CAPITAL UNIVERSITY OF SCIENCE AND
TECHNOLOGY, ISLAMABAD

Exploring the Impact of Drought Stress on *Oryza sativa* L.

by

Syed Khalilullah

A thesis submitted in partial fulfillment for the
degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2024

I dedicate my work to my family, friends, and to my teachers. Specially, with a feeling of gratitude, I dedicate this work to my loving parents, my two 2 sisters (Amna Waris and Maimoona Waris) and my brothers (Syed Abdul Bais and Syed Safiullah) for their generous support they provided me throughout my entire life and particularly through the process of pursuing the Master's degree and gave me valuable advice whenever I needed it the most. I thank him from the bottom of my heart of their unconditional love and prayers.

CERTIFICATE OF APPROVAL

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Acknowledgement

In the name of **Allah**, the most gracious and the most merciful; and prayers and peace be upon **Muhammad**, His servant and messenger. First and foremost, I would like to acknowledge my limitless thanks to **Allah Almighty** and to his **last Prophet Muhammad (S.A.W)** without their guidance this work would have never become completed. I owe a deep depth of gratitude to our University, **Capital University of Science & Technology (CUST)** for giving me such an opportunity to complete this work. A special thanks to the **Dean & Head** of the Department of Bioinformatics and Biosciences **Dr. Sahar Fazal & Dr. S. Marriam Bakhtiar** for encouraging and the most of all patience throughout the entire process.

I would like to acknowledge and very grateful to my **Supervisor Dr. Shaukat Iqbal Malik** who has been always generous during all phases of the research and for all the valuable lessons that I learned during this period, I thank her from the bottom of my heart. I must acknowledge my special thanks **Dr. Sahar Fazal** to being a mentor, who has always been a source of motivation and guidance.

A special thanks to all others who helped me during my research especially. I would like a warm thanks to my beloved friends, **Nouman Shahzad Malik**, **Waleed Asad Khan** and **Aiman Fatima** who have been supportive along the way of doing my research work.

Last but not least, I want to express my wholehearted thanks to my **family specially my Parents, Brothers and Sisters** who have provided me generous support throughout my life and specially in doing my research. Because of their unconditional love, support and prayers, I have completed this thesis.

(Syed Khalilullah)

Abstract

Rice (*Oryza sativa* L.) is a crucial staple food for over half of the world's population, but its production is significantly hindered by biotic and abiotic stresses, exacerbated by climate change. This study aims to explore the molecular mechanisms underlying drought tolerance in rice by examining the transcriptomes of two cultivars: Dhagaddeshi (drought-resistant) and IR20 (drought-sensitive) under drought conditions.

Using microarray analysis, we identified differentially expressed genes (DEGs) and their associated metabolic pathways that aid in drought adaptation. Our findings highlight a complex hormonal regulatory network involving abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene, mediated by key transcription factors like MYC2, ATAF1/ERD15, and heat shock factors (HSFs). Additionally, mechanisms for ROS accumulation and scavenging are crucial for maintaining cellular homeostasis under stress.

Dhagaddeshi's robust response involves both ABA-dependent and independent pathways, indicating a comprehensive adaptation strategy. The identified DEGs and pathways provide valuable insights for breeding programs to enhance drought resilience in high-yielding rice cultivars. This research underscores the importance of integrating various stress response pathways to develop rice varieties with improved drought tolerance, contributing to global food security amidst climate challenges.

Keywords: *Oryza sativa*, Biotic and Abiotic stresses, Climate Change, DEGs, ROS, ABA Pathways..

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Abbreviations

ABA	Abscisic Acid
DEG	Differentially Expressed Genes
GPXs	Glutathione Peroxidases
GSH	Glutathione
H202	Hydrogen Peroxide
HSP	Heat Shock Proteins
KEGG	Kyoto Encyclopedia of Genes and Genomes
MDA	Malondialdehyde
QTL	Quantitative Trait Loci
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase

Chapter 1

Introduction

1.1 Background

Rice (*Oryza sativa* L.), an essential food, is consumed by over half of the world's inhabitants. The movement of the Green Revolution in a number of nations predicted an increase in the production of this grain. 350 million people worldwide rely mostly on rice as a dietary source. By 2050, there will be 10 billion people on the planet, which means that 852 million tons of rice will be needed by 2035 [1]. However, as with Unlike other crops, rice is impacted by biotic and abiotic variables in its growth and development, reducing Its final product. In addition, the stagnation of the harvest of rice varieties and the threats of climate change cause great concern for global food security. An estimated 55 million people worldwide lose their livelihoods due to drought each year, according to estimates from the World Health Organisation (WHO) [2]. According to WHO projections, by 2030, around 700 million people might face relocation as a result of drought [3].

Historically, rice varieties were flourishing in regions that were watered mostly through floods. Because rice needs a lot of water to grow, it is more sensitive to fluctuations in soil moisture compared to crops such as maize and wheat.. Thus, across more than 20 million hectares of rainfed land in South and Southeast Asia, drought is the biggest burden on rice production [4]. The well-known rice cultivars cultivated in these areas, such as Swarna, IR64, and MTU1010, suffer

from their high yields and tolerance to drought [2, 5]. The emphasis has shifted from identifying rice types with enhanced performance in drought conditions to enhancing the water efficiency of rice varieties, as global food grain production faces increasing pressure. In recent years, several countries have initiated research efforts to discover rice types that exhibit greater resilience to specific environmental stress factors [6–10].

Even though Pakistan's traditional rice cultivars have a lot going for them, not all of them can be considered resistant to biotic and abiotic stress. Native cultivars, such as Dhagaddeshi and Nagina22, are drought-resistant while having lower yields than commercial cultivars. Identifying and introducing drought-tolerant QTLs impacting grain output is one breeding strategy to increase drought tolerance. [11, 12], and [13]. Due to its shallow root structure, short height, and potential for large yields, the Indica cultivar IR20 is a top genotype for crop production. Its ability to tolerate protracted dry spells has been questioned, though, considering how susceptible it is to moisture stress. Due to its susceptibility to moisture stress, there are concerns about its ability to withstand prolonged periods of dryness. Since some of the fundamental mechanisms may have similarities, it is important to identify the molecular mechanisms or mechanisms that are mainly responsible for developing a cultivar that is either resistant or susceptible to different abiotic stresses, such as drought. This also applies to many other rice cultivars. Analysis of the rice transcriptome under various abiotic conditions has revealed numerous stress-responsive genes [14, 15].

Numerous transcription factors, genes encoding for the production of osmolytes, genes scavenging reactive oxygen species (ROS), and additional metabolic processes have been identified by these studies. These findings may help in the selection of potential genes for the development of crop plants that are more adapted to abiotic stress conditions [16]. The two categories of these genes are signalling and functional components [17]. To gain a better understanding of the rice abiotic stress regulatory networks, efforts have been made to better characterise these stress-responsive genes.While a wide range of methodologies have been used to expand the body of information already in existence, very few studies have attempted

to investigate the underlying mechanisms that function under stress. Therefore, a more comprehensive approach is needed to properly comprehend the intricate structure of the regulatory networks associated with rice's abiotic stress response.

The current work uses a microarray approach to analyze the transcriptomes of two types of seedlings—Dhagaddeshi, which is drought resistant, and IR20, which is drought sensitive—that were subjected to conditions of drought stress for varied lengths of time. Even with the advent of more advanced technology, microarray analysis of transcriptome data continues to be a reliable and efficient approach. This is particularly relevant to rice research since high-quality rice genomes were utilized to create the microarray chips. The laboratory was involved in the rice genome sequencing project (IRGSP 2005) and made significant use of first-generation rice microarray chips for gene discovery related to the regulation of reproductive growth, hormone signaling, and response to abiotic stress [18, 19]. Examine the signaling networks of several drought-responsive cultivars by employing a range of downstream techniques to provide a concise summary. In due course, it should be possible to determine which genes are unique to each cultivar, confirm these genes in transgenic animals, and employ these genes in molecular methods of breeding.

1.2 Problem Statement

The population that consumes rice is growing by 1.8% a year. By 2025, the current 560 million tons of rice produced annually must rise to 850 million tons. Global agriculture is greatly impacted by climate change and drought stress, which has been shown to lower average rice plant yields by more than 50%.

1.3 Aim and Objectives

This study aim to anticipate how rice plants will react to the stress of drought and climate change using differential gene expression and their metabolic pathway.

- To map the rice plants' differential expression of genes (DEG) under drought stress.
- To functional annotate drought stress differential expressed genes (DEG) by Using heat mapping, Venn diagrams, and KEGG pathways.

Chapter 2

Literature Review

2.1 Impact of Climate Change on Agriculture

According to present climate prediction models, an expected 3-5 degree Celsius increase in surface temperatures on average over the next 50–100 years will have a considerable influence on the global agricultural sector [20]. This is going to happen concurrently with an increase in heat waves, floods, and droughts [20, 21]. Mid-continental regions like central Africa and Europe are expected to experience warmer, drier summers, leading to extensive salinization due to rising sea levels. These areas may also see a reduction in their growing seasons and a decrease in the amount of land suitable for agriculture. Changes in rainfall and temperature could negatively impact agricultural yields and the nutritional quality of crops [22–24].

Additionally, climate change is anticipated to alter the range of The increase in temperatures may lead to the spread of infections, affecting pests, diseases, and their habitats. This makes agricultural plants more susceptible to a wider range and increased volume of environmental stressors, which can be harmful when they occur at the same time. As a result of changing climatic conditions and the growing pressure on global food production due to population growth, there is now a greater demand for crop varieties that can withstand stress [25]. Consequently, agricultural plants are susceptible to a greater range and volume of environmental

stressors, which can be harmful if they occur simultaneously. The combination of shifting climatic circumstances and increasing strain on global food output due to population increase has led to a greater need than ever for crop types that can tolerate stress [25]. Thus, in order to provide prospects for the creation of broad-spectrum stress-tolerant crop, it is imperative to understand the processes behind plant responses to several stressors happening simultaneously.

2.2 Drought Stress in Rice Cultivation

Drought is the most common of the severe abiotic pressures that threaten Asia's rice-growing regions. Rice is a drought-prone crop due to its short root structure, and minor water stress causes stomatal closure to accelerate. Consequently, reduced photosynthesis, the accumulation of organic acids and osmolytes, and changes to the metabolism of carbohydrates are the usual physiological and biochemical reactions to drought stress [26]. Throughout the vegetative development, blooming, and final stages of rice cultivation, drought stress may delay floret initiation, which can result in spikelet infertility or grain filling [27]. During reproductive development, rice is especially susceptible to drought stress; even moderate stress can drastically lower grain production [28, 29]. According to [30], tillering, daytime photosynthesis, and leaf growth are all inhibited by dry stress. Because of early senescence, it also reduces leaf area and photosynthetic rate. A multitude of morphological, physiological, and biochemical changes, including a significant reduction in cell growth and elongation, leaf curling, stomata closing, the leaf's tip dry out or even dies, and changes in leaf shape, occur in the rice plant during drought stress [31].

Both fresh and dried leaf weights are decreased, along with the levels of chlorophyll a, b, a/b, total chlorophyll content, carotenoid content, Fv/Fm, relative water content, membrane stability, and photosynthetic inhibition. Reduced leaf water potential, disrupted gas exchange, hygienic loading and assimilate transport, increased relative electrolyte permeability, and altered cytokinin and auxin (IAA) distribution are also seen. Proline is accumulating. The activities of glutathione

reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), CAT, SOD, and GR are all elevated.

MDA and H2O2 concentrations rise along with significant alterations in nitrogen metabolism indicators, which include protein content, glutamine synthetase, and nitrogen concentration, and carbohydrate metabolism indexes, which include soluble sugar and starch content [32].

Although soil drying is bad for plant development, if the plant can withstand drought stress before grain filling starts, it might not be bad for grain output. Thus, in areas with rainfall in particular, drought poses a considerable threat to rice farming productivity and is a major production obstacle. Therefore, in order to reduce the poor yield and guarantee future food supply, it is strategically crucial for rice to boost crop stability under various levels of drought circumstances. Through the use of physiological approaches such as seed-priming, transgenic synthesis, or conventional breeding and selection, the oxidative stress tolerance of crops is genetically modified for agricultural improvement [33].

2.3 Seed Priming Techniques for Stress Tolerance

One method of addressing environmental stress concerns in seeds is pre-sowing priming therapy, which is easy, affordable, low-risk, and efficient [34, 35]. Redrying is done after priming, a regulated hydration period that comes before radical immersion to allow for the continuation of metabolic activity [36]. Numerous priming strategies, including hormonal, hydro, halo, and osmo priming, can cause pre-germination changes. These changes usually have a major effect on the consistency of the emergence of seeds and germination rate, especially under stressed situations [37]. Significant field outcomes after priming the seeds have been reported in the literature. Potassium salt seed priming improved emergence and production of winter wheat that was planted deep into summer fallow [38]. Hydropriming improved lentils seed development and the growth of seedlings in the

field, whereas exogenous injection of H2O2 increased red bean seedlings' tolerance to cold [39].

However, the mechanisms underlying stress tolerance remain unclear. Certain components of the antioxidant defense system have been found by a research describing the effects of seed-priming on Sorghum seeds during salt stress [40] and in the seeds of rice during floods [41].

Brassica juncea seedlings exposed to stress were shown to respond better to hydropriming compared to chemical or hormonal priming after 15 days of development under normal conditions [42]. However, experimental data on the connection among oxidative stress and growth of seedlings during drought stress following seed priming is often lacking. Finding the genetic markers that react to seed priming is very desirable in order to have a greater knowledge of the process.

2.4 Oxidative Stress and Antioxidant Defense Mechanisms

The production of free radicals of reactive oxygen species (ROS) by drought stress causes stress-dependent lipid peroxidation and membrane deterioration [43]. Lipid peroxidation has been identified as a potential marker of increased oxidative damage [44]. Products of lipid peroxidation are made from polyunsaturated precursors, which include related compounds such as malondialdehyde (MDA) and small hydrocarbon fragments like ketone bodies. Green beans' drought resistance may be determined by looking at their MDA content. The antioxidative defense mechanisms may be able to control ROS through antioxidant glutathione (GSH), a low-molecular-weight, soluble in water antioxidant. By lowering organic lipid hydroperoxides and H2O2, glutathione peroxidases (GPXs), a broad family of distinct isozymes, can help plants strengthen their defenses against oxidative stress [45]. Transgenic plants' resilience to abiotic stress is increased when GPX is overexpressed, as evidenced by previous research [46]. GPXs are efficient scavenger of H2O2 and lipid hydroperoxides, using GSH as a reducing agent. However, it has

been additionally suggested that the ideal oxidant in plants is thioredoxin [47]. Superoxide dismutase (SOD), comprising enzymes like Chloramphenicol acetyltransferase (CAT) and products from the ascorbate–glutathione cycle, is one of the main scavenging processes. When a seed germinates to produce seedlings, several physiological and biochemical processes occur, including There is a disruption in the water balance, harm to membrane transport, decreased ATP synthesis, inhibition of respiration, and poor and delayed seed germination. The activity of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) are altered, and free proline accumulates [48]. Superoxide dismutates into oxygen and H2O2 and is the first ROS to be formed, making it the primary enzyme in a ROS attack. However, it is also swiftly scavenged. Plants normally include Cu/Zn-SOD in their cytosol, Fe-SOD in their chloroplasts, and Mn-SOD in their mitochondria [49].

Research on drought-tolerant durum wheat suggests that mitochondria could have a crucial role in how cells respond to abiotic stress. According to [50], plant mitochondria are a significant contributor to ROS production and oxidative damage during two types of abiotic stress.: salinity and drought. Proteomics research carried out during the development of transgenic rice also suggested that upregulating Mn-SOD expression could enhance drought resistance. Tetrameric heme-containing enzymes called catalases directly change H2O2 into H2O and O2. In times of stress, they are crucial to the detoxification of ROS [51]. Under stress, the peroxisome-specific protein CAT may function as an extensive amount absorber of excess ROS generation. Extreme stress from drought could be the only condition that increases its activity because of its reduced attraction for H2O2. In wheat, CAT activity increased significantly after a severe drought, per [52]. Molecular chaperones provide extra resistance towards heat and other stimuli by restoring denatured proteins. Heat shock proteins (HSPs), including HSP70, HSP101, and minor HSP classes, were expressed more frequently in dry-rooted cuttings of Loblolly pine [53]. The most common and well-studied member of the heat shock protein family is the 70-kDa HSP (HSP70). Plants with overexpressed HSP70 genes have better resistance to salt, water, and high temperatures, which has a positive association with an increase of thermos tolerance [54]. In the years to come, crop plants that have figured out how to scavenge and/or regulate

the quantity of cellular ROS through seed priming may be useful in coping with adverse environmental circumstances. Our identification of the molecular components of rice's antioxidant defense system—a major crop plant in many regions of the world—may greatly advance our knowledge of how to enhance crop plant growth and yield [55]. When a plant's typical reaction to a subsequent stress is changed as the outcome of an acclimation reaction brought on by the existence of a beginning or prior stress, the stress elements are said to interact. The harmful combination effect of two different abiotic stresses on crops, that has been the subject of much research, was reviewed recently by . He [56] at and drought stress particularly can damage crops disproportionately if compared with each stress alone [57].

