mean±S.E.	Total Chlorophyll Stress (mg/g) Mean±S.E.	Proline Stress (µ mol/g f.wt) Mean±S.E.	MDA stress (µmols / g f.wt) Mean±S.E.
GP-145-103	2.173±0.003	2.300±0.000	4.970±0.000	0.447±0.008	1.128±0.004
SL-62	2.340±0.000	1.290±0.000	3.630±0.000	0.095±0.003	0.585±0.027
DagadDeshi	1.720±0.000	0.837±0.015	2.560±0.012	0.380±0.003	1.563±0.006
Nagina 22	2.550±0.000	1.240±0.000	3.790±0.000	1.402±0.000	0.512±0.009
Swarna	0.523±0.003	0.323±0.003	0.853±0.003	1.567±0.000	1.617±0.002
GP-145-55	1.140±0.000	0.620±0.000	1.760±0.000	2.044±0.001	0.326±0.000
CGZR-1	2.743±0.003	1.107±0.003	3.890±0.000	1.975±0.001	0.432±0.004
Annada	2.680±0.000	0.051±0.000	3.210±0.000	1.903±0.000	0.265±0.002
Pornima	1.760±0.000	0.410±0.000	2.163±0.003	1.906±0.001	0.945±0.006
Karma mahsuri	2.547±0.003	1.800±0.000	4.343±0.003	0.404±0.000	0.748±0.004
ARB-6-11	2.203±0.003	0.480±0.000	2.687±0.003	1.941±0.001	1.271±0.006
GP-145-40	1.290±0.000	0.340±0.000	1.930±0.000	1.667±0.006	1.684±0.004
MTU 1010	1.540±0.000	0.340±0.000	1.623±0.003	0.921±0.000	1.430±0.007
RRF-127	0.933±0.003	0.203±0.003	1.133±0.003	0.246±0.001	0.901±0.004
Mean	1.874	0.873	2.747	1.213	0.922
Minimum	0.523	0.203	0.853	0.095	0.265
Maximum	2.743	2.300	4.970	2.044	1.684
CD (*p*=0.05)	0.006	0.012	0.011	0.009	0.025
SE(m)±	0.002	0.004	0.004	0.003	0.009
SE(d)±	0.003	0.006	0.005	0.004	0.012
C.V.	0.202	0.83	0.238	0.435	1.634

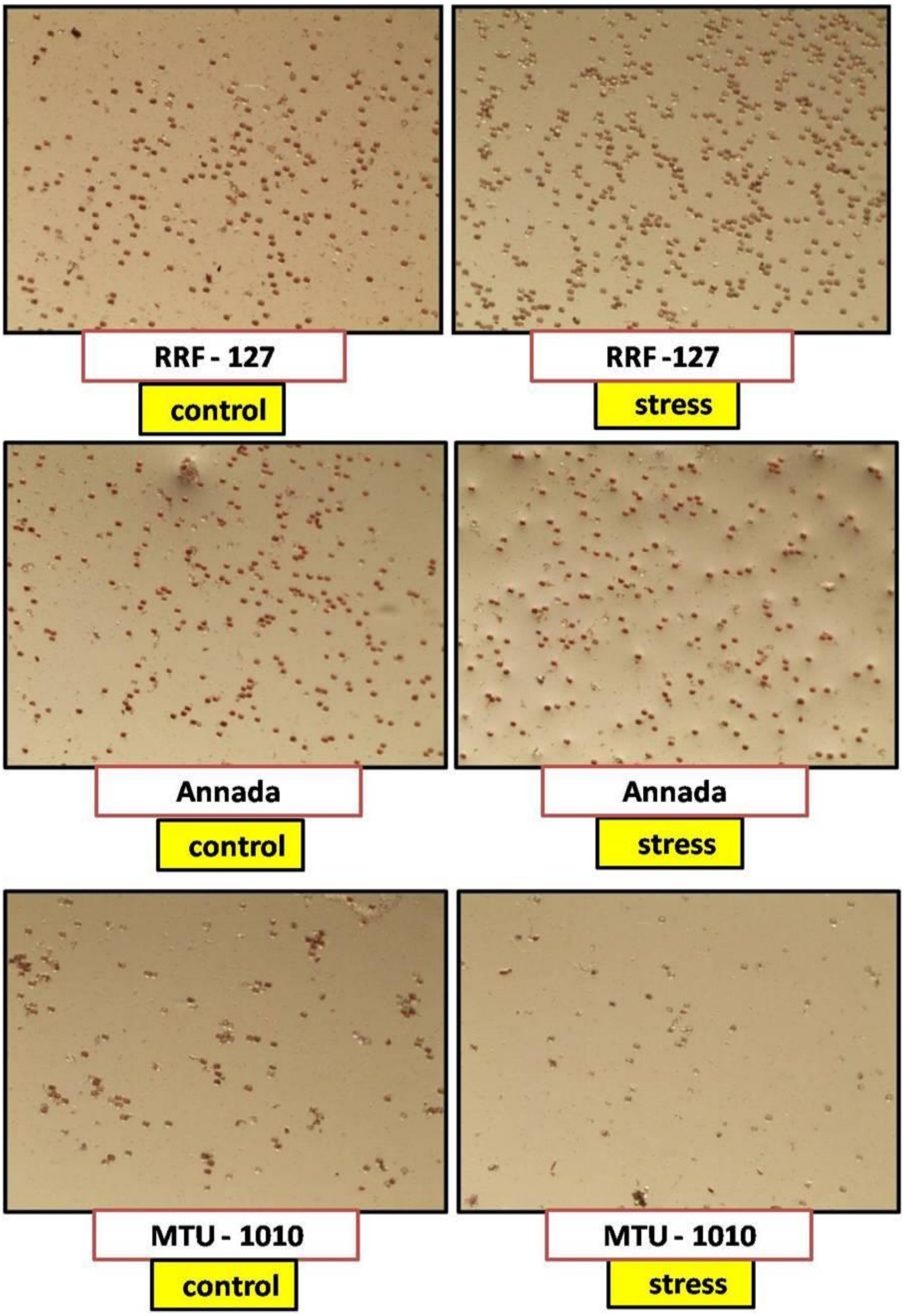


Fig 4.4: Fertile (red/stained) pollen and sterile (transparent/unstained) pollen in rice genotypes under control and stress conditions.

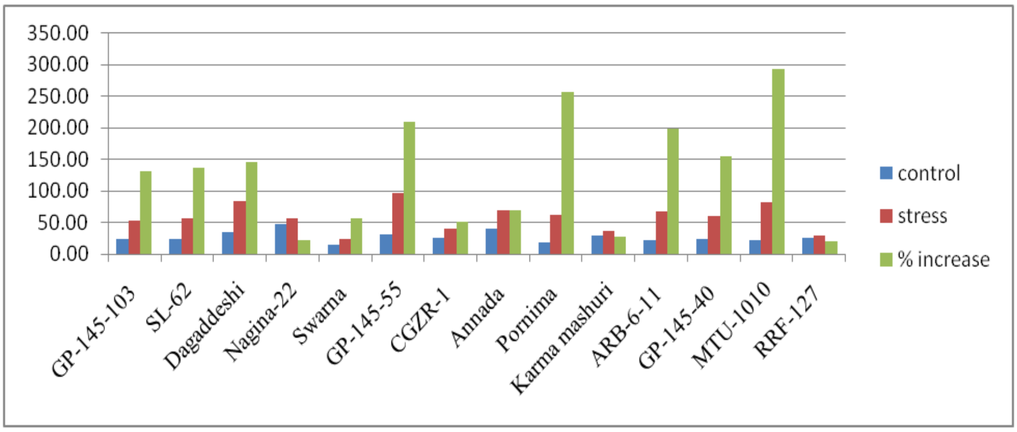


Fig 4.3: Membrane Stability Index (MSI) of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

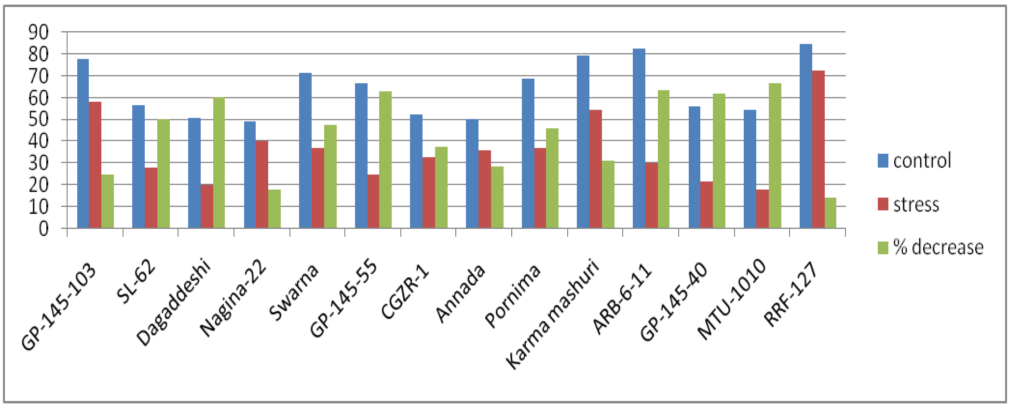


Fig 4.5: Pollen fertility of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

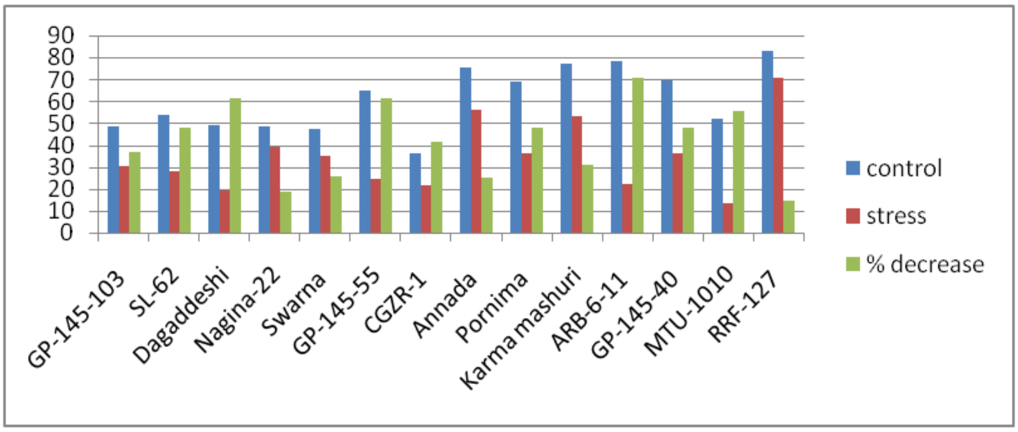


Fig 4.6: Spikelet fertility of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

