

Ribosomal Protein Genes as Potential Targets for Manipulating Stress Tolerance in Rice

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

by

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August, 2021



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I, **Anusree Saha**, hereby declare that this thesis entitled “**Ribosomal Protein Genes as Potential Targets for Manipulating Stress Tolerance in Rice**” submitted by me under the guidance and supervision of **Prof. P. B. Kirti** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Parts of the thesis has been:

A. Published in the following publications:

1. **Saha, A., Das, S., Moin, M., Dutta, M., Bakshi, A., Madhav, M. S., & Kirti, P. B.** (2017). Genome-wide identification and comprehensive expression profiling of ribosomal protein small subunit (RPS) genes and their comparative analysis with the large subunit (RPL) genes in rice. *Frontiers in plant science*, 8, 1553.
2. **Moin, M*, Saha, A*, Bakshi, A., MS, M., & PB, K.** (2021). Study on Transcriptional Responses and Identification of Ribosomal Protein Genes for Potential Resistance against Brown Planthopper and Gall Midge Pests in Rice. *Current Genomics*, 22(2), 98-110. (*equal contribution)
3. **Moin, M., Saha, A., Bakshi, A., Madhav, M. S., & Kirti, P. B.** (2021). Constitutive expression of Ribosomal Protein L6 modulates salt tolerance in rice transgenic plants. *Gene*, 789, 145670.

B. Presented in the following conferences:

1. Presented E-poster at the “**4th Edition of Global Conference on Plant Science and Molecular Biology**” during September 19-21, 2019 at London, UK.
2. Presented poster at the “**2018 International Plant Molecular Biology Conference**” held during 5th-10th August, 2018 at Montpellier, France.

3. Presented poster at the “**3rd International conference on Bioresource and stress management**” during November, 2017, Jaipur, India.

Further, the student has passed the following courses toward the fulfillment of the coursework requirement for Ph.D. degree.

S.no	Course	Name	Credits	Pass/fail
1.	AS801	Analytical Techniques	4	Pass
2.	AS802	Research Ethics, Data Analysis and Biostatistics	3	Pass
3.	AS803	Lab work and record	5	Pass

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Prof. P. B. Kirti

Supervisor

To my parents, to whom I owe everything.

Acknowledgements

It is often said, “It takes a village to raise a child”. In my case, it has taken a village to complete this thesis. So many kind people around me have contributed directly and indirectly to my endeavour to get this degree. I want to begin by acknowledging those people who made sure I did not give up and the people who made this moment possible.

*First and foremost, I want to extend my heartfelt gratitude towards my supervisor, **Prof. P. B. Kirti**. His support, patient guidance, encouragement, constructive criticism have helped me immensely throughout this journey. I want to thank him for allowing his students to explore new ideas independently without restricting their thoughts and creativity. The enthusiasm he has for science and research has motivated me and always kept me on my toes. I want to express my gratefulness for timely and very carefully correcting the manuscripts. His thoughtful and invaluable inputs paved the way for designing experiments, writing manuscripts, and completing this thesis. I will forever be grateful for his faith in me right from the beginning of my admission. I will never forget how he took care of us, backed us up and had our best interests in his mind during hard times. This work, indeed, would not have been possible without him.*

*Besides my supervisor, I would like to thank my Doctoral Committee members: **Prof. G. Padmaja** and **Dr J. S. S. Prakash**, for their insightful comments and encouragement and the questions that incited me to widen my research from various perspectives.*

*I thank the former Deans, **Prof. P. Reddanna**, **Prof. M. N.V Prasad**, **Prof. N. Siva Kumar**, **Prof. Ramaiah** and the present Dean, **Prof. S. Dayananda**, School of Life Sciences and former Head, **Prof Ch. Venkata Ramana**, and current Head, **Prof. G. Padmaja**, Dept. of Plant Sciences, for their support in all possible ways. The school and the Department have gone out of their ways to help me finish my thesis, for which I will be forever grateful. I would like to especially thank **Dr Santosh R Kanade** for sharing the lab space and letting us work peacefully during the end of our tenure. My sincere thanks to the entire Faculty of the Department of Plant Sciences and School of Life Sciences for helping me either directly or indirectly in my study.*

*A very special thanks to **Prof. Christine A. Raines**, University of Essex, the UK, for hosting me in her lab during my Newton Bhabha PhD placement. Her constant motivation, appreciation, flexibility and belief in me have helped me tide over tough times. I am thankful to her entire research group, specially **Patty**, for her kind support while grasping Golden gate cloning and CRISPR-Cas9 strategies. I cannot thank her enough for making time from her busy schedule for hours of discussion and for making me feel at home in a foreign land.*

I am thankful to all the teachers I came across in my life who directly and indirectly instilled scientific aptitude and good moral and ethical values in me.

*I thank the **Department of Biotechnology (DBT)** for providing Research fellowship in the form of JRF and SRF. Thanks are also due to **DBT, CSIR, DST-FIST, UGC-SAP and DBT-CREB** for funding in the form of projects and Fellowships to our lab and the Department. I am thankful to **British Council**, London and DBT, India, for funding my Newton Bhabha PhD placement; and **DST-SERB** for travel grants to attend international conferences.*

*I want to express my appreciation to my outstanding labmates (present and former) **Dr Deepanker Yadav, Dr Naveen Singh, Dr Pawan Shukla, Dr Mazahar Moin, Dr Achala Bakshi, Dr Sakshi Rampuria, Dr Trishla Jain, Dr Ranjana Gautam, Venkat Reddy, Dr Prasanna Boyidi, Dr Kiranmayee, Shubhajit and Hali Krishna** for their cooperation and excellent lab environment.*

*I extend my heartfelt appreciation to the previous and present lab attendants **Kishan Anna, Mahesh, Satish, Ramesh, Susheelam, Naresh, and Manohar**, who have done a very tough job in the field, taking care of the plants, and in the lab, providing the things necessary for my work.*

My friends have been blessings in my life, and they are instrumental in making me what I am today. My friends from school, college, university have always stood by me through good and bad times.

***Mouboni** has been my rock, wind beneath my wings and my grounding force all at once. I couldn't have asked for a better companion to share my college, university, PhD and hostel life with. She made everything better and less traumatic. Had it not been for her, this journey would have been far more harsh and unbearable. I am sure we will keep making memories together, but for now, I want to take a moment to thank her for being there with me through thick and thin. I am glad that things we have gone through have only made us stronger and I wish her all the best for all the wonderful things she will achieve in future.*

*The friends I made in UoH will always be special. We have started our doctoral journey together and dealt with so many ups and downs. Without their support and help at every turn of my journey, I cannot imagine reaching this far. In this regard, I want to especially thank **Anirban** for his practical suggestions and constructive comments on my presentation, manuscripts and work; and **Indu** for her unwavering support and encouragement. Both of them made my time in campus enjoyable and pleasant. I am also grateful for my girls' gang (Pina colada), including **Mouboni, Indu, Anusha, Divya and Smita**, for being my pillar of strength and inspiration. Our impromptu dinner plans, trips, shopping and movie nights have saved me from monotony and dullness most people in academia suffer from. It has been such a pleasure knowing them all and seeing them grow as scientists and in personal lives. We have come a long way and I can't wait to see them shine brighter. Thank you for being the best companions and cheerleaders all this while.*

*I am incredibly thankful to my senior **Mastan sir**, for all the life lessons, guidance and inspiration. All my batchmates, seniors and juniors from SLS have helped in one way or the other including **Payal, Tamna, Rutuparna, Mounica, Rajesh Sir, Suresh Sir, Tinni, Haritha, Saumashish, Prodosh, Supriyo**.*

*Specially thankful to **Arunabha** for years of friendship and the special bond that never seems to weaken. Genuinely grateful for having **Sneha** in my life, who has never given up on me. I thank the rest of my M.Sc friends, specially **Argha** and **Jyotirmoy**, for making this place feel like home. I want to thank my school, college and work friends **Sukanya, Pallabi, Trisha, Adrija, Arpita, Sonali, Subrata, Rajatava, Dhriti** for being my support system for years. I am blessed to have them in my life.*

*Special thanks to my friends in the UK, **Pachecho, Giorgos** and **Olga**, for gifting me the most beautiful four months of my life. I will be indebted to them for their love, friendship and affection. Hope we will always be in touch. During my Newton Bhabha placement, I had the opportunity to meet some great people, and I feel fortunate to have found precious friendships in them. A very special thanks to my partner in crime, **Nilapratim**, for all the fun rendezvous we had in the UK. Grateful to have met the other folks, including **Shyamali, Mrunal, Bhumika, Debarati, Kamal, Ketaki, Dinesh, Debaki, Anurag, Parag, Saurav, Prachi, Prosenjit**.*

*I cannot begin to express my gratitude to **Pritam**, who never for once wavered in his support. I thank him for constantly bearing with my Tech-challenged self, spending endless hours fixing my manuscripts, making my graphs in R and for everything else.*

*I cannot close without acknowledging the invaluable gifts I received from **my parents**, who ultimately made all of this possible by raising me. Their sacrifices, profound belief in my abilities, unparalleled support and patience have been my only fuel for all these years. I owe every bit of my success to **Maa and Baba**. Thank you for never burdening me with your expectations, for always cheering me up, for letting me follow my dreams and for always being the two most loved people I can turn to during the dark and desperate times. Apart from my parents, the rest of the family has always played an instrumental role in my life. I am forever indebted to my Uncles and Aunts (**B.B. Saha, Pishi, Chhoto masi, Chhoto mama, Fulmami, Kakima, Amal Kaku, Puno kaku, Boro, Kakimani, Kutti aunty, Mini, Mejo Mesho**) for always believing in me and to my cousins (**Ankit, Joy, Jui, Jiya, Suvom, Rivu**) for their love, affection and respect. I want to especially thank my sister **Pooja** for being the person I can run to with my problems. Her unconditional love and patience can never be underestimated. Last but not least, I regret not being able to thank my **Grandmother** in person. I wish she were alive to see me get the degree.*

“To all those who pushed me towards my dream”

- Anusree

Abbreviations

Abbreviation	Full form
μg	Microgram
μl	Microliter
μM	Micro molar
ABA	Absciscic Acid
CAMV	Cauliflower mosaic virus
cDNA	Complementary DNA
cm	Centimeter
CTAB	Cetyl trimethylammonium bromide
dai	Days after infection
hai	Hours after infection
d	Day
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	hours
Kb	Kilobases
KDa	Kilodalton
LB	Luria Bertani
M	Molar
min	Minutes
ml	Milliliter
MS	Murashige and Skoog
ng	Nano gram
O.D	Optical density
PCR	Polymerase Chain Reaction
pI	Isoelectric point
RNA	Ribo Nucleic Acid
Rpm	Revolutions Per Minute
RP	Ribosomal proteins
RPL	Ribosomal protein large subunit
RPS	Ribosomal protein small subunit
WT	Wild type
WUE	Water-use efficiency

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Chapter 1

Introduction and literature review

1.1 Introduction

1.1.1 Ribosomal Proteins

The ribosome plays a crucial role in every cell of an organism. The biogenesis of ribosome is one of the most coordinated and energy requiring cellular activities. Ribosomes are the primary machinery that functions at the core of the translation apparatus responsible for converting information coded in an mRNA into corresponding proteins. Basically, ribosomes act as machines for a steady supply of cellular proteins (Barakat et al., 2001). Ribosome is a ribonucleic complex comprised of rRNA and ribosomal proteins. Ribosomal proteins play vital roles in maintaining structural and functional integrity of rRNA, protection of rRNA from RNAase, and general protein synthesis (Cherepneva et al., 2003). Ribosomal protein genes are a class of highly expressed genes in most cell types and are directly involved in ribosome synthesis. The entire mechanism of controlling the expression of RP genes is regulated to balance the supply of new ribosomal proteins with the demand for new ribosomes. Eukaryotic ribosomes have a sedimentation coefficient of the 80S with the large 60S subunits having 25S, 5.8S, 5S rRNA, and the small 40S subunit consisting of 18S rRNA. The number of Ribosomal proteins varies in different organisms, with eukaryotes and prokaryotes reporting up to 80 and 54 RPs, respectively, in both subunits. Within eukaryotes, the number of RPs varies as well. In Yeast RPs (32 of the small subunit and 46 of the large subunit) are encoded by 137 genes. Two hundred and forty-nine genes encode 80 RPs of *Arabidopsis*, among which 32 are small subunit, and 48 are large subunit proteins (Barakat et al., 2001). Plant ribosomal proteome is far more complex and complicated than mammals and fungi because plants contain a third type of ribosomal proteins in plastids apart from the cytosolic and mitochondrial proteins. Heterogeneity is common in plants, as observed in the *Arabidopsis* genome; none of the 80 different r-protein families are encoded by a single-copy gene. Plants, being sessile organisms, need more adaptability

to environmental variations. This noticeable heterogeneity is most likely attributable to their potential to avoid environmental fluctuations (Carroll, 2013). Most of the ribosomal proteins bind and interact with RNA, contributing to stabilizing the RNA structure. However, some of these proteins are also involved in protein-protein interactions, which indicates the potential for extra-ribosomal functions (Aseev and Boni, 2011).

1.1.2 Ribosomal proteins and extra-ribosomal activities

Ribosomal proteins (RPs) are the primary components of the ribosome, and they are in charge of maintaining the rRNA structure in the ribosome to ensure that protein synthesis is as effective as possible. RPs have significant roles in ribosome biogenesis, protein synthesis, cell growth, development, and apoptosis. Gradually the association of ribosomal proteins with other physiological activities like protection against radiation and pathogens is elucidated. Increasing evidence indicates that mutations in certain ribosomal protein genes and ribosome assembly components in fungi, plants, and mammals can cause specific physiological abnormalities. This might be attributed to a generalized decrease in protein synthesis. However, the fact that there is evidence of physiological defects indicates that the ribosomes have regulatory roles to play. In one of the studies, a list of criteria has been proposed based on which we can conclude if the RP is acting in an extra-ribosomal capacity. These criteria being interactions between the ribosomal proteins with non-ribosomal components of the cells (RNA or protein), a manifestation of these interactions as a physiological effect on the organism and the occurrence of such interactions at sites away from the ribosomes (Warner and McIntosh, 2009). Several ribosomal proteins have been reported to be engaged in modulating their own expression or expression pattern of other genes, and in doing so, these ribosomal proteins can govern the cell cycle and apoptosis. Many ribosomal proteins have been engaged in DNA repair processes in both prokaryotes and eukaryotes, including the SOS repair mechanism, cell protection from genotoxic stress, oxidative damage repair, and ionising radiation damage repair. Regulation of expression pattern of ribosomal protein impacts developmental processes in many organisms. In *Arabidopsis*, mutated forms of some ribosomal protein genes are lethal to embryo development, suggesting their role in developmental processes (Bhavsar et al., 2010). Regulation of expression pattern of genes coding for r-proteins occurs at the transcriptional, post-transcriptional, translational, and post-translational levels depending on the organisms. Ribosomal protein genes have been identified to be upregulated in response to cold treatment, UV- radiation, and other stressed conditions. This has given a new dimension to ribosome research, opening up new avenues for ribosomal proteins to exhibit extra-ribosomal functions. Ribosomal protein counterparts vary significantly among the class of organisms. Bacterial ribosomal proteins, archaeal ribosomal proteins, and eukaryotic ribosomal proteins estimate

have been around 57, 68, 80 in number, respectively. E.g.- In *E.coli*, ribosomes contain 55 proteins (21 in small and 31 in large subunits), *Saccharomyces cerevisiae* has at least 78 r-proteins (32 in the small and 46 in large subunit), the mammalian ribosome has 80 r-proteins (33 in small and 47 in large subunit). Plant ribosomal subunits have been reported to contain 75-92 r-proteins. There is a vast diversity among genes that encode ribosomal proteins. These ribosomal protein genes are found in multigene families. Many ribosomal proteins have been engaged in DNA repair processes in both prokaryotes and eukaryotes, including the SOS repair mechanism, cell protection from genotoxic stress, oxidative damage repair, and ionising radiation damage repair. The rate of translation is high in growing plants and may require more than one ribosomal protein gene belonging to each family to be expressed at all times. The expressed isoforms might be present at different developmental tissues at a particular time point. Expression of a multigene family is also an indication that some members of the ribosomal protein genes serve functions other than the one related to ribosome assembly and biogenesis. It might also indicate ribosome heterogeneity (Chang et al., 2005). As seen in other organisms, an equal proportion of ribosomal proteins is present within the ribosome assembly. This is due to a cumulative action of several regulatory processes, including transcriptional, post-transcriptional, translational, and post-translational modifications of each r-protein gene. What makes this regulation extremely complicated and complex in plants is that in plants, there are families of expressed genes for individual ribosomal protein. Each of these gene families has diverse expression patterns (Wu et al., 1995; Barakat et al., 2001). Transcript levels of ribosomal protein genes vary in plants during different developmental stages, like there is increased expression of certain r-proteins in meristems and other growing tissues of plants. Also, mechanical injury, phytohormone treatment, and induction of stress lead to increased transcript levels of numerous ribosomal protein genes with auxin, cytokinin, and abscisic acid having a powerful impact on the expression pattern of these genes. The impact of environmental stresses on the expression pattern of ribosomal protein genes is probably due to the adaptability mechanism of plants to withstand changing environments in their sessile form.

1.1.3 Importance of functional genomics research in Rice

According to the 2020 World Population Data Sheet, the global population is expected to grow from 7.8 billion in 2020 to 9.9 billion in 2050 (PRB data sheet, 2020). This level represents an increase of more than 25% from the year 2020. One way to tackle and feed this growing population is through ensuring food security which calls for limited constraints on agricultural production. Under present circumstances, many biotic and abiotic stress factors are deteriorating the global agricultural scenario by negatively affecting plant growth and productivity. Rice is the principal source of carbohydrate

and a staple cereal crop for more than two billion population in Asia and thus accounts for more than 40% of the calorie consumption of most Asians. Also, rice cultivation has been considered as one of the vital agricultural activities and a major source of income in several Asian and African countries. Increasing demand necessarily requires a proportionate increase in the production of rice. The growth in rice production with stability has been a matter of concern to achieve food security, particularly in developing countries Bandumula (2018). Rice production is alarmingly affected by biotic and abiotic stresses. Abiotic stress includes salinity, drought, extreme climates, and biotic stress comprising pest, weeds, bacterial and viral pathogen attack, causing huge impediments towards effortless rice production across the world (Das et al., 2017).

Apart from rice being a staple food crop providing calories for a major section of the world's population, there are various reasons for rice to emerge as a model plant species for genomic characterization. There are several characteristics based on which rice is considered as a model monocot crop for genomic studies. Rice has one of the smallest genome sizes among other crop plants, and its genome is sequenced with high precision. Moreover, high-frequency rice transformation strategies are available. A rich germplasm resource for rice, vast varieties of cultivated rice and its wild relatives have been maintained in the germplasm bank. Hence a large number of genetic stocks are available for conducting research. Lastly, rice genome exhibits similarities with members of other cereal crops like corn and wheat. The knowledge of rice genome sequence is a step forward towards understanding the function of the genome in producing phenotypes and decoding the information hidden in gene sequences, sequences of regulatory elements. One of the most essential purposes of this effort is to characterize genes that control important agronomic traits like productivity, quality, resistances against biotic and abiotic stresses and nutrient-use-efficiency. So far, the development of enabling tools and genetic resources led to deciphering functions of genes that encode several transcription factors, proteins, enzymes, transporter proteins, receptors, kinases, NBS-LRRs, hormone response factors in providing resistance against insects, pests, abiotic stresses, or better phenotypic vigour leading to better yield, productivity and N and P use efficiency (Jiang et al., 2012).

In the present study, we have analyzed the differential expression pattern of ribosomal protein small subunit genes (one from each gene family) under abiotic and biotic stress and also a comparative analysis of both the RPL and RPS genes in rice has been provided Saha et al. (2017). These genes including *RPL6*, *RPL23A*, *RPS6A*, *RPS4*, *RPS13A*, *RPS18A*, etc., have exhibited interestingly high expression patterns under most of the stressed conditions. We have also focused on the salt-tolerant behavior of *RPL6* in independent transgenic plants overexpressing *RPL6*. Although there are no significant reports on the contribution of RP genes in conferring resistance to biotic invaders, in our study, we have noticed several RPL genes got considerably upregulated

in rice plants infected with *Xanthomonas oryzae*, among which *RPL10*, *RPL6*, *RPL11*, *RPL15*, *RPL24a* were significant. *RPL10* and *RPL6* are also activated in shoot samples treated with MeJA and SA (the critical hormones involved in plant defense mechanism under insect and pathogen attack) Moin et al. (2016a). We could demonstrate the upregulation of 60% and 63% of the RPS genes to be upregulated in the rice plants infected with *Xanthomonas oryzae* and *Rhizoctonia solani*, respectively. Among these, *RPS6a*, *RPS9*, *RPS4* and *RPS5* are significant. Also, most of the RPL and RPS genes have biotic stress-responsive elements and their repeats in the respective putative promoter regions, which further suggests their involvement in providing biotic stress tolerance. Based on these findings, we are keen on finding the differential expression patterns of all the RPL and RPS genes in susceptible and resistant rice plants infected with Brown plant hopper and Gall midge in different time points post-infection. In this paper, we report a transcript analysis of the complete RP gene family in susceptible (BPT-5204) and resistant (RPNF05) *indica* rice genotypes challenged by two major insect pests, BPH and GM. We have also identified the candidate RP genes specifically or commonly upregulated in one or both the genotypes Moin et al. (2021b).

1.2 Review of literature

1.2.1 Impact of abiotic and biotic stress on Rice cultivation

One-third of the Asian economy depends on rice production, its marketing and consumption. Asia, being the largest producer of rice, contributes to 91% of global rice production. Among South Asian countries, India ranks second in rice production after China, while India is the fourth-largest rice-consuming nation and the largest exporter. Rice is a staple crop for 70% of the Indian population. Hence it plays a critical role both in the Indian diet and economy. In recent years, there has been a decrease in Rice yield in India due to various reasons. Despite the demand, India's rice yield is lower than that of Bangladesh and Srilanka. Since rice is a predominant crop in South Asian countries, food security is a clear reflection of rice security in this part of the world (Verma, 2017). Therefore, any stress condition can have a direct devastating impact on the economic status of the nation. Although rice is better suited to endure harsh environmental conditions compared to many other crops, there is still a huge need for a better rice yield to meet the demand of the constantly growing global population. Abiotic stress conditions like salinity, drought, high temperature, low temperature, UV radiation can jeopardize rice production and yield.

Salinity is one of the significant perils for crop production globally and it is estimated to impair 20% of all agricultural land and 50% of all irrigated land, lowering agricultural productivity and reducing soil fertility. Salt stress affects 21.5 million

hectares of land in Asia. By the mid-twentieth century, it is estimated that roughly half of the accessible land would have been affected. Although rice is sensitive to salinity, a considerable tolerance level has been noticed in rice under salt stress condition (Ijaz et al., 2019). Despite being susceptible, rice can survive salinity to a certain extent because the waterlogged condition in rice cultivation can dissolve salt molecules and leach them down, thereby decreasing the salt concentration in rice fields. But high salinity levels can lead to decreased dry matter, reduced leaf size, shoot length, inhibition in germination, and delayed flowering, thus negatively impacting yield. Rice is mainly sensitive to salt stress during seedling and reproductive stages (Arif et al., 2019; Lafitte et al., 2004; Ijaz et al., 2019). Comparatively, fewer attempts have been made to examine salt tolerance in rice plants. Recently, many QTLs have been identified related to salt tolerance in rice. Subsequently, screening differentially expressed genes led to identification of several promising potential candidate genes associated with salt tolerance. The salt-tolerant genotypes of rice adapt better mechanisms to tolerate salt stress. The differentially expressed genes in the tolerant genotypes can be grouped in terms of the following categories- a. Genes that are involved in sensing and signalling of salt stress encode for pectinesterase, Ser/Thr phosphatase, chitinase, CIPK. b. Transcriptional regulation- salinity responsive transcription factors (*TIFY*, *MYB*, *HSF*, *HOX*, *WRKY*, *AP2*, and *GRAS* families) play an important role in salt tolerance in rice. c. Ionic and osmotic homeostasis- genes coding for transporters are also identified as promising candidate genes like *HKT1* (Na^+/K^+ transporter), *NCX* (Sodium/calcium exchanger), and *TIP2-1* (aquaporin). 4. ROS scavenging- genes coding for hydrolase, oxidoreductase, and peroxidase are among the differentially expressed genes (Mansuri et al., 2020). In one study by Quan et al. 2018, the team explored the genes in wild rice varieties as potential source for abiotic stress resistance. Candidate genes in the QTL region are identified in wild rice varieties NJ16, DJ15, Koshikikari, and Dongxiang. The candidate genes identified from these wild varieties are *OsSKC1*, *OsHAK6*, *MYB*, Zinc finger, *AP2/ERF*, and protein kinases. The team has introgressed wild rice fragments into a rice cultivar that is severely affected by salt stress. The introgression line obtained from this resulted in better salinity tolerance after rounds of selection (Quan et al., 2018).