2.5 Molecular Responses of Plants to Combined Stressors

Research on the molecular reactions of plants to diverse stressors has often focused on overlapping transcriptional patterns. Numerous research have been carried out in this respect to examine the patterns of gene expression of different plant groupings that are concurrently exposed to different stressors. Subsequently, it is discovered that overlap gene sets that are controlled by both stressors may be a symptom of a cross-talk between signalling pathways or a generalised stress response [58]. Rumour has it that these genes may be exploited to increase the stress tolerance of agricultural plants [59]. For instance, through Affymetrix has ATH1 micro arrays to compare the transcriptome to the reaction of plants such as Arabidopsis to 9 distinct abiotic stresses, it was discovered that 67 genes had been consistently regulated by each stress, indicating that a common component of the response to each circumstance existed [60]. A related study looked at the effects of various abiotic stresses, infections, or hormone therapy on cytochrome P450 gene expression. It was shown that several genes were frequently activated by both biotic and abiotic stress, indicating that they may be important regulators of pathway cross-talk [61]. The response times of chickpea to biotic and abiotic stresses were compared

using a 758-probe microarray. Many common reactions were observed, especially between a fungal infection as well as a high salinity stress [62]. However, these studies may not be particularly useful in comprehending the way plants respond to multiple simultaneous stresses [63] because current transcriptome analysis in tobacco and Arabidopsis has shown that plants' molecular reactions to concurrent heat and drought stress are not additive. Understanding each stress individually would not have allowed one to predict the novel variation in gene regulation that arises from a confluence of stressors, leading to the stimulation of several genes. The heat shock proteins (HSPs), proteases, lipid biosynthesis enzymes, starch-degrading enzymes, MYB TFs, protein kinases, and defence protein that regulate the oxidative stress defence belong to the genes that are specifically regulated by the two stressors in Arabidopsis [63]. More transcriptome research on a range of species has revealed genes, hormones, as well as pathways crucial for regulating how plants respond to various biotic or abiotic stresses, which has suggested goals for enhancing stress tolerance [64]. In [64], the combination of bright light and high temperatures activated a particular group of genes with differential expression.

2.6 Plant Responses to Combined Stressors

Wheat underwent a non-additive structure of HSP expression in response to concurrent heat stress and drought, which was unexpected from the analysis of any stress alone [65]. The combined impacts of drought and heat stress on wheat rates of photosynthesis were more harmful to the physiological state of the plant than each one of the conditions operating alone. A transcriptome analysis conducted after tobacco was infected by two herbivorous insects, a chewing hornworm (*Manduca sexta*) and a sap-feeding mirid (*Tupiocoris notatus*), revealed a different transcriptional response when the two herbivores were administered together compared to independent of each other.

This supports the existence of trans-activating elements, which change the expression of genes in response to various stresses. Furthermore, applying the herbivores in a sequential manner produced different gene expression patterns than applying

them simultaneously. This suggests a potential priming the mechanism whereby the transcriptome evolves over time in reaction to biotic stress and serves as a kind of immunological memory. In a different investigation, *Aspergillus parasiticus*-infected peanut plants that had been subjected to drought were investigated [66]. Drought-stricken peanut plants infected with *Aspergillus parasiticus* were the subject of another experiment [66].

Utilizing two expressed sequence tag (EST) cDNA libraries, it was demonstrated that 42 genes were up-regulated in response to the fungus and the drought at the same time, whereas 52 genes were up-regulated only in response to the drought. Drought-induced root damage is beneficial to this pathogen, hence it is hypothesized that the fungus could be able to reduce drought signaling and ABA in order to increase infection rates.

The above-described non-linear consequences of numerous shocks on plant stress resistance can be explained molecularly by these sorts of transcriptome data. Rather of generating a stock reaction to any infectious agent or abiotic stress, plants start a novel gene expression programme in response to particular environmental circumstances. However, no study has yet shown whole-genome transcriptomic changes brought on by a specific stress combination, despite growing interest in the effects of concurrent abiotic and biotic stressors on plants.

Transcriptome research yields a great deal of information, but its conclusions are not always complete in assessing changes in cellular protein activity [67]. There isn't much evidence linking TF to target gene mRNA, which suggests that phosphorylation or post-transcriptional regulation controls most of TF activity [68]. Furthermore, there is an exceptionally low correlation between transcript abundance as determined by microarrays and proteomic data to acquire a more precise depiction of the elements of the proteomic, metabolic, and stress signaling networks. This correlation is R $\frac{1}{4}$ 0.24. Combining this data with findings from transcriptome analyses will offer the most effective method for describing these intricate plant processes [69]. Furthermore, our knowledge has been significantly improved by never ending research on hormone mutants providing insight on the mechanism of action.

2.7 Hormonal Interactions in Stress Responses

While the antagonistic interaction among the salicylic acid (SA) as well as jasmonic acid (JA)/ethylene signalling pathways determines defence against diverse biotic attackers, the hormone ABA is principally responsible for controlling abiotic stress responses. However, recent studies show that ABA interacts both antagonistic and complimentary ways with biotic stress signalling, creating a complicated network of pathways with different amounts of cross-talk (Fig 2.1).

It follows that ABA probably has a major influence on modulating responses seen to concurrent biotic and abiotic stressors. Pathogens such *B. cinerea* and *Erwinia chrysanthemi* in tomatoes, *Phytophthora infestans* and *Cladosporium cucumerinum* in the potato and *Magnaportha grisea* blast fungus in rice are among those that are more susceptible to treatment with ABA [70]. He thinks that an ABA shortage may produce a greater amount of pathogen.

The tomato sitiens mutant, for example, showed reduced ABA levels and was unable to resist the oomycete *Peronospora parasitica* as well as the fungal pathogen *Fusarium oxysporum*, accordingly, but the ABA-insensitive Arabidopsis mutants abi1-1 along with abi2-1 showed increased accumulation of SA-dependent defence transcripts, including PR1, and enhanced tolerance to *B. cinerea* [72].

Treatment with ABA inhibits the SAR pathway in tobacco and Arabidopsis, including upstream and downstream of SA induction. Furthermore, it inhibits the accumulation of vital defence molecules such as lignins and phenylpropanoids (Fig 2.1) [73]. Furthermore, SA could block the abiotic stress signal. The exogenous administration of SA causes maize to become susceptible to drought [74].

On the other hand, in the presence of experimentally produced SAR, Arabidopsis exhibits decreased abiotic stress responses. Rice resistance against the rice blast fungus, *M. grisea*, is mediated by a perfect balance between the levels of ABA and SA. ABA also inhibits JA and ethylene defence signalling, as evidenced by the ABA-mediated regulation of defence genes such as PDF1.2, a result that is irreversible upon administration of JA or ethylene.

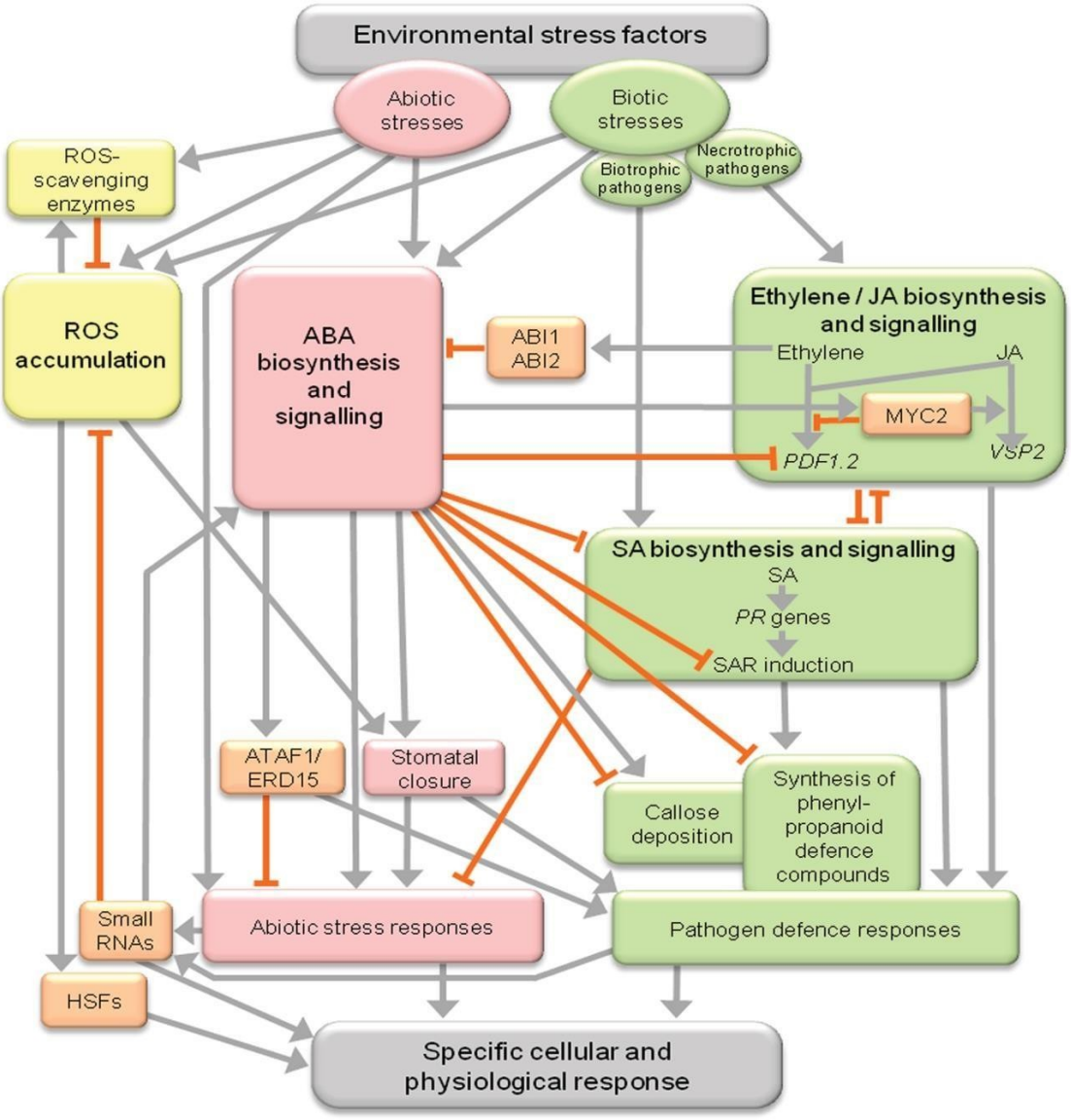

FIGURE 2.1: Plant hormones control how stress from both abiotic and interact with one another. The interaction of hormones, transcription factors, and other regulatory elements under simultaneous biotic and abiotic stresses is depicted schematically in the figure. Because of their intricate network of relationships, plants are able to react to the precise mix of environmental stressors in a very specific way. Orange bars indicate repression or inhibition, and grey arrows indicate induction or positive regulation. Events illustrating reactions to biotic stress are displayed in green, whereas events illustrating reactions to abiotic stresses are displayed in pink.Orange boxes indicate the transcription factors in addition to regulatory genes. Pathogenesis-related (PR) factors include heat shock factor (HSF), ABA (abscisic acid), JA (jasmonic acid), SA (salicylic acid), ROS (reactive oxygen species), and systemic acquired resistance (SAR) [71].

Ethylene treatment subsequently activates ABI1 and ABI2, the two negative regulators of ABA signalling [72]. ABA has been demonstrated to have positive impacts on pathogen defence pathways notwithstanding these findings [72]. It was shown that plants employed the defensive mechanism of ABA-induced stomatal closure—which also needed intact SA signaling—to combat microbial invasion through stomata that were open. Furthermore, ABA is necessary for the callose deposition caused by b-aminobutyric acid (BABA) during defence against fungal infections [74–76]. Interestingly, though, ABA can also prevent bacteria from inducing callose formation. A new model explaining the intricate function that ABA performs in pathogen response has been developed [75]. According to this model, the degree of influence that ABA has depends on the type of invader and the length of the infection.

The model mentions three distinct phases of pathogen infection. In the first, ABA inhibits the openings of the stomata, enhancing the body's defensive system and fortifying the barrier against microbes like bacteria. ABA is currently sparingly using resources by resisting the impact associated with the SA, JA, and ethylene pathways because they are not yet needed. The deposition of callose to strengthen cell walls is the primary focus of the subsequent stage of post-invasion defenses [76].

When a bacterial infection occurs, ABA inhibits this process; yet, when a fungal infection occurs, it facilitates it. Throughout the third stage of an infection, PAMPs (pathogen-associated molecular pattern) stimulate hormones such as SA, JA, and ethylene as well as long-distance signals that regulate a variety of defense chemicals. The ABA-inducible genes ATAF1 and ERD15 have been shown to act as switches that may prefer ABA-dependent biological stress responses to abiotic ones [75].

Abiotic stressor-induced elevations in ABA levels have the potential to block the SA, JA, as well as ethylene responses even during phase three. This idea explains contradictory historical evidence and provides a mechanism for controlling ABA through biotic and abiotic factors stress signaling [72]. To further complicate matters, recent research has shown that ABA can either positively or negatively

affect bacteria-induced callose deposition, depending on other growth factors such as light and glucose levels [52]. This implies that the environment can affect how ABA affects the defense response. These investigations show that while making judgments regarding pathogen resistance, care must be taken.

Numerous research investigating the effects of single and combined stressors have contributed to the intricate network of molecular interactions that regulate plant stress responses. Plants may respond to adverse environmental conditions by initiating both specialised and generic stress responses. This allows them to adapt as best they can to the specific challenges they face while simultaneously storing assets for future growth. Signal specificity is determined by the precise interactions between the components of each pathway, which include short RNAs, TFs, HSFs, ROS, and hormones such as ABA, SA, and JA. With so many interacting factors, it is easy to see how different pressures might have unexpected effects on one resistance's response. Historically, plant stress factors have been investigated studied as distinct stimuli that start linear signalling cascades. Given how closely related both abiotic and biotic stress pathways are to one another in a complex web of molecular interactions, it is clear that this paradigm is no longer appropriate. One of the primary objectives of crop stress research is to identify targets for improving the stress tolerance of agricultural plants. Due to anticipated climatic changes, many parts of the world are projected to face new stress combines in agricultural systems, which will provide new challenges in the development of multiple resilient to stress crops [22]. The creation of such plants will need an understanding of critical stress-regulatory pathways and the potential effects of different combinations of unfavourable environments. Studies of the diverse responses to stress in Arabidopsis along with other species have revealed a plethora of possible research areas.

2.8 Biotic and Abiotic Signalling Pathways

The antagonistic nature of biotic and abiotic signalling pathways may be explained by their shared characteristics. Master regulatory genes are involved in both the

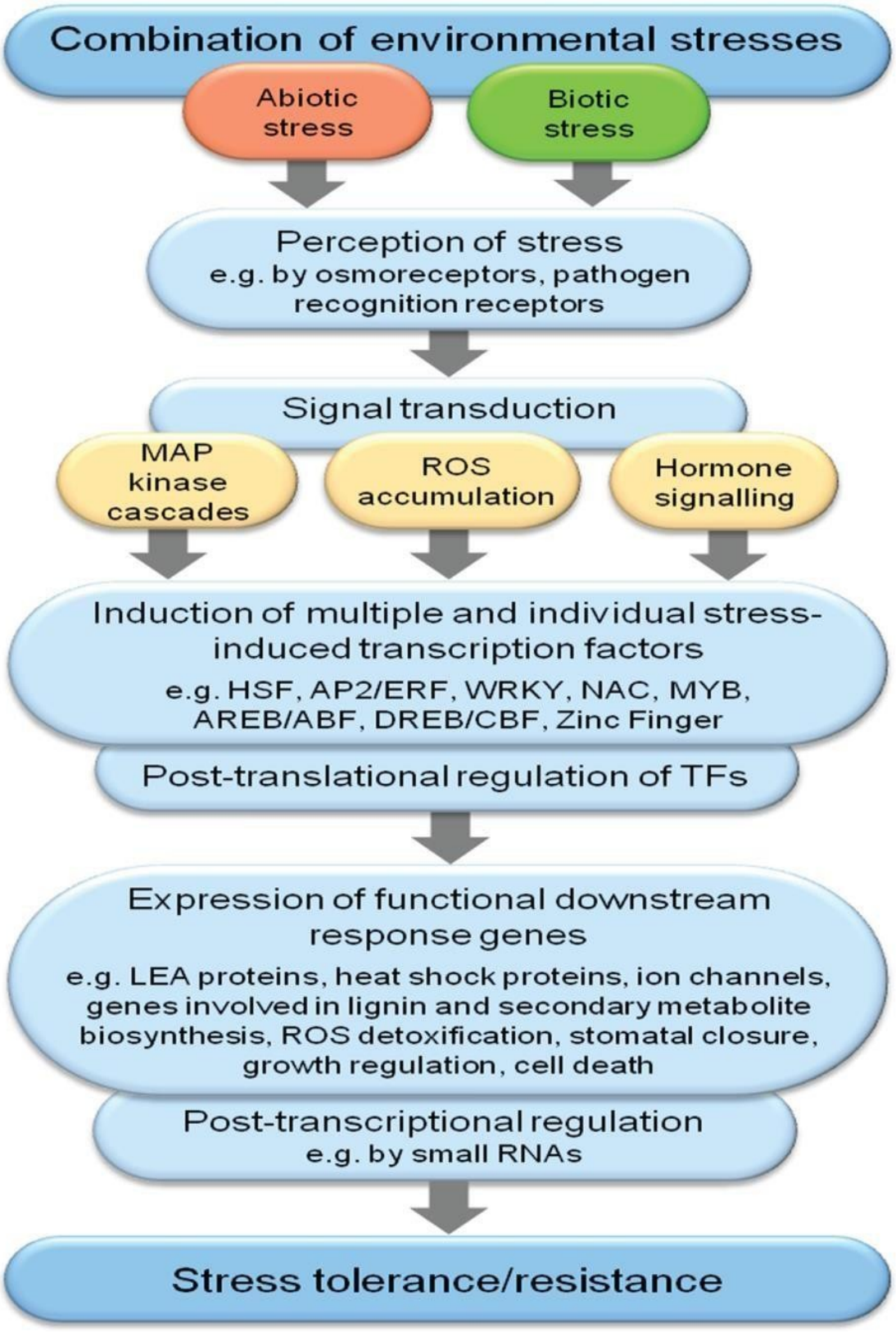

FIGURE 2.2: Significant events in the signal transduction pathway that are brought on by biotic and abiotic stressors in combination [71].

abiotic and biotic stress response systems, and they may be used to efficiently control stress tolerance [77].