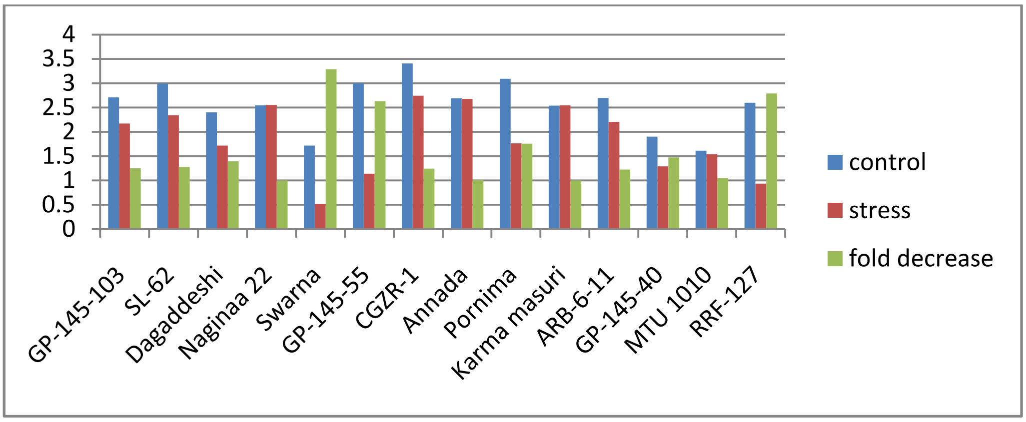


Fig 4.7a: Chlorophyll –a content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

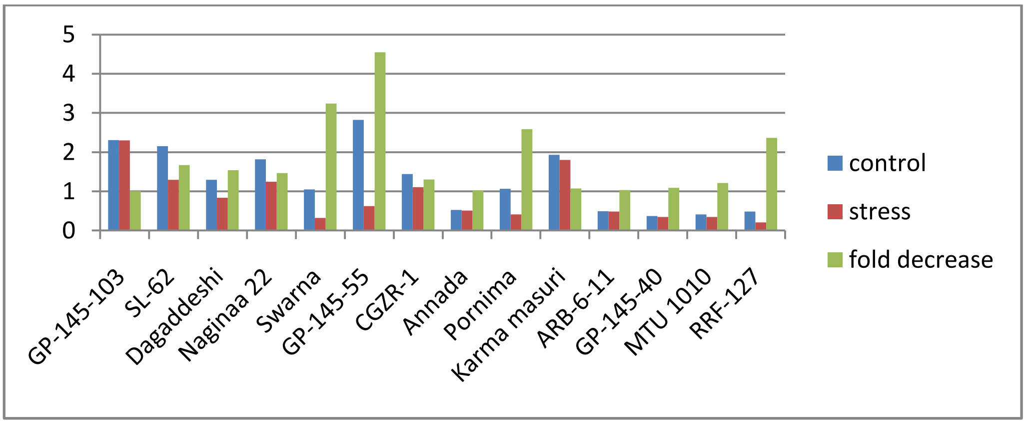


Fig 4.7b: Chlorophyll –b content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

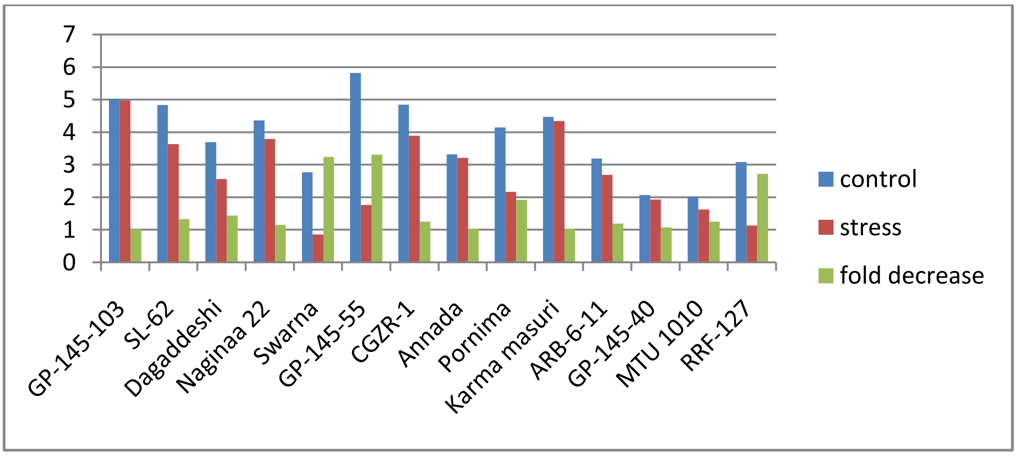


Fig 4.7c: Total Chlorophyll content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

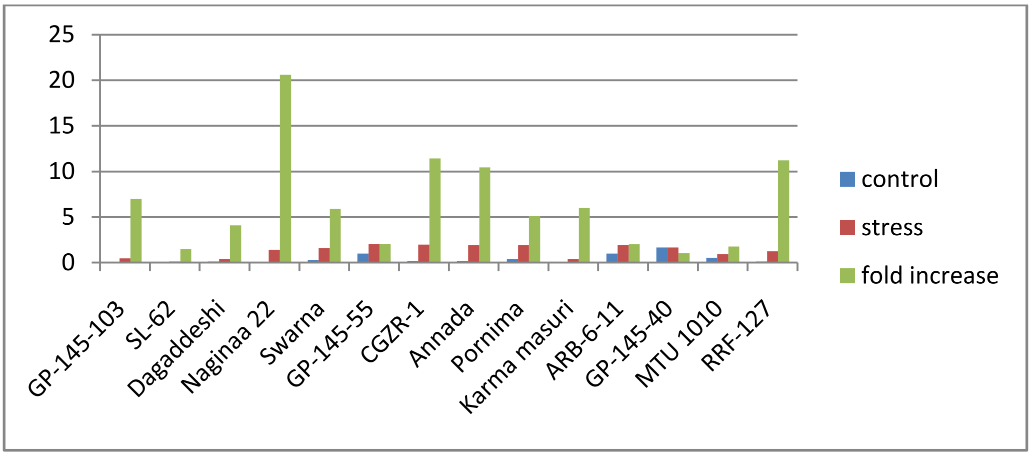


Fig 4.8: Proline content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

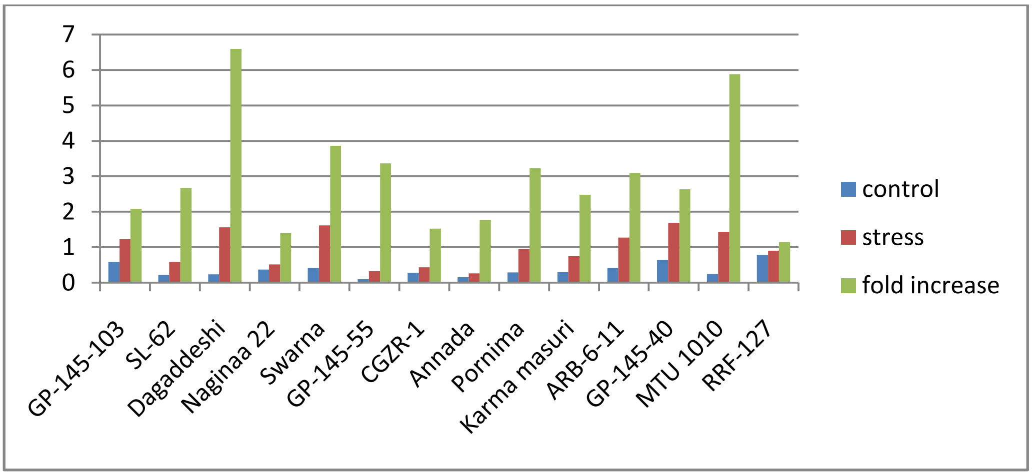


Fig 4.9: MDA content of fourteen rice genotypes under control and stress conditions at vegetative stage before panicle initiation

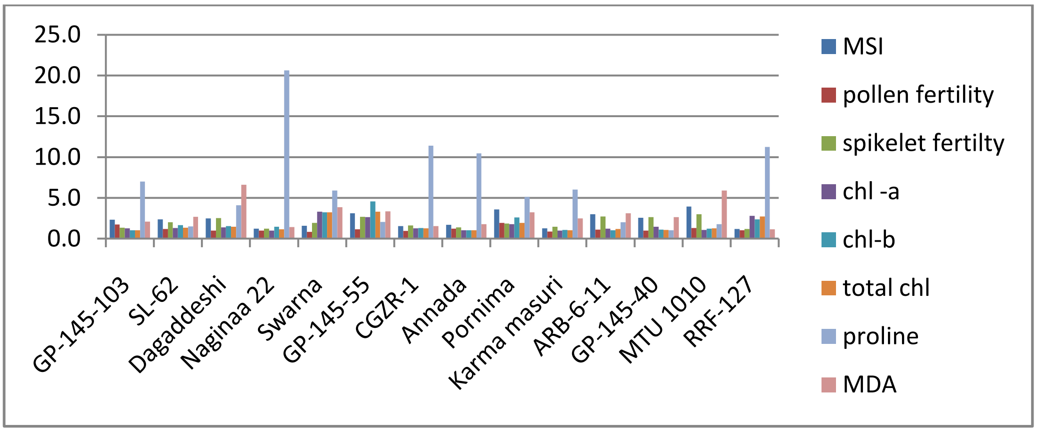


Fig 4.10: Overall Fold Increase or Decrease in all phenotypic and biochemical characters of fourteen rice genotypes under stress conditions at vegetative stage before panicle initiation.

4.2. Expression analysis of heat responsive genes using semi-quantitative RT-PCR

As mentioned earlier, the fourteen rice genotypes were grown both under control and stress conditions. The stress treatment of 42°C was given at day time continuously for 6 days by maintaining the temperatures in green house, at vegetative stage before panicle initiation. After 6 days of stress treatment, the leaf samples were harvested from both control and stress plants in liquid nitrogen and the they are preserved in 80 °C for RNA isolation. RNA should be extracted immediately after the sample collection as RNA degrades very fast. Isolation of total RNA was done using TRIzol (Invitrogen, USA) manufacturer's protocol including some minor modifications. The concentration of the isolated RNA was quantified using Nanodrop spectrophotometer ND-1000 ® (NanoDrop Technologies, USA). From this isolated RNA , synthesis of cDNA was done using BIORAD iScriptTM cDNA Synthesis Kit as per manufacturer's instruction. Then the expression pattern of five different genes was analysed using Semi quantitative RT-PCR. These genes include OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon (Table 3.6).