Drought stress is one of the most damaging abiotic stresses influencing crop growth, resulting in a sharp decrease in yield. Decreased precipitation and a high rate of evapotranspiration contribute to drought conditions in agricultural land. When an adequate quantity of water is not available for the rice plants to continue their life cycle, the impact of agricultural drought can be observed in rice (Rahman et al., 2019). Rice grown in rainfed lowland where the water supply is mainly rainfall, rice cultivation is often critically impacted by water scarcity. Even in irrigated lowlands, there is a massive shortage of water. Drought stress is caused by a number of reasons including decreas-

ing water supplies, poor water quality owing to pollution, salinization, damaged irrigation systems, and rising demand for water by businesses and urban families (Arif et al., 2019). According to an estimate, 20 million hectares will be affected by insufficient water supply (Bouman, 2007).

Some of the primary impacts of drought on rice crops are decreased rate of cell division and expansion; reduced leaf size; interrupted stem elongation and ramification of roots; and decreased stomatal conductance. Plant water intake capability, nutrient and water usage efficiency are also compromised under drought stress. Rice, like other crops, tends to manage the devastating influence of drought by either of these mechanisms: i) drought escape- plants escape the complications induced by drought by rapidly growing and completing their life cycle, ii) by avoidance, which includes increased intake of water through roots and decreased rate of transpiration, and iii) drought tolerance- where plants regulate growth vigour via adjusting osmotic potential difference. QTL analysis has helped to design strategies towards drought tolerance in rice. Changes in the expression of specific genes when rice is under drought stress have been revealed. These genes include MAP kinases, DREB, CDPKs, translation elongation factors, glutathione reductase, genes associated with aquaporins, signalling kinases, membrane integrity proteins (LEA), carbohydrate metabolism, and so on. Altering the expression of these genes can modulate the response of rice to water deficit or dehydration stress (Arif et al., 2019; Rahman et al., 2019).

A temperature increase that exceeds the tolerance limit can exert heat stress that can impair yield, productivity, growth, pollination, germination, quality of grains, flowering, etc in plants. During reproductive and ripening stages, rice plants are more sensitive to high temperatures. It can also cause spikelet sterility in rice (Rahman et al., 2019). It has been estimated that a 1% increase in temperature in tropical and subtropical countries can cause a 10% decrease in yield. Overall, 41% yield loss is expected by the end of the 21st century due to increasing heat stress. Plant response to heat stress can again be grouped into three classes-i) heat escape- where plants grow faster and complete their life cycle prior to the start of acute stress, ii) heat avoidance- strategies are adopted to minimize the devastation caused by heat stress by stomatal closure, reduced leaf area, senescence of older leaves, increase in growth of root and iii) heat tolerance- plants recover and maintain its function during severe heat stress. (Arif et al., 2019).

Like heat stress, any temperature below the optimum temperature can bring about low-temperature stress. Low-temperature stress can be categorized into- chilling stress and freezing stress. Low temperature can have a drastic influence on structural and functional integrity of the cellular membrane and cytoskeleton structures. It can also severely damage the protein synthesis machineries. The structural damage of the membrane is mainly due to acute dehydration associated with freezing during cold stress. This can greatly impact membrane permeability. Low temperature can negatively influ-

ence both light and dark reactions of photosynthesis and increase accumulation of free radical species which can prove to be detrimental for rice crops (Rahman et al., 2019).

As the ozone layer depletes and the emission of chlorofluorocarbons into the atmosphere increases, all plants become more susceptible to UV radiation, reducing crop yield and productivity. Increased exposure to UVB radiation can negatively influence biological processes as several important biological components like protein, DNA can absorb UVB radiation. UVB radiation can have a destructive influence on rice plants by forming reactive oxygen species (Rahman et al., 2019).

1.2.2 Salt stress in rice

Plants are primarily categorized into two sections on the basis of impact of salinity on growth and development- i) Glycophytes- crop species that are vulnerable to soil salinity and ii) Halophytes- the plants that can thrive in water containing high salt concentration and can endure the same (Tuteja et al., 2011). Salinity seems to impact rice cultivation extensively by affecting plant adaptation and other stress responses. Salinity leads to decrease in dry matter, reduction in leaf size, shoot and root length, number of tillers per plant, spikelet number, etc. If the stress is imposed during pollination and fertilization, it can severely induce sterility by inhibiting germination, causing delayed flowering and seed set, damaged fertility, partial or complete loss of grains, increased percentage of sterile florets production, thus negatively effecting panicle development and yield. Salt stress also interferes with stomatal conductance by modulating stomatal opening, reducing stomatal size and density, which consequently hampers the rate of transpiration and photosynthesis (Reddy et al., 2017).

The region indicating maximum evaporation and low rainfall i.e., arid and semi-arid zones, are most afflicted due to salt accumulation over ages. Salinity occurs naturally near seashores by seawater flooding. Irrigation and land clearing can be pointed out as other artificial reasons for salinity. Higher salt concentration can occur in subsoil either naturally due to accumulation of salt over a long period in arid and semi-arid regions or through secondary salinization by excessive use of manure, compost or inorganic fertilizers and irrigation with brackish water (Reddy et al., 2017).

Salinity causes two types of stresses in plants. Initially, it gives rise to osmotic stress by increasing the osmotic potential of the rhizosphere caused by high salt concentration. Later by imparting ionic stress which is due to toxicity induced by high ionic concentration. NaCl is the most predominant salt; therefore, most plants have well-equipped adaptability to regulate NaCl concentration. It occurs either by selective uptake of other ions like K^+ and HNO_3^- or via exclusion of Na^+ and Cl^- from roots (Ghosh et al., 2016). Resistance to salinity is a polygenic trait. Chloroplast and mitochondria are most affected by ionic stress induced by salinity. The accumulated salt has

an inhibitory effect on photosynthetic efficiency and has a negative impact on vascular bundles. An increased leaf relative water content level is observed in rice plants under salt stress, indicating the presence of osmoprotectants in the cells to protect the cellular machinery from dehydration. One of the ways through which plants tend to adjust the difference in osmotic potential is by accumulating a high concentration of inorganic or organic solutes termed osmolytes. These are compatible molecules, mainly sugar, organic acids, polyols, amides, imino acids, amino acids, proteins, and ammonium compounds observed to be accumulated in higher plants under salt stress. The toxic effect of salinity has been reported to be neutralized by the accumulation of prolines and glycine betaines in rice plants (Ghosh et al., 2016; Jaiwal et al., 1997). The plant's overall defensive response against salinity can be divided into three phases- a) Plants tend to adjust osmotic potential to alleviate the osmotic stress. This aspect is related to the plant's capability to withstand the drought stress induced by salinity and, by doing so controlling leaf expansion and stomatal conductance; b) Plants tend to exclude Na^+ ions from leaf blade by preferential ion uptake mechanism with the help of specific transporters on the cell membrane like H^+ pump and by Na^+/H^+ antiporters, high-affinity K^+ uptake. This helps the plants regulate Na^+ uptake at the molecular level; c) Tissue tolerance- plants tolerate the accumulated Na^+ and Cl^- ion in their tissue by compartmentalizing these ions in vacuoles or other organelles. Plants also transport toxic ions to the older leaves and leaf sheath to protect the younger leaves. Constant transport of toxic ions to older transpiring leaves increases the concentration of Na^+ and Cl^- in these leaves and eventually leads to the death of these leaves. The rate of death of old leaves and survival of new ones is essential to assess the capability of the plant to withstand salinity (Munns and Tester, 2008).

Another way to prevent the toxic effect of salt stress by the plants is to alter or re-configure protein synthesis and accumulation in the cell. Exposure to salt stress often increases protein synthesis, resulting in high concentrations of specific proteins in the cells. Hence protein synthesis is believed to be an important response to control the difference in osmotic potential induced by salinity (Singh et al., 1987). The initial reaction of plants to prevent the harmful effects of salt stress is primarily by their ability to perceive osmotic stress, which can be quantified by the Na^+/K^+ ratio. Since tolerance to salinity is a polygenic trait, several regulatory genes and pathways have been identified to be involved in this purpose, including CDPKs (calcium-dependent protein kinases: *OsCDPK7*); MAPKs (Mapkinases: *OsMAPKs*); SOS pathway (*SOS1*, *SOS2*, *SOS3*) (Xiong and Yang, 2003).

Apart from osmotic and ionic stress, plants also undergo oxidative stress due to inhibition of photosynthesis inflicted by extreme salinity. This is mitigated in plants by the upregulation of genes that code for superoxide dismutase (SODs), peroxidase, catalase, etc that regulate several metabolic pathways (Ghosh et al., 2016). We can

evaluate salt tolerance in plants by quantifying the percentage of biomass production in salt stress conditions compared to the normal state over time. Also, plants show growth vigour under stress, an avoidance mechanism to avoid detrimental effects of salinity stress. Stress signalling molecules like Brassinosteroids play a crucial role in reducing the effect of salinity stress on growth by reinstating the levels of pigments and enhancing nitrate reductase activity. Stress-induced increase in endogenous ABA levels helps close the guard cells, thereby keeping the stomata shut to acclimate the plants under dehydration stress. Jasmonates play a role in modulating ABA-dependent regulation of salinity-responsive genes (Reddy et al., 2017).

1.2.3 Biotic stress in rice

Rice production is alarmingly impacted by biotic stress, which comprises pests, weeds, bacterial and viral pathogen attacks. Major loss in rice production by biotic stressors occurs due to bacterial blight, blast diseases, and insect pests which together account for the loss of about 52% of the total global rice production, out of which 21% is solely attributed to insect pests (Prahallada et al., 2017). Among the insect pests, Asian Rice Gall Midge (*Orseolia oryzae*), which is a severe pest affecting rice production in China, India, Srilanka, and Brown Plant Hopper (*Nilaparvata lugens*), which has been one of the calamitous pests in Vietnam and Asia (Das et al., 2017). The rice brown planthopper (BPH), *Nilaparvata lugens* is a destructive monophagous insect pest that causes direct damage to rice crops by feeding on the plant (phloem) sap, often resulting in hopper burn. The feeding behaviour of BPH includes insertion of the stylet to penetrate the rice tissue, saliva secretion, suction of the phloem sap, and excretion of honeydew. It can also indirectly cause harm to the plants by transmitting viral diseases like rice grassy stunt and ragged stunt. The rice varieties IT64 and MTU 1010 are resistant to BPH to some extent in the adult stage, whereas TN1 is susceptible. In resistant varieties, when phloem sucking is not suitable for feeding, the insects can shift to the xylem, and hence in resistant varieties, xylem feeding is also observed (Sunil et al., 2018). In some resistant varieties, a different proportion of secondary metabolites such as flavonoids can be detected compared to the susceptible lines, which can determine the feeding behavior of *N. lugens*. Moreover, the pest excretes less honeydew when fed on to nitrogen-deficient or resistant rice plants. The amino acid content of the honeydew has been observed to be decreased significantly compared to a susceptible plant. The use of nitrogen fertilisers can increase the supply of free amino acids in the phloem, compromising the plants' capacity to resist disease (Zhu et al., 2017). It became a serious pest in India after 1973, when high-yielding short-duration fertilizers were introduced. The application of pesticides has been the regular practice to control BPH, but pesticides misuse is harmful to the environment and health. In addition to this, the application of pesticides

disrupts the natural control mechanism of the pest by predators and thus intensifying the pest population (Prahalada et al., 2017). In this regard, several efforts have been made to search for genes conferring resistance to BPH. Large-scale screening of rice genotypes under green-house conditions led to the recognition of several genotypes which are less affected by BPH. Some of the resistance is caused by enhanced photosynthesis that compensates for the loss caused by the pest. So far, around thirty genes conferring resistance to BPH have been identified and mapped to six of the 12 chromosomes (2, 3, 4, 6, 11, and 12) of rice. Among those, only 17 genes (*BPH1*, *BPH2*, *BPH6*, *BPH9*, *BPH12*, *BPH14*, *BPH15*, *BPH17*, *BPH18*, *BPH19*, *BPH25*, *BPH26*, *BPH27*, *BPH28*, *BPH29*, *BPH30*, and *BPH32*) have been fine-mapped, and only seven genes (*BPH14*, *BPH17*, *BPH18*, *BPH26*, *BPH29*, *BPH9*, and *BPH32*) have been cloned and characterized. But none of these genes can provide strong resistance against all the biotypes (Prahalada et al., 2017).

Apart from the Brown planthopper, Gall midge (*Orseolia oryzae*) is another major dipteran pest of Rice that has caused havoc in several rice-growing areas of India, South East Asia, and Southern China (Mohan et al., 1994). In India, Gall midge is considered the third most devastating rice pest after stem borers and planthoppers (Bentur et al., 2016). The larvae of this pest feed of the shoot apical meristem of rice plants, causing a tubular leaf sheath gall called silver shoot, and this also interferes with tiller development. The pupae drill a hole on the gall, allowing the emergence of the adult fly (Bentur and Kalode, 1996). Among the other detrimental pests, GM can extensively manipulate the plant defence mechanisms to acquire nutrients from the host plant and reorganize the hormone signalling to exploit host cell morphology leading to the occurrence of galls. The formation of galls can essentially inhibit normal tiller formation causing tremendous economic loss. When fed upon resistant varieties, the larvae enter the apical meristem, but post-hatching, it cannot survive more than 2-4 days because the resistant rice genotypes can react strongly to kill invading maggots within 24-48 hours of initial feeding. This prevents the formation of galls (Bentur et al., 2016).

Eleven genes have been identified against GM, and eight out of these 11 R genes have been tagged and mapped to different chromosomes (*Gm1*, *Gm2*, *gm3*, *Gm4*, *Gm6*, *Gm7*, *Gm8*, and *Gm11*). Candidate genes *Gm3* and *Gm4* have been characterized and validated functionally. Both have been seen to belong to NBS-LRR (Nucleotide-Binding Site-Leucine-Rich Repeat) class of R genes and is highly indicated in resistance of plants against pathogen and insects (Bentur et al., 2016). But none of these 11 genes confers resistance against all the seven biotypes of GM (Divya et al., 2018).

Moreover, the evolution of the virulent biotypes of GM in order to overcome host resistance is of enormous concern. These two biotic invaders can cause significant loss of yield in the major rice-growing nations of China, thus impacting the economic status of these countries to a great extent. Plant's immediate response to minimize the damage

inflicted by insect infection can be categorized into i) Antibiosis- which helps in reducing the chance of insect survival, growth rate, and reproduction after it invades the host tissues, ii) Antixenosis- this reduces the chance of colonization of insects by repelling and disturbing the pests and iii) tolerance- this is related to the ability of the plants to modify its metabolism and functions in a way so that it can maintain high-quality yield despite the insect invasion. Any insect resistance is achieved by either or a combination of these defence mechanisms (Alam and Cohen, 1998). Hence, manipulating the host plant resistance can be instrumental in the effective management of BPH and GM. Discovering novel and broad-spectrum candidate R genes and alleles for overexpression in rice for enhanced resistance to pests is of immediate need in this context.

1.2.4 Role of Ribosomal proteins in mediating stress

The critical functional centres of the ribosome are conserved, whereas the molecular weight and number of components etc., vary in different organisms. Eukaryotic ribosome contains 20-30 more ribosomal proteins than the prokaryotic ribosome, and many components of both eukaryotic and prokaryotic ribosome are primarily conserved. The less conserved proteins and rRNA components of ribosomes render the functional uniqueness of higher organisms (Doudna and Rath, 2002). The essential roles of Ribosomal proteins in the development and physiology of organisms have been pointed out in several studies where the abnormal or reduced level of these proteins has been observed to cause a detrimental effect on those organisms. Distinct developmental defects were observed involving leaf patterning, inflorescence, floral meristem, and seed due to semi-dominant mutation of cytoplasmic ribosomal protein RPL27aC in *Arabidopsis* (Szakonyi and Byrne, 2011). In *Arabidopsis*, *PFL* codes for ribosomal protein S18 and mutation at *PFL* locus results in developmental abnormalities involving reduced fresh weight, pointed first leaves, and stunted growth, indicating the importance of its gene product in meristem activity. The phenotypic change on *PFL* mutation signifies the vital role of the protein in normal meristematic activity, and it has also been confirmed by cloning and complementation that the normal phenotype can be restored from the mutant phenotype. This study indicated the *RPS18A* gene is highly correlated with transcription, and the S18 protein is associated with meristem activity and, to some extent, related to translation efficiency and a number of ribosomes in that tissue (Van Lijsebettens et al., 1994). Ribosomal proteins have been found to play a multifunctional role in sexual plant reproduction as well, where mutations in *S6*, *S11*, *L2*, *L8*, *L23*, *L19*, and *L40* lead to lethal embryo development. This points out the essential role of ribosomal protein in embryogenesis. Another ribosomal protein, RPL18aB, functions at the initial stage of embryo development and orientation of cell division. RPL18aB has been concluded to play a significant role in cell fate determination. The mutation in

RPL18aB leads to hindered growth of pollen grains in style (Yan et al., 2016).

A recessive mutation in one of the genes that code for a cytoplasmic ribosomal protein S10e causes suppression in excessive shoot branching and the formation of lateral shoot organs. Hence this protein is essential in promoting branching in a shoot by enhancing axillary shoot development (Stirnberg et al., 2012). In another study, ribosomal proteins RPL4A, RPL4D, and RPL5A could regulate auxin responses through controlling the translational mechanism of ARF containing 5'-leader sequences in *Arabidopsis* (Rosado et al., 2012). Mutation in several ribosomal proteins manifests into severe developmental error and abnormalities. These mutations must have created overall dysfunction of ribosomal subunits or partial ribosomal damage due to deficiency of specific ribosomal proteins. Mutation in certain genes like *rpl4d*, *rps28b*, *rps6a* showed severe abnormalities in proper leaf development leading to the formation of pointed leaves, reduced growth, abaxialization, and establishment of leaf polarity (Horiguchi et al., 2011). Genes coding for cytoplasmic ribosomal proteins L10a, L9, and L5, play an important role in determining leaf adaxial and abaxial fate during development via their interaction with adaxial HD-ZIPIII gene *REVOLUTA* and abaxial *KANADI* gene (Pinon et al., 2008). T-DNA insertion mutagenesis in one of the RPS genes (*ARS27A*) leads to potent growth inhibition and induced the formation of tumour-like structure in place of auxiliary roots in the presence of methyl methane sulfate (MMS) in the growth medium. This peculiar outgrowth in response to genotoxic MMS was observed in mutant seedlings only. In contrast, the wild-type seedlings exhibited no such tumour-like outgrowth in the presence of a lethal dose of MMS (Revenkova et al., 1999). In *Arabidopsis*, delayed cell division and complete developmental arrest were observed in RPS5 heterozygous and homozygous mutants, respectively (Weijers et al., 2001). When mutated in *Arabidopsis*, a key translation initiation factor, RPL24, led to perturbed gynoecium development, including diminished ovary and lengthened gynophore (Nishimura et al., 2004).

Apart from several instances where RP genes play crucial roles in developmental processes in plants, considerable efforts have been made to focus on their expression pattern in response to several external stimuli. Phytohormones like ABA and cytokinin regulate ribosomal protein genes expression. ABA and cytokinin have an opposite regulatory role in the expression of *RPS14*, *RPS16*, *RPS13a*, and *RPL30*, where ABA repressed and cytokinin induced the expression of these genes at both the transcript and the protein levels (Cherepneva et al., 2003). UV-B light is one of the external stimuli that can severely damage ribosomal functioning. Consequently, in the presence of UV-B radiation, *RPL10* and *RPL10C* expression were induced in maize and *Arabidopsis*, respectively, and expression of *RPL10B* was repressed in *Arabidopsis* (Falcone Ferreyra et al., 2010). Even in *Saccharomyces cerevisiae* expression of ribosomal protein genes is in correlation with the immediate environment. Ribosomal protein genes ex-

hibit high expression under the shift of nutritional state or active growth phase, and conversely, these genes are downregulated under heat stress, starvation, or stationary phase (Warner, 1989).

In a salt-tolerant variety of rice, *RPS4*, 7, 8, 9, 10, 19, 26 and *RPL2*, 5, 18, 44 were among the early responsive genes that were up-regulated under salt stress. Nutrition deficiency also resulted in the differential expression pattern of RP genes in *Arabidopsis*. Comparison of differentially expressed RP genes under Fe and Pi deficiency in *Arabidopsis* roots revealed that both the RPS and RPL genes were differentially expressed under Fe deficiency. In contrast, only RPL genes were differentially expressed under Pi deficiency (Wang et al., 2013). This gives us an idea that differentially expressed RP genes in response to stress leads to differential accumulation of Ribosomal proteins in the ribosomal apparatus, which might be the impetus for ribosome remodelling and selective translation to cope with the nutrition deficiency and other unfavourable conditions. Induction of soybean ribosomal proteins RPS13, RPS6 and RPL37 gene expression as a response to cold treatment gave a clue to their potential role in secondary signalling under low-temperature conditions (Kim et al., 2016). Transcription of Ribosomal proteins has been induced considerably in maize by UV-B radiation. Expression pattern of *L13* from *Brassica napus* and *S13*, *S6*, and *L37* from soybean caused by cold treatment and the reverse happened in the expression of *S7* genes in rye (Casati and Walbot, 2003).

There are also reports of ribosomal proteins contributing to biotic stress tolerance. *L13a* of *Solanum torvum* (eggplant) is highly responsive to a fungal pathogen *Verticillium dahlia* which causes leaf yellowing, curling, and stem wilt in plants. *StoL13a*, when expressed in the potato, which is sensitive to this particular pathogen, showed resistance to infection, thus validating the role of *L13a* in the defence mechanism in plants (Yang et al., 2013). The target of Rapamycin kinase (TOR) is a key regulator which modulates several biological processes in response to external stimuli, and there is a correlation between the TOR complex and the translation of proteins. To investigate the effect of TOR inactivation on the expression of *Arabidopsis* ribosomal protein, it has been observed that there is a powerful influence of TOR inactivation on the expression pattern of nuclear-encoded Ribosomal Proteins in plastids. The main phosphorylation was identified to be a conserved Ser240 residue in a 40S ribosomal protein RPS6. This TOR-dependent C-terminal RPS6 phosphorylation is conserved in plants like other eukaryotes (Dobrenel et al., 2016). A study shows that *Arabidopsis* lacking Glutamate synthase, also termed as Fd-GOGAT1, that does not produce glutamate and some other amino acids shows altered expression of certain genes coding for enzymes involved nitrogen assimilation and amino acid biosynthesis. Apart from these enzymes, there has been a significant upregulation of genes coding for ribosomal proteins. This upregulation indicates that during glutamate deficiency, the plant needed to keep up

with the optimal protein synthesis, and this is achieved by overexpression of ribosomal protein genes (Muñoz-Nortes et al., 2017). In a salinity susceptible pearl millet line (ICMB 081), thirty nine ribosomal-associated genes were downregulated, whereas, in a tolerant line (ICMB 01222), seventeen ribosomal-associated genes were downregulated. The authors concluded that the downregulation of a large number of ribosomal protein genes is one of the reasons for its salinity susceptibility (Shinde et al., 2018). Through activation tagging carried out for enhanced water use efficiency in *indica* rice, our research group could identify some Ribosomal Protein Large Subunit (RPL) genes (*RPL23A* and *RPL6*) to be significantly up-regulated (Moin et al., 2016b). In another study by our research group, the rice ribosomal protein large subunit gene, *OsRPL23A*, is observed to be involved in salinity stress tolerance, as *RPL23A*-overexpressing transgenic rice lines showed a significant increase in fresh weight, root length, proline, and chlorophyll contents under salinity stress (Moin et al., 2017a).

Chapter 2

Materials and Methodology

2.1 Materials

2.1.1 Chemicals

All the chemicals used in the present study were obtained from Clontech, Takara Biotech, Japan; Roche, Germany; Fermentas, Germany; Sigma-Aldrich Corporation, USA; Hi-media Chemicals, Mumbai, India; Invitrogen, USA; SRL India.

2.1.2 Bacterial strains

All the activation tagging binary vectors used in the study were stored as glycerol stocks in *Escherichia coli*, DH5 α strain. The *Agrobacterium tumefaciens* strain, EHA105, was used for rice transformation.

2.1.3 Plasmid DNA vectors

2.1.3.1 pTZ57R (MBI Fermentas, Germany)

The pTZ57R vector was used for cloning PCR amplified products with poly-A overhangs for sequence confirmation of the amplicons. The positive clones were identified based on Ampicillin resistance and blue/white screening.

2.1.3.2 pCAMBIA1300 (CAMBIA, Australia)

pCAMBIA1300 has the *hptII* gene as a plant selection marker.

2.1.3.3 pRT100 (Addgene Vector Database)

pRT100 vector contains CaMV35S promoter and Poly-A tail. It was used for cloning of the genes identified for high water-use efficiency to release the expression cassette.

The expression cassette carrying gene under CaMV35S promoter and Poly-A tail was further cloned in plant binary vector at appropriate cloning sites. The pRT100 contains *ampR* for bacterial selection.

2.2 Methodology

2.2.1 Bacterial growth conditions

The DH5 α of strain *E.coli* was grown at 37°C in LB (Luria Broth) or LA (Luria Agar) medium, while *Agrobacterium* strain, EHA 105, was cultured at 28°C. The LB and LA media required for bacterial growth were obtained from (Himedia, Mumbai, India). The strains with vectors were maintained at -80°C in 35-50% glycerol (v/v).

