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Modulation of stress response in Rice via genome editing of splicing factors

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Modulation of Stress Response in Rice via Genome Editing of Splicing Factors

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Abstract

Serine/arginine-rich (SR) proteins are a conserved family of RNA-binding proteins that act as key modulators of alternative splicing. While their functional relevance in plants remains largely unknown, a mounting evidence suggests a central role for these proteins in the response to various stresses. The work presented in this thesis sheds light on the functional significance of OsRS29 and OsRS33, two SR (arginine/serine subfamily) genes in rice as a model.

In this study, genome editing using CRISPR/Cas9 system followed by RNA-seq were utilized to target two splicing factors in rice, Os-RS29 and Os-RS33 to examine the transcriptome-wide effects of these double mutants before and after salt (NaCl) treatment. Functional enrichment of the differentially expressed transcripts as well as the differentially spliced genes was done to further understand how plants are affected by salt-stress and the interplay between stress and alternative splicing. Under normal growth conditions, when compared to the wild type, the differentially expressed genes in heterozygous Os-RS29 / homozygous Os-RS33 double mutant were enriched in oxidation-reduction processes, response to stress and various plant hormone signaling pathways. On the other hand, the homozygous Os-RS29 / homozygous Os-RS33 double mutant, showed a greater impact on the expression of many genes involved in biotic and abiotic stress responsive as well as mRNA modification. After salt treatment (250mM), both double mutants displayed significant down-regulation of critical salt responsive genes rendering a sensitive response towards salt stress especially in the homozygous Os-RS29 / homozygous Os-RS33 double mutant.

Since the double mutants involved two members of splice factor proteins, the alteration in the landscape of constitutive and alternative splicing (AS) was investigated. The homozygous Os-RS29 / homozygous Os-RS33 double mutant showed more decrease in the total no. of AS events than the heterozygous Os-RS29 / homozygous Os-RS33 double mutant and the wild-type. Upon exposure to salt stress, however, the no. of AS events increased dramatically in the homozygous Os-RS29 / homozygous Os-RS33 double mutant compared to the other double mutant heterozygous Os-RS29 / homozygous Os-RS33 and the wild-type. The isoform shifts under different growth conditions suggest that Os-RS33 and Os-RS29 mediate stress responses via modulating the splicing of various salt stress-responsive genes. Among the genes that showed an altered splicing the homozygous Os-RS29 / homozygous Os-RS33 double mutant are the

Eukaryotic Initiation Factors (eIFs) along with some mRNA processing and splice factors. These factors were found to interact with a number of WD40-repeat proteins whose expression is changed after salt treatment.

In conclusion, transcriptomic analyses of the two double mutants showed that both splicing factors play important roles in regulating various stress responses during early plant development. Further investigations of the roles of tandem repeat domain proteins in stress will provide more understanding of the mechanisms by which the plant responds to various stresses.

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List of Abbreviations

CS	Constitutive Splicing
AS	Alternative Splicing
RNP	Ribonucleoprotein
snRNP	Small nuclear ribonucleoprotein
pre-mRNA	Precursor messenger ribonucleic acid
5′ and 3′ SS	5′ and 3′ splice sites
SR	Serine/Arginine-rich
U2AF	U2 auxiliary factor
BPS	Branch point sequence
IR	Intron retention
CE	Cassette exon
MXE	Mutually exclusive exon
A5SS	5′ Alternative splicing
A3SS	3′ Alternative splicing
AFE	Alternative first exon
ALE	Alternative last exon
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
hnRNPs	Heterogeneous nuclear ribonucleoproteins
PTC	Premature termination codons
NMD	Nonsense-mediated decay
RBPs	RNA-binding proteins
RRM	RNA recognition motifs
RS	Arginine/Serine-rich
ESR	Exonic splicing regulators
ISR	Intronic splicing regulators
ABA	Abscisic acid

ROS	Reactive oxygen species
VHA	Vacuolar type H^+-ATPase
HKT	Histidine kinase transporter
NHX	Na^+/H^+ antiporters
JA	Jasmonate
ET	Ethylene
RLKs	Receptor-like kinases
TFs	Transcription factors
TPR	Tetratricopeptide repeats
ANK	Ankyrin
PPR	Pentatricopeptide repeats
LRR	Leucine rich repeats
GA	Gibberellin
SRWD	Salt Responsive WD40 repeats
CRISPR	Clustered regularly interspaced short palindromic repeat
Cas9	CRISPR-associated protein 9 nuclease
gRNA	guide RNA
sgRNA	single guide RNA
crRNA	CRISPR RNA
tracrRNA	trans-activating CRISPR RNA
PAM	Protospacer-adjacent motif
DSB	Double strand break
NHEJ	Non-homologous end-joining
ORF	Open reading frame
HDR	Homology-directed repair
PTG	Polycistronic tRNA-gRNA gene
RNA-seq	RNA-sequencing
TPM	Transcript per million reads mapped
DETs	Differentially expressed transcripts
DSG	Differentially spliced genes
FDR	False rate ratio

CARMO	Comprehensive Annotation of Rice Multi-Omics
ASATP	Alternative Splicing Analysis Tool Package

Chapter 1: Introduction

1.1.Abiotic stress in plants

Plants are continuously subjected to and strongly influenced by changing environmental conditions and they have to adapt to various types of stresses. These unfavorable stresses include biotic stress, like pathogen infection and herbivore attack, as well as abiotic stress, such as salinity, dramatic temperature changes, drought, nutrient deficiency, and increase of soil toxic metals like cadmium, aluminum and arsenate. Abiotic stress factors, especially salinity, drought, and extreme temperatures, are the main cause of crop loss around the world, reducing the productivity for most major crop plants -up to 50% by 2050- , thus threatening food security. [62][63]

Unfortunately, the undesirable effects of these abiotic stresses are expected to be aggravated by climate change, and are predicted to be more extreme and less predicted in the future. It became inevitable to prevent substantial crop yield loss especially in the face of a fast growing world population, which constitutes an enormous pressure on humans to produce 70% more food crop to feed an additional 2.3 billion people by 2050 worldwide [64][65][66][67]. Consequently, a major goal in plant science is to investigate and understand the underlying mechanisms by which plants respond to and survive environmental stresses efficiently. Abiotic stresses trigger several genetic, biochemical, molecular, physiological and morpho-anatomical changes as well as adaptive responses that affect various cellular and whole-plant processes [68]

1.2. Constitutive and alternative splicing in plants

Most of the eukaryotic genes consist of coding sequences (exons) interrupted by stretches of non-coding sequences (introns). During transcription, precursor messenger ribonucleic acids (pre-mRNAs) transcripts are produced. By means of splicing, an essential step in eukaryotic gene expression, introns are removed and the flanking exons are ligated together to form the mature mRNA. Multi-exon genes are transcribed into pre-mRNAs that can produce a single mature mRNA, Constitutive Splicing (CS) or multiple mature mRNAs, Alternative Splicing (AS). [1] [2]

In constitutive splicing, within a particular gene, constitutive exons are recognized, joined together and are always included in the mature mRNA. On the other hand, more than 60% of intron-containing genes in plants undergo alternative splicing. [3]
In AS, pre-mRNAs from a gene can be spliced by different ways, leading to the production of multiple isoforms or variants from a single gene. This is achieved by attaining different combinations of copy and paste, of whole or part of exons and introns. By being alternatively spliced, a single gene can lead to the production of more than one polypeptide. Thus, it has been suggested that one of the main purposes of alternative splicing is to upscale the proteome coding capacity of genes. [3] [4]

Generally, eukaryotic genes undergo both constitutive and alternatively spliced events. Alternative splicing coordinates the expression of the optimal version of an mRNA transcript in a spatial-temporal manner. Data generated from several RNA-sequencing studies showed that splicing decisions are influenced by tissue, cell-type and developmental stage specific splicing factors. Moreover, plants grown under different conditions like biotic or abiotic stresses, showed higher rates of alternatively spliced genes. Owing to their sessile nature, plants should adapt immediately to the environmental changes so that they could survive. [Reviewed in [5]]
Alternative splicing showed a crucial role in the control of several physiological and developmental processes, where it acts during photosynthesis, starch metabolism, defense responses, circadian clock control, flowering, as well as in hormone signaling, besides other functions. [5] [6]
In humans, more than 95% of multiexonic genes are estimated to undergo alternative splicing.[7]
In plants, on the other hand, under control conditions, approximately 61% of the genes of *Arabidopsis thaliana* [8], 50% of rice (*Oryza sativa*) [7], and 40% of maize (*Zea mays*) [9] undergo AS.

1.3. Spliceosome, the splicing machinery

The splicing process is performed by the fascinating multimegadalton ribonucleoprotein (RNP) complex, the spliceosome. This huge splicing machinery is found in the nucleus and is assembled from several small non-coding ribonucleoacids (snRNAs) and hundreds of small and non-small nuclear ribonucleoproteins (snRNPs and non-snRNPs). It has a highly dynamic

composition and conformation allowing the splicing machinery to be flexible and accurate. It assembles around the splice sites in the introns of the target pre-mRNA and then it catalyzes the removal of introns by two successive phosphodiester transfer reactions. [6] [10] [11] [12]

In plants, there are two types of spliceosomes, major (U2-type) and minor (U12-type) differing in their composition. The U2-dependent spliceosome is more abundant, splices U2-dependent introns, and is composed of five small nuclear RNPs (named U1, U2, U4/U6, and U5 snRNPs). [13][14]

On the other hand, the U12-dependent spliceosome is less frequent, performs splicing of the rare U12-type class of introns and is assembled from U11, U12, and U5, U4atac / U6atac snRNPs. [10][12]

1.4. Mechanism of pre- mRNA splicing

During splicing, exon and intron sequences of the target pre-mRNA have to be distinguished efficiently. In addition, the 5′ and 3′ splice sites (5′ and 3′ SS) have to be marked and juxtaposed before the catalytic step begins. The 5′ and 3′ splice sites, which identify the borders of each intron in a pre-mRNA together with the branch site -a consensus sequence-containing region located near the 3′SS- are recognized by the uridine (U)-rich snRNPs, U1,U2, U5 and U4/U6, in addition to several non-snRNP splicing factors, like U2AF65, U2AF35, and serine/arginine-rich (SR) proteins. Together, these factors assemble to form the splicing machinery, which performs the two transesterification reactions needed for the excision of introns and ligation of the selected exons. [15][16][17]

The splicing machinery assembly on a target pre-mRNA is initiated by the recognition of specific intronic sequences by distinct snRNPs so that the two ends of an intron would get near to each other for the transesterification reactions to occur (See Figure 1.1). This is accomplished by base-pairing interactions of U1 with 5′ SS and U2 auxiliary factor (U2AF) with the 3′ splice site, thus E complex formation. Then, the A complex is formed upon the interaction of U2 with the branchpoint region. Afterwards, the tri-snRNPs (U4/U6/U5) and other several proteins, including SR proteins are added leading to the formation of B complex. After a subsequent

remodeling of the B complex, C complex, the mature and active form of the splicing machinery is formed allowing the splicing reaction to take place. [18][19][20][21][22]

The two transesterification -phosphodiester bonds formation- reactions are rather simple chemical reactions that involve three functional groups from reactive regions in the target pre-mRNA. [17]

In the first reaction, 2′-hydroxyl of an adenosine of the intronic branch point sequence (BPS) performs a nucleophilic attack on the phosphate group at the 5′ splice site, generating a phosphodiester bond and, subsequently, generating an intermediate structure known as lariat, leaving a free 3′ hydroxyl group at the 5′ exon. In the second step, the free 3' hydroxyl group of the 5′ exon attacks the phosphodiester bond at the 3′ splice site (See Figure 1.2). The splicing process is completed when the cleaved exons are ligated, the lariat intron is excised, leading to the formation of a mature mRNA. [7][20]

As previously discussed, there are two types of splicing events: constitutive and AS events. In constitutive splicing events, the splice sites are recognized by the splicing machinery and each pre-mRNA from a certain gene is spliced in the same way. On the other hand, in AS events, the recognition and joining of a 5′ and 3′ SS pair are in competition with at least one other 5′ or 3′ SS, permitting different rearrangements of the gene's coding fragments, generating multiple forms of mature mRNA from the same pre-mRNA molecule [Reviewed in [21]].

There are seven classes of AS events that were observed in eukaryotes (Figure 1.3).

These include intron retention (IR), exon skipping - an exon is either included or excluded, also known as cassette exon (CE), mutually exclusive exons (MXE) -when an exon is included, it prevents inclusion of adjacent exon-, 5′ alternative splicing (A5SS), 3′ alternative splicing (A3SS), alternative first exons (AFE), and alternative last exons (ALE). [4][7]

In plants, intron retention is the most prevalent AS event, constituting more than 40% and 47% of all AS events in *Arabidopsis* and rice, respectively. [3] [7]

On the contrary, in humans, intron retention is the least occurring (<5%) AS event and exon skipping is the most frequent (>42%).[3] [22]

Two or more of these AS events can take place simultaneously to generate other types of AS in a pre-mRNA, for instance, cassette exon and intron retention or occurrence of both alternative 5′ and 3′ splice sites. [Reviewed in [4]].

1.5. Regulation of the splicing process

The assembly of the spliceosome on introns in pre-mRNAs is controlled by different splicing signals. First, the assembly is directed by sequence features in the pre-mRNA, in particular, introns are bordered by short consensus sequences: the GU and AG, respectively at the 5´ and 3´ splice sites and the branch point, a sequence near the 3´ SS. Besides, the Uridine/Arginine (UA) richness of the introns is essential for their recognition and hence for efficient splicing.
Second, the use of the splice sites is also regulated by *cis*-regulatory sequences and *trans*-acting factors. The *cis*-regulatory sequences are short consensus sequences (approximately 10 nucleotides), including exonic splicing enhancers and silencers (ESE, ESS), as well as intronic splicing enhancers and silencers (ISE, ISS). They differ in their locations on the pre-mRNA and in the way they control the use of a splice site. These *cis*-regulatory elements act by recruiting further RNA-binding proteins during the assembly and the catalytic cycle of the spliceosome.[23] [24]
Furthermore, *trans*-acting splicing factors which include the members of serine/arginine (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins families -besides tissue-specific factors- function by binding to these splicing enhancer and silencer elements, controlling the splice site choice. [Reviewed in [24]]
Alternative splicing enables a single gene to produce multiple mRNAs, leading to the synthesis of several proteins with different sequences, accordingly, those proteins would have a potentially different subcellular localization, function or stability. Additionally, AS can also regulate transcript levels by introducing premature termination codons (PTCs) - translational stop codon found in transcripts upstream of the authentic stop codon- that then let the mRNAs undergo degradation by nonsense-mediated decay (NMD), a quality control mechanism that precludes accumulation of aberrant and potentially injurious proteins. [23][25]
Numerous splice variants were found to contain a PTC upstream of an exon–exon junction. In animal systems, transcripts with a PTC located at more than 50 nucleotides upstream of an exon–exon junction are targeted for degradation by NMD. [26][27]
In a similar manner, the recognition of NMD substrates in plants rely on the distance from the PTC to the 3´ end of the transcript or downstream the splice junction. However, in some cases, certain transcripts comprising a PTC were not turned over by NMD, for instance, some

transcripts with retained introns -or parts of introns- were unaffected by NMD, proposing that not all NMD trigger signals or transcript arrangements are fully understood. [28][29][30]

1.6. Plant serine/arginine proteins

The serine and arginine-rich (SR) protein family is one of the most evolutionarily conserved families of RNA-binding proteins (RBPs), a key splicing factors that play a vital role in constitutive and alternative splicing. [31] [32]

They have a characteristic multi-domain structure, consists of one or two N-terminal RNA recognition motifs (RRMs) and C-terminal arginine/serine-rich (RS) domain, that can be phosphorylated at multiple serine residues [33] [34]. RRMs are responsible for recognizing and binding to a specific RNA sequences in the target pre-mRNA, which are mostly purine-rich sequences. Whereas, RS domains are involved in protein–protein interactions that enhance the recruitment of key components of the spliceosome to nearby splice sites, besides they are also able to modulate RNA binding as well. [35] [36][37]

Based on the recent updated definition for plant SR proteins, *Oryza sativa* and *Arabidopsis thaliana* genomes encode for 22 and 18 SR proteins, respectively, while humans have 12 SR proteins. [38]

SR genes in plants are grouped into seven subfamilies, (SR, SC, SCL, RS, RSZ, RS2Z and SR45), four of these subfamilies are specific to the plant systems (RS, RS2Z, SR45 and SCL) (See Figure 1.4) [39][38]. Regarding the plant-specific SR subfamilies, the RS members were first identified in Arabidopsis [40] , their RS domain is highly rich in arginine residues rather than serine-arginine dipeptides. Also, they are characterized by having two RRM domains while lacking a characteristic signature of the SR family (SWQDLKD heptapeptide) in their second RRM. Four RS subfamily genes were reported in *Arabidopsis* [40] [41], while only two in rice [42].The RS2Z subfamily proteins have an RRM, two Zn-knuckles, an SR domain followed by a region enriched in serine and proline residues. SR45 subfamily members have two RS domains separated by one RRM. The members of the SCL -were also previously known as SC35- protein subfamily are characterized by possessing a single RRM followed by an RS domain, besides they have a short charged N-terminal extension. [43] [44] [45]

1.7. Plant serine/arginine proteins and alternative splicing

SR proteins are key AS regulators, they can bind to the *cis*-acting regulatory elements, including exonic or intronic splicing enhancers or silencers on pre-mRNA and influencing the splice site choice by interaction with spliceosome components at the 5' and 3' splice sites (See Figure 1.5). Upon binding to a specific sequences in exons -exonic splicing regulators (ESRs), SR proteins can recruit and stabilize the binding of U1 snRNP to the 5′ splice site, U2AF complex to the 3′ splice site and U2 snRNP to the branch point. They also mediate interaction between the U2AF complex and U1 snRNP on exons. On the other hand, they bind to sequences in introns -intronic splicing regulators (ISRs)- in introns to mediate interaction between the U2AF complex and U1 snRNP on introns. [35] [43]

Regulation of AS by SR proteins is tissue-specific and stress-responsive. The pre-mRNAs encoding for SR proteins are themselves often subjected to alternative splicing, and these AS events can be crucial for the regulation of AS of other pre-mRNAs. [46]

In the AS process, Alternative exons are usually shorter in length and possess weak 5' splice sites, hence SR proteins favors the inclusion of alternative exons by increasing their weak splice sites recognition by spliceosome. A special feature of alternative splice sites is that they tend to be weaker than typical constitutive strong splice sites. Owing to their crucial regulatory roles, weak alternative splice sites were found to be more conserved than constitutive splice sites, hence they got preserved during evolution. [47] [48]

Furthermore, regulation of AS by RNA binding proteins is context- and position-dependent, thus the location of the SR protein and RNA interaction affect the splicing outcome. [21]

In a context-dependent manner, upon binding with other RNA binding proteins, SR proteins may function as activators or repressors. In addition, the position of SR protein-RNA binding determines their function, for instance, intron-bound SR proteins can act as suppressors, whereas exon-bound SR proteins function as enhancers. [21][49][50]

Phosphorylation/dephosphorylation of the RS domain of SR proteins by numerous kinases and phosphatases is an essential mechanism in the regulation of their activity, because it determines their ability to interact with RNA and other splicing factors as well as determining their subcellular localization [45][52] [50][53][54]. Moreover, the accurate positioning and activity of SR proteins are affected the methylation of arginine residues by protein arginine methyltransferases (PRMTs). [55]

Besides their roles in constitutive and AS splicing, SR proteins also have roles in post-splicing processes like RNA stability, mRNA export, mRNA quality control (NMD), as well as translation.[56][57]

PTC-containing SR proteins are expected to be degraded by the NMD pathway [26][27]. Interestingly, studies in mammalian systems suggested that a significant fraction of AS events produce PTC-containing SR isoforms may regulate the level of the functional transcripts through a mechanism so-called regulated unproductive splicing and translation (RUST) [58]. In plants, RUST may be involved in the regulation of the level of functional SR transcripts as it was noticed that accumulation of PTC-containing SR transcripts -that were not removed entirely by the NMD pathway- was associated with reduction in the amount of functional transcript, suggestive of a strong connection between the level of PTC-containing transcripts and functional mRNA encoding the protein. [58][59][60][61]

Also, some PTC-containing transcripts may escape NMD, yielding proteins with altered functions. SR proteins are consisting of multiple functional domains, a truncated versions that are deficient of one or more domains may have altered functions.[43]

1.8.Role of alternative splicing and serine/arginine proteins in plants stress responses

Alternative splicing in plants is known to be frequently associated with environmental conditions including biotic and abiotic stresses that are influencing plant growth and development and productivity[69][70]. One of the key actions by which plants can tolerate several stresses is through reprogramming their transcriptome, via inducing particular genes and repressing others [71][72][73]. In various plant species, stress-responsive genes with regulatory functions are mostly prone to AS. Furthermore, many plant genes that encode for transcription factors undergo AS in a stress-dependent manner, by this means they accurately ensure the proper expression downstream stress-related genes. [74][75][76][77]

Contrary to transcriptional control, post transcriptional control via AS acts by altering the structure of transcripts, thus influencing almost all aspects of protein function, including its enzymatic activity, binding properties, stability and intracellular localization. In addition, alternative splicing may also be coupled to NMD to regulate the levels of functional transcripts [37][58]. It was reported that a proportion of unproductive isoforms of some vital regulatory

genes -having premature termination codons- can be regulated by the NMD surveillance mechanism under environmental stresses [70][78]

Numerous splicing factors have roles in the abiotic stress response including SR proteins. As described earlier, besides their pivotal role in both constitutive and alternative splicing, studies showed that the AS of plant SR pre-mRNAs themselves is dramatically influenced by abiotic stresses [39][70][79][80]. SR proteins possess a multi-domain structure that can allow AS to produce isoforms differing in their domain organization and thus in function [38][81]. Stress signals were also shown to control both the phosphorylation state of plant SR protein as well as their subcellular localization. [82]

Interestingly, NMD can function as a negative feedback mechanism to regulate the amounts of functional SR proteins by shifting their gene splicing patterns in response to stress[70].

SR proteins were shown to have key roles in mediating responses to various environmental stresses in plants.

In Arabidopsis, arginine/serine-rich domain containing protein 40 (rs40) and arginine/serine-rich domain containing protein 41 (rs41) mutants displayed more sensitivity to abscisic acid (ABA) and salt treatments compared to wild type [83]. In addition, SCL30a mutant, a member of the SC35-like (SCL) subfamily exhibited hypersensitivity to ABA treatment during seed germination [84]. A loss-of-function SR45 mutant was shown to be hypersensitive to ABA [85], while mutation of SR34b leads to increased sensitivity to cadmium (Cd) stress [86].

Over 4000 target RNAs were found to be associated with the SR45 protein, and SR45 is suggested to be involved in the alternative splicing of about 30% of ABA responses in Arabidopsis. [87]

In rice, mutants of RS29 and RS33, an arginine/serine splicing factors resulted in impaired manganese (Mn) and zinc (Zn) accumulation in rice shoots. [88]

On the other hand, RS29 and U2AF35A splicing factors were AS-regulated under Cd stress. Interestingly, RS29 exhibited significantly increased intron inclusive level which suggested that various AS self-regulatory circuits of splicing factors possess an unexpected role Cd stress response in plant. [89]

1.9. Plants response to salinity stress

Salinity is one of the most brutal environmental stresses that affects the quality and quantity of agricultural crops [74][90]. More than 20% of cultivated land worldwide (approximately 45

hectares) is affected by salt stress and is expanding day by day [91]. It is reported that more than 9 billion ha of lands are affected by salinity and increasing at the rate of about 2 million ha (around 1%) of the world agricultural land yearly. [92]

Soil salinity can be caused by natural along with anthropogenic activity in the environment. Natural activity includes weathering of parent rock and deposition of oceanic salt carried by rain and wind. On the other hand, anthropogenic activity includes practices of heavy crop production, recurrent irrigation by canal systems and water logging leading to accumulation of salt on land surface. [74]

On the basis of adaptive evolution, plants can be categorized into two major classes according to their salinity tolerance: the halophytes, which can tolerate salinity and the glycophytes –include most of the major crops-, which cannot tolerate salinity and die eventually.[93]

Salt stress involves alterations in several physiological and metabolic processes, depending on severity and duration of the stress, and eventually hinders crop production. [94][95]

Salinity stress triggers primary and secondary stress signals. [62]

The primary signals include both osmotic and ionic toxicity on cellular level. In the early phases of salinity stress, the capacity of water absorption by root systems declines leading to accelerated water loss from leaves due to high salt accumulation, that's why salinity stress is considered as hyperosmotic stress [95]. Hyperosmotic stress is followed by ionic stress, where high concentrations of the major form of soil salt (NaCl) lead to accumulation of sodium (Na^+) and chloride (Cl^-) ions in plant tissues. Entry of both Na^+ and Cl^- into the cells leads to severe disturbance in the balance of essential nutrients causes ionic toxicity [96]. High Na^+ concentration inhibits potassium (K^+) ions influx -which is essential for plant growth and development- leading to osmotic imbalance and loss of stomatal functioning. The limited supply of K+ also leads to lower plant productivity and might eventually lead to death. [97]

The secondary signals are complex and include metabolic dysfunction, oxidative stress -due to the production of reactive oxygen species (ROS)- that would induces damage to cellular components like proteins, nucleic acids and membrane lipids. [62][91]

Consequently, in order to survive high salt concentrations in the soil, plants adopt several biochemical and physiological mechanisms. The main mechanisms are ion homeostasis, biosynthesis of compatible solutes (osmolytes) and osmoprotectants, synthesis and activation of

antioxidants, hormone regulation, nitric oxide (NO) generation and synthesis of polyamines. [Reviewed in [91]]

1.10. Rice and salinity

Rice (*Oryza sativa* Japonica), a member of family Gramineae, with a diploid genome consisting of approximately 370 megabases across 12 chromosomes [98]. It has been recognized for being easily genetically modified, owing to its relatively small genome, therefore it has been adopted as a model organism for studying cereal biology [99][100][101]

Rice is the second most important cereal in the world after wheat. A huge proportion of world's population depends on rice as food and it was found to constitute up to 20% of calories consumed by humans worldwide. Asia, is the main rice producer accounting for 90% of the total world yield [102]. Unfortunately, biotic and abiotic stresses threaten rice production and hinder its optimum yields, leading to massive losses in many areas all over the world, even in the most productive irrigated lands. High salinity is one of the main stresses that cause rice yield loss worldwide. At present, a vast areas of land that are supposed to be suitable for rice production in Asia and Africa are not used due to of high salt concentration in the soil caused by rising sea levels near coasts and excessive irrigation without proper drainage. [103]

Compared to other cereals, like wheat and barley, rice is not very tolerant to salinity and classified as a sensitive species that cannot survive under low salt conditions.[90][104][105]

The japonica cultivar Nipponbare was described as salt susceptible. [106][107]

Here, highlighted some of the aspects of rice response to salt stress.

1.10.1. Ion homeostasis

Maintaining ion homeostasis by controlling the transport of Na+ ion and its compartmentation is a crucial process for growth during salt stress [108]. Plants cannot tolerate high salt concentration in their cytoplasm. Thereby, the excess salt is either transported to the vacuole or sequestered in older tissues which ultimately are sacrificed, thus protecting the plant from salt stress.[109][110]

The Na+ ion that enters the cytoplasm is then transported to the vacuole via Na+/H+ antiporter. Vacuolar type H+-ATPase (VHA), present in the vacuolar membrane is the most dominant H+

pump present within the plant cell. Under stressed condition, plant's survival depends on the activity of V-ATPase. [111]

Plants maintain a high level of K^+ within the cytosol for cytoplasmic enzyme activities. K^+ plays an essential role in maintaining the turgor in the cell. It is transported via K+ transporters and membrane channels into the plant cell against the concentration gradient. Whereas, a very low concentration of Na^+ ion is maintained in the cytosol. During salinity stress, increased concentration of Na^+ in the soil makes Na^+ ion competes with K^+ for the transporter since both ions share the same mechanism of transport, leading to reduced uptake of K^+. [90][112]

A large number of genes and proteins, such as HKT (histidine kinase transporter) and NHX (Na^+/H^+ antiporters), encoding K+ transporters and channels have been identified. During salt stress expression of some low abundance transcripts is enhanced which are found to be involved in K^+ uptake. [113]

HKT family are Na^+/K^+ symporter, which are located on the plasma membrane. They play an important role in salt tolerance by regulating transportation of Na^+ and K^+. Class 1 HKT transporters have been observed in rice to remove excess Na+ from xylem, hence protecting the photosynthetic leaf tissues from the damaging effect of Na+ [114].

Intracellular NHX proteins are Na^+, K^+ /H^+ antiporters involved in K^+ homostasis, endosomal pH regulation, and salt tolerance. A study by Barragan et al. revealed that NHX proteins (NHX1 and NHX2) localized in the tonoplast are crucial for active K^+ uptake at the tonoplast, for turgor regulation, and for stomatal function. [115]

In addition, the expression of OsNHX1-5 is differently regulated in rice tissues and is upregulated by salt stress, hyperosmotic stress, and abscisic acid (ABA) [116].

1.10.2. Plant hormone signaling

In order to understand the molecular basis of salinity stress tolerance, it is essential to identify the main functional and regulatory genes in the involved signaling pathways. Key signaling pathways such as abscisic acid (ABA), jasmonate (JA), ethylene (ET) and salicylic acid (SA) pathways are involved in salt stress tolerance.

Abscisic acid (ABA) biosynthesis is increased by salinity-induced osmotic stress, hence regulating ABA-dependent stress response pathway [117]. Transgenic rice overexpressing some ABA-responsive genes demonstrated a significant enhanced salinity tolerance. [118]

Application of exogenous ABA was shown to enhance the salinity tolerance in rice [119]. Also, endogenous ABA content was shown to be higher in salinity-tolerant than in salinity-sensitive rice cell lines. [120]

Moreover, sensitive rice cultivars exhibited only a slight, delayed, and temporary increase in ABA content after exposure to salt treatment, while tolerant cultivars exhibited a large increase and a rapid increase in ABA [121].

ABA was shown to effectively reduce Na^+ and Cl^- contents in rice [119]. The capability of plant cells to maintain cytoplasmic ion homeostasis is crucial for salt stress tolerance. To handle the excessive Na+, cells get it out from the cytoplasm, this requires costs a lot of metabolic energy that can be provided by H+ gradients produced by membrane-bound H+ pumps. [119]

It was found that under salt stress, ABA acts synergistically with salinity on H+-pumping and antagonistically on the activation of Na^+/H^+ antiport. Thus, it was suggested that ABA is involved in tolerance responses to salt stress by maintaining cytoplasm ion homeostasis in rice [122]. Furthermore, genes coding for protein kinases such as receptor-like kinases (RLKs), Ca^{2+} dependent protein kinases (CDPKs), SNF1-related protein kinases (SnRKs), Mitogen activated protein kinases (MAPKs), transcription factors (TFs), and reactive oxygen species (ROS) are all involved in the salt stress tolerance via ABA-dependent pathway. [117]

The biological significance of jasmonate (JA) signaling in salt stress in rice was investigated in many studies. JAs are considered as positive regulators of salt tolerance [123][124]. Remarkably, endogenous JA contents in rice were observed to be higher in salt-tolerant cultivar than in salt-sensitive cultivar [125]. In addition, application of exogenous JA dramatically decreased Na^+ ions in the salt-tolerant cultivar. [125]

In wheat, JAs enhances the activity of antioxidant enzymes such as superoxide dismutase, catalase and ascorbate peroxidase as well as salinity tolerance. [124]

JA plays a role in plant development and defense responses by regulating jasmonate ZIM-domain (JAZ) transcription factors. JAZ is a subfamily of TIFY genes which are responsive several abiotic stresses including salinity. Interestingly, several rice JAZ proteins such as

OsTIFY1, 6, 9, 10 and 11 were recognized as salt-inducible genes[126]. MYC2 transcription factors function as a gene expression regulator of JA, ABA and salt stress. JA enhances proteolysis of JAZ -which represses MYC2-, thus allowing MYC2 transcription factors to activate the expression of downstream target genes [127].

Ethylene (ET) signaling pathway can positively or negatively affect salt stress tolerance. Upon exposure to salt, large numbers of ET-responsive genes show alterations [128].

ET demonstrated a negative effect on salt tolerance because an elevated ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) levels were correlated with reduced salt tolerance in Arabidopsis [129].