HSFs regulate a large number of downstream stress-responsive genes, such as those belonging to the HSFA1 and HSFA2 families, which have shown promise in offering resistance to a range of pressures. Members of the MYC, MYB, as well as NAC TF families may be of relevance because they modulate the antagonistic relationship among hormone-mediated abiotic stresses and pathogen response pathways.

Previous research has shown that altering them can provide resistance to biotic and abiotic stresses in a variety of animals. Regulating the genes involved in ROS homeostasis may also affect tolerance to a range of stimuli, considering the function that ROS play during both abiotic stresses and pathogen stress. Transgene pyramiding has been shown to effectively give resistance to particular stresses, including drought in wheat [78], nematode in potatoes, insects in chickpeas, as well as salt stress in tobacco. Genetically modified crops are permitted to utilise as many as three pyramided transgenes that give resistance to two different biotic stressors (e.g., Monsanto's potatoes that is immune to both viruses as well as insects).

Crops with pyramided trans-genes (practice of stacking multiple genes with desirable traits into a single organism) are being grown on a growing amount of land. Thus, the pyramiding of many genes may lead to further prospects for the development of plants that are stress-tolerant throughout a broad spectrum [79]. We also know from extensive study on crop stress responses that ideal settings for studies should resemble those seen in the field or in the natural world. Plants may generate drastically varied transcriptional patterns in response to different stressors because they are able to recognise and respond to a broad range of environmental stimuli [80]. Variations in growth conditions like light and sugar levels can have a significant influence on the priorities of a plant's stress response, as evidenced by differences in the results of comparable tests [52]. Therefore, an integrated strategy that attempts to mimic a field stress condition in terms of development media, stress duration and intensity, stress timing, and availability of nutrients should be employed to offer a model for crop responses to stress [21].

In addition, it is now clear that plant types that have been produced with greater stress resistance should be evaluated under a variety of stress interactions which are likely to be encountered in the field, rather than just individual stressors. Recent years have shown that the focus of plant abiotic stress studies has to change. Growth or yield penalties are often associated with both natural and intentionally produced stress tolerance, which makes them undesirable for agricultural usage. This is because plants possess restricted resources which must be divided between growth and defence against stressors [80]. Therefore, rather than developing crops that can resist excessively stressful environments, it could be more desirable to focus on generating stress-tolerant crops that yet have significant amounts of the process of photosynthesis growth rates, and yield. Arabidopsis that has the C24 genotype exhibits these traits [81].

While exhibiting constitutive expression of SA-induced defences that permit substantial levels of tolerance against *P. syringae* and *Hyaloperonospora arabidopsidis*, C24 exhibits no yield loss in contrast to mutant genotypes with constitutive SA responses. It also requires less water to generate the same seed output than other accessions since it is more drought resistant and has a greater water usage efficiency. With additional study being done to determine the underlying genetics of this beneficial trait, it may be possible to generate broad-spectrum stress tolerance in plants without sacrificing yield, as this discovery suggests [81]. The equilibrium of energy levels links stress reactions with the maintenance of growth. Plants under most stress conditions experience energy shortage in addition to nutritional deficiency and darkness. The Arabidopsis kinase protein SnRK1 detects when energy is being used up and employs a complex transcriptional reprogramming process to bring the plant back into balance, enabling the plant to continue growing and metabolising [78]. Its targets include a large number of general stress-responsive genes implicated in pathogens and abiotic stress tolerance. Therefore, maintaining photosynthesis and production under stressful conditions may depend on controlling energy sensors like SnRK1. Targeted miRNAs involved in the control of growth and development as well as stress responses may provide further options [82]. Plant scientists will have a challenge in the 21st century to establish stable various stress tolerance characteristics in agronomically essential crop

plants. This will increase yields and promote global food security, particularly in regions with unfavourable climatic circumstances.

2.9 Gap Analysis

- The absence of a study and comparative analysis of transcriptome responses to drought stress in two rice cultivars (Dhagaddeshi and IR20) represents a research gap.
- Comprehending these particular pathways may offer significant perspectives for breeding initiatives targeted at enhancing drought resilience in rice cultivars with elevated yields.

2.10 Research Questions

- What are the specific molecular mechanisms underlying the drought tolerance observed in traditional Indian rice cultivars like Dhagaddeshi compared to modern high-yielding cultivars like IR20?
- Which metabolic pathways are enriched in the transcriptome data of Dhagaddeshi under drought stress, and how do they contribute to drought tolerance?

Chapter 3

Research Methodology

The method opted for this research can be noted in Fig 3.1.

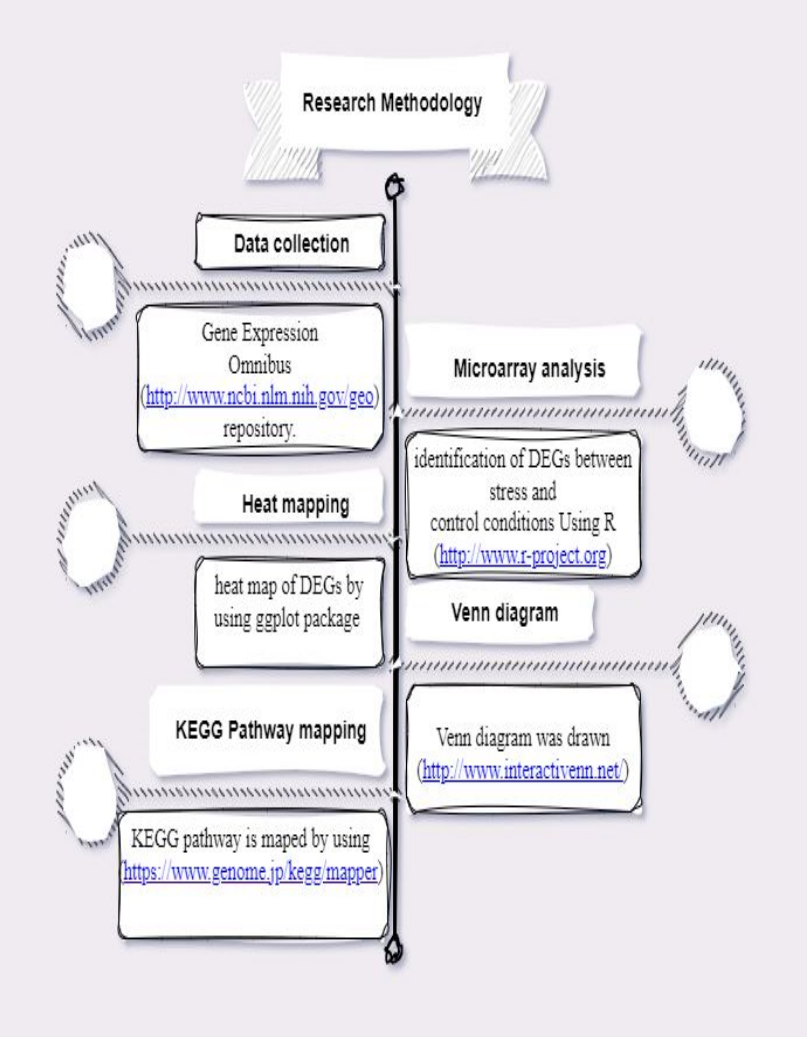

FIGURE 3.1: Research Methodology for this research project.

3.1 Data Collection

3.1.1 Source Gene Expression Omnibus (GEO)

3.1.1.1 Overview of GEO

The Gene Expression Omnibus (GEO) is a essential public repository for gene expression data, including data from microarray and next-generation sequencing experiments. Managed by the National Center for Biotechnology Information (NCBI), GEO provides access to a vast collection of genomic studies for researchers.

3.1.1.2 Search Strategy

To identify relevant datasets for *O. sativa* (rice), we employed a systematic search using specific keywords: "*O. sativa*," "abiotic stress," "gene expression microarray," and "genome." These keywords were chosen to narrow down the search to studies examining gene expression changes under abiotic stress conditions, such as drought, in rice.

3.1.1.3 Selection Criteria

We focused on datasets that clearly differentiated between stress conditions and control conditions. Only those studies that provided detailed experimental conditions and were available in CEL format (raw microarray data) were selected. This ensured the datasets were suitable for normalization and further analysis.

3.1.1.4 Access and Retrieval

The GEO repository at http://www.ncbi.nlm.nih.gov/geo was used to access selected data. Downloaded the data in CEL format, which is necessary for preprocessing steps such as normalization, quality control, and subsequent analysis.

3.2 Microarray Analysis Using R studio

3.2.1 Data Normalization Using Bioconductor 'affy' Package

3.2.1.1 Introduction to Bioconductor and 'affy' Package

Bioconductor is an open-source software project that provides tools for analyzing and understanding genomic data. The 'affy' package within Bioconductor is specifically designed for the processing and analysis of Affymetrix microarray data. It includes functions for reading CEL files, background correction, normalization, and summarization.

3.2.1.2 Normalization Process

Normalization is a critical step to adjust for systematic technical differences between arrays, which could include variations in hybridization, sample preparation, and array manufacturing. Using the 'affy' package, which applied robust multi-array average (RMA) normalization, which involves background correction, quantile normalization, and summarization of probe intensities.

3.2.2 Reducing Batch Effects and Data Integration

3.2.2.1 Combining Multiple Libraries

Batch effect refers to systematic non-biological differences between batches of data that can introduce variability and potentially confound the results of an analysis. Batch effects are a common problem in high-throughput experiments, such as microarray, RNA-seq, proteomics, and metabolomics studies. To mitigate batch effects — which are non-biological variations introduced during different experimental runs — the data is combined from multiple studies. This approach helps

in harmonizing the datasets and reducing inconsistencies that could arise from different experimental conditions or processing times.

3.2.2.2 Using Bioconductor 'RankProd' Package

The 'RankProd' package is used to identify DEGs by ranking gene expression changes. By applying 'RankProd', which increased the statistical power to detect DEGs and minimized the residual effects specific to each study.

3.2.3 Statistical Analysis for DEG Identification

3.2.3.1 P-value Calculation

Differential expression analysis involves comparing gene expression levels between stress and control conditions. To calculate p-values for each gene, identifying those with significant changes in expression. A threshold p-value of less than 0.05 was set to define statistically significant DEGs.

3.2.3.2 Detection of DEGs

The analysis resulted in a list of genes that showed differential expression in response to drought stress. These DEGs are critical for understanding the molecular mechanisms underlying stress responses in rice and can be targeted for further functional studies or breeding programs.

3.3 Heat Mapping

3.3.1 Introduction to Heat Mapping

Heat maps are a graphical representation of data where individual values are represented as colors. They are particularly useful in genomics for visualizing

large-scale data such as gene expression levels, allowing for the identification of patterns, clusters, and outliers.

3.3.2 ggplot2 Package in R

ggplot2 is a versatile and powerful data visualization package in R, designed for creating complex and multi-layered graphics. It allows for extensive customization and produces high-quality plots suitable for publication.

3.3.3 Creating the Heat Map

The ggplot2 package to create heat maps of DEGs. This involved filtering the data to include only significant DEGs and mapping their expression levels across different conditions. The heat map provides a visual summary of gene expression patterns, helping to identify genes with similar expression profiles under stress conditions.

3.4 Venn Diagram

3.4.1 Purpose of Venn Diagrams

Venn diagrams are used to show the logical relationships between different sets of items. In the context of genomics, they are useful for comparing lists of DEGs from different conditions or experiments, highlighting shared and unique genes.

3.4.2 Tool

Interactive Venn which is available at http://www.interactivenn.net, is an online tool that allows for the creation of Venn diagrams. It supports the visualization of intersections between multiple sets, making it easy to identify common and unique elements.

3.4.3 Creating the Venn Diagram

Interactive Venn is used to compare DEGs between drought stress-intolerant and sensitive rice cultivars. The resulting Venn diagram highlighted the overlap of DEGs, providing insights into the common and distinct genetic responses of the two types of cultivars to drought stress.

3.5 KEGG Pathway Mapping

3.5.1 Overview of KEGG

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive resource for understanding biological pathways, systems, and processes. KEGG pathways map molecular interactions and reaction networks, providing insights into the functional context of genes and proteins. It is a widely used bioinformatics resource that integrates genomic, chemical, and systemic functional information, and has very easy user interface.

3.5.2 Functional Orthologs

KEGG mapping involves assigning genes to functional ortholog groups within the KO (KEGG Orthology) database. These groups are based on functional equivalency rather than sequence similarity, allowing for the transfer of functional information across species.

3.5.3 Mapping DEGs to Pathways

Given the lack of direct KEGG IDs for rice, DEG genes are converted from rice to their homologs in Arabidopsis using the Plant Ensemble website available at https://plants.ensembl.org/biomart. Arabidopsis is a model organism with well-annotated pathways, facilitating the functional annotation of rice genes.

3.5.4 Using KEGG Mapper

The KEGG Mapper tool https://www.genome.jp/kegg/mapper is utilized to link DEGs to relevant pathways. This process involved inputting the Arabidopsis homologs of rice DEGs into KEGG Mapper, which provided pathway maps highlighting the biological processes and networks affected by drought stress. These pathway maps are essential for understanding the broader biological implications of DEGs and identifying potential targets for genetic intervention to improve stress tolerance.

Chapter 4

Results

4.1 Identifying DEGs Under Stress and Control Conditions

Based on the two cultivars' comparative transcriptome study and their selection for a comprehensive gene expression analysis. Furthermore, genes associated in the initial signalling reactions to a 50% decrease in water content during drought stress exposure were discovered during the 3-hour stress interval from the list of probe sets obtained after microarray data analysis in both cultivars.

Genes expressed during unstressed conditions were eliminated by comparing the highlighted genes for each cultivar during three hours of stress to the appropriate control list. After that, the list of genes that both cultivars shared during three hours of stress was further altered using the equation 4.1 to get relative fold change (RFC) values:

$$RFC = \frac{FC \cdot 3h \cdot DD}{FC \cdot 3h \cdot IR20} \tag{4.1}$$

Where:

FC stands for fold change.

Fig. 4.1 displays a volcano plot. The results of the differential expression analysis of "control vs. Drought Stress 3h, Padj¡0.05" show how the expression of genes differs in the control and drought-stressed samples after three hours. The analysis focuses on genes whose adjusted p-values (Padj) are less than 0.05, which highlights changes that are statistically significant. The plot's x-axis displays the fold difference in gene expression between the two conditions that is log2-transformed. In drought-stressed samples, upregulation is shown by positive values on this axis (greater expression), whereas downregulation is indicated by negative values (lower expression). Higher numbers indicate more importance. The y-axis shows the negative log10-transformed p-value, which measures the statistical significance of these changes.