2.2.2 Restriction endonuclease treatments

Various restriction enzymes were employed in the study for cloning and Southern-blot hybridization analysis. For plasmid digestion, 20 μ l reaction mixture carrying 10X buffer, template, and 5 U of restriction enzyme/ μ g of DNA was incubated at 37°C for 2-3 h. For digestion of genomic DNA, incubation was carried out overnight. The double digestion reactions were carried either separately or using compatible buffers in the same reaction mix.

2.2.3 Retrieval of RPS gene sequences

A list of all ribosomal protein families (both large and small subunits) was obtained using a keyword search, “ribosomal,” in the RGAP-DB and Phytozome v11 databases. From the list, a total of 56 ribosomal small subunit genes (the 40S) were shortlisted by their names, starting with the letter “S” as a prefix (S for Small subunit). Nucleotide sequences of all the 56 genes were retrieved from RGAP-DB. These 56 sequences were further confirmed for their ribosomal origin through nucleotide and protein BLAST search in the NCBI and Hidden Markov Model (HMM) of Pfam databases, respectively. The availability of only 34 ribosomal small subunit proteins, which are coded by 56 genes, indicated the existence of paralogs of the genes coding for more than one RPS protein. For the gene expression analysis, we selected 34 RPS genes, one from each group representing their corresponding paralogous groups. Identical gene copies were, thus, excluded from further analyses. The sequences of RPS genes were retrieved from RGAP-DB (a Nipponbare database). Our analysis of gene expression is performed in an *indica* rice variety (Samba Mahsuri). Hence, we cross-checked the sequence similarity of the coding and the 5'-upstream regions of the genes selected for analysis in the present investigation in both *indica* and *japonica* sequence databases by doing a detailed

BLAST analysis of the *japonica* sequences on *indica* genome databases, OryGenesDB and EnsemblPlants. Further, we have looked for the similarity of rice (40S) RPS genes with RPS genes in *Arabidopsis* and carried out a BLAST search of the Rice sequences in TAIR database.

2.2.4 Structural features and Chromosomal locations of RPS genes

To obtain the genome-wide chromosomal distribution of all RPS genes on 12 chromosomes of rice, we had submitted the locus numbers (obtained from RGAP-DB) of 56 RPS genes as an input to the OryGenesDB. Based on the OryGenesDB outputs, all RPS gene locations were indicated on the chromosome maps, and the number of genes on each chromosome has also been shown. To predict the gene structure features such as intron and exon number, size, etc., we had submitted the genomic and corresponding cDNA sequences of the selected 34 genes to the Gene Structure Display Server (GSDSv2).

2.2.5 Putative Promoter Analysis of RPS genes

Since all the 34 genes exhibited differential expression under multiple stress treatments, we checked for the presence of putative *cis*-elements in the promoter regions of the corresponding genes. To verify this, we have used an *in-silico* approach. The nucleotide sequence of about ≤ 1 kb upstream of each of the 34 RPS genes was retrieved from RGAP-DB. The obtained sequences were cross-checked for their identity/ deviation with the *indica* genome OryGenesDB and EnsemblPlants databases. Further, the sequences that exhibited the highest similarity with *indica* genome in the databases were submitted to PlantCARE (*Cis*-Acting Regulatory Elements) database for identifying the regulatory elements (Rombauts et al., 1999; Dhadi et al., 2009; Ding et al., 2011). We have compared the promoter regions of some of the significant genes in two other databases, such as PlantPAN 2.0 and the JASPAR, and found the functional similarity of the elements with respect to the PlantCare database confirming our observations' authenticity.

2.2.6 Plant growth and Abiotic and Biotic stress treatments

Oryza sativa L. sp. *indica* var. Samba Mahsuri (BPT-5204) seeds were surface sterilized using 70% ethanol for 50 sec followed by washing with 4% sodium hypochlorite for 15-20 min and 5-6 washes with sterile double-distilled water. After this, the seeds were blot dried and placed on solid MS media at $28 \pm 2^\circ\text{C}$ and 16 h light/8 h dark photoperiod cycle (Moin et al., 2016a).

For native expression studies, the surface-sterilized seeds were allowed to germinate on a solid MS medium. Some of the seeds were soaked for 16 h to collect the embryo and endosperm tissues using a sterile surgical blade. Shoot and root apical meristems of 3 d old seedlings and roots and shoots of 7 d old seedlings were collected for the expression studies. After transferring the seedlings from MS medium to soil in the pots; roots, shoots, leaves, root-shoot transition region, flowers, grains, and panicles were collected (Moin et al., 2016a).

For abiotic stress treatments, 7 d old seedlings were exposed to ABA (100 μ M), PEG (10%), NaCl (250 μ M), and H₂O₂ (10 μ M) by dipping them in the respective solutions, and around five seedlings in replicates were collected after 15 min, 3 h, 6 h, 12 h, 24 h, 48 h, and 60 h duration. Roots and shoots were collected separately for RNA isolation and cDNA synthesis. The untreated seedlings in water were used as control samples for normalization in qRT-PCR (Moin et al., 2016a).

To analyze the expression of the RPS genes under biotic stress, rice samples infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Rhizoctonia solani* were collected 20 and 25 d post-infection, respectively. For the biotic stress experiments, one-month-old rice plants were used for treatments. The untreated samples of the same age were used as corresponding controls. The suspension of *Xoo* bacterium was applied at the edge of the leaves using a sterile blade (Moin et al., 2016a), and agar blocks of *Rhizoctonia solani* were placed on the leaf sheaths (Park et al., 2008) as per the standard protocols and maintained under controlled culture room conditions.

2.2.7 Total RNA isolation

Total RNA was extracted from the tissue samples using TriReagent (Takara Bio, UK) following the manufacturer's protocol. The RNA pellet was suspended in nuclease-free water, and all the RNA isolation procedures were conducted at 4°C. The quality of RNA was verified by running the samples on 1% agarose gel in TBE (Tris-borate-EDTA) buffer and quantity was checked by using Nanospectrophotometer. The first-strand cDNA was synthesized using reverse transcriptase enzyme (Takara Bio, UK) from 2g of total RNA.

All the samples were collected as three biological and technical replicates, and qRT-PCR was performed with their respective control samples. Rice specific act1 and β -tub genes were used as internal reference genes for normalization, and the mean values of relative fold change were calculated as per $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). For native expression studies, the normalization was performed with only rice-specific act1 and β -tub genes (ΔC_T). Bar diagrams and heat maps were generated by Sigmaplot V11 and Morpheus programs, respectively using the means of the fold change that were calculated from the qRT-PCR data.

2.2.8 DNA and RNA quantification

The quality and quantity of DNA and RNA were analyzed in a Nano-drop spectrophotometer (ND-1000, USA). For Southern-blot hybridization, DNA free from protein and salt contamination with values 1.8 and 2.1 at OD_{260/280} and OD_{260/230}, respectively, were selected at a concentration of 10-15 µg. Similarly, the values ranging within 1.9-2.1 were selected for RNA as per the standard protocols.

2.2.9 Synthesis of cDNA first strand

According to the manufacturer's protocol, total RNA (2 µg) was used to synthesize the first strand cDNA using MMLV reverse transcriptase (Takara Bio, UK). The cDNA was diluted in 1:7 proportions and 2 µl of it was used in qRT-PCR.

2.2.10 Quantitative real-time PCR (qRT-PCR)

The synthesized cDNA was diluted ten times, and 2 µl of it was used to perform qRT-PCR, and 10 µM of the primers for each gene were used per reaction. The sequences of forward and reverse primers are listed in Table 2.1 and 2.2. The amplification protocol included an initial denaturation at 94°C for 2 min, with 40 cycles of 94°C for 30 sec, primer specific annealing temperature for 25 sec, an extension of 72°C for 30 sec. This was followed by constructing a melt curve to estimate each gene's amplification efficiency. The fold change was calculated as per $\Delta\Delta C_T$ method, and the mean of these values was considered as the final fold change of the transcript levels (Moin et al., 2016a). Means of relative fold change were plotted in SigmaPlot V11 with standard error to produce the bar diagrams for all the genes in a specific stress.

2.2.11 Designing over-expression cassette of *RPL6* and transformation in Rice

The full-length cDNA sequence of the *RPL6* gene that got considerably over-expressed in activation tagged mutant line in our previous study was obtained from the RGAP-DB. The sequence was then confirmed through nucleotide and protein BLAST searches in NCBI, Hidden Markov Model (HMM) of Pfam databases. For the cloning process, the entire expression cassette of *RPL6* and promoter and poly A tail as a *Pst*I fragment was cloned into a T-DNA of binary vector, pCAMBIA 1300. This binary vector carrying the overexpression cassette of *RPL6* was mobilized into *Agrobacterium tumefaciens* strain EHA105 for genetic transformation in rice. Overexpression cassette of *RPL6* is shown in Figure 2.1.



Figure 2.1: Overexpression cassette of *RPL6*

The genetic transformation was done in BPT-5204 of *indica* rice variety. The 35S:*RPL6* cassette was transformed in rice using *in planta* transformation as reported in Moin et al., 2016b. After the *in planta* transformation, the transformed seeds were transferred to the soil under controlled greenhouse conditions ($30 \pm 2^\circ\text{C}$ with 16/8 h of light/ dark photoperiod), and we got T_0 lines. The cloning and subsequent generation of T_0 putative transgenic lines were already available in our laboratory. This part of the thesis work was continued using this material.

2.2.12 Genomic DNA isolation

Genomic DNA of the transgenic plants was isolated following CTAB method. At first, 150 mg of leaf sample was ground to a fine powder in clean autoclaved mortar and pestle with the help of liquid nitrogen. The powder was immediately transferred to 1 ml of CTAB buffer + 20 μl - mercapto-ethanol and the vials were incubated at 65°C . After one hour of incubation, the samples were centrifuged at 11,000 rpm for 15 min, and the supernatant obtained was transferred to a fresh vial and an equal proportion of phenol-chloroform : Isoamyl alcohol (25:24:1) was added and mixed. These vials were incubated at 4°C for 5 min followed by centrifugation at 5000 rpm for 8 min. An equal volume of chloroform: Isoamyl alcohol (24:1) was then added to the supernatant, which was further incubated at 4°C for 15 min with gentle shaking followed by centrifugation at 12000 rpm for 12 min. This step was repeated twice, and the upper clear liquid phase was separated out into a fresh vial to which an equal volume of iso-propanol was added and incubated for 8-12 h at -20°C . Genomic DNA was pelleted down, washed with 70% ethanol, air-dried, and dissolved in 100 μl nuclease free water.

2.2.13 Selection of T_1 generation of transgenic plants

The plants grown from each of the *Agrobacterium tumefaciens* transformed seeds are regarded as individual lines because of the independent integration of T-DNA. The expression cassette carried the *hptII* gene for plant selection. After T_0 plants are grown, T_1 seeds are subsequently harvested and grown on MS selection medium containing the antibiotic Hygromycin (50 mg l^{-1}). The seedlings that germinated on the selection medium containing the antibiotic were transferred to pots in the greenhouse. These

putative transgenic plants were further confirmed by PCR amplification with primers specific to full length *hptII* cDNA and forward and reverse ends of 35S promoter sequence. The transgenic nature and copy number of T-DNA integration were further verified by Southern-Blot hybridisation.

2.2.14 Polymerase Chain reaction

PCR amplification was performed for the confirmation of the transgenic plants. Primers specific for *hptII* gene with forward and reverse ends of 35S promoter sequence in the T-DNA were designed using OligoCalc and Primer3 tools. The final volumes of 25 μ l of PCR reaction contained 20-25 pmol/U forward and reverse primers, 100-150 mg of genomic DNA or appropriate template, 200 μ M of dNTP mix, 1.5 mM of $MgCl_2$, and 2.5 U of recombinant Taq DNA polymerase (Invitrogen, USA). PCR was performed in Eppendorf Master Cycler Gradient (Germany) and/or Bio-Rad Laboratories Inc., (USA). The reaction conditions for PCR included an initial denaturation at 94°C for 3-5 min followed by 35 repeated cycles of 94°C for 30-60 sec, an appropriate annealing temperature ranging 56°C -61.9°C for 50 sec, extension at 72°C for 1 min. This was followed by a final extension of 72°C for 10 min. The PCR products were then visualized on 1% agarose gel under a UV trans-illuminator. The sequence of primers were listed in Table 2.1, 2.2, 2.3.

2.2.15 Southern-Blot Hybridisation

To confirm the transgenic nature and determine the copy number of T-DNA integration in rice transgenic plants, T_3 generation PCR positive plants were analysed by Southern-Blot Hybridisation using the approach of DIG-DNA labelling and detection kit (Roche Life-Sciences, Germany). Genomic DNA (15-20 μ g) was isolated from the rice leaves, and the DNA samples were subjected to overnight digestion by restriction enzyme *Bam*HI (the enzymes that were chosen for Southern Blot did not contain any site within the T-DNA region) at 37°C. The completely digested DNA fragments were separated on 0.8% agarose gel by electrophoresis at 20V overnight. The gel was then subjected to alkali (NaOH) denaturation and transferred to Hybond N⁺ nylon membrane (GE Healthcare Life Sciences), which was followed by UV cross-linking (120KJ/cm²). The PCR amplified product of the *hptII* gene using Klenow polymerase was labelled with DIG-UTP and used as a probe. After hybridisation and stringent washing steps, the binding of the probe was detected using anti-DIG-alkaline phosphatase enzyme and NBT/BCIP substrate according to the manufacturer's protocol (Roche Life Sciences, Germany).

2.2.16 Semi-quantitative (Semi-Q) and Quantitative real time PCR (qRT-PCR)

Semi-Q and qRT-PCR analyses were performed on T₃ generation of *RPL6* over-expression rice transgenic plants to separate low, medium, and high expression lines of *RPL6*. Total RNA was isolated using the standard Trizol method (Sigma-Aldrich, US) from the root and shoot tissues of two-week old seedlings of negative control and 35S:*L6* transgenic lines before and after treatment with different concentrations of NaCl. About 2 µg of total RNA was used for first-strand cDNA synthesis using SMARTTM MMLV Reverse Transcriptase (Takara Bio, Clontech, USA). The synthesized cDNA was diluted seven times (1:7), and 2 µl of this dilution was used for analysing the transcript level of *RPL6* gene. Semi-Q PCR was performed using primers specific to *OsRPL6* gene. The conditions for semi-Q PCR included initial denaturation at 94°C for 30 sec, 56°C for 25 sec, and 72°C for 30 sec with a final extension for 5 min at 72°C. The *OsActin* was used as an endogenous reference gene in both Semi-Q and qRT-PCR analyses. Based on the band intensity observed on the 1.5% agarose gel after semi-Q PCR, the transgenic lines are categorized as low and high expression lines. This was further confirmed with qRT-PCR. Similar methods were used to isolate total RNA and prepare cDNA first strand. The 1:7 dilution of the synthesized cDNA was used to analyse *RPL6* transcript level. Primers specific to *RPL6* gene were used to perform semi-Q PCR and qRT-PCR analyses. Rice specific actin, *act1* was used as an endogenous reference gene in both semi-Q and qRT-PCR analyses.

The qRT-PCR reaction conditions included an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 56°C for 25 sec, and 72°C for 45 sec. The last step was followed by a melting curve. The qRT-PCR data was analysed according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

2.2.17 Plant growth and salt stress treatment

Based on the semi-Q and qRT-PCR analyses, T₃ seedlings were selected taken into consideration three low and three high expression lines for subjecting them to salt stress screening and comparison with respect to negative control (NC). The seeds of NC and the transgenic lines were grown on solid MS medium for 14 days. The seeds were germinated in replicates of five. After 14 days on solid MS medium, the seedlings were shifted to liquid MS medium prepared with two different NaCl concentrations, 150 mM and 200 mM. NaCl treatment for each concentration was applied for up to 5 days. Significant differences in visible phenotype were not observed after 3 days in 150 mM concentration. Hence, several phenotypic parameters were checked after 5 days for 150 mM salt stress and after both 3 and 5 days for 200 mM salt stress. After the treatments,

the seedlings (both transgenic and negative controls) were allowed to revive by shifting them to plain liquid MS medium devoid of salt (NaCl).

2.2.18 Phenotypic parameters of the transgenic line post stress

Two week old seedlings both untreated and under salt stress were measured for several phenotypic parameters. These parameters were compared with their corresponding NC under the same treatment conditions. The root length, shoot length, and fresh weight were measured after treatment with 150 mM and 200 mM salt stress at different time points. Each time, the measurements of the transgenic lines were compared with the corresponding negative controls. Similar parameters were also recorded after the seedlings were allowed to revive and grow in salt-free liquid MS media after 3 and 5 days of exposure to 150 mM and 200 mM salt stress. Each reading was recorded in biological replicates of three, and the measurements were plotted as bar diagrams.

2.2.19 Measurement of Chlorophyll fluorescence and Photosystem II

The fluorescence of chlorophyll (Chl) was detected using a small pulse-amplitude modulated photosynthesis yield analyzer (MINI-PAM) based on the manufacturer's instructions (Heinz Walz, Germany). The Chl fluorescence is a key indicator of the overall photosynthetic performance of a plant, which also indicates whether a plant is experiencing stress (Murchie and Lawson, 2013). It also elucidates how photosystem II (PSII) utilises excitation energy. Chlorophyll is triggered to a minimum level (F_o) when leaves that have been acclimated to a brief dark period are subjected to a beam of low intensity light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$). The use of a saturating pulse ($8000 \mu\text{mol m}^{-2} \text{s}^{-1}$) leads in the creation of the highest feasible fluorescence yield (F_m). Variability in fluorescence (F_v) is computed as the difference between F_m and F_o , and the functional quantum yield of PSII equals F_v/F_m (Murchie and Lawson, 2013). Plants growing under normal circumstances with no stress have a quantum efficiency of 0.83-0.84. Plants with lower F_v/F_m levels are stressed, whereas those with higher values have a higher quantum yield (Batra et al., 2014).

The measurements were recorded in dark acclimated leaves of NC and lines of 35S:L6 plants (T_3) under unstressed conditions and after revival from the application of 200 mM NaCl stress for 5 days. All readings were recorded in three replicates, and the mean of F_v/F_m observations were plotted as a histogram.

2.2.20 Estimation of chlorophyll content in the transgenic lines post stress

Using 100 mg of leaf tissue in 80% acetone, the chlorophyll was extracted as stated by Zhang et al. (2009). An UV spectrophotometer was used to detect the extract's absorbance at OD₆₆₃ and OD₆₄₅ nm using an UV spectrophotometer. The concentrations of Chl-a, Chl-b, and total chlorophyll were then calculated using the equation as described earlier (Arnon, 1949).

The chlorophyll content was measured in untreated healthy plants and over-expression line, which were revived after 5 days of exposure under 150 mM and 200 mM salt stress.

2.2.21 Proline estimation in RPL6 overexpression lines under salt stress

Proline estimation was carried out following a method described by Bates et al. (1973). About 100 mg of seedlings were homogenized in 5 ml of 3% Sulfo-salicylic acid. The homogenate was then centrifuged at 12000 rpm for 15 min, and the 400 µl of the upper aqueous phase was extracted and separated into a fresh 2 ml tube. To this supernatant, 400 µl of acid ninhydrin and 400 µl of glacial acetic acid were added and kept in a hot water bath (100°C) for 1 hour. Acid ninhydrin was prepared in 6N orthophosphoric acid. The tubes were placed on ice after 1 hour of incubation to halt the process. 0.8 mL toluene was used to extract the reaction mixture. Finally, using toluene as a blank, absorbance of the samples was taken in a spectrophotometer at 520 nm wavelength. A standard curve was constructed using proline dilution, and proline content in samples was determined using the standard curve as stated by Bates et al. (1973).

The proline content was measured in untreated healthy plants and over-expression lines, which were revived after 5 days of exposure under 150 mM and 200 mM salt stress.

2.2.22 Transcript analysis of stress-responsive genes in transgenic lines

Since we have observed better phenotypic characters of transgenic plants overexpressing *RPL6* under medium and high salt stress (150 mM and 200 mM), we examined the expression level of some selected stress responsive genes (*OsALDH2a*, *OsAOX1a*, *OsZIP23*, *OsLEA3-1*, *OsWRKY72*, *OsDREB2B*, *OsSOS1*) based on the previous published reports, which stated that these stress responsive genes were up-regulated during abiotic stress conditions, notably in salinity stress.

We have checked the transcript levels of these genes in the transgenic lines and

NC under untreated condition and after collecting samples post 3 days exposure to 200 mM concentration. The samples were collected from both the shoot and root of the seedlings. Total RNA was isolated, cDNA first strand was synthesized following the protocol mentioned earlier. The qRT-PCR with primers specific to genes (*OsALDH2a*, *OsAOX1a*, *OsZIP23*, *OsLEA3-1*, *OsWRKY72*, *OsDREB2B*, *OsSOS1*) was performed, and the values were plotted as bar diagrams following protocol discussed before. The list of primer sequences specific to these genes was mentioned in Table 2.3.

2.2.23 Plant growth and infestation with Insect pests

BPT-5204 and RPNF05 were the two rice genotypes that have been used for this part of the experiment. RPNF05 is moderate to highly resistant against BPH (Brown Plant Hopper), and GM (Gall Midge) pests, and this genotype of rice was collected from the Agri Biotech Foundation, Hyderabad, India. BPT-5204 is susceptible to both BPH and GM, and this variety was obtained from the Indian Institute of Rice Research, Hyderabad, India. RPNF05 was used as a positive control. It is a derivative of BPT-5204, which is acquired by systemic introgression of bacterial blight (*Xa21*, *xa13*, and *xa5*), fungal blast (*Pi54*), and GM resistance genes (*Gm1*, *Gm3*, *Gm4*, and *Gm8*). The plant samples were grown (BPT-5204 and RPNF05) and subsequently infested with BPH and GM following the protocol reported by (Divya et al., 2018). The rice seeds of both the genotypes were sown in plastic trays (60 x 40 x 7 cm), and plants were allowed to grow under controlled greenhouse conditions ($30 \pm 2^\circ\text{C}$, 16 h light/ 8 h dark photoperiods). Three weeks (25 days) old plants were challenged with Gall midge belonging to gall midge biotype 1 (GMB1) for 24-48 h. After that, plants were shifted to a humidity chamber for the next 48 h to enhance the hatching of eggs. Post 120 h, some of the plants were observed to have maggots at the apical meristematic region through plant dissection, which validated the successful infestation. Stem samples of 2 cm length above the soil level were cut at 24 and 120 h after infestation (hai) and stored in liquid nitrogen for subsequent RNA isolation. Sample collected from healthy uninfected plants of a similar age under other similar growth conditions were used as controls for double normalization. Similarly, 25 days old plants were infected with BPH nymphs, and lower sheath samples were collected at 6 and 12 hai. All the samples were collected in biological and technical triplicates for transcript analysis. The procedure for plant growth is depicted in Figure 2.2.

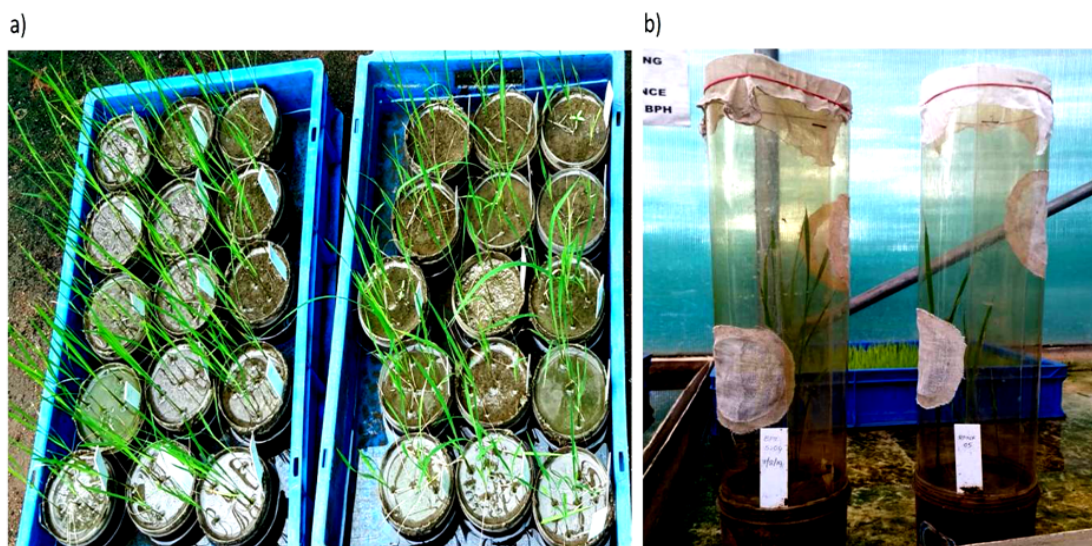


Figure 2.2: Growth of BPT-5204 and RPNF05 seedlings and infestation with BPH pest
 a) Rice seeds of BPT-5204 and RPNF05 were allowed to germinate in plastic trays and plants were raised under controlled greenhouse conditions ($30 \pm 2^\circ\text{C}$ with 16/8 h of light/ dark photoperiod).
 b) Infestation of three-week old plants with BPH nymphs.