Remarkably, increased ET levels due to a loss-of-function mutation of ethylene overproducer 1 (eto1-1) in Arabidopsis is associated with enhanced salt tolerance, which is suggested to occur through an improved Na^+/K^+ homeostasis by reducing root influx and shoot delivery of Na^+. This salt-tolerance phenotype was also affected by ET-dependent ROS production mediated by the respiratory burst oxidase homolog f (RBOHF) NADPH oxidase. [130]

Mitogen-activated protein kinases (MAPKs) are key players in ET signaling [131]. MPK3/ MPK6 pathway is activated by MKK9, this activation positively regulates DNA-binding protein ethylene insensitive3 (EIN3) resulting in an increase in ET biosynthesis [132]. MKK9 was shown to be a positive regulator of salt tolerance [129]. Furthermore, gain- and loss-of-function studies of two ethylene-activated transcription factors, EIN3 and EIL1 (EIN3-LIKE 1), showed that they are essential and sufficient for enhancing salt tolerance. [133]

Salt Overly Sensitive (SOS) pathway is known to regulate Na^+/K^+ homeostasis in Arabidopsis under salt stress [134][135][136]. Interestingly, EIN3 and SOS2 were demonstrated to act synergistically to modulate plant salt tolerance. [137]

On the other hand, overexpression of lectin receptor-like kinase salt intolerance 1 (SIT1), a positive regulator of ET accumulation, elicited salt sensitivity in rice. SIT1, activates the MPK3/6 pathway which increases ROS production. Also, SIT1 enhances the activity of ACS, a ET biosynthetic enzyme, resulting in increased ET accumulation [138]

Salicylic acid (SA) is an important plant endogenous signal molecule that modulates plant responses to stress[139]. It can enhance salt tolerance by inducing a cascade of endogenous hormone signaling pathways. SA was suggested to enhancing salinity resistance in tomato by

regulating and balancing osmotic potential, improving photosynthesis, inducing the metabolism of compatible osmolytes, and minimizing membrane damage. [140]

SA is synthesized in plants through two different pathways: the phenylpropanoid and the isochorismate pathways [141]. Phenylpropanoid pathway starts by converting phenylalanine to trans-cinnamic acid (t-CA), which is catalyzed by phenylalanine ammonia lyase (PAL) [142] Recently, the increased expression of genes involved in SA biosynthesis pathway (OsPAL, OsCM and OsICS) in rice was associated with enhanced tolerance under saline conditions. [143]

1.10.3. Transcription factors

Transcription factors (TFs) are considered as most essential regulators that control gene expression. WRKY, bZIP, NAC, DREB, MYB, HSF, BHLH and zinc finger genes families comprise a large number of stress-responsive members. These TFs are capable of regulating the expression of numerous target genes by binding to the specific cis-acting element in the promoters of these genes.[144][145]

Transgenic rice overexpressing OsbZIP23 showed significantly enhanced tolerance to high-salinity stress, while a null mutant of this gene showed significantly decreased tolerance to high-salinity stress. [118]

Overexpression of a NAC transcription factor in rice showed improved salinity tolerance [146][147]. In addition, OsNAC5 and ZFP179 were up-regulated under salinity stress, which is suggested to regulate the synthesis and accumulation of sugar, proline and LEA proteins that subsequently play a pivotal role in stress tolerance. [148]

Several studies demonstrated that transgenic rice plants overexpressing OsDREB1A, OsDREB1F and OsDREB2A showed enhanced tolerance to salt stress. [149][150]

1.10.4. Repeat domain gene families

Genomes of higher plants, like *Arabidopsis* and rice encode for numerous repeat domain gene families, such as WD40, Tetratricopeptide repeats (TPR), Ankyrin (ANK), Pentatricopeptide repeats (PPR) Armadillo, HEAT, Kelch and Leucine rich repeats (LRR). [151]

In rice, 152 genes representing and 28 Kelch-repeat family were reported. [152][153]. Remarkably, several rice Armadillo-repeat containing genes (OsARM) displayed a differential expression upon exposure to salinity stress. [152]

In addition, various databases have predicted nearly 290 TPR containing domains in the rice genome [151]. An increased abundance of STI1, a stress-responsive protein that comprises two heat shock chaperonin-binding motifs and three TPR, was revealed in salt-treated rice [154]. This finding suggested that a large regulatory network is affected by salinity stress as TPR-containing proteins were identified to be involved in several processes like Heat shock protein 90 (HSP90) signalling, mitochondrial protein transport and gibberellin (GA) signalling. [155][156][157]

The number of ANK proteins has been found to be 175 in rice [158]. Interestingly, during salt stress, a gene encoding for a protein with ANK repeats was found to be up-regulated in a salt tolerant cultivar [159]. The ANK repeat is a common protein-protein interaction motif that has various functional roles, including ion transport. In plants, the ANK repeat domain was identified in some of the inward-rectifying potassium channels. [160]

Another large gene family with closely 200 representatives in rice were found to be potential WD40 repeat gene family members [161]. A subfamily of WD40 proteins, SRWD (Salt Responsive WD40 repeats), was identified in rice. The expression profiling of these genes showed that they are differentially expressed during salinity stress [162]

Stress regulatory LRR-RLK members have been characterized in rice. OsSIK1, LRR kinase, was observed to be induced and its overexpression displays higher tolerance toward salt and drought stresses [163]. Similarly, the transcripts levels of OsGIRL1 (Oryza sativa gamma-ray induced LRR- RLK1) were significantly increased under salt and osmotic stress conditions in rice. [164]

1.10.5. Other salinity responses in rice

Abiotic stress perception by the cell wall involves members of different receptor-like kinases (RLKs), a large family of integral proteins in the plasma membrane. Many of the genes encoding for receptor-like kinases are induced by abiotic stress itself, consequently amplifying the signal essential for stress adaption response. RLKs can sense different environmental stimuli and transmit their signal to downstream intercellular signaling networks, using either second

messengers or ROS. They also have roles in regulation of absicisic acid signalling and phosphorylation of various transcription factors. [165][166]

Stress-associated protein 11 (OsSAP11) and receptor-like cytosolic kinase 253 (OsRLCK253) was shown to improve the water-insufficiency and salt stress tolerance by affecting signaling pathways. [167]

The plant cell wall consists of cellulose fibrils cross-linked by hemicellulose chains, such as xyloglucan and arabinoxylan. The cell wall also contains phenolics; pectin esterases, peroxidases -which cross-link phenolic residues to the hemicellulose polymers- and other enzymes; expansins, extensins as well as calcium (Ca^{+2}). Osmotic stress caused by salt or drought increases reactive oxygen species (ROS) production and other changes in the plant cell wall. Key players in this process are the formation of ROS and peroxidases [168].

The buildup of ROS, which are a strong oxidizing compounds, leads to crosslinking of phenolics and glycoproteins in the cell wall such as extensins, resulting in cell wall stiffening. On the other hand, stress upregulates the expression of expansins and xyloglucan-modifying enzymes that aid in cell wall remodelling [168]. It was demonstrated that the expression of rice expansin-3 (OsEXPA3) is upregulated by salt stress, suggestive of having a great potential in improving salt tolerance of rice [169]. Also, salt stress can cause the wall to lose Ca2+.

At initial stage, salinity-induced osmotic stress plants decrease the stomatal aperture to prevent the excessive loss of water. Subsequently, internal carbon dioxide (CO_2) concentration decreases, slowing down the reduction of CO_2 by the Calvin cycle, which will lead to the increase of the electron leakage to O_2 and formation of superoxide radical (O_2^-). Moreover, the toxicity of Na^+/Cl^- triggered by the salt stress can disrupt the photosynthetic electron transfer, increases the respiration rate and consequently stimulate respiratory electron leakage to O_2 [170].

Enzymatic antioxidant action involves superoxide dismutase -one of the main scavenging enzymes- that converts O_2^- to hydrogen peroxide (H_2O_2) and then coordinates the action of other enzymes catalase, ascorbate peroxidase, glutathione peroxidase and proxy-redoxins, inducing the conversion of H_2O_2 to O_2 and H_2O. [171]

Among many plant nutrients required for the growth and development of plant cells, nitrogen (N) ions. Saline conditions can influence various steps of N metabolism, such as nitrate NO_3^- uptake and reduction and protein synthesis. Uptake of nitrate is limited by high salinity in soil. The uptake reduction was attributed to displacement of Ca^{2+} from the cell membranes by Na^+

under salinity conditions which disrupts membrane integrity, leading to alterations in nitrate transport ability. High-affinity nitrate transport system such as NRT2.2, NRT2.3 were shown to be up-regulated under salt conditions. [Reviewed in[172]]

1.11. Crispr/Cas9 system and plant genome editing

At present, multiple challenges are affecting agriculture, including rapid population growth, climate change and other environmental threats. Therefore, there is a crucial need to have more specific and effective genome editing technologies through non-transgenic approaches, which can develop improved food crops having a higher yield potential, better abiotic stress tolerance, and superior resistance to pests and pathogens. [173][174][175]

In the past few years, CRISPR (clustered regularly interspaced short palindromic repeat) and Cas9 (CRISPR-associated protein 9 nuclease), CRISPR/Cas9 system, has been recognized as a cutting-edge genome editing approach that is particularly simple and does not involve protein engineering [176]. CRISPR/Cas9 system's progress nowadays as gene editing tool can be traced back to the late 1980s when Yoshizumi Ishino and his colleagues accidentally cloned part of a CRISPR- in form of interrupted clustered repeats was not identified at the time- along with the iap (inhibitor of apoptosis) gene, the gene of interest [177]. Afterwards, it was discovered to be microbial adaptive immune system and its progress till date is the outcome of the work of numerous researchers around the globe [178][179]. In 2013, the first application of CRISPR/Cas9 was reported in plants [180][181][182], after which it spread widely applied to several other plant species.

Genome editing in plants can be accomplished by introducing two CRISPR/Cas9 components into the same cell (illustrated in Figure 1.6): a guide RNA (gRNA) and the Cas9 nuclease. The first component is gRNA (sometimes referred to as single guide RNA (sgRNA)) which consists of two parts, crRNA (CRISPR RNA) and tracrRNA (trans-activating CRISPR RNA). The crRNA, the guide, is a repeat sequence and a variable spacer sequence that is complementary to the target gene sequence [183][184]. The tracrRNA, is a short RNA sequence and is complementary to the CRISPR repeat and acts as a structural bridge between the crRNA and Cas9. [173][174]

Cas9 is an RNA-guided DNA nuclease, transported to the target DNA sequence by forming a complex with a scaffold structure in the gRNA [174]. Cas9 is able to target a DNA sequences of

(5′-N(20)-NGG-3′) -that is complementary to the protospacer- where N can be any base followed by 20 nucleotides, and juxtaposed to NGG (N, any base and G is guanine), the protospacer-adjacent motif (PAM) [185][186][187]. The protospacer, is also known as gRNA-spacer, is found in the 5′ region of the gRNA, which guides Cas9 to its particular targets [176]. After the gRNA spacer region can anneal to the complementary target DNA, Cas9 can then interact and create a double strand break (DSB) in the target DNA 3 base pairs upstream of the PAM site. [186][188]

DSBs are then repaired by DNA repair pathways. Non-homologous end-joining (NHEJ) is most common pathway for DSB repair [189], which can introduce of insertion or deletion (indel) in the coding region of the target gene. These mutations can create a premature stop codon in the open reading frame (ORF) of the gene, leading to NMD of its transcript. Otherwise, homology-directed repair (HDR) can also repair DSBs by introducing a new sequences when a DNA template with homology to the target sequence is present.[174]

The CRISPR/Cas9 system has been shown to be effective in creating point mutations and short insertion-deletions in various plant species [190][191][192]. Nevertheless, gene's functional redundancy is common of the complex plant genome owing to the presence of many gene families. Hence, the functional characterization of genes in complex systems requires a concurrent targeting of multiple genes [193]. This simple RNA-guided system is remarkably successful in targeting several genomic sites, and if more than one gRNA is present, CRISPR-Cas9 system enables editing of multiple sites simultaneously (multiplexing). [176][194]

Multiplexing can be carried out by combining Cas9 with several sgRNAs specific for different target genes. The expression of these sgRNAs can be achieved by assembling their expression system with each having its own promoter [195][196]. Furthermore, multiple sgRNAs can also be produced inside the cell by a single polycistronic tRNA-gRNA gene (PTG) system that comprises sgRNA sequences between the tRNA sequences. The PTG is processed by endogenous tRNA system in the cell, producing multiple sgRNAs by recognizing and cleaving tRNA end sequences [194]. Many studies utilized multiplex plant genome editing to incorporate point mutations at several target sites simultaneously. [194][197][198][199]

1.12. RNA-sequencing in plants

Deep sequencing using high throughput Next-Generation Sequencing (NGS) technologies, RNA-sequencing (RNA-seq), have revolutionized the study of transcriptomics at a cellular level. RNA-seq provides a potent and cost-effective tool to characterize transcriptomes aiming at gene discovery and quantifying gene expression. Thus, making it possible to unravel the complexity of plant transcriptomes by obtaining high-throughput expression data at a single-base resolution. [118][201]

The absolute quantitation of gene expression using RNA-seq is more insightful, and accurate than microarray [202][203][204]. For this reason, RNA-seq is superseding the microarray-based approaches for studying expression levels of particular genes, allele-specific expression of transcripts and differential splicing which can be precisely determined by RNA-Seq experiments [205]. In addition, when RNA-seq is combined with appropriate bioinformatics tools, it provides a superior approach that allow a global scale investigation of gene expression dynamics in various cellular and biological contexts. [206]

In plants, transcriptome sequencing at single-base resolution revealed the alternative splicing of 42 and up to 48% of the intron-containing genes in *Arabidopsis* and rice, respectively [70][207][208].

Plants evolved various mechanisms to cope with different aspects of salt stress but how plants respond to or tolerate salt stress is not fully understood. Consequently, it became inevitable to investigate mechanisms of salt stress responses and the layers of regulation responsible for tolerance, thus providing valuable information for the production of salinity-resistant crops. [209][210]

RNA-seq enables a global analysis of AS to dissect its functional regulation roles in stress responses including salt stress. AS has been defined to influence many stress-related genes including protein kinases, transcription factors and splicing regulators like serine/arginine-rich (SR) proteins. [79][211]

RNA-seq experiments enable thorough exploration of the molecular/genetic basis for plant response to various stresses including salinity [212]. High-coverage RNA-seq data of *Arabidopsis* seedlings treated with salt (NaCl) showed a significant increase of AS upon exposure to salt stress compared to unstressed conditions; most differentially spliced genes might

not be regulated by salt stress but are linked to specific functional pathways associated with stress responses and RNA splicing, SR splicing factors. [213]

1.13. Study design and objectives

The main objective of this project is to study the effect of CRISPR/Cas9-mediated mutagenesis of two rice serine/arginine rich (SR) proteins (RS-subfamily), Os-RS29 (LOC_Os04g02870) and Os-RS33 (LOC_Os02g03040), on pre-mRNA splicing regulation with or without the exposure to salt stress using RNA-sequencing. There were two double mutants according to the mutation status, heterozygous Os-RS29 / homozygous Os-RS33 and homozygous Os-RS29 / homozygous Os-RS33.

The effect of this mutagenesis will be investigated by: 1) Reporting the transcriptome (sequencing data) analysis results, 2) Identifying the differentially expressed transcripts among the different conditions and controls, 3) Overviewing the global alterations of alternative splicing and detecting the differentially spliced genes (DSGs) among the different conditions and controls and 4), 4) Functional annotation, gene and pathway enrichment analysis of both differentially expressed transcripts and DSGs.

To achieve those objectives, paired-end RNA-sequencing of 12 plant samples (2 replicates of control wild-type, 2 replicates of salt (NaCl)-treated wild-type, 2 replicates of control Cas9 overexpression only, 2 replicates of NaCl-treated Cas9 overexpression only, 1 control homozygous Os-RS29 / homozygous Os-RS33 double mutant, 1 NaCl-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant, 1 control heterozygous Os-RS29 / homozygous Os-RS33 double mutant, 1 NaCl-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant) were carried out. Differential expression analysis using Kallisto and Deseq2. The sequencing data was further analyzed for alternative splicing changes using STAR, cufflinks and IsoformSwitchAnalyzeR was used for identifying isoform switches among different conditions. Finally, functional enrichment analysis of the differentially expressed transcripts and DSGs was done using CARMO (Comprehensive Annotation of Rice Multi-Omics) database and STRING database was used to build the protein-protein interaction network for a significant group of genes. In conclusion, this study aims at elaborating the role of SR proteins (RS-rich subfamily members) on the global AS regulation especially in response to salt stress.

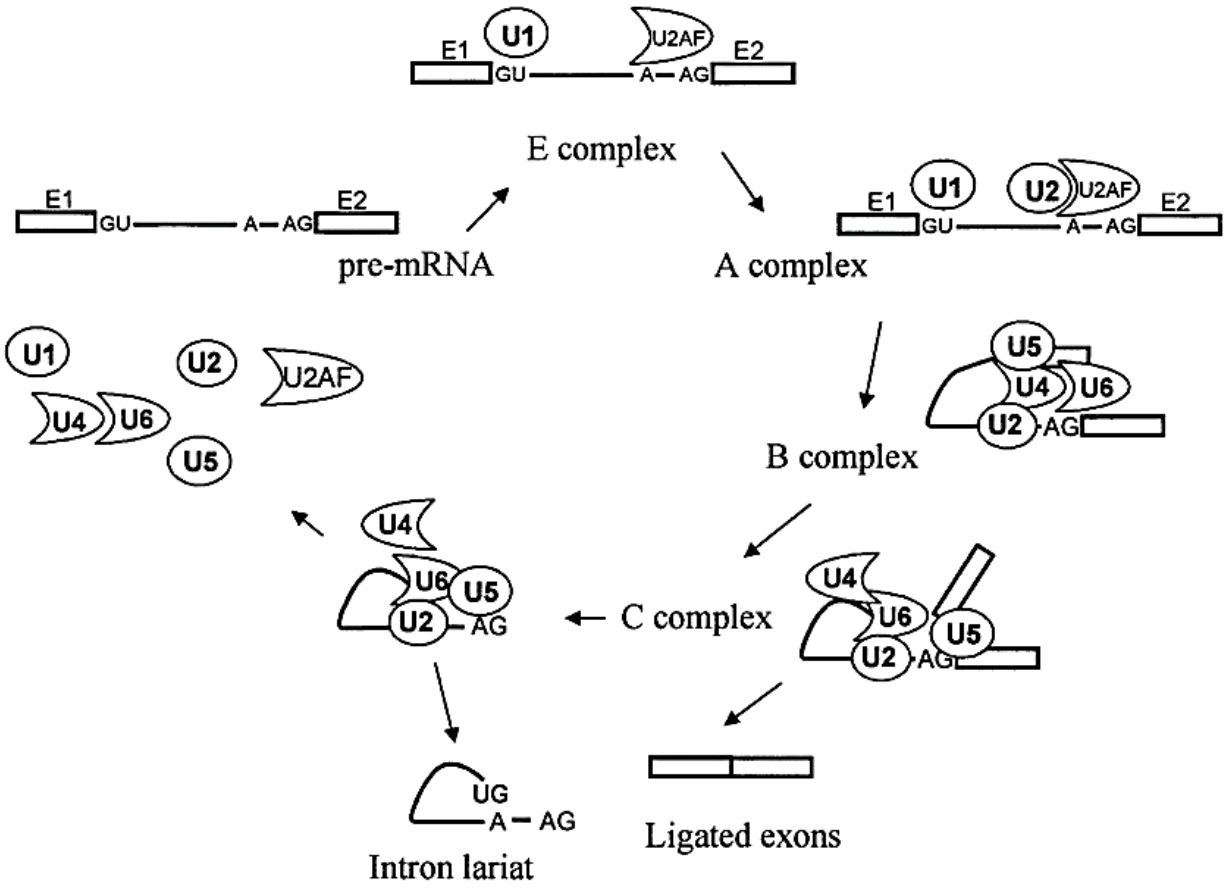


Figure 1.1. A schematic of the steps of the major spliceosome assembly and disassembly cycle. The assembly is initiated by the recognition intronic sequences of the pre-mRNA U1 with 5′ SS and U2 auxiliary factor (U2AF) with the 3′ splice site, thus E complex formation. A complex is formed upon the interaction of U2 with the branchpoint region. Three snRNPs (U4/U6/U5) shaped into the B complex. After remodeling of the B complex, C complex, the active form of the splicing machinery is formed allowing the splicing reaction to take place, releasing the ligated exons and an intron lariat. (Adopted from [19])

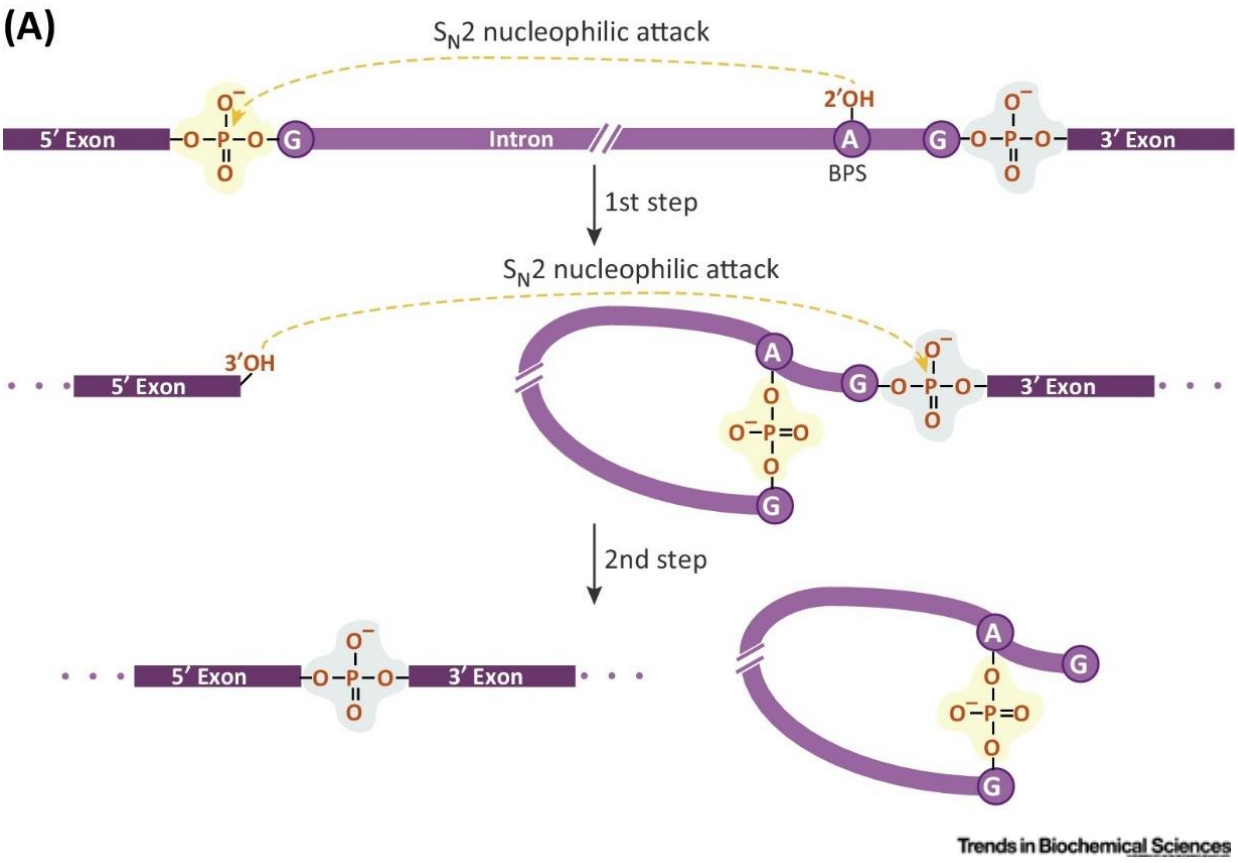


Figure 1.2. Mechanism of the Splice Reaction. (Adopted from [20])

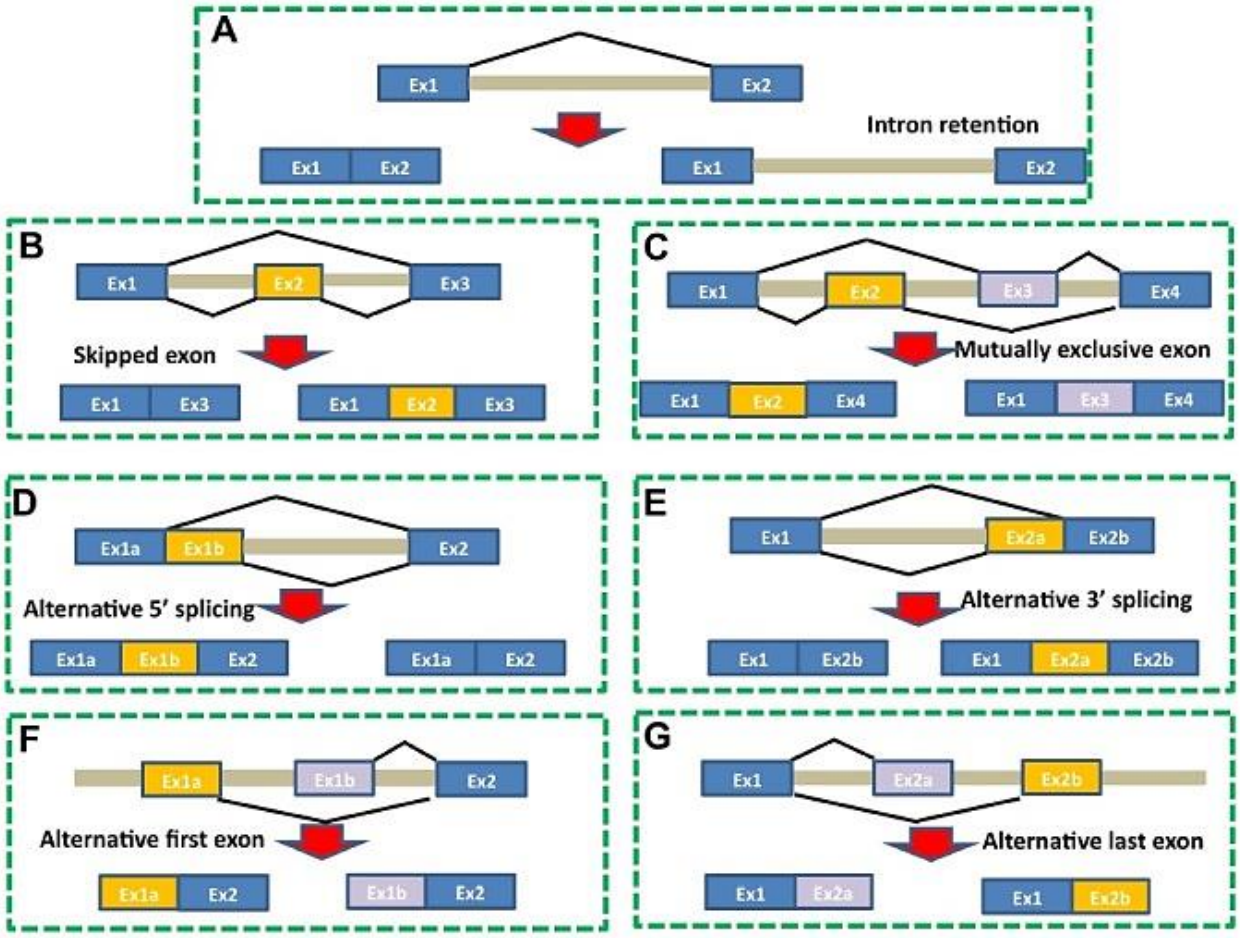


Figure 1.3. Different types of alternative splicing are shown: (A) Retained intron, (B) Exon skipping (cassette exon), (C) Mutually exclusive exons, (D) alternative 5′splice sites, (E) Alternative 3′splice sites, (F) Alternate first exon and (G) Alternate last exon.(Adopted from [7])

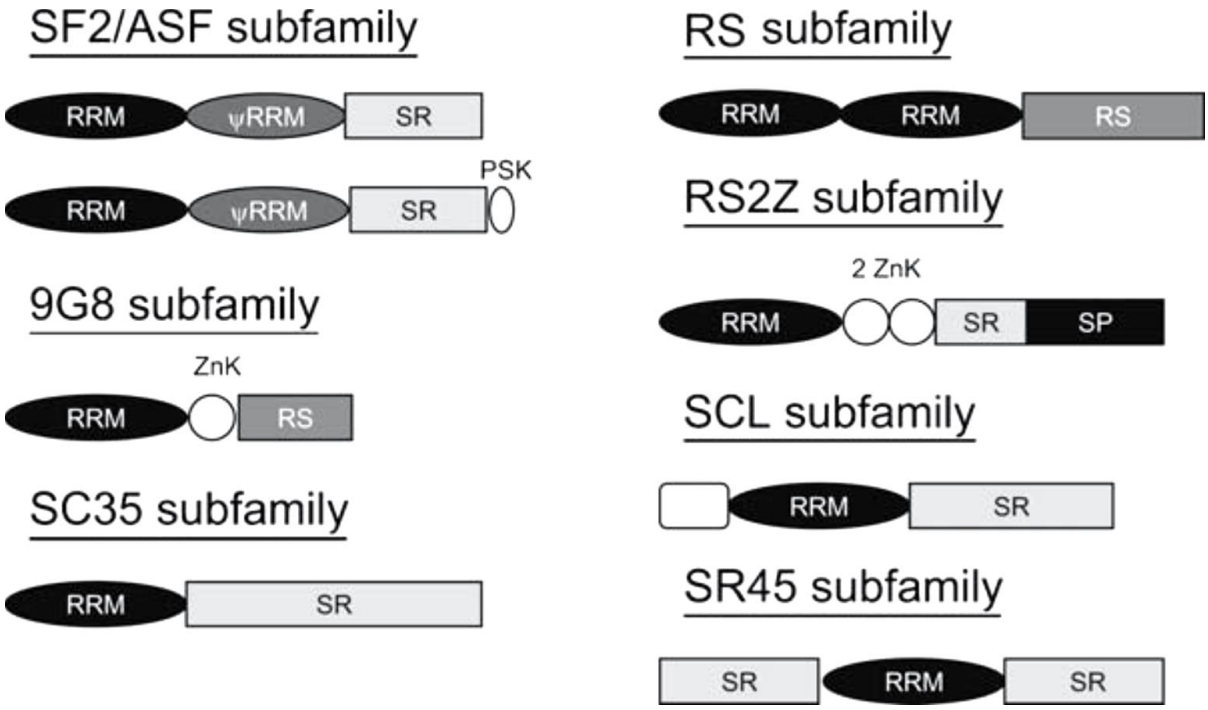


Figure 1.4. Plant SR proteins subfamilies. Subfamilies in the left column are found in both plant and animal kingdoms. While, the ones in the right panel are plant-specific.
RRM (RNA recognition motif), ΨRRM (contains the SWQDLKD heptapeptide), SR (domain rich in serine-arginine dipeptides) RS (domain rich in arginine and serine residues), ZnK (zinc knuckle), SP (domain rich in serine and proline residues) and PSK (region rich in proline, serine, and lysine residues). SCL proteins have an N-terminal extension rich in arginine, proline, serine, glycine, and tyrosine residues. (Adopted from [45])

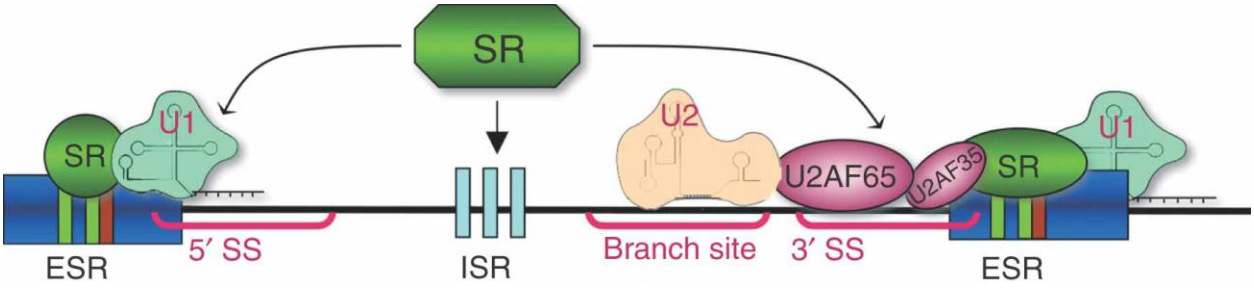


Figure 1.5. Roles of plant serine/arginine-rich (SR) proteins in pre-mRNAs splicing. SR proteins can bind to the cis-acting regulatory elements, including exonic or intronic splicing enhancers or silencers on pre-mRNA and influencing the splice site choice by interaction with spliceosome components at the 5' and 3' splice sites. Upon binding to specific sequences in exons -exonic splicing regulators (ESRs), SR proteins can recruit and stabilize the binding of U1 snRNP to the 5′ splice site, U2AF complex to the 3′ splice site and U2 snRNP to the branch point. They also mediate interaction between the U2AF complex and U1 snRNP on exons. In addition, they bind to sequences in introns -intronic splicing regulators (ISRs)- in introns to mediate interaction between the U2AF complex and U1 snRNP on introns. (Adopted from [43])

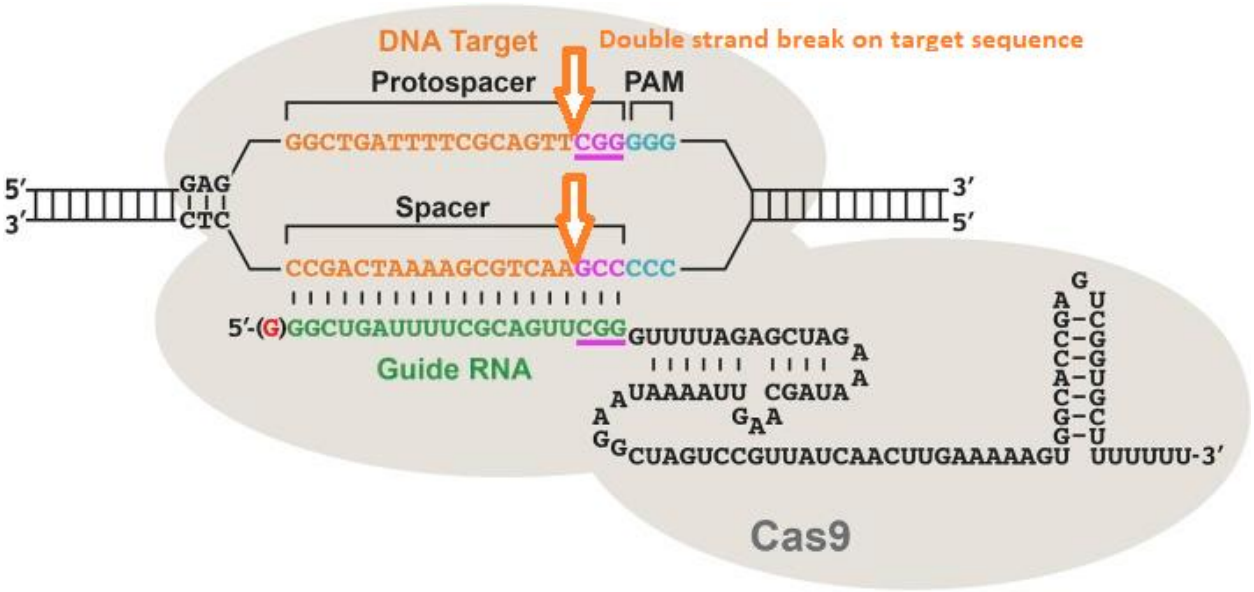


Figure 1.6. CRISPR / Cas9 system. The target DNA sequence (orange-colored) is shown with the two strands separated. The sense strand of target sequence has protospacer adjacent motif (PAM) at its 3' end (blue-colored NGG). The cleavage site (pointed to by two red arrows) is located at 3 nucleotides upstream of the PAM. The spacer region of the guide RNA gRNA (20 nucleotides green-colored) recognizes the target sequence. After the gRNA spacer region can anneal to the complementary target DNA, nuclease Cas9 (gray-colored) can then interact and create a double strand break. (Image modified from [188]).

Chapter 2: Methods

2.1. Experimental setup

2.1.1. Plant material and vector construction

Rice (*Oryza sativa* L. ssp. japonica cv. Nipponbare) was used in this study. The plasmid vector pRGE32 was used for callus transformations (for details about plasmid vector construction see [194]) . The expression of Cas9 endonuclease from Streptococcus
Pyogenes and the chimeric sgRNAs (cgRNA) were driven by the OsUbiquitin and OsU3 promoters, respectively. The first target is the exon 3 of Os-RS29, the mutation will cause 6 nucleotides deletion causing deletion of P62 and G63 amino acids (in the RRM1 domain). The second target is the exon 2 of Os-RS33, the mutation is 1 nucleotide insertion causing premature termination at 32. The sequences of sgRNAs, PAMs, mutations and genes structures are shown in (Table 2.1).

2.1.2. Rice transformation and treatment

Agrobacterium-mediated rice transformation was performed using rice mature seed-derived calli according to this protocol [214]?.Wild-type plants are used as controls. As these mutant plants have Cas9 protein, so Cas9 overexpression plants with no gRNA are used as controls as well. Seeds were germinated in 1/2MS media for 7 days, then the 7-days old seedlings were Mock- and NaCl-treated (with 250mM NaCl) for 6-hours. Samples are summarized in (Table 2.2).

2.1.3. RNA extraction, library preparation and sequencing

Whole seedlings have been used for subsequent RNA extraction and sequencing. For RNA extraction, total RNA was extracted using Trizol method. Library preparation for RNA-seq was performed using (TruSeq Stranded mRNA, Illumina). Paired end sequencing was performed on Illumina Hiseq2000 sequencing platform (Illumina Inc., California, USA).
12 samples were sequenced in this study the output included 24 fastq files (paired-end).