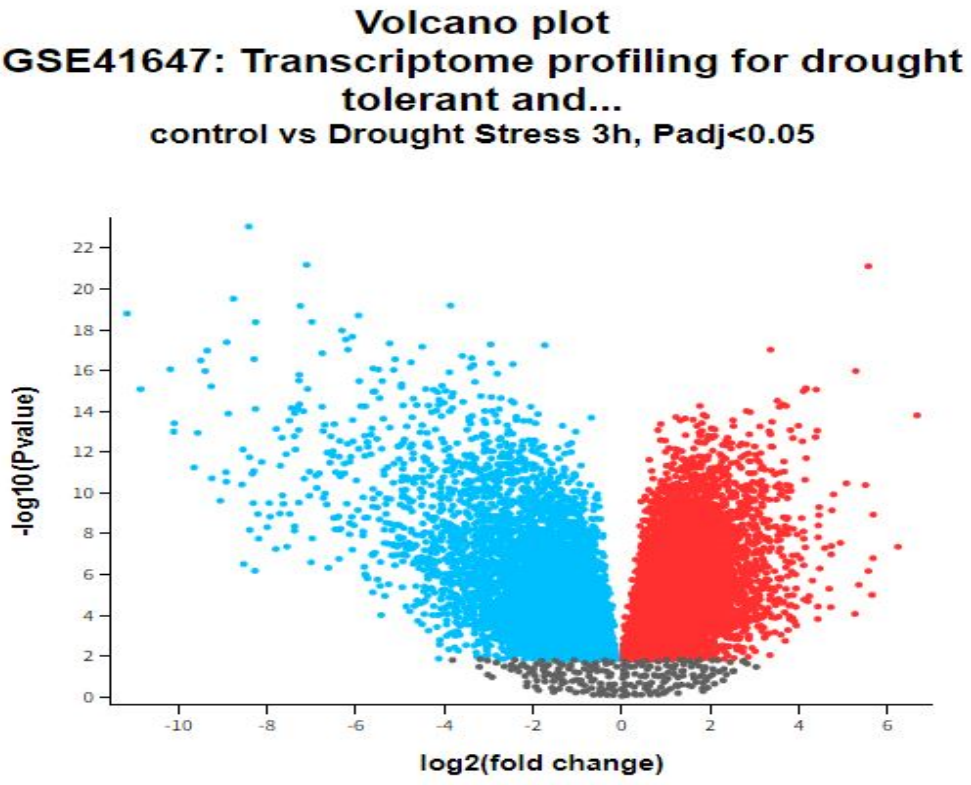

FIGURE 4.1: Volcano Plot between Control and Drought Stress 3h.

Data points on the plot, Genes that are significantly upregulated (red dots) in response to drought stress (positive log2(fold change) and Padj < 0.05), significantly downregulated (blue dots) in response to drought stress (negative log2(fold change) and Padj < 0.05), and not significantly differentially expressed (grey dots) are denoted by Padj ≥ 0.05.

This volcano plot effectively highlights genes that are significantly influenced by drought stress, either upregulated or downregulated, thereby providing valuable insights into the biological responses to this condition. The use of distinct colors facilitates quick identification of the most relevant genes for further investigation.

The Fig 4.2 shows a volcano plot, display the results of differential gene expression analysis of "Drought 6h vs control, Padj <0.05" indicates that the plot compares gene expression levels between samples subjected to drought stress for six hours and control samples. The criterion "Padj<0.05" signifies that only genes with adjusted p-values less than 0.05, indicating statistical significance, are included in the plot.

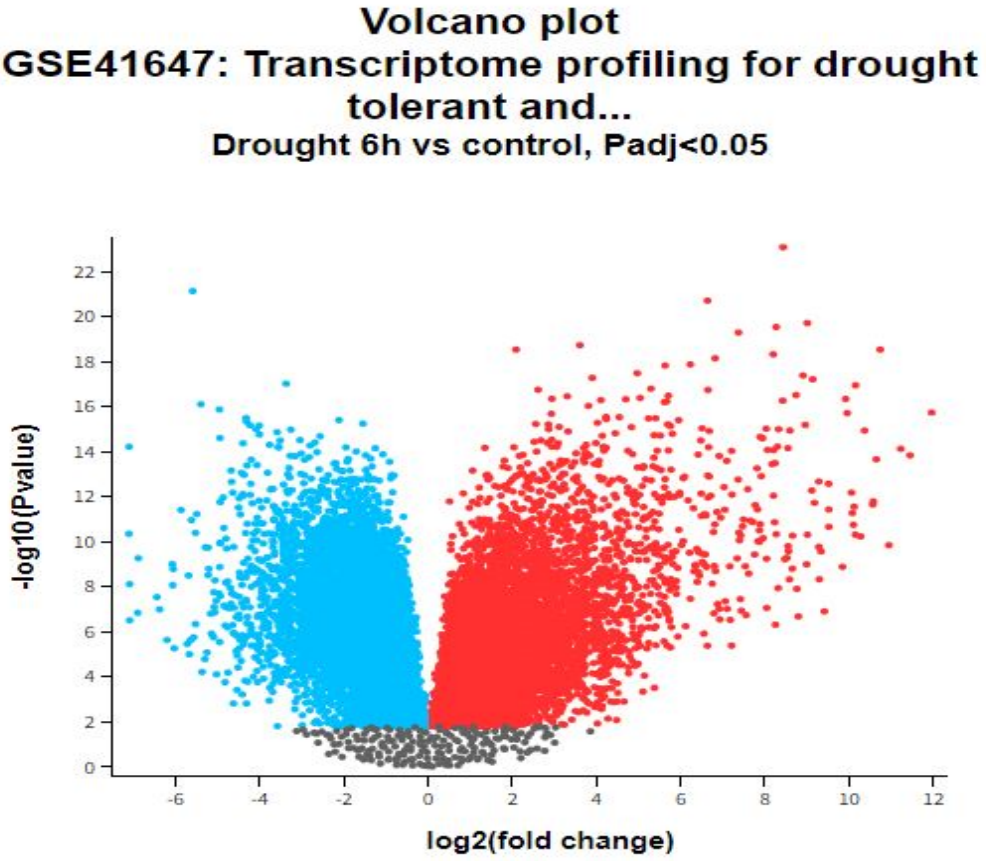

FIGURE 4.2: Volcano Plot between Control and Drought Stress 6h

The x-axis of the plot represents the log2-transformed fold change in gene expression between the two conditions. Positive values on this axis correspond to genes that are upregulated in response to drought stress, whereas negative values indicate downregulation. The y-axis depicts the negative log10-transformed p-value, which

measures the statistical significance of the changes in gene expression. Higher values on the y-axis reflect greater statistical significance.

The data points in the plot are color-coded to differentiate between significantly upregulated, significantly downregulated, and non-significantly changed genes. Red dots represent genes that are significantly upregulated in response to drought stress, as they exhibit positive log2(fold change) and meet the significance threshold (Padj < 0.05).

Blue dots indicate genes that are significantly downregulated, characterized by negative log2(fold change) and Padj < 0.05. Gray dots correspond to genes that do not show significant differential expression (Padj ≥ 0.05).

This volcano plot effectively identifies genes that are significantly affected by drought stress, either through upregulation or downregulation. The distinct color-coding facilitates the rapid identification of the most relevant genes for further biological and functional analysis.

This visualization underscores the differential gene expression dynamics in response to drought stress over a six-hour period, providing insights into the molecular mechanisms underlying the stress response.

In the gene expression analysis, a total of 18,249 genes were examined to identify regulatory patterns. The findings indicate that 10,009 of these genes exhibit down-regulation, suggesting a decrease in their expression levels under drought stress conditions. This down-regulation may be associated with specific biological processes or pathways that are inhibited or less active. Conversely, 8,240 genes are up-regulated, indicating an increase in their expression levels. This up-regulation could be linked to processes or pathways that are activated or more active under drought stress conditions. These differential expression patterns provide insights into the molecular mechanisms underlying the biological response to drought stress. Notably, the metabolic pathways associated with these up-regulated genes help the plants cope with drought stress. These differential expression patterns provide valuable insights into the molecular mechanisms underlying the biological response to drought stress.

Table 4.1 explains the expression levels of key genes involved in drought stress responses in two rice varieties, DD and IR20, under drought conditions. This differential gene expression analysis was conducted under the conditions of a three-hour drought stress period compared to control samples. The analysis revealed significant differences in gene expression between the two varieties, with DD exhibiting a more pronounced response than IR20.

TABLE 4.1: Expression levels of key genes involved in drought stress responsein DD and IR20.

Genes	**MSU Locus ID**	**Fold Change [3hDD] Vs [CtrlDD]**	**Fold Change [3hIR20] vs [CtrlIR20]**
OsDREB1A	LOC_Os09g35030	7.51	2.36
OsDREB1B	LOC_Os09g35010	7.56	9.74
OsDREB1F	LOC_Os01g73770	—	5.85
OsDREB2A	LOC_Os01g07120	14.94	3.89
OsDREB2B	LOC_Os05g27930	12.81	2.97
OsLEA1a	LOC_Os01g06630	21.26	4.87
OsLEA3-2	LOC_Os03g20680	324.57	288.03
OsLEA3-1	LOC_Os05g46480	779.56	21.13
OsLEA4	LOC_Os06g02040	258.72	26.50
OsNAC19/ SNAC1/ OsNAC9	LOC_Os03g60080	52.71	19.11
OsNAC2/ OsTIL1	LOC_Os04g38720	3.41	—
OsNAC4	LOC_Os01g60020	13.64	6.50
OsNAC5	LOC_Os11g08210	8.53	7.32
OsNAC6/ SNAC2	LOC_Os01g66120	13.33	5.75

Table 4.1: Expression levels of key genes (Continued).

Genes	**MSU Locus ID**	**Fold Change [3hDD] Vs [CtrlDD]**	**Fold Change [3hIR20] vs [CtrlIR20]**
OsNAC10	LOC_Os11g03300	183.01	31.62
OsVP1	LOC_Os01g68370	9.89	—
OREB1/ OsABI5/ OsABF1/ OsbZIP10	LOC_Os01g64000	3.18	—
OsbZIP12	LOC_Os01g64730	25.66	7.25
NAM	LOC_Os01g66120	13.33	5.75
OsRDCP1	LOC_Os04g44820	7.76	2.85
OsOAT	LOC_Os03g44150	3.01	—
DRO1	LOC_Os09g26840	3.73	—
OSISAP1	LOC_Os09g31200	6.64	—
OsNCED1	LOC_Os02g47510	4.26	—
OsNCED4	LOC_Os07g05940	601.40	309.87
OsNCED5	LOC_Os12g42280	41.99	138.42
ABA8ox1	LOC_Os02g47470	10.18	3.0273

After three hours of drought stress, DD had 10,901 probe sets expressing differently, almost twice as many as the 5,502 probe sets seen in IR20, according to the differential expression analysis, which used a threshold of $p < 0.05$ and a larger than two-fold change. This indicates that DD has a stronger early transcriptional reaction to dehydration stress.

The amount of differentially expressed probe sets after six hours of drought stress was 8,601 in IR20 and 11,041 in DD, highlighting the more substantial and prolonged transcriptional activity in DD as opposed to IR20, as DEGs reveal in (Fig 4.1, 4.2).

4.2 Gene-Specific Analysis

The table presents specific genes and their corresponding fold changes in expression levels under three-hour drought stress conditions compared to controls for both varieties. Notably, OsDREB1A (LOC_O09g35030) showed substantial upregulation in DD with a fold change of 7.51, whereas it was 2.36 in IR20, indicating a more significant response in DD. Similarly, OsDREB1B (LOC_Os09g35010) exhibited fold changes of 7.56 in DD and 9.74 in IR20, reflecting its critical role in drought stress response in both varieties.

Extremely high fold changes were observed for genes such as OsLEA3-1 (LOC_Os05 g46480), which had a fold change of 779.76 in DD and 21.13 in IR20, and OsLEA3-2 (LOC_Os03g20680), with fold changes of 324.57 in DD and 288.03 in IR20. These genes are associated with late embryogenesis abundant (LEA) proteins, which are crucial for protecting cellular structures under stress conditions.

Other significant genes include OsNAC10 (LOC_Os11g03300), with fold changes of 183.01 in DD and 31.62 in IR20, and OsNCED4 (LOC_Os07g05940), showing 601.40 in DD and 309.87 in IR20. These genes are involved in transcriptional regulation and abscisic acid biosynthesis, respectively, indicating their pivotal roles in drought response mechanisms.

Interestingly, the gene OsNCED1 (LOC_Os02g47510) showed a negative fold change of -4.26 in DD, suggesting downregulation under three-hour drought stress, while no data is available for IR20. This highlights a potential difference in the regulatory mechanisms between the two varieties.

This analysis underscores that DD exhibits a more significant and rapid transcriptional response to initial drought stress compared to IR20, as evidenced by the greater number of differentially expressed genes and higher fold changes in key stress-responsive genes. The data provides valuable insights into the molecular mechanisms underlying drought tolerance in rice, suggesting that DD may activate more robust initial stress responses within the first three hours of exposure to dehydration. These findings are critical for understanding the genetic basis of

drought tolerance and can inform breeding programs aimed at developing more resilient rice varieties.

4.3 Heat Mapping

For Heat Mapping study, all genes falling within - 2 < x > 2 RFC were chosen.

The heatmap in Figure 4.3 provides a comprehensive visualization of differential gene expression across various experimental conditions, specifically under IR2D and DD treatments. The heatmap displays gene expression levels, where the color gradient ranges from green (indicating down-regulation) to red (indicating up-regulation), with intensity corresponding to expression magnitude, scaled from 1.5 to 15.37. Columns represent distinct experimental conditions, including control (Ctrl IR2D and Ctrl DD) and treatment after three hours (3h IR2D and 3h DD). Rows correspond to individual genes.

Hierarchical clustering is employed to group genes (rows) and conditions (columns) based on expression pattern similarities. The left dendrogram distinctly categorizes up-regulated genes in red clusters and down-regulated genes in green clusters. Additionally, a zoomed-in section provides a detailed view of specific gene clusters. This hierarchical organization underscores the significant changes in gene expression induced by the treatments, facilitating the identification of key regulatory genes involved in the cellular response. This visualization method elucidates the complex gene expression dynamics, contributing to our understanding of the molecular mechanisms underlying the treatments' effects. After comparing the signal values obtained in the stressed condition with the expression values of these genes in the unstressed conditions, the lists of genes (table 4.1) that were uniquely up- or down-regulated for each cultivar at 3 hours of stress were sourced.

The Fig 4.4 presented is a hierarchical clustering heatmap, which shows the down-regulation of a specific cluster of genes under distinct experimental conditions related to drought stress. The conditions illustrated in the heatmap include control (Ctrl/IR20), 3 hours Drught stress (3h IR20), control under drought conditions

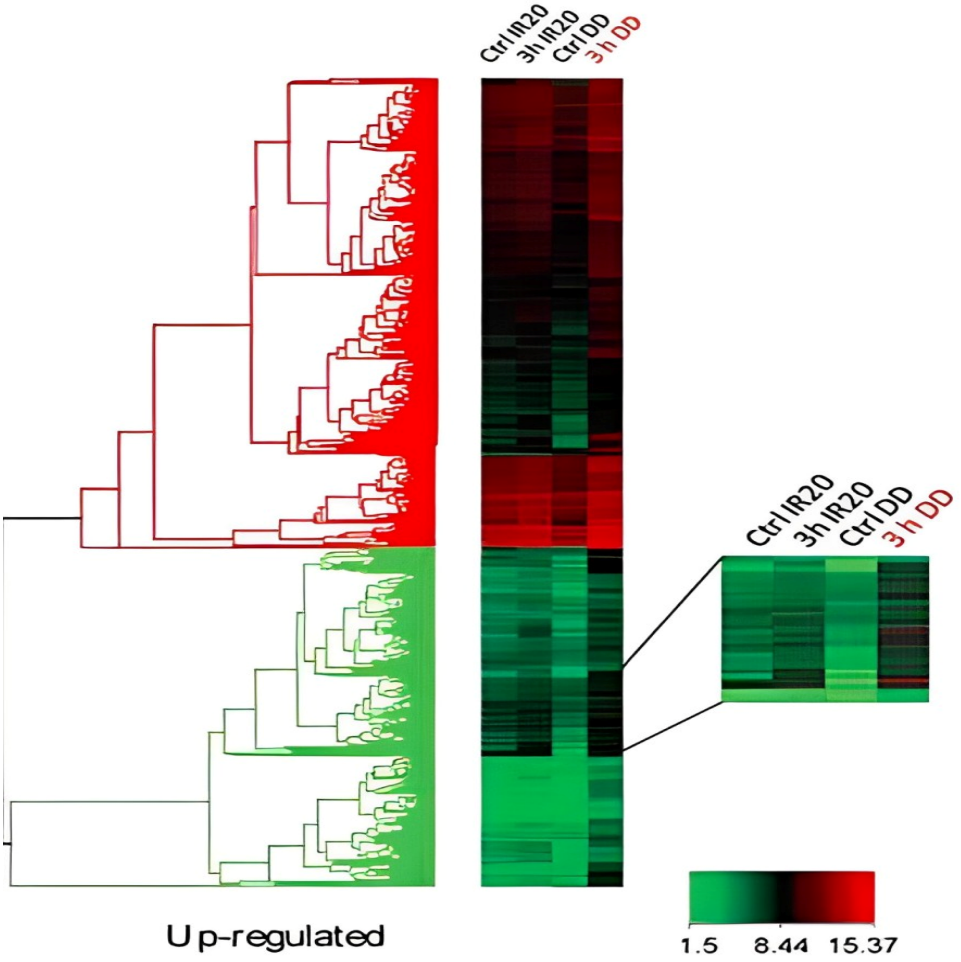

FIGURE 4.3: Genes upregulated in DD arranged hierarchically. A group of genes that are up-regulated in DD at 3 hours in comparison to IR20 are displayed in the inset.