4.2.1. Standardization of RT-PCR parameters for expression profiling of heat responsive genes

Considering the concentration of RNA, assessed by Nanodrop ND1000 spectrophotometer, initially samples were diluted to 1500ng/µl for normalization. After cDNA synthesis, normalization was checked by using Os-ACTIN gene (house-keeping gene) as internal control. Further relative quantification of selected 5 genes was done by conducting semi-quantitative RT-PCR analysis by using gene specific primers on by comparing the florescence intensities of individual bands observed in each well. NRCPB, New Delhi provides the primer sequences. The melting temperature, template DNA and other parameters related to RT-PCR, of selected genes were standardized for expression analysis. The first-strand cDNA was obtained from 1 µg of total RNA in a 20 µl reaction mixture, and 20 ng/µl of synthesized cDNA was used as template for PCR reaction (94°C for 2 min, 15 s at 94°C, 30 s at 60-64°C, and 45 s at 72°C, then 72°C for 7 min followed by 4°C).

4.2.2. Semi-quantitative expression profiling of heat stress responsive genes in rice genotypes under heat stress

Gene expression analysis by semi-quantitative RT-PCR was undertaken to calculate the transcript abundance differences among both control and stress (high temperatures) conditions in genotypes. In the present study, the semi-quantitative RT-PCR analysis of five heat stress responsive genes was performed for all the fourteen genotypes (-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) under stress and control conditions at vegetative stage before panicle initiation. The differential expression of five heat stress responsive genes (OsHSP 26.7, OsHSP 16.9, OsHSP 18, OsHSP DnaJ and 60kDa chaperon) in all the fourteen rice genotypes under both control and stress conditions was indicated in the figure 4.16. The genotype specific fold increase and decrease of heat stress responsive genes in rice genotypes under stress as compared with control conditions are given in table 4.9. The results are discussed below in detail.

Note: The increase or decrease in the Fold value was calculated by measuring the band intensity and size using GelQuantNET software.

In this study, semi quantitative RT-PCR of OsHSP 26.7 gene has shown up-regulation under heat stress conditions in all the rice genotypes. Table 4.9 shows the fold increase in all the fourteen rice genotypes. The rice genotype RRF-127 showed the highest up-regulation of 14.3 fold increase followed by Annada (13.9 fold), Karma mahsuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold) as shown in figure 4.11. Previous studies reported that OsHSP 26.7 gene belongs to sHSP (small heat shock proteins) class of genes and showed strong up-regulation under heat stress conditions in comparison to control (Chandel *et al.*, 2013).

Semi quantitative RT-PCR of OsHSP 16.9 gene, in the present investigation has shown up-regulation in almost all rice genotypes under heat stress conditions. Table 4.9 shows fold increase in all genotypes except for DagadDeshi. The rice genotype RRF-127 showed the highest up-regulation of 10.0

fold increase followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mahsuri (3.1 fold) as shown in figure 4.12. Its been reported that OsHSP 16.9 gene belongs to sHSP (small heat shock proteins) class of genes and showed slight up-regulation under heat stress conditions when compared with control (Chandel *et al.,* 2013).

In this study, semi quantitative RT-PCR of OsHSP DnaJ gene, has shown almost the same expression under control and stress conditions and no convincing difference was observed in heat stress. Table 4.10 shows the quantified expression of the gene under both and stress conditions. Consistent expression was seen in the genotypes like GP-145-103, DagadDeshi, GP-145-55, CGZR-1, and Annada as shown in figure 4.13. As per the reports of previous work done, the gene OsHSP DnaJ also showed no significant change in the expression under control and stress (Chandel *et al.,* 2013) and this gene also belongs to sHSP (small heat shock proteins) family.

OsHSP 18 gene expression was also studied in the present investigation using semi quantitative RT-PCR and it showed up-regulation in almost all genotypes of rice under heat stress. Table 4.9 shows fold increase in all genotypes of rice. The rice genotype GP-145-103 has shown highest up-regulation of 17.7 fold increase followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and Poornima (8.9 fold) as shown in the figure 4.14. This gene also belongs to the family sHSP (small heat shock proteins) class of genes and showed up-regulation under heat stress conditions (Chandel *et al.,* 2013).

In the present study, semi quantitative RT-PCR of 60Kda chaperon gene was studied and it has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. Table 4.9 shows fold increase in all the genotypes of rice. The rice genotype Annada has shown highest up-regulation of 1.9 fold increase followed by CGZR-1 (1.5 fold), Nagina -22 (1.4 fold), GP-145-103 (1.3 fold) and Karma mahsuri (1.3 fold) as shown in figure 4.15. 60Kda chaperon was also reported to be up-regulated by minimal levels under heat stress but it is lately induced under stress (Chandel *et al.,* 2013).

Heat stress triggers few defence mechanisms like obvious gene expression, which results in the increased synthesis of particular protein groups called "heat shock proteins (HSPs)". Generally HSPs are classified into 5 evolutionarily conserved families based on their molecular weight, which include HSP 60, 70, 90, 100 and small HSPs (Krishna 2003). As the present investigation studied the expression of five different genes mentioned above, most of them belong to the small HSPs family. Brief explanation about the small HSPs and their mechanism is explained under 4.2.3.

Table 4.9: Fold increase of all up-regulated genes in rice genotypes under high temperature stress compared with control conditions

				Fold increase		
S.No	**Rice Genotypes**	**OsHSP 26.7**	**OsHSP 16.9**	**OsHSP 18**	**OsHSP DnaJ**	**60kDa chaperon**
1	GP-145-103	8.6	3.2	17.7	1.0	1.3
2	SL – 62	1.7	2.3	8.4	0.9	1.0
3	DagaDeshi	2.4	1.5	5.9	1.0	1.1
4	Nagina -22 (Check)	3.0	2.9	4.8	0.8	1.4
5	Swarna	3.6	1.4	3.3	0.9	1.2
6	GP – 145-55	3.3	1.3	11.9	1.0	1.2
7	CGZR -1	3.7	3.4	14.1	1.0	1.5
8	Annada	13.9	3.7	13.8	1.0	1.9
9	Poornima	1.6	1.3	8.9	0.9	1.2
10	Karma mahsuri	11.5	3.1	6.1	1.1	1.3
11	ARB – 6-11	3.0	1.7	1.2	0.9	1.2
12	GP -145 -40	2.4	1.3	4.8	0.7	1.2
13	MTU-1010	2.3	1.4	2.0	1.1	1.1
14	RRF-127	14.3	10.0	5.2	1.1	1.1

4.2.3 Small heat shock protein family (smHSPs)

Small heat shock proteins (sHSPs) are the largest ubiquitous HSP subgroup whose molecular weight ranges from 12 t0 42 KDa. "Small HSPs sequence analysis indicates that members belonging to this family includes evolutionarily divergent N-terminal part, which is followed by a conserved α-crystallin domain and a short C-terminal tail (de Jong *et al.,* 1993)". Along the evolutionary scale, sHSPs genes number increases where as bacteria had only one or two sHSPs and multi cellular organisms have large no.of sHSPs in their genome. Its been recognized that ten separate sHSPs families have been conserved in both monocots and dicot plants, which indicates the diversity for sHSPs mechanisms (Siddique *et al.,* 2008; Scharf *et al.,* 2001). Among the ten, sHSPs encoded by 4 families are localized to cytoplasm and other 6 families are localized to different cellular organelles like nucleus, chloroplasts, endoplasmic reticulum, mitochondria and peroxisomes (Basha *et al.*, 2010). In vivo studies reported that sHSPs function as molecular chaperons. Plants synthesize large amounts of sHSPs when they are exposed to high temperature suggesting that they play a major role for enduring thermo-tolerance in plants (Charng *et al.,* 2006). Higher plants have at least 20 sHSPs and chances of presence of 40 kinds of these sHSPs in one plant species has been reported (Vierling 1991). Some regulatory proteins called heat stress transcription factors (Hsfs) control the transcription of heat shock protein genes. Plants have at least 21 Hsfs, each one having its own specific role in regulation. They also cooperate in all phases of responses due to periodical stress like triggering, maintenance and recovery. "The smHsps cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, preventing irreversible unfolding or wrong protein aggregation (Mogk, *et al.,* 2003)". "Recent findings showed that the smHSPs 18.1 isolated from *Pisum sativum*, as well as the sHsps 16.6 from *Synechocystis* sp. PCC6803 under *in vitro* conditions, binds to unfolded proteins and allows further refolding by HSP70/HSP100 complexes (Mogk *et al.*, 2003)". A very strong positive qualitative relation was noticed between the thermo-tolerance and accumulation of sHSPs in plastids.

"The current model for sHSP chaperone activity was defined based on studies of a cytosolic sHSP family named as Class I sHSPs (sHSP-CI), which represent the most abundant sHSP in plants (Guan *et al.*, 2004)". According to this model, a large homo-oligomer is formed by the assembly of sHSPs, which is binded to denatured proteins by ATP-independent fashion, and keeps them in folding competent state. Then this complex cooperates with molecular chaperons like HSP70 and HSP90, which are ATP-dependent to refold those denatured proteins. “Notably, sHSP has a much larger binding stoichiometry than other molecular chaperones, which has led to the speculation that sHSP functions as a reservoir to stabilize the flood of denatured proteins in response to stress (Lee *et al.,* 2000; Ehrnsperger *et al.,* 1997)”. “Its been proposed that major mechanism through which sHSPs expose normally inaccessible, hydrophobic client binding surfaces by heat-induced oligomer dissociation, Nevertheless, the details about the interactions between sHSP and non-native proteins and how these non-native proteins are subsequently refolded are still lacking. This is partially due to limited knowledge on the molecular structure of sHSPs (Haslbeck *et al.,* 2005)”. “Among the few solved crystallographic structures of sHSPs is a wheat TaHsp16.9- CI, the basic building block of wHSP16.9 is a dimer, which further assembles as a 12-mer consisting of two trimers of dimmers, in solution to this wHSP16.9 can dissociate into smaller oligomeric states in a temperature dependent manner (van Montfort *et al.,* 2001)”. Based on this mechanism it is observed that heat-induced sHSPs oligomers dissociation may expose the hydrophobic patches that were buried in the oligomeric interface, which leads to binding and stabilization of denatured proteins under heat stress and imparts thermo-tolerance.