2.2. Computational analysis

2.2.1. Differential gene expression analysis

Quantification and normalization of transcripts abundance -read counts and TPM (transcript per million reads mapped)- were carried out using Kallisto (default parameters) with 100 bootstrap (http://pachterlab.github.io/kallisto) (v0.44.0). Kallisto quantifies read files directly without alignment, but it performs a process called pseudoalignment, which requires processing a transcriptome file to create a transcriptome index. This pseudoalignment method is computationally less demanding while achieving similar or better accuracies compared to other methods [215] [216]

Complete gene sequence and annotation information for *Oryza sativa* (*Oryza_sativa*.IRGSP-1.0.34) were retrieved from ensemble plants (ftp://ftp.ensemblgenomes.org/pub/plants/release-34/fasta/oryza_sativa/).

Kallisto was used to create an index for quantification using the ensemble gtf file, by the default settings. Afterwards, kallisto was used to quantify all putative transcripts. The resulting abundance data were imported into the R statistical environment (version R-3.4.4)

R package (tximport) [217] was then used to import transcript-level abundance, estimated counts and transcript lengths generated by kallisto, and summarize them into gene-level count matrices to be used by downstream gene-level analysis packages. Descriptive statistics and differential expression analysis were performed with R/Bioconductor package DESeq2 [218]. The statistically significant differentially expressed transcripts (DETs) were extracted at an FDR <0.01 and a logFC cutoff of 4. (Log_2 fold change was greater than 2 or less than -2 with an adjusted p-value of less than 0.05.)

Heatmaps of top DETs of each comparison were generated by Heatmapper [219]. Clustering of genes was done using the default parameters in the tool, where the clustering method is Average Linkage and the distance measurement method is Euclidean. In addition, CCTop tool was used to predict off targets. [220]

2.2.2. Alternative splicing analysis

For AS analysis, fastq files were mapped against a reference genome using STAR, a splice-aware aligner [221] . STAR was run with option (--outSAMstrandField intronMotif) to generate the XS strand attribute for spliced alignments. For transcriptome assembly from the mapped RNA-Seq reads, Cufflinks (v2.2.1) [222] was used.

Alternative Splicing Analysis Tool Package (ASATP) [223] was used to identify the AS events using the gtf files produced by Cufflinks. ASRecovist tool is a program that was used for alternative splicing recognition and visualization, it detects alternative splicing events from a gene annotation and classified them into different types (IR, CE, MXE, A5SS, A3SS, AFE and ALE). IsoformSwitchAnalyzeR was used for identifying isoform switches among different conditions. [224]

2.2.3. Enrichment Analysis and network analysis

Comprehensive functional enrichment analysis of the differentially expressed transcripts and the differentially spliced genes was held using CARMO (Comprehensive Annotation of Rice Multi-Omics) database [225].

STRING was used to build the protein-protein interaction network for a significant group of genes with default settings.[226]

The workflow of this study is summarized in (Figure2.1).

Table 2.1. Target rice loci, sequences of sgRNAs, PAMs, mutations and their gene structures.

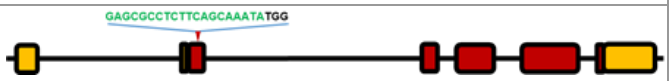
Targeted rice locus	sgRNA sequence	PAM	Mutation	Gene Structure
LOC_Os04g02870 (Os-RS29)	GCTATCCTTTTGGCCCTGGG	AGG	6nt deletion causing deletion of P62 and G63 amino acids (RRM1 domain)	
LOC_Os02g03040 (Os-RS33)	GAGCGCCTCTTCAGCAAATA	TGG	1nt Insertion causing PTC at 32	

Table 2.2. Samples summary. WT, Wild type. C, Control (mock treated). Cas9, Cas9 overexpression only. RS_Het_C, heterozygous Os-RS29 / homozygous Os-RS33 double mutant. RS_Homo_C, Control homozygous Os-RS29 / homozygous Os-RS33 double mutant. RS_Homo_NaCl, NaCl-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant. RS_Het_NaCl, NaCl-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant.

Sample ID	**Sample Name**		
1	WT-C-1		
2	WT-C-2		
3	WT-NaCl-1		
4	WT-NaCl-2		
5	Cas9-C-1		
6	Cas9-C-2		
7	Cas9-NaCl-1	**Mutation status**	
8	Cas9-NaCl-2	**RS29**	**RS33**
9	RS_Het_C	+/- (heterozygous mutation)	-/-
10	RS_Homo_C	-/- (homozygous mutation)	-/-
11	RS_Homo_NaCl	-/-	-/-
12	RS_Het_NaCl	+/-	-/-

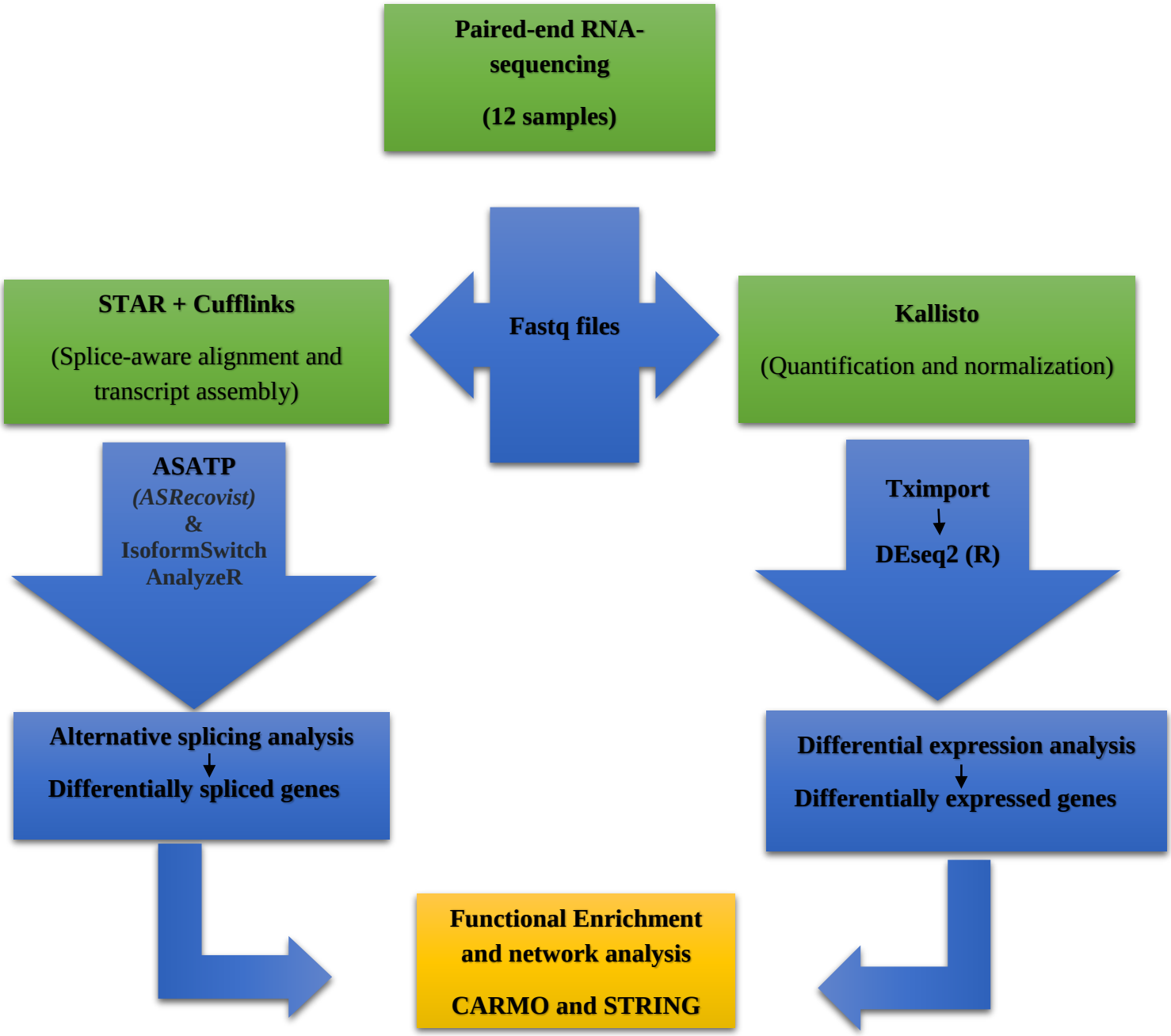


Figure 2.1. Summary of the study workflow.

Chapter 3: Results and Discussion

3.1. Differential expression analysis and functional enrichment of the differentially expressed transcripts

Off-target mutations were not detected in other gene' regions that are highly homologous to the guide-RNA sequences verified by CCTop tool.

Dendogram is a hierarchical clustering plot where samples that are most similar reside in closer positions in the tree, whereas samples that are less similar are separated by higher number of branch points [227]. Cas9 over expression samples were closely related to the wild-type than RS_Het and RS_Homo samples with and without salt treatment (see Figure 3.1).

Volcano plots were utilized to display the results of the RNA-sequencing experiment. It is a type of scatterplot that generated by plotting statistical significance (negative log p-value) against magnitude of change (log fold change). It allows quick identification of genes with large fold changes with high statistical significance. The most down-regulated genes are towards the left, the most up-regulated genes are towards the right, and the most statistically significant genes are towards the top [228][229]. The volcano plots of –log10 (p-value) vs. log fold- change, showing both the statistical and biologically significant genes (see Figure 3.2).

A total outcome of 42346 gene transcripts were analyzed for differential expression. Transcripts are considered significantly up- or down-regulated , if the log2-fold-change is greater than 2 or less than -2, respectively, and the Benjamini-Hochberg (BH) [230] adjusted p-value is less than 0.05 (below a false rate ratio (FDR) cutoff of 0.05). Functional enrichment of the differentially expressed genes was done. Significant function categories are those with p-value is less than 0.02.

Seven comparisons were made to identify the significantly differentially expressed transcripts (DETs). The top most DETs in each comparison as well as the top enriched functional categories will be discussed in this section. The number of the total differentially expressed transcripts as well as the number of significantly up- and down-regulated ones is summarized in (Table 3.1).

3.1.1. Control wild-type *versus* control heterozygous Os-RS29 / homozygous Os-RS33 double mutant

There were 98 differentially expressed transcripts in the heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_C) compared to the wild-type (WT_C) (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.3).

Up-regulated:

Up-regulated genes in this comparison were enriched in response to stress (p-value=4.74E -4) and oxidation-reduction process (p-value= 5.47E-4) (see Figure 3.10).

Metallothioneins (MTs) are low molecular mass and cysteine-rich metal-binding proteins known to be mainly involved in maintaining metal homeostasis and stress responses. Previous studies revealed that the expression of plant MTs are regulated by various factors, such as salt stress, heat shock and wounding [231][232][233]. It was also shown that the expression of OsMT3 (p-value= 5.51E-19) in rice was increased by osmotic and cold stress, indicating that OsMT3 might play a role in scavenging the reactive oxygen species (ROS) which brought about by those stresses. [233]

OsbHLH008 (p-value=2.27E-08) is a member of the basic/helix-loop-helix (bHLH) gene family, one of the largest transcription factor families in plants that are involved in a wide and diverse range of biological processes and characterized by the bHLH motif that possesses a DNA-binding and dimerization capabilities [234][235]. bHLHs were demonstrated to play essential roles in response to various abiotic stresses in plants such as salt, drought and cold through ABA and jasmonic signaling pathways. [236][237]

OsAmy1A (p-value= 2.43E-08) is one of the α-amylase genes that are indispensable for seed germination where they function by hydrolyzing starch into sugars, in order to nourish the young seedlings. Gibberellins were shown to control the expression of the α-amylase genes which were observed to be significantly up-regulated by GA treatment of wild-type rice seeds. [238][239]

Gibberellin 20-oxidase catalyses successive oxidation steps in the late part of GA biosynthetic pathway [240]. OS20OX2 ((p-value= 4.13E-05) is encoded by semi-dwarf 1 (SD1) which was demonstrated to play an important role in GA biosynthesis and plant growth regulation. [241]

Hyperosmolality-Gated Calcium-Permeable Channel 1.2 (OsOSCA1.2) (p-value= 4.51E-07) was also up-regulated in this comparison. Hyperosmolality-gated calcium-permeable channels were identified as an osmosensor, which act by receiving and responding to exogenous and endogenous osmotic changes in order to sustain plant growth and development. [242]

Hence, in this comparison the double mutant imitates the effect of abiotic stress on the cells and some of the up-regulated genes are involved in hormone signaling pathways.

Down-regulated:

In this comparison, down-regulated genes were not enriched in any biological process.

Cationic amino acid transporter 5 (OsCAT5)(p-value =4.95E-17) and amino acid transporter-like 15 (OsATL15) (p-value 6.24E-07) were observed to be down regulated in this comparison. Both are amino acid transporters which act by transporting amino acids across cellular membranes [243]. They play an essential role in several processes of plant growth and development, such as long distance amino acid transport, abiotic stresses, and response to pathogen. [244]

Rice Terminal Flower 1(TFL1)/Centroradialis(CEN) Homolog 1 (RCN1) (p-value=1.10E-05) gene, belongs to the phosphatidyl-ethanolamine-binding protein (PEBP) family in rice [245]. TFL1/CEN-like genes play a vital role in determining plant architecture by controlling the timing of phase transition during plant development [246]. It has been demonstrated that RCN1 overexpression caused delayed phase transition - from vegetative to reproductive stage- in rice development as well as altered panicle morphology. [247]

3.1.2. Control wild-type versus control homozygous Os-RS29 / homozygous Os-RS33 double mutant

There were 886 differentially expressed transcripts in the homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_C) compared to the wild-type (WT_C) (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.4).

The double homozygous mutations of both RS genes (Os-RS29 and Os-RS33) were found to activate the expression of genes responsive to several biotic and abiotic stresses. Similar results were obtained by repressing splicing machinery via splicing inhibitors producing gene expression patterns that resemble response to abiotic stress treatment.[248][249]

Up-regulated:

Genes responding to various stresses were up-regulated. Response to cadmium ion (p-value=4.06E-09), defense response to bacterium (p-value=0.0003), response to cold (p-value=0.001) and response to abscisic acid (p-value=0.008) were among the top biological processes enriched by the up-regulated genes in this comparison (see Figure 3.11).

A wide variety of salt stress responsive genes displayed increased expression in this comparison such as NA+/H+ antiporters (OsNHX), high-affinity potassium(K+) transporters (OsHAK), Vacuolar H-ATPases (OsVHAs), receptor-like cytoplasmic kinases (OsRLKs), Catalase A (OsCatA) (p-value=3.51E-05)and superoxide dismutase (SOD) (p-value=1.73E-11).

Salt-responsive gene OsSalT (p-value=0.001) was also up-regulated in this comparison. Previous study showed that the expression of OsSalT is regulated by salinity as well as ABA and gibberellic acid. [250]

Submergence stress constitutes a state of oxygen deprivation which is associated with anaerobic carbohydrate catabolism, mainly ethanol fermentation [251][252]. This tolerance mechanism is aided by Alcohol dehydraogenase 1 (ADH1) (p-value=0.00092), which has a pivotal role in sugar metabolism [253]. Furthermore, ADH1 was demonstrated to play an important role in cold stress where it can protect plant cells from freezing damage by producing C1 to C9 alcohols. [254]

The expression of heat shock proteins (HSPs) including OsHSP80.2 (p-value=1.32E-10), HSP70 (p-value=1.46E-08), OsHsp70CP2 (p-value=8.82E-08) and OsHsp90 (p-value=1.32E-10) was highly elevated. The accumulation of HSPs is believed to play an essential role in abiotic stress responses in plants [255]. The levels of HSPs are usually reported to be elevated by high temperature [256]. However, some rice HSP genes exhibited significant expression profiles in response to salinity and ABA stress. For example, the expression of OsHSP80.2 was induced by high salt stress. [257]

OsWD40-178/OsPUB72 (p-value=3.33E-13), OsWD40-138/OsSERL7 (p-value=8.24E-07), OsWD40-181 (p-value=6.05E-06), OsWD40-171(p-value=1.56E-05), OsWD40-59 (p-value=2.41E-05), OsWD40-29 (p-value=0.0001), OsWD40-36 (p-value=0.0002), OsWD40-130 (p-value=0.0008) and OsWD40-194 (p-value=0.0009) were up-regulated. They all belong to

WD40-repeat protein family which have been involved in a broad spectrum of essential functions in eukaryotes [258]. They participate in the regulation of various key cellular pathways, such as RNA processing, signal transduction, cytoskeleton dynamics, cell division, and are principally prevalent in chromatin modification and transcriptional regulation [258][259][260]. In addition, WD40 proteins play a role in abiotic stress responses in plants such as ABA, salt and osmotic stress [261]. SRWD2 (OsWD40-52) (p-value=3.51E-11) is a member of SRWD (Salt Responsive WD40 repeats) subfamily known to be regulated by salinity in rice. [162]

Trehalose 6-phosphate phosphatase 1 (OsTPP1) (p-value=4.29E-13) was demonstrated to have an elevated expression under salt, osmotic and cold stresses as well as ABA treatment. Furthermore, overexpression of OsTPP1 triggered abiotic stress responsive genes, which proposes a potential transcriptional regulation pathway in stress-induced reprogramming is initiated by OsTPP1. [262]

Aquaporins in the tonoplast were suggested to be involved in vacuolar functions. The rice genome contains 33 aquaporin genes, 10 of which encode tonoplast intrinsic proteins (TIPs). OsTIPs showed different expression patterns under various abiotic stress conditions including dehydration, high salt and ABA treatments [263]. In this comparison, OsTIP1;1 (p-value=0.00014) was up-regulated.

Cellulose biosynthetic process (p-value=1.49E-05) was also enriched by several genes involved in cellulose synthesis such as Cellulose synthase A (OsCESA1-5), Cellulose Synthase-Like H1 (OsCSLH1) (p-value=3.89E-13) and UDP-Arabinopyranose Mutase 2 (OsUAM2) (p-value=0.001).

In addition, biological processes enriched by chloroplast-related genes such protein targeting to chloroplast (p-value=0.002921), thylakoid membrane organization (p-value=0.0056) and chlorophyll biosynthetic process (p-value=0.0055) were observed. This is consistent with a recent study proposing a role of alternative splicing in plant's response to light and promotion of photo-respiration. [264]

Down-regulated:

As expected, mRNA modification (p-value= 0.001) was the top enriched BP by the down-regulated genes in this comparison, followed by N-terminal protein myristoylation (p-value=0.003), carbohydrate metabolic process (p-value=0.004) and regulation of meristem growth (p-value=0.005) (see Figure 3.11).

N-myristoylation is an irreversible protein lipidation, which is recognized as a key modification because it is suggested to involve almost 2% of all plant proteins. This lipid modification is believed to mediate the control of the redox imbalances resulting from various stresses in plants as well as enhance the control of the damages induced by environmental changes.[265][266]

Among the top down-regulated genes in this comparison is Ion channel POLLUX (p-value=2.64E-10), a symbiotic cation channel that is known to play an important role in symbiosis signaling pathway and plant nutrition. POLLUX was shown to be essential for inducing the nuclear calcium spiking, an early crucial step plant- symbiotic partner recognition. [267]

Two pentatricopeptide repeat (PPR) domain containing proteins Os09g0423300 (p-value=0.0006) and Os02g0697500 (p-value=4.43E-07) were down-regulated in this comparison. Os02g0697500 was previously reported to be differentially expressed in drought salt and cold stresses. [268]

Yellow Stripe-Like (YSL) proteins belong to the oligopeptide transporter family and have been implicated in metal transport and homeostasis in different plant species. OsYSL6 (p-value=6.20E-08) was found to be involved in the detoxification of excess manganese in rice. [269]

A spliceosome-associated protein, Cwf15/Cwc15 cell cycle control protein family protein (p-value=9.03E-08), was reported to be involved in RNA processing. [270]

3.1.3. Control wild-type versus control Cas9 overexpression only

CRISPR-Cas is a defense system in the bacteria and archaea against invading genetic elements. Cas9 protein targets and cleaves DNA which is complementary to a guide RNA (gRNA) after the recognition of a protospacer adjacent motif (PAM) sequence in the target DNA. In this

comparison, the overexpression of Cas9 only without a specific guide RNA was found to affect the expression of a set of genes involved in stress responses when compared to the wild type control. This finding couldn't be fully explained, yet, it was reported that Streptococcus pyogenes Cas9 (SpyCas9) is able to cleave DNA without a guide RNA in the presence of divalent metal ions like manganese (Mn^{2+}) ions.[271]

There were 122 differentially expressed transcripts in the Cas9 overexpression only (Cas9_C) compared to the wild-type (WT_C) (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.5).

The differentially expressed transcripts in this comparison were not enriched in any biological processes. So, some of the top differentially expressed transcripts will be discussed in this section.

Up-regulated:

Many Late Embryogenesis Abundant (LEA) genes are expressed in response to ABA, drought, and salinity stress [272][273]. OsLEA27 (p-value= 3.72E-05), also known as RAB16C (RESPONSIVE TO ABA GENE 16C), was shown to be down-regulated by ABA while up-regulated by GA [274]. Also, OsLEA14 (p-value= 0.000171) and OsLEA25 (p-value= 2.79E-05) were up-regulated in this comparison.

Pathogenesis-related gene 1b (OsPR1b) (p-value= 7.35E-06) play a key role in defense signaling pathway [275][276]. It was also demonstrated that OsPR1b was induced by JA and upregulated by salicylic acid (SA) and ABA treatments.[277]

Phi Glutathione S-Transferase 4 (OsGSTF4) (p-value= 2.94E-06) belongs to the Glutathione S-transferases family that comprise a large number of genes encode crucial defense enzymes against xenobiotic toxicity. [278]

Down-regulated:

The elimination of damaged proteins can be accomplished by autophagy, a key process and a highly conservative protein degradation system in eukaryotic cells [279]. Recent studies revealed that autophagy play a role in plant responses to environmental stresses like salt, hypoxia, drought and osmotic stress[280][281][282]. Autophagy Associated Gene 18c (OsATG18c) (p-value=

1.50E-08), also known as OsWD40-30, a gene involved in the autophagic process was down-regulated in this comparison.

Cinnamyl Alcohol Dehydrogenase 3 (OsCAD3) (p-value= 2.61E-08) is an enzyme important in lignin biosynthesis [283]. OsCADs were displayed to be involved in the defense response of rice against biotic and abiotic stresses. [284]

Plants respond to various environmental threats by initiating many signaling processes that usually involves different protein kinases, such as calcineurin B-like protein-interacting protein kinases (CIPKs). OsCIPK17 (p-value= 6.79E-06) was previously reported to be expressed under several stresses like drought, salinity and ABA treatment. [285]

3.1.4. Control wild-type *versus* salt-treated wild-type

There were 1174 differentially expressed transcripts in the salt-treated (WT_NaCl) compared to the control wild-type (WT_C) samples (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.6).

In this comparison, a variety of stress responsive genes were differentially expressed in response to salinity (see Figure 3.12).

Up-regulated:

Response to oxidative stress (p-value=6.17E-15), cell growth (p-value=1.21E-12), anatomical structure morphogenesis (p-value=2.31E-10), lipid transport (p-value=3.45E-08), oxidation-reduction process (p-value=5.33E-07), plant-type cell wall organization (p-value=1.51E-05) and metal ion transport (p-value=0.00011) were among the top biological processes enriched by the up-regulated genes in this comparison (see Figure 3.12).

In response to oxidative stress, a wide range (31 genes) of peroxidase activity-related genes (p-value=2.67E-16) were found to be among the most up-regulated genes in this comparison in response to salt stress as previously reported [286].

Salinity suppress growth by affecting cell wall loosening. Cell wall-related genes and expansins promote wall loosening and play roles in regulating salt stress tolerance. [287]
Cell wall organization or biogenesis related genes (p-value=1.51E-05) that encode for many enzymes that aid in the cell wall remodeling including expansins (alpha and beta expansins) and

xyloglucan endotransglucosydase/hydrolase (OsXTH19) were also found to be upregulated in this comparison.

Wall-associated kinases (WAKs) gene family, one of RLK gene families in plants, are plasma membrane localized and serve as cell wall receptors and also bind to pectin, one of the polymers constituting the cell wall [288]. Efficient communication between the plant cell wall and the cytoplasm is essential in plant development and response to biotic and abiotic stresses, WAKs play an important role in this communication[289]. They play a vital roles in cell expansion, pathogen resistance and stress responses [290]. In this study, a group of rice polysaccharide-binding genes (p-value=0.005406) including WAKs (OsWAK37, OsWAK47 and OsWAK71) and receptor-like cytoplasmic kinase (OsRLCK378) were found to be up-regulated in response to salt stress.

Lipid-binding genes ((p-value=8.40E-07) hybrid proline- or glycine-rich (HyP/GRP) were up-regulated. HyP/GRP plant-specific cell-wall/plasma membrane-associated proteins that are characterized by having two characteristic domains: a variable hydrophilic N-terminal domain and a conserved hydrophobic C-terminal domain which is related to non-specific lipid transfer proteins. They are believed to have various functions in plant development and responses to various stresses including salinity. [291][292]

Rice stress/ABA-activated protein kinase (OsSAPK8) was highly up-regulated (p-value= 2.05E-06). Hyperosmotic stress activates all the members of the SnRK2 protein kinase family, among which subclass III of SAPKs (SAPK8, SAPK9, and SAPK10) are activated by ABA, inducing osmotic stress tolerance by regulation of stomatal closure. [293]

High-affinity nitrate transport system (NRT2.2) (p-value= 0.002613) was up-regulated under salt conditions to compensate for the decreased nitrate uptake as previously reported. [172]

High-Affinity K+ Transporter 1(OsHKT1) (p-value=5.50E-10) and (OsHKT7) (p-value=0.0009) were up-regulated in this comparison. HKT protein family have been shown to be essential for salinity tolerance, as it acts by eliminating excess Na+ ions from sensitive shoot tissues in plants. [294]

Aquaporins, including OsTIPs (OsTIP1;1) ((p-value=8.39E-06) (OsTIP2;1) ((p-value=2.67E-16) (OsTIP4;2) ((p-value=0.0003) were suggested to contribute to salinity tolerance as they play an

important role in facilitating water flux and maintaining the water potential in various plant tissues and cells. [295]

Down-regulated:

Response to water deprivation (p-value= 2.46E-05), transmembrane transport (p-value= 0.00022), response to heat (p-value= 0.0011), response to growth hormone (p-value= 0.004) and response to abscisic acid (p-value= 0.006) were among the top biological processes enriched by the down-regulated genes in this comparison (see Figure 3.12).

WRKY DNA-binding protein 70 (OsWRKY70) (p-value= 2.43E-11) is among the top down-regulated genes. In *Arabidopsis*, WRKY70 and WRKY54 were demonstrated to negatively regulate stomatal closure, thus attenuating osmotic stress tolerance. Moreover, the inactivation of WRKY70 and WRKY54 enhanced the plant tolerance to osmotic stress. [296]

Guard cell outward rectifying k+ channel (OsGORK) (p-value=5.04E-07) was down-regulated in this comparison as well. Demidchik and collegues observed that salinity-induced osmotic stress leads to increased ROS production and activated K^+- efflux channels (OsGORK), resulted in a decrease of cystolic K^+ levels and induction of cell death. [297]

ABA Insensitive 5 (OsABI5) (Adjusted p-value=0.005306) is a bZIP-type transcription factor. Its gene expression was shown to be induced by high salinity. Overexpression of OsABI5 in rice is correlated with high sensitivity to salt stress while its repression enhanced stress tolerance. [298]

NA+/H+ antiporter (OsNHX1) (p-value= 2.74E-07) was down-regulated in this comparison. Overexpression of OsNHX1 enhanced salt tolerance in transgenic rice cells and plants. [299]

In literature, Late Embryogenesis Abundant Protein (LEA) proteins were reported to accumulate during the salinity-triggered growth arrest in seedlings [300]. OsLEA16 (p-value= 1.09E-05) OsLEA22 (p-value=1.20E-06) OsLEA3-1 (p-value= 2.41E-05) OsLEA33 (p-value= 0.001) OsLEA9 (p-value= 6.57E-05) showed down-regulation in this comparison.

3.1.5. Control heterozygous Os-RS29 / homozygous Os-RS33 double mutant versus salt-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant

There were 409 differentially expressed transcripts in the salt-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_NaCl) compared to the control (RS_Het_C) (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.7).

Up-regulated:

In this comparison, oxidation-reduction process (p-value= 3.64E-09) and response to oxidative stress (p-value=3.07E-06) were the most enriched processes by the up-regulated genes (see Figure 3.13). Genes with peroxidase activity (p-value= 3.41E-12) were highly up-regulated similar to WT_C versus WT_NaCl comparison.

Metabolic pathways were also enriched. Genes involved in phenylalanine metabolism (p-value=4.30E-05) phenylpropanoid biosynthesis (p-value=0.00015) were up-regulated as a response to salt stress as previously reported. [210]

Plant-type cell wall organization (p-value=0.004136259) was enriched by the cell wall modifying proteins expansins (OsEXPA11, OsEXPA14, OsEXPA22, OsEXPB6 and OsEXPB7) which were found to be up-regulated in this comparison. Expansins are involved in plant cell growth, proliferation, senescence and adaptation to stress conditions as well as response to variety of plant hormones [301].

OsbZIP74 (p-value=3.16E-05) encodes a basic leucine–zipper transcription factor that plays an important role in endoplasmic reticulum (ER) stress regulation. It has been associated with response to heat stress and salicylic acid, a crucial plant hormone in systemic acquired resistance against pathogens. [302]

Down-regulated:

Response to water (p-value=1.87E-07) was enriched by the down-regulated genes of this comparison (see Figure 3.13).

A variety of stress responsive genes were down-regulated in this comparison.

Basic helix-loop-helix protein 008 (OsbHLH008) also referred to as OsMYC2-like protein 2 (OsMYL2) (p-value=1.29E-07) that play a role in JA signaling [303]. OsbHLH008 was highly

down-regulated in this comparison (-24.96 folds) while it was up-regulated in the untreated WT_C vs RS_Het_C comparison. In a similar manner, the α-amylase gene (OsAmy1A) (p-value=0.0004) was shown to be down-regulated in this comparison yet up-regulated in the WT_C vs RS_Het_C comparison. Suggesting that the mutation in the Os-RS33 affects the expression of these genes in response to salt treatment.

In plants, osmotic stress triggers opening of the osmosensitive channels, allowing a rapid downstream signaling cascade initiated by elevated cytosolic calcium. Members of the hyperosmolality-gated calcium-permeable channels (OSCA) family have been proposed to play a pivotal role during the initial phase of hyperosmotic stress response [304]. OsOSCA1.2 (p-value=1.12E-05) was highly (-21 folds) down-regulated in this comparison.

Ethylene Response Factor 118 (OsERF118) (p-value=0.0005) was down-regulated. ERF subfamily belongs to the AP2/EREBP transcription factor family [305]. They function in plant stress tolerance by regulating the stress-responsive genes. Many ERF genes are induced by various abiotic stresses, such as high salinity, osmotic stress, drought and cold and it was shown that their over-expression improved abiotic stress tolerance in transgenic plants. [306][307]

3.1.6. Control homozygous Os-RS29 / homozygous Os-RS33 double mutant versus salt-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant

There were 2089 differentially expressed transcripts in the salt-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_NaCl) compared to the control (RS_Homo_C) (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.8).

Up-regulated:

A wide range of stress responsive biological processes and pathways were enriched by the up-regulated transcripts in this comparison among them is response to oxidative stress (p-value=3.68E-07), carotenoid biosynthetic process (p-value=3.42E-05), response to blue light (p-value=0.0003), chlorophyll biosynthetic process (p-value=0.00852556) and response to water deprivation (p-value=0.01) (see Figure 3.14).

GDSL Esterase/Lipase Proteins (OsGELP10, OsGELP107, OsGELP31, OsGELP43, OsGELP7 and OsGELP82) (p-value=0.01) expression was displayed to be modulated by biotic and abiotic

stresses as well as chemical and hormonal treatments. OsGELP107 (p-value=1.60E-05) was up-regulated under salinity stress. [308]

Laccases family genes are widely distributed in plant genomes where they function by oxidizing mono-lignols for producing a higher-order lignin that is involved in plant development and stress responses. OsLACs were found to be induced by salt, drought, hormones and heavy metals stresses. OsLAC4 OsLAC7, OsLAC11, OsLAC13, OsLAC14 and OsLAC15 were upregulated in this comparison. OsLAC7 (p-value=0.0003) and OsLAC14 (p-value=1.37E-12) were previously reported to be induced by salt stress.[309][310]

Receptor-Like Cytoplasmic Kinase (RLCKs) are known to be regulated by various abiotic stresses such as drought, salt and cold. They act by perceiving extracellular signals and consequently activate the downstream signaling pathway via phosphorylating specific targets [311]. Several RLCKs were up-regulated in this comparison (OsRLCK78, OsRLCK79, OsRLCK90, OsRLCK157, OsRLCK185, OsRLCK256, OsRLCK265 and OsRLCK319).

Yellow Strip-Like Gene 6 (OsYSL6) (p-value=4.93E-05) was shown to play a role in detoxification of excess manganese (Mn) in rice hence enhancing Mn tolerance.[269] [312]

On the other hand, OsYSL6 was down-regulated in WT_C versus WT_NaCl and WT_C versus RS_Homo_C comparisons.

Down-regulated:

Stress response biological processes were enriched such as response to cadmium ion (p-value=1.45E-08), response to abscisic acid (p-value=2.49E-05), cellulose biosynthetic process (p-value=0.00042), response to heat (p-value=0.0013), defense response to bacterium, incompatible interaction (p-value=0.0001), response to cold (p-value=0.0032), cellular homeostasis (p-value=0.004), response to water deprivation (p-value=0.005835964) and defense response to bacterium (p-value=0.006) (see Figure 3.14).

Several salt stress responsive genes were down-regulated including Dehydration-Responsive Element-Binding Protein 1C (OsDREB1C) (p-value=1.50E-07) and OsDREB1G (p-value=2.39E-05), High-Affinity K+ Transporter 4 (OsHKT4) (p-value=0.00089), Na+/H+ Antiporter 1 (OsNHX1) (p-value=1.68E-10) and OsNHX2 (p-value=0.00063), Vacuolar H-

ATPase A (OsVHA-A) (p-value=1.34E-05) and OsVHA-B (p-value=3.01E-05) and Trehalose-6-Phosphate Phoshphatase 1 (OsTPP1) (p-value=0.001), rendering a sensitive response towards salinity stress. [111][114][115][313]

Those genes displayed up-regulation in RS_Homo_C double mutant compared to WT_C before salt treatment.