(Ctrl DD), and 3 hours under drought/drought conditions (3h DD). Each row in the heatmap represents a gene, and each column represents a specific condition. The color intensity reflects the level of gene expression, with green indicating down-regulation and red indicating up-regulation.

The heatmap reveals a prominent cluster of genes that are significantly down-regulated in the 3h DD condition, marked in red. This specific cluster is visibly distinct from the gene expression patterns observed in the other conditions, indicating a unique transcriptional response produced by prolonged exposure to drought conditions. The hierarchical clustering on the left side of the heatmap organizes the genes based on their expression profiles across the different conditions, allowing for the identification of genes with similar expression patterns. The inset

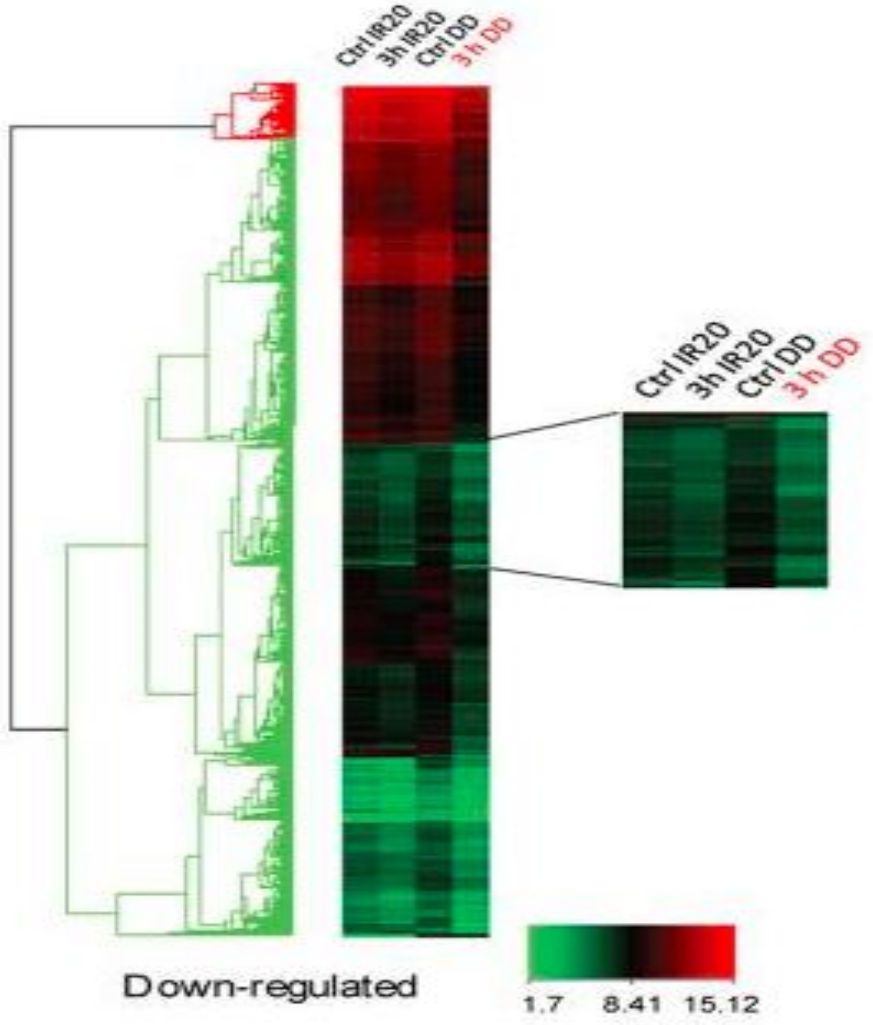

FIGURE 4.4: A group of genes that were down-regulated after three hours

magnification within the heatmap provides a focused view of the down-regulated genes, emphasizing their expression levels under the different experimental conditions. This detailed view underscores the contrast between the 3h DD condition and the other conditions, highlighting the specific genes that exhibit significant down-regulation in response to the drought environment.

The different down-regulation observed in the 3h DD condition suggests a unique molecular response to prolonged drought stress. This response may involve the suppression of specific pathways or processes that are sensitive to water deficit. Understanding these differential gene expression patterns is crucial for explaining the molecular mechanisms underlying the response to prolonged drought stress. This data provides a foundation for further investigation into the specific genes and pathways involved in the adaptive response to extended drought conditions.

The Fig 4.5 is a hierarchical clustering heatmap illustrating the expression patterns of common genes across different experimental conditions related to drought stress. The conditions showed include control (Ctrl/IR20), 3 hours Drought Stress (3h IR20), 6 hours Drought Stress (6h IR20), control under drought conditions (Ctrl DD), 3 hours under drought conditions (3h DD), and 6 hours under drought conditions (6h DD). In this heatmap, each row represents a gene, and each column represents a specific condition. The color intensity reflects the level of gene expression, with green indicating down-regulation and red indicating up-regulation. The hierarchical clustering on the left side groups genes based on similarities in their expression profiles across the various conditions, enabling the identification of genes with similar regulatory responses.

The heatmap reveals distinct expression patterns of common genes under the different experimental conditions, particularly highlighting significant changes at the 3-hour mark under drought conditions (3h DD) compared to the 6-hour mark (6h DD). The inset magnifies a section of the heatmap, providing a clearer view of these differences in gene expression. At 3 hours of drought stress, there are clear differences in the expression levels of common genes between the two cultivars, as highlighted by the variation in color intensity. These differences diminish by the 6-hour mark, indicating a more uniform expression pattern.

At the 3-hour mark under drought stress, there is a pronounced difference in the expression levels of common genes. This early response is characterized by significant up-regulation (red) and down-regulation (green) of various genes, suggesting that the plants are actively adjusting their gene expression in response to the initial onset of drought stress. By the 6-hour mark, these differences tend to reduce, resulting in a more uniform expression pattern. This indicates that the plants may be undergoing a process of adaptation or acclimation, leading to stabilization in gene expression. The reduced variability at this time point implies that the plants have adjusted to the prolonged drought conditions, resulting in a more consistent transcriptional response.

Understanding these chronological dynamics in gene expression under drought stress is crucial for explaining the molecular mechanisms of stress response and

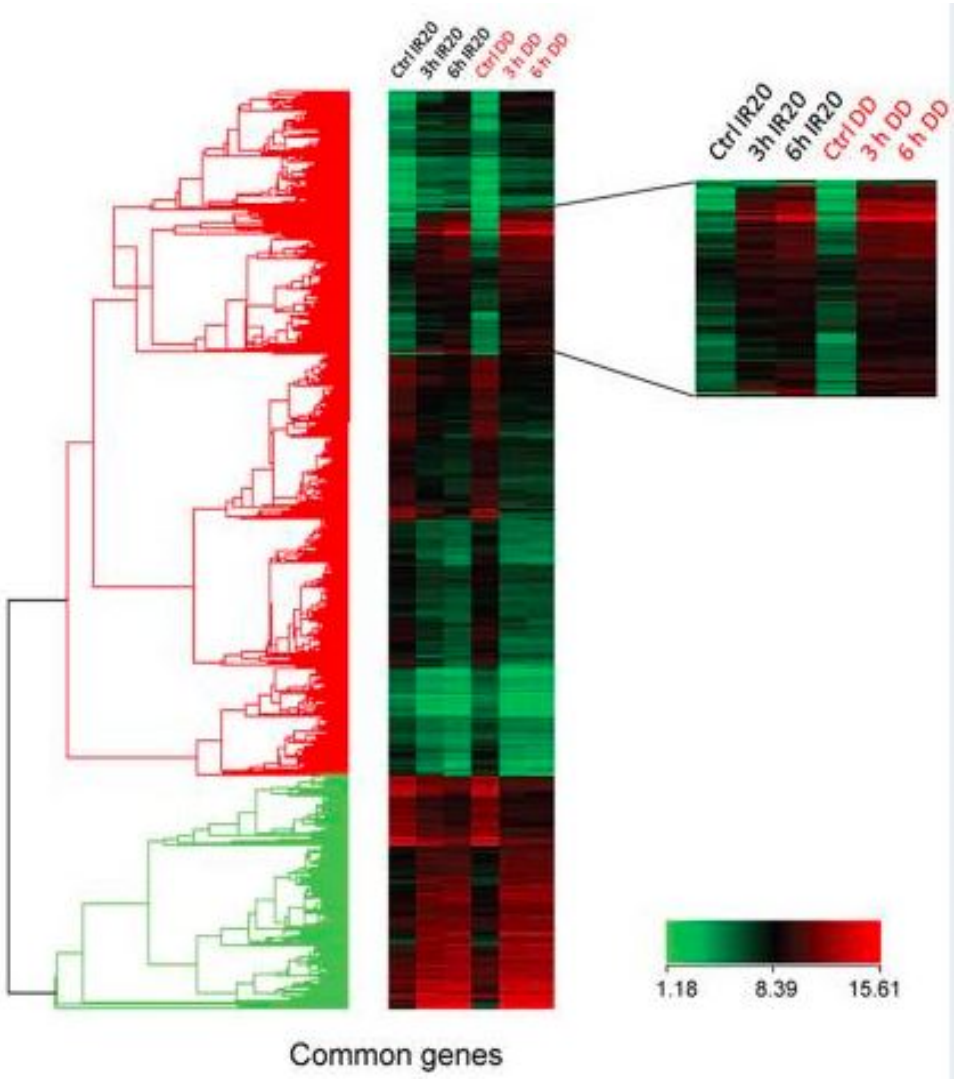

FIGURE 4.5: In both cultivars, common genes are indicated. Even for common genes, the variations in the rate of expression at three hours are displayed in the inset. But after six hours of stress, the differences disappear (third and sixth columns from the right.

adaptation. The initial significant transcriptional changes at 3 hours may represent an acute stress response, while the subsequent stabilization at 6 hours suggests an acclimation process. The hierarchical clustering heatmap provides a comprehensive view of the differential gene expression patterns under various conditions of drought stress. The different early response at 3 hours and the subsequent stabilization at 6 hours highlight the complex regulatory mechanisms that plants employ to cope with drought. These findings contribute to a deeper understanding of plant stress physiology and have significant implications for agricultural biotechnology and crop improvement strategies aimed at enhancing drought tolerance.

4.4 Venn Diagram

Venn diagrams were generated using the web application Venny v2.1 once the individual microarray data set had normalizedlised and fold change estimates were calculated. DEGs with a fold change value less than two were deemed to be downregulated, whereas those with a fold change value greater than two were considered upregulated. With a list of selected reference IDs, the online tool that displays the DEGs in Control vs. Drought stress 6h, Control vs. Drought stress 3h, and Drought stress 3h vs. Drought stress 6h in GSE41647 microarray data was completed. Venn diagrams showing the relationships between the following were made: Control, 3 and 6 hours of drought stress.

The Venn diagram (Fig 4.6) illustrates the overlap and uniqueness of gene expression changes under different drought stress conditions compared to control conditions. The three sets of conditions compared are control versus 6 hours of drought stress, control versus 3 hours of drought stress, and 3 hours of drought stress versus 6 hours of drought stress. Each circle represents a set of differentially expressed genes under these specific conditions, with the numbers indicating the count of unique and overlapping genes between the sets.

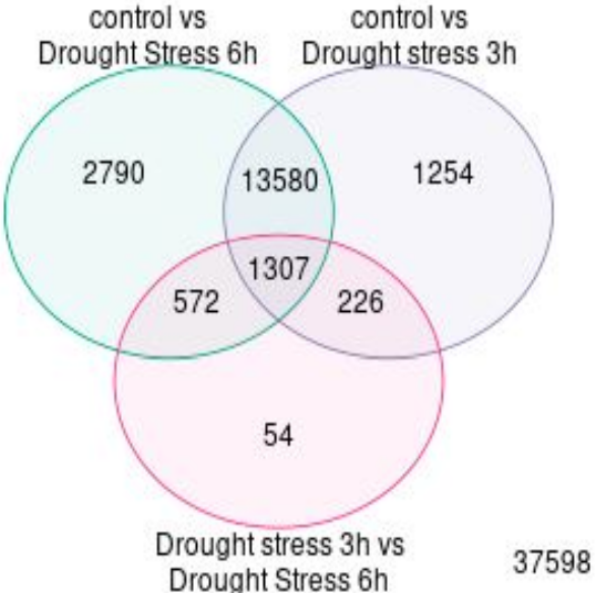

FIGURE 4.6: Venn diagrams that summarise the study of microarray data: It displays many DEG genes in rice under drought stress. For this study, only genes having a log2 fold change greater than 2 were taken into account.

The green circle represents the comparison between control and 6 hours of drought stress, highlighting 2,790 genes that are uniquely differentially expressed under this condition. This suggests that a significant number of genes respond specifically to prolonged drought stress, indicating the activation of certain stress-response pathways after an extended period. The purple circle represents the comparison between control and 3 hours of drought stress, revealing 1,254 genes that are uniquely differentially expressed in this early response phase. These genes indicate a distinct set of molecular mechanisms that are activated or repressed shortly after the onset of drought stress.

The pink circle represents the comparison between 3 hours and 6 hours of drought stress, with 54 genes uniquely differentially expressed when comparing these two time points. The small number of genes in this category indicates that while the majority of stress response genes are common to both time points, there are some genes whose expression levels specifically change as the duration of drought stress extends.

The overlap between the green and purple circles shows 13,580 genes that are differentially expressed under both control versus 6 hours and control versus 3 hours of drought stress conditions. This substantial overlap indicates a core set of genes consistently involved in the drought response, regardless of the stress duration. Additionally, the overlap between the green and pink circles includes 572 genes differentially expressed when comparing control versus 6 hours of drought stress and 3 hours versus 6 hours of drought stress. These genes may represent those that are continuously regulated as the stress persists. The overlap between the purple and pink circles shows 226 genes differentially expressed when comparing control versus 3 hours of drought stress and 3 hours versus 6 hours of drought stress. These genes might be involved in the transition phase from initial to prolonged stress response.

At the center of the diagram, where all three circles intersect, there are 1,307 genes that are commonly differentially expressed across all three comparisons. These genes likely play crucial roles in the general response to drought stress, being responsive both in the initial and prolonged phases of the stress condition.

The Venn diagram effectively illustrates the complexity of the rice plant's gene expression response to drought stress. A large core set of genes (13,580) is consistently involved in the drought response, highlighting fundamental pathways and mechanisms activated under stress.

The unique sets of genes differentially expressed at 3 hours (1,254) and 6 hours (2,790) of drought stress indicate specific responses to the duration of stress. The relatively small number of genes unique to the comparison between 3 and 6 hours of stress (54) suggests that most gene expression changes occur early and stabilize as the stress persists. This analysis underscores the dynamic nature of the plant's transcriptional response to drought and provides insights into the temporal regulation of stress-responsive genes.

4.5 KEGG Pathways Analysis

4.5.1 Metabolic Response of Drought-Tolerant Rice Under Stress

The distinct metabolic adaptations exhibited by the drought-tolerant rice cultivar DD compared to the drought-sensitive IR20 under water scarcity conditions. Microarray expression data were integrated with metabolic pathways from the Gramene RiceCyc database (version 3.3) to establish a metabolic profile based on differentially expressed genes (DEGs).

4.5.2 Metabolic Reprogramming Under Control Conditions

Analysis of DEGs revealed significant enrichment of pathways associated with amino acid degradation, carbohydrate metabolism, and biosynthesis of secondary metabolites like phenypropanoid derivatives in both cultivars under control conditions (Figures 4.7). This suggests a basal metabolic configuration geared towards stress tolerance in both genotypes.

4.5.3 Enhanced Metabolic Response in DD Under Stress

Following 3 hours of drought stress, DD displayed a more pronounced enrichment of DEGs related to amino acid metabolism, hormone biosynthesis, redox homeostasis, and secondary metabolite production compared to IR20 (Figures 4.7). This finding indicates a more robust and multifaceted metabolic reprogramming in DD to combat drought stress.

4.5.4 Metabolic Pathway Highlights

A schematic diagram (Figure 4.8) illustrates the key metabolic pathways regulated during drought stress in DD, highlighting the interconnected nature of these pathways. Notably, several pathways were shared by both cultivars, including those for the biosynthesis of ABA, jasmonate, polyamines, stachyose, and proline.