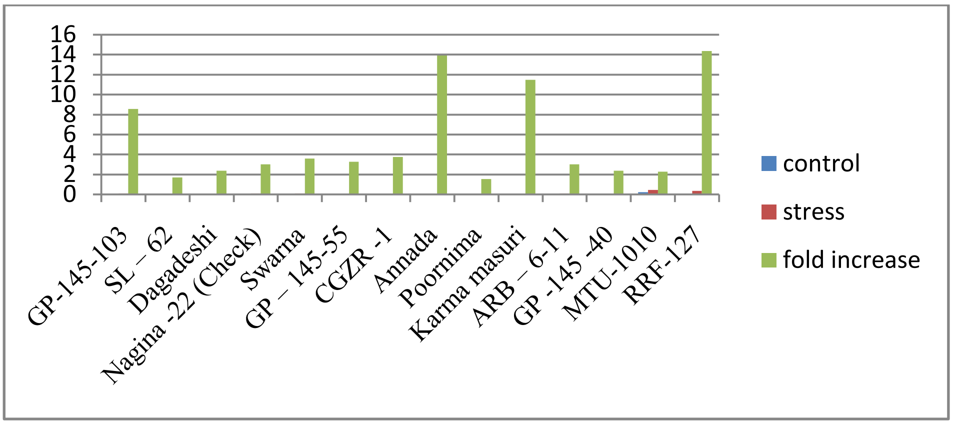


Fig 4.11: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP26.7 gene (strongly up-regulated)

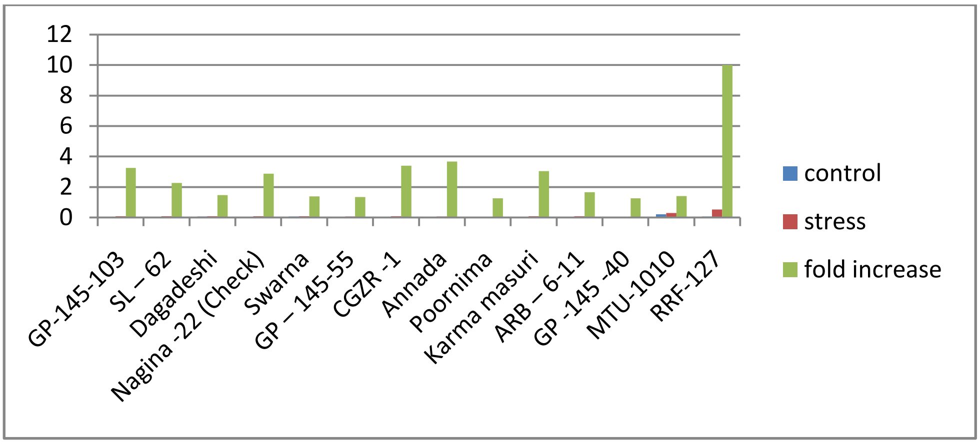


Fig 4.12: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP16.9 gene (up-regulated)

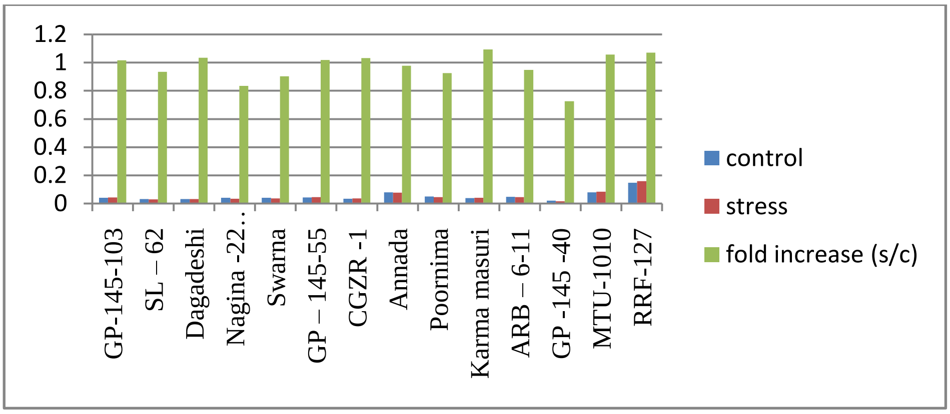


Fig 4.13: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP DnaJ gene (consistent expression)

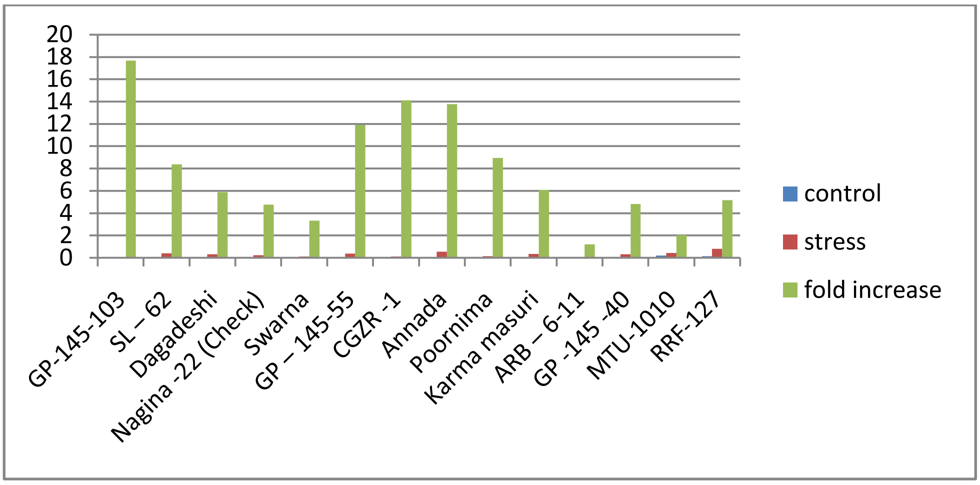


Fig 4.14: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on OsHSP18 gene (up-regulation

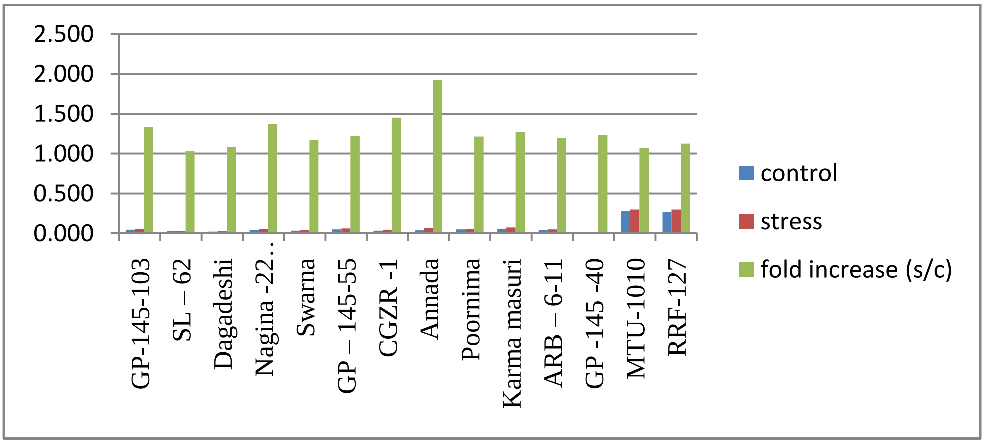


Fig 4.15: Effect of heat stress on the gene expression in fourteen rice genotypes at vegetative stage before panicle initiation on 60Kda chaperon gene (slight up-regulation)

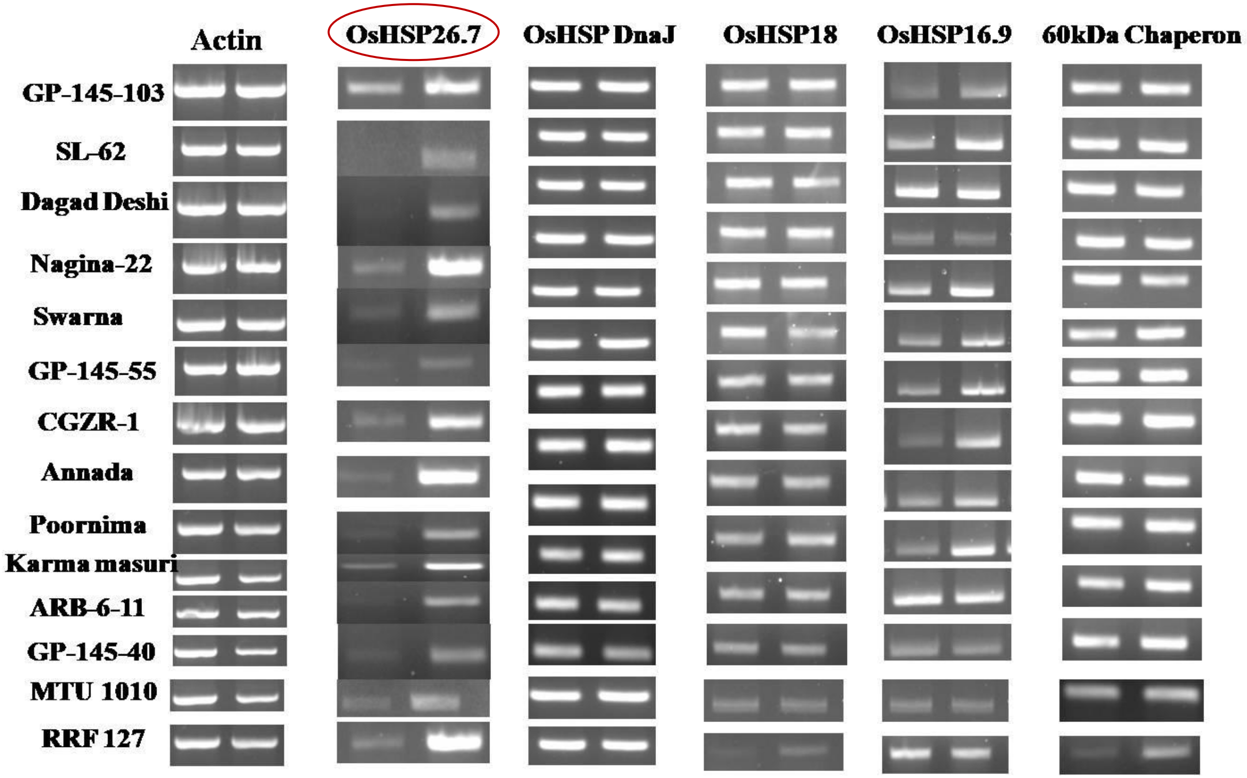


Fig 4.16: Semi quantitative RT-PCR Analysis of five heat responsive genes for fourteen rice genotypes (GP-145-103, SL-62, DagadDeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mahsuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) under control and stress condition