2.2.24 Transcript analysis of RPL and RPS genes in BPT5204 and RPNF05 under BPH and GM infestation

Total RNA was isolated from shoot samples of infested and uninfested BPT-5204 and RPNF05 plants using the Trizol (Sigma-Aldrich, US) method. About $2\ \mu\text{g}$ of total RNA was used to synthesize the first-strand cDNA with reverse transcriptase (Takara Bio, Clontech, USA). The cDNA was diluted in 1:7 ratio with sterile Milli-Q water, and $2\ \mu\text{l}$ of this diluted cDNA was used for analyzing the transcript levels of RPL and RPS genes in the two genotypes. The rice-specific RPL and RPS primers, designed through the primer-3 tool was used in qRT-PCR. The qRT-PCR was performed using SYBR Green R Premix (Takara Bio, USA). The qRT-PCR cyclic conditions included an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, an annealing temperature specific to each RP gene for 25 s, and an amplicon extension step at 72°C for 30s. This was followed by a melting curve step to analyze the specificity of each amplicon. The qRT-PCR was performed as three technical and biological repeats and the fold change was calculated using the standard $\Delta\Delta\text{C}_\text{T}$ double normalization method.

The fold change in each BPH and GM infested samples were normalized with the corresponding uninfected samples using two reference genes, *act-1* and *β -tub* that are specific to rice. The mean fold change of each replicate obtained after normalization with these two genes was considered as the final fold change. In this study, genotype and the infestation time were the major experimental factors. A two-way analysis of variance (ANOVA) was employed for each gene on all replicates to look for effects

of genotype and infestation time, and their interactions. Tukey's pairwise comparisons were used to calculate the statistical significance. Probability, $P < 0.05$, was considered as statistically significant. SigmaPlot v.11 and R program v.3.6.3 were used for statistical analyses. The P values obtained from two-way ANOVA were adjusted with a Bonferroni correction in Microsoft excel v.2013 following the instructions available on the web tool.

Table 2.1: List of RPL gene primer sequences in 5'-3' direction

Primer Name	Sequence	Primer Name	Sequence
RPL genes (qRT-PCR analysis)		RPL genes (qRT-PCR analysis)	
L3 RT FP	TGGACTTGTGGCCTATGTGA	L22 RT FP	GAGGTGAAAGGTCTGGATGTT
L3 RT RP	CCGGCATCGCTATCATACTT	L22 RT RP	TCACTGGTTCCTCTCTCTG
L4 RT FP	AAGAAGCTCGACGAGGTGTA	L23A RT FP	GACCAAAGACCCTGAAGAAGG
L4 RT RP	CCACATTCTTCAGAGGGTTC	L23A RT RP	ACGATGAAGACAAGGGTGTG
L5 RT FP	GATCTTGGCATCAAGTACGAC	L24b RT FP	GTTGGTGCTACACTGGAAGTT
L5 RT RP	GACACCCTCATACTTGACCTG	L24b RT RP	CCTTCGACTGTGTCTTCTGAG
L6 RT FP	GTTCTCAAGCAGCTCAAAT	L26.1 RT FP	ACAAGTACAACGTGGTGAGG
L6 RT RP	CTTCTGCTTCTGTCCCTAGA	L26.1 RT RP	GTCCTTGTGCGAGCTTGAGTT
L7 RT FP	TACCCAAACCTGAAGAGTGTC	L27.3 RT FP	CTTCCTCAAGCTCGTCAACT
L7 RT RP	GACAGTCATGATCTCGTGGA	L27.3 RT RP	CTTGGTGAAGAACCACCTGT
L8 RT FP	ACTACGCCATCGTCATCAG	L28 RT FP	TAGACGAATACCTCTGAAGA
L8 RT RP	GGTACTTGTGGTAGGCGTTT	L28 RT RP	AAACCCTGTTGATCTTAGTC
L10 RT FP	AGAAGAAGCCTGGATTAGAGC	L29 RT FP	CCCAACAAGCTCTCCAATATA
L10 RT RP	ATATCCTGCTGGAGGACTTG	L29 RT RP	AGAAACAGAAGCATTCCCTG
L11 RT FP	AAGAAGATCGGTGAGGACATC	L30e RT FP	GAGCAAGAAGAAGAACAAGTC
L11 RT RP	TCTTGACCTTCTTCTGTCC	L30e RT RP	GCTTCATCCATATCTTTCCG
L12 RT FP	GCTCATTGTACAGCACAGAG	L31 RT FP	TCAAGGAGATCAGGAAGTTTG
L12 RT RP	TTGGTTCAGTCTGAGAAGGAG	L31 RT RP	AACAGTGACCAGAGAGTAGAG
L13a RT FP	GAACTACCACGACACCATCAG	L32 RT FP	GCCTAATATTGGCTATGGTTC
L13a RT RP	GGGGCCAAAATATCTATCTG	L32 RT RP	CTTCTTCGTTGAGACATTGTG
L13b RT FP	AAGCACTGGCAGAAGTATGTC	L34 RT FP	GAAGAAGATCCAGGAATTCC
L13b RT RP	CCCTCGACTTCATGTTGTACT	L34 RT RP	CACAATCTTCTGCTCTTCAAC
L14 RT FP	GTGAAGTACGGCAAGGACTAC	L35a.3 RT FP	CTACGTCTACAAGGCCAAG
L14 RT RP	TAACATCAGCCTCCTCCATAG	L35a.3 RT RP	TGCTGGGGTACATGAAGA
L15 RT FP	ACAAGTACGTGTCGGAGCTAT	L36.2 RT FP	GGAAAAGTACCAAGAGAGTGA
L15 RT RP	GACACGGTAAACCACATAACC	L36.2 RT RP	CTTCTTCTTGTCTCTTGTG
L18a RT FP	TCCAAGTTCTGGTACTTCCTG	L37 RT FP	CTTCCACCTGCAGAAGAG
L18a RT RP	GTTGTGGTAACCTGTTCTGCT	L37 RT RP	CCCCTCTCTGAAGTTACTCT
L18p RT FP	TGGGGAGGACTACTATGTTGA	L38 RT FP	CACGAGATCAAGGACTTCC
L18p RT RP	AAACCTCTGTCACTGTGAGG	L38 RT RP	AAAGGTGGATGAAATGTAGGC
L19.3 RT FP	AGTATCGTGAGGCCAAGAAG	L44 RT FP	AAGAAGACCTACTGCAAGAAC
L19.3 RT RP	CTTAGCCTCAAACCTGGTCAGA	L44 RT RP	CCTTACCCTTCTTGTACTGAG
L21.2 RT FP	CTGAGGAAGATCAAGAACGAC	L51 RT FP	GTGACAGAGTTAGTCCGTGGA
L21.2 RT RP	AACCACCCTTGAGATCATTG	L51 RT RP	TCTCAGCTTACCACCTTCCT

Table 2.2: List of RPS gene primer sequences in 5′- 3′ direction

Primer Name	Sequence	Primer Name	Sequence
RPS genes (qRT-PCR analysis)		RPS genes (qRT-PCR analysis)	
S3a RT FP	TGAAGCCCATGTGGATGTTA	S17 RT FP	AACAAGAAGGTGCTGGAGGA
S3a RT RP	TCACATGTTGATGCCTGGTT	S17 RT RP	TCCTGGAGCTTGAGGGAGAT
S4 RT FP	CAGGTTGAAGTATGCGCTGA	S18 RT FP	CGACATCGACATGAACAAGC
S4 RT RP	AGCGACCCCTGGTGTATAG	S18 RT RP	TCAGCCTCTCAAGGTCATCC
S4a RT FP	AGCGGCATGTTATGGTTGAT	S18a RT FP	CTCACCTCCATCAAGGGTGT
S4a RT RP	GGACCTTGCAGAGCTTGAAC	S18a RT RP	ACCTCCCGTCCTTGTAGTCC
S5 RT FP	ATATCCCTCGCCGACTACCT	S18b RT FP	AAGGATGGGAGGTTCTCTCAG
S5 RT RP	TGATCTTCTTGCCGTTGTTG	S18b RT RP	TCTTGAGACACCGACAGTCT
S5a RT FP	CAACGGGAAGAAGATCATGG	S19 RT FP	CAAGATGGAGCTCCCTGAGT
S5a RT RP	GACCCTCCTCAAGGGAGAGA	S19 RT RP	GGACGTGAGCCATTCTCT
S6 RT FP	GCTACGGCATCAAGAAGCTC	S21 RT FP	TCAGATGGTGGACCTCTACG
S6 RT RP	CAAGTGCCGTGTCAAAAGT	S21 RT RP	AGAGCACTGTCGGCGTCT
S6a RT FP	CATGAAGCAGGGTGTGCTTA	S23 RT FP	GAGCCATCTTGGCAATGAAT
S6a RT RP	TCAGTCAAGCCAGGAAGGTC	S23 RT RP	CGATGAAGTTCAAGCAACCA
S7 RT FP	TGTGCAAGGCATTCAGAAA	S23a RT FP	TTTTGGGAGAAAAAGGCTTG
S7 RT RP	TCCAAGATTCCATCATGAACAG	S23a RT RP	CCTCTGCTTCTTCTCTCTTG
S7a RT FP	AGAAGAAGTTCAGCGCAAG	S24 RT FP	GTCTCCAAGGCTGAGCTGAA
S7a RT RP	GCACCATCCAGACGGTATCT	S24 RT RP	CTTGGGCTCGTACTTCTTCG
S9 RT FP	TTATCAGGCAGCACCATC	S25 RT FP	GGGAAAGCAAAAGGAGAAGG
S9 RT RP	GGCCTTCTGGTTCTTCTCT	S25 RT RP	GATTAGGCCCCGTGTCATTA
S9-2 RT FP	GAGCTGTGGCGTGTTCAGTA	S25a RT FP	CGGAGGCAAGCAGAAGAAG
S9-2 RT RP	AACAGTGAGGGCAAGGACAT	S25a RT RP	CAAGATCCTTGATGGCCTGT
S10 RT FP	CCCTCAAGAAGCTGCCAAG	S26 RT FP	CAAGGCGATCAAGAGGTTTC
S10 RT RP	ACCAAAATCACCTGGAGCAC	S26 RT RP	GACGATGTGAGCATGGATTG
S10a RT FP	GCATTGAGCACCTGAGGAAT	S27 RT FP	CGGAGCTGGAGAAGCTCA
S10a RT RP	AACCTTGGTCTGTCCCTTC	S27 RT RP	CTGGCCTTCCCACCAGTAG
S13 RT FP	AGGTGGAGGAGATGATCGTG	S27a RT FP	CGAAGATCCAGGACAAGGAG
S13 RT RP	GACGGCCTTCTTGATGAGG	S27a RT RP	CTTGGGCTTGGTGTACGTCT
S13a RT FP	GTGGTGCTCCGTGACCAG	S28 RT FP	GGATACCCAGGTCAAACCTGC
S13a RT RP	TGGAGTCCTTGTCCTTCCTG	S28 RT RP	CTGGCCTCCCTCTCAGACT
S15 RT FP	ATGACCTCGTCCAGCTCTTC	S29 RT FP	CACTCCAACGTGTGGAATC
S15 RT RP	CCGATCATCTCAGGGACAAT	S29 RT RP	CGGTACTTGATGAAGCCAATG
S15a RT FP	CCGACCCTCATCAAAGGTTA	S30 RT FP	GAAGGTGAGGGGGCAGAC
S15a RT RP	TCCTTGACACCAACATCGAA	S30 RT RP	GACGAAACGGCGGTTGTACT

Table 2.3: List of Primer sequences for other genes

Primer Name	Sequence
Stress responsive genes	
OsLEA3-1 RT RP	GCGAGTGAGCAGGTGAAGA
OsLEA3-1 RT RP	GTGGCAGAGGTGTCCTTGTT
OsAOX1a RT FP	GATGTTTGTCTACTGCCGAGGATTT
OsAOX1a RT RP	ATGTTAGTATATATAACTCAGCTGCC
OsDREB2B RT FP	ATCCACAGGGTCCAAAGAAG
OsDREB2B RT RP	CACACCACGGAAGTCACAAC
Oszip23 RT FP	CACATCCCACCTCTCCTCAG
Oszip23 RT RP	CTCACCCAACCAACCAATC
OsWRKY72 RT FP	CGAGAAATCCAACGACAACCTT
OsWRKY72 RT RP	GCTGAAGGGAAGAGAGGTGAG
OsAldh2a RT FP	TAGCTGTAGTAATCGATC
OsAldh2a RT RP	TGTACAAAAGATTGCCCG
OsSOS1 RT FP	AAGAGGACGAGGACAACCTC
OsSOS1 RT RP	TTGGCTGGTCCAACAATTAC
Screening of Transgenic plants	
CaMV35S FP	AAGCTTGATCCCCAACATGGTGGAGC
CaMV35S RP	TTCGGATCTAGATATCACATCAATC
hptII FP	TATTTCTTTGCCCTCGGACGAGTGCTG
hptII RP	ATGAAAAAGCCTGAACTCACCGCG
Actin-1 FP	CTCCCCATGCTATCCTTCG
Actin-1 RP	TGAATGAGTAACACGCTCCG
Tub6 FP	ACCACTCCTAGCTTTGGTGATCTG
Tub6 RP	AGGTTCACTGCGAGCTTCCTCA

Chapter 3

RPS genes identified to have Stress responsive roles

3.1 Introduction

Discerning the function of plant genes is mainly achieved by raising mutant populations. Mutants are generated by using chemical agents, radiations, and T-DNA/ Transposable elements that disrupt the function of genes in the genome. Disrupting the function of genes often gives rise to loss-of-function mutagenesis, which is not always desirable as it does not allow us to understand the role of redundant genes. Thus, gain-of-function mutagenesis techniques are evolving as an efficient substitute to loss-of-function mutagenesis (Moin et al., 2016b). In gain-of-function mutagenesis technique, transcriptional enhancers, generally derived from 35S gene of the Cauliflower Mosaic Virus, are randomly introduced into the plant genome. This enhancer integration can randomly activate genes when inserted at their neighbouring sites. In this strategy, the problem of redundant genes getting masked will be solved (Moin et al., 2017b; Jeong et al., 2002). One of the most efficient gain-of-function mutagenesis strategies is Activation tagging, in which a T-DNA/ transposable element carrying an enhancer system can activate any gene randomly when integrated into its genome, which is situated as far as 10 kb on either side of the integration site. Hence, the expression level of generally one of the genes in the corresponding region is induced several times automatically than its native level of expression. However, activation tagging requires the development of a huge number of transgenic plants in which this enhancer system has been integrated at random sites in the genome. The genome should be well saturated with this sort of integration to elucidate the functions of as many genes as possible. This population is a gold mine, and it can then be screened for several agronomically important traits with suitable selection screens (Moin et al., 2017b; Jeong et al., 2002; Ayliffe and Pryor, 2007).

In our previous studies, we have developed a large-scale activation tagged mutant population in a widely used *indica* variety of rice, BPT-5204 (known as Samba Mahsuri) (Moin et al., 2016b). Several thousand enhancer-based-activation tagged plants and stable *Ds*-enhancer plants were raised. Moreover, around 3000 mutants were screened for water-use efficiency by analyzing traits like photosynthetic quantum efficiency and other traits related to yield like tillering, plant height, panicle number, number of productive panicles, and seed yield under water limiting conditions. Around 200 such mutants were selected that were analyzed for the flanking sequences of the enhancer integration sites in them. This has led to the identification of plants showing better yielding traits and water use efficiency. Subsequently, Moin et al. (2016b) could identify significant upregulation of Ribosomal Protein Large Subunit genes, *RPL6* and *RPL23A*, by the integrated enhancers with the help of TAIL PCR. Apart from ribosomal protein genes, several transcription factors like *WRKY*, *GRAS*, helicases like *XPB2* and *SEN1* Dutta et al. (2021), and genes involved in ubiquitination like *Cullins* were identified to be activated under water limiting conditions. This subsequently persuaded us to study the regulatory roles of all the RPL (Ribosomal Large Subunit) genes under different external stimuli (Moin et al., 2016a). This study also led another group to identify the insect-resistant role of *NIRPL5* in rice as an essential responsive gene in resistance to the Brown planthopper, *Nilaparvata lugens* (Zhu et al., 2017). The objective of the present study is to ascertain whether RPS (Ribosomal protein Small Subunit) genes are also stress-responsive like the RPL genes as analyzed by our group earlier (Saha et al., 2017).

Ribosomal proteins (RPs) constitute the protein part of the ribosomes and have a significant role in ribosome biogenesis, protein synthesis, cell growth, development, and apoptosis (Ramakrishnan and White, 1998; Naora and Naora, 1999; Maguire and Zimmermann, 2001; Wang et al., 2013). RPs have long been known primarily for their housekeeping functions (Warner and McIntosh, 2009). But, there are several reports on their extra-ribosomal functions in animal systems (Uechi et al., 2006; Danilova et al., 2008 Gazda et al., 2008). Several studies have demonstrated the important roles of RPs in the development and physiology of organisms as their reduced level in these organisms resulted in detrimental effects (Szakonyi and Byrne, 2011 Van Lijsebettens et al., 1994 Yan et al., 2016). In addition to their crucial roles in developmental processes in plants, several reports also focused on their expression pattern in response to several external stimuli (Cherepneva et al., 2003 Kawasaki et al. Wang et al.). Cumulatively, these reports point toward the differential expression of RP genes in response to stress treatments leading to differential accumulation of RPs in the ribosomal apparatus, which might help ribosome remodelling and selective translation to cope with nutrition deficiency or other unfavourable conditions (Wang et al., 2013). In this section, we have attempted to analyse the differential expression patterns of ribosomal

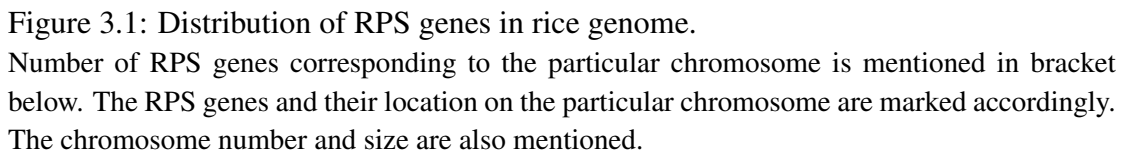
protein small subunit genes (RPS genes, one from each family) for their spatiotemporal and stress-induced regulation along with a comparative analysis with the large subunit counterparts and *in-silico* studies on their promoter sequences, gene structures, protein properties, and phylogeny. These observations are reported here.

3.2 Distribution of Ribosomal Protein Small Subunit genes on Rice chromosome

From RGAP-DB and Phytozome v11 databases, we enlisted a total of 56 ribosomal small subunit genes (40S). The sequence of these genes, as retrieved from RGAP-DB, confirmed their ribosomal origin through nucleotide and protein BLAST search in NCBI and Hidden Markov Model (HMM) of Pfam databases, respectively. Only 34 ribosomal small subunit proteins are available, which are coded by 56 genes. This indicates the existence of paralogs of the genes coding for more than one RPS protein. We selected 34 RPS genes for our detailed study, one from each group representing the corresponding paralogous groups. To obtain the genome-wide chromosomal distribution of all RPS genes on 12 chromosomes of rice, we had submitted the locus numbers (obtained from RGAP-DB) of 56 RPS genes as an input to the OryGenesDB. Rice genome has 12 chromosomes carrying 56 RPS genes. Based on the OryGenesDB output, the RPS genes were distributed throughout the rice genome covering all 12 chromosomes. Both the arms of the chromosome randomly carried the RPS genes. Each chromosome carried at least one member of the RPS gene family. Most of the genes are located on chromosomes 3, 1, 7, and 11, with chromosome 3 bearing the highest number of eleven RPS genes. Both chromosomes 1 and 7 carried a total of eight and nine genes, respectively. Chromosome 9 is the only example with only one RPS gene present near the telomere region of its long arm. Chromosomes 10 and 1 carried three and eight RPS genes, respectively. The distribution of RPS genes appeared to be throughout the rice genome. Each of the 12 chromosomes carried at least one RPS gene family member, with chromosome 3 exhibiting a maximum of 11 RPS genes. The distribution of RPS genes in rice genome is depicted in Figure 3.1.

3.3 Structural prediction of RPS genes

To predict the gene structure features, we had submitted the genomic and corresponding cDNA sequences of the selected 34 genes to the Gene Structure Display Server (GS-DSv2). RPS genes, like other eukaryotic genes, showed the organization of introns, exons and UTRs. On submitting the nucleotide sequences of the 34 RPS genes in the Gene Structure Display Server (GSDS), we have obtained the predicted gene structure



showing all exons, introns, UTRs for each gene and the number and relative position of each of the components. The BLAST analysis of RGAP-DB sequences with *indica* variety database showed that the coding regions of the 34 genes in both the varieties were similar. The percent similarity of the coding and the 5'-upstream regions of the genes found from the BLAST analysis has been listed in Table 3.2. The similarity of RPS genes in rice with the corresponding RPS genes in *Arabidopsis* has also been searched with the help of BLAST analysis in TAIR database. All the 34 RPS genes studied in this report exhibited very high similarities with their counterparts in *Arabidopsis*. The minimum similarity percentage is 78 and, the maximum is 95. Results from the GSDS suggested that most of the RPS genes consisted of both introns and exons in Figure 3.2. *RPS25a* has a long stretch of intronic sequence covering most of its length. It is also the longest gene among the selected genes with a total length of approximately 6 kb. *RPS17* and *RPS30* lacked introns and possessed a single and small CDS flanked by UTRs. *RPS6*, *RPS19*, *RPS23a*, and *RPS7* lacked UTRs and are only composed of intronic and exonic sequences. *RPS23a* is the smallest gene with a total size of 0.5 kb. The number of exons also varied from a minimum of two in *RPS23a* and *RPS29* to a maximum of three exons in *RPS3a*. The details of properties related to the gene structure have been provided in Table 3.1.

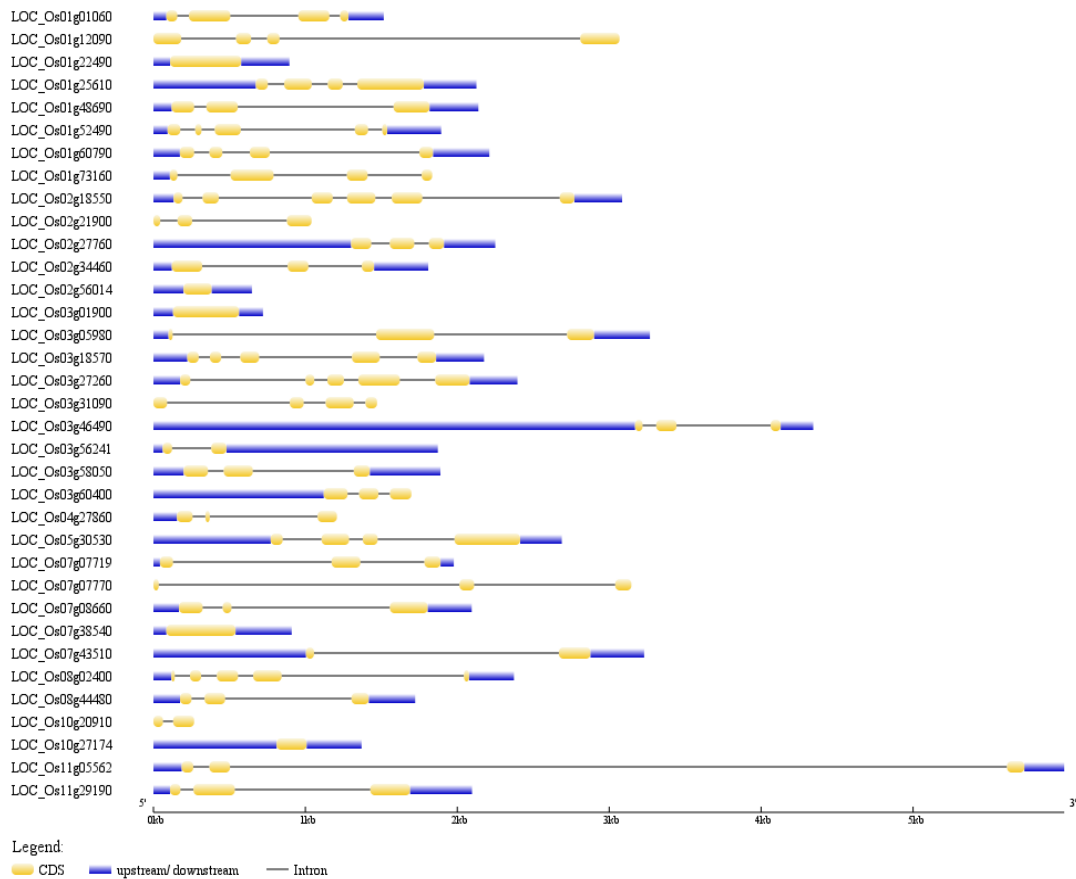


Figure 3.2: Gene structure of 35 RPS genes-

This diagram shows the inronic, exonic and UTRs of the RPS genes. Blue, yellow and line regions represent the upstream/downstream regions, exon and intron of the individual genes respectively.