Moreover, despite being up-regulated in RS_Homo_C double mutant compared to WT_C, many WD40-repeat protein family members (OsWD40-130, OsWD40-138, OsWD40-143, OsWD40-17, OsWD40-171, OsWD40-178, OsWD40-181, OsWD40-199, OsWD40-29, OsWD40-36, OsWD40-59 and OsWD40-76) were down-regulated after salt treatment. WD40 proteins were identified to play a pivotal role in various protein-protein interactions by acting as scaffolding molecules and hence assisting the proper activity of the proteins. [314]

The conservation of mechanisms to globally inhibit protein synthesis concomitant to mRNA translation reprogramming under different stresses points out to the fundamental importance of translation regulation during the response to abiotic stresses in eukaryotes. After salt treatment of this double mutant, Eukaryotic Initiation Factor 4a (eIF-4a) (p-value=1.36E-05), eIF-3e (p-value=0.0014) and eIF-3m (p-value=0.0015) were found to be down-regulated indicating an impaired mRNA translation reprogramming which was previously demonstrated to be an essential translation regulation mechanism in response to abiotic stresses in eukaryotes. [315]

Protein folding and disaggregation are essential processes for the survival of plant cells under stressful conditions. These processes are supported by a collaborative action between molecular chaperones, heat shock proteins (Hsps), and co-chaperones, like DnaJ domain proteins [316]. Heat shock proteins were shown to be stimulated in response to a wide range of stress conditions and execute a vital role in protecting plants against various abiotic stresses [317]. In this comparison several heat shock proteins (OsHsp17.3 OsHsp70CP2 OsHsp80.2 OsHsp90) (p-value=8.46E-05) and DnaJ domain proteins (OsDjA1, OsDjA6, OsDjB7 and OsDjC74) (p-value=0.008) were down-regulated.

Therefore, it can be inferred that the tolerance to salt stress decreased in this double mutant (with the homozygous mutation of Os-RS29) via affecting the expression of a subset of salt responsive

genes. This finding also suggest that Os-RS29 plays a pivotal role in controlling the expression of many stress responsive genes and that the first RRM is crucial for its action.

3.1.7. Control Cas9 overexpression only versus salt-treated Cas9 overexpression only

There were 1245 differentially expressed transcripts in the salt-treated Cas9 overexpression (CAS9_NaCl) compared to the control (CAS9_C) samples (see Table 3.1). Heatmap of the top 50 differentially expressed transcripts is shown in (Figure 3.9).

Up-regulated:

Like the previous comparisons, after treatment, the most up-regulated processes are response to oxidative stress (p-value=8.88E-11) and oxidation-reduction process (p-value=4.04E-10) (see Figure 3.15).

Metal ion transport (p-value=0.00033) as well as different metal binding activities such as heme binding (p-value=1.92E-11), iron ion binding (p-value=5.21E-10) and copper ion binding (p-value=1.99E-05) were up-regulated in this comparison.

Down-regulated:

Stress responsive processes including response to water (p-value=2.01E-06), response to abscisic acid (p-value=1.79E-05), response to heat (p-value=3.72E-05), response to water deprivation (p-value=7.27E-05), response to cold (p-value=0.00062) and response to biotic stimulus (p-value=0.00012) were down-regulated (see Figure 3.15).

Late Embryogenesis Abundant Proteins Group 2, also referred to as dehydrins (OsLEA22, OsLEA25, OsLEA27, OsLEA29) (p-value= 2.01E-06) which were down-regulated in this comparison. Dehydrins were demonstrated to be induced by dehydration-related stresses such as drought, high salinity and low temperature [318]. Furthermore, they were associated with enhance tolerance to salinity and osmotic stress in various plants. [319]

Metabolic processes such as proline biosynthetic process (p-value=0.0016) and secondary metabolic process (p-value=0.00094) were also down-regulated.

Thus, the response to salt stress in this condition resembles that of the wild-type.

3.3. Alternative splicing analysis

Since the double mutants involved two splicing factors (Os-RS29 and Os-RS33), the landscape of constitutive and alternative splicing is proposed to be altered. Therefore, it was interesting to investigate the isoform shift associated with each double mutant and identify the functional enrichment of the genes with such changes. Isoform shift occurs when a gene has two or more isoforms, but only one is the most abundant compared to the others. Under certain circumstances, the distribution of isoforms is altered, where the dominant isoform is shifted to be less abundant compared to the other isoform(s) that were formerly less abundant. Isoform shift can be employed to produce proteins with different or modified function or regulate gene expression via nonsense-mediated decay (NMD) in response to changes associated with growth, development and various environmental stresses.

In this study, 15181, 11836, 17264, 4764, 12701, 18730, 14866 and 20408 alternative splice events have been detected from WT_C, CAS9_C, RS_Het_C, RS_Homo_C, WT_NaCl, CAS9_NaCl, RS_Het_NaCl and RS_Homo_NaCl conditions, respectively by ASATP (ASRecovist tool) (See Table 3.2). Seven types of Alternative splicing (AS) events have been identified in the analysis including intron retention (IR), exon skipping-cassette exon (CE)-, mutually exclusive exons (MXE), 5′ alternative splicing (A5SS), 3′ alternative splicing (A3SS), alternative first exons (AFE), and alternative last exons (ALE). The count of each AS event type per condition was calculated and plotted to infer the effect of the double mutations on AS landscape (See Fig).

Intron retention (IR) found to be the most predominant AS pattern among samples, this is consistent with the intron-retention background in rice that was recently reported [320]. IR was followed by 3′ alternative splicing then 5′ alternative splicing which were relatively more abundant compared with the other types of AS events. This finding resembles the results of a recent study where splicing was inhibited by Pladienolide B in Arabidopsis. [248]

The RS_Homo_C (homozygous Os-RS29/homozygous Os-RS33 double mutant) showed more decrease in the total no. of AS events than RS_Het_C (heterozygous Os-RS29/homozygous Os-RS33 double mutant) compared to the wild-type control WT_C. Upon exposure to salt stress, however, the no. of AS events increased dramatically in the treated RS_Homo_NaCl compared to the other double mutant RS_Het_NaCl and the wild-type WT_NaCl. Summary of all the

numbers of alternative splice events, genes underwent AS and transcripts produced per condition is shown in Table 3.2.

The mechanism by which OsRS29 and OsRS33 affect alternative splicing in rice is not fully understood. In this study, double mutants of those splicing factors where produced to investigate the effect of these mutations on the rice transcriptome before and after exposure to salt stress. Modulation of splice site selection was shown to be held through RNA recognition motif (RRM)-mediated binding to exonic splicing enhancers (ESEs) and RS domain-mediated protein-protein and protein-RNA interactions during the spliceosome assembly. [321]
Furthermore, RRM domain was displayed to determine the substrate specificity, whereas RS domains are functionally substitutable and are not contributing to substrate specificity [322]. A study by Isshiki and colleagues [42], indicated that Os-RS29 and Os-RSZ23 enhance splicing and favor different 5´ splice sites of the same intronic region. In addition, domain-swapping experiments revealed that the first RRM is essential for Os-RS29's efficient splicing activity [42]. In this study, the first mutation targeted the first RRM domain (6 nucleotides deletion causing deletion of P62 and G63 amino acids) of the Os-RS29, hence it affected the interaction of the splicing factor with its target genes. In addition, it required a homozygous mutation to exhibit this effect. The second target was exon 2 of Os-RS33, the insertion of 1 nucleotide caused PTC at position 32 early at the mRNA sequence. Thus, it can be suggested that it was subjected to degradation by a pathway like NMD.

The functional consequences of double mutants on AS was studied through functional enrichment of the genes with altered splicing among the different comparisons. The biological processes (p-value<0.03) enriched as well as the top most differentially spliced genes will be discussed in the following section.

3.3.1. Control wild-type versus control heterozygous Os-RS29 / homozygous Os-RS33 double mutant

Lipid catabolic process (p-value=0.00036) and lipid metabolic process (p-value=0.0037) were enriched by the top DSG in RS_Het_C compared to the wild type (See Figure 3.17).

Patatin-like phospholipase family proteins (OspPLAIIIalpha, OspPLAIIeta and OspPLAIIIzeta) were among the top differentially spliced genes. They were demonstrated to be involved in various stress responses, hormone signaling, and plant development. [323][324]

OsCPS1 (ent-copalyl diphosphate (CDP) synthase1) showed aberrant splicing in this comparison. It was reported to participate in gibberellin biosynthesis. A loss-of-function OsCPS1 mutants showed a severe dwarf phenotype [325]

Protein phosphorylation (p-value=0.019) was also enriched. Different kinases were differentially spliced. NIMA (Never In Mitosis Gene A)-related kinases (NEKs) are members of serine/threonine kinases family that was linked to cell-cycle regulation in fungi and mammals. The expression profiles of plant NEKs proposed that they are involved in plant development processes[326]. NEKs were associated to osmotic stress response and involved in regulation of plant growth [327][328]. OsNEK3 displayed aberrant splicing compared to the wild type.

3.3.2. Control wild-type versus control homozygous Os-RS29 / homozygous Os-RS33 double mutant

In this comparison, response to osmotic stress (p-value=0.00015), response to salt stress (p-value=0.00016) and response to abscisic acid (p-value=0.0018) were enriched by the top DSG (See Figure 3.18). These findings are consistent with previous studies which demonstrated that repressing the spliceosome machinery by using splicing inhibitors could trigger abiotic response in *Arabidopsis*. [248][249]

Protein ubiquitination (p-value=0.007) was found to be enriched as well. Protein regulation by the ubiquitin/proteasome system has been investigated as a target mechanism to improve rice adaptation and tolerance to different abiotic stresses. Ubiquitination is involved in the regulation of the phytohormones levels, stomatal opening, cell membrane integrity, along with the regulation of heavy metals levels and reactive oxygen species. [329]

Moreover, the alteration of the targeted SR proteins activity lead to alteration in other RNA processing and splicing factors owing to the cross-regulation among splicing factors.

RNA-binding proteins which regulate gene expression at the post-transcriptional level involving processes like pre-mRNA splicing, mRNA transport, mRNA stability and decay, as well as translation. [330]

In this comparison, splicing factors like glycine-rich RNA-binding proteins, especially (OsGRP3) showed an altered splicing. GRPs are RNA-binding proteins which are characterized by having RRMs at the N-terminus and a glycine-rich region at the C-terminus. GRPs are found

in many plant species and their expression is regulated by a number of external stimuli [331]. However, the exact physiological functions of GRPs in rice currently are mostly unknown.

Pre-mRNA-processing factor 19 (PRP19) also showed aberrant splicing. PRP19 is U-box and WD repeat containing protein with a ubiquitin ligase activity which is mainly involved in pre-mRNA processing and DNA repair [332]. It is also known as OsPUB72 that plays a role in the protein ubiquitination pathway.

Eukaryotic Translation Initiation Factor 4G protein (eIF4G) is another gene with altered splicing. eIF4G was displayed to play a vital role in the pre-mRNA splicing and mRNA surveillance by recruiting important splicing factors and critical mRNA decay factors to the pre-mRNA/mRNA. [333][334]

Os06g0608300 is a putative elongation factor 2 that was also found to have altered splicing in the RS_Homo_C double mutant. Interestingly, Os06g0608300, PRP19, OsGRP3 and eIF4G was found to interact with a several WD40 domain containing proteins that were shown to be up-regulated in the RS_Homo_C double mutant compared to the wild type WT_C while they were down-regulated in the RS_Homo_C versus RS_Homo_NaCl comparison. This network of protin-protein interaction (See Figure 3.24.A) was enriched in RNA transport (p-value=0.0048) and Spliceosome (p-value=0.0097) pathways (See Figure 3.24.B).

Therefore, it can be suggested that both Os-RS29 and Os-RS33 affects the splicing of the splicing factors that control the expression of genes possessing these repeat motif features that might have a role in stress response in rice. Future research is required to investigate the potential biological functions of these WD40 domain containing proteins.

3.3.3. Control wild-type versus control Cas9 overexpression only

Overexpression of Cas9 resulted in altered AS of genes which were enriched in various processes including lipid metabolic process (p-value=0.00014), response to blue light (p-value=0.001), protein glycosylation (p-value=0.0023), fatty acid beta-oxidation (p-value=0.0032) and cell death (p-value=0.0044) (See Figure 3.19).

It’s unclear and not previously reported how Cas9 can edit the genome without a gRNA. Furthermore, it could affect the splicing of different genes. This finding warrants further investigation.

3.3.4. Control wild-type versus salt-treated wild-type

rRNA processing (p-value=0.0024), systemic acquired resistance (p-value=0.0048) and pentose-phosphate shunt (p-value=0.014) were enriched by the top DSG of this comparison (See Figure 3.20).

Chloroplasts were demonstrated to fine-tune pathways for salinity response. Salt-responsive genes encoding chloroplast-localized proteins were identified. These genes are involved in several essential pathways in chloroplasts in response to salinity, such as chloroplast ROS scavenging [335]. Os11g0521500 (Acyl carrier protein, chloroplast) was one of the chloroplast-related genes with altered splicing in this comparison.

Calcineurin B-Like Protein-Interacting Protein Kinase 23 (OsCIPK23) was reported to participate in signaling pathways in response to different abiotic stresses including salinity [336][337]

3.3.5. Control heterozygous Os-RS29 / homozygous Os-RS33 double mutant versus salt-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant

Pollen development (p-value=0.0025), mRNA splicing, via spliceosome (p-value=0.0356) and cellular homeostasis (p-value=0.0359) were enriched by the top DSG in this comparison (See Figure 3.21).

Pollen development is a sensitive process that is affected by several abiotic stress [338][339]. Salinity was shown to have a severe effect on the flowering stage in rice, leading to unviable pollen, decreasing the pollen germination rate and fertilization ability, and ultimately decrease the grain yield. [340]

Members of the protein disulfide isomerase (PDI) family catalyze disulfide bonds formation, which is required for the stability and function of numerous proteins [341][342]. The correct disulfide bonds are formed during folding of peptides to produce native proteins in eukaryotic cells, via oxidative protein folding, which mainly takes place in the endoplasmic reticulum (ER) [343]. Furthermore, PDI family plays a crucial role in both the formation and reduction of disulfides for correct folding of proteins entering the ER [343]. However, under stress, the demand for protein folding exceed the protein folding machinery in the ER capacity. Under these conditions, unfolded proteins or misfolded accumulate in the ER, triggering an unfolded protein

response which in return trigger a signal that activates specific genes transcription [341]. These activated genes induce the expression of other genes can either increase the folding or enhance the degradation of unfolded proteins, such as ER-localized PDIs, attempting to maintain homeostasis of the ER [341][344]. Recently, member of PDI-like proteins (OsPDILs) was demonstrated to have a role in protein folding during rice development and in rice resistance to abiotic stress [345]. However, little is known about how plant PDIs function or how they are regulated in rice in response to stress. In this comparison, OsPDIL5-3 showed AS alteration where it exhibited increased intron retention after salt treatment.

A conserved hypothetical protein (Os06g0148000) that is involved in the splicing via the spliceosome showed aberrant AS in response to salt stress in this comparison. Further investigation will be required to characterize this protein and its role in AS regulation under salt stress.

3.3.6. Control homozygous Os-RS29 / homozygous Os-RS33 double mutant versus salt-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant

Many stress responsive genes were found to have altered AS in this comparison. Response to cold (p-value=0.002), response to abscisic acid (p-value=0.006) and response to salt (p-value=0.011) were enriched by the top DSG (See Figure 3.22).

Plant calcineurin B-like (CBL)-interacting protein kinases (CIPKs) (OsCIPK17 and OsCIPK33) exhibited altered AS in this comparison. OsCIPKs were displayed to have important roles in plant-specific calcium signaling. Furthermore, OsCIPKs are involved in various stress response pathways in plants. They were induced by biotic stresses and abiotic stresses such as bacterial infection, heavy metal, high salinity, ABA and cold stresses. [346]

Another family of PKs known as dual specificity PKs (OsDPK), also show response to biotic and abiotic stresses. OsDPK1 -which showed aberrant splicing in this comparison- along with OsDPK2 and OsDPK3 are all induced by exogenous application of ABA, drought, salinity and in response to the rice blast fungus. [347]

Thus, AS alteration of a protein kinases can further suggest a significant coupling between AS and phosphorylation especially in response to stress.

Importin β1 showed aberrant splicing. Importin β proteins are characterized by having a series of similar helical HEAT repeats in their structure and they mediate the nuclear transport of particular cargoes [348][349]. Furthermore, importin β1 is known to be involved in ABA response and it enhances drought tolerance in *Arabidopsis* [350]. Interestingly, importin β1 was found to be up-regulated in WT_C vs RS_Homo_C comparison while down-regulated in RS_Homo_C vs RS_Homo_NaCl comparison.

In plants, initiation of translation is a main target of the translation regulation in response to abiotic stress [315]. Translational regulation of preexisting mRNAs offers a quick and alternative approach to control gene expression in response to stress, compared to other slower cellular processes like mRNA transcription, processing, and transport to cytoplasm. [351]

The splicing of Eukaryotic Initiation Factor 3M (eIF-3m) and Eukaryotic Translation Initiation Factor 4G (eIF4G) was also altered in this comparison.

In addition, as previously mentioned, several eukaryotic translation initiation factors were found to be significantly down-regulated in RS_Homo_C versus RS_Homo_NaCl comparison. This finding suggests that initiation of translation another layer of regulation of gene expression besides alternative splicing that is affected by this the double mutant when exposed to salt stress.

3.3.7. Control Cas9 overexpression (CAS9_C) versus salt-treated Cas9 overexpression only (CAS9_NaCl)

Cell redox homeostasis (p-value=0.0016), pollen development (p-value=0.002) and rRNA processing (p-value=0.003) were enriched by the top DSG in this comparison (See Figure 3.21). These results were very similar to the results of the wild type comparison after treatment (WT_C versus WT_NaCl) suggesting that even if the overexpression of Cas9 altered the expression of a set of genes, it did not change the response of the plant to salt stress.

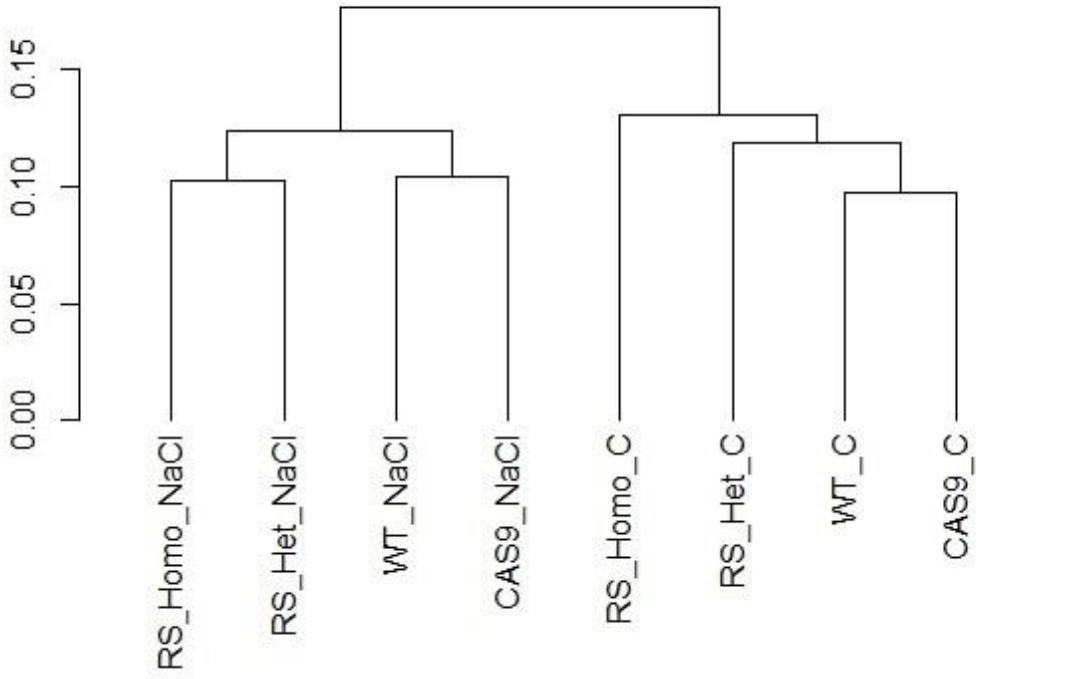


Figure 3.1. Hierarchical clustering (Dendogram) of normalized counts across the samples.

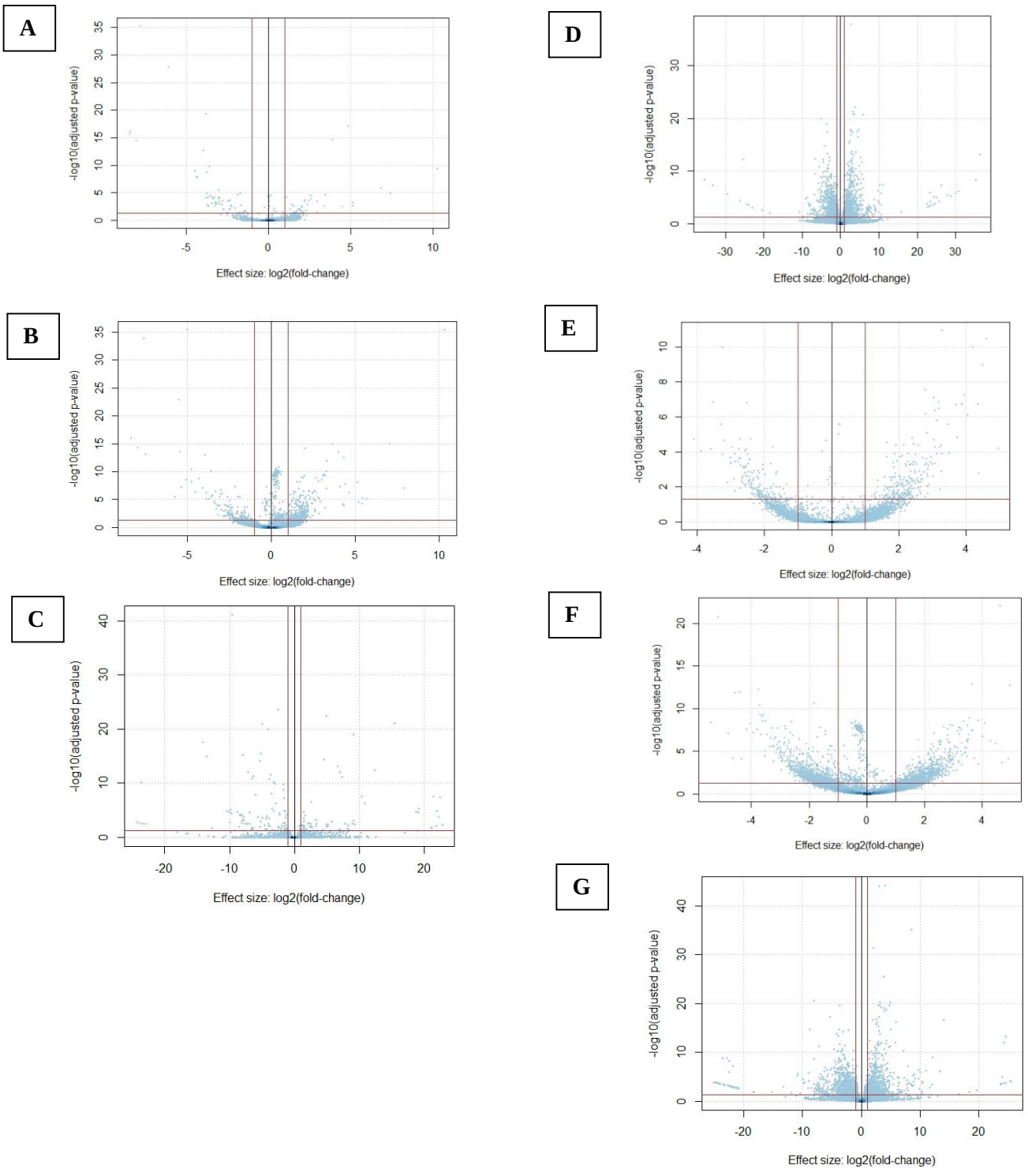


Figure 3.2. Volcano plots of expression alterations upon exposure to ? mM NaCl. Vertical lines indicate fold-change of two. Horizontal line indicates p-value less than 0.05. (A) WT_C and RS_Het_C (B) WT_C and RS_Homo_C (C) WT_C and Cas9_C (D) WT_C and WT_NaCl (E) RS_Het_C and RS_Het_NaCl (F) RS_Homo_C and RS_Homo_NaCl (G) Cas9_C and Cas9_NaCl

Table 3.1. Differentially expressed transcripts (DETs) among the different conditions. Transcripts are considered significantly up- or down-regulated, if the log2 fold change is greater than 2 or less than -2, respectively, and adjusted *p*-value is < 0.05.

Pair-wise comparison	*Total DETs*	*Up-regulated DETs*	*Down-regulated DETs*
WT_C versus RS_Het_C	*98*	*46*	*52*
WT_C versus RS_Homo_C	*886*	*722*	*164*
WT_C versus CAS9_C	*122*	*52*	*70*
WT_C versus WT_NaCl	*1174*	*556*	*618*
RS_Het_C versus RS_Het_NaCl	*409*	*234*	*175*
RS_Homo_C versus RS_Homo_NaCl	*2089*	*854*	*1235*
CAS9_C versus CAS9_NaCl	*1245*	*533*	*712*

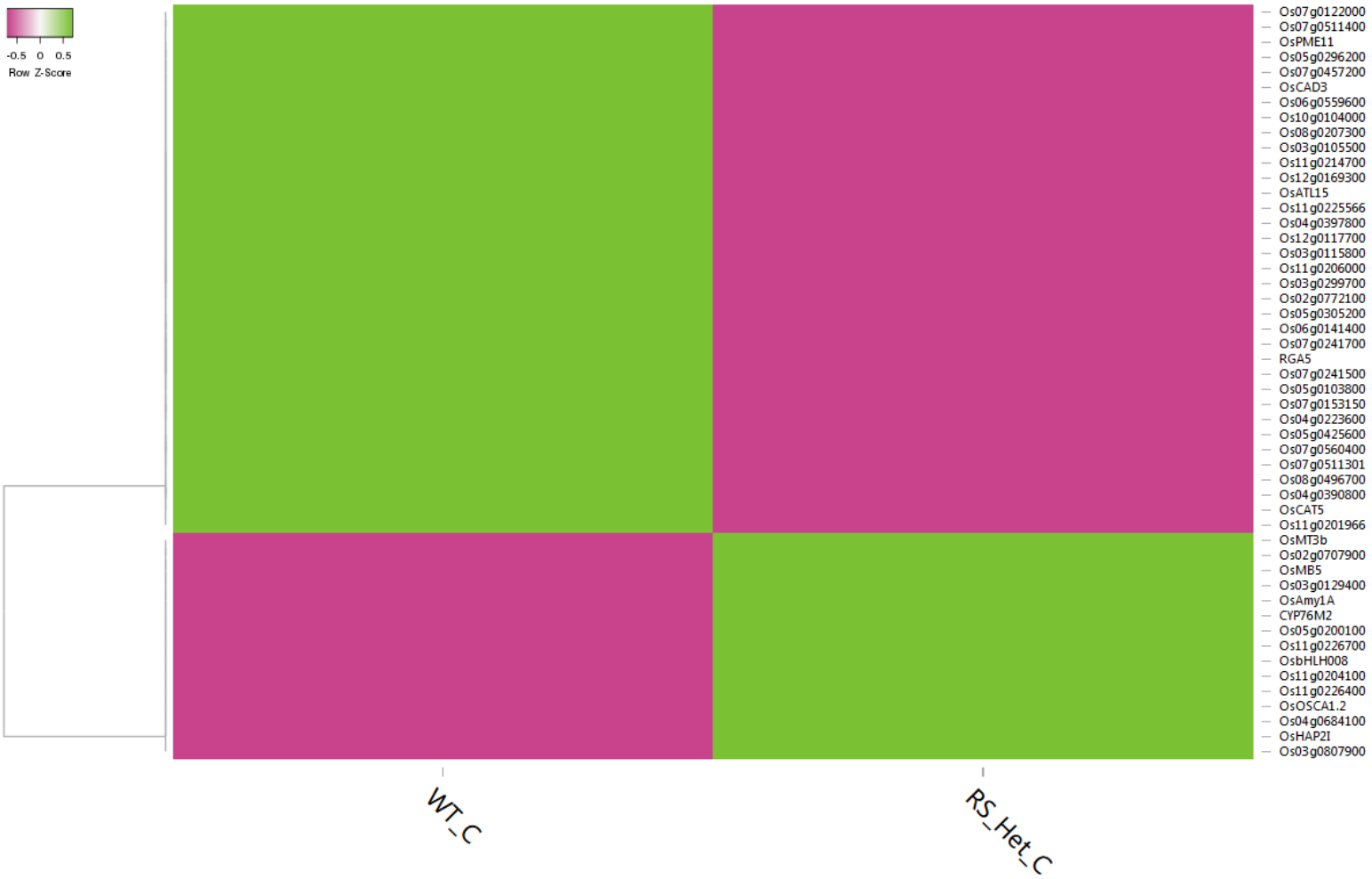


Figure 3.3. Heatmap of top 50 differentially expressed transcripts between controls of the wild-type (WT_C) and heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_C). Clustering of genes was done using the default parameters in the Heatmapper tool.

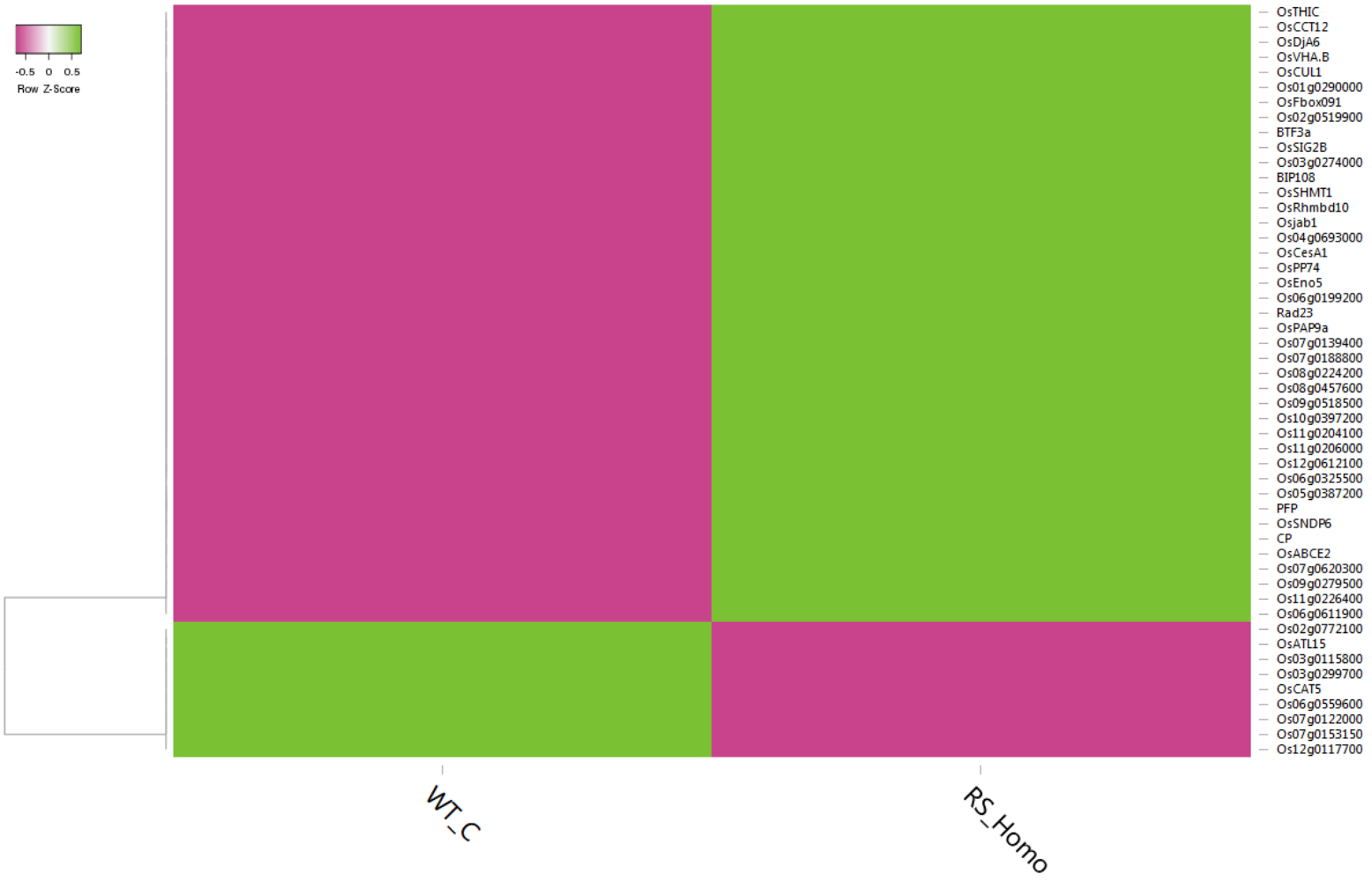


Figure 3.4. Heatmap of top 50 differentially expressed transcripts between the controls of the wild-type (WT_C) and the homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_C). Clustering of genes was done using the default parameters in the Heatmapper tool.