4.3 Correlation between Gene expression pattern and various phenological, biochemical traits

High temperature is a severe environmental stress and the major constraint on plant productivity with an obvious effect on plant growth. The aim of this work was to study the response of rice genotypes under high temperature stress at the phenological, biochemical and molecular levels. Characterization of rice genotypes contrasting in their response to high temperature stress was performed at the phenological and biochemical level by determination of MSI, Pollen fertility, Spikelet fertility, chlorophyll content, free proline content and MDA content under temperature stress condition. Pollen fertility and Spikelet fertility were positively correlated and MSI was negatively correlated with the yield per plant which was significant. The remaining traits showed non-significant correlation with yield per plant. In this study rice genotypes RRF-127 and Nagina-22 showed minimum decrease in MSI, pollen fertility and spikelet fertility. Chlorophyll-a degradation was positively correlated with chlorophyll-b and total chlorophyll content and it was negatively correlated with MDA levels and was significant, which correctly indicated the increase in MDA levels under high temperatures with decreased chlorophyll content. Chlorophyll-b degradation was positively correlated with chlorophyll-a, total chlorophyll content and it was negatively correlated with proline content and was significant, which indicated the increase in proline accumulation under high temperature with decreased chlorophyll content.

The expression level of five high temperature responsive genes (OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon) was also analyzed by reverse transcription–polymerase chain reaction (RT-PCR). The result revealed that the all genes are up regulated under high temperature stress treatment in rice genotypes expect OsHSP DnaJ which showed consistent expression both under control and stress conditions. Very prominent result was observed for OsHSP26.7 gene in all rice genotype under high temperature, and the genotypes RRF-127 and Annada which were screened as heat stress tolerance genotype on the basis of biochemical and phonological studies carried out in this work showed the highest fold increase. Positive correlation was found among the expression of OsHSP26.7, OsHSP16.9, 60kDa chaperon and it was significant. Rice genotype RRF-127 showed a higher level of up regulation for gene OsHSP26.7 (14.3 fold), followed by OsHSP16.9 (10.0 fold). Annada showed higher level of up-regulation for gene OsHSP26.7 (13.9 fold), followed by OsHSP16.9 (3.7 fold) and 60kDa chaperon (1.9 fold). Based on the expression pattern all five genes (OsHSP26.7, OsHSP16.9, OsHSP18, OsHSP DnaJ and 60kDa chaperon) under study were identified as positively up regulated under high temperature in rice genotypes. These genes are not expressed in the control plants, but only in the stressed plants. This result was used as a basis to select for the gene OsHSP26.7 as the most responsive gene under high temperature and genotypes RRF-127 and Annada as the heat-tolerant genotypes on the basis of study at phenological, biochemical and molecular levels. The table 4.10 given below shows the correlation among all the phenological, biochemical traits and the gene expression

Table 4.10: Correlation between various phenological and biochemical traits.

	MSI	Pollen fertility	spikelet fertility	chl a	chl b	chl total	proline	MDA	Yield/plant
MSI	1								
Pollen fertility	-0.503NS	1							
spikelet fertility	-0.499NS	0.999**	1						
chl a	0.054NS	0.076NS	0.048NS	1					
chl b	-0.142NS	0.173NS	0.157NS	0.653*	1				
chl total	-0.079NS	0.130NS	0.108NS	0.867**	0.931**	1			
proline	0.095NS	0.104NS	0.099NS	-0.147NS	-0.552*	-0.394NS	1		
MDA	-0.054NS	-0.344NS	-0.340NS	-0.543*	-0.305NS	-0.397NS	-0.215NS	1	
Yield/plant	-0.640*	0.642*	0.643*	0.202NS	0.016NS	0.101NS	0.072NS	-0.396NS	1

* (significance at .05), ** (significance at .01) NS (non-significant)

CHAPTER – V
SUMMARY AND CONCLUSIONS

All abiotic stress conditions like drought, salinity, cold and high temperatures effects the agricultural production as they hinder the expression of genes to their full potential. Among them heat stress is the major constraint in the present days, due to increasing earth's surface temperature as a result of global warming. Hence its necessary to produce crops with higher heat stress tolerance to combat yield loss. The first step in generating the tolerant crops is to understand the complete mechanisms involved in response to high temperatures in plants. Research done at the level of genes (Genomics), proteins (Proteomics) and metabolites (Metabolomics) is fundamental understanding the plant responses to high temperatures. Identification of key regulators through gene expression patterns related to stress interactions is the important aspect of using functional genomics tools. So to study the effect of heat stress on the expression of genes that respond to high temperatures, that lead to heat-tolerance, the present investigation was conducted with two major objectives:

1. **Phenological and Biochemical characterization of rice genotypes under heat stress.**
   - Genotypes RRF-127 , Nagina-22, Karma mashuri, CGZR-1 and Annada had recorded lower electrolyte leakage i.e membrane stability index under stress when compared with control as 19.8%, 21.1%, 27.5%, 51.7% and 69.8% respectively.
   - Lowest decrease in pollen fertility under stress conditions when compared to control was observed in the genotypes RRF-127, Nagina-22, GP-145-103, Annada and CGZR-1 as 14.4%, 18.1%, 24.9%, 28.3% and 37.9% respectively.
   - Spikelet fertility decrease under stress conditions when compared to control was found to be lowest in the genotypes RRF-127, Nagina-22, Annada, Karma mashuri and CGZR-1 as 14%, 19%, 25%, 31% and 42% respectively.

- When comparing the fold increase in proline content for stress over control among all the fourteen genotypes, Nagina-22 showed highest fold increase of 20.6 folds followed by CGZR-1 (11.4 fold), RRF-127 (11.2 fold), Annada (10.5 fold), GP-145-103 (7.0 fold) and Karma mashuri (6.0 fold).
- In chlorophyll content, Annada has the lowest fold decrease of 1.0 folds for all chla, chlb and total chlorophyll content followed by Karma mashuri (1.0 fold) under stress when compared with control
- When comparing the fold increase in MDA content for stress over control among all fourteen genotypes, the lowest fold increase in MDA content was shown by RRF-127 with 1.1 fold increase followed by Nagina-22 (1.4 fold), CGZR-1 (1.5 fold), Annada (1.8 fold), GP-145-103 (2.1 fold) and Karma mashuri (2.5 fold).

1. **Semi quantitative expression analysis of candidate genes responsible for heat stress among different rice genotypes.**

Wet lab conformation using RT-PCR analysis for the expression pattern of five selected genes showed a genotype specific banding pattern under control and heat stress conditions. A significant level of up-regulation was observed in all the five genotypes under stress as these belong to small HSPs family, whose genes become responsive in all abiotic stress conditions.

- OsHSP 26.7 gene has shown strong up-regulation under heat stress conditions in all the rice genotypes. The rice genotype RRF-127 showed the highest up-regulation of 14.3 fold increase followed by Annada (13.9 fold), Karma mashuri (11.5 fold), GP-145-103 (8.6 fold) and CGZR-1 (3.7 fold).
- OsHSP 16.9 gene has shown up-regulation in almost all rice genotypes under heat stress conditions. The rice genotype RRF-127 showed the highest up-regulation of 10.0 fold increase followed by Annada (3.7 fold), CGZR-1 (3.4 fold), GP-145-103 (3.2 fold) and Karma mashuri (3.1 fold).

- OsHSP DnaJ gene, has shown almost the same expression under control and stress conditions and no convincing difference was observed in heat stress.
- OsHSP 18 gene showed up-regulation in almost all genotypes of rice under heat stress. The rice genotype GP-145-103 has shown highest up-regulation of 17.7 fold increase followed by CGZR-1 (14.1 fold), Annada (13.8 fold), GP-145-55 (11.9 fold) and poornima (8.9 fold).
- 60Kda chaperon gene has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. The rice genotype Annada has shown highest up-regulation of 1.9 fold increase followed by CGZR-1 (1.5 fold), Nagina -22 (1.4 fold), GP-145-103 (1.3 fold) and Karma mashuri (1.3 fold).

CONCLUSIONS

- one of the effective biochemical changes that takes place during heat stress condition is the over accumulation of proline, which acts as osmo-protectant. The membrane stability index (MSI), chlorophyll content (chla-a, chl-b and total chl) of leaves in all the genotypes of rice decreased remarkably when subjected to high temperatures. A significant increase in the levels of proline and MDA content was observed under high temperatures, revealing that accumulation of proline and MDA content takes place during stress conditions.
- High temperature stress imposed at late vegetative stage before panicle initiation, reduced the pollen fertility and spikelet fertility in all rice genotypes revealing the impaired pollen development and fertilization under stress.
- A positive correlation was recorded for yield per plant and pollen fertility and spikelet fertility under stress and it was significant, where as yield is negatively correlated with Membrane stability index (MSI).
- Phenological and biochemical studies (MSI, pollen fertility, spikelet fertility, chlorophyll content, proline and MDA content) on rice genotypes resulted in identifying some of the tolerant and susceptible genotypes for

heat stress. Among all the fourteen genotypes selected for the study, RRF-127, Annada, Karma mashuri, CGZR-1 and GP-145-103 showed a positive regulation towards heat stress and genotypes like MTU-1010, ARB-6-11 and GP-145-55 showed negative regulation towards heat stress.

- There are a number of genes involved in heat stress mechanisms that regulate the expression of genome in response to environment and physiological signals under high temperature conditions. In wet lab conformation using RT-PCR analysis of 5 genes belonging to the family of small HSPs (OsHSP 26.7, OsHSP 16.9, OsHSP DnaJ, OsHSP 18 and 60Kda chaperon), there was a correlation in between phonological and biochemical traits with the genotypes (RRF-127, Annada, Karma mashuri and CGZR-1) which had shown a significant level of up-regulation among all the genes under stress conditions.
- OsHSP26.7 gene is strongly up-regulated and it is recorded as one of the most responsive genes under high temperature stress.
- Genotypes RRF-127 and Annada showed a positive regulation for almost all the phonological and biochemical traits which are positively correlated along with significant up-regulation in the expression analysis of all the five genes. By this we can say that, these genotypes are tolerant towards high temperatures.