3.4 Putative promoter analysis

In accordance with the observations regarding the stress-responsive differential expression and tissue-specific expression of several RPS genes, we tried to correlate the transcript levels with the presence of stress-responsive elements in the 5' upstream regions of the individual genes. Among the 5' regions of the RPS genes, we have shortlisted some common stress-responsive elements present in single or multiple copies. We have used an *in-silico* approach. The nucleotide sequence of about ≤ 1 kb upstream of each of the selected RPS genes was retrieved from RGAP-DB. The sequences were cross-checked for their identity/ deviation with the *indica* genome OryGenesDB and EnsemblPlants databases. Further, the sequences that exhibited the highest similarity with the *indica* genome in the databases were submitted to PlantCARE (*Cis*-Acting Regulatory Elements) Database to identify the regulatory elements. All the RPS genes have at least two stress-responsive elements in their immediate upstream regions (up to 1 kb). *RPS30* has one copy of ABRE and TGACG, and *RPS18b* has one copy of LTR and

TC rich repeat as their *cis*-acting regulatory elements (CAREs). Other than these two genes, putative promoter regions of the rest of the genes have more than two elements. SARE, EIRE, motifIb, DRE, and ERE are responsive to SA, elicitor, ABA, dehydration, and ethylene, respectively, and are present in single copies in the putative promoter regions of some of the genes. *RPS23* showed five repeats of ABREs, three copies of the TGACG motif, three copies of CGTCA motif, and one copy of TGA, Box W1, W box, and GARE elements. *RPS4a* exhibited three copies of ABREs, two repeats each of TGA, TGACG, and CGTCA elements. *RPS15* has two repeats of MBS. Similarly, the genes (*RPS4*, *10a*, *6a*, and *5*) that have exhibited remarkable up-regulation under biotic stress conditions have multiple MeJa responsive elements, and some of them contain box W1 and TCA elements that are related to responsiveness under pathogen attack. All the stress-responsive *cis*-acting elements were pictorially represented by their physical mapping in Figure 3.3 and Table 3.3, mentioning the function of the corresponding motifs. We have listed the elements that might have significance in tissue specificity of expression in Table 3.4. Most of the genes have elements for meristem and endosperm specificity in their promoter regions.

3.5 Properties and Phylogeny of RPS proteins

The submission of the protein sequences from RGAP-DB to the ExPasyProtParam server revealed many putative properties. Their molecular weights (MW) varied from approximately 7 to 30 kDa, with *RPS4* having the highest MW of 29.8 kDa and *RPS30* with the lowest MW of 6.9 kDa. Following the MW, the *RPS4* is the largest protein with a sequence of 265 amino acids, while *RPS30* is the smallest with 62 amino acids. *In-silico* analysis using the ExPasyProtParam server helped us get the theoretical *pI*-values of each RPS. Most of them were in the range of 9 to 12, with very few exceptions out of this range. For example, *RPS6* has a very low *pI* of 4.86. Most of the RPS proteins are rich in positively charged amino acids like Arg and Lys compared to negatively charged amino acids like Asp or Glu. The only exceptions are the *RPS21*, which has an equal number of both the types, while the *RPS6* exhibited more acidic amino acids. The percentage composition of secondary protein structures (disordered protein, alpha-helix and beta-sheet) has variations within the RPS family. *RPS4* has a minimum value of 14% while the *RPS30* has a maximum value of 76%, respectively; *S10a* exhibited 10%, and *S29* and *S13* have 64% α -helical content. The range on the percentage of β -sheet content also varied with *RPS13*, *13a* having 0% and *S27* showing 49% of the β -sheet content.

The Grand Average of Hydrophobicity (GRAVY) indices were below the value of zero for all 34 RPS proteins suggesting the hydrophobic nature of RPS proteins. We have also identified some of the interacting ligands of these RPS proteins from the

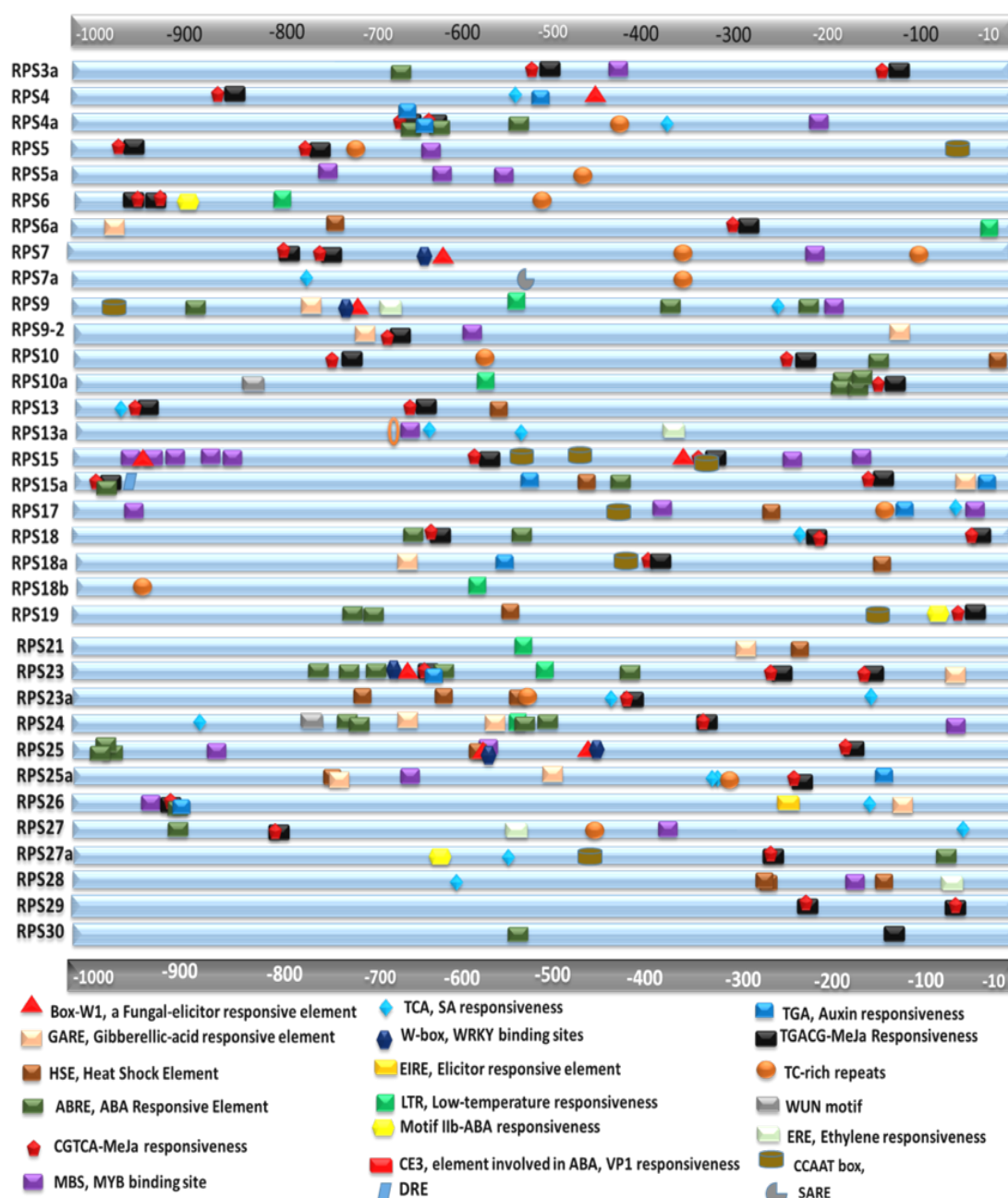


Figure 3.3: *In-silico* analysis for the presence of *cis*-regulatory elements in the putative promoter regions of RPS genes.

About ≤ 1 kb upstream region of each gene is retrieved and searched for *cis*-regulatory elements that might have reported roles in stress responsiveness. Different elements have been demonstrated in different shapes and colours, and they are approximately placed according to their actual location in the genome. The functions of each of the elements are mentioned below the figure.

3DLigandSite. The metal and non-metal ligands included IF-1, FMN, FAD, Mg^{2+} , Zn^{2+} etc. and the specific residues involved in the ligand binding have been listed in Tables 3.5 and 3.6. Most of the RPS proteins exhibited Low Complexity Region (LCR) motifs, which are the stretches of biased amino acid sequences having a long

repetition of single or few amino acids. They have been connected to many protein functions and interactions. LCRs located terminally in a protein has been considered to be translationally enriched and have stress responsive roles.

Three dimension protein structures from 3D ligand site have been included in Figure 3.4. The ribosomal protein sequences were submitted to the SMART database, and it was found that all of them exhibited ribosomal domains. Some of the proteins also contain the KOW domain (e.g., *RPS4*, *RPS4a*). *RPS6* has an SPT2 domain; *RPS5a* has a HLH domain, a CARD domain and an ANTAR domain. *RPS18a* has a SANT domain, which is a MYB- related domain that binds to DNA and helps in transcriptional regulation, and it also has a homeobox domain HALZ.

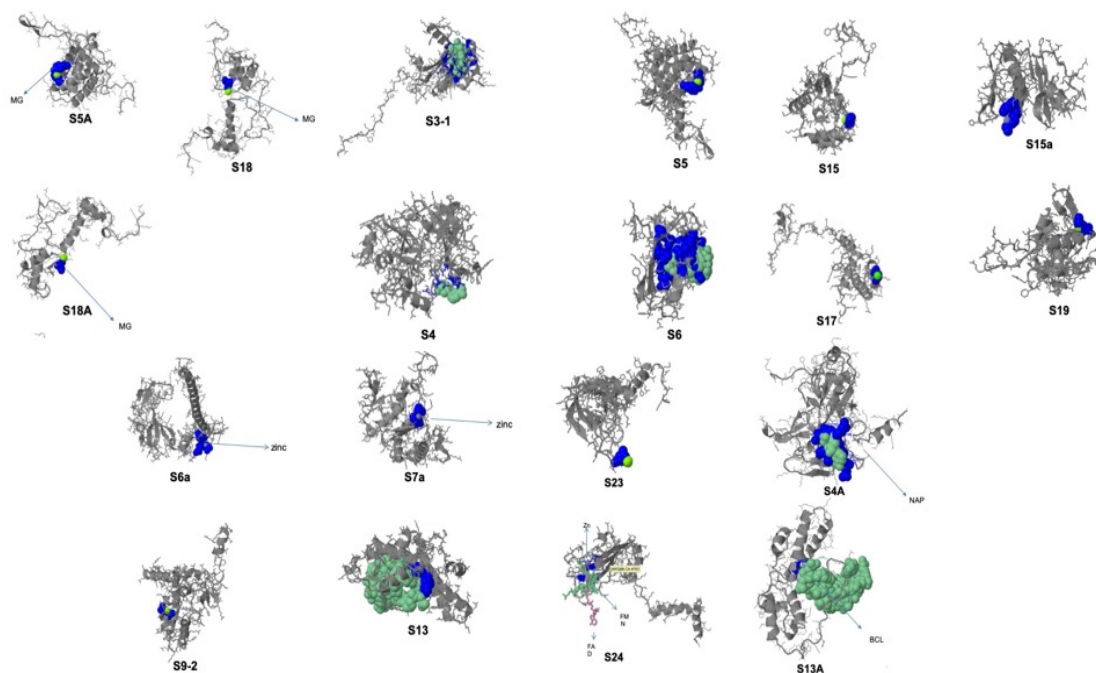


Figure 3.4: Predicted secondary structure and ligand binding sites of the RPS proteins To predict the three dimensional structure and to check for the presence of ligand binding sites on each RPS, we have used the Phyre2 and 3DLigandSite programs respectively. This figure shows the predicted secondary structure and ligand binding sites of the 19 RPS proteins with the metal ligands and cofactors mentioned in the images.

Protein sequence data was exploited to construct an unrooted phylogenetic tree of 34 RPS proteins in the MEGA6 software. Alignment with ClustalW employing a neighbour-joining algorithm, which resulted in the generation of a tree that showed the evolutionary relationship between the RPS proteins. The tree suggested that a considerable number of cladogenesis events occurred during evolution in the RPS gene family, reflected in their protein sequence similarity and diversity. The earliest divergence separated the proteins into two major clades, of which one contains five proteins (*RPS13*, *13a*, *3a*, *19*, and *2,1*), and the other major clade carried all the other proteins, which has further diverged several times. These five RPS genes were checked for similarity

with their paralogs, and the result was depicted as a phylogenetic tree in Figure 3.5. While *RPS19* and *RPS21* have no paralogous members, *RPS13* and *RPS3a* have two paralogous members, and they have shown similarity with their corresponding group. Node, from where some pairs of genes were diversified, showed the bootstrap value of 100. Such a high value indicates the high significance of the clade. The phylogenetic tree showing the evolutionary relationships among the RPSs is shown in Figure 3.6

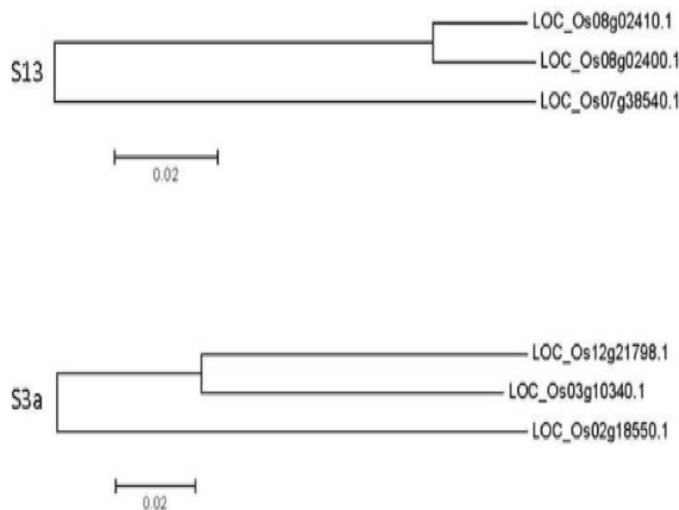


Figure 3.5: Phylogenetic tree of the RPS13 and RPS3a

Among the divergent genes (*RPS13a*, 13, 3a, 19 and 21) from the above phylogenetic tree, *RPS13* and *RPS3a* have three orthologous groups shown in the form of phylogenetic trees.

3.6 Native expression profiling of RPS genes in different tissues

To gain insights into the expression patterns of RPS genes in rice development to provide a comparative analysis between RPs of both the large and small subunits, we performed qRT PCR of 34 RPS genes in 13 different tissues. The mean values were utilized to construct heat maps to demonstrate the native expression pattern of the RPS genes in Figure 3.7. The tissues used to study the native expression pattern included embryo and endosperm from 16 h old germinating seed, plumule and radicle from 3 d old seedlings; shoot and root of 7 d old seedling; shoot, root, flag leaves, panicle, flower, grains and root-shoot transition of mature 20 d old plants (after transfer to the greenhouse). The complete tissue-specific up-regulation pattern of the RPS genes in native conditions has been summarized in Table 3.7 and in the form of heat maps in Figure 3.7. The up-

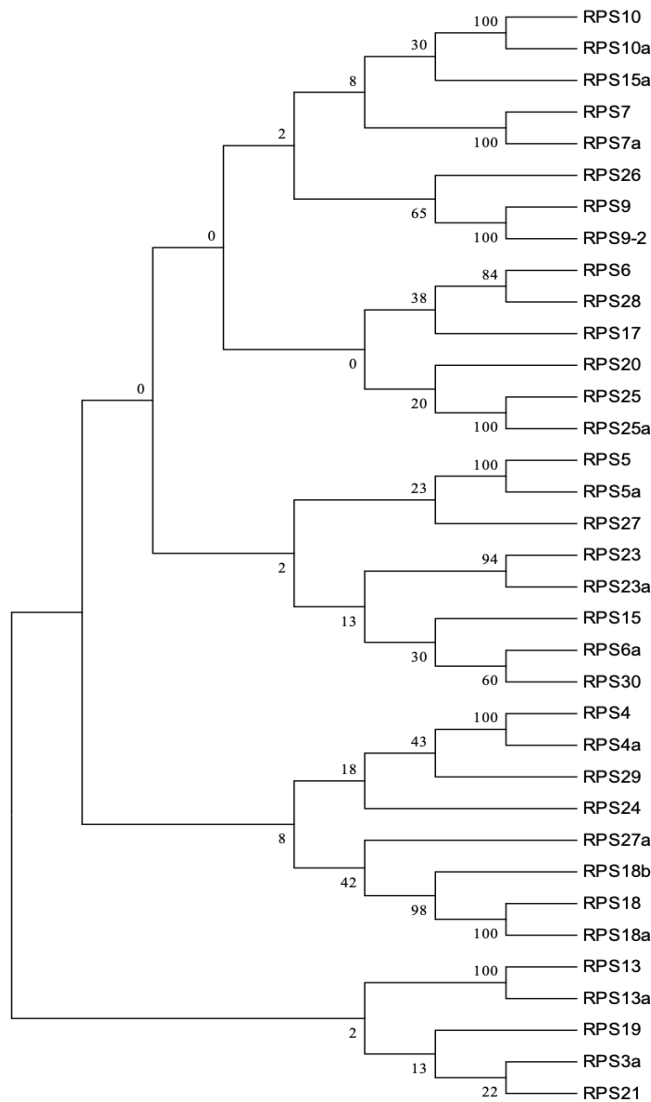


Figure 3.6: Phylogenetic tree showing relations within the RPS proteins.

Phylogenetic relationships among the RPS proteins were deciphered by multiple sequence alignment in the MEGA6 (Molecular Evolutionary Genetics Analysis) platform, which constructed an unrooted phylogenetic tree showing evolutionary sequence similarity among the proteins with bootstrap values of 100.

regulation of RPS genes was significant in mature leaves (25 genes), shoots (21 genes), 7d shoots (26 genes), plumules (21 genes), and root-shoot transition (23 genes). Apart from these, four, seven and 15 genes were up-regulated in seedling radicles, 7 d old root and mature roots, respectively. An analogous pattern of gene expression was observed under different stress conditions. More RPS genes were up-regulated in the shoot than in the root tissues showing that the majority of the RPS genes were up-regulated in shoots, leaves and plumules compared to the other organs.

About 17 RPS genes showed expression in rice grains and panicles, indicating their involvement in inflorescence development and grain filling. None of the genes was expressed in florets implying that RPS proteins have probably no significant roles in the

development of this meiotically active tissue. About 18 and 15 RPS genes were up-regulated in embryo and endosperm, respectively showing that these genes might have a role in seed development and maturity. *RPS23a*, *RPS27a*, *RPS10a*, and *RPS7* have exhibited no significant up-regulation in any of the tissues and *RPS23* is up-regulated only in root-shoot transition. A considerable up-regulation of *RPS28* and *RPS29* was observed in roots under different stresses. *RPS4*, *RPS5a*, *RPS7a*, *RPS9*, *RPS9-2*, *RPS13*, *RPS6*, *RPS17*, *RPS18b*, *RPS24*, and *RPS29* were up-regulated in most of the tissues, while *RPS13a*, *RPS18a*, *RPS4* were up-regulated in shoots throughout the duration of all the stress conditions. Similarly, these genes were also expressive in shoots, leaves, plumules and native conditions as well.

The RPS genes are prevalently up-regulated under native conditions in 13 different tissues in 45 d old mature leaves, shoots and root-shoot transition and 7 d shoots and plumules. Also, several of them were up-regulated in roots, panicles, embryos, endosperm and grains. But, none of them was found to be expressive in flowers. The role of RPS genes might be presumed to be involved in modulating developmental stages in vegetative tissues, mainly shoots and not to a greater extent in reproductive tissues. The activity of the *RPS18A* promoter of *Arabidopsis* was seen in meristematic tissues, and embryonic stage and mutations in the corresponding loci resulted in the formation of pointed leaves. This can be correlated with the transcriptional up-regulation of *RPS18*, *RPS18a*, and *RPS18b* in embryo, plumules, shoots, leaves, and root-shoot transition in the present study.

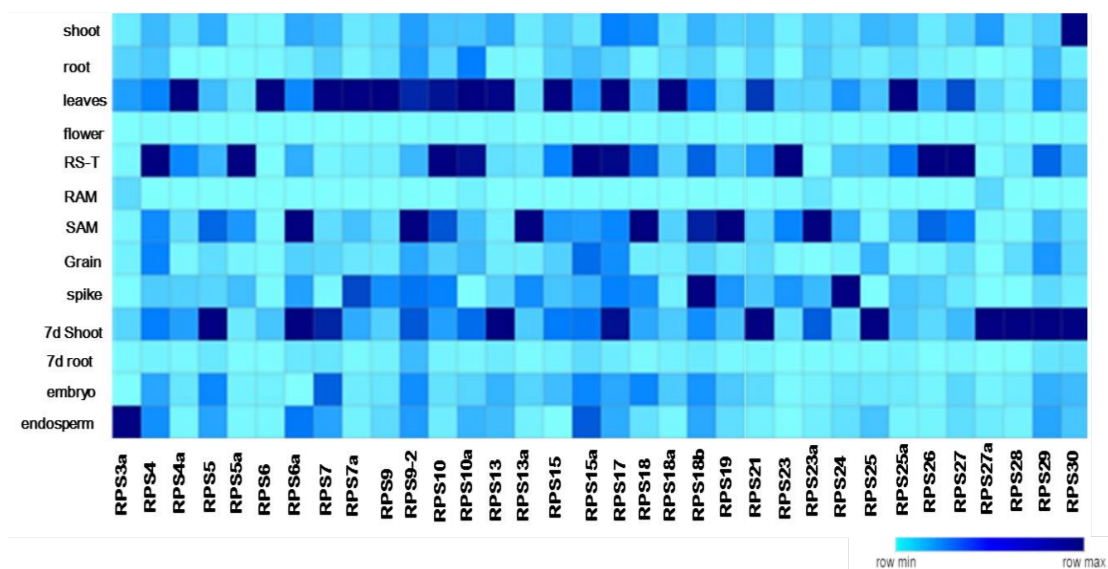


Figure 3.7: Tissue-specific expression of the RPS genes.

The native expression of 34 RPS genes in 13 tissues is determined by qRT-PCR with single normalization with rice actin. The mean values of the fold change of the biological and technical replicates are represented in a heat map.

3.7 Differential expression analysis of RPS genes in response to Abiotic stress treatments

Our findings on screening a large pool of activation tagged mutant population for high water-use efficiency revealed the significant up-regulation of RPL genes (*RPL6* and *RPL23A*) by the integrated enhancers in two of the mutant plants with sustained productivity under limited water conditions (Moin et al., 2016b). In the follow-up study, an extensive tissue-specific and differential expression study of the entire RPL gene family was carried out, which showed significant up-regulation of certain RPL genes under abiotic and biotic stresses (Moin et al., 2016a). These studies prompted us to further investigate the expression of 40S Ribosomal Protein genes in response to abiotic and biotic stress conditions and to arrive at a proper combination of RPS and RPL genes for subsequent genetic manipulation. For studying their differential expression, we have selected 34 RPS candidate genes from the respective paralogous groups (Saha et al., 2017) in response to four abiotic stresses (ABA, PEG, NaCl, and H₂O₂) at seven time points and two biotic stresses (BLB and SB). ABA, PEG, NaCl, and H₂O₂ induce a stress similar to osmotic, drought, salinity and oxidative stress, respectively. Treatment of the plants with these stress-inducing agents would mimic the corresponding stress situations and activate related stress-responsive pathways. Also, we also analyzed their native expression pattern in different tissues.

The genes that exhibited ≥ 3 fold transcript level on the log₂ scale were considered as up-regulated. The pattern of expression of the genes depended mainly on the type of stress signal and the tissue in which the expression was studied. We have categorized the genes in terms of their timing and the intensity of response. Some of the genes were up-regulated as early as 15 min- 3 h after the onset of the stress, while a few of them responded between 3 and 12 h after exposure. The others were up-regulated 12 h after the application of the stress. We have differentiated them as Immediate Early (IE), Early (E), and Late (L) expressing genes, respectively. Since the level of expression varied from 3 to several 100 folds, we have further categorized the up-regulated genes as highly expressive genes (≥ 30 fold), moderately expressive (between 10 and 30 fold) and low-expressive (between 3 and 10 fold). The expression of the RPS genes in the shoot samples has been observed to be higher compared to the corresponding root samples under almost all the abiotic stresses at different time intervals. Under some conditions, the up-regulation of the genes in the shoot samples has been detected to be several 100 folds, which is not the case for the corresponding root samples. In the case of shoot samples, all the genes showed significant up-regulation for all the stress conditions for at least one time point, and they were predominantly IE type in their response, except under H₂O₂ treatment.