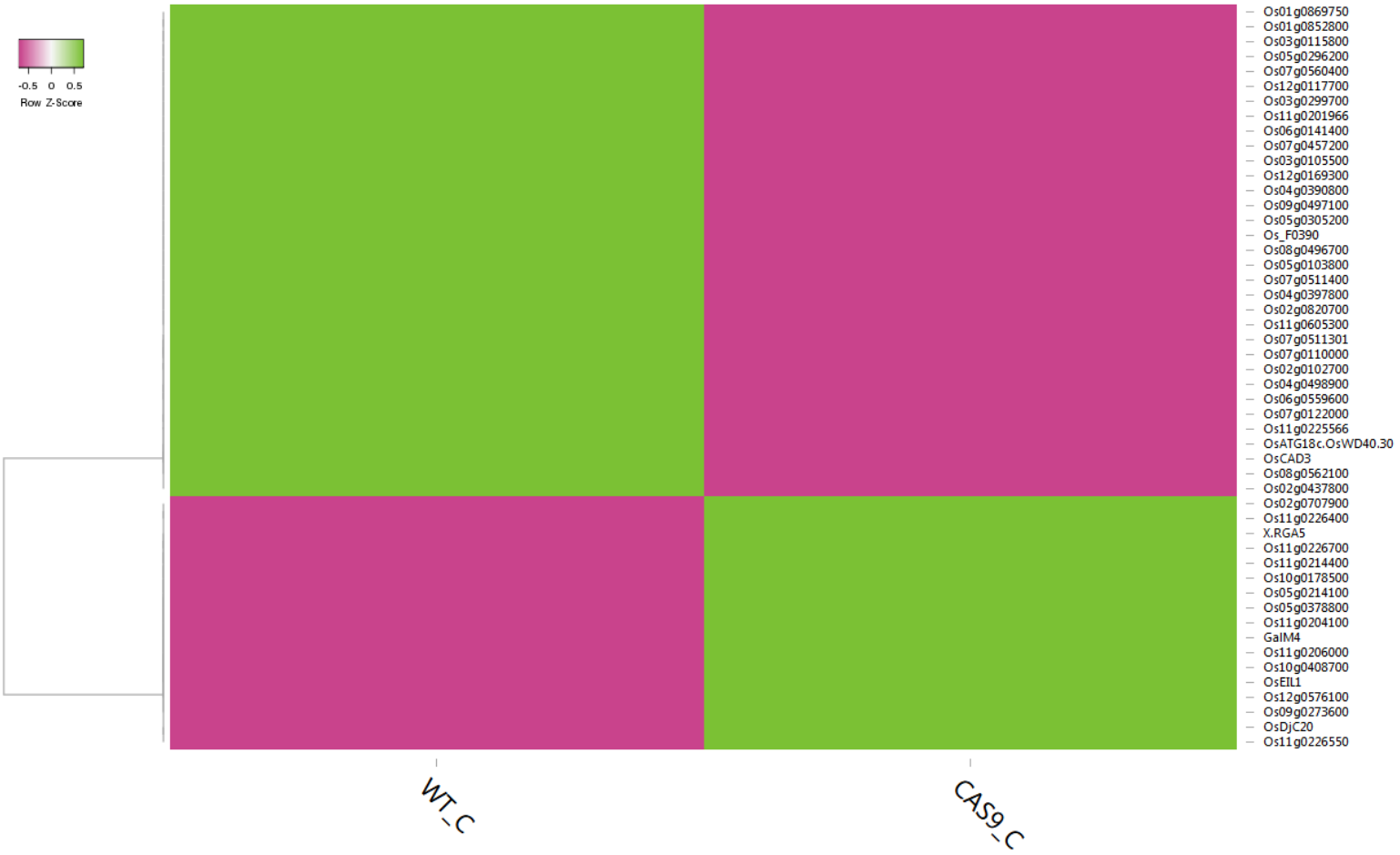


Figure 3.5. Heatmap of top 50 differentially expressed transcripts between the controls of the wild-type (WT_C) and the Cas9 overexpression (Cas9_C). Clustering of genes was done using the default parameters in the Heatmapper tool.

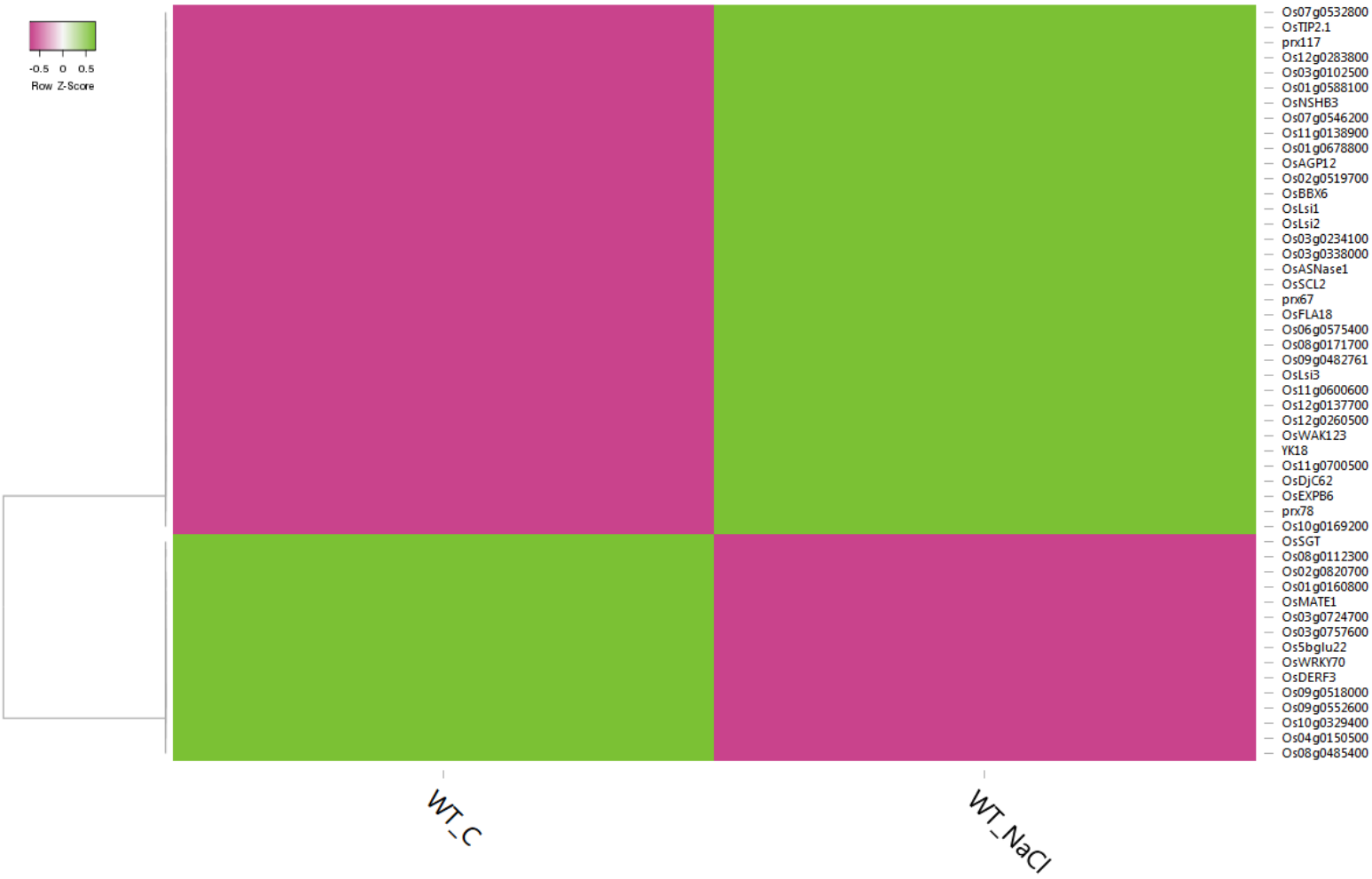


Figure 3.6. Heatmap of top 50 differentially expressed transcripts between the control (WT_C) and the salt-treated (WT_NaCl) wild-type samples. Clustering of genes was done using the default parameters in the Heatmapper tool.

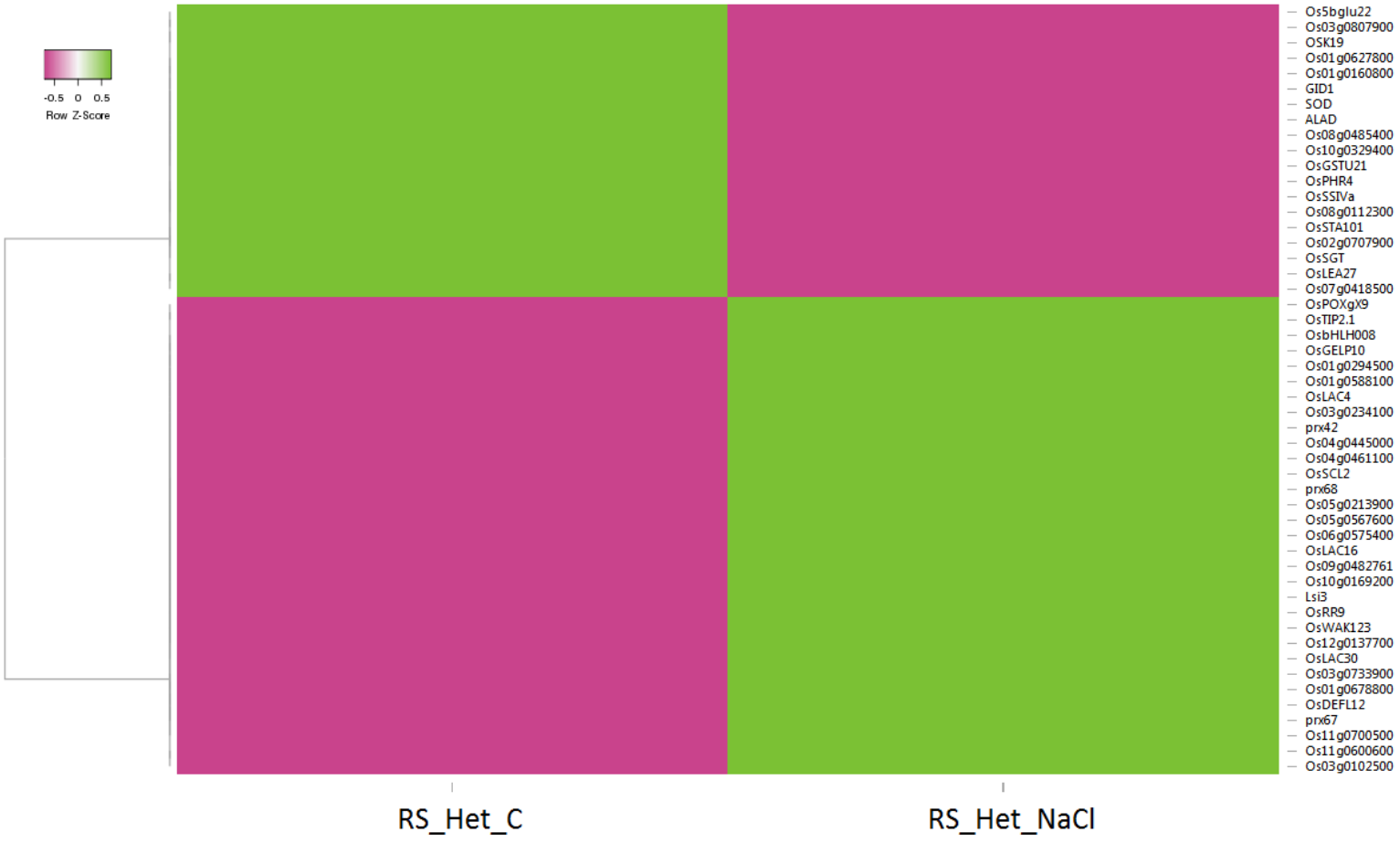


Figure 3.7. Heatmap of top 50 differentially expressed transcripts between the control (RS_Het_C) and the salt-treated (RS_Het_NaCl) heterozygous Os-RS29 / homozygous Os-RS33 double mutant samples. Clustering of genes was done using the default parameters in the Heatmapper tool.

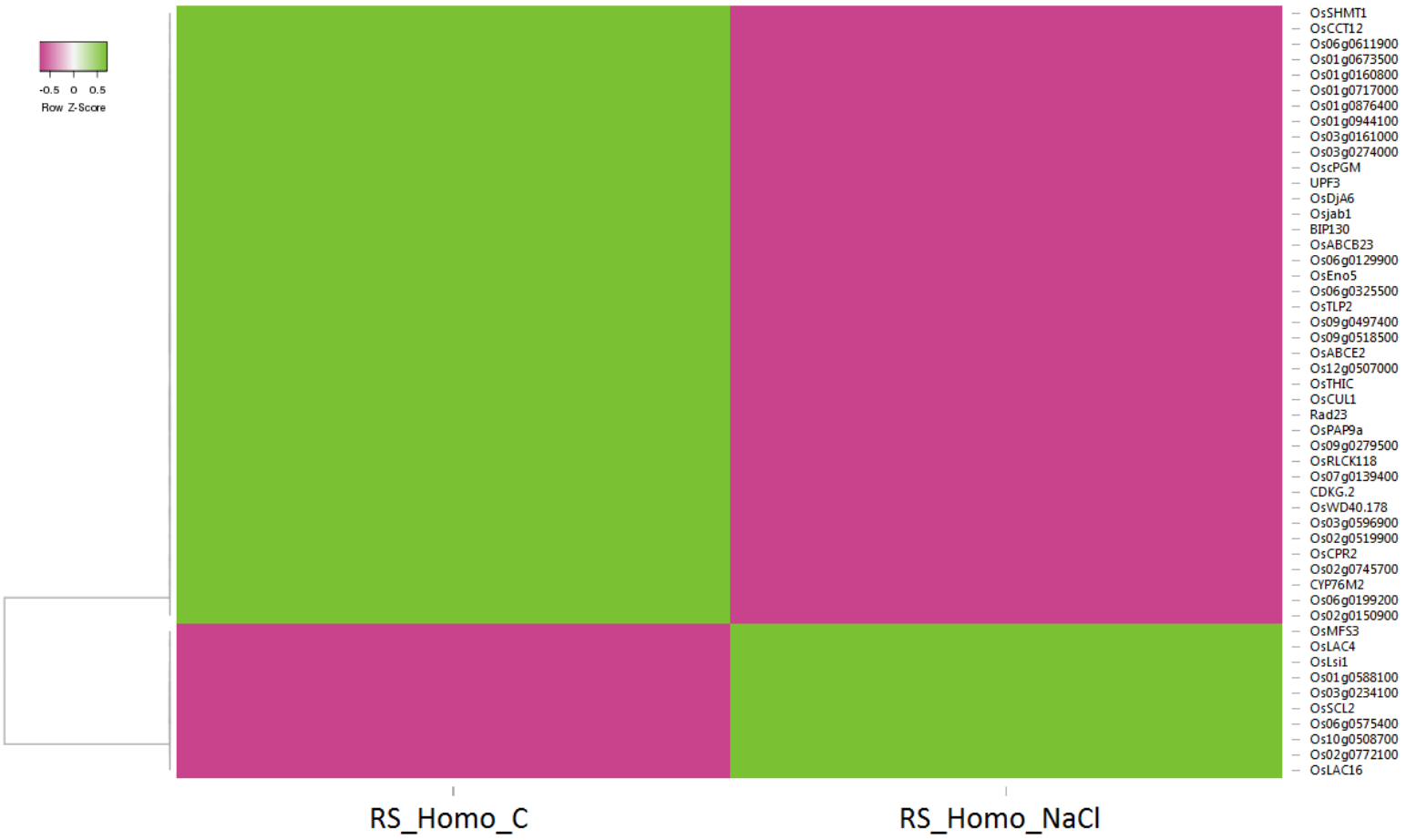


Figure 3.8. Heatmap of top 50 differentially expressed transcripts between the control (RS_Homo_C) and the salt-treated (RS_Homo_NaCl) homozygous Os-RS29 / homozygous Os-RS33 double mutant samples. Clustering of genes was done using the default parameters in the Heatmapper tool.

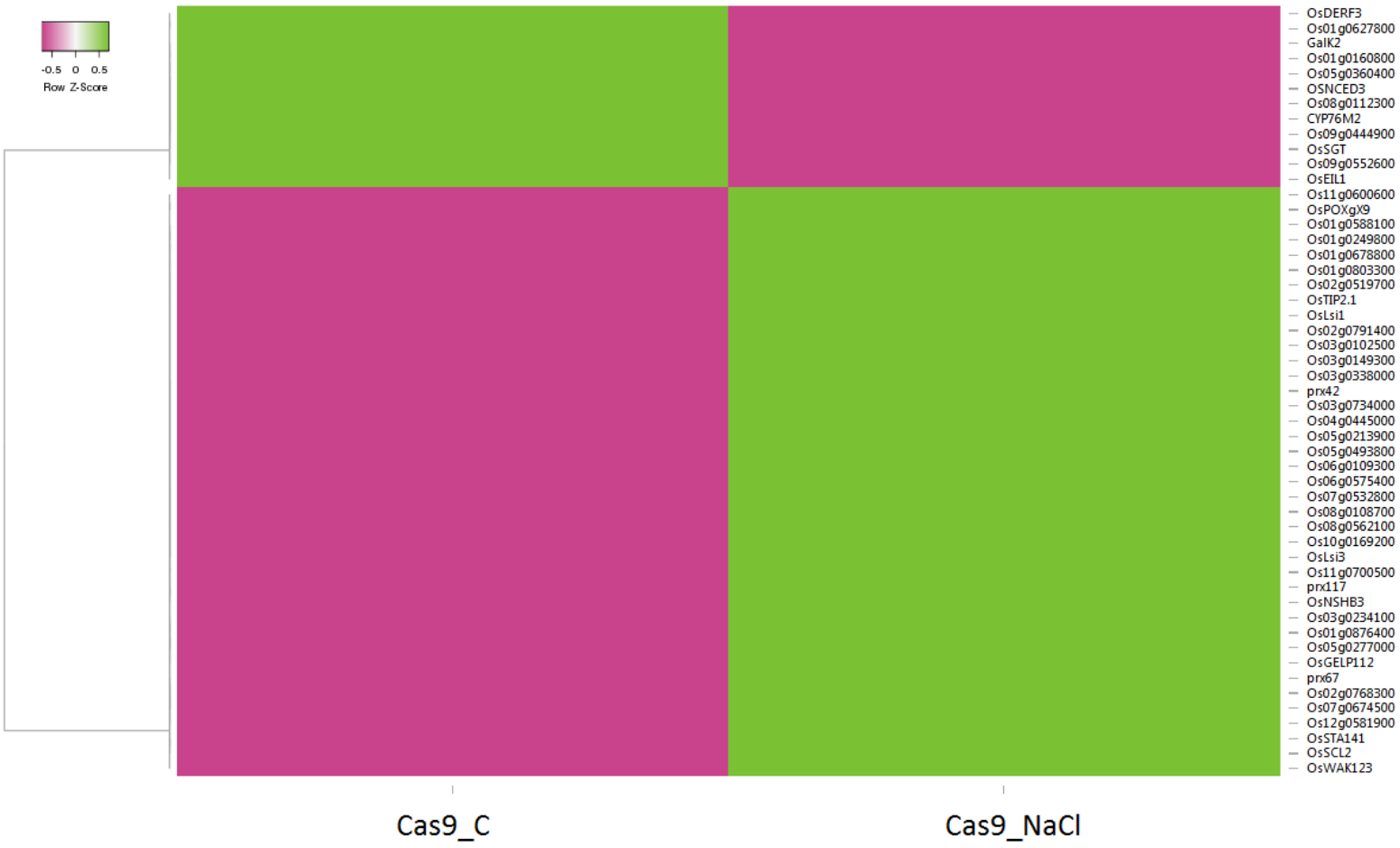


Figure 3.9. Heatmap of top 50 differentially expressed transcripts between the control (Cas9_C) and the salt-treated (Cas9_NaCl) Cas9 overexpression samples. Clustering of genes was done using the default parameters in the Heatmapper tool.

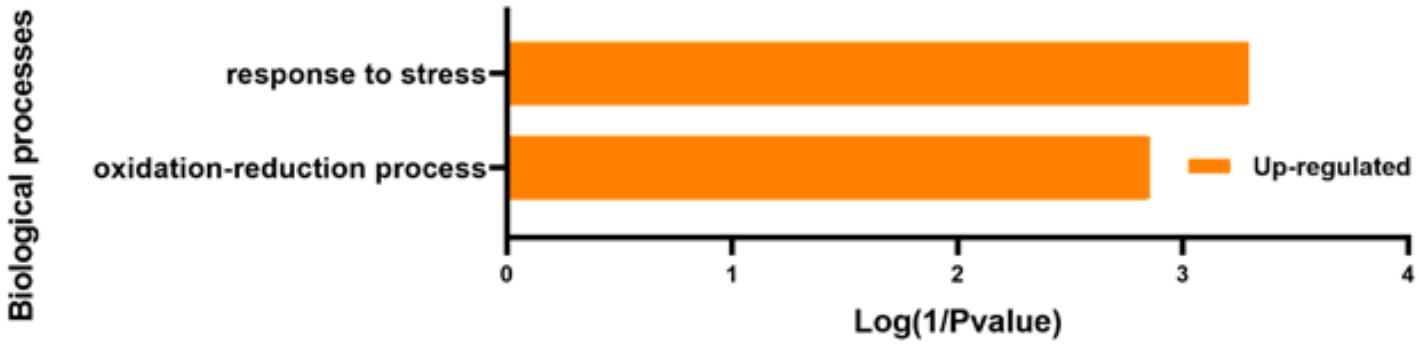


Figure 3.10. Top biological processes (BPs) enriched by the differentially expressed transcripts between the controls of the wild-type (WT_C) and the heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_C) using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

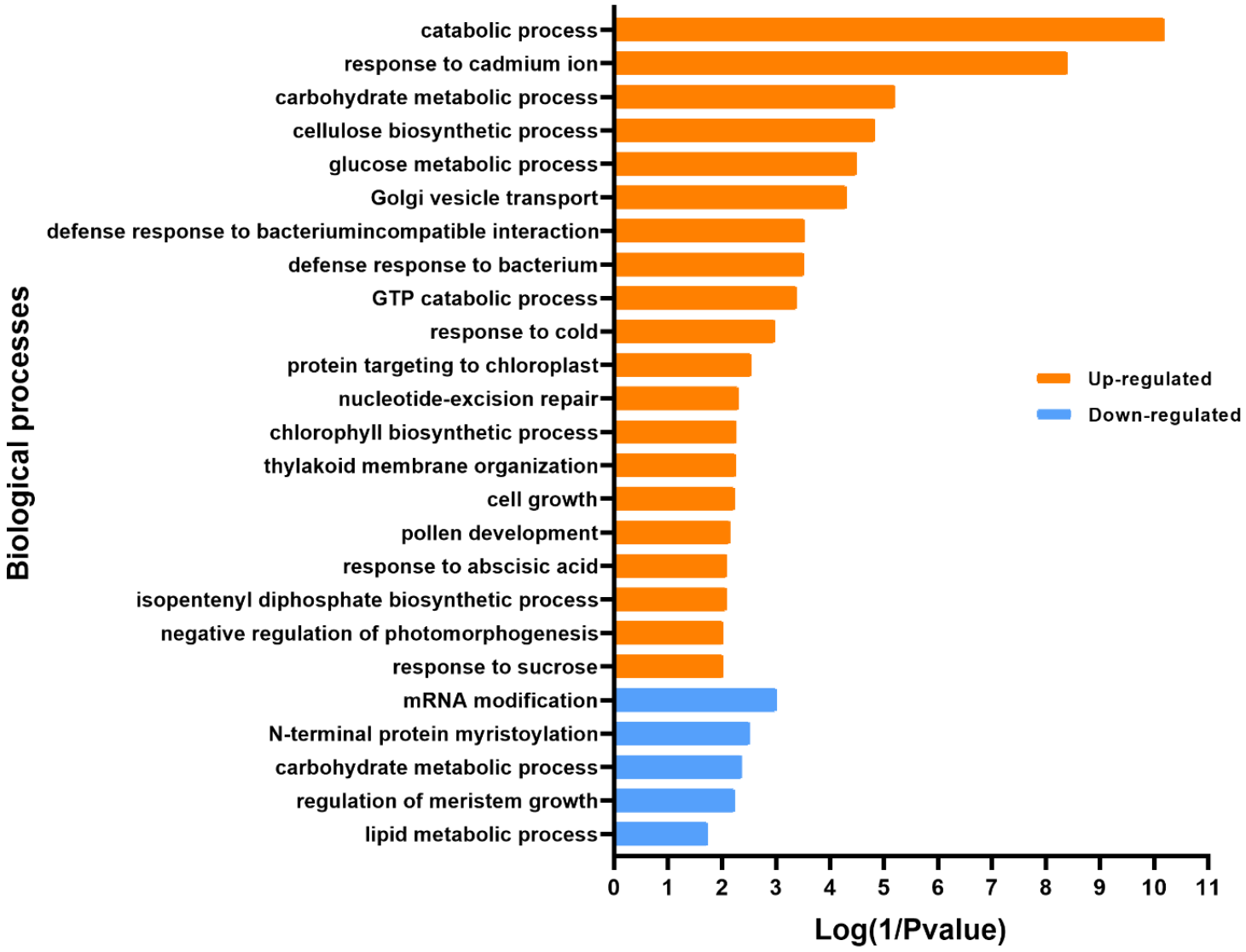


Figure 3.11. Top biological processes (BPs) enriched by the differentially expressed transcripts between the controls of the wild-type (WT_C) and the homozygous Os-RS29 / homozygous Os-RS33 double mutant (Rs_Homo_C) using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

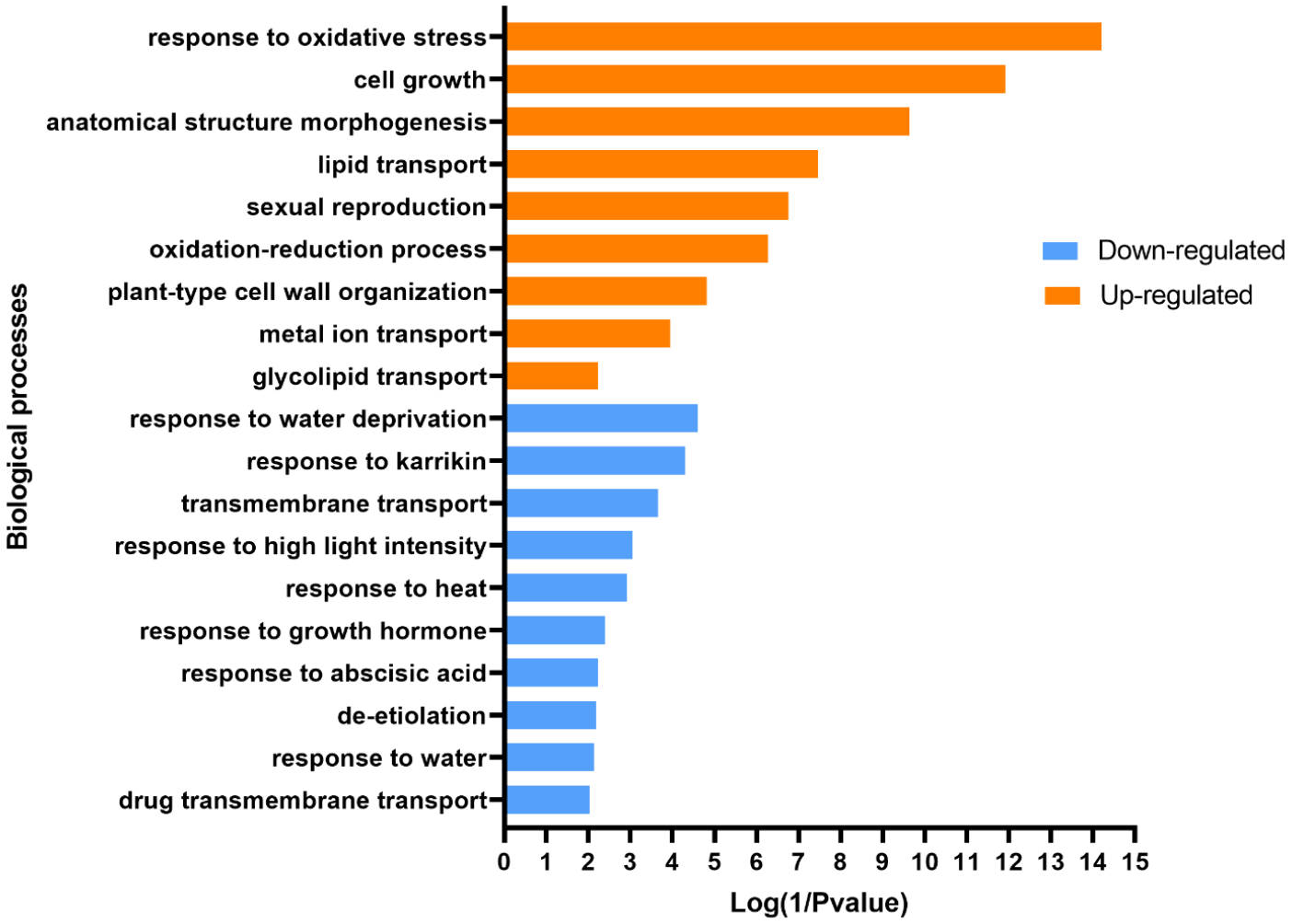


Figure 3.12. Top biological processes (BPs) enriched by the differentially expressed transcripts between the control (WT_C) and the salt-treated (WT_NaCl) wild-type samples using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

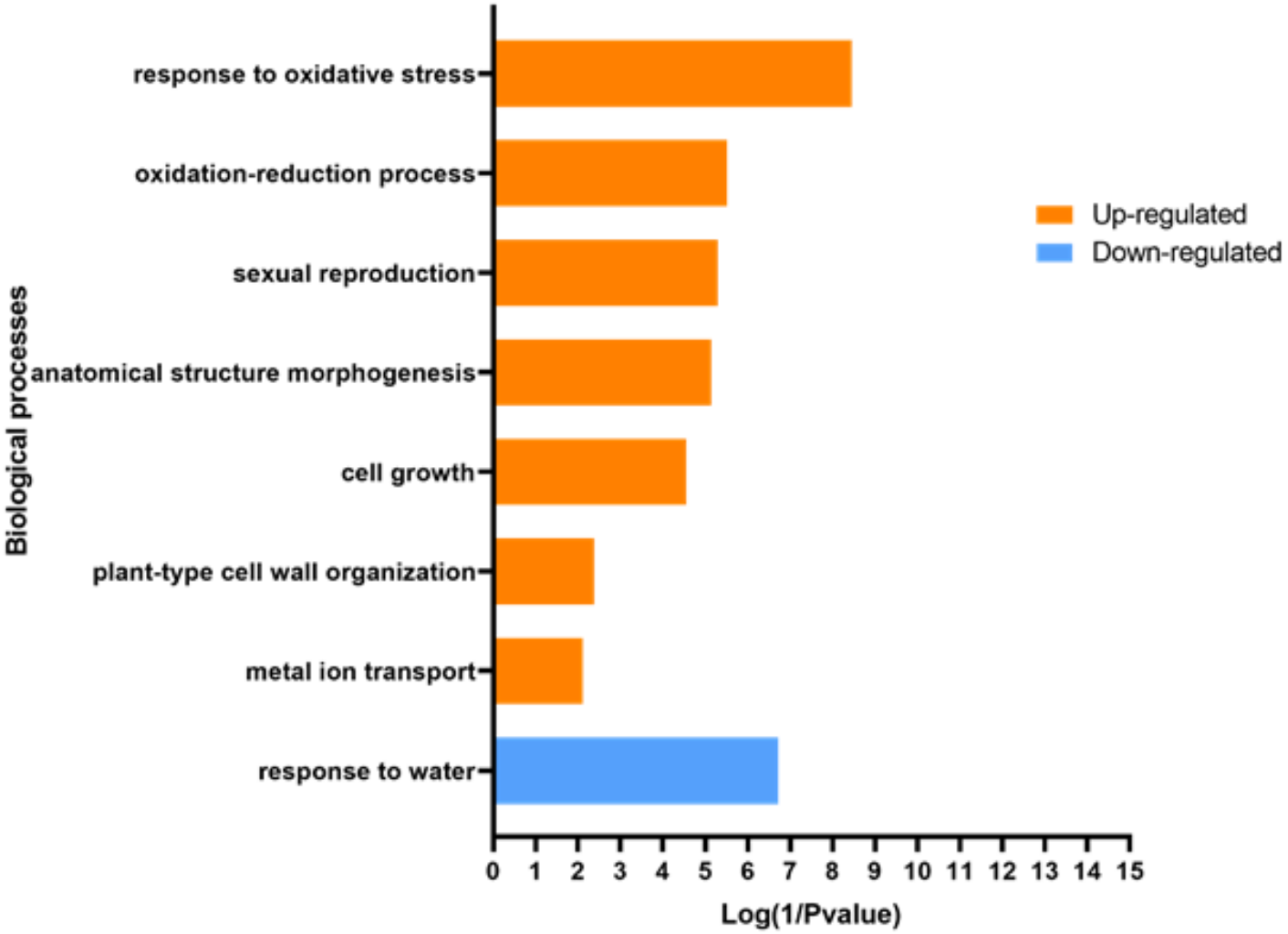


Figure 3.13. Top biological processes (BPs) enriched by the differentially expressed transcripts between the control (RS_Het_C) and the salt-treated heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_NaCl) samples using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

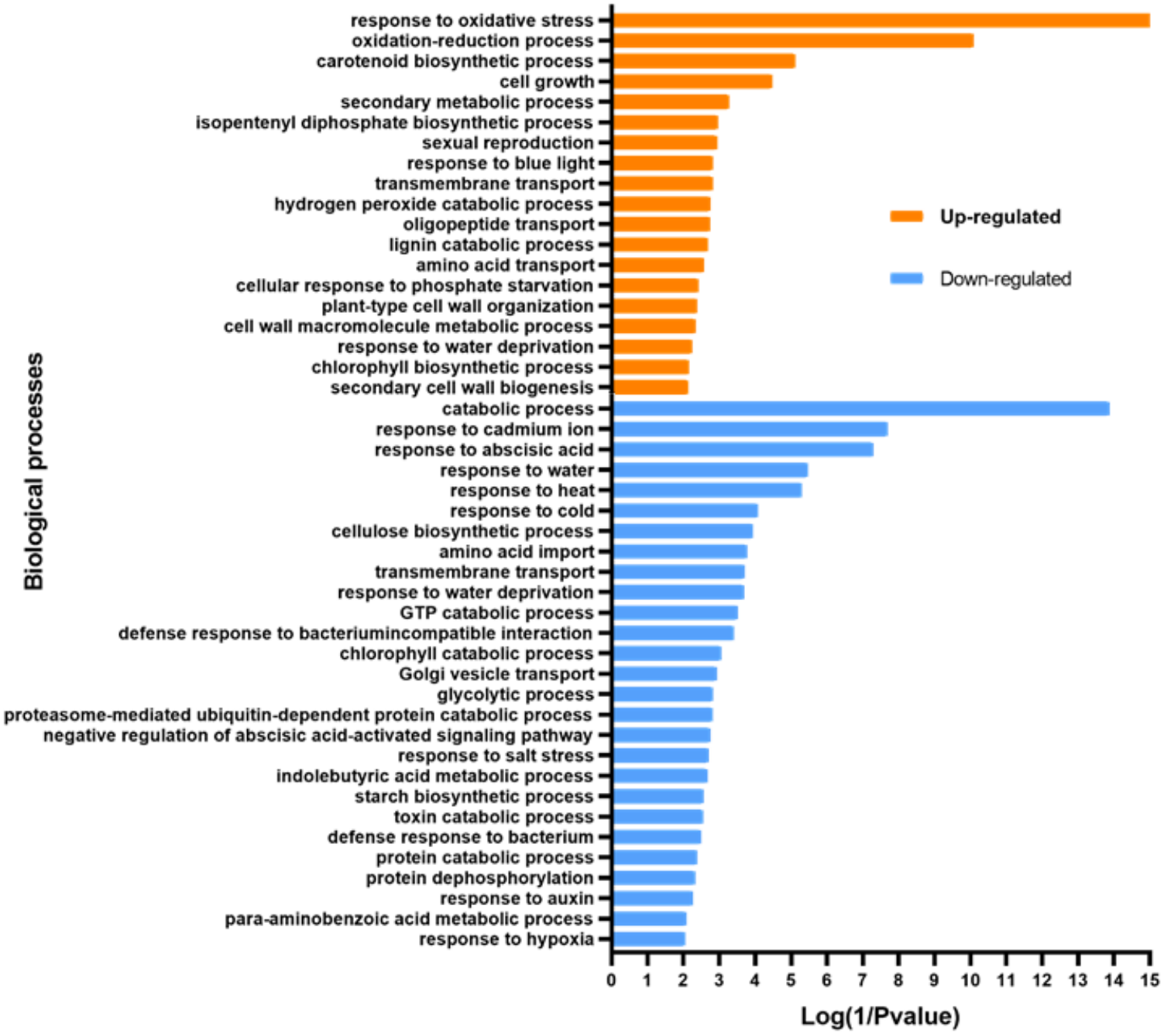


Figure 3.14. Top biological processes (BPs) enriched by the differentially expressed transcripts between the control (RS_Homo_C) and the salt-treated homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_NaCl) using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

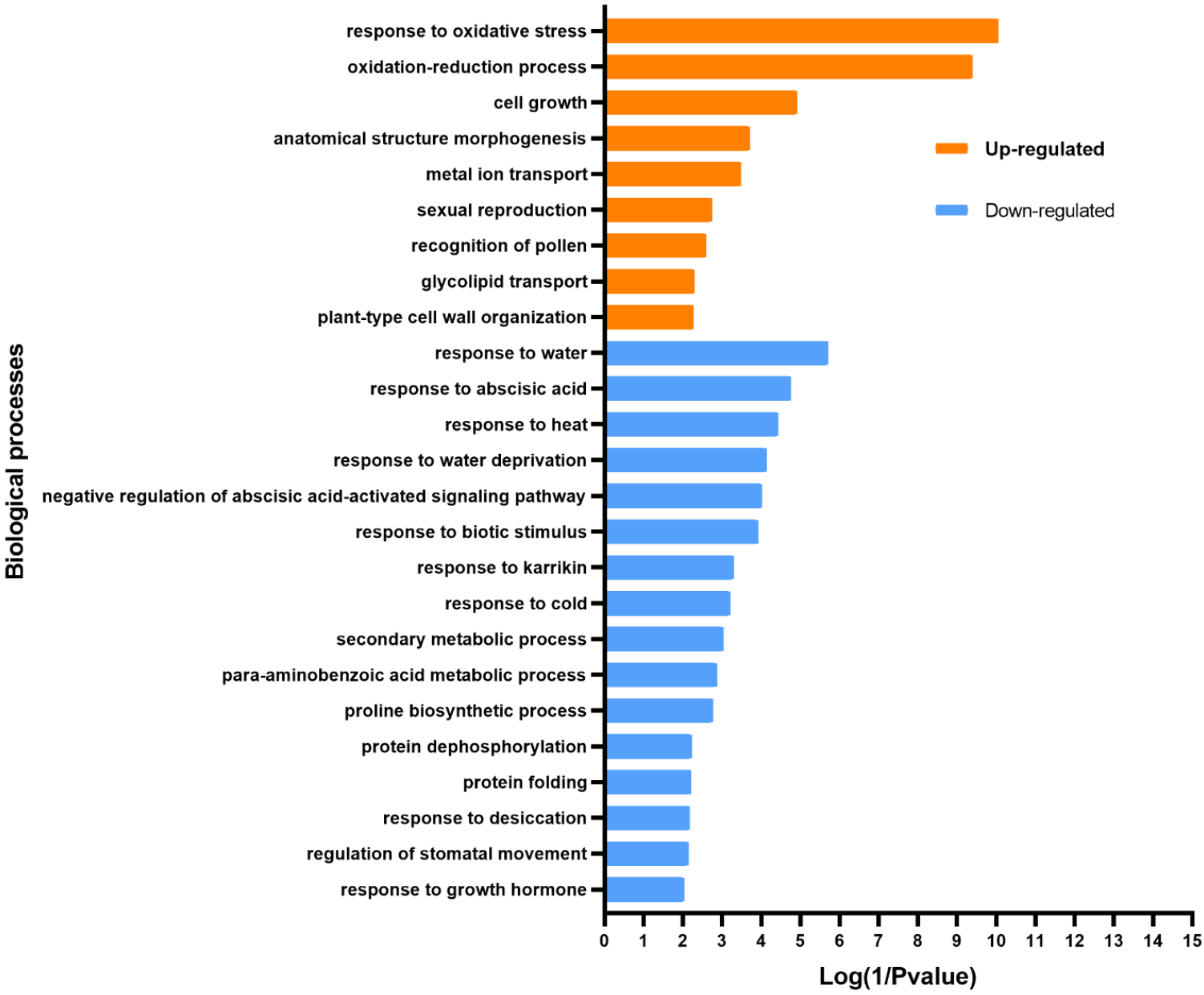


Figure 3.15. Top biological processes (BPs) enriched by the differentially expressed transcripts between the control (Cas9_C) and the salt-treated (Cas9_NaCl) Cas9 overexpression samples using CARMO. BPs plotted exceed Log (1/p-value) of 2 that is equivalent to p-value less than 0.01.