However, DD exhibited distinct responses in pathways like tryptophan, fructan, phenylpropanoid, diterpenoid, momilactone, oryzalexin C, phytocassane, chalcone, and glutathione redox biosynthesis. Additionally, transcripts for genes involved in gluconeogenesis, citrulline, and phytocassane biosynthesis were upregulated in DD but downregulated in IR20 under stress.

4.5.5 Differential Amino Acid Metabolism

Interestingly, DD displayed increased transcript abundance for genes related to tryptophan, histidine, ornithine, and γ-aminobutyric acid (GABA) under stress, while glutamate, lysine, and homoserine transcripts decreased. This suggests a shift in amino acid metabolism towards stress adaptation in DD.

4.5.6 Enhanced Redox Homeostasis in DD

Under drought stress, DD seedlings exhibited significantly higher transcript levels for genes encoding antioxidant metabolites like glutathione and ascorbate, along

with enzymes involved in their biosynthesis (glutathione peroxidase, ascorbate peroxidase 1, catalase isozyme B, etc.). These findings suggest a more efficient redox homeostasis system in DD compared to IR20, crucial for mitigating stress-induced oxidative damage. Notably, DD displayed stronger gene induction for the methylglyoxal degradation pathway leading to glutathione production, potentially indicating a more efficient detoxification mechanism.

4.5.7 Osmolyte Regulation and Carbohydrate Metabolism

The downregulation of proline dehydrogenase, an enzyme involved in proline breakdown, suggests reduced proline catabolism in DD under stress, potentially contributing to osmotic adjustment. Additionally, our study identified genes associated with the biosynthesis of fructose, sucrose, glycine betaine, stachyose, and raffinose in DD during dehydration.

4.5.8 Hormonal Regulation and Membrane Stability

Phenylalanine ammonia-lyase (PAL), crucial for plant defense responses, showed downregulated transcripts under control conditions in both cultivars. However, DD exhibited higher transcript levels for PAL after stress exposure. This suggests a potential role for PAL in DD's stress response, possibly leading to increased auxin (indole-3-acetic acid) synthesis.

Furthermore, differential expression of genes involved in the metabolism of ABA, jasmonate, ethylene, gibberellin (GA), and polyamines like spermidine and putrescine was observed. Under stress, DD displayed a more pronounced induction of genes associated with jasmonate, ethylene, putrescine, and auxin production compared to IR20, suggesting a more complex hormonal signaling network in DD.

Finally, the analysis of DEGs revealed enrichment of pathways related to fatty acid oxidation, glycolipid, and phospholipid desaturation. These pathways likely contribute to maintaining membrane stability under dehydration stress, which aligns with physiological observations of reduced ion leakage in DD under stress.

Figure 4.7 explains the regulation of metabolic pathways in Dhagaddeshi under a 3-hour drought stress period, highlighting the distinct metabolic shifts that occur between control and drought-stressed conditions. Under control conditions, several key metabolic pathways are predominantly active, including starch biosynthesis, sucrose degradation, flavonoid biosynthesis, phenylpropanoid derivatives biosynthesis, ethanol fermentation to acetate, and the degradation of methionine and phenylalanine. Starch biosynthesis is vital for the conversion of glucose into starch, providing an essential energy reserve. Sucrose degradation ensures a consistent supply of glucose and fructose for energy production and metabolic processes. Flavonoid biosynthesis contributes to UV filtration, symbiotic nitrogen fixation, and serves as antioxidants. Phenylpropanoid derivatives biosynthesis produces essential compounds for plant defense and structural integrity. Ethanol fermentation to acetate supports energy production under anaerobic conditions, while methionine and phenylalanine degradation play critical roles in maintaining amino acid balance and energy homeostasis.

In response to drought stress, there is a prominent shift in metabolic activity towards pathways that enhance stress tolerance and adaptation. Glutamate biosynthesis, fatty acid oxidation, removal of superoxide radicals, indole-3-acetic acid (IAA) biosynthesis, and the biosynthesis of polyamines such as putrescine and spermidine are significantly upregulated. Glutamate functions as an osmoprotectant and a precursor for other amino acids, aiding in nitrogen metabolism. Fatty acid oxidation becomes crucial for energy production when carbohydrate reserves are low. The removal of superoxide radicals through enzymes like superoxide dismutase (SOD) is essential to mitigate oxidative damage caused by drought-induced reactive oxygen species (ROS).

Moreover, tryptophan biosynthesis, glycolipid and phospholipid desaturation, glutathione synthesis, jasmonate biosynthesis, and phytoalexin biosynthesis are also upregulated under drought conditions. Tryptophan serves as a precursor for several vital metabolites, including auxins and alkaloids, which are important for stress response and metabolic adjustments. Glycolipid and phospholipid desaturation help maintain membrane fluidity and integrity, which is crucial for cellular

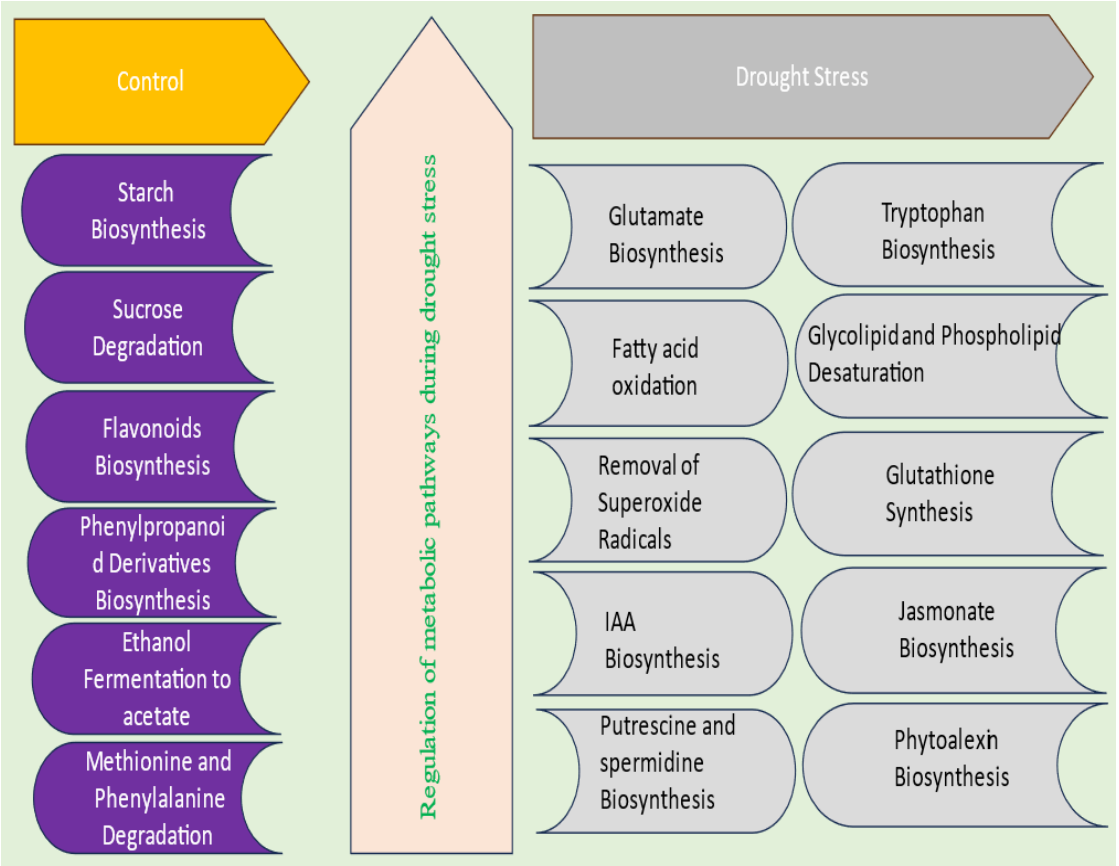

FIGURE 4.7: Regulation of metabolic pathways in response to drought stress. The metabolic pathways that are enriched in Dhagaddeshi genes that are differently expressed after a 3-hour drought stress are shown.

function during stress. Glutathione synthesis enhances the antioxidant capacity of the plant, protecting it from oxidative damage. Jasmonate biosynthesis mediates stress responses, growth, and development, while phytoalexin biosynthesis bolsters the plant's defense against pathogens.

This detailed analysis of metabolic pathway regulation underscores the plant's sophisticated and dynamic response to drought stress. By rearranging metabolic resources and activating specific pathways, Dhagaddeshi enhances its ability to survive and adapt to adverse conditions, demonstrating a well-coordinated physiological response to environmental stress. These findings contribute to a deeper understanding of the molecular mechanisms underlying plant stress tolerance, offering potential targets for improving crop resilience through genetic and biotechnological interventions.

Figure 4.8 presents an complex schematic representation of the major metabolic

pathways regulated in Dhagaddeshi under 3 hours of drought stress, highlighting the expression changes in differentially expressed genes. The pathways are depicted with arrows of varying colors to indicate the direction and extent of gene regulation: red arrows denote up-regulated genes, blue arrows indicate down-regulated genes, and black arrows represent genes with unchanged or unrepresented expression levels. This figure comprehensively maps the metabolic adjustments the plant undertakes in response to drought stress.

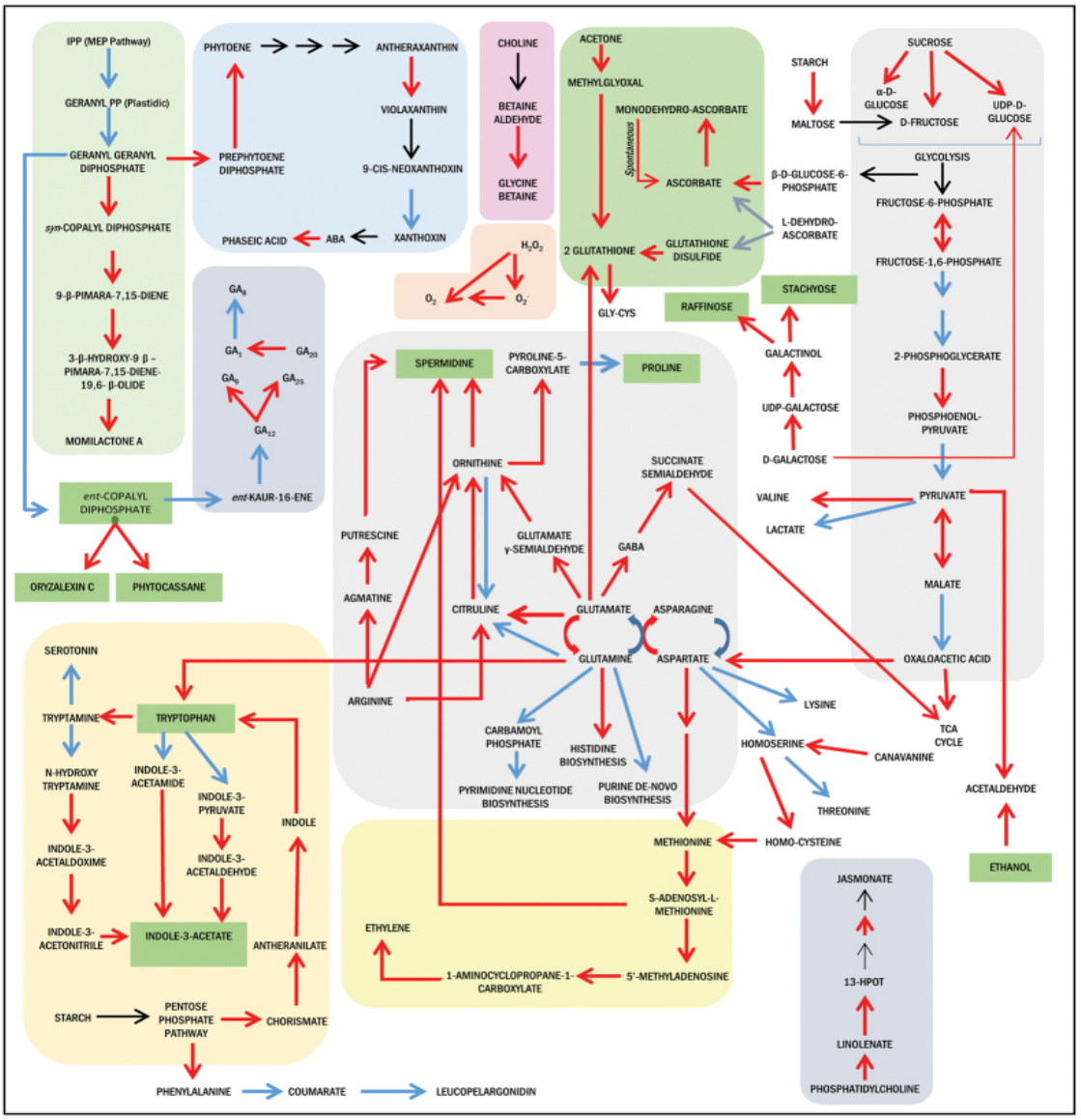

FIGURE 4.8: Diagram showing the main metabolic pathways that are controlled under drought conditions. Under three hours of drought stress, the metabolic pathways of Dhagaddeshi are enriched in genes that are differentially expressed. The expression level of gene loci is shown by arrows that are underlined in red, blue, or black; these indicate that the gene locus is up-regulated, down-regulated, or unchanged/not represented, respectively.

4.5.9 Carbon Metabolism

In the context of central carbon metabolism, starch and sucrose pathways are critical for maintaining energy homeostasis. The up-regulation of genes involved in starch degradation and sucrose metabolism, as evidenced by the red arrows, suggests an increased mobilization of stored carbohydrates to meet the heightened energy demands imposed by drought conditions. Furthermore, the glycolytic pathway and the tricarboxylic acid (TCA) cycle, which are fundamental for ATP production and the provision of metabolic intermediates, show significant up-regulation. This indicates an enhanced breakdown of carbohydrates, reinforcing the plant's energy supply during stress.

4.5.10 Amino Acid Metabolism

Amino acid metabolism, particularly involving glutamate and proline, is notably adjusted. Glutamate, a key amino acid in nitrogen metabolism and a precursor for proline synthesis, plays a crucial role during drought stress.

Proline accumulation, facilitated by the up-regulation of biosynthetic genes, functions as an osmoprotectant, helping the plant cells maintain osmotic balance. Additionally, the biosynthesis of polyamines such as spermidine and putrescine is up-regulated, reflecting their roles in stabilizing cellular membranes and protecting against oxidative damage.

4.5.11 Reactive Oxygen Species (ROS) Management

The management of reactive oxygen species (ROS) is critical under drought stress, with glutathione and ascorbate biosynthesis pathways being significantly upregulated. These antioxidants are essential for scavenging ROS, thereby protecting cellular components from oxidative stress. The up-regulation of these pathways underscores their vital role in mitigating the detrimental effects of drought-induced oxidative stress.

4.5.12 Secondary Metabolite Biosynthesis

The biosynthesis of secondary metabolites, particularly through the phenylpropanoid pathway, is differentially regulated. This pathway produces a variety of compounds, including flavonoids and lignin, which are crucial for plant defense and structural integrity.

The up-regulation of jasmonate biosynthesis further emphasizes its involvement in modulating stress responses and signaling pathways, indicating a complex regulatory mechanism that enhances the plant's ability to withstand drought stress.

4.5.13 Lipid Metabolism

Lipid metabolism, specifically fatty acid oxidation, is another crucial aspect of the plant's response to drought. The up-regulation of fatty acid oxidation genes suggests an increased reliance on lipids as an alternative energy source, particularly under conditions where carbohydrate reserves are depleted. This metabolic shift also contributes to membrane remodeling, which is essential for maintaining cellular integrity during stress.

4.5.14 Hormone Biosynthesis and Signaling

The regulation of hormone biosynthesis and signaling pathways, including indole-3-acetic acid (IAA) and ethylene, reflects their significant roles in adapting growth and developmental processes under stress conditions. The up-regulation of these pathways highlights the complex hormonal interplay that facilitates the plant's adaptive responses to drought stress. Figure 4.8 provides a detailed and comprehensive overview of the major metabolic pathways regulated in Dhagaddeshi under drought stress. The differential expression of genes, as indicated by the colored arrows, illustrates the plant's metabolic flexibility and its capacity to physiological and biochemical pathways to enhance stress tolerance. This detailed map of metabolic regulations offers valuable insights into the molecular mechanisms

underlying plant stress responses and serves as a foundation for future research aimed at improving crop resilience through targeted genetic and biotechnological interventions.

4.6 Differential Gene Expression and Abiotic Stress Signaling Networks

Plants respond to various abiotic stresses, like drought and salinity, by activating a complex and interconnected signaling network. Research has identified numerous genes encoding kinases, DREBs, NAC, WRKY, and MYB transcription factors as crucial players in stress response pathways [83]. This section explores the differential expression of these key genes in drought-tolerant (DD) and drought-sensitive (IR20) rice cultivars (Table 4.1).

Building upon existing literature and our own investigations, we examined the components of the plant abiotic stress signaling network. Studies like [83] have categorized functional and regulatory protein groups induced by drought stress. Here, we categorized genes based on prior research and known functions of their orthologs in other species. Genes with multifaceted roles were assigned to a broader "Metabolism" category. Additionally, Pfam-Bs (short peptides), DUFs (Domains of Unknown Function), and genes not fitting established categories were excluded as outliers from further analysis.