SUGGESTIONS FOR FUTURE RESEARCH WORK

- The study delineates and employs the strategy for discovering useful genes responsive to heat stress in rice genotypes. Same can be carried out for the identification of other key genes responsible for heat tolerance in rice as well as other food crops. The same strategy can also be employed for other agricultural important crops.
- High temperature these days are turning out to be one of the serious challenges for crop production. There are many stress related genes that are expressed under high temperatures and today there is an immense need for examining and analyzing stress related genes, their products and mechanisms under multiple environmental conditions.

- There is a great need to understand the signal transduction pathways role along with post-transcriptional and translational regulatory mechanisms in both biotic and abiotic stress responses to examine and understand the function of various stress related genes in order to improve stress tolerance towards heat in rice as well as other crops.
- Identification of genes and mechanisms of general stress processes as well as stress-specific responses and identifying the function of genes whose functions are currently unknown will help in determining their role in stress tolerance.
- Hundreds of heat specific transcription factors respond to heat stress, understanding the function of these Hsfs will help to unravel the mechanisms of heat stress tolerance.
- More attempts should be made for the validation of genes for abiotic stresses (salinity, drought, cold, nutrients) with genotypes of diverse genetic background in order to identify the key genes responsible for different abiotic traits.
- Genes that are significantly up and down regulated in heat tolerant cultivars when compared to a susceptible cultivar can serve as a candidate genes for heat stress tolerance.
- Genes which are screened from the expression analysis studies of rice could be used for narrow down process and transcriptome sequencing to measure the transcript abundance at varying heat stress levels.

REFERENCES

Agarie, S., Hanaoka, N., Kubota, F., Agata, W. and Kaufman, P.B., 1995. Measurement of cell membrane stability evaluated by electrolyte leakage as a drought and heat tolerance test in rice (Oryza sativa L.). *Journal of the Faculty of Agriculture, Kyushu University*, *40*(1), pp.233-240.

Agarwal, M., Sahi, C., Katiyar-Agarwal, S., Agarwal, S., Young, T., Gallie, D.R., Sharma, V.M., Ganesan, K. and Grover, A., 2003. Molecular characterization of rice hsp101: complementation of yeast hsp104 mutation by disaggregation of protein granules and differential expression in indica and japonica rice types. *Plant molecular biology*, *51*(4), pp.543-553.

Ahmed, J.U. and Hassan, M.A., 2011. Evaluation of seedling proline content of wheat genotypes in relation to heat tolerance. *Bangladesh Journal of Botany*, *40*(1), pp.17-22.

Alisoltani, A., Shiran, B., Sarvestani, N.R., Fallahi, H., Feto, N.A. and Ebrahimie, E., 2018. Changes in Microsatellite Motifs in Response to Abiotic Stresses: a Case Study Using Wheat and Rice RNA-sequencing Data. *Asian Journal of Scientific Research*, *11*, pp.12-21.

Arnon, D.I., 1949. Copper enzyme polyphenoloxides in isolated chloroplast in Beta Vulgaris Plant physiology, 24: 1–15.

Ashraf, M.Y., Azmi, A.R., Khan, A.H. and Ala, S.A., 1994. Effect of water stress on total phenols, peroxidase activity and chlorophyll content in wheat [Triticum aestivum L.]. *Acta physiologiae plantarum*, *16*(3).

Bahuguna RN, Jagadish KS (2015) Temperature regulation of plant phenological development. Environ Exp Bot 111:83–90

Bahuguna RN, Pal JJ, Shah D, Lawas LM, Khetarpal S, Jagadish KS (2015) Physiological and biochemical characterization of NERICA rice: a novel source of heat tolerance at the vegetative and reproductive stages in rice. Physiol Plant 154(4):543–559

Barakat MM, Al-Doss AA, Elshafei AA, Moustafa KA (2011) Identification of new microsatellite marker linked to the grain filling rate as indicator for heat tolerance genes in F2 wheat population. AJCS 5(2):104–110. ISSN:1835-2707

Basha, E., Jones, C., Wysocki, V. and Vierling, E., 2010. Mechanistic differences between two conserved classes of small heat shock proteins found in the plant cytosol. *Journal of Biological Chemistry*, *285*(15), pp.11489-11497.

Bates, L., Waldren, R.P. and Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. Plant and Soil, 39: 205–207.

Blum, A. and Ebercon, A., 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat 1. *Crop Science*, *21*(1), pp.43-47

Borriboon, W., Lontom, W., Pongdontri, P., Theerakulpisut, P. and Dongsansuk, A., 2018. Effects of Short-and Long-Term Temperature on Seed Germination, Oxidative Stress and Membrane Stability of Three Rice Cultivars (Dular, KDML105 and Riceberry). *Pertanika Journal of Tropical Agricultural Science*, *41*(1).

Cao, Y., Zhang, Q., Chen, Y., Zhao, H., Lang, Y., Yu, C. and Yang, J., 2013. Identification of differential expression genes in leaves of rice (Oryza sativa L.) in response to heat stress by cDNA-AFLP analysis. *BioMed research international*, *2013*.

Ceccarelli, S., Grando, S., Maatougui, M., Michael, M., Slash, M., Haghparast, R., Rahmanian, M., Taheri, A., Al-Yassin, A., Benbelkacem, A. and Labdi, M., 2010. Plant breeding and climate changes. *The Journal of Agricultural Science*, *148*(6), pp.627-637.

Chandel, G., Dubey, M. and Meena, R., 2013. Differential expression of heat shock proteins and heat stress transcription factor genes in rice exposed to different levels of heat stress. *Journal of plant biochemistry and biotechnology*, *22*(3), pp.277-285.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Hsu, F.C. and Ko, S.S., 2006. Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. *Plant Physiology*, *140*(4), pp.1297-1305.

Chen, C., Begcy, K., Liu, K., Folsom, J.J., Wang, Z., Zhang, C. and Walia, H., 2016. Heat stress yields a unique MADS box transcription factor in determining seed size and thermal sensitivity. *Plant physiology*, *171*(1), pp.606-622.

Cheng, W., Sakai, H., Yagi, K. and Hasegawa, T., 2009. Interactions of elevated [CO2] and night temperature on rice growth and yield. *Agricultural and forest meteorology*, *149*(1), pp.51-58.

Cui, L., Li, J., Fan, Y., Xu, S. and Zhang, Z., 2006. High temperature effects on photosynthesis, PSII functionality and antioxidant activity of two Festuca arundinacea cultivars with different heat susceptibility. *Botanical Studies*, *47*(1), pp.61-69.

Das, S., Krishnan, P., Nayak, M. and Ramakrishnan, B., 2014. High temperature stress effects on pollens of rice (Oryza sativa L.) genotypes. *Environmental and Experimental Botany*, *101*, pp.36-46.

de Jong, W.W., Leunissen, J.A. and Voorter, C.E., 1993. Evolution of the alpha-crystallin/small heat-shock protein family. *Molecular biology and evolution*, *10*(1), pp.103-126.

Dexter, S.T., Tottingham, W.E. and Graber, L.F., 1932. Investigations of the hardiness of plants by measurement of electrical conductivity. *Plant Physiology*, *7*(1), p.63.

Driedonks N, Rieu I, Vrizen WH (2016) Breeding for plant heat tolerance at vegetative and

Ehrnsperger, M., Gräber, S., Gaestel, M. and Buchner, J., 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The EMBO journal*, *16*(2), pp.221-229.

ElBasyoni, I., Saadalla, M., Baenziger, S., Bockelman, H. and Morsy, S., 2017. Cell membrane stability and association mapping for drought and heat tolerance in a worldwide wheat collection. *Sustainability*, *9*(9), p.1606.

El-Esawi, M.A. and Alayafi, A.A., 2019. Overexpression of rice Rab7 gene improves drought and heat tolerance and increases grain yield in rice (Oryza sativa L.). *Genes*, *10*(1), p.56.

Fahad, S., Ihsan, M.Z., Khaliq, A., Daur, I., Saud, S., Alzamanan, S., Nasim, W., Abdullah, M., Khan, I.A., Wu, C. and Wang, D., 2018. Consequences of high temperature under changing climate optima for rice pollen characteristics-concepts and perspectives. *Archives of Agronomy and Soil Science*, *64*(11), pp.1473-1488.

Fitter, A.H.; Hay, R.K.M. *Environmental Physiology of Plants*, 3rd ed.; Academic Press:London, UK, 2002.

Fu, G., Feng, B., Zhang, C., Yang, Y., Yang, X., Chen, T., Zhao, X., Zhang, X., Jin, Q. and Tao, L., 2016. Heat stress is more damaging to superior spikelets than inferiors of rice (Oryza sativa L.) due to their different organ temperatures. *Frontiers in plant science*, *7*, p.1637.

Gosavi, G.U., Jadhav, A.S., Kale, A.A., Gadakh, S.R., Pawar, B.D. and Chimote, V.P., 2014. Effect of heat stress on proline, chlorophyll content, heat shock proteins and antioxidant enzyme activity in sorghum (Sorghum bicolor) at seedlings stage.

Guan, J.C., Jinn, T.L., Yeh, C.H., Feng, S.P., Chen, Y.M. and Lin, C.Y., 2004. Characterization of the genomic structures and selective expression profiles of nine class I small heat shock protein genes clustered on two chromosomes in rice (Oryza sativa L.). *Plant molecular biology*, *56*(5), pp.795-809.

Han, Y., Fan, S., Zhang, Q. and Wang, Y., 2013. Effect of heat stress on the MDA, proline and soluble sugar content in leaf lettuce seedlings. *Agricultural Sciences*, *4*(05), p.112.

Harsant, J., Pavlovic, L., Chiu, G., Sultmanis, S. and Sage, T.L., 2013. High temperature stress and its effect on pollen development and morphological components of harvest index in the C3 model grass Brachypodium distachyon. *Journal of experimental botany*, *64*(10), pp.2971-2983.

Harsh, A., Sharma, Y.K., Joshi, U., Rampuria, S., Singh, G., Kumar, S. and Sharma, R., 2016. Effect of short-term heat stress on total sugars, proline and some antioxidant enzymes in moth bean (Vigna aconitifolia). *Annals of Agricultural Sciences*, *61*(1), pp.57-64.

Hasan, M.A., Ahmed, J.U., Bahadur, M.M., Haque, M.M. and Sikder, S., 2007. Effect of late planting heat stress on membrane thermostability, proline content and heat susceptibility index of different wheat cultivars. *Journal of the National Science Foundation of Sri Lanka*, *35*(2).