Table 3.2. Summary of all the numbers of alternative splice events, genes underwent AS and transcripts produced per condition.

Condition	Total no. of AS events	No. of genes with AS events	No. of transcripts
WT_C	15181	9832	25064
CAS9_C	11836	8026	19885
RS_Het_C	17264	11639	28961
RS_Homo_C	4764	3147	7840
WT_NaCl	12701	8327	21063
CAS9_NaCl	18730	12390	31446
RS_Het_NaCl	14866	9912	25069
RS_Homo_NaCl	20408	13493	34395

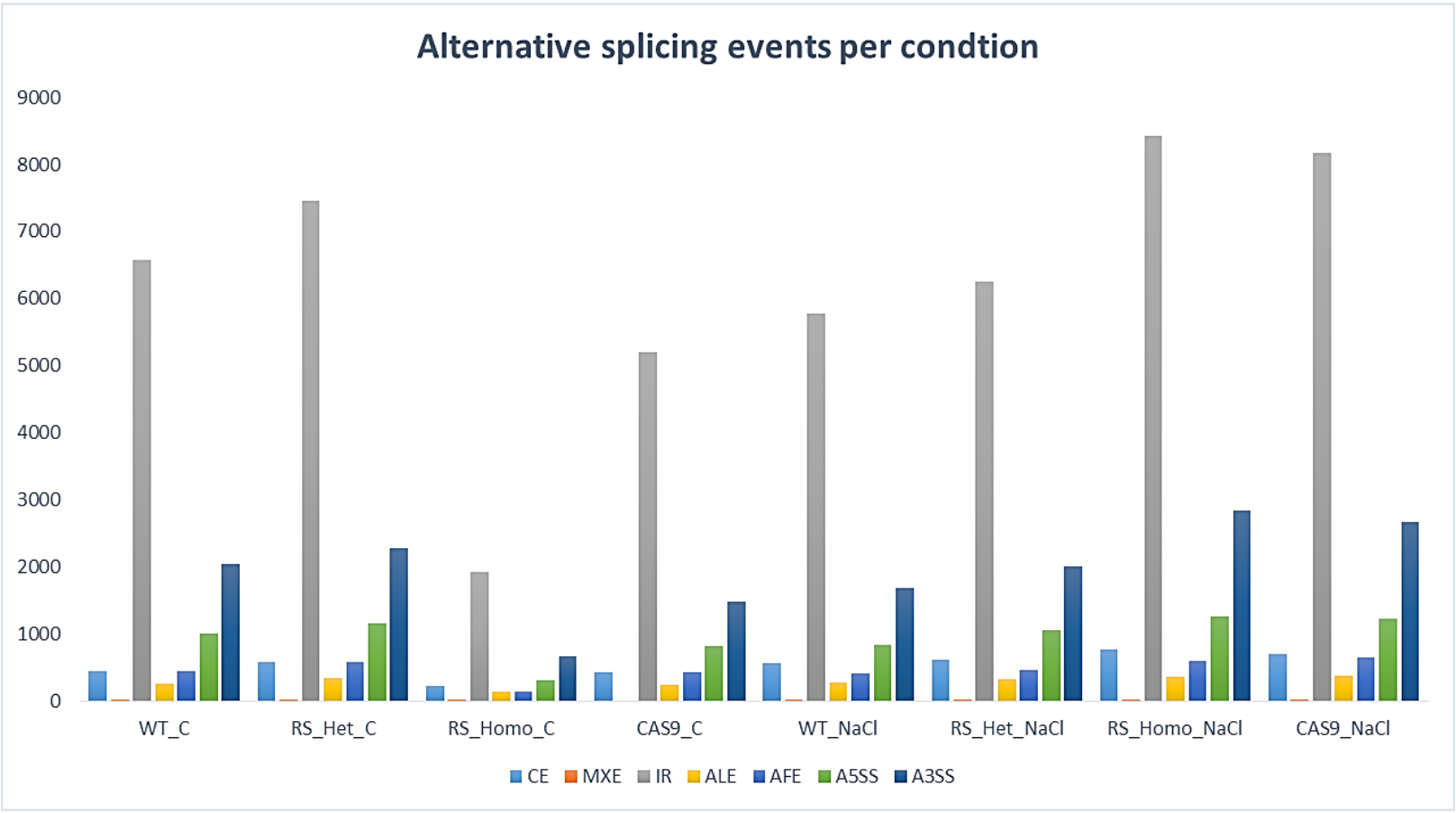


Figure 3.16. The count of alternative splicing events types per condition. Exon skipping-cassette exon (CE)-, mutually exclusive exons (MXE), intron retention (IR), alternative last exons (ALE), alternative first exons (AFE), 5´ alternative splicing (A5SS) and 3´ alternative splicing (A3SS).

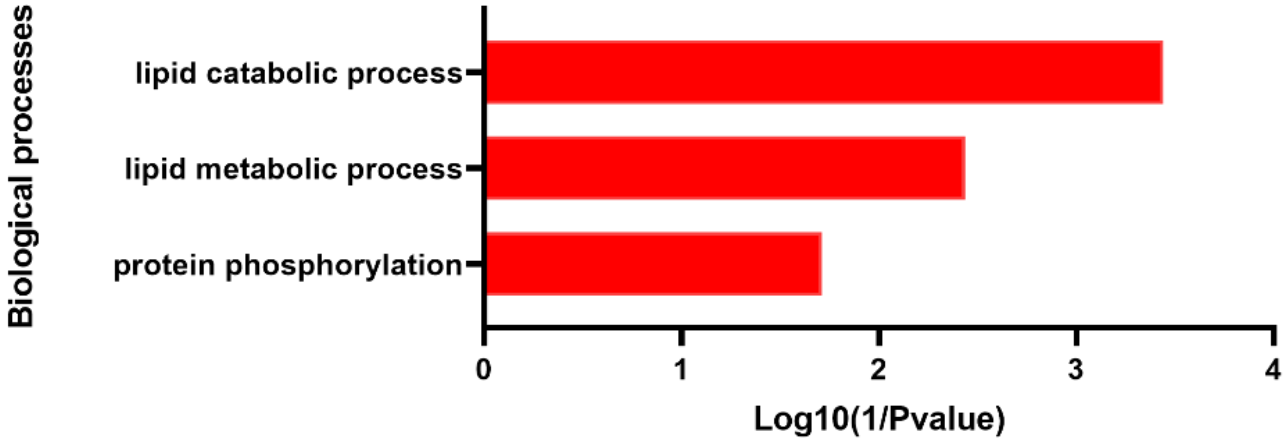


Figure 3.17. Biological processes (BPs) enriched by the top differentially spliced genes between the controls of the wild-type (WT_C) and the heterozygous Os-RS29 / homozygous Os-RS33 (RS_Het_C) double mutant using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

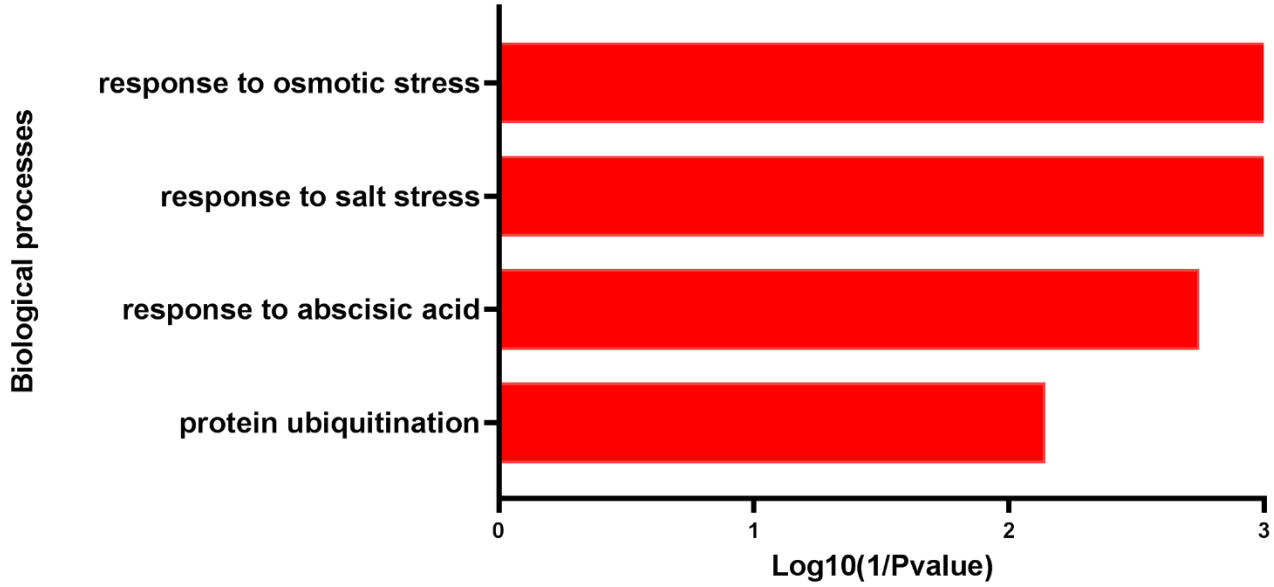


Figure 3.18. Biological processes (BPs) enriched by the top differentially spliced genes between the controls of the wild-type (WT_C) and the homozygous Os-RS29 / homozygous Os-RS33 (RS_Homo_C) double mutant using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

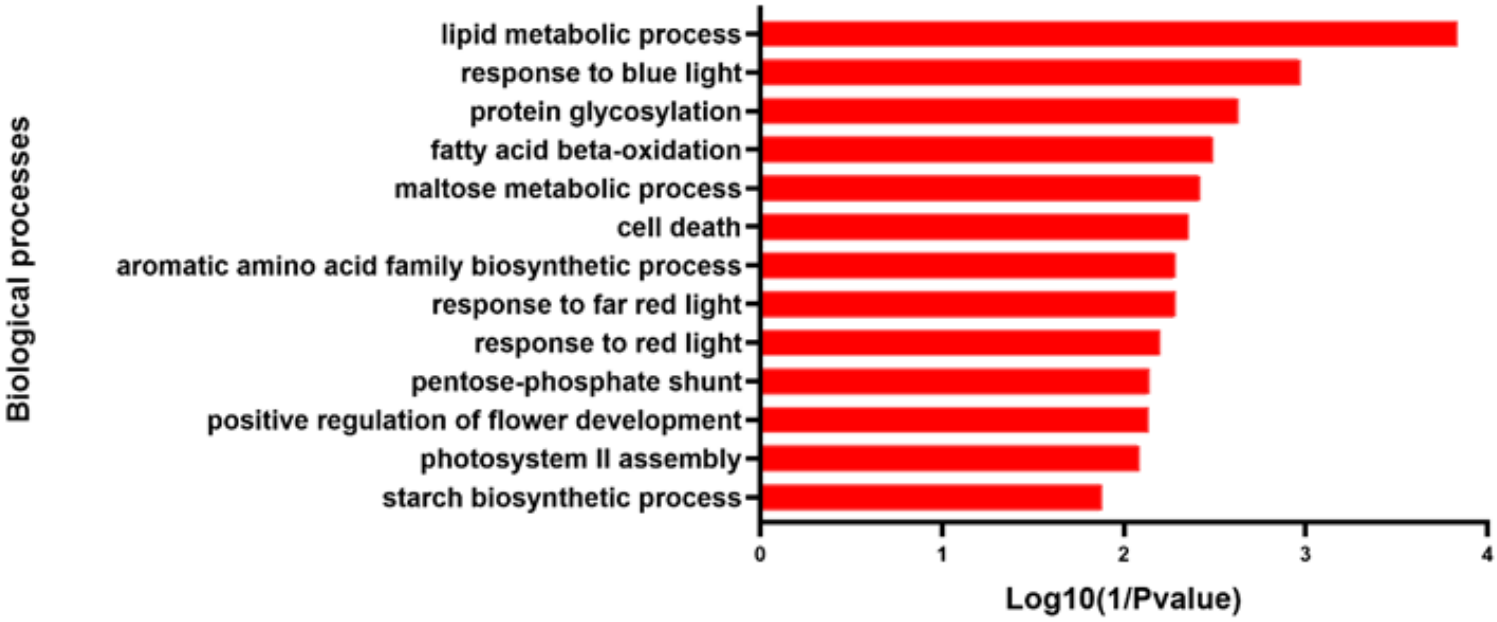


Figure 3.19. Biological processes (BPs) enriched by the top differentially spliced genes between the controls of the wild-type (WT_C) and the Cas9 overexpression (Cas9_C) double mutant using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

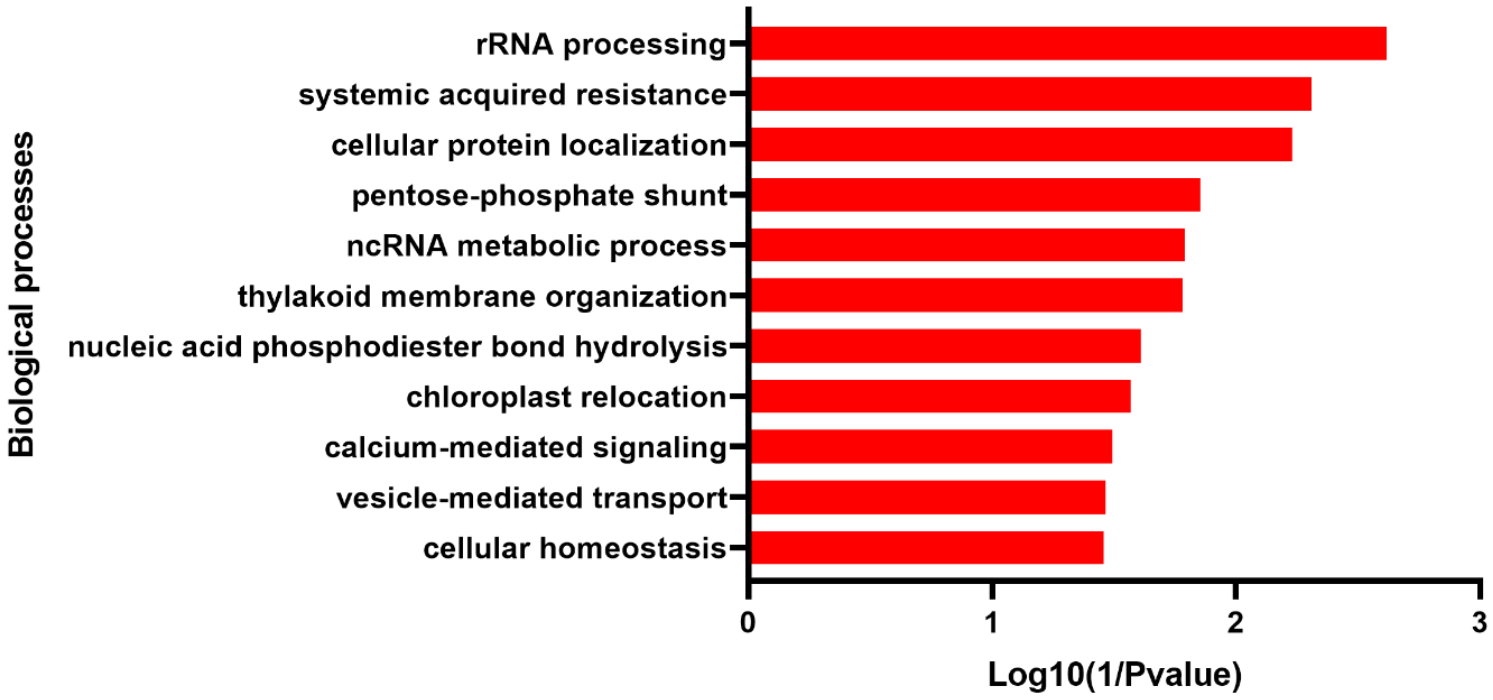


Figure 3.20. Biological processes (BPs) enriched by the top differentially spliced genes between the control (WT_C) and the salt-treated (WT_NaCl) wild-type using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

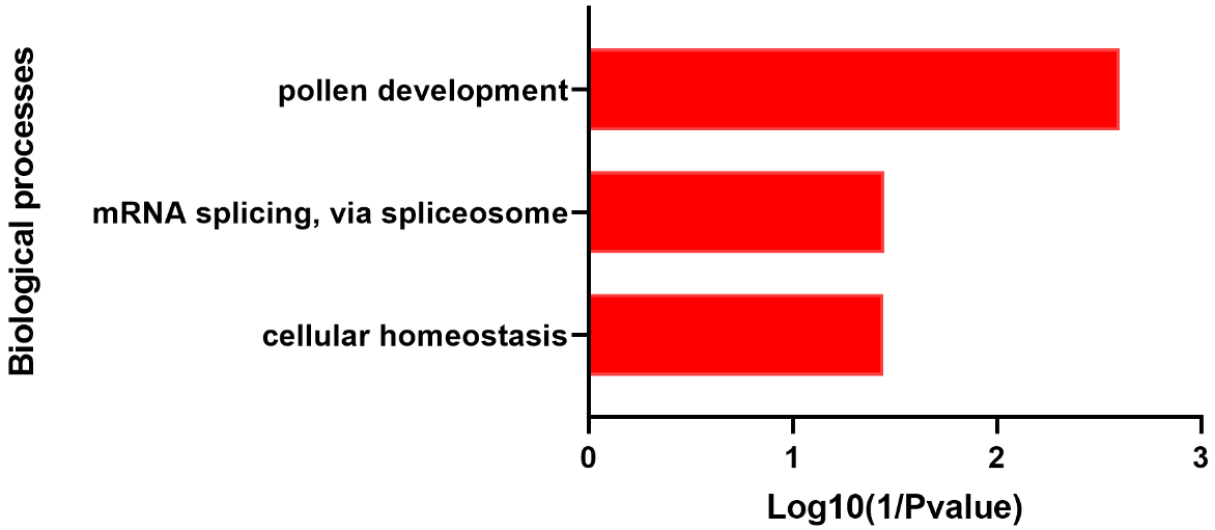


Figure 3.21. Biological processes (BPs) enriched by the top differentially spliced genes between the control (RS_Het_C) and the salt-treated (RS_Het_NaCl) heterozygous Os-RS29 / homozygous Os-RS33 double mutant using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

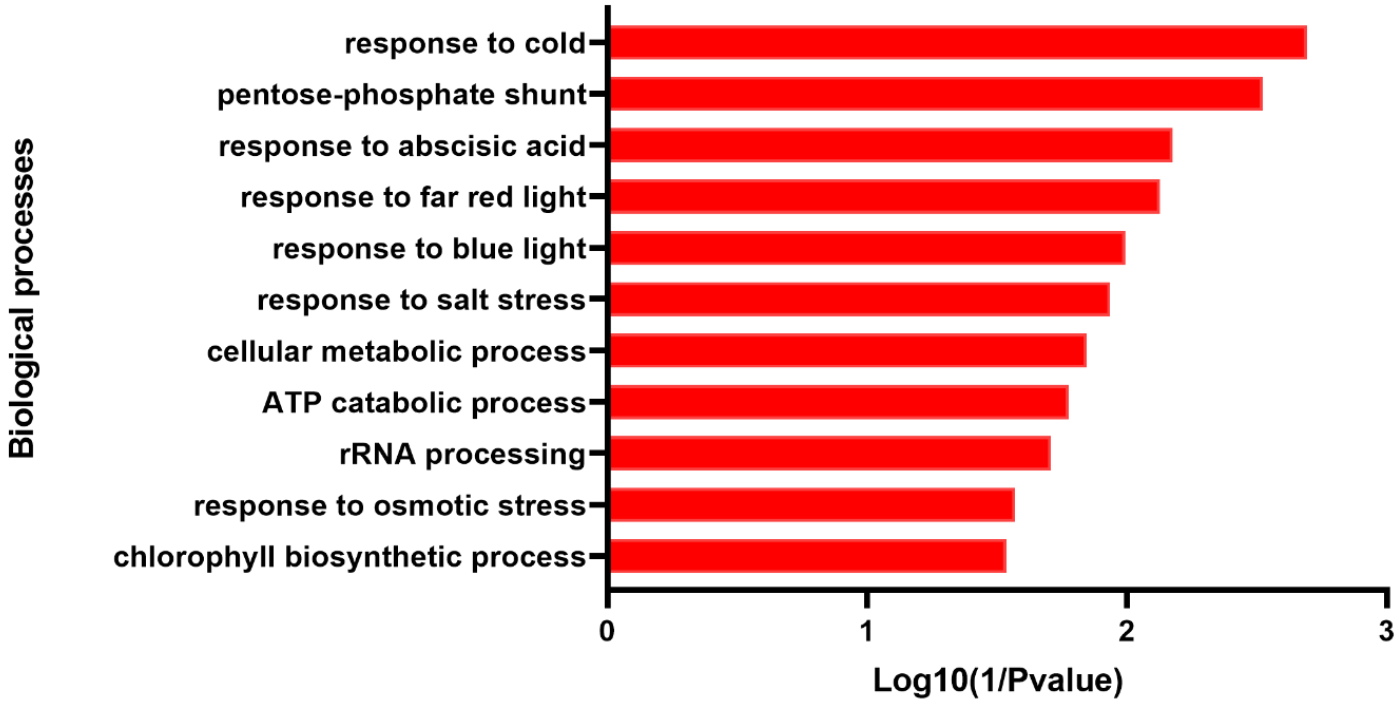


Figure 3.22. Biological processes (BPs) enriched by the top differentially spliced genes between the control (RS_Homo_C) and the salt-treated (RS_Homo_NaCl) homozygous Os-RS29 / homozygous Os-RS33 double mutant using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

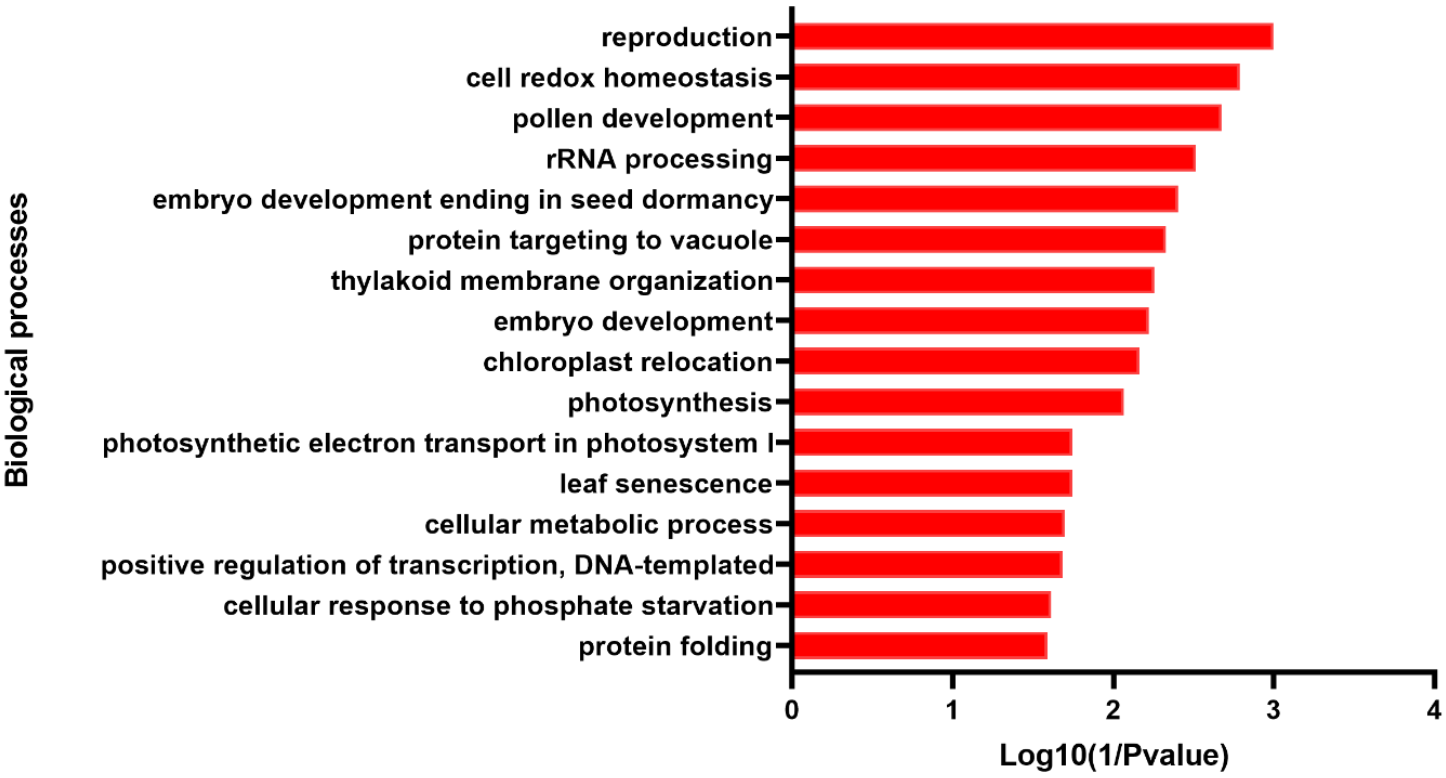


Figure 3.23. Biological processes (BPs) enriched by the top differentially spliced genes between the control (Cas9_C) and the salt-treated (Cas9_NaCl) Cas9 overexpression samples using CARMO. BPs plotted exceed Log (1/p-value) of 1.5 that is equivalent to p-value less than 0.03.

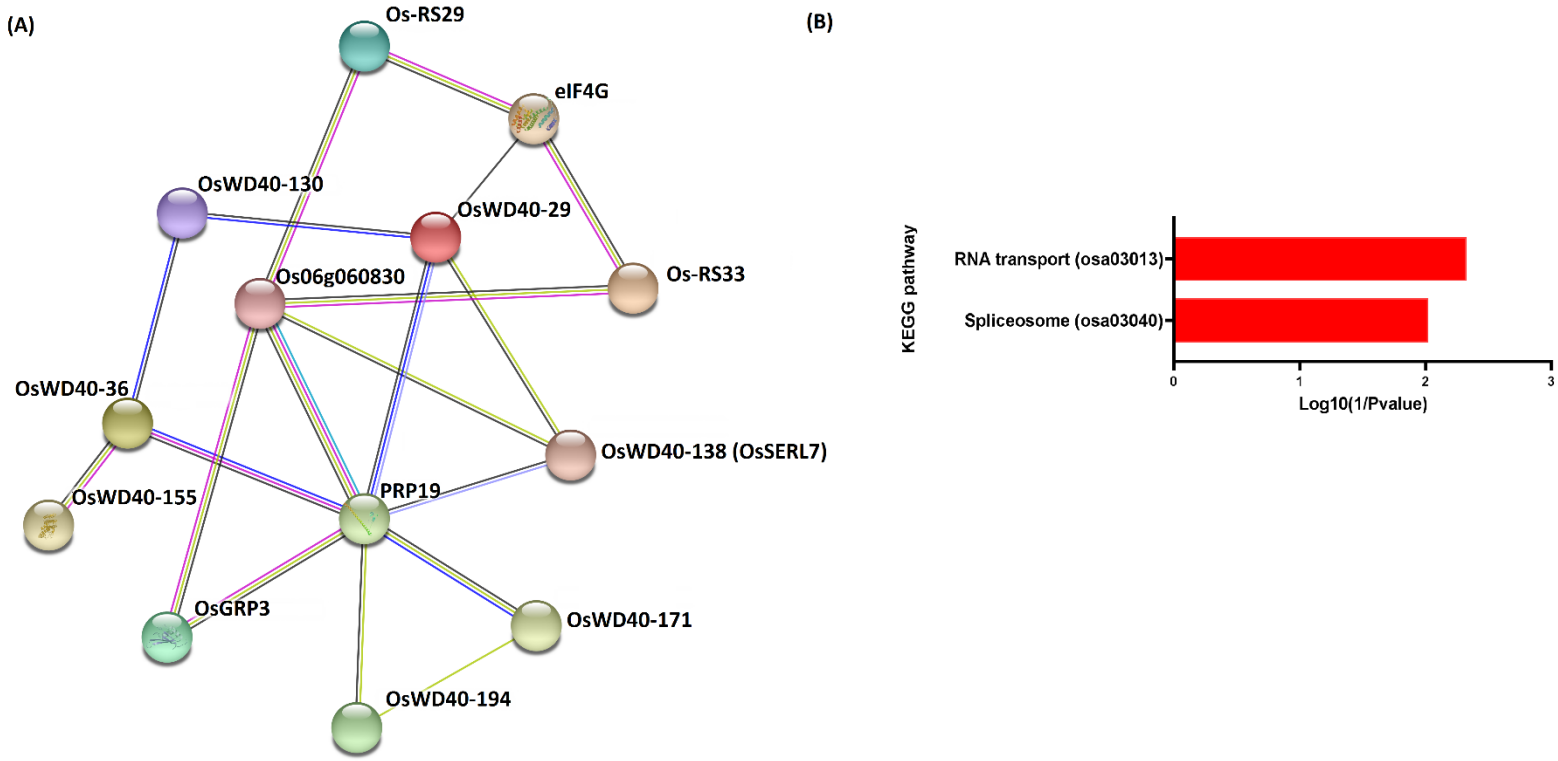


Figure 3.24. Altered splicing of mRNA processing and splicing factors affects the expression of several WD40 domain containing proteins in the homozygous Os-RS29 / homozygous double mutant. (A) protein-protein interaction network of Os06g0608300, PRP19, OsGRP3 and eIF4G which showed altered splicing in RS_Homo_C double mutant and several WD40 domain containing proteins that were shown to be up-regulated in the RS_Homo_C double mutant compared to the wild type WT_C while they were down-regulated in the RS_Homo_C versus RS_Homo_NaCl comparison. (B) The interacting genes in the network were enriched in RNA transport and spliceosome pathways.

Chapter 4: Conclusion and future perspectives

In this study, RNA-seq data were analyzed to investigate effect of double mutants of two splice factors (Os-RS29 and Os-RS33) on the rice transcriptome as a model to further understand their roles in stress response.

Under normal conditions, when compared to the wild type (WT_C), the differentially expressed genes in heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_C), were enriched in oxidation-reduction processes and response to stress involving plant hormone signaling pathways like gibberellin (GA) and jasmonic (JA) signaling pathways. On the other hand, the homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_C), exhibited a greater impact on the expression of many biotic and abiotic stress responsive genes for example response to cadmium ion, cold, abscisic acid, salt and defense response to bacterium. Interestingly, genes belonging to the WD40-repeat protein family which are known to be involved in many stress responses were significantly up-regulated in this double mutant. Thus, it can be suggested that the homozygous mutation of the Os-RS29 splicing factor affected its function profoundly reflected on the variety of stress-related genes affected by this double mutant. Furthermore, the Cas9 overexpression only without a guide RNA interestingly showed to affect the expression of a subset of genes like the Late Embryogenesis Abundant (LEA) genes (How do you interpret this?).