Although all the genes were up-regulated under all the stress conditions in shoots,

their mode of response varied with the nature of the stress. For example, ABA-induced the up-regulation of 26% of the genes (*RPS7*, *15a*, *18a*, *25a*, *4*, *7a*, *10*, *10a*, and *13a*), and these maintained a significant expression (≥ 3 -fold) until the last time point used (60 h). Of these, 50% were highly expressive (*RPS7*, *15a*, *S8a*, and *25a*) with >30 -fold transcript level throughout the stress treatment up to 60 h time point. *RPS7* and *RPS18a* showed up-regulation up to several 100 folds. PEG and NaCl also induced the up-regulation of most genes in shoots until 60 h time point, the exceptions being *RPS7a* and *RPS23* and *RPS5*, *RPS10a*, and *RPS23a* under PEG and NaCl treatments, respectively, which became down-regulated. Some of the RPS genes maintained remarkably high transcript levels (≥ 100 fold). These included *RPS4a*, *RPS9*, *RPS9-2*, *RPS13*, *RPS17*, *RPS19*, *RPS27*, and *RPS30* under PEG treatment and *RPS4a*, *RPS7*, *RPS9-2*, *RPS18a*, and *RPS23a* under NaCl treatment. The up-regulation of *RPS4*, *RPS7*, *RPS9*, *RPS10*, *RPS19*, and *RPS26* under salt treatment was in agreement with the earlier studies (Kawasaki et al., 2001). Unlike in ABA, PEG, and NaCl treatments, several RPS genes were late responding in shoots under H_2O_2 treatment. Moreover, the level of up-regulation was also not as high as in other treatments. About 60% of the genes were up-regulated 15 min post-treatment. About 30 genes have been found to maintain a considerable level of expression, and among these, *RPS4a*, *RPS9-2*, *RPS15a*, *RPS18*, and *RPS28* have exhibited ≥ 100 fold up-regulation. In roots, *RPS19* was down-regulated persistently in response to all the abiotic stresses at all the time intervals. *RPS10a*, *RPS18*, *RPS18a*, *RPS18b*, *RPS24*, *RPS27a*, and *RPS6a* were also observed to maintain a very low transcript level. Some of the genes that were initially up-regulated became down-regulated subsequently with the progression of the stress treatment. The expression pattern of all the genes under different stress treatments in the shoot and root tissues has been represented in the form of heat maps in Figure 3.8.

Detailed lists of genes that were up/down-regulated in different stress conditions in the shoot and root tissues separately has been presented in Tables 3.8, 3.9, 3.10 and 3.11. The percentage of genes over-expressed at specific time points under the stress conditions in shoot and root has been represented in the form of Pie chart in Figure 3.9 and 3.10. The genes are tabulated in the tables that accompanied the figures. The genes commonly up-regulated under all the treatments, particularly during the early response, may have significance in stress-related signalling as the early responsive genes play a crucial role in defence against environmental stresses.

Around 60 and 20% of the genes were up-regulated immediately after the treatments in shoots and roots, respectively, under all the stress conditions. Up-regulation of these genes might fall under immediate response to the stress. These genes could be presumed to encode non-specific master regulators needed for plant core environmental stress response proteins. The immediate-early response to certain stress might sometimes be non-specific, and responses with continuous up-regulation are considered to be specific

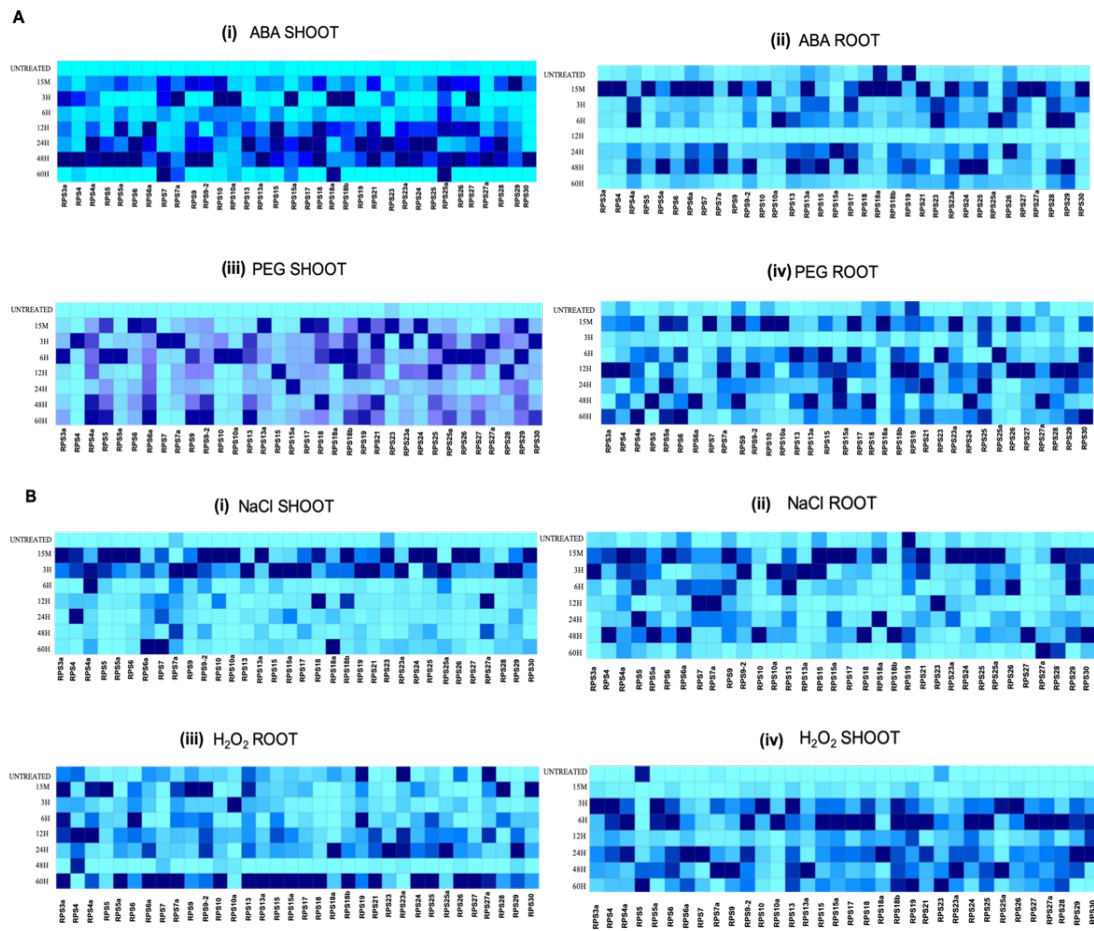


Figure 3.8: Heat map representation of RPS genes in response to ABA, PEG, NaCl and H_2O_2 treatments

The 7d old seedlings were treated with ABA(100 μ M), PEG (10%), NaCl (250 μ M) and H_2O_2 (10 μ M) and qRT-PCR was used to determine the level of expression of 34 RPS genes in the shoot and the root tissues at the time points mentioned on the left side. The fold change has been normalized using the $\Delta\Delta C_T$ method with respect to the unstressed seedlings maintained in water. Rice specific *act1* and β -*tub* genes were used as endogenous reference genes. The mean values of the fold changes of the biological and technical replicates were considered for preparing the heat maps. A-(i), (ii), (iii) and (iv) represent the expression of the genes in shoot and root tissues under ABA and PEG, respectively and B- (i), (ii), (iii) and (iv) represent the expression of the genes in shoot and root tissues under NaCl and H_2O_2 respectively. The darkest colour depicts the highly up-regulated genes, whereas the lightest colour represents the less up-regulated and down-regulated genes.

ones (Kilian et al., 2007). And, the genes that are up-regulated at around 1 h time points can be assumed to have a pivotal role in early defence signalling and might function in reconstructing the protein synthesis apparatus (Ouyang et al., 2007). This has led us to look for continuity in the up-regulation of immediate-early responsive RPS genes. There are many genes for which the up-regulation was observed throughout the duration of the stress, for example, *RPS13a*, *18a*, *4* and *4a*. Subsequently, *RPS29* showed a remarkably high transcript level (≥ 100 fold) in roots under ABA and PEG treatments

and showed a moderate level of expression (11-fold) under H_2O_2 treatment. *RPS28* has consistently displayed a moderately high level of expression in all the stress conditions in root tissues.

On the other hand, *RPL23A*, 6, 8, *L19.3*, and *L38* among the members of large subunit genes showed consistent up-regulation under ABA, PEG, NaCl treatments. *RPL18a*, *L24a*, *L24b*, *L30*, and *L34* were significantly overexpressed under oxidative stress. Moreover, *RPL23A*, one of the candidate genes that were identified using an activation tagging approach in water use efficiency (Moin et al., 2016b), might have a potential role to be utilized in modifying abiotic stress tolerance in rice orchestrating the responses of other candidate genes from both the subunits. Among the RPS genes, *RPS3a*, *RPS6*, *RPS15a*, *RPS17*, *RPS18a*, *RPS23a*, *RPS24*, *RPS25*, *RPS27a*, and *RPS28* are substantially up-regulated in shoots under a minimum of two abiotic stress conditions.

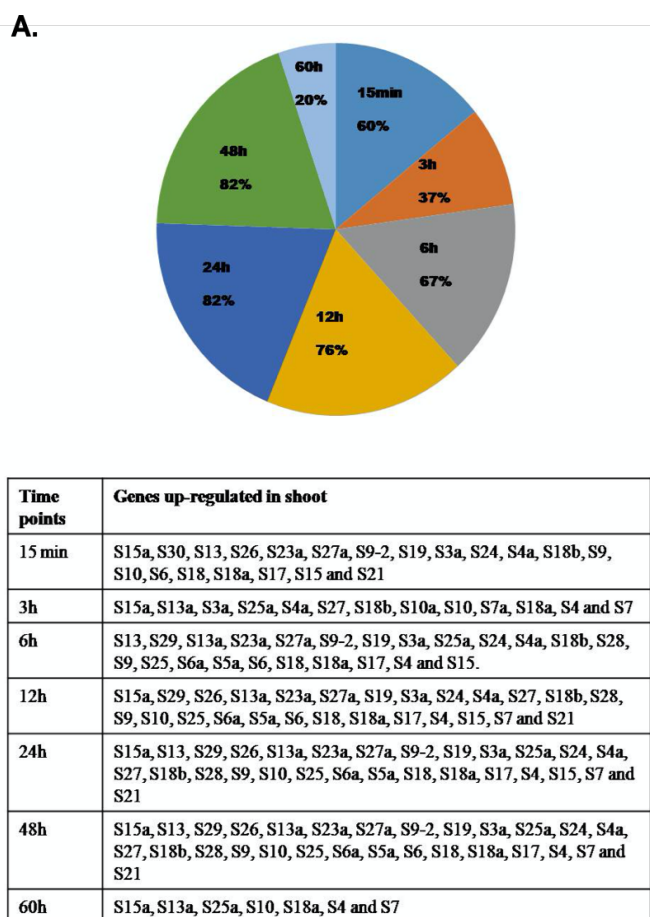


Figure 3.9: Percentage of genes up-regulated in shoot under all the stresses at different time points

The percentage of genes up-regulated in shoot at different time intervals after the application of the stress is represented in the form of pie charts with the genes enlisted in the respective tables.

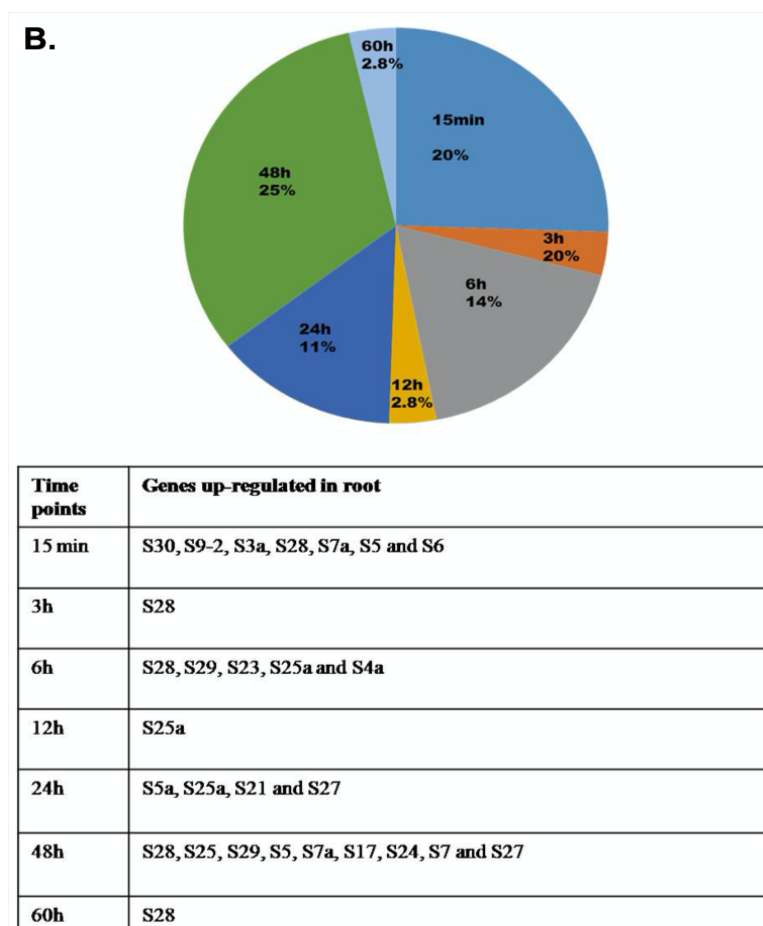


Figure 3.10: Percentage of genes up-regulated in root under all the stresses at different time points

The percentage of genes up-regulated in root at different time intervals after the application of the stress is represented in the form of pie charts with the genes enlisted in the respective tables.

3.8 Differential Expression of RPS genes upon infection with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Rhizoctonia solani*

Apart from the four abiotic stresses, the expression level of 34 RPS genes was also studied in response to the treatments with *Xoo* and *R. solani* pathogens that cause severe Bacterial Leaf Blight (BLB) and Sheath Blight (SB) diseases, respectively in rice. In the BLB infected samples, 14 (40%) genes were down-regulated, and the rest were up-regulated (60%). *RPS6a* (26 fold), *RPS9* (18 fold), *RPS10a* (29 fold), and *RPS4* (13 fold) showed a high level of transcript upregulation when compared with the other up-regulated genes.

The qRT-PCR analysis of 34 RPS genes was performed from both the shoot and the leaf samples infected with *R. solani*. The up-regulation of genes was observed mainly in the shoot tissues for a majority of the RPS genes. Thirteen (37%) genes were

down-regulated in response to this pathogen in the shoot region, whereas the 22 RPS (63%) genes were up-regulated. Among the genes that were expressive, *RPS4* and *RPS5* exhibited 22 and 14 fold up-regulation, respectively, which is the highest compared with the other genes. The expression pattern of all the genes under two biotic stresses has been depicted graphically in Figure 3.11 a and b.

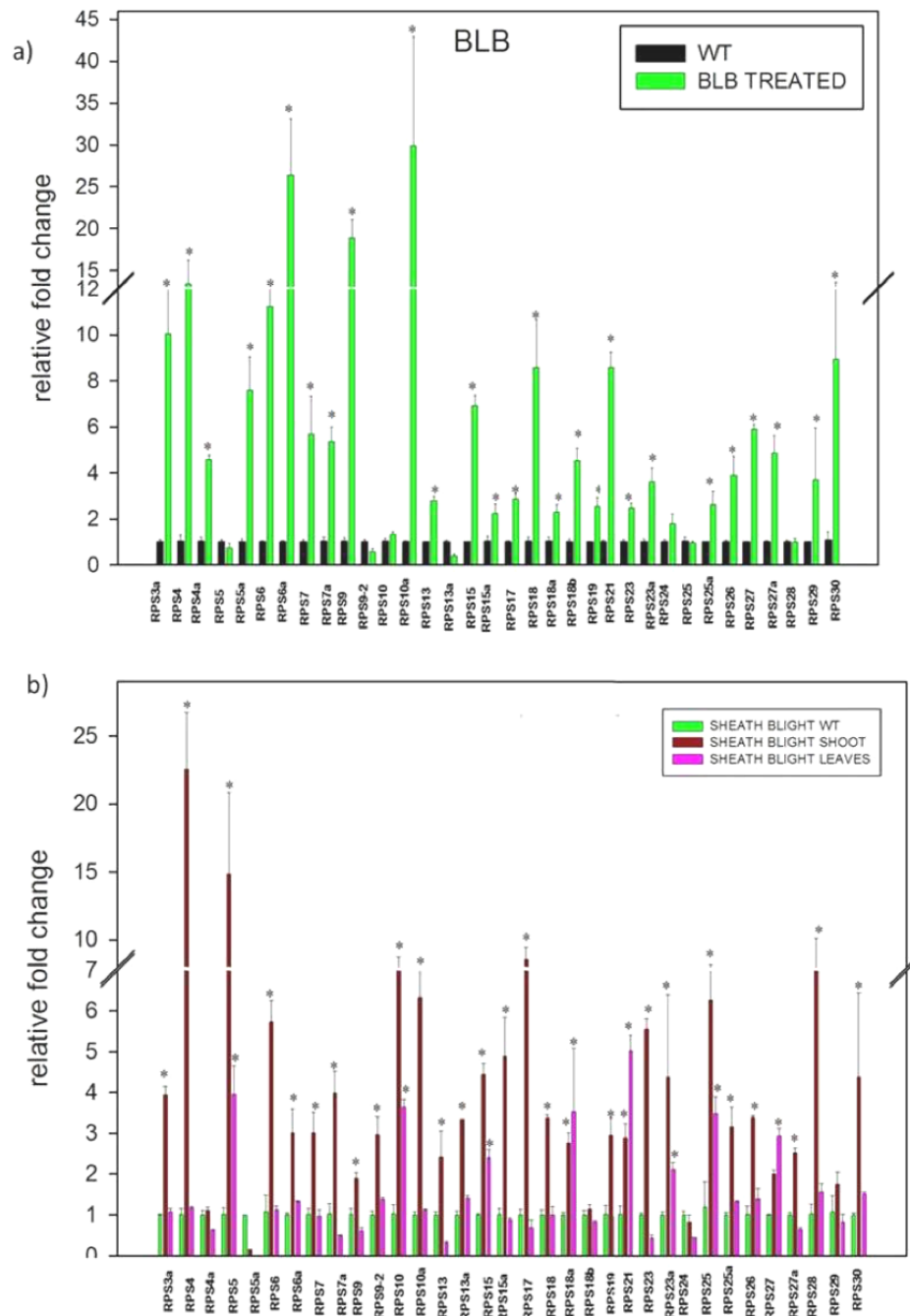


Figure 3.11: Differential expression of RPS genes in response to BLB and SB

Differential expression of RPS genes in response to *Xanthomonas oryzae* pv. *oryzae* (Xoo) (A) and *Rhizoctonia solani* (B), which cause Bacterial Leaf Blight (BLB) and Sheath Blight (SB) respectively- Expression of 34 RPS genes was determined in BLB and SB infected leaf samples after 20 and 25 days, respectively, after infection. The expression was normalized with corresponding untreated samples in identical conditions.

3.9 Comparative Expression Analysis of RPL and RPS Genes

Previously available information about the RPL genes (Moin et al., 2016a) has been utilized and subsequently combined with the information obtained from the current study on the expression patterns of RPS genes and these details regarding their stress responsiveness and tissue-specific expression pattern under native condition have been summarized in a tabular form to select suitable combinations of RPS and RPL genes for stress alleviation in transgenic plants. All the RPL and RPS genes up-regulated in response to four individual stress treatments were depicted in Table 3.12 and 3.13. Very high expressing RPL and RPS genes in each of the 13 different tissues under native conditions have been represented in Figure 3.12. Four heat maps combining the expression of RPL and RPS genes under four abiotic stresses have been illustrated separately in Figure 3.13. Figure 3.14 shows the comparative expression analysis of RPL and RPS genes under 13 different tissues in the form of a heat map.

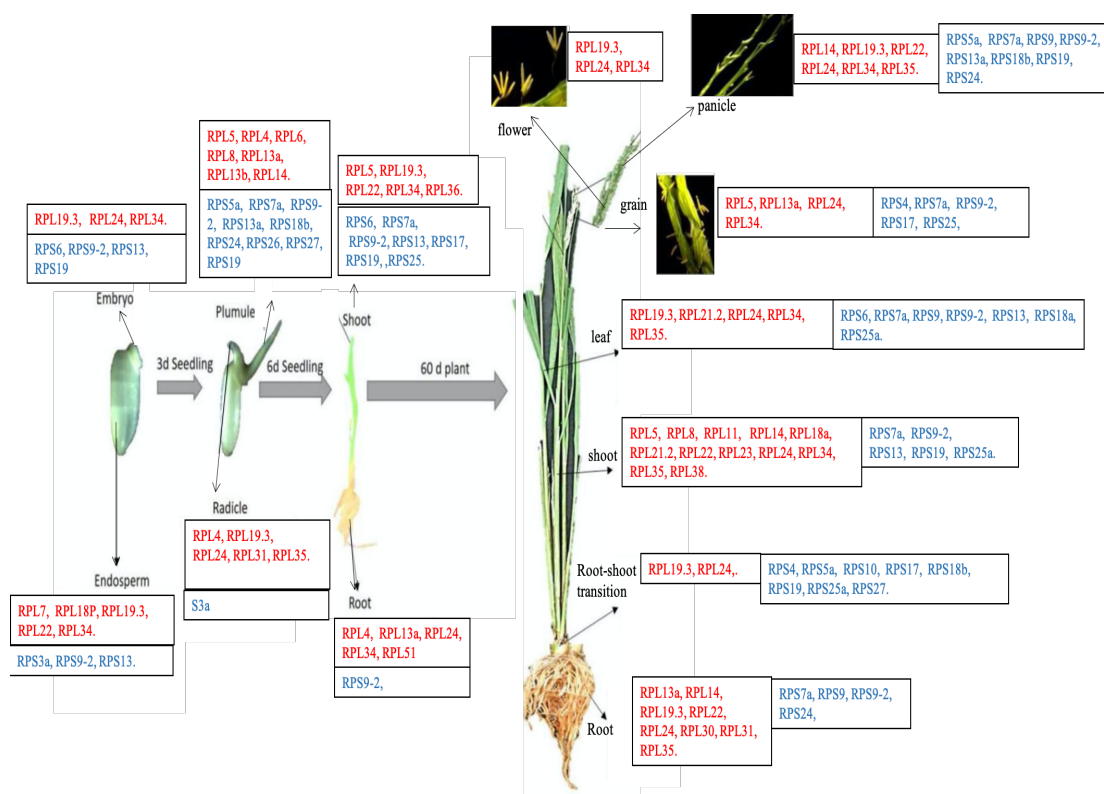


Figure 3.12: Native expression of RPL and RPS genes in different tissues

Some of the RPL and RPS genes showing considerably high expression than the other genes in each of the 13 different tissues under native conditions have been listed in the boxes (Moin et al. 2016b). In each box, the names of the genes mentioned in red font belong to the RPL gene family, and the names of the genes mentioned in blue font belong to the RPS gene family.

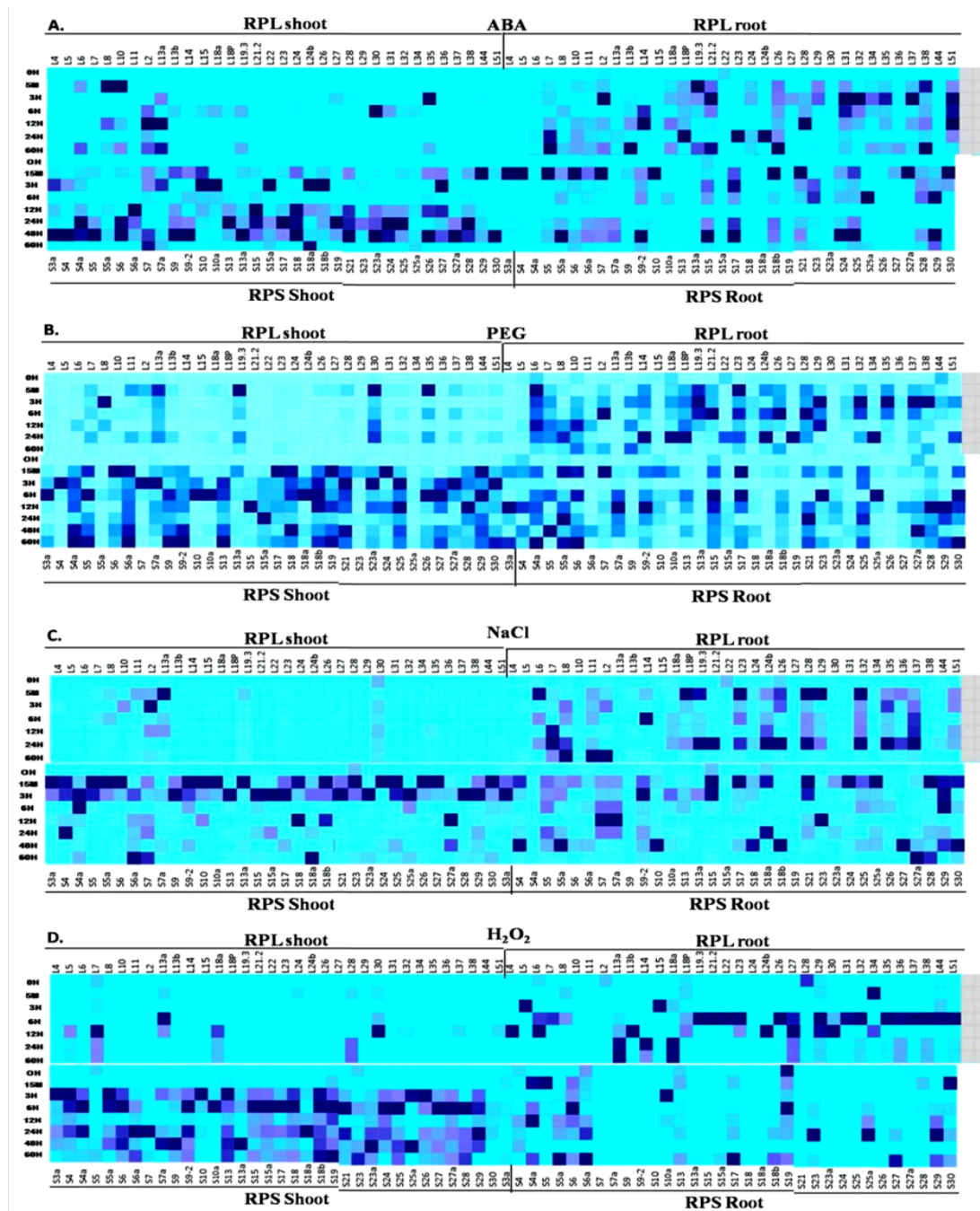


Figure 3.13: Combined heat maps depicting differential expression of RPL and RPS genes under 4 abiotic stresses in shoot and root tissues

Differential expression of all the RPL and RPS genes under four abiotic stress is depicted together in 4 heat maps (a.- ABA, b.- PEG, c.- NaCl and d.- H_2O_2). The mean of the fold change values of the biological and technical replicates is represented in heat maps. The darkest colour depicts the highly up-regulated genes, whereas the lightest colour represents the less up-regulated and down-regulated genes.