Hasanuzzaman, M., Nahar, K., Alam, M., Roychowdhury, R. and Fujita, M., 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International journal of molecular sciences*, *14*(5), pp.9643-9684.

Haslbeck, M., Franzmann, T., Weinfurtner, D. and Buchner, J., 2005. Some like it hot: the structure and function of small heat-shock proteins. *Nature structural & molecular biology*, *12*(10), p.842.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives in Biochemistry and Biophysics* 125**,** 189–198.

Hemantaranjan, A., Bhanu, A.N., Singh, M.N., Yadav, D.K., Patel, P.K., Singh, R. and Katiyar, D., 2014. Heat stress responses and thermotolerance. *Adv. Plants Agric. Res*, *1*(12), pp.10-15406.

Herrero, M.P. and Johnson, R.R., 1980. High Temperature Stress and Pollen Viability of Maize 1. *Crop science*, *20*(6), pp.796-800.

HorieT, Matsui T, Nakagawa H, Omasa K.1996. Effect of elevated CO_2 and global climate change on rice yield in Japan. In: Omasa K, Kai K, Toda H, Uchijima Z, Yoshino M, eds. *Climate change and plants in East Asia*. Tokyo: Springer-Verlag, 39–56.

Hu, W., Hu, G. and Han, B., 2009. Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. *Plant Science*, *176*(4), pp.583-590.

IPCC, 2014. Intergovernmental Panel on Climate Change. Fifth Assessment Report of the Intergovernmental Panel on Climate Change: the Impacts, Adaptation and Vulnerability. Cambridge University Press, United Kingdom and New York, NY, USA.

Ishimaru, T., Hirabayashi, H., Sasaki, K., Ye, C. and Kobayashi, A., 2016. Breeding efforts to mitigate damage by heat stress to spikelet sterility and grain quality. *Plant Production Science*, *19*(1), pp.12-21.

Jagadish, S.V.K., Craufurd, P.Q. and Wheeler, T.R., 2007. High temperature stress and spikelet fertility in rice (Oryza sativa L.). *Journal of experimental botany*, *58*(7), pp.1627-1635.

Jagadish, S.V.K., Cairns, J., Lafitte, R., Wheeler, T.R., Price, A.H. and Craufurd, P.Q., 2010. Genetic analysis of heat tolerance at anthesis in rice. *Crop Science*, *50*(5), pp.1633-1641.

Jagadish, S.V.K., Murty, M.V.R. and Quick, W.P., 2015. Rice responses to rising temperatures–challenges, perspectives and future directions. *Plant, Cell & Environment*, *38*(9), pp.1686-1698.

Jung, K.H., Ko, H.J., Nguyen, M.X., Kim, S.R., Ronald, P. and An, G., 2012. Genome-wide identification and analysis of early heat stress responsive genes in rice. *Journal of Plant Biology*, *55*(6), pp.458-468.

Kazim, A.M., Abid, A. and Saddia, G., 2013. Response of rice (Oryza sativa L.) under elevated temperature at early growth stage: Physiological markers. *Russian Journal of Agricultural and Socio-Economic Sciences*, *20*(8).

Kilasi, N.L., Singh, J., Vallejos, C.E., Ye, C., Jagadish, S.K., Kusolwa, P. and Rathinasabapathi, B., 2018. Heat stress tolerance in rice (Oryza sativa L.): Identification of quantitative trait loci and candidate genes for seedling growth under heat stress. *Frontiers in plant science*, *9*.

Kondamudi R, Swamy KN, Chakravarthy DVN, Vishnuprasanth V, Rao YV, Rao PR, Sarla N,Subrahmanyam D, Voleti SR (2012) Heat stress in rice – physiological mechanisms and adaptation strategies page under the book crop stress and its management perspective and strategies. In: Venkateswarlu B, Shanker AK, Maheswari M (eds) Crop stress and its management: perspectives and strategies. Springer, Dordrecht, pp 193–223

Krishna, P., 2003. Plant responses to heat stress. In *Plant responses to abiotic stress* (pp. 73-101). Springer, Berlin, Heidelberg.

Kumar, N., Kumar, N., Shukla, A., Shankhdhar, S.C. and Shankhdhar, D., 2015. Impact of terminal heat stress on pollen viability and yield attributes of rice (Oryza sativa L.). *Cereal research communications*, *43*(4), pp.616-626.

Kumar, N., Suyal, D.C., Sharma, I.P., Verma, A. and Singh, H., 2017. Elucidating stress proteins in rice (Oryza sativa L.) genotype under elevated temperature: a proteomic approach to understand heat stress response. *3 Biotech*, *7*(3), p.205.

Kumari S, Roy S, Singh P, Singla-Pareek SL, Pareek A (2013) Cyclophilins: proteins in search of function. Plant Signal Behav 8:e22734

Larcher, W. *Physiological Plant Ecology*; Springer: Berlin, Germany, 1995.

Lawas, L.M., Bheemanahalli, R., Solis, C.A. and Krishna Jagadish, S.V., 2018. Sheathed Panicle Phenotype (cv. Sathi) Maintains Normal Spikelet Fertility and Grain Filling under Prolonged Heat Stress in Rice. *Crop Science*, *58*(4), pp.1693-1705.

Lee, G.J. and Vierling, E., 2000. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiology*, *122*(1), pp.189-198.

Li, Y.F., Wang, Y., Tang, Y., Kakani, V.G. and Mahalingam, R., 2013. Transcriptome analysis of heat stress response in switchgrass (Panicum virgatum L.). *BMC plant biology*, *13*(1), p.153.

Maclean, J., Hardy, B. and Hettel, G., 2013. *Rice Almanac: Source book for one of the most important economic activities on earth*. IRRI.

Martineau, J.R., Specht, J.E., Williams, J.H. and Sullivan, C.Y., 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability 1. *Crop Science*, *19*(1), pp.75-78.

Mishra, N., Srivastava, A.P., Esmaeili, N., Hu, W. and Shen, G., 2018. Overexpression of the rice gene OsSIZ1 in Arabidopsis improves drought-, heat-, and salt-tolerance simultaneously. *PloS one*, *13*(8), p.e0201716.

Mogk, A., Schlieker, C., Friedrich, K.L., Schönfeld, H.J., Vierling, E. and Bukau, B., 2003. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *Journal of Biological Chemistry*, *278*(33), pp.31033-31042.

Mohammed, A.R. and Tarpley, L., 2010. Effects of high night temperature and spikelet position on yield-related parameters of rice (Oryza sativa L.) plants. *European journal of agronomy*, *33*(2), pp.117-123.

Mohammed, A.R. and Tarpley, L., 2011. Morphological and physiological responses of nine southern US rice cultivars differing in their tolerance to enhanced ultraviolet-B radiation. *Environmental and experimental botany*, *70*(2-3), pp.174-184.

Mottaleb, K.A., Rejesus, R.M., Mohanty, S., Murty, M.V.R., Li, T., Valera, H.G. and Gumma, M.K., 2012. *Ex ante impact assessment of a drought tolerant rice variety in the presence of climate change* (No. 323-2016-11905).

Nagarajan, S., Jagadish, S.V.K., Prasad, A.H., Thomar, A.K., Anand, A., Pal, M. and Agarwal, P.K., 2010. Local climate affects growth, yield and grain quality of aromatic and non-aromatic rice in northwestern India. *Agriculture, Ecosystems & Environment*, *138*(3-4), pp.274-281.

Nevame, A.Y.M., Emon, R.M., Malek, M.A., Hasan, M.M., Alam, M., Muharam, F.M., Aslani, F., Rafii, M.Y. and Ismail, M.R., 2018. Relationship between High Temperature and Formation of Chalkiness and Their Effects on Quality of Rice. *BioMed research international*, *2018*.

Nguyen, D.N., Lee, K.J., Kim, D.I., Anh, N.T. and Lee, B.W., 2014. Modeling and validation of high-temperature induced spikelet sterility in rice. *Field crops research*, *156*, pp.293-302.

Pareek A, Singla SL, Grover A (1997) Short-term salinity and high temperature stress-associated ultrastructural alterations in young leaf cells of Oryza sativa L. Ann Bot 80:629–639

Prasad, P.V.V., Boote, K.J., Allen Jr, L.H., Sheehy, J.E. and Thomas, J.M.G., 2006. Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field crops research*, *95*(2-3), pp.398-411.

Pressman, E., Peet, M.M. and Pharr, D.M., 2002. The effect of heat stress on tomato pollen characteristics is associated with changes in carbohydrate concentration in the developing anthers. *Annals of Botany*, *90*(5), pp.631-636.

Qu, A.L., Ding, Y.F., Jiang, Q. and Zhu, C., 2013. Molecular mechanisms of the plant heat stress response. *Biochemical and biophysical research communications*, *432*(2), pp.203-207.

Rajaram, H., Chaurasia, A.K. and Apte, S.K., 2014. Cyanobacterial heat-shock response: role and regulation of molecular chaperones. *Microbiology*, *160*(4), pp.647-658.

Rang, Z.W., Jagadish, S.V.K., Zhou, Q.M., Craufurd, P.Q. and Heuer, S., 2011. Effect of high temperature and water stress on pollen germination and spikelet fertility in rice. *Environmental and Experimental Botany*, *70*(1), pp.58-65.

Reis, S.P., Lima, A.M. and de Souza, C.R.B., 2012. Recent molecular advances on downstream plant responses to abiotic stress. *International Journal of Molecular Sciences*, *13*(7), pp.8628-8647.

Sailaja, B., Subrahmanyam, D., Neelamraju, S., Vishnukiran, T., Rao, Y.V., Vijayalakshmi, P., Voleti, S.R., Bhadana, V.P. and Mangrauthia, S.K., 2015. Integrated physiological, biochemical, and molecular analysis identifies important traits and mechanisms associated with differential response of rice genotypes to elevated temperature. *Frontiers in plant science*, *6*, p.1044.

Sairam, R.K., Deshmukh, P.S., and Shukla, D.S., 1997. Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. Journal of Agronomy and Crop Science, 178: 171–178.

Sakata, T. and Higashitani, A., 2008. Male sterility accompanied with abnormal anther development in plants–genes and environmental stresses with special reference to high temperature injury. *Int. J. Plant Dev. Biol*, *2*(4).