After salt treatment, the wild-type (WT_NaCl) and the Cas9 overexpression (Cas9_NaCl) samples showed a similar profile of the differentially expressed genes responding to the salt stress involved in oxidative stress, metal ion transport and response to water deprivation and response to abcisic acid. On the other hand, the heterozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Het_NaCl) displayed significant down-regulation of critical salt responsive genes like hyperosmolality-gated calcium-permeable channel (OsOSCA1.2) and Ethylene Response Factor 118 (OsERF118). In addition, the homozygous Os-RS29 / homozygous Os-RS33 double mutant (RS_Homo_NaCl) showed a wide expression of many stress responsive genes with a significant down-regulation of core salinity responsive genes including High-Affinity K+ Transporters, Na+/H+ Antiporters, Vacuolar H-ATPases and Trehalose-6-Phosphate Phosphatase, rendering a sensitive response towards salt stress.

In addition, the alteration in the landscape of constitutive and alternative splicing (AS) was investigated. A marked decrease in the total number of AS events in RS_Homo_C compared to WT_C suggesting that this double mutant affected the whole splicing machinery significantly. While, the number of AS events increased dramatically in the salt treated RS_Homo_NaCl compared to the other double mutant RS_Het_NaCl and the wild-type WT_NaCl, inferring that the altered splicing machinery responded to the salt stress in an oversensitive manner.

In RS_Het_C, the top differentially spliced genes (DSG) were enriched in protein phosphorylation and lipid metabolism. Whereas, the top DSGs in RS_Homo_C were enriched in many stress responses like osmotic stress, salt stress and abscisic acid (ABA) suggesting that Os-RS29 mediates stress responses via modulating the splicing of various stress-responsive genes. After exposure to salinity stress, the top DSGs in RS_Het_NaCl were enriched in pollen development, cellular homeostasis and mRNA splicing. While, many stress responsive genes were found to have altered AS in RS_Homo_NaCl involved in response to cold, ABA and salt.

Among the genes that showed an altered splicing the homozygous Os-RS29 / homozygous Os-RS33 double mutant are the Eukaryotic Initiation Factors along with some mRNA processing and splicing factors. Those factors were found to interact with a number of WD40-repeat proteins whose expression is changed in this double mutant. Suggesting that Os-RS29 and Os-RS33 regulate the expression of those repeat domain proteins genes by affecting splicing of the factors involved in their mRNA processing.

In conclusion, Os-RS29 and Os-RS33 were demonstrated to play a role in stress response in rice by controlling the expression as well as the splicing of many stress responsive genes.

Further investigations should be done to examine how the overexpression of Cas9 only without guide RNA can affect the expression and splicing of a subset of genes within the plant cell.

Also, future research should be conducted to further investigate the exact role of tandem repeat domain proteins in rice stress response.

References

[1] M. J. Moore and N. J. Proudfoot, “Pre-mRNA Processing Reaches Back toTranscription and Ahead to Translation,” *Cell*, vol. 136, no. 4, pp. 688–700, 2009.

[2] P. A. Sharp, “The discovery of split genes and RNA splicing,” in *Trends in Biochemical Sciences*, 2005, vol. 30, no. 6, pp. 279–281.

[3] A. S. N. Reddy, Y. Marquez, M. Kalyna, and A. Barta, “Complexity of the Alternative Splicing Landscape in Plants,” *Plant Cell*, vol. 25, no. 10, pp. 3657–3683, 2013.

[4] S. D. Wagner and J. A. Berglund, “Alternative pre-mRNA splicing,” *Methods Mol. Biol.*, vol. 1126, pp. 45–54, 2014.

[5] R. F. Carvalho, C. V. Feijão, and P. Duque, “On the physiological significance of alternative splicing events in higher plants,” *Protoplasma*, vol. 250, no. 3, pp. 639–650, 2013.

[6] K. Greenham and C. R. McClung, “Integrating circadian dynamics with physiological processes in plants,” *Nature Reviews Genetics*. 2015.

[7] E. Zhiguo, L. Wang, and J. Zhou, “Splicing and alternative splicing in rice and humans,” *BMB Rep.*, vol. 46, no. 9, pp. 439–447, 2013.

[8] Y. Marquez, J. W. S. Brown, C. Simpson, A. Barta, and M. Kalyna, “Transcriptome survey reveals increased complexity of the alternative splicing landscape in Arabidopsis,” *Genome Res.*, 2012.

[9] S. R. Thatcher *et al.*, “Genome-Wide Analysis of Alternative Splicing in Zea mays: Landscape and Genetic Regulation,” *Plant Cell*, 2014.

[10] W. Chen and M. J. Moore, “Spliceosomes,” *Curr. Biol.*, 2015.

[11] Y. Ru, B. B. Wang, and V. Brendel, “Spliceosomal proteins in plants,” *Current Topics in Microbiology and Immunology*, vol. 326. pp. 1–15, 2008.

[12] C. L. Will and R. Lührmann, “Spliceosome Structure and Function,” *Cold Spring Harb. Monogr. Arch.*, 2006.

[13] N. Behzadnia *et al.*, “Composition and three-dimensional EM structure of double affinity-purified, human prespliceosomal A complexes,” *EMBO J.*, 2007.

[14] J. Deckert *et al.*, “Protein Composition and Electron Microscopy Structure of Affinity-Purified Human Spliceosomal B Complexes Isolated under Physiological Conditions,” *Mol. Cell. Biol.*, 2006.

[15] D. N. Richardson *et al.*, “Comparative analysis of serine/arginine-rich proteins across 27 eukaryotes: Insights into sub-family classification and extent of alternative splicing,” *PLoS One*, vol. 6, no. 9, 2011.

[16] M. S. Jurica and M. J. Moore, “Pre-mRNA splicing: Awash in a sea of proteins,”

Molecular Cell, vol. 12, no. 1. pp. 5–14, 2003.

[17] M. C. Wahl, C. L. Will, and R. Lührmann, "The Spliceosome: Design Principles of a Dynamic RNP Machine," *Cell*. 2009.

[18] J. D. Kohtz *et al.*, "Protein-protein interactions and 5'-splice-site recognition in mammalian mRNA precursors," *Nature*, 1994.

[19] T. Rauch and I. Kiss, "How to find the real one (at the level of pre-mRNA splicing)," *Acta Biol. Hung.*, vol. 54, no. 3–4, pp. 219–231, 2003.

[20] P. Papasaikas and J. Valcárcel, "The Spliceosome: The Ultimate RNA Chaperone and Sculptor," *Trends in Biochemical Sciences*. 2016.

[21] X. D. Fu and M. Ares, "Context-dependent control of alternative splicing by RNA-binding proteins," *Nature Reviews Genetics*. 2014.

[22] E. Kim, A. Magen, and G. Ast, "Different levels of alternative splicing among eukaryotes," *Nucleic Acids Res.*, vol. 35, no. 1, pp. 125–131, 2007.

[23] N. H. Syed, M. Kalyna, Y. Marquez, A. Barta, and J. W. S. Brown, "Alternative splicing in plants - coming of age," *Trends Plant Sci.*, vol. 17, no. 10, pp. 616–623, 2012.

[24] S. Naftelberg, I. E. Schor, G. Ast, and A. R. Kornblihtt, "Regulation of Alternative Splicing Through Coupling with Transcription and Chromatin Structure," *Annu. Rev. Biochem.*, 2015.

[25] O. Kelemen *et al.*, "Function of alternative splicing," *Gene*, vol. 514, no. 1, pp. 1–30, 2013.

[26] L. E. Maquat, "Nonsense-mediated mRNA decay: Splicing, translation and mRNP dynamics," *Nature Reviews Molecular Cell Biology*. 2004.

[27] Y.-F. Chang, J. S. Imam, and M. F. Wilkinson, "The Nonsense-Mediated Decay RNA Surveillance Pathway," *Annu. Rev. Biochem.*, 2007.

[28] M. Kalyna *et al.*, "Alternative splicing and nonsense-mediated decay modulate expression of important regulatory genes in Arabidopsis," *Nucleic Acids Res.*, 2012.

[29] Z. Kerényi *et al.*, "Inter-kingdom conservation of mechanism of nonsense-mediated mRNA decay," *EMBO J.*, 2008.

[30] S. Kertész *et al.*, "Both introns and long 3′-UTRs operate as cis-acting elements to trigger nonsense-mediated decay in plants," *Nucleic Acids Res.*, 2006.

[31] W. Mei, L. Boatwright, G. Feng, J. C. Schnable, and W. Brad Barbazuk, "Evolutionarily conserved alternative splicing across monocots," *Genetics*, vol. 207, no. 2, pp. 465–480, 2017.

[32] M. Kalyna, S. Lopato, V. Voronin, and A. Barta, "Evolutionary conservation and regulation of particular alternative splicing events in plant SR proteins," *Nucleic Acids Res.*, vol. 34, no. 16, pp. 4395–4405, 2006.

[33] J. L. Manley and A. R. Krainer, "A rational nomenclature for serine/arginine-rich protein

splicing factors (SR proteins)," *Genes and Development*. 2010.

[34] C. F. Bourgeois, F. Lejeune, and J. Stévenin, "Broad Specificity of SR (Serine{plus 45 degree rule}Arginine) Proteins in the Regulation of Alternative Splicing of Pre-Messenger RNA," *Prog. Nucleic Acid Res. Mol. Biol.*, 2004.

[35] J. C. Long and J. F. Caceres, "The SR protein family of splicing factors: master regulators of gene expression," *Biochem. J.*, 2009.

[36] H. Shen and M. R. Green, "RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans," *Genes Dev.*, 2006.

[37] T. Laloum, G. Martín, and P. Duque, "Alternative Splicing Control of Abiotic Stress Responses," *Trends in Plant Science*. 2018.

[38] A. Barta, M. Kalyna, and A. S. N. Reddy, "Implementing a Rational and Consistent Nomenclature for Serine/Arginine-Rich Protein Splicing Factors (SR Proteins) in Plants," *Plant Cell*, vol. 22, no. 9, pp. 2926–2929, 2010.

[39] N. Tanabe, K. Yoshimura, A. Kimura, Y. Yabuta, and S. Shigeoka, "Differential expression of alternatively spliced mRNAs of Arabidopsis SR protein homologs, atSR30 and atSR45a, in response to environmental stress," *Plant Cell Physiol.*, 2007.

[40] S. Lopato, "Characterization of a Novel Arginine/Serine-Rich Splicing Factor in Arabidopsis," *PLANT CELL ONLINE*, 1996.

[41] M. Kalyna and A. Barta, "A plethora of plant serine/arginine-rich proteins: redundancy or evolution of novel gene functions?," *Biochem. Soc. Trans.*, 2004.

[42] M. Isshiki, "The Serine/Arginine-Rich Protein Family in Rice Plays Important Roles in Constitutive and Alternative Splicing of Pre-mRNA," *Plant Cell Online*, vol. 18, no. 1, pp. 146–158, 2006.

[43] A. S. N. Reddy and G. Shad Ali, "Plant serine/arginine-rich proteins: Roles in precursor messenger RNA splicing, plant development, and stress responses," *Wiley Interdiscip. Rev. RNA*, vol. 2, no. 6, pp. 875–889, 2011.

[44] M. Golovkin and A. S. N. Reddy, "An SC35-like protein and a novel serine/arginine-rich protein interact with Arabidopsis U1-70K protein," *J. Biol. Chem.*, 1999.

[45] A. Barta, M. Kalyna, and Z. J. Lorković, "Plant SR proteins and their functions," *Current Topics in Microbiology and Immunology*. 2008.

[46] K. Iida and M. Go, "Survey of conserved alternative splicing events of mRNAs encoding SR proteins in land plants," *Mol. Biol. Evol.*, vol. 23, no. 5, pp. 1085–1094, 2006.

[47] C. W. Sugnet, W. J. Kent, M. Ares, and D. Haussler, "Transcriptome and genome conservation of alternative splicing events in humans and mice.," *Pacific Symp. Biocomput.*, 2004.

[48] S. Jeong, "SR Proteins: Binders, Regulators, and Connectors of RNA," *Mol. Cells*, 2017.

[49] J. Han *et al.*, "SR Proteins Induce Alternative Exon Skipping through Their Activities on

the Flanking Constitutive Exons," *Mol. Cell. Biol.*, 2011.

[50] M. Shen and W. Mattox, "Activation and repression functions of an SR splicing regulator depend on exonic versus intronic-binding position," *Nucleic Acids Res.*, 2012.

[51] Z. J. Lorković, S. Lopato, M. Pexa, R. Lehner, and A. Barta, "Interactions of Arabidopsis RS domain containing cyclophilins with SR proteins and U1 and U11 small nuclear ribonucleoprotein-specific proteins suggest their involvement in pre-mRNA splicing," *J. Biol. Chem.*, 2004.

[52] P. Duque, "A role for SR proteins in plant stress responses," *Plant Signal. Behav.*, vol. 6, no. 1, pp. 49–54, 2011.

[53] V. Tripathi *et al.*, "The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation," *Mol. Cell*, 2010.

[54] S. Stamm, "Regulation of alternative splicing by reversible protein phosphorylation," *Journal of Biological Chemistry*. 2008.

[55] M. C. Yu, "The Role of Protein Arginine Methylation in mRNP Dynamics," *Mol. Biol. Int.*, 2011.

[56] Y. Huang, T. A. Yario, and J. A. Steitz, "A molecular link between SR protein dephosphorylation and mRNA export," *Proc. Natl. Acad. Sci.*, 2004.

[57] R. Reed and H. Cheng, "TREX, SR proteins and export of mRNA," *Current Opinion in Cell Biology*. 2005.

[58] L. F. Lareau, M. Inada, R. E. Green, J. C. Wengrod, and S. E. Brenner, "Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements," *Nature*, 2007.

[59] S. G. Palusa and A. S. N. Reddy, "Extensive coupling of alternative splicing of pre-mRNAs of serine/arginine (SR) genes with nonsense-mediated decay," *New Phytol.*, 2010.

[60] R. E. Green *et al.*, "Widespread predicted nonsense-mediated mRNA decay of alternatively-spliced transcripts of human normal and disease genes," in *Bioinformatics*, 2003.

[61] Y. Kurihara *et al.*, "Genome-wide suppression of aberrant mRNA-like noncoding RNAs by NMD in Arabidopsis," *Proc. Natl. Acad. Sci.*, 2009.

[62] J. K. Zhu, "Abiotic Stress Signaling and Responses in Plants," *Cell*. 2016.

[63] S. Mahajan and N. Tuteja, "Cold, salinity and drought stresses: An overview," *Archives of Biochemistry and Biophysics*. 2005.

[64] N. V. Fedoroff *et al.*, "Radically rethinking agriculture for the 21st century," *Science*. 2010.

[65] J. Bailey-Serres *et al.*, "Making sense of low oxygen sensing," *Trends in Plant Science*. 2012.

[66] I. Ahuja, R. C. H. de Vos, A. M. Bones, and R. D. Hall, "Plant molecular stress responses

face climate change," *Trends in Plant Science*. 2010.

[67] Food and Agriculture Organization of the United Nations, "How to Feed the World in 2050: High Level Expert Forum," 2009.

[68] W. Wang, B. Vinocur, and A. Altman, "Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance," *Planta*. 2003.

[69] K. Iida *et al.*, "Genome-wide analysis of alternative pre-mRNA splicing in Arabidopsis thaliana based on full-length cDNA sequences," *Nucleic Acids Res.*, 2004.

[70] S. A. Filichkin *et al.*, "Genome-wide mapping of alternative splicing in Arabidopsis thaliana," *Genome Res.*, 2010.

[71] T. Hirayama and K. Shinozaki, "Research on plant abiotic stress responses in the post-genome era: Past, present and future," *Plant J.*, 2010.

[72] M. F. Thomashow, "PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms," *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1999.

[73] N. Sreenivasulu, S. K. Sopory, and P. B. Kavi Kishor, "Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches," *Gene*. 2007.

[74] J. Kumar *et al.*, "Transcriptional regulation of salinity stress in plants: A short review," *Plant Gene*, vol. 11, no. December 2016, pp. 160–169, 2017.

[75] B.-B. Wang and V. Brendel, "Genomewide comparative analysis of alternative splicing in plants," *Proc. Natl. Acad. Sci. Unitied States Am.*, vol. 103, no. 18, pp. 7175–7180, 2006.

[76] G. S. Ali and A. S. N. Reddy, "Regulation of alternative splicing of pre-mRNAs by stresses," *Current Topics in Microbiology and Immunology*. 2008.

[77] K. Kazan, "Alternative splicing and proteome diversity in plants: The tip of the iceberg has just emerged," *Trends in Plant Science*. 2003.

[78] S. A. Filichkin *et al.*, "Environmental stresses modulate abundance and timing of alternatively spliced circadian transcripts in Arabidopsis," *Mol. Plant*, 2015.

[79] P. Zhang, H. Deng, F. M. Xiao, and Y. S. Liu, "Alterations of alternative splicing patterns of Ser/Arg-Rich (SR) genes in response to hormones and stresses treatments in different ecotypes of rice (Oryza sativa)," *J. Integr. Agric.*, vol. 12, no. 5, pp. 737–748, 2013.

[80] S. G. Palusa, G. S. Ali, and A. S. N. Reddy, "Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: Regulation by hormones and stresses," *Plant J.*, 2007.

[81] A. S. N. Reddy, "Alternative Splicing of Pre-Messenger RNAs in Plants in the Genomic Era," *Annu. Rev. Plant Biol.*, vol. 58, no. 1, pp. 267–294, 2007.

[82] G. S. Ali, M. Golovkin, and A. S. N. Reddy, "Nuclear localization and in vivo dynamics of a plant-specific serine/arginine-rich protein," *Plant J.*, vol. 36, no. 6, pp. 883–893, 2003.

[83] T. Chen, P. Cui, H. Chen, S. Ali, S. Zhang, and L. Xiong, "A KH-Domain RNA-Binding

Protein Interacts with FIERY2/CTD Phosphatase-Like 1 and Splicing Factors and Is Important for Pre-mRNA Splicing in Arabidopsis," *PLoS Genet.*, 2013.

[84] T. M. D. Cruz, R. F. Carvalho, D. N. Richardson, and P. Duque, "Abscisic acid (ABA) regulation of arabidopsis SR protein gene expression," *Int. J. Mol. Sci.*, 2014.

[85] R. F. Carvalho, S. D. Carvalho, and P. Duque, "The Plant-Specific SR45 Protein Negatively Regulates Glucose and ABA Signaling during Early Seedling Development in Arabidopsis," *PLANT Physiol.*, 2010.

[86] W. Zhang, B. Du, D. Liu, and X. Qi, "Splicing factor SR34b mutation reduces cadmium tolerance in Arabidopsis by regulating iron-regulated transporter 1 gene," *Biochem. Biophys. Res. Commun.*, 2014.

[87] D. Xing, Y. Wang, M. Hamilton, A. Ben-Hur, and A. S. N. Reddy, "Transcriptome-Wide Identification of RNA Targets of Arabidopsis SERINE/ARGININE-RICH45 Uncovers the Unexpected Roles of This RNA Binding Protein in RNA Processing," *Plant Cell*, 2016.

[88] C. Dong *et al.*, "Alternative Splicing Plays a Critical Role in Maintaining Mineral Nutrient Homeostasis in Rice (*Oryza sativa*)," *Plant Cell*, 2018.

[89] F. He, Q. Liu, L. Zheng, Y. Cui, Z. Shen, and L. Zheng, "RNA-Seq Analysis of Rice Roots Reveals the Involvement of Post-Transcriptional Regulation in Response to Cadmium Stress," *Front. Plant Sci.*, vol. 6, no. December, pp. 1–16, 2015.

[90] R. Munns and M. Tester, "Mechanisms of Salinity Tolerance," *Annu. Rev. Plant Biol.*, 2008.

[91] B. Gupta and B. Huang, "Mechanism of salinity tolerance in plants: Physiological, biochemical, and molecular characterization," *International Journal of Genomics*. 2014.

[92] T. J. Flowers, "Improving crop salt tolerance," in *Journal of Experimental Botany*, 2004.

[93] J. M. Cheeseman, "The evolution of halophytes, glycophytes and crops, and its implications for food security under saline conditions," *New Phytol.*, 2015.

[94] J. Rozema and T. Flowers, "Ecology: Crops for a salinized world," *Science*. 2008.

[95] R. Munns, "Genes and salt tolerance: Bringing them together," *New Phytologist*. 2005.

[96] R. Munns, R. A. James, and A. Läuchli, "Approaches to increasing the salt tolerance of wheat and other cereals," in *Journal of Experimental Botany*, 2006.

[97] R. A. James, C. Blake, C. S. Byrt, and R. Munns, "Major genes for Na+ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na+ accumulation in bread wheat leaves under saline and waterlogged conditions," *J. Exp. Bot.*, 2011.

[98] Y. Kawahara *et al.*, "Improvement of the oryza sativa nipponbare reference genome using next generation sequence and optical map data," *Rice*, 2013.

[99] S. A. Goff, "Rice as a model for cereal genomics," *Curr. Opin. Plant Biol.*, 1999.

[100] N. Saraswathy, P. Ramalingam, N. Saraswathy, and P. Ramalingam, "Genomes of model

organisms," in *Concepts and Techniques in Genomics and Proteomics*, Woodhead Publishing, 2011, pp. 29–48.

[101] D.-K. Lee *et al.*, "Production of insect-resistant transgenic rice plants for use in practical agriculture," *Plant Biotechnol. Rep.*, vol. 10, no. 6, pp. 391–401, Nov. 2016.

[102] D. Breviario, "Stress Response in Rice," *Rice Res. Open Access*, vol. 2, no. 1, pp. 1–2, 2014.

[103] R. C. Venu *et al.*, "Deep transcriptome sequencing reveals the expression of key functional and regulatory genes involved in the abiotic stress signaling pathways in rice," *J. Plant Biol.*, 2013.

[104] G. J. Maas, E.V.; Hoffman, "Crop Salt Tolerance-Current Assessment.," *J. Irrig. Drain. Div.*, vol. 103, no. IR2, pp. 115–134, 1977.

[105] K. Nagamiya *et al.*, "Enhancement of salt tolerance in transgenic rice expressing an Escherichia coli catalase gene, katE," *Plant Biotechnol. Rep.*, 2007.

[106] R. Karan, T. DeLeon, H. Biradar, and P. K. Subudhi, "Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes," *PLoS One*, 2012.

[107] M. R. Hossain, J. Pritchard, and B. V. Ford-Lloyd, "Qualitative and quantitative variation in the mechanisms of salinity tolerance determined by multivariate assessment of diverse rice (Oryza sativa L.) genotypes," *Plant Genet. Resour. Characterisation Util.*, 2016.

[108] P. M. Hasegawa, "Sodium (Na+) homeostasis and salt tolerance of plants," *Environ. Exp. Bot.*, 2013.

[109] J. K. Zhu, "Regulation of ion homeostasis under salt stress," *Current Opinion in Plant Biology*. 2003.

[110] M. P. REDDY, S. SANISH, and E. R. R. IYENGAR, "PHOTOSYNTHETIC STUDIES AND COMPARTMENTATION OF IONS IN DIFFERENT TISSUES OF SALICORNIA-BRACHIATA ROXB UNDER SALINE CONDITIONS," *Photosynthetica*, 1992.

[111] K. J. Dietz *et al.*, "Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level," in *Journal of Experimental Botany*, 2001.

[112] R. K. Sairam and A. Tyagi, "Physiology and molecular biology of salinity stress tolerance in plants," *Current Science*. 2004.

[113] H. E. Yen, S. M. Wu, Y. H. Hung, and S. K. Yen, "Isolation of 3 salt-induced low-abundance cDNAs from light-grown callus of Mesembryanthemum crystallinum by suppression subtractive hybridization," *Physiol. Plant.*, 2000.

[114] J. I. Schroeder *et al.*, "Using membrane transporters to improve crops for sustainable food production," *Nature*. 2013.

[115] V. Barragan *et al.*, "Ion Exchangers NHX1 and NHX2 Mediate Active Potassium Uptake

into Vacuoles to Regulate Cell Turgor and Stomatal Function in Arabidopsis," *Plant Cell*, 2012.

[116] A. Fukuda, A. Nakamura, N. Hara, S. Toki, and Y. Tanaka, "Molecular and functional analyses of rice NHX-type Na+/H+ antiporter genes," *Planta*, 2011.

[117] K. Kumar, M. Kumar, S. R. Kim, H. Ryu, and Y. G. Cho, "Insights into genomics of salt stress response in rice," *Rice*. 2013.

[118] Y. Xiang, N. Tang, H. Du, H. Ye, and L. Xiong, "Characterization of OsbZIP23 as a Key Player of the Basic Leucine Zipper Transcription Factor Family for Conferring Abscisic Acid Sensitivity and Salinity and Drought Tolerance in Rice," *PLANT Physiol.*, 2008.

[119] A. R. Gurmani, A. Bano, N. Ullah, H. Khan, M. Jahangir, and T. J. Flowers, "Exogenous abscisic acid(ABA) and silicon (Si) promote salinity tolerance by reducing sodium (Na+) transport and bypass flow in rice (Oryza sativa indica)," *Aust. J. Crop Sci.*, 2013.

[120] L. Perales, V. Arbona, A. Gómez-Cadenas, M. J. Cornejo, and A. Sanz, "A relationship between tolerance to dehydration of rice cell lines and ability for ABA synthesis under stress," *Plant Physiol. Biochem.*, 2005.

[121] A. Moons, G. Bauw, E. Prinsen, M. Van Montagu, and D. Van der Straeten, "Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant Indica rice varieties.," *Plant Physiol.*, 1995.

[122] R. Pons, M. J. Cornejo, and A. Sanz, "Is ABA involved in tolerance responses to salinity by affecting cytoplasm ion homeostasis in rice cell lines?," *Plant Physiol. Biochem.*, 2013.

[123] W. Dong *et al.*, "Wheat Oxophytodienoate Reductase Gene TaOPR1 Confers Salinity Tolerance via Enhancement of Abscisic Acid Signaling and Reactive Oxygen Species Scavenging," *PLANT Physiol.*, 2013.

[124] Z. Qiu, J. Guo, A. Zhu, L. Zhang, and M. Zhang, "Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress," *Ecotoxicol. Environ. Saf.*, 2014.

[125] D. J. Kang *et al.*, "Jasmonic acid differentially affects growth, ion uptake and abscisic acid concentration in salt-tolerant and salt-sensitive rice cultivars," *J. Agron. Crop Sci.*, 2005.

[126] H. Ye, H. Du, N. Tang, X. Li, and L. Xiong, "Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice," *Plant Mol. Biol.*, 2009.

[127] K. Kazan and J. M. Manners, "JAZ repressors and the orchestration of phytohormone crosstalk," *Trends in Plant Science*. 2012.

[128] X. Shen *et al.*, "Transcriptomic profiling revealed an important role of cell wall remodeling and ethylene signaling pathway during salt acclimation in Arabidopsis," *Plant Mol. Biol.*, 2014.

[129] J. Xu *et al.*, "Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in Arabidopsis," *J. Biol. Chem.*, 2008.

[130] C. Jiang, E. J. Belfield, Y. Cao, J. A. C. Smith, and N. P. Harberd, "An Arabidopsis Soil-

Salinity-Tolerance Mutation Confers Ethylene-Mediated Enhancement of Sodium/Potassium Homeostasis," *Plant Cell*, 2013.

[131] Q. Zhao and H. W. Guo, "Paradigms and paradox in the ethylene signaling pathway and interaction network," *Molecular Plant*. 2011.

[132] S. D. Yoo, Y. H. Cho, G. Tena, Y. Xiong, and J. Sheen, "Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4signalling," *Nature*, 2008.

[133] J. Peng *et al.*, "Salt-Induced Stabilization of EIN3/EIL1 Confers Salinity Tolerance by Deterring ROS Accumulation in Arabidopsis," *PLoS Genet.*, 2014.

[134] J. Liu, "The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance," *Proc. Natl. Acad. Sci.*, 2000.

[135] J. Liu and J. K. Zhu, "A calcium sensor homolog required for plant salt tolerance," *Science (80-.).*, 1998.

[136] H. Shi, M. Ishitani, C. Kim, and J.-K. Zhu, "The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter," *Proc. Natl. Acad. Sci.*, 2000.

[137] R. Quan, J. Wang, D. Yang, H. Zhang, Z. Zhang, and R. Huang, "EIN3 and SOS2 synergistically modulate plant salt tolerance," *Sci. Rep.*, 2017.

[138] C.-H. Li *et al.*, "The Receptor-Like Kinase SIT1 Mediates Salt Sensitivity by Activating MAPK3/6 and Regulating Ethylene Homeostasis in Rice," *Plant Cell*, 2014.

[139] B. Singh and K. Usha, "Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress," *Plant Growth Regul.*, 2003.

[140] H. Mimouni *et al.*, "Does Salicylic Acid (SA) Improve Tolerance to Salt Stress in Plants? A Study of SA Effects On Tomato Plant Growth, Water Dynamics, Photosynthesis, and Biochemical Parameters," *Omi. A J. Integr. Biol.*, 2016.

[141] D. A. Dempsey, A. C. Vlot, M. C. Wildermuth, and D. F. Klessig, "Salicylic Acid Biosynthesis and Metabolism," *Arab. B.*, 2011.

[142] A. Rohde, "Molecular Phenotyping of the pal1 and pal2 Mutants of Arabidopsis thaliana Reveals Far-Reaching Consequences on Phenylpropanoid, Amino Acid, and Carbohydrate Metabolism," *PLANT CELL ONLINE*, 2004.

[143] J. D and J. B, "Salicylic Acid Mediated Salt Tolerance at Different Growth Stages of Oryza sativa l. and its Effect on Salicylic Acid Biosynthetic Pathway Genes," *Biotechnol. An Indian J.*, vol. 13, no. 2.

[144] D. Golldack, I. Lüking, and O. Yang, "Plant tolerance to drought and salinity: Stress regulating transcription factors and their functional significance in the cellular transcriptional network," *Plant Cell Reports*. 2011.

[145] R. Mirdar Mansuri, Z. S. Shobbar, N. Babaeian Jelodar, M. R. Ghaffari, G. A. Nematzadeh, and S. Asari, "Dissecting molecular mechanisms underlying salt tolerance in rice: a comparative transcriptional profiling of the contrasting genotypes," *Rice*, 2019.

[146] H. Hu *et al.*, "Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice," *Proc. Natl. Acad. Sci.*, 2006.

[147] K. Nakashima *et al.*, "Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice," *Plant J.*, 2007.

[148] S. Y. Song, Y. Chen, J. Chen, X. Y. Dai, and W. H. Zhang, "Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress," *Planta*, 2011.

[149] Q. Wang, Y. Guan, Y. Wu, H. Chen, F. Chen, and C. Chu, "Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both Arabidopsis and rice," *Plant Mol. Biol.*, 2008.

[150] G. Mallikarjuna, K. Mallikarjuna, M. K. Reddy, and T. Kaul, "Expression of OsDREB2A transcription factor confers enhanced dehydration and salt stress tolerance in rice (Oryza sativa L.)," *Biotechnol. Lett.*, 2011.

[151] M. Sharma and G. K. Pandey, "Expansion and Function of Repeat Domain Proteins During Stress and Development in Plants," *Front. Plant Sci.*, 2016.

[152] M. Sharma *et al.*, "Comprehensive expression analysis of rice armadillo gene family during abiotic stress and development," *DNA Res.*, 2014.

[153] Y. Sun, X. Zhou, and H. Ma, "Genome-wide analysis of Kelch repeat-containing F-box family," *J. Integr. Plant Biol.*, 2007.

[154] A. D. Dooki, F. J. Mayer-Posner, H. Askari, A. A. Zaiee, and G. H. Salekdeh, "Proteomic responses of rice young panicles to salinity," *Proteomics*, 2006.

[155] K. Kosová, I. T. Prášil, and P. Vítámvás, "Protein contribution to plant salinity response and tolerance acquisition," *International Journal of Molecular Sciences*. 2013.

[156] N. C. Chan, V. A. Likić, R. F. Waller, T. D. Mulhern, and T. Lithgow, "The C-terminal TPR Domain of Tom70 Defines a Family of Mitochondrial Protein Import Receptors Found only in Animals and Fungi," *J. Mol. Biol.*, 2006.

[157] A. Izhaki, S. M. Swain, T. S. Tseng, A. Borochov, N. E. Olszewski, and D. Weiss, "The role of SPY and its TPR domain in the regulation of gibberellin action throughout the life cycle of Petunia hybrida plants," *Plant J.*, 2001.

[158] J. Huang, X. Zhao, H. Yu, Y. Ouyang, L. Wang, and Q. Zhang, "The ankyrin repeat gene family in rice: Genome-wide identification, classification and expression profiling," *Plant Mol. Biol.*, 2009.

[159] H. Walia *et al.*, "Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage.," *Plant Physiol.*, 2005.

[160] Y. Cao *et al.*, "Multiple Genes, Tissue Specificity, and Expression-Dependent Modulation Contribute to the Functional Diversity of Potassium Channels in Arabidopsis thaliana," *Plant Physiol.*, 1995.

[161] Y. Ouyang, X. Huang, Z. Lu, and J. Yao, "Genomic survey, expression profile and co-expression network analysis of OsWD40 family in rice," *BMC Genomics*, 2012.

[162] J. Huang, M. M. Wang, Y. M. Bao, S. J. Sun, L. J. Pan, and H. S. Zhang, "SRWD: A novel WD40 protein subfamily regulated by salt stress in rice (OryzasativaL.)," *Gene*, 2008.

[163] S. Q. Ouyang *et al.*, "Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (Oryza sativa) plants," *Plant J.*, 2010.

[164] S. J. Park, J. C. Moon, Y. C. Park, J. H. Kim, D. S. Kim, and C. S. Jang, "Molecular dissection of the response of a rice leucine-rich repeat receptor-like kinase (LRR-RLK) gene to abiotic stresses," *J. Plant Physiol.*, 2014.

[165] Y. Osakabe, K. Yamaguchi-Shinozaki, K. Shinozaki, and L. S. P. Tran, "Sensing the environment: Key roles of membrane-localized kinases in plant perception and response to abiotic stress," *Journal of Experimental Botany*. 2013.

[166] H. Lindner, L. M. Müller, A. Boisson-Dernier, and U. Grossniklaus, "CrRLK1L receptor-like kinases: Not just another brick in the wall," *Current Opinion in Plant Biology*. 2012.

[167] J. Giri, S. Vij, P. K. Dansana, and A. K. Tyagi, "Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic Arabidopsis plants," *New Phytol.*, 2011.

[168] R. Tenhaken, "Cell wall remodeling under abiotic stress," *Front. Plant Sci.*, 2015.

[169] S. Qiu *et al.*, "Repression of OsEXPA3 expression leads to root system growth suppression in rice," *Crop Sci.*, 2014.