This study focuses on the upregulation and downregulation patterns observed within each category for commonly and differentially expressed genes in DD and IR20. The percentage contribution from each category is calculated and visualized in stacked graphs for interpretation. The Fig 4.9 shows a comparative analysis of the differential expression of various pathway categories in the rice variety DD relative to IR20 under unstressed conditions. This bar chart describes the proportion of genes that are upregulated and downregulated across distinct functional categories, offering a detailed insight into the baseline molecular differences between the two rice varieties.

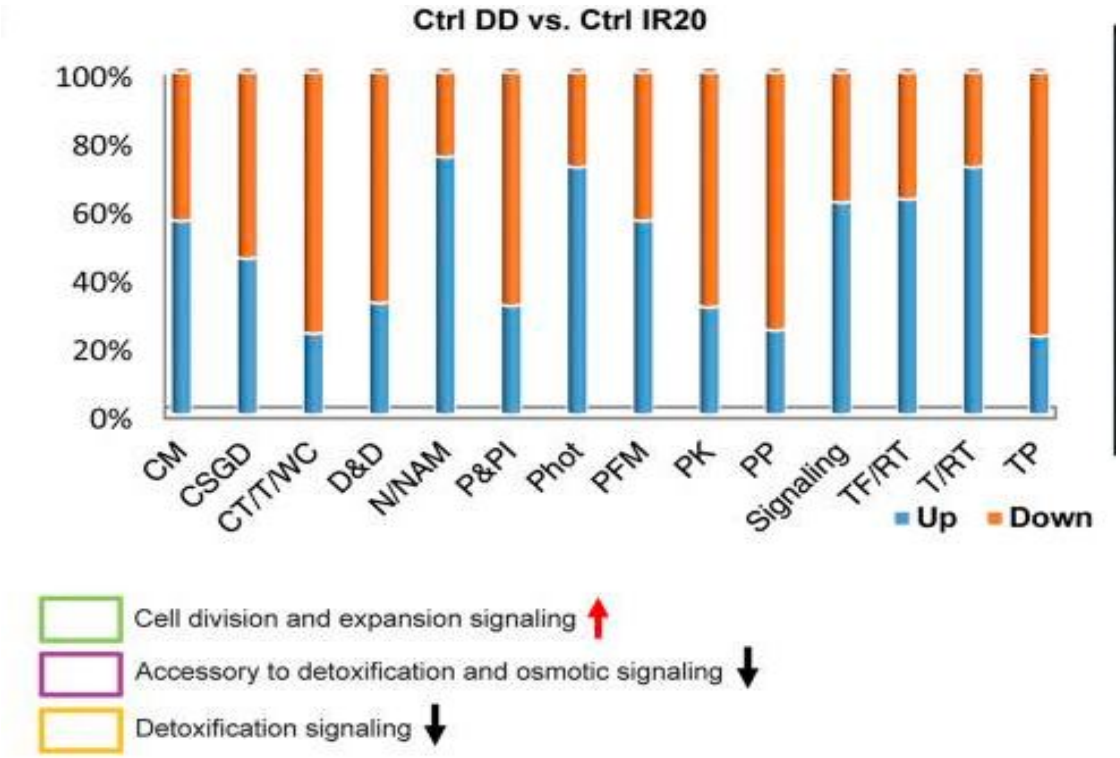

FIGURE 4.9: The percentage of the route categories in DD compared to IR20 in unstressed situations.

In the category of carbohydrate metabolism (CM), which is essential for energy production and storage, the figure reveals a slight predominance of upregulation in DD. This indicates that DD may possess a more active carbohydrate metabolism pathway compared to IR20. Similarly, in the category of cell structure, growth, and dynamics (CSGD), there is a notable proportion of genes being upregulated, suggesting enhanced cellular integrity and growth dynamics in DD.

The analysis of cellular transport, transporters, and water channels (CTT/WC) highlights a substantial upregulation in DD, implying a more robust system for nutrient and water transport compared to IR20. The degradation and detoxification (D&D) category shows a mixed response with both upregulated and downregulated genes, indicating a balanced regulatory mechanism in DD for maintaining cellular homeostasis. Nucleoproteins and nucleic acid modifiers (N/NAM) exhibit a predominance of upregulation, indicating potentially higher rates of nucleic acid processing and modification in DD. In the photosynthesis (Phot) category, there is a significant proportion of genes upregulated, suggesting that DD may have a more efficient photosynthetic apparatus compared to IR20.

Proteases and protease inhibitors (P&PI) demonstrate a balance between upregulation and downregulation, reflecting a finely tuned protein regulatory system in DD. The protection factors of macromolecules (PFM) category shows upregulation, suggesting that DD may have a better protective mechanism against cellular damage.

In the protein kinases (PK) category, crucial for signal transduction, there is notable upregulation, indicating a more active signaling network in DD. Protein phosphatases (PP), involved in dephosphorylation processes, show balanced regulation, suggesting a stable protein dephosphorylation system in DD.

The signaling category reveals upregulation, implying enhanced cellular communication and response capabilities in DD. Transcription factors and regulation of transcription (TF/RT) show significant upregulation, suggesting higher transcriptional activity in DD. In the translation and regulation of translation (T/RT) category, the data indicates a balance between upregulation and downregulation, reflecting stable translational regulation in DD. Lastly, the transmembrane proteins (TP) category shows balanced expression, indicating a stable presence of transmembrane proteins in DD.

Overall, the comparative analysis under unstressed conditions reveals that DD exhibits notable upregulation in key categories such as carbohydrate metabolism, cell structure and dynamics, cellular transport, photosynthesis, and signaling. This suggests that DD may possess a more robust baseline physiological and metabolic state, potentially contributing to its resilience under stress conditions.

These findings provide a deeper understanding of the intrinsic molecular characteristics of DD compared to IR20, offering valuable insights for further research and breeding programs aimed at improving stress tolerance in rice.

The fig 4.10 showing the performance of various pathway categories under a 3-hour stress condition, comparing two specific treatments: DD and IR20. The pathways are categorized and color-coded to denote three key types of signaling: cell division and expansion signaling (green), accessory to detoxification and osmotic signaling (purple), and detoxification signaling (orange).

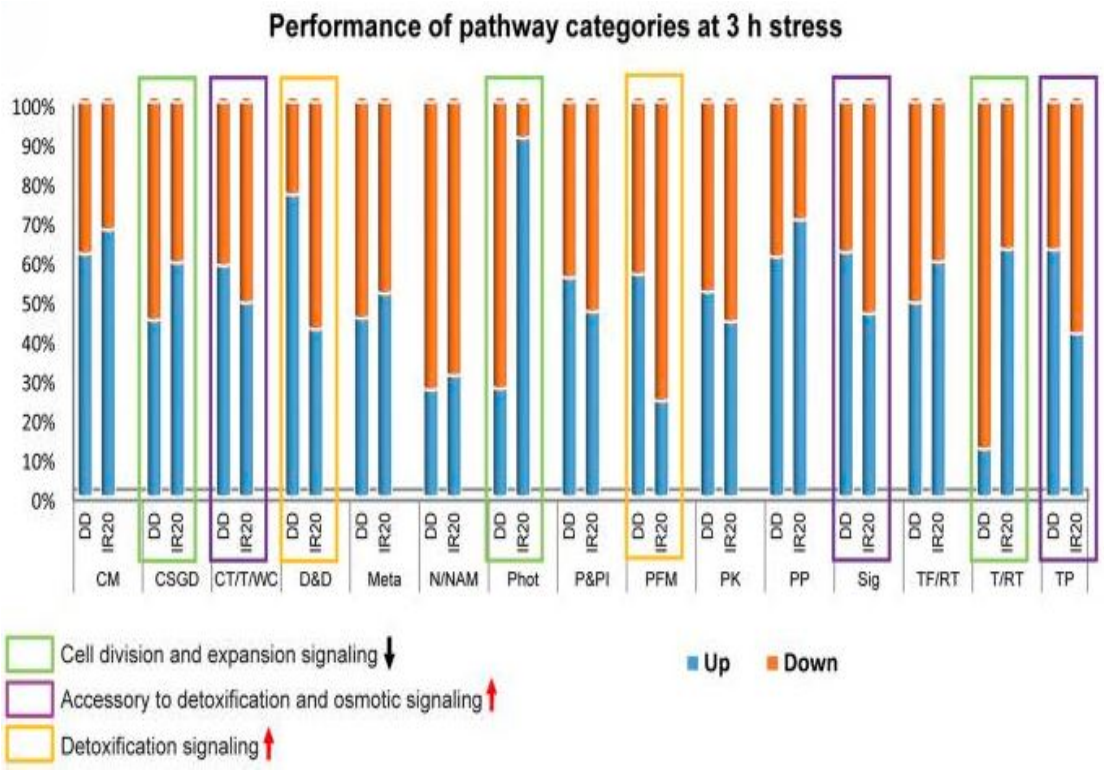

FIGURE 4.10: Graph showing stacked categories in DD and IR20

Each pathway category is represented by a set of two bars, one for DD and one for IR20, with the height of the bars indicating the percentage performance. The bars are further divided into segments showing the proportions of upregulated (blue) and downregulated (orange) genes within each category. The categories examined include CM, CSGD, CT/T/WC, D&D, Meta, N/NAM, Phot, P&PI, PFM, PK, PP, Sig, TF/RT, T/RT, and TP.

The data suggests differential gene expression patterns in response to stress, with notable variations between the DD and IR20 treatments across different pathway categories. For instance, categories such as CT/T/WC and TP, marked in purple, indicate their roles in detoxification and osmotic signaling. Meanwhile, categories like D&D and Phot, marked in orange, are primarily involved in detoxification signaling. Categories such as CSGD and T/RT, marked in green, highlight their involvement in cell division and expansion signaling. The visual representation emphasizes the intricate biological responses to stress, underscoring the complexity of the underlying molecular mechanisms.

The fig 4.11 categories of common genes expressed at 3-hour stress, focusing on

the relative expression within the DD treatment. The graph categorizes genes into various functional groups, including CM, CSGD, CT/T/WC, D&D, N/NAM, Phot, P&PI, PFM, PK, PP, Signaling, TF/RT, T/RT, and TP. Each category is represented by a single bar, indicating the proportion of upregulated (blue) and downregulated (orange) genes.

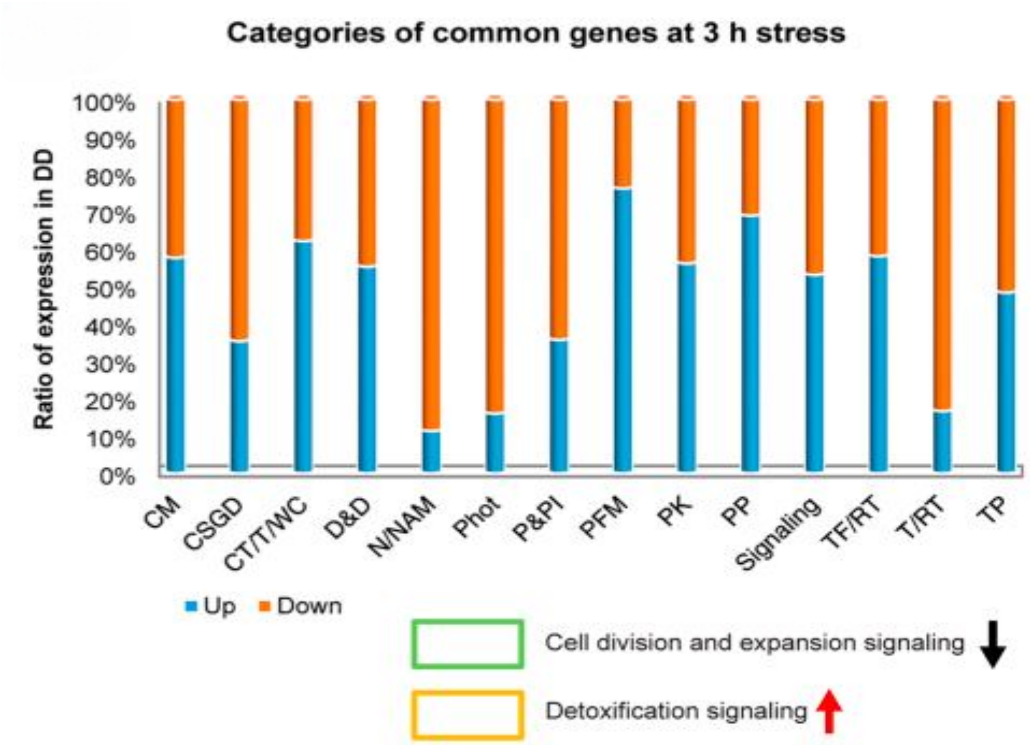

FIGURE 4.11: Different groups are created based on the relative expression of common genes in DD and IR20.

The height of each bar reflects the percentage ratio of gene expression within the DD treatment, providing insights into the dynamic changes in gene expression profiles under stress conditions. The categorization of genes into functional groups allows for a detailed understanding of the biological processes affected by stress.

In this representation, certain categories like CM and N/NAM show a more balanced ratio of upregulated and downregulated genes, suggesting a complex regulatory mechanism in response to stress. Conversely, categories such as CT/T/WC and P&PI exhibit a higher proportion of downregulated genes, indicating a suppression of specific pathways under the stress condition.

This detailed examination of gene expression ratios in different categories highlights the differential regulatory responses and potential stress adaptation mechanisms. The identification of upregulated and downregulated genes in specific functional categories provides valuable information for understanding the molecular basis of stress responses, which could inform further research into stress mitigation strategies and genetic resilience in various organisms.

The fig 4.12 show details the contributions of accessory categories involved in cell integrity and stress perception, focusing on three specific categories: lipid metabolism, secondary metabolite, and stress-induced responses. The graph compares the expression profiles of genes within these categories under two treatments: DD and IR20, providing a comprehensive overview of the molecular adjustments in response to stress.

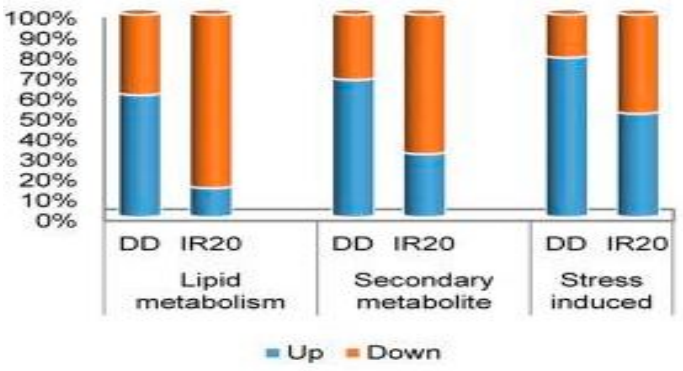

FIGURE 4.12: The roles of accessory categories in stress sensing and cell integrity.

4.6.1 Lipid Metabolism

In the lipid metabolism category, the DD treatment shows a balanced ratio of upregulated (blue) and downregulated (orange) genes. This indicates a dynamic regulation of lipid metabolic pathways, essential for maintaining cell membrane integrity and function under stress conditions. The IR20 treatment, however, exhibits a higher proportion of downregulated genes, suggesting a potential suppression of lipid metabolism pathways, which may affect the fluidity and permeability of cell membranes during stress.

4.6.2 Secondary Metabolite

The secondary metabolite category reveals a notable difference between the two treatments. Under the DD treatment, there is a predominant upregulation of genes involved in secondary metabolite biosynthesis. This upregulation highlights the role of secondary metabolites in providing protective functions, such as antioxidant activity and stress signaling. Conversely, the IR20 treatment displays a significant downregulation of these genes, indicating a reduced synthesis of secondary metabolites, which could impact the plant's ability to counteract oxidative stress and other stress-related damages.

4.6.3 Stress-Induced Responses

The stress-induced category provides insights into the immediate molecular responses to stress. The DD treatment shows a substantial proportion of upregulated genes, emphasizing the activation of stress-responsive pathways that aid in stress perception and signal transduction.

This upregulation is critical for initiating downstream defense mechanisms. In contrast, the IR20 treatment has a relatively balanced expression profile, with a slight inclination towards upregulation, indicating a moderate activation of stress-induced pathways.

4.6.4 Comparative Analysis

The comparative analysis of these accessory categories under the DD and IR20 treatments underscores the complexity of the stress response at the molecular level. The differential expression patterns reflect the specific regulatory mechanisms adopted by plants to cope with varying stress intensities and types.

The observed downregulation in the IR20 treatment across these categories may suggest a strategic suppression of certain pathways to conserve energy or redirect resources towards more critical stress responses.

4.6.5 Differential Gene Expression in Drought Tolerant (DD) and Drought-Sensitive (IR20) Rice Cultivars Under Stress Conditions

This section explores the contrasting transcriptional responses of drought-tolerant (DD) and drought-sensitive (IR20) rice cultivars under unstressed and drought-stressed conditions. The analysis focuses on gene categories associated with cellular processes critical for stress tolerance.