Sangwan, S., Ram, K., Rani, P., Munjal, R., 2018. Effect of Terminal High Temperature on Chlorophyll Content and Normalized Difference Vegetation Index in Recombinant Inbred Lines of Bread Wheat. *International Journal of Current Microbiology and Applied Sciences, 7(6),* PP. 1174-1183.

Sarkar, J., Chakraborty, B. and Chakraborty, U., 2016. Temperature stress induced antioxidative and biochemical changes in wheat (Triticum aestivum L.) cultivars. *Journal of Plant Stress Physiology*, pp.22-30.

Sarsu, F., Ghanim, A.M., Das, P., Bahuguna, R.N., Kusolwa, P.M., Ashraf, M., Singla-Pareek, S.L., Pareek, A., Forster, B.P. and Ingelbrecht, I., 2018. *Pre-Field Screening Protocols for Heat-Tolerant Mutants in Rice*. Springer.

Satake, T. and Yoshida, S., 1978. High temperature-induced sterility in indica rices at flowering. *Japanese Journal of Crop Science*, *47*(1), pp.6-17.

Scharf, K.D., Siddique, M. and Vierling, E., 2001. The expanding family of Arabidopsis thaliana small heat stress proteins and a new family of proteins containing α-crystallin domains (Acd proteins). *Cell stress & chaperones*, *6*(3), p.225.

Sehgal, A., Sita, K., Siddique, K.H., Kumar, R., Bhogireddy, S., Varshney, R.K., HanumanthaRao, B., Nair, R.M., Prasad, P.V. and Nayyar, H., 2018. Drought or/and Heat-Stress Effects on Seed Filling in Food Crops: Impacts on Functional Biochemistry, Seed Yields, and Nutritional Quality. *Frontiers in plant science*, *9*.

Shah F, Huang J, Kul K, Nie L, Shah T, Chen C (2011) Impact of high-temperature stress on rice plant and its traits related to tolerance. J Agric Sci 149:545–556

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C. and Wang, K., 2011. Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, *149*(5), pp.545-556.

Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C. and Wang, K., 2011. Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, *149*(5), pp.545-556.

Sharma, D., Rachhoya, H.K., Sharma, M. and Agarwal, R., 2018. Effect of Rising Temperature Stress on Growth and Physiology of Domestic Crops of

Rajasthan, India. *International Journal of Current Microbiology and Applied Sciences*, 7(7), p. 1426-1440.

Sharma, R., Sharma, R., Sudan, J., Salgotra, R.K. and Singh, R., 2017. Research Article Alterations in cellular membrane stability due to heat stress in different genotypes of bread wheat. *Electronic Journal of Plant Breeding*, *8*(4), pp.1022-1027.

Siddique, M., Gernhard, S., von Koskull-Döring, P., Vierling, E. and Scharf, K.D., 2008. The plant sHSP superfamily: five new members in Arabidopsis thaliana with unexpected properties. *Cell Stress and Chaperones*, *13*(2), pp.183-197.

Soda, N., Gupta, B.K., Anwar, K., Sharan, A., Singla-Pareek, S.L. and Pareek, A., 2018. Rice intermediate filament, OsIF, stabilizes photosynthetic machinery and yield under salinity and heat stress. *Scientific reports*, *8*(1), p.4072.

Song, L., Yue, L., Zhao, H. and Hou, M., 2013. Protection effect of nitric oxide on photosynthesis in rice under heat stress. *Acta physiologiae plantarum*, *35*(12), pp.3323-3333.

Tiwari, A., Prasad, S., Jaiswal, B., Gyanendra, K., Singh, S., Singh, K.N., 2017. Effect of Heat Stress on Yield Attributing Traits in Wheat (Triticum aestivum L.). *International Journal of Current Microbiology and Applied Sciences* , 6(12), p. 2738-2744.

Tonini, A. and Cabrera, E., 2011. *Opportunities for global rice research in a changing world* (No. 2215-2019-1630).

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A. and Latif, M.A., 2015. Expression of target gene Hsp70 and membrane stability determine heat tolerance in chili pepper. *Journal of the American Society for Horticultural Science*, *140*(2), pp.144-150.

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A. and Latif, M.A., 2015. Expression of target gene Hsp70 and membrane stability determine heat tolerance in chili pepper. *Journal of the American Society for Horticultural Science*, *140*(2), pp.144-150.

Usman, M.G., Rafii, M.Y., Ismail, M.R., Malek, M.A., Latif, M.A. and Oladosu, Y., 2014. Heat shock proteins: functions and response against heat stress in plants. *Int. J. Sci. Technol. Res*, *3*(11), pp.204-218.

van Montfort, R.L., Basha, E., Friedrich, K.L., Slingsby, C. and Vierling, E., 2001. Crystal structure and assembly of a eukaryotic small heat shock protein. *Nature Structural & Molecular Biology*, *8*(12), p.1025.

Van Oort, P.A.J., Saito, K., Zwart, S.J. and Shrestha, S., 2014. A simple model for simulating heat induced sterility in rice as a function of flowering time and transpirational cooling. *Field Crops Research*, *156*, pp.303-312.

Veerasamy, M., He, Y. and Huang, B., 2007. Leaf senescence and protein metabolism in creeping bentgrass exposed to heat stress and treated with cytokinins. *Journal of the American Society for Horticultural Science*, *132*(4), pp.467-472.

Vierling, E., 1991. The roles of heat shock proteins in plants. *Annual review of plant biology*, *42*(1), pp.579-620.

Vishwakarma, H., Junaid, A., Manjhi, J., Singh, G.P., Gaikwad, K. and Padaria, J.C., 2018. Heat stress transcripts, differential expression, and profiling of heat stress tolerant gene TaHsp90 in Indian wheat (Triticum aestivum L.) cv C306. *PloS one*, *13*(6), p.e0198293.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–233

Wahid, A., Gelani, S., Ashraf, M. and Foolad, M.R., 2007. Heat tolerance in plants: an overview. *Environmental and experimental botany*, *61*(3), pp.199-223.

Wang, Q.L., Chen, J.H., He, N.Y. and Guo, F.Q., 2018. Metabolic reprogramming in chloroplasts under heat stress in plants. *International journal of molecular sciences*, *19*(3), p.849.

Wang, Q.L., Chen, J.H., He, N.Y. and Guo, F.Q., 2018. Metabolic reprogramming in chloroplasts under heat stress in plants. *International journal of molecular sciences*, *19*(3), p.849.

Wilson, R.A., Sangha, M.K., Banga, S.S., Atwal, A.K. and Gupta, S., 2014. Heat stress tolerance in relation to oxidative stress and antioxidants in Brassica juncea. *Journal of environmental biology*, *35*(2), p.383.

Wu, C., Cui, K., Wang, W., Li, Q., Fahad, S., Hu, Q., Huang, J., Nie, L. and Peng, S., 2016. Heat-induced phytohormone changes are associated with disrupted early reproductive development and reduced yield in rice. *Scientific reports*, *6*, p.34978.

Xue DW, Jiang H, Hu J, Zhang XQ, Guo LB, Zeng DL, Dong GJ, Sun GC, Qian Q, 2012. Characterization of physiological response and identification of associated genes under heat stress in rice seedlings. Plant Physiol Biochem 61:46–53

Yoshida S (1981) Fundamentals of rice crops science. International Rice Research Institute, Los Banos, The Philippines

Yoshida, S., Satake, T. and Mackill, D.S., 1981. High-temperature stress in rice [study conducted at IRRI, Philippines]. *IRRI Research Paper Series (Philippines)*.

Zafar, S.A., Hameed, A., Khan, A.S. and Ashraf, M., 2017. Heat shock induced morpho-physiological response in indica rice (Oryza sativa L.) at early seedling stage. *Pakistan Journal of Botany*, *49*(2), pp.453-463.

Zafar, S.A., Hameed, A., Nawaz, M.A., Wei, M.A., Noor, M.A. and Hussain, M., 2018. Mechanisms and molecular approaches for heat tolerance in rice (Oryza sativa L.) under climate change scenario. *Journal of Integrative Agriculture*, *17*(4), pp.726-738.

Zhang, C., Li, G., Chen, T., Feng, B., Fu, W., Yan, J., Islam, M.R., Jin, Q., Tao, L. and Fu, G., 2018. Heat stress induces spikelet sterility in rice at anthesis through inhibition of pollen tube elongation interfering with auxin homeostasis in pollinated pistils. *Rice*, *11*(1), p.14.

Zou, J., Liu, C. and Chen, X., 2011. Proteomics of rice in response to heat stress and advances in genetic engineering for heat tolerance in rice. *Plant Cell Reports*, *30*(12), pp.2155-2165.

APPENDIX–A:

Department of Agricultural Meteorology
Indira Gandhi Agricultural University, Raipur (C.G.) - 492 006

Station: Labhandi **Monthly Meteorological Data: 2018**

Month	Max. Temp. (°C)	Min. Temp. (°C)	RainFall (mm)	Rainy days	Relative Humidity (%)		Vapour pressure (mm)		Wind Velocity (Kmph)	Evaporation (mm)	Sun Shine (hours)
					I	**II**	**I**	**II**			
Jan.	28.5	10.1	0	0	83	23	8.1	6.4	2.2	103.2	8.7
Feb.	31.5	15	16.4	3	81	30	10.9	9.4	2.8	112.6	8.3
Mar	36.3	19.6	1.2	0	64	21	11.8	8.8	3.2	186.7	11.1
Apr.	39.3	23.8	41.4	4	61	23	15.2	11.3	4.7	235.7	8.7
May	41.8	27.4	15.4	3	62	31	19.4	17.4	6.7	312.4	8.9
Jun.	36.9	26.3	141.5	9	78	46	21.8	19.6	6.7	226.9	6.4
Jul.	31.2	25.4	328	11	90	74	22.7	22.7	7.5	100.5	1.4
Aug.	29.8	24.8	560.2	15	93	81	22.8	23.3	5.6	92.2	1.5
Sep.	31.3	24.5	84.8	7	92	65	22.3	20.7	3.7	96.9	4.9
Oct.	32.9	21.4	0	0	88	45	18.4	16.3	1.5	116.8	8.1
Nov.	31.3	16	0	0	87	34	12.9	11.2	1.3	100.7	8.5
Dec.	25.9	12.4	47.2	2	88	42	10.2	9.8	1.5	70.6	4.7
Total			**1236.1**							**1755.2**	
Average	**33.1**	**20.6**			**81**	**43**	**16.4**	**14.7**	**4**		**6.8**

Source: http://igau.edu.in/agromet.htm

RESUME

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2 messages

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