Table 3.1: Structure of RPS genes

Gene ID	Chr-number	Coded protein	Gene size	GC%	Intron	Exon	Alternative splice forms	Orientation
LOC_Os02g18550	Os2	RPS3a	3085	42	5	6	1	3'-5'
LOC_Os01g25610	Os1	RPS4	2126	45	3	4	1	5'-3'
LOC_Os05g30530	Os4	RPS4a	2688	42	3	4	1	5'-3'
LOC_Os01g01060	Os1	RPS5	1516	46	3	4	1	5'-3'
LOC_Os11g29190	Os11	RPS5a	2097	50	2	3	5	3'-5'
LOC_Os01g12090	Os1	RPS6	3068	40	3	4	1	3'-5'
LOC_Os03g27260	Os3	RPS6a	2396	43	4	5	1	3'-5'
LOC_Os02g21900	Os2	RPS7	1041	39	2	3	1	3'-5'
LOC_Os03g18570	Os3	RPS7a	2176	45	4	5	2	5'-3'
LOC_Os07g43510	Os7	RPS9	3230	42	1	2	1	3'-5'
LOC_Os03g05980	Os3	RPS9-2	3268	40	2	3	2	5'-3'
LOC_Os01g73160	Os1	RPS10	1835	43	3	4	1	3'-5'
LOC_Os02g34460	Os2	RPS10a	1809	40	2	3	1	5'-3'
LOC_Os07g38540	Os7	RPS13	910	55	0	1	1	5'-3'
LOC_Os08g02400	Os8	RPS13a	2373	42	4	5	1	3'-5'
LOC_Os07g08660	Os7	RPS15	2095	43	2	3	1	3'-5'
LOC_Os02g27760	Os2	RPS15a	2250	41	2	3	1	5'-3'
LOC_Os03g01900	Os3	RPS17	721	61	0	1	1	3'-5'
LOC_Os03g58050	Os3	RPS18	1889	47	2	3	1	3'-5'
LOC_Os07g07719	Os7	RPS18a	1977	41	2	3	1	5'-3'
LOC_Os07g07770	Os7	RPS18b	3146	41	2	3	1	5'-3'
LOC_Os03g31090	Os3	RPS19	1472	45	3	4	1	5'-3'
LOC_Os03g46490	Os3	RPS21	4343	41	2	3	1	3'-5'
LOC_Os03g60400	Os3	RPS23	1698	42	2	3	1	3'-5'
LOC_Os10g20910	Os10	RPS23a	268	44	1	2	1	3'-5'
LOC_Os01g52490	Os1	RPS24	1894	45	4	5	1	3'-5'
LOC_Os08g44480	Os8	RPS25	1723	45	2	3	2	5'-3'
LOC_Os11g05562	Os11	RPS25a	5995	41	2	3	1	5'-3'
LOC_Os01g60790	Os1	RPS26	2211	44	3	4	1	5'-3'
LOC_Os04g27860	Os4	RPS27	1208	42	2	3	1	5'-3'
LOC_Os01g22490	Os1	RPS27a	895	55	0	1	1	3'-5'
LOC_Os10g27174	Os10	RPS28	1370	47	0	1	1	3'-5'
LOC_Os03g56241	Os3	RPS29	1872	40	1	2	1	3'-5'
LOC_Os02g56014	Os2	RPS30	648	52	0	1	1	3'-5'

Table 3.2: BLAST analysis of the coding and the 5'-upstream region of the RPS genes in OryGenesDB and EnsemblPlants databases to check the similarity of the sequences in *indica* and *japonica* variety.

Gene				5' upstream region		
Gene name	ODB (<i>japonica</i>) Query coverage	ODB (<i>indica</i>) Query coverage	Ensembl (<i>Indica</i>)	ODB (<i>japonica</i>) Query coverage	ODB (<i>indica</i>) Query coverage	Ensembl (<i>Indica</i>)
S3a- LOC_Os02g18550.1	101.40	101.40	100.00%	99.90	99.90	98.20%
S4- LOC_Os01g25610.1	99.75	99.75	100.00%	98.50	98.50	99.50%
S5 LOC_Os01g01060.1	101.99	101.99	100.00%	100.00	100.00	100.00%
S6 LOC_Os01g12090.1	101.13	101.13	100.00%	99.90	99.90	100.00%
S6a LOC_Os03g27260.1	100.27	99.19	100.00%	99.90	99.90	98.00%
S7 LOC_Os02g21900.1	100.99	100.99	99.40%	99.90	99.90	98.90%
S7a LOC_Os03g18570.1	101.55	101.55	100.00%	99.90	99.90	99.10%
S9 LOC_Os07g43510.1	101.15	101.15	96.30%	99.90	99.90	98.50%
S9-2 LOC_Os03g05980.1	95.58	95.58	99.20%	99.90	99.90	98.60%
S10 LOC_Os01g73160.1	100.00	100.00	100.00%	99.90	99.90	100.00%
S13 LOC_Os07g38540.1	99.78	99.78	99.80%	99.90	99.90	99.00%
S15 LOC_Os07g08660.1	100.00	100.00	99.60%	99.90	99.80	98.00%
S15a LOC_Os02g27760.1	101.27	101.27	99.40%	99.90	99.90	99.00%
S17 LOC_Os03g01900.1	99.77	99.77	99.50%	99.90	99.90	98.80%
S19 LOC_Os03g31090.1	100.00	100.00	100.00%	67.00	47.00	98.50%
S21 LOC_Os03g46490.1	100.80	100.80	100.00%	99.90	99.90	100.00%
S23 LOC_Os03g60400.1	98.60	98.60	100.00%	62.00	63.00	98.65%
S23a LOC_Os10g20910.1	100.00	100.00	100.00%	99.88	106.70	97.50%
S24 LOC_Os01g52490.1	94.00	94.00	100.00%	99.90	99.90	99.40%
S25 LOC_Os08g44480.1	99.39	99.39	100.00%	76.00	69.00	100.00%
S26 LOC_Os01g60790.1	102.49	102.49	100.00%	99.90	99.90	99.20%
S27 LOC_Os04g27860.1	88.89	88.39	100.00%	99.90	99.90	98.90%
S27a LOC_Os01g22490.1	99.79	99.79	100.00%	99.89	99.89	100.00%
S28 LOC_Os10g27174.1	99.49	99.49	100.00%	99.90	99.90	99.70%
S29 LOC_Os03g56241.1	94.15	94.15	100.00%	99.92	99.92	99.30%
S30 LOC_Os02g56014.1	97.88	97.88	99.50%	99.89	99.89	99.30%
S10a LOC_Os02g34460.2	100.24	100.24	100.00%	99.90	99.90	98.77%
S4a LOC_Os05g30530.1	100.38	100.38	98.80%	99.80	98.30	98.80%
S13a LOC_Os08g02400.1	89.25	89.25	100.00%	99.50	99.40	100.00%
S25a LOC_Os11g05562.1	98.80	98.80	100.00%	99.90	99.90	100.00%
S5a LOC_Os11g29190.1	101.49	101.49	100.00%	-	-	98.60%
S18 LOC_Os03g58050.1	100.44	100.44	100.00%	99.80	99.80	99.20%
S18a LOC_Os07g07719.1	100.79	100.79	100.00%	99.90	-	-
S18b LOC_Os07g07770.1	89.03	89.30	99.00%	99.90	86.72	-

Table 3.3: Functions of the different stress-responsive elements found in the promoter regions of the RPS genes

Name of the elements	Function
ABRE (ABA responsive elements)	osmotic stress-mediated transcriptional regulation (Kims et al., 2011)
DRE (dehydration responsive elements)	
HSE (heat stress element)	tolerance to heat stress
LTR (low temperature responsive)	Cold responsive
MBS (MYB binding site)	drought induced expression of genes defense (Sazegari et al., 2015)
	involved in defense (Sazegari et al., 2015)
W-box- binding site of WRKY transcription factors	pathogen induced response (Rushton et al., 2002)
Box-W1-fungal-elicitor responsive element	involved in defense signaling (Hernandez-Garcia et al., 2014).
TCA and SARE	responsive to salicylic acid
TGACG and CGTCA	responses related to methyl jasmonate
ERE (ethylene responsive elements)	crucial for stress signaling
TGA and AuxR	auxin responsive (Sutoh et al., 2003, Bastian et al., 2010, Sazegari et al., 2015)
motif IIb and CE3 (coupling element 3)	Involved in ABA mediated osmotic stress signaling (Hobo et al., 1999).
WUN motif (wound specific)	involved in wound response (Pandey et al., 2015).

Table 3.4: Tissue specific elements observed in the upstream sequences of RPS genes

Tissue specific elements	Reported function	Sequence	Genes
RY-Element	seed specific regulation	CATGCATG	S3a
Skn1 motif	endosperm expression	CTCAT	S4, S5, S6, S7, S7a, S9, S10, S13, S15, S15a, S19, S23, S23a, S24, S27a, S28, S2, S5a, S18a, S4a, S18b, S10a, S13a
CAT-box	meristem expression	GCCACT	S5, S6, S7, S9, S9-2, S15, S23, S25, S26, S30, S10a, S4a, S25a
AC-II	xylem expression	CTCACCAACCCC	S6a, S27a, S18a
CCGTCC-Box	meristem specific activation	CCGTCC	S7, S9-2, S13, S15, S15a, S19, S21, S25, S27, S27a
GCN4	endosperm expression	TGAGTCA	S7, S9-2, S21, S25, S18, S18a
ATGCAAT	endosperm expression	ATGCAAT	S7a, S21
as-2 box	shoot specific expression	CATAATGATG	S13, S5a, S18b, S25a
dOCT	meristem specific activation	CACGGATC	S9-2, S23

Table 3.5: Properties of RPS proteins

Protein	Length (aa)	MW (Da)	pI	LCR	Disordered protein %	Alpha helix %	Beta- Strand %	GRAVY
RPS3a	261	29759.56	9.85	2	29	30	28	-0.516
RPS3-1	233	25999.36	9.63		26	39	24	-0.195
RPS4	265	29807.84	10.13	2	14	16	45	-0.417
RPS5	200	22226.65	9.74		28	54	9	-0.227
RPS6	206	24013.16	4.86	1	43	58	8	-0.46
RPS6a	245	28058.7	10.65	2	23	44	16	-0.897
RPS7	100	11409.51	9.75		31	41	31	0.144
RPS7a	192	22182.78	9.79		23	41	27	-0.515
RPS9	86	9541.93	10.26	1	51	20	41	-0.563
RPS9-2	195	22673.18	10.29	1	38	60	5	-0.71
RPS10	179	19910.46	9.81	2	51	31	9	-0.892
RPS13	151	16934.02	10.54		21	64	0	-0.236
RPS15	154	17415.44	10.19		35	31	25	-0.47
RPS15a	130	14818.38	9.94		15	28	39	-0.135
RPS17	144	16615.17	10.34		46	65	2	-0.73
RPS19	146	16386.81	10.04		16	52	4	-0.557
RPS21	82	9283.4	6.71		34	23	33	-0.662
RPS23	142	15660.37	10.43		35	20	26	-0.561
RPS23a	66	7302.83	10	1	24	30	21	0.309
RPS24	138	15718.58	10.57	2	31	36	28	-0.771
RPS25	108	12068.04	10.57	1	35	56	15	-0.898
RPS26	133	14991.3	11.02	2				-0.907
RPS27	86	9558.14	9.06	1	33	14	49	-0.256
RPS27a	155	17668.52	9.79	1	35	12	31	-0.772
RPS28	65	7463.76	11.16	1	31	17	42	-0.429
RPS29	112	12810.93	10.08		61	64	4	-0.623
RPS30	62	6931.16	12.03	1	76	61	0	-1.281
RPS10a	138	14952.46	9.84	2	68	10	12	-1.036
RPS4a	263	29662.71	10.15	1	11	16	44	-0.396
RPS13a	151	17115.28	10.53		26	62	0	-0.437
RPS25a	108	11918.86	10.41		35	56	15	-0.854
RPS5a	200	22493.03	9.69		28	55	10	-0.133
RPS18	152	17662.58	10.74		15	51	8	-0.751
RPS18a	126	14740.19	11		15	52	2	-0.88
RPS18b	78	9294.84	11.23		18	37	17	-1.183

Table 3.6: Interactions of RPS proteins

Protein	Ligands	Ligand binding residues	Specific interactions
RPS3a			
RPS3-1	HEM, Protoporphyrin	PHE19 GLU22 LEU23 ASN24 MET26 LEU27 TYR36 VAL39 VAL41 ARG42 VAL43 THR44 MET46 ARG47 THR48 GLU49 ILE50 ILE51 ILE52 THR72 VAL74 VAL75 ARG78 PHE79 PHE81 ASN84 GLY85 VAL86 GLU87 LEU88 TYR89 ALA90	IF-1
RPS4	NAP	GLY185 GLY186 ARG187 ASN188 ALA217 PHE218 ALA219 THR220 ASN224	RNA BINDING SITE
RPS5	Mg ⁺	HIS75 TYR148	S11 interface, S25 interface, S9 interface, rRNA binding site
RPS6	FMN	LEU8 VAL10 LEU22 ARG25 VAL26 ALA27 ARG29 MET127 MET129 LEU139 LEU142 ASN143 LEU148 TRP151	
RPS6a	Zn ⁺	ASP154 ARG156 LEU183	IF-1
RPS7			
RPS7a	Zn ⁺	HIS64 THR98	
RPS9			
RPS9-2	Mg ⁺	ARG55	IF-1
RPS10			
RPS13	BCL	ASP87 PHE90	IF-1, RNA binding domain
RPS15	Mg ⁺	TYR24	IF-1
RPS15a	Zn ⁺	LYS32 ILE35	
RPS17	Ca ⁺	GLU36	IF-1, DNA Binding site
RPS19	Mg ⁺	GLN111	
RPS21			
RPS23	Mg ⁺	ASN62	aminoacyl-tRNA interaction site (A-site), 18S rRNA interaction site, 28S rRNA interaction site, streptomycin interaction site, eEF2 interaction site
RPS23a			IF-1
RPS24	FMN, FAD, Zn ⁺	ARG15 GLU31 LYS74 THR76	
RPS25			
RPS26			
RPS27			
RPS27a			Ubq - E2 interaction site, Ubq - CUE interaction site, Ubq - UCH interaction site
RPS28			RNA binding site
RPS29			IF-1
RPS30			
RPS10a			
RPS4a	NAP	GLY185 GLY186 ARG187 ASN188 GLN217 PHE218 ALA219 THR220 ASN224	
RPS13a	BCL	ASP87 PHE90	
RPS25a			
RPS5a	Mg ⁺	HIS74 TYR147	
RPS18	Mg ⁺	SER32	
RPS18a	Mg ⁺	SER6	
RPS18b			

Table 3.7: RPS genes expressing in each tissue specifically

Embryo	Endosperm	Plumule	Radicle	7D-S	7D-R	Root	Shoot	Leaf	Panicle	Grain	Flower	R-S transition
	S3a		S3a	S3a		S3a	S3a	S3a				
S4	S4	S4		S4		S4	S4	S4	S4	S4		S4
								S4a				
S5a	S5a	S5a	S5a	S5a		S5a	S5a	S5a	S5a	S5a		S5a
S6	S6	S6	S6	S6	S6	S6	S6	S6	S6			
		S6a		S6a								
S7a	S7a	S7a		S7a	S7a	S7a	S7a	S7a	S7a	S7a		S7a
S9	S9	S9		S9	S9	S9	S9	S9	S9	S9		S9
S9-2	S9-2	S9-2		S9-2	S9-2	S9-2	S9-2	S9-2	S9-2	S9-2		S9-2
		S10		S10		S10	S10	S10	S10	S10		S10
								S10a				S10a
S13	S13	S13		S13	S13		S13	S13	S13	S13		S13
S13a		S13a	S13a	S13a			S13a	S13a		S13a		S13a
S15		S15		S15		S15	S15	S15	S15	S15		S15
S15a	S15a	S15a		S15a				S15a	S15a	S15a		S15a
S17	S17	S17		S17		S17	S17	S17	S17	S17		S17
S18		S18		S18			S18	S18	S18			S18
S18a		S18a		S18a		S18a	S18a	S18a		S18a		S18a
S18b	S18b	S18b		S18b		S18b	S18b	S18b	S18b	S18b		S18b
				S21				S21				S21
												S23
S24	S24	S24		S24		S24	S24	S24	S24	S24		S24
	S25			S25			S25	S25		S25		S25
S25a		S25a		S25a		S25a	S25a	S25a	S25a			S25a
		S26		S26			S26	S26	S26			S26
S27	S27	S27		S27			S27	S27	S27	S27		S27
				S28	S28							
S29	S29	S29		S29	S29	S29	S29	S29		S29		S29

Table 3.8: Regulation of RPS genes in shoots and roots under ABA treatment

ABA						
Gene	Regulation	Response	Max. Fold change	Regulation	Response	Max. Fold change
	Shoot			Root		
RPS3a	UP	IE (47.23)	267.24	UP	IE (55.89)	55.89
RPS4	UP	IE (10.22)	280.43	UP	IE (19.29)	19.29
RPS4a	UP	IE (39.545)	197.2	UP	IE (6.06)	7.084
RPS5	UP	IE (25.13)	114.8852	UP	IE (45.03)	45.035
RPS5a	UP	IE (21.81)	55.41	UP	IE (7.235)	16.32
RPS6	UP	IE (29.16)	140.87	UP	IE (29.55)	29.55
RPS6a	UP	IE (31.25)	187.72	UP	IE (3.31)	3.315
RPS7	UP	IE (132.89)	245.67	UP	IE (7.805)	7.805
RPS7a	UP	IE (4.243)	12.568	UP	IE (6.48)	34.015
RPS9	UP	IE (39.066)	58.97	UP	IE (21.89)	21.89
RPS9-2	UP	IE (38.20)	73.3	UP	IE (4.59)	4.59
RPS10	UP	IE (54.88)	71.79	UP	IE (14.77)	14.77
RPS10a	UP	IE (5.646)	56.49	UP	E (9.458)	9.458
RPS13	UP	IE (3.57)	76.67	UP	E (4.612)	6.664
RPS13a	UP	IE (22.51)	90.8	DOWN	---	---
RPS15	UP	IE (5.99)	28.99	UP	L (4.159)	4.159
RPS15a	UP	IE (5.23)	224.545	UP	L (10.142)	10.142
RPS17	UP	IE (35.00)	186.78	UP	IE (11.17)	17.22
RPS18	UP	IE (44.34)	75.63	UP	IE (7.28)	7.28
RPS18a	UP	IE (34.18)	278.54	DOWN	--	--
RPS18b	UP	IE (54.40)	216.73)	UP	IE (6.34)	6.34
RPS19	UP	IE (16.54)	153.23	DOWN	--	--
RPS21	UP	IE (53.26)	105.17)	UP	IE (21.06)	21.06
RPS23	UP	IE (11.64)	35.78	UP	IE (6.63)	7.728
RPS23a	UP	IE (18.30)	114.56	UP	IE (5.55)	5.55
RPS24	UP	IE (34.23)	382.71	UP	IE (3.43)	8.76
RPS25	UP	IE (21.79)	106.15	UP	IE (14.5)	19.99
RPS25a	UP	IE (41.42)	56.23	UP	IE (4.34)	58.42
RPS26	UP	IE (16.23)	32.28	UP	L (3.121)	3.121
RPS27	UP	IE (25.71)	49.96	UP	IE (26.53)	26.53
RPS27a	UP	IE (6.57)	302.66	UP	IE (41.93)	41.93
RPS28	UP	IE (61.53)	167.88	UP	IE(5.5)	9.73
RPS29	UP	IE (128.60)	128.6	UP	IE (52.64)	213.33
RPS30	UP	IE (23.244)	113.63	UP	IE (57.34)	57.34

Table 3.9: Regulation of RPS genes in shoots and roots under PEG treatment

PEG						
Gene	Regulation	Response	Max. Fold change	Regulation	Response	Max. Fold change
	Shoot			Root		
RPS3a	UP	IE (26.5)	226.24	UP	IE (5.79)	13.56
RPS4	UP	IE (16.58)	467	DOWN	--	--
RPS4a	UP	IE (213.06)	641.1959	UP	IE (9.24)	15.29
RPS5	UP	IE (36.84)	46.53	UP	IE (4.62)	47.36
RPS5a	UP	IE (8.42)	140.56	UP	IE (5.07)	5.55
RPS6	UP	IE (182.15)	182.15	UP	IE (19.90)	24.11
RPS6a	UP	IE (61.71)	66.33	UP	L (12.40)	12.4
RPS7	UP	IE (19.53)	531.87	UP	IE (18.66)	18.66
RPS7a	UP	IE (3.14)	11.51	UP	IE (6.75)	36.64
RPS9	UP	IE (32.99)	100.11	DOWN	--	--
RPS9-2	UP	IE (364.83)	689.87	UP	IE (8.03)	14.98
RPS10	UP	IE (14.67)	287.6	UP	IE (3.256)	3.256
RPS10a	UP	IE (4.94)	72.6	UP	IE (11.54)	11.54
RPS13	UP	IE (8.46)	96.86	UP	E (10.53)	10.53
RPS13a	UP	IE (91.59)	91.59	DOWN	--	--
RPS15	UP	IE (6.14)	142.01	UP	E (4.58)	4.58
RPS15a	UP	IE (21.99)	333.14	UP	E (7.59)	8.97
RPS17	UP	IE (413.73)	413.73	UP	IE (20.17)	20.17
RPS18	UP	IE (134.83)	144.28	UP	L (3.07)	3.07
RPS18a	UP	IE (34.17)	315.89	DOWN	--	--
RPS18b	UP	IE (49.35)	91.722	UP	E (3.81)	4.9
RPS19	UP	IE (202.27)	202.27	DOWN	--	--
RPS21	UP	IE (36.49)	58.4	UP	IE (15.15)	29.51
RPS23	UP	IE (6.05)	6.05	UP	IE (6.62)	33.39
RPS23a	UP	IE (71.23)	218.64	UP	IE (7.91)	7.91
RPS24	UP	IE (262.68)	262.68	UP	L (5.42)	5.42
RPS25	UP	IE (26.27)	110.35	UP	IE (7.62)	9.43
RPS25a	UP	IE (13.69)	41.51	UP	IE (26.88)	230.74
RPS26	UP	IE (7.09)	70.88	UP	IE (3.29)	3.4
RPS27	UP	IE (94.99)	131.19	UP	IE (9.57)	22.27
RPS27a	UP	IE (38.79)	251.68	DOWN	--	--
RPS28	UP	IE (110.33)	264.44	UP	IE (9.41)	32.11
RPS29	UP	IE (33.12)	33.56	UP	IE (5.32)	146.67
RPS30	UP	IE (33.43)	127.31	UP	IE (5.98)	9.07