[170] I. Lesak, Z. Miszalski, B. Karpinska, E. Niewiadomska, R. Ratajczak, and S. Karpinski, "Redox control of oxidative stress responses in the C3-CAM intermediate plant Mesembryanthemum crystallinum," in *Plant Physiology and Biochemistry*, 2002.

[171] M. Kordrostami, B. Rabiei, and H. H. Kumleh, "Different physiobiochemical and transcriptomic reactions of rice (Oryza sativa L.) cultivars differing in terms of salt sensitivity under salinity stress," *Environ. Sci. Pollut. Res.*, 2017.

[172] J. Yao, W. M. Shi, and W. F. Xu, "Effects of salt stress on expression of nitrate transporter and assimilation-related genes in tomato roots," *Russ. J. Plant Physiol.*, 2008.

[173] S. K. Upadhyay, J. Kumar, A. Alok, and R. Tuli, "RNA-Guided Genome Editing for Target Gene Mutations in Wheat," *G3: Genes|Genomes|Genetics*, 2013.

[174] J. W. Paul and Y. Qi, "CRISPR/Cas9 for plant genome editing: accomplishments, problems and prospects," *Plant Cell Reports*. 2016.

[175] R. Mishra, R. K. Joshi, and K. Zhao, "Genome Editing in Rice: Recent Advances, Challenges, and Future Implications," *Front. Plant Sci.*, vol. 9, no. September, 2018.

[176] V. Srivastava, J. L. Underwood, and S. Zhao, "Dual-targeting by CRISPR/Cas9 for precise excision of transgenes from rice genome," *Plant Cell. Tissue Organ Cult.*, vol. 129, no. 1, pp. 153–160, 2017.

[177] Y. Ishino, H. Shinagawa, K. Makino, M. Amemura, and A. Nakatura, "Nucleotide

sequence of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in Escherichia coli, and identification of the gene product," *J. Bacteriol.*, 1987.

[178] J. E. Garneau *et al.*, "The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA," *Nature*, 2010.

[179] P. Horvath and R. Barrangou, "CRISPR/Cas, the immune system of Bacteria and Archaea," *Science*. 2010.

[180] V. Nekrasov, B. Staskawicz, D. Weigel, J. D. G. Jones, and S. Kamoun, "Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease," *Nat. Biotechnol.*, vol. 31, no. 8, pp. 691–693, Aug. 2013.

[181] J.-F. Li *et al.*, "Multiplex and homologous recombination–mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9," *Nat. Biotechnol.*, vol. 31, no. 8, pp. 688–691, Aug. 2013.

[182] Q. Shan *et al.*, "Targeted genome modification of crop plants using a CRISPR-Cas system," *Nat. Biotechnol.*, vol. 31, no. 8, pp. 686–688, Aug. 2013.

[183] C. R. Hale *et al.*, "RNA-Guided RNA Cleavage by a CRISPR RNA-Cas Protein Complex," *Cell*, 2009.

[184] S. J. J. Brouns *et al.*, "Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes," *Science (80-.).*, vol. 321, no. 5891, pp. 960–964, Aug. 2008.

[185] G. Gasiunas, R. Barrangou, P. Horvath, and V. Siksnys, "Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria," *Proc. Natl. Acad. Sci.*, 2012.

[186] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," *Science (80-.).*, 2012.

[187] L. Cong *et al.*, "Multiplex genome engineering using CRISPR/Cas systems," *Science (80-.).*, 2013.

[188] B. Farboud and B. J. Meyer, "Dramatic enhancement of genome editing by CRISPR/cas9 through improved guide RNA design," *Genetics*, 2015.

[189] H. Puchta, "The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution," *Journal of Experimental Botany*. 2005.

[190] D. P. Weeks, M. H. Spalding, and B. Yang, "Use of designer nucleases for targeted gene and genome editing in plants," *Plant Biotechnol. J.*, 2016.

[191] K. Chen and C. Gao, "Targeted genome modification technologies and their applications in crop improvements," *Plant Cell Reports*. 2014.

[192] N. J. Baltes and D. F. Voytas, "Enabling plant synthetic biology through genome engineering," *Trends in Biotechnology*. 2015.

[193] N. Soda, L. Verma, and J. Giri, "CRISPR-Cas9 based plant genome editing: Significance,

opportunities and recent advances," *Plant Physiology and Biochemistry*. 2018.

[194] K. Xie, B. Minkenberg, and Y. Yang, "Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system," *Proc. Natl. Acad. Sci.*, 2015.

[195] X. Ma *et al.*, "A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants," *Mol. Plant*, 2015.

[196] S. Schiml, F. Fauser, and H. Puchta, "The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny," *Plant J.*, 2014.

[197] L. Bortesi and R. Fischer, "The CRISPR / Cas9 system for plant genome editing and beyond," *Biotechnol. Adv.*, vol. 33, no. 1, pp. 41–52, 2015.

[198] Z. Zhang, Y. Mao, S. Ha, W. Liu, J. R. Botella, and J. K. Zhu, "A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in Arabidopsis," *Plant Cell Reports*. 2016.

[199] X. Gao, J. Chen, X. Dai, D. Zhang, and Y. Zhao, "An Effective Strategy for Reliably Isolating Heritable and *Cas9* -Free Arabidopsis Mutants Generated by CRISPR/Cas9-Mediated Genome Editing," *Plant Physiol.*, 2016.

[200] O. Morozova, M. Hirst, and M. A. Marra, "Applications of New Sequencing Technologies for Transcriptome Analysis," *Annu. Rev. Genomics Hum. Genet.*, 2009.

[201] R. Garg and M. Jain, "RNA-Seq for transcriptome analysis in non-model plants," *Methods Mol. Biol.*, 2013.

[202] P. A. C. 't Hoen *et al.*, "Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms," *Nucleic Acids Res.*, 2008.

[203] F. Ozsolak and P. M. Milos, "RNA sequencing: Advances, challenges and opportunities," *Nature Reviews Genetics*. 2011.

[204] J. C. Marioni, C. E. Mason, S. M. Mane, M. Stephens, and Y. Gilad, "RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays," *Genome Res.*, 2008.

[205] V. Costa, C. Angelini, I. De Feis, and A. Ciccodicola, "Uncovering the complexity of Transcriptomes with RNA-SEQ," in *The Role of Bioinformatics in Agriculture*, 2014.

[206] M. Jain, "Next-generation sequencing technologies for gene expression profiling in plants," *Brief. Funct. Genomics*, 2012.

[207] T. Lu *et al.*, "Function annotation of the rice transcriptome at single-nucleotide resolution by RNA-seq," *Genome Res.*, vol. 20, no. 9, pp. 1238–1249, 2010.

[208] G. Zhang *et al.*, "Deep RNA sequencing at single base-pair resolution reveals high complexity of the rice transcriptome," *Genome Res.*, 2010.

[209] H. Wei *et al.*, "Alternative splicing complexity contributes to genetic improvement of

drought resistance in the rice maintainer HuHan2B," *Sci. Rep.*, vol. 7, no. 1, pp. 1–13, 2017.

[210] E. Formentin *et al.*, "Transcriptome and Cell Physiological Analyses in Different Rice Cultivars Provide New Insights Into Adaptive and Salinity Stress Responses," *Front. Plant Sci.*, vol. 9, no. March, pp. 1–17, 2018.

[211] A. M. Mastrangelo, D. Marone, G. Laidò, A. M. De Leonardis, and P. De Vita, "Alternative splicing: Enhancing ability to cope with stress via transcriptome plasticity," *Plant Science*. 2012.

[212] V. V. Mironova, C. Weinholdt, and I. Grosse, "RNA-seq data analysis for studying abiotic stress in horticultural plants," in *Abiotic Stress Biology in Horticultural Plants*, 2015.

[213] F. Ding, P. Cui, Z. Wang, S. Zhang, S. Ali, and L. Xiong, "Genome-wide analysis of alternative splicing of pre-mRNA under salt stress in Arabidopsis," pp. 1–14, 2014.

[214] Y. Hiei and T. Komari, "Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed," *Nat. Protoc.*, 2008.

[215] C. Zhang, B. Zhang, L. L. Lin, and S. Zhao, "Evaluation and comparison of computational tools for RNA-seq isoform quantification," *BMC Genomics*, 2017.

[216] N. L. Bray, H. Pimentel, P. Melsted, and L. Pachter, "Near-optimal probabilistic RNA-seq quantification," *Nat. Biotechnol.*, 2016.

[217] C. Soneson, M. I. Love, and M. D. Robinson, "Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences," *F1000Research*, 2016.

[218] M. I. Love, W. Huber, and S. Anders, "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2," *Genome Biol.*, 2014.

[219] S. Babicki *et al.*, "Heatmapper: web-enabled heat mapping for all," *Nucleic Acids Res.*, 2016.

[220] M. Stemmer, T. Thumberger, M. del Sol Keyer, J. Wittbrodt, and J. L. Mateo, "CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool," *PLoS One*, vol. 10, no. 4, p. e0124633, Apr. 2015.

[221] A. Dobin *et al.*, "STAR: Ultrafast universal RNA-seq aligner," *Bioinformatics*, 2013.

[222] C. Trapnell *et al.*, "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks," *Nat. Protoc.*, 2012.

[223] "ASATP." [Online]. Available: http://systbio.cau.edu.cn/asatp/. [Accessed: 15-Mar-2018].

[224] K. Vitting-Seerup and A. Sandelin, "IsoformSwitchAnalyzeR: Analysis of changes in genome-wide patterns of alternative splicing and its functional consequences," *Bioinformatics*, 2019.

[225] J. Wang, M. Qi, J. Liu, and Y. Zhang, "CARMO: A comprehensive annotation platform for functional exploration of rice multi-omics data," *Plant J.*, 2015.

[226] D. Szklarczyk *et al.*, "STRING v11: Protein-protein association networks with increased

coverage, supporting functional discovery in genome-wide experimental datasets," *Nucleic Acids Res.*, 2019.

[227] A. E. Loraine, I. C. Blakley, S. Jagadeesan, J. Harper, G. Miller, and N. Firon, "Analysis and visualization of RNA-Seq expression data using rstudio, bioconductor, and integrated genome browser," in *Plant Functional Genomics: Methods and Protocols: Second Edition*, 2015.

[228] X. Cui and G. A. Churchill, "Statistical tests for differential expression in cDNA microarray experiments," *Genome Biology*. 2003.

[229] W. LI, "VOLCANO PLOTS IN ANALYZING DIFFERENTIAL EXPRESSIONS WITH mRNA MICROARRAYS," *J. Bioinform. Comput. Biol.*, 2012.

[230] Y. Hochberg and Y. Benjamini, "More powerful procedures for multiple significance testing," *Stat. Med.*, 1990.

[231] H. M. Hsieh, W. K. Liu, and P. C. Huang, "A novel stress-inducible metallothionein-like gene from rice," *Plant Mol. Biol.*, 1995.

[232] H. L. Wong, "Down-Regulation of Metallothionein, a Reactive Oxygen Scavenger, by the Small GTPase OsRac1 in Rice," *PLANT Physiol.*, 2004.

[233] G. H. Zhu, J. J. Zhang, J. S. Chen, and X. X. Peng, "Characterization of a rice metallothionein type 3 gene with different expression profiles under various nitrogen forms," *Biol. Plant.*, vol. 52, no. 4, pp. 668–673, Dec. 2008.

[234] K. Wei and H. Chen, "Comparative functional genomics analysis of bHLH gene family in rice, maize and wheat.," *BMC Plant Biol.*, vol. 18, no. 1, p. 309, Nov. 2018.

[235] C. Murre, P. S. McCaw, and D. Baltimore, "A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins," *Cell*, 1989.

[236] J.-S. Seo *et al.*, "OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice," *Plant J.*, vol. 65, no. 6, pp. 907–921, Mar. 2011.

[237] R. Le Hir, M. Castelain, D. Chakraborti, T. Moritz, S. Dinant, and C. Bellini, "AtbHLH68 transcription factor contributes to the regulation of ABA homeostasis and drought stress tolerance in Arabidopsis thaliana," *Physiol. Plant.*, vol. 160, no. 3, pp. 312–327, Jul. 2017.

[238] M. Nakata *et al.*, "High Temperature-Induced Expression of Rice α-Amylases in Developing Endosperm Produces Chalky Grains," *Front. Plant Sci.*, 2017.

[239] K. Yano, K. Aya, K. Hirano, R. L. Ordonio, M. Ueguchi-Tanaka, and M. Matsuoka, "Comprehensive Gene Expression Analysis of Rice Aleurone Cells: Probing the Existence of an Alternative Gibberellin Receptor," *Plant Physiol.*, 2015.

[240] T. Oikawa, M. Koshioka, K. Kojima, H. Yoshida, and M. Kawata, "A role of OsGA20ox1, encoding an isoform of gibberellin 20-oxidase, for regulation of plant stature in rice," *Plant Mol. Biol.*, 2004.

[241] X. Qin, J. H. Liu, W. S. Zhao, X. J. Chen, Z. J. Guo, and Y. L. Peng, " Gibberellin 20-

Oxidase Gene OsGA20ox3 Regulates Plant Stature and Disease Development in Rice ," *Mol. Plant-Microbe Interact.*, 2012.

[242] Y. Li *et al.*, "Genome-wide survey and expression analysis of the OSCA gene family in rice," *BMC Plant Biol.*, 2015.

[243] H. Zhao, H. Ma, L. Yu, X. Wang, and J. Zhao, "Genome-Wide Survey and Expression Analysis of Amino Acid Transporter Gene Family in Rice (Oryza sativa L.)," *PLoS One*, 2012.

[244] M. Tegeder, "Transporters for amino acids in plant cells: Some functions and many unknowns," *Current Opinion in Plant Biology*. 2012.

[245] D. Bradley, R. Carpenter, L. Copsey, C. Vincent, S. Rothstein, and E. Coen, "Control of inflorescence architecture in Antirrhinum," *Nature*, 1996.

[246] S. Zhang *et al.*, "TFL1/CEN-like genes control intercalary meristem activity and phase transition in rice," *Plant Sci.*, 2005.

[247] M. Nakagawa, K. Shimamoto, and J. Kyozuka, "Overexpression of RCN1 and RCN2, rice Terminal Flower 1/Centroradialis homologs, confers delay of phase transition and altered panicle morphology in rice," *Plant J.*, 2002.

[248] Y. Ling *et al.*, "Pre-mRNA splicing repression triggers abiotic stress signaling in plants," *Plant J.*, vol. 89, no. 2, pp. 291–309, 2017.

[249] S. AlShareef, Y. Ling, H. Butt, K. G. Mariappan, M. Benhamed, and M. M. Mahfouz, "Herboxidiene triggers splicing repression and abiotic stress responses in plants," *BMC Genomics*, 2017.

[250] A. B. Garcia *et al.*, "The expression of the salt-responsive gene salT from rice is regulated by hormonal and developmental cues," *Planta*, 1998.

[251] B. Mohanty, H. Takahashi, B. G. de los Reyes, E. Wijaya, M. Nakazono, and D. Y. Lee, "Transcriptional regulatory mechanism of alcohol dehydrogenase 1-deficient mutant of rice for cell survival under complete submergence," *Rice*, 2016.

[252] J. Gibbs and H. Greenway, "Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism," *Functional Plant Biology*. 2003.

[253] H. Takahashi, H. Greenway, H. Matsumura, N. Tsutsumi, and M. Nakazono, "Rice alcohol dehydrogenase 1 promotes survival and has a major impact on carbohydrate metabolism in the embryo and endosperm when seeds are germinated in partially oxygenated water," *Ann. Bot.*, 2014.

[254] Y. Song, L. Liu, Y. Wei, G. Li, X. Yue, and L. An, "Metabolite Profiling of adh1 Mutant Response to Cold Stress in Arabidopsis," *Front. Plant Sci.*, 2017.

[255] W. Wang, B. Vinocur, O. Shoseyov, and A. Altman, "Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response," *Trends in Plant Science*. 2004.

[256] H. J. Koo, X. Xia, and C. B. Hong, "Genes and expression pattern of tobacco mitochondrial small heat shock protein under high-temperature stress," *J. Plant Biol.*, vol.

46, no. 3, pp. 204–210, Sep. 2003.

[257] J. Zou *et al.*, “Expression analysis of nine rice heat shock protein genes under abiotic stresses and ABA treatment,” *J. Plant Physiol.*, 2009.

[258] T. F. Smith, C. Gaitatzes, K. Saxena, and E. J. Neer, “The WD repeat: A common architecture for diverse functions,” *Trends in Biochemical Sciences*. 1999.

[259] T. F. Smith, “Diversity of WD-Repeat proteins,” in *The Coronin Family of Proteins*, 2008.

[260] S. van Nocker and P. Ludwig, “The WD-repeat protein superfamily in Arabidopsis: Conservation and divergence in structure and function,” *BMC Genomics*. 2003.

[261] D. Kong, M. Li, Z. Dong, H. Ji, and X. Li, “Identification of TaWD40D, a wheat WD40 repeat-containing protein that is associated with plant tolerance to abiotic stresses,” *Plant Cell Rep.*, 2014.

[262] L. F. Ge, D. Y. Chao, M. Shi, M. Z. Zhu, J. P. Gao, and H. X. Lin, “Overexpression of the trehalose-6-phosphate phosphatase gene OsTPP1 confers stress tolerance in rice and results in the activation of stress responsive genes,” *Planta*, 2008.

[263] G. W. Li *et al.*, “Transport functions and expression analysis of vacuolar membrane aquaporins in response to various stresses in rice,” *J. Plant Physiol.*, 2008.

[264] H. Shikata, K. Hanada, T. Ushijima, M. Nakashima, Y. Suzuki, and T. Matsushita, “ Phytochrome controls alternative splicing to mediate light responses in Arabidopsis ,” *Proc. Natl. Acad. Sci.*, 2014.

[265] J. A. Traverso, T. Meinnel, and C. Giglione, “Expanded impact of protein N-myristoylation in plants,” *Plant Signal. Behav.*, 2008.

[266] T. Meinnel, “Protein lipidation meets proteomics,” *Front. Biosci.*, 2008.

[267] M. Venkateshwaran *et al.*, “The Recent Evolution of a Symbiotic Ion Channel in the Legume Family Altered Ion Conductance and Improved Functionality in Calcium Signaling,” *Plant Cell*, 2012.

[268] G. Chen, Y. Zou, J. Hu, and Y. Ding, “Genome-wide analysis of the rice PPR gene family and their expression profiles under different stress treatments.,” *BMC Genomics*, vol. 19, no. 1, p. 720, Oct. 2018.

[269] A. Sasaki, N. Yamaji, J. Xia, and J. F. Ma, “OsYSL6 Is Involved in the Detoxification of Excess Manganese in Rice,” *PLANT Physiol.*, 2011.

[270] M. Collado-Romero, E. AlÃ^{3}s, and P. Prieto, “Unravelling the proteomic profile of rice meiocytes during early meiosis,” *Front. Plant Sci.*, 2014.

[271] R. Sundaresan, H. P. Parameshwaran, S. D. Yogesha, M. W. Keilbarth, and R. Rajan, “RNA-Independent DNA Cleavage Activities of Cas9 and Cas12a,” *Cell Rep.*, 2017.

[272] E. A. Bray, “Plant responses to water deficit,” *Trends in Plant Science*. 1997.

[273] K. Shinozaki and K. Yamaguchi-Shinozaki, “Molecular responses to dehydration and low

temperature: Differences and cross-talk between two stress signaling pathways," *Current Opinion in Plant Biology*. 2000.

[274] X. S. Wang, H. B. Zhu, G. L. Jin, H. L. Liu, W. R. Wu, and J. Zhu, "Genome-scale identification and analysis of LEA genes in rice (Oryza sativa L.)," *Plant Sci.*, 2007.

[275] M. S. Chern, H. A. Fitzgerald, R. C. Yadav, P. E. Canlas, X. Dong, and P. C. Ronald, "Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis," *Plant J.*, 2001.

[276] X. Liu, X. Bai, X. Wang, and C. Chu, "OsWRKY71, a rice transcription factor, is involved in rice defense response," *J. Plant Physiol.*, 2007.

[277] G. K. Agrawal, R. Rakwal, and N. S. Jwa, "Rice (Oryza sativa L.) OsPR1b gene is phytohormonally regulated in close interaction with light signals," *Biochem. Biophys. Res. Commun.*, 2000.

[278] N. Soranzo, M. Sari Gorla, L. Mizzi, G. De Toma, and C. Frova, "Organisation and structural evolution of the rice glutathione S-transferase gene family," *Molecular Genetics and Genomics*. 2004.

[279] K. Yoshimoto, Y. Takano, and Y. Sakai, "Autophagy in plants and phytopathogens," *FEBS Letters*. 2010.

[280] L. Chen *et al.*, "Autophagy contributes to regulation of the hypoxia response during submergence in Arabidopsis thaliana," *Autophagy*, 2015.

[281] S. Slavikova, S. Ufaz, T. Avin-Wittenberg, H. Levanony, and G. Galili, "An autophagy-associated Atg8 protein is involved in the responses of Arabidopsis seedlings to hormonal controls and abiotic stresses," *J. Exp. Bot.*, 2008.

[282] Y. Liu, Y. Xiong, and D. C. Bassham, "Autophagy is required for tolerance of drought and salt stress in plants.," *Autophagy*, 2009.

[283] C. M. Tobias and E. K. Chow, "Structure of the cinnamyl-alcohol dehydrogenase gene family in rice and promoter activity of a member associated with lignification," *Planta*, vol. 220. Springer, pp. 678–688, 2005.

[284] H. Park, T. Kim, S. Bhoo, T. Lee, S.-W. Lee, and M.-H. Cho, "Biochemical Characterization of the Rice Cinnamyl Alcohol Dehydrogenase Gene Family," *Molecules*, vol. 23, no. 10, p. 2659, Oct. 2018.

[285] Y. Xiang, Y. Huang, and L. Xiong, "Characterization of Stress-Responsive CIPK Genes in Rice for Stress Tolerance Improvement," *Plant Physiol.*, vol. 144, no. 3, pp. 1416–1428, 2007.

[286] J. Wang *et al.*, "Comparative transcriptome analysis reveals molecular response to salinity stress of salt-tolerant and sensitive genotypes of indica rice at seedling stage," *Sci. Rep.*, vol. 8, no. 1, pp. 1–13, 2018.

[287] C. H. Kao, "Mechanisms of Salt Tolerance in Rice Plants : Cell Wall-Related Genes and Expansins," vol. 66, no. 2, pp. 87–93, 2017.

[288] A. Nakhamchik *et al.*, "A comprehensive expression analysis of the Arabidopsis proline-rich extensin-like receptor kinase gene family using bioinformatic and experimental approaches," *Plant Cell Physiol.*, 2004.

[289] B. D. Kohorn, "WAKs; cell wall associated kinases," *Current Opinion in Cell Biology*. 2001.

[290] S. Zhang *et al.*, "Evolutionary expansion, gene structure, and expression of the rice wall-associated kinase gene family.," *Plant Physiol.*, vol. 139, no. 3, pp. 1107–24, Nov. 2005.

[291] K. Fujino, M. Obara, and K. Sato, "Diversification of the plant-specific hybrid glycine-rich protein (HyGRP) genes in cereals," *Front. Plant Sci.*, 2014.

[292] L. Dvořková, M. Srba, Z. Opatrny, and L. Fischer, "Hybrid proline-rich proteins: Novel players in plant cell elongation?," *Ann. Bot.*, 2012.

[293] Y. Kobayashi, S. Yamamoto, H. Minami, Y. Kagaya, and T. Hattori, "Differential Activation of the Rice Sucrose Nonfermenting1–Related Protein Kinase2 Family by Hyperosmotic Stress and Abscisic Acid," *Plant Cell*, 2004.

[294] S. Waters, M. Gilliham, and M. Hrmova, "Plant High-Affinity Potassium (HKT) Transporters Involved in Salinity Tolerance: Structural insights to Probe Differences in ion Selectivity," *International Journal of Molecular Sciences*. 2013.

[295] C. H. Kao, "Mechanisms of salt tolerance in rice plants: Compatible solutes and aquaporins," *Crop. Environ. Bioinforma.*, 2015.

[296] J. Li *et al.*, "Defense-related transcription factors WRKY70 and WRKY54 modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis," *New Phytol.*, 2013.

[297] V. Demidchik *et al.*, "Arabidopsis root K+-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death," *J. Cell Sci.*, 2010.

[298] M. Zou, Y. Guan, H. Ren, F. Zhang, and F. Chen, "A bZIP transcription factor, OsABI5, is involved in rice fertility and stress tolerance," *Plant Mol. Biol.*, 2008.

[299] A. Fukuda *et al.*, "Function, Intracellular Localization and the Importance in Salt Tolerance of a Vacuolar Na+/H+ Antiporter from Rice," *Plant Cell Physiol.*, 2004.

[300] K. Chourey, S. Ramani, and S. K. Apte, "Accumulation of LEA proteins in salt (NaCl) stressed young seedlings of rice (Oryza sativa L.) cultivar Bura Rata and their degradation during recovery from salinity stress," *J. Plant Physiol.*, 2003.

[301] H. T. Choe and D. J. Cosgrove, "Expansins as agents in hormone action," in *Plant Hormones: Biosynthesis, Signal Transduction, Action!*, 2010.

[302] S. J. Lu, Z. T. Yang, L. Sun, L. Sun, Z. T. Song, and J. X. Liu, "Conservation of IRE1-regulated bZIP74 mRNA unconventional splicing in rice (Oryza sativa L.) involved in ER stress responses," in *Molecular Plant*, 2012.

[303] S. Ogawa *et al.*, "OsMYC2, an essential factor for JA-inductive sakuranetin production in

rice, interacts with MYC2-like proteins that enhance its transactivation ability," *Sci. Rep.*, 2017.

[304] X. Liu, J. Wang, and L. Sun, "Structure of the hyperosmolality-gated calcium-permeable channel OSCA1.2," *Nat. Commun.*, 2018.

[305] K. J. Dietz, M. O. Vogel, and A. Viehhauser, "AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signalling," *Protoplasma*, 2010.

[306] Z.-S. Xu, M. Chen, L.-C. Li, and Y.-Z. Ma, "Functions of the ERF transcription factor family in plants," *Botany*, 2008.

[307] H. Wang, H. Wang, H. Shao, and X. Tang, "Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology," *Front. Plant Sci.*, 2016.

[308] H. Chepyshko, C. P. Lai, L. M. Huang, J. H. Liu, and J. F. Shaw, "Multifunctionality and diversity of GDSL esterase/lipase gene family in rice (Oryza sativa L. japonica) genome: New insights from bioinformatics analysis," *BMC Genomics*, 2012.

[309] P. V. Turlapati, K. W. Kim, L. B. Davin, and N. G. Lewis, "The laccase multigene family in Arabidopsis thaliana: Towards addressing the mystery of their gene function(s)," *Planta*, 2011.

[310] Q. Liu, L. Luo, X. Wang, Z. Shen, and L. Zheng, "Comprehensive analysis of rice laccase gene (OsLAC) family and ectopic expression of OsLAC10 enhances tolerance to copper stress in Arabidopsis," *Int. J. Mol. Sci.*, 2017.

[311] L. L. Gao and H. W. Xue, "Global analysis of expression profiles of rice receptor-like kinase genes," *Mol. Plant*, 2012.

[312] Y. Ishimaru *et al.*, "Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese," *Plant J.*, vol. 62, no. 3, pp. 379–390, Feb. 2010.

[313] J. G. Dubouzet *et al.*, "OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression," *Plant J.*, 2003.

[314] A. K. Mishra, S. Puranik, and M. Prasad, "Structure and regulatory networks of WD40 protein in plants," *J. Plant Biochem. Biotechnol.*, 2012.

[315] A. Muñoz and M. M. Castellano, "Regulation of Translation Initiation under Abiotic Stress Conditions in Plants: Is It a Conserved or Not so Conserved Process among Eukaryotes?," *Comp. Funct. Genomics*, 2012.

[316] N. K. Sarkar, U. Thapar, P. Kundnani, P. Panwar, and A. Grover, "Functional relevance of J-protein family of rice (Oryza sativa)," *Cell Stress Chaperones*, 2013.

[317] A. M. Timperio, M. G. Egidi, and L. Zolla, "Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP)," *Journal of Proteomics*. 2008.

[318] T. J. Close, “Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins,” in *Physiologia Plantarum*, 1996.

[319] M. Hanin, F. Brini, C. Ebel, Y. Toda, S. Takeda, and K. Masmoudi, “Plant dehydrins and stress tolerance: Versatile proteins for complex mechanisms,” *Plant Signaling and Behavior*. 2011.

[320] Z. Zhang and B. Xiao, “Comparative alternative splicing analysis of two contrasting rice cultivars under drought stress and association of differential splicing genes with drought response QTLs,” *Euphytica*, 2018.

[321] S. Lin and X.-D. Fu, “SR proteins and related factors in alternative splicing.,” *Adv. Exp. Med. Biol.*, 2007.

[322] S. D. Chandler, A. Mayeda, J. M. Yeakley, A. R. Krainer, and X.-D. Fu, “RNA splicing specificity determined by the coordinated action of RNA recognition motifs in SR proteins,” *Proc. Natl. Acad. Sci.*, 2002.

[323] G. Liu, K. Zhang, J. Ai, X. Deng, Y. Hong, and X. Wang, “Patatin-related phospholipase A, pPLAIIIα, modulates the longitudinal growth of vegetative tissues and seeds in rice,” *J. Exp. Bot.*, 2015.

[324] G. F. E. Scherer, S. B. Ryu, X. Wang, A. R. Matos, and T. Heitz, “Patatin-related phospholipase A: Nomenclature, subfamilies and functions in plants,” *Trends in Plant Science*. 2010.

[325] T. Sakamoto, “An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice,” *PLANT Physiol.*, 2004.

[326] F. Vigneault, D. Lachance, M. Cloutier, G. Pelletier, C. Levasseur, and A. Séguin, “Members of the plant NIMA-related kinases are involved in organ development and vascularization in poplar, Arabidopsis and rice,” *Plant J.*, 2007.

[327] B. Zhang *et al.*, “NIMA-related kinase NEK6 affects plant growth and stress response in Arabidopsis,” *Plant J.*, 2011.

[328] Y. Ning, Q. Xie, and G.-L. Wang, “OsDIS1-mediated stress response pathway in rice.,” *Plant Signal. Behav.*, vol. 6, no. 11, pp. 1684–6, Nov. 2011.

[329] A. Dametto, G. Buffon, É. Aparecida, D. Reis, A. Dametto, and G. Buffon, “Ubiquitination pathway as a target to develop abiotic stress tolerance in rice Ubiquitination pathway as a target to develop abiotic stress tolerance in rice,” vol. 2324, no. October, pp. 1–7, 2015.

[330] G. G. Simpson and W. Filipowicz, “Splicing of precursors to mRNA in higher plants: Mechanism, regulation and sub-nuclear organisation of the spliceosomal machinery,” *Plant Mol. Biol.*, 1996.

[331] G. Sachetto-Martins, L. O. Franco, and D. E. De Oliveira, “Plant glycine-rich proteins: A family or just proteins with a common motif?,” *Biochimica et Biophysica Acta - Gene Structure and Expression*. 2000.

[332] L. R. Zeng, C. H. Park, R. C. Venu, J. Gough, and G. L. Wang, “Classification, expression pattern, and E3 Ligase Activity Assay of Rice U-box-containing proteins,” *Mol. Plant*, 2008.

[333] P. Kafasla *et al.*, “Interaction of yeast eIF4G with spliceosome components: implications in pre-mRNA processing events.,” *RNA Biol.*, 2009.

[334] S. Das, U. Saha, and B. Das, “Cbc2p, Upf3p and eIF4G are components of the DRN (Degradation of mRNA in the Nucleus) in Saccharomyces cerevisiae,” *FEMS Yeast Res.*, 2014.

[335] J. Suo, Q. Zhao, L. David, S. Chen, and S. Dai, “Salinity response in chloroplasts: Insights from gene characterization,” *International Journal of Molecular Sciences*. 2017.

[336] J. Li *et al.*, “The Os-AKT1 Channel Is Critical for K+ Uptake in Rice Roots and Is Modulated by the Rice CBL1-CIPK23 Complex,” *Plant Cell*, 2014.

[337] K.-N. Kim, J.-S. Lee, H. Han, S. A. Choi, S. J. Go, and I. S. Yoon, “Isolation and characterization of a novel rice Ca2+-regulated protein kinase gene involved in responses to diverse signals including cold, light, cytokinins, sugars and salts.,” *Plant Mol. Biol.*, vol. 52, no. 6, pp. 1191–202, Aug. 2003.

[338] J. Yu, M. Jiang, and C. Guo, “Crop Pollen Development under Drought: From the Phenotype to the Mechanism,” *Int. J. Mol. Sci.*, 2019.

[339] K. D. Sharma and H. Nayyar, “Regulatory Networks in Pollen Development under Cold Stress,” *Front. Plant Sci.*, 2016.

[340] Tran Thi Huong Sen, Phan Thi Phuong Nhi, and Trinh Thi Sen, “Salinity Effect at Seedling and Flowering Stages of Some Rice Lines and Varieties (Oryza sativa L.),” *J. Agric. Sci. Technol. A*, 2017.

[341] J.-X. Liu and S. H. Howell, “Endoplasmic Reticulum Protein Quality Control and Its Relationship to Environmental Stress Responses in Plants,” *Plant Cell*, vol. 22, no. 9, pp. 2930–2942, 2010.

[342] A. Rietsch and J. Beckwith, “THE GENETICS OF DISULFIDE BOND METABOLISM,” *Annu. Rev. Genet.*, 2002.

[343] N. J. Bulleid and L. Ellgaard, “Multiple ways to make disulfides,” *Trends in Biochemical Sciences*. 2011.

[344] R. J. Kaufman, “Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls,” *Genes and Development*. 1999.

[345] K. Xia *et al.*, “Formation of Protein Disulfide Bonds Catalyzed by OsPDIL1;1 is Mediated by MicroRNA5144-3p in Rice,” *Plant Cell Physiol.*, 2018.

[346] X. F. Chen, Z. M. Gu, F. Liu, B. J. Ma, and H. S. Zhang, “Molecular Analysis of Rice CIPKs Involved in Both Biotic and Abiotic Stress Responses,” *Rice Sci.*, 2011.

[347] Z. Gu, J. Wang, J. Huang, and H. Zhang, “Cloning and characterization of a novel rice

gene family encoding putative dual-specificity protein kinases, involved in plant responses to abiotic and biotic stresses," *Plant Sci.*, 2005.

[348] K. Tamura and I. Hara-Nishimura, "Functional insights of nucleocytoplasmic transport in plants," *Front. Plant Sci.*, 2014.

[349] K. Lott and G. Cingolani, "The importin β binding domain as a master regulator of nucleocytoplasmic transport," *Biochimica et Biophysica Acta - Molecular Cell Research*. 2011.

[350] Y. Luo *et al.*, "An Arabidopsis homolog of importin β1 is required for ABA response and drought tolerance," *Plant J.*, 2013.

[351] T. E. Graber and M. Holcik, "Cap-independent regulation of gene expression in apoptosis," *Mol. Biosyst.*, 2007.