4.6.6 Downregulation of Growth-Related Genes in DD Under Stress

Transcript levels of genes assigned to cellular transport, transmembrane proteins, degradation and detoxification, and protein phosphatases were significantly lower in DD compared to IR20 under unstressed conditions (Figure 4.3).

Conversely, DD exhibited upregulation of genes related to cell division and expansion regulation (CDER), including nucleoproteins/nucleic acid modifiers, photosynthesis, and translation/regulation of translation (Figure 4.3). Following stress application, DD displayed a further downregulation of CDER-related genes compared to IR20 (Figure 4.5). This suggests a potential prioritization of stress response mechanisms over cell growth in DD.

4.6.7 Upregulation of Dehydration and Osmotic Signaling in DD

Interestingly, DD exhibited a marked increase in transcripts associated with dehydration signaling (DS) and osmotic signaling (OS) pathways, particularly those involved in ribosome assembly, compared to IR20 (Figure 4.4). This observation suggests a more rapid activation of stress response pathways in DD, potentially leading to faster recovery from stress.

4.6.8 Enhanced Lipid Metabolism and Damage Control in DD

Furthermore, DD displayed significantly higher expression of genes involved in lipid metabolism compared to IR20 (Figure 4.5). These genes play a crucial role in maintaining cell membrane stability under stress conditions. This finding suggests a potential advantage for DD in preserving cellular integrity during drought stress.

4.6.9 Comparative Analysis of Shared Genes

The analysis of differentially expressed genes (DEGs) identified through relative fold change (RFC) calculations provides an opportunity to compare the kinetics of gene activation/suppression for specific categories shared by both cultivars (Figure 4.5). This analysis could reveal variations in the timing and magnitude of transcriptional responses between DD and IR20.

4.6.10 Prioritization of Cellular Protection in DD

Consistent with the previous observations, categories like cell structure, growth and dynamics (CSGD), nucleoproteins/nucleic acid modifiers (N/NAM), photosynthesis, and translation/regulation of translation (T/RT) displayed significant downregulation in DD compared to IR20 under stress conditions (Figure 4.5). Conversely, genes associated with protection factors for macromolecules (PFM) were upregulated in DD. This suggests a strategic adjustment in gene expression by DD, prioritizing cellular protection and stress response mechanisms over growth processes under stress.

4.6.11 Differential Signaling Network Kinetics

The observed differences in gene expression patterns between DD and IR20, under both stressed and unstressed conditions, as well as the analysis of shared DEGs

(Figure 4.5), can be attributed to potential variations in the underlying signaling network kinetics. These variations likely play a key role in determining the contrasting drought tolerance phenotypes observed in the two rice cultivars.

Chapter 5

Discussion

The subcontinent is home to a vast array of native rice cultivars with innate resistance to both biotic and abiotic stressors. But these cultivars also have the unfavourable characteristic of having poor yields, while the stress-sensitive cultivars have great yields. Transcriptome data for N22 and IR64 cultivars are readily accessible in public sources, and several research have been conducted with these. Some native cultivars, nevertheless, have not received as much research. To better understand the molecular machinery at work in these uncharted rice cultivars and to comprehensively analyse the metabolic network associated with stress, this study performed the comparison the transcriptome examination of Dhagaddeshi and IR20 rice varieties using microarray technology according to the control and drought-related stress at the seedling stage. To do a comprehensive analysis of the nature of the pathways operating under drought stress in these cultivars, the GO classifications were first extended to include the previously identified signalling networks [83].

Our microarray study demonstrated that the amount of expression of genes expressing elements implicated in the *DS* as well as *CDER* smaller networks was dramatically altered in the various cultivars under three hours of drought stress. The components of the *DS* networking were activated in Dhagaddeshi sooner than in IR20 in response to the induced dehydration stress, and the processes controlling cell growth and expansion were stopped more rapidly in DD than in IR20.

DD reacted to dehydration at the molecular level by upregulating genes that encode proteins (e.g., LEA, DREBs, NACs); enzymes involved in the metabolism of carbohydrates; and antioxidants that scavenge reactive oxygen species (ROS).

According to the theory, disruptions in the ionic and osmotic balance under situations of salt and drought stress activate the networks that help plants achieve ionic and osmotic homeostasis and stress tolerance. Injuries brought on by water stress can cause the *DS* and *CDER* networks to become active simultaneously. Damage control and repair are brought about by the DS network, whereas growth inhibition is the outcome of activating the *CDER*.

All of these networks collaborate closely to reduce stress, and because of their complex nature of how they operate, a significant number of their regulators overlap, which makes it very challenging to identify the main actors with clarity. Nonetheless, our findings revealed a noticeable shift in the regulation of the genes expressing several detoxification network components after three hours of stress.

Despite this, the expression data obtained at this specific moment between the two genotypes was nearly identical, considering that they had lost more than 70% of their water content after a 6-hour dry stress. Therefore, it made sense to disregard the information gathered at this specific time.

Consequently, it was shown that DD seedlings were able to identify drought stress significantly sooner than IR20. They responded by altering the expression of genes related to growth control and turning on the signalling networks for detoxification as well as osmotic regulation, which helped them build resistance to the current drought stress long before IR20.

These findings corroborate other findings that resistant cultivars of N22 and Pokkali had greater expression levels of genes related to antioxidant defence and cell wall remodelling compared to susceptible cultivar IR64. Because high levels of early responsive to dehydration (*ERD1*) is known to be rapidly activated during drought stress, our idea that DD seedlings sense and respond to dehydration stress more quickly is corroborated by this preferential expression.

It was found that DD accumulated anthocyanins (observed phenotypically) even under control conditions (data not shown). The transcript levels of leucoanthocyanidin dioxygenase (*LDOX*), a gene essential for proanthocyanidin and anthocyanin synthesis in Arabidopsis, were shown to be greater in unstressed DD seedlings. It has been shown that plants with high levels of anthocyanins increase in response to cold stress and drought, and that these plants are more drought-tolerant. This lends credence to the belief that Dhagaddeshi is naturally equipped to counteract drought conditions and react swiftly.

Both the tolerant and susceptible cultivars shared a large number of previously known genes associated to drought stress, as shown in Table 4.1, although their expression levels differed greatly. Our findings support earlier studies on the role of TF-encoding genes in the abiotic stress response, including *OsDREB, OsLEA, OsWRKY, OsMYB, and OsbZIP. During a 3-hour water deficiency stress, the expression of several genes, including OsDREB2A, OsDREB2B, OsLEA3-1, OsLEA4, SNAC1, SNAC2, OsNAC4, OsNAC10,* and *OsbZIP12*, was much greater in DD than in IR20. The D/D signalling pathway's hitherto unidentified component, *OsFBK1*, was discovered to be unique to IR20. Transgenic plants lacking *OsFBK1* were shown to be more resistant to prolonged ABA treatment as compared to untransformed plants.

Although the plants with excessive expression were also shown to impart some tolerance, the knock-down or mutant lines outperformed the control group. Several other non-stress studies have already documented the same responses of excessive expression as well as functional loss lines. The *OsFBK1* transgenics have also been shown to have a variety of phenotypic changes. The transgenic rice cultivars that excessively express *OsDREB2A* and *OsDREB2B* have been shown to be more drought tolerant and to have greater amounts of free proline and soluble sugars in their seedling stage.

According to our research, both cultivars showed increased transcript levels of the genes raffinose and stachyose, which are involved in the production of soluble sugars. Similarly, it was discovered that both cultivars accumulated free proline similarly when there was a water shortage. Additionally, LEA proteins stabilise

membranes and other proteins. Compared to IR20, DD seedlings demonstrated lower ionic leakage or greater membrane stability under stress. It's interesting to note that *OsLEA3-1* cDNA is selectively induced by ABA, and excessive expression of the associated gene improves drought tolerance in rice. It was also demonstrated that *OsLEA4* (FC = 258.7 vs control) and *OsLEA3-1* (FC = 779.7 vs control) had considerably higher expression levels in DD. *OsNAC2, OsVP1, OsABI5/OsABF1/OsbZIP10, DRO1*, and *OSISAP1* were all overexpressed in DD alone. *OsFBK1*, a gene specific to IR20, has dehydration kinetics that illustrate how DD responds to stress more quickly than IR20. This suggests that DD has a cultivar-specific mechanism that governs signalling and metabolic pathways differently in DD than in IR20.

Remarkably, *OsFBK1*'s functional validation in the vulnerable cultivar PB1 shows that IR20 has genes whose expression may be adjusted to bring about stress tolerance. However, compared to the tolerant DD, the overall number of these genes activated under pressure is noticeably lower, indicating that IR20 lacks the defence mechanisms required to withstand stressful environments. Therefore, transcriptome analyses like ours may act as a roadmap for subsequent trials and applications aimed at producing stress-tolerant cultivars for marketable purposes, as users may utilise this knowledge to make informed decisions when choosing potential genes from either cultivar. The phytohormone ABA also induces most, but not all, of the genes that are inducible by dehydration. In rice, five genes—*OsNCED1* through 5—have been found.

According to our findings, dehydration caused the genes involved in ABA production to be activated in both cultivars. On the other hand, transcript levels for the ABA biosynthesis-related OsNCED4 (LOC_Os07g05940) gene (after normalisation with corresponding control tissues) were nearly twice as high in DD (> 600 FC Vs Control) as they were in IR20 (> 300 FC Vs control). It was discovered that salt stress significantly up-regulated OsNCED4 transcripts. *OsNCED5* (LOC_Os12g42280) exhibited > 3

Relative FC increase in IR20 (> 100 FC vs control) in contrast to other ABA biosynthesis genes when compared to DD. It has been documented that *OsNCED5*

functions as an osmotic sensor and is stimulated by elevated glucose levels.

Under conditions of dehydration stress, accumulation of glucose, fructose, and sucrose has also been reported. which, in our data, shows an up-regulation of the genes encoding the enzymes involved in the breakdown of starch, sucrose, and glucose. Moreover, it was shown that DD is the only condition in which *OsNCED1* (LOC_Os02g47510) levels are down-regulated. Additionally, it was discovered that DD had greater transcript levels of *OsABA8ox1* (LOC_Os02g47470), a gene implicated in ABA inactivation, than IR20. Under extended drought stress, plants may benefit from the reduction in ABA levels caused by *OsABA8ox1*'s breakdown of ABA. Reduced ROS levels may also be partially attributed to the activation of this gene. As a result, the *OsNCED* gene transcript levels decreased and stabilised at the same time as the estimated total ABA content, which peaked at three hours of stress. It also explains why, in DD seedlings under stress, ABA levels grow steadily but in IR20, ABA levels rise quickly.

Given the number of differently expressed genes and their expression levels, it seems that DD also has unique regulation of ABA-independent drought signalling network pathways. There were significantly higher transcript levels of *OsLEA3-1, OsLEA4* and *OsLEA1a*, the genes directly influenced by the ABA levels, as well as indirectly controlled *OsDREB* genes within DD.

Our findings suggest that, at least in the early stages of drought stress imposition, solely ABA-dependent pathways might be primarily controlled in IR20. Dehydration stress damages cellular structures by increasing the production of reactive oxygen species (ROS) and ROS-related peroxidation reactions.

The balance between the creation and scavenging of ROS is crucial for maintaining the amount of ROS, which is important for cellular signalling. Therefore, the tissue's capacity to adjust to the energy imbalance brought on by elevated ROS levels is reflected in the kinetics of ROS detoxification.

According to a genome-scale metabolic pathway study, genes involved in the production of antioxidant agents and metabolites are up-regulated in DD. Some metabolic pathways, such as the ascorbate glutathione cycle, glutathione-dependent

redox activities, and genes involved in removing superoxide radicals, were expressed more in DD after three hours of dehydration stress. Other phytoalexins, such as momilactone, oryzalexin, and phytocassanes, may also quench ROS, which could explain their production in response to different biotic and abiotic stressors.

Reduced violaxanthin de-epoxidase (LOC_Os04g31040) and elevated zeaxantin epoxidase 1 (LOC_Os04g37619) are important for maintaining plant redox homeostasis and controlling the xanthophyll cycle. These genes' transcript levels also suggest a rise in reduced ascorbate levels and a faster loss of NADPH, which is a decreasing equivalent.This favours the preservation of lower levels of oxidative stress in individuals with Down syndrome. A higher sugar content, such as glucose, also aids in the production of ascorbate, an antioxidant metabolite that quenches reactive oxygen species (ROS).

Additionally, sugars may directly scavenge ROS54. It is discovered that oxidative, abiotic, and amino acid deprivation conditions all trigger the tryptophan production pathway. Additionally linked to stress tolerance, ROS scavenging, and carbon-nitrogen balance is GABA metabolism. Several WRKY family TFs have been shown to be influenced by salicylic acid (SA), jasmonic acid (JA), and ABA. These TFs confer resistance against infections caused by bacteria in rice by stimulating the production of PR proteins and the previously mentioned phytoalexins, such as momilactones, oryzalexin, and phytocassanes. Our data indicate that just two among the WRKY TF-encoding genes had been particularly increased in DD, while eight of those genes were shared by both cultivars.Since this family of TFs is well recognised for its function in mediating biotic and abiotic stressors, it also suggests that, in contrast to IR20, DD's resistance to drought stress may be mostly attributed to ROS-mediated redox pathways.As was previously reported, Components of the detoxifying signalling system were active in DD earlier than in IR20.

These included glutathione as a conjugation in antioxidants defence responses and redox metabolites. Our study revealed that certain glutathione-S-transferases, notably those belonging to the Detoxification Signalling group, are specifically regulated in DD. Of these, 7(5.6%) were common GST-encoding genes in DD as

well as shared by both cultivars, 28(8.3%) were up-regulated, and 11(10%) were down-regulated. It is important to remember that ABA, JA, auxin, and abiotic stressors can all affect these GSTs. It has also been found that the over-expression of GST-encoding the genes improves stress tolerance in tobacco transgenic and Arabidopsis plants. Interestingly, the transcripts of some auxin-responsive genes (log2 FC $>$ 10, RiceXPro Version 3.0) were notably up-regulated in DD, while the majority of ABA-sensitive genes were expressed similarly in both cultivars. excluding from GSTs. Numerous genes linked to the metabolism of auxin and indole were also found by pathway analysis (Fig 4.7 and 4.8). Auxin's complicated involvement, which may entail interaction with ROS and redox pathways, has been suggested by functional genomic investigations, even if its exact function under abiotic stressors is yet unknown. Actually, oxidative stress can cause alterations in the distribution and concentration of auxin in Arabidopsis seedlings, leading to a wide range of auxin-like actions.

The pathway that converts glucose to methylglyoxal and then to pyruvate is known as the methylglyoxal route. It is a byproduct of glycolysis. is an important detoxification process controlled by glutathione. The formation of methylglyoxal is cytotoxic and does not result in the creation of ATP. It is eliminated by the glyoxalase pathway. Two enzymes make up this detoxification route. The first one uses glutathione to create an intermediate, and the second one regenerates it in the next process. According to our research, DD had much higher levels of methylglyoxal pathway-related genes than IR20 did, suggesting that the tolerant cultivar activated the detoxification system sooner and more selectively (Fig 4.8). Thus, it is plausible to suggest that the ABA, JA, auxin, and redox pathways interact to form the first line of defense in Dhagaddeshi, with ROS serving as input signals to initiate the osmotic stress and detoxification signaling.

Chapter 6

Conclusion and Future Work

Eventually, the conclusion that can be drawn is that the DD cultivar is likely drought tolerant because of its quicker sensing mechanism and kinetics, which enable it to successfully activate its detoxification signaling network. This network involves both ABA-dependent as well as independent pathways, including JA, auxin, and ROS signaling. The significant number and functional characteristics of DEGs following three hours of stress suggest that, although some genes specific to IR20 may contribute to drought tolerance, the response to stress by DD seems to be an evolved mechanism to cope with water deficit stress. This response is likely modulated by the protection of cell organelles and membranes from oxidative stress, along with the simultaneous induction of stress-responsive genes. While focusing solely on ABA-inducible genes could lead to heightened stress responses, it is crucial to have strong control over both ABA-dependent and ABA-independent pathways to enhance rice's ability to adapt to drought. It is believable that over time, native rice cultivars resistant to drought have encouraged these pathways, leading to more rapid stress adaptation.

Furthermore, the information gathered from this study may be utilised to pinpoint genes (from both cultivars) that may be altered genetically to increase or create the drought resistance of the vulnerable but highly productive rice cultivars.

Our research also demonstrates the need for caution and likely informed decision-making when choosing genes from different cultivars to increase agricultural plants'

ability to respond to stress, as stress-effective genes may be found within either parent instead of just in the tolerant cultivar. A portion of these genes that correspond to the identified DEGs may be verified and applied to marker-assisted breeding to give stress resistance to vulnerable yet highly productive rice cultivars.

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