Table 3.10: Regulation of RPS genes in shoots and roots under NaCl treatment

NaCl						
Gene	Regulation	Response	Max. Fold change	Regulation	Response	Max. Fold change
	Shoot			Root		
RPS3a	UP	IE (394.95)	394.95	UP	IE (10.16)	17.009
RPS4	UP	IE (94.08)	107.34	UP	IE (4.21)	5.32
RPS4a	UP	IE (209.01)	754.56	UP	IE (14.40)	14.4
RPS5	UP	IE (64.99)	64.99	UP	IE (7.89)	8.65
RPS5a	UP	IE (233.41)	233.41	UP	IE (16.07)	36.43
RPS6	UP	IE (132.97)	132.97	UP	IE (5.44)	5.44
RPS6a	UP	IE (7.03)	47.95	DOWN	--	--
RPS7	UP	IE (87.15)	135.3	UP	IE (13.69)	32.01
RPS7a	UP	IE (5.44)	5.44	UP		
RPS9	UP	IE (167.46)	325.91	UP	IE (3.75)	3.75
RPS9-2	UP	IE (1357.75)	1357.75	UP	IE (7.14)	11.27
RPS10	UP	IE (380.49)	380.49	UP	L (8.40)	8.4
RPS10a	UP	IE (129.63)	129.63	UP	IE (19.8)	19.8
RPS13	UP	IE (25.66)	364.99	UP	IE (4.61)	7.37
RPS13a	UP	IE (187.85)	187.85	UP	IE (3.52)	53.19
RPS15	UP	IE (10.02)	88.46	UP	IE (3.18)	3.18
RPS15a	UP	IE (103.02)	724.23	UP	IE (6.01)	6.01
RPS17	UP	IE (145.12)	252.99	UP	IE (18.42)	18.42
RPS18	UP	IE (819)	885.39	UP	IE (3.49)	9.76
RPS18a	UP	IE (266.91)	1068.83	UP	IE (3.62)	3.91
RPS18b	UP	IE (318.11)	318.11	UP	IE (4.55)	84.05
RPS19	UP	IE (166.16)	332.04	DOWN	--	--
RPS21	UP	IE (53.78)	96.45	UP	IE (13.65)	13.65
RPS23	UP	IE (3.37)	3.37	UP	IE (23.68)	164.02
RPS23a	UP	IE (58.99)	439.57	UP	IE (5.46)	5.46
RPS24	UP	IE (238.23)	238.23	UP	IE (32.20)	32.2
RPS25	UP	IE (129.19)	129.19	UP	IE (14.58)	14.58
RPS25a	UP	IE (91.95)	91.95	UP	IE (34.99)	34.99
RPS26	UP	IE (143.50)	143.5	UP	IE (3.25)	6.94
RPS27	UP	IE (552.55)	552.55	UP	IE (22.45)	22.45
RPS27a	UP	IE (216.54)	1420.6	UP	L (9.35)	9.35
RPS28	UP	IE (26.19)	543.29	UP	IE (33.77)	33.77
RPS29	UP	IE (24.27)	47.44	UP	IE (42.79)	50.48
RPS30	UP	IE (65.93)	65.93	UP	IE (13.00)	15.4

Table 3.11: Regulation of RPS genes in shoots and roots under H₂O₂ treatment

H ₂ O ₂						
Gene	Regulation	Response	Max. Fold change	Regulation	Response	Max. Fold change
	Shoot			Root		
RPS3a	UP	IE (8.8)	83.86	UP	IE (3.46)	3.46
RPS4	UP	IE (29.68)	29.68	DOWN	--	--
RPS4a	UP	IE (18.75)	487.002	UP	IE (9.86)	10.73
RPS5	DOWN	--	--	UP	IE (22.29)	22.29
RPS5a	UP	IE (33.87)	33.87	UP	E (6.49)	14.245
RPS6	UP	IE (6.60)	53.1	UP	IE (8.03)	14.35
RPS6a	UP	IE (12.95)	69.88	DOWN	--	--
RPS7	UP	IE (16.64)	180.48	UP	L (3.00)	3
RPS7a	UP	IE (10.30)	15.94	UP	IE (7.42)	9.804
RPS9	UP	IE (4.76)	269.65	DOWN	--	--
RPS9-2	UP	IE (33.16)	329.89	UP	IE (5.18)	5.18
RPS10	UP	IE (10.29)	93.11	UP	L (5.65)	5.65
RPS10a	UP	IE (16.59)	87.74	UP	IE (11.69)	11.69
RPS13	UP	IE (11.59)	69.7	DOWN	--	--
RPS13a	UP	IE (31.08)	137.24	DOWN	--	--
RPS15	UP	IE (5.98)	51.09	UP	E (3.31)	6.49
RPS15a	UP	IE (23.67)	298.48	UP	L (94.29)	4.29
RPS17	UP	IE (4.18)	154.85	UP	L (7.78)	7.78
RPS18	UP	IE (15.43)	187.73	UP	L (6.04)	6.04
RPS18a	UP	IE (4.07)	491.19	DOWN	--	--
RPS18b	UP	IE (6.82)	62.92	UP	L (4.56)	4.56
RPS19	UP	IE (18.42)	71.76	DOWN	--	--
RPS21	UP	IE (3.07)	81.9	UP	E (8.869)	11.5
RPS23	UP	L (3.08)	3.08	UP	E (3.14)	14.99
RPS23a	UP	IE (4.10)	164.71	DOWN	--	--
RPS24	UP	IE (5.27)	66.26	UP	IE (10.69)	23.09
RPS25	UP	IE (30.38)	58.89	UP	E (5.60)	7.79
RPS25a	UP	IE (146.44)	155.55	UP	IE (4.65)	34.66
RPS26	UP	IE (4.00)	47.17	DOWN	--	--
RPS27	UP	IE (27.00)	66.18	UP	L (7.89)	23.34
RPS27a	UP	IE (3.54)	146.72	DOWN	--	--
RPS28	UP	IE (19.92)	137.66	UP	IE (78.78)	78.78
RPS29	UP	IE (22.81)	37.3	UP	E (4.293)	16.65
RPS30	UP	IE (3.12)	30.58	UP	IE (9.01)	9.01

Table 3.12: List of up-regulated RPL and RPS genes under ABA and PEG

ABA- Shoot		ABA- Root	
RPL	RPS	RPL	RPS
L4(24.34), L5(3.8), L6(124.30), L7(13.78), L8(239.07), L10(143.47), L11(8.87), L12(280.52), L13a(16.18), L13b(14.76), L15(16.25), L18a(18.92), L18P(13.13), L19.3(33.48), L22(4.35), L23(23.68), L24(12.5), L24b(17.58), L27(36.0), L28(111.9), L30(63.95), L31(48.24), L32(68.54), L34(21.16), L35(248.90), L38(13.93), L44(25.83), L51(4.31)	S3a(267.2), S4(10.22), S4a(39.54), S5(2513), S5a(55.41), S6(140.87), S6a(187.72), S7(245.67), S7a(12.56), S9(58.97), S9-2 (73.3), S10(71.79), S10a(56.49), S13(76.67), S13a(90.80), S15(28.99), S15a(224.54), S17(186.78), S18(75.63), S18a(278.54), S18b(216.73), S19(153.23), S21(105.17), S23(35.78), S23a(114.56), S24(382.71), S25(106.15), S25a(56.23), S26(32.28), S27(49.96), S27a(302.66), S28(167.88), S29(128.60), S30(113.63)	L5(4.76), L6(3.08), L7(62.58), L8(13.78), L10(6.43), L11(9.52), L12(3.18), L13b(58.14), L14(99.05), L18a(8.06), L18P(32.94), L19.3(52.83), L21.2(41.50), L23(906.26), L24(6.37), L24b(170.11), L26(14.54), L28(164.60), L29(25), L31(10.01), L32(39.87), L34(5.27), L35(16.43), L36(24.77), L37(58.50), L38(75.46), L51(16.22).	S3a(55.89), S4(19.29), S4a(7.08), S5(45.03), S5a(16.32), S6(29.55), S6a(3.31), S7(7.8), S7a(34.01), S9(21.89), S9-2 (4.59), S10(14.77), S10a(9.45), S13(6.66), S15(4.5), S15a(10.14), S17(17.22), S18(7.28), S18b(6.34), S21(21.06), S23(7.72), S23a(5.55), S24(8.76), S25(19.99), S25a(58.42), S26(3.12), S27(26.53), S27a(41.93), S28(9.73), S29(213.33), S30(57.34)
PEG- Shoot		PEG-Root	
RPL	RPS	RPL	RPS
L4(11.73), L5(3.08), L6(145.15), L7(13.91), L8(876.21), L10(19.04), L11(26.71), L12(163.70), L13a(9.32), L13b(15.48), L15(14.45), L18a(9.51), L18P(4.91), L19.3(213.12), L21.2(4.56), L23(34.75), L24(7.87), L24b(3.9), L26(10.68), L27(24.01), L28(39.22), L30(22.09), L31(20.60), L32(34.26), L34(5.44), L35(496.90), L37(30.05), L38(21.45), L44(36.39)	S3a(226.24), S4(467), S4a(641.19), S5(36.84), S5a(8.42), S6(182.15), S6a(61.71), S7(19.53), S7a(3.14), S9(32.99), S9-2(364.83), S10(14.67), S10a(72.60), S13(8.46), S13a(91.59), S15(142.01), S15a(333.14), S17(413.73), S18(134.83), S18a(34.17), S18b(91.7), S19(202.2), S21(58.40), S23(6.05), S23a(218.64), S24(262.88), S25(110.35), S25a(41.51), S26(70.88), S27(131.12), S27a(251.68), S28(264.44), S29(33.12), S30(127.31)	L4(6.11), L7(8.42), L8(36.97), L10(4.71), L11(8.09), L12(36.03), L13b(22.88), L14(19.53), L18a(5.28), L18P(29.56), L19.3(19.68), L21.2(27.58), L23(48.31), L24(4.02), L24b(13.38), L26(18.25), L27(13.05), L28(19.96), L29(65.33), L31(7.57), L32(15.23), L34(30.72), L35(12.10), L36(30.72), L37(57.82), L38(57.09), L51(22.46)	S3a(13.56), S4a(15.29), S5(47.36), S5a(5.55), S6(24.11), S6a(12.40), S7(18.66), S7a(36.64), S9-2 (14.98), S10(3.25), S10a(11.54), S13(10.53), S15(4.58), S15a(8.97), S17(20.17), S18(3.07), S18b(4.9), S21(29.51), S23(33.39), S23a(7.91), S24(5.42), S25(9.43), S25a(230.74), S26(3.40), S27(22.27), S28(32.11), S29(146.67), S30(9.07)

Table 3.13: List of up-regulated RPL and RPS genes under NaCl and H₂O₂

NaCl-Shoot		NaCl-Root	
RPL	RPS	RPL	RPS
L4(40.24), L5(5.29), L6(27.13), L7(10.62), L8(48.49), L10(60.93), L11(18.13), L12(170.39), L13a(20.55), L13b(10.08), L18a(3.22), L18P(4.89), L19.3(48.40), L23(17.61), L27(10.50), L32(4.94), L35(17.04), L37(3.47)	S3a(394.95), S4(107.34), S4a(754.56), S5(64.99), S5a(233.41), S6(132.97), S6a(47.95), S7(135.30), S7a(5.44), S9(325.91), S9-2(1357.75), S10(380.49), S10a(129.63), S13(364.99), S13a(187.85), S15(88.46), S15a(724.23), S17(252.99), S18(885.39), S18a(1068.83), S18b(318.11), S19(332.04), S21(96.45), S23(3.37), S23a(439.57), S24(238.23), S28(543.29), S29(47.44), S30(65.93) S25(129.19), S25a(91.95), S26(143.50), S27(552.5), S28(543.29), S29(47.44), S30(65.93) S27a(1420.60)	L6(5.09), L7(139.01), L8(13.56), L10(4.4), L11(10.20), L12(7.79), L13b(4.62), L14(51.41), L18P(32.98), L19.3(25.54), L21.2(106.62), L23(41.92), L24(4.19), L24b(11.00), L26(3.12), L28(18.98), L29(30.50), L31(13.14), L32(12.93), L34(3.35), L35(22.74), L36(25.45), L37(26.13), L38(69.29), L51(16.45)	S3a(17.0), S4(5.32), S4a(14.40), S5(8.65), S5a(36.43), S6(5.44), S7(32.01), S7a(), S9(3.75), S9-2(11.27), S10(8.40), S10a(19.8), S13(7.33), S13a(53.19), S15(3.18), S18a(3.91), S18b(84.05), S21(13.65), S23(164.02), S23a(5.46), S24(32.20), S25(14.58), S25a(34.99), S26(6.94), S27(22.45), S27a(9.35), S28(33.77), S29(50.48), S30(15.40)
H ₂ O ₂ -Shoot		H ₂ O ₂ -Root	
RPL	RPS	RPL	RPS
L4(3.9), L5(15.25), L6(7.24), L7(4.07), L14(12.20), L18a(62.41), L19.3(7.7), L21.2(3.25), L22(21.25), L24(36.86), L28(81.50), L29(3.10), L30(3.80), L31(18.96), L32(11.61), L34(8.6), L37(14.58), L38(15.93), L44(14.47), L51(8.48)	S3a(83.86), S4(29.68), S4a(487.00), S5a(33.87), S6(53.10), S6a(69.88), S7(180.48), S7a(15.94), S9(269.65), S9-2(329.89), S10(93.11), S10a(87.74), S13(69.70), S13a(137.24), S15(51.09), S15a(298.48), S17(154.85), S18(187.73), S18a(491.19), S18b(62.92), S19(71.76), S21(81.90), S23(3.08), S23a(164.71), S24(66.26), S25(58.89), S25a(155.55), S26(47.17), S27(66.18), S27a(146.72), S28(137.66), S29(37.30), S30(30.58)	L4(126.92), L5(18.09), L6(4.92), L7(8.16), L8(1.25), L13a(222.42), L14(57.69), L15(215.27), L18a(43.28), L18P(6.36), L19.3(4.36), L21.2(17.24), L22(80.45), L23(18.12), L24b(194.48), L26(148.92), L27(5.95), L29(6.27), L30(188.155), L31(13.89), L32(25.11), L34(241.73), L35(142.88), L36(33.39), L37(47.87), L38(26.64), L44(6.86), L51(343.61)	S3a(3.46), S4a(10.73), S5(22.29), S5a(14.24), S6(14.35), S7(3.00), S7a(9.80), S9-2(5.18), S10(5.65), S10a(11.69), S15(6.49), S15a(4.29), S17(7.78), S18(6.04), S18b(4.56), S21(11.50), S23(14.99), S24(23.09), S25(7.79), S25a(34.66), S27(23.34), S28(78.78), S29(16.65), S30(9.01)

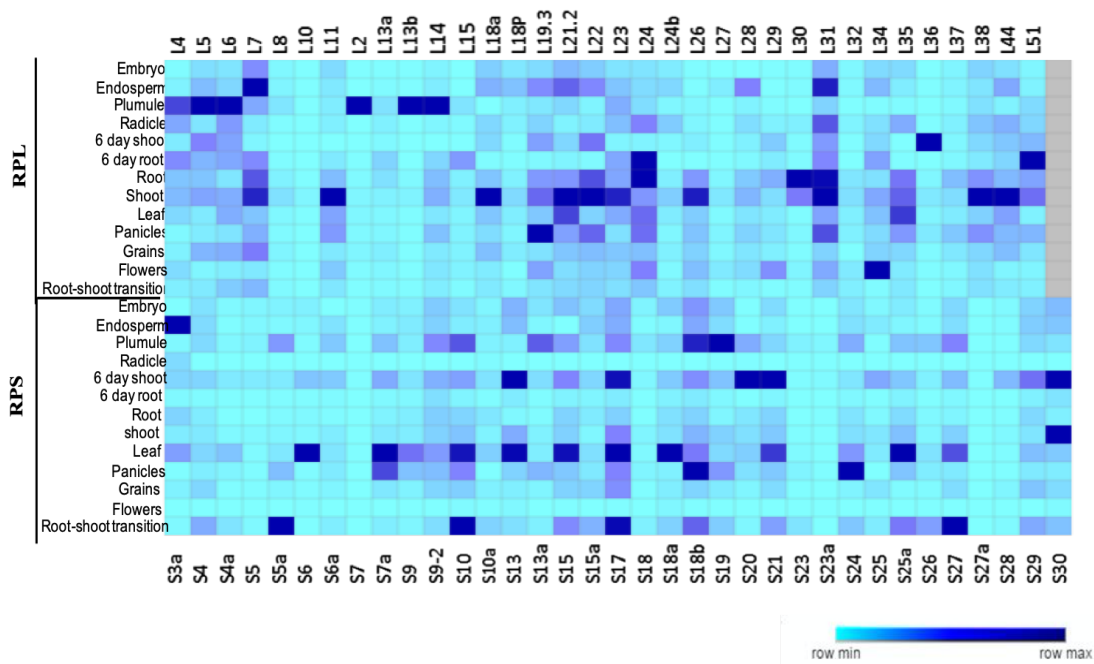


Figure 3.14: Combined heat map depicting the native expression of RPL and RPS genes in 13 different tissues

Expression pattern of RPL and RPS genes in different tissues. The mean of the fold change values of the biological and technical replicates is represented in heat maps.

Chapter 4

Characterization of one of the candidate genes, RPL6, and its involvement in regulating salt stress tolerance in rice

4.1 Introduction

Soil salinity is recognized as one of the critical problems that has a negative influence on crop productivity among numerous abiotic stressors. It impairs chloroplast activity and stomatal conductance, which is how it affects photosynthesis (Tuteja et al., 2011). Furthermore, it has an influence on plant metabolism and protein expression, and extreme stress can potentially put the plants' survival in jeopardy. Salinity can, thus, be one of the major impediments in crop production for sustaining the booming human population. According to FAO, 2006, 45 billion ha out of the current 230 billion ha of irrigated land will be impacted by salinity. This accounts for a total of 19.5% of the world's total irrigated land area. Rice is a staple food for half of the world's population. It is the most important staple cereal crop for more than two billion population in Asia and accounts for more than 40% of the calorie consumption of most Asians. India ranks first in area and second in rice production, following China. Increasing population necessarily demands a proportionate increase in the production of rice.

Salt absorption directly does not interfere with plant growth. Salinity stress triggers both osmotic and ionic stress in plants (Munns and Tester, 2008). It initially elevates the osmotic potential of the rhizosphere, thus affecting cell expansion and cell division. Consequently, it imparts toxicity due to high salt concentration, which leads to symptoms manifesting the injuries caused by the accumulation of salt in the cell wall or cytoplasm as vacuole is unable to sequester any more salt. High salinity re-

sults in osmotic stress and a condition equivalent to drought, making the acquisition of nutrients by plants extremely difficult (Munns and Tester, 2008). Salt salinity has a tremendous effect on the growth and sustenance of several salt-sensitive plant species known as glycophytes, compared to salt-tolerant species known as halophytes. Since rice is a glycophyte, it is susceptible to salinity, and thus, salt accumulation leads to drastic responses in rice cultivation. Different plants respond differently to salt stress. Rice is relatively vulnerable to salinity stress. Hence, salt accumulation is observed to disrupt both biochemical and physiological functions in plant cells to greatly hinder plant growth and yield (Hu, 2008; Tuteja et al., 2011). Salinity seems to impact rice cultivation extensively by affecting plant adaptation and other stress responses. Salinity leads to a decrease in dry matter, reduction in leaf size, shoot and length, number of tillers/plant, spikelet number, etc. If the stress is imposed during pollination and fertilization, it can severely induce sterility by inhibiting germination, causing delayed flowering and seed set, damaged fertility, partial or complete loss of grain, increased percentage of sterile florets production, thus negatively affecting panicle development and yield (Reddy et al., 2017). Salt stress also interferes with stomatal conductance by modulating stomatal opening, reducing stomatal size and density, which consequently hampers the rate of transpiration and photosynthesis (Reddy et al., 2017).

Higher salt concentration can occur in subsoil strata either naturally due to accumulation of salt over extended periods in arid and semi-arid regions or through secondary salinization by excessive use of chemical manures, compost or inorganic fertilizers and irrigation with brackish water. The ability to tolerate salt stress is an essential aspect of sustaining plant health and productivity. Deciphering ways and means of developing salinity tolerant crops can be a solution to mitigate the devastating plight of deteriorating crop yields due to salt accumulation (Reddy et al., 2017).

In this context, finding new candidate genes and alleles for overexpression in crops to improve salt tolerance while maintaining a steady yield is critical. Ribosomal proteins are known to be important in the establishment of protein synthesising machinery and the formation of rRNA structure. They are also necessary for all beings to thrive and develop. As salt stress may alter protein synthesis, overexpression of genes coding for ribosomal proteins in stressed plants has been found in a number of instances, suggesting that it could lead to reconstructing optimized protein synthesizing machinery (Moin et al., 2021a).

There have been sporadic reports of better salt tolerance under stressed conditions due to the expression/upregulation of genes encoding ribosomal proteins. In an attempt to analyze transcript abundance and expression profiling in salt-tolerant Pokkali variety of Rice by DNA microarrays, several ribosomal protein genes (including *RPS4*, *RPS7*, *RPS8*, *RPS9*, *RPS10*, *RPS19*, *RPS26*; *RPL2*, *RPL5*, *RPL18*, *RPL44*) were among the early responsive genes to be significantly upregulated during salt stress (Dai Yin et al.,

2005; Sahi et al., 2006). Salinity stress leads to compromised chloroplast functioning. It has been observed that salt stress impairs the protein synthesizing machinery in plastids. Salt stress-dependent upregulation of plastidial ribosomal protein gene *PRPL11* has been reported, which can be beneficial in obtaining better photosynthetic ability during the initial phase of salt stress in the plant (Omidbakhshfard et al., 2012). The increased expression level of 40S ribosomal proteins *S20*, *S24* and ribosomal protein *L34e* was also observed under NaCl treatment in the root tissues of *Tamarix hispida* (Li et al., 2009). Several ribosomal protein genes have also been induced in a salt-tolerant rice variety under NaCl treatment by subtractive suppression hybridization. This indicates that these genes might have roles in the salt adaptation/ tolerance process. In another attempt to identify functional and genetic orthologs of stress-responsive genes common to cyanobacteria, fungi and higher plants, a gene expression profiling was performed using salt-shock experiments to mimic salt stress conditions (Bohnert et al., 2001). In the root cDNA libraries comprising salt-stressed corn and rice samples, ribosomal proteins constituted one of the large groups of ESTs. Also, a remarkable number of RP genes, including *RPL34B*, *RPL23A*, *RPS24A*, *RPS13C*, *RPS6A*, *RPL9A* etc., were induced in yeast within 10-30 min post-stress. This comparison was thought to point out that the synthesis of ribosome and increase in transcripts for protein turnover and reconstructing protein synthesizing machinery to be the immediate consequence of salt shock in cells (Bohnert et al., 2001). Again, several RP genes were observed to be highly expressed in a comprehensive analysis of differential expression of genes expressed under Pi and Fe deficiency in *Arabidopsis* roots, which were, thus, predicted to be involved in salt stress recovery in plants (Wang et al., 2013). Ribosomal proteins are also involved in the efficient reconstruction of protein synthesizing machinery. Since salt stress leads to a massive adjustment of gene regulation and protein synthesis, ribosomal proteins play a crucial role in normalizing ribosome biosynthesis in such stressed conditions for the normal functioning of cell machinery and unhindered protein synthesis.

4.2 Generation, screening and selection of 35S:L6 lines

After the identification of the involvement of *RPL6* in enhancing WUE in rice (Moin et al., 2016b), the full-length cDNA sequence (681bp) of the *RPL6* gene (LOC_Os04g39700; accession, XM_015781380) was obtained from the Rice Genome Annotation Project database (RGAP-DB). The retrieved sequence was further verified through nucleotide and protein BLAST searches in RGAP-DB, NCBI and Hidden Markov Model (HMM) of Pfam databases. When the sequence of *RPL6* from all the databases showed identical matches, the primers were synthesized by incorporating *XhoI* and *NcoI* restriction sites at the forward and reverse ends of the cDNA sequence, respectively, for subsequent cloning steps. The *RPL6* sequence was PCR amplified from the cDNA of the

rice variety, BPT-5204. Initially, the pRT100 (Addgene, A05521) vector was double-digested with *Xho*I and *Nco*I restriction enzymes. In the next step, the *Xho*I and *Nco*I-treated *RPL6* sequence was ligated into the double-digested pRT100 vector. This step brought the *RPL6* in a transcriptional fusion with CaMV35S promoter and poly-A tail of pRT100 at its 5' and 3' ends, respectively, resulting in the *RPL6* plant expression cassette. The entire expression cassette with the promoter and poly-A tail was taken out as a *Pst*I fragment from pRT100 and cloned into the binary vector, pCAMBIA1300. This binary vector (pCAMBIA1300: *RPL6*) carrying the expression cassette of *RPL6* was mobilized into the *Agrobacterium tumefaciens* strain EHA105 for genetic transformation of rice. After confirmation of the vector and subsequent transformation in the EHA105 strain of *Agrobacterium tumefaciens*, it was used to transform a rice variety (BPT-5204) following an *in planta* approach of rice genetic transformation. The seedlings that grew from these transformed seeds are T₀ lines. The seeds from this T₀ plants were considered to constitute the T₁ generation, which was further advanced to T₂ and T₃ generations through appropriate selfing. The transformed seeds were allowed to grow on MS media containing the selection antibiotic hygromycin since the binary vector pCAMBIA1300 carried hygromycin as a marker for transformed plant selection. The plants growing on MS media and hygromycin were again confirmed by PCR with hygromycin resistance gene-specific primers. Since *L6* is a gene endogenous to the rice genome, *hptII* was used to confirm the transgenic nature of the plants. The plants showing an expected band of around 1.2 kb, shown in Figure 4.1, were considered putative positive ones and maintained for further analysis. Some of the positive plants of the T₃ generation were confirmed by Southern blot- hybridization to verify the number of T-DNA insertions in the host genomes. Among the eleven lines chosen for Southern-blot hybridization, six were observed to be positive with a single copy of T-DNA insertion (Figure 4.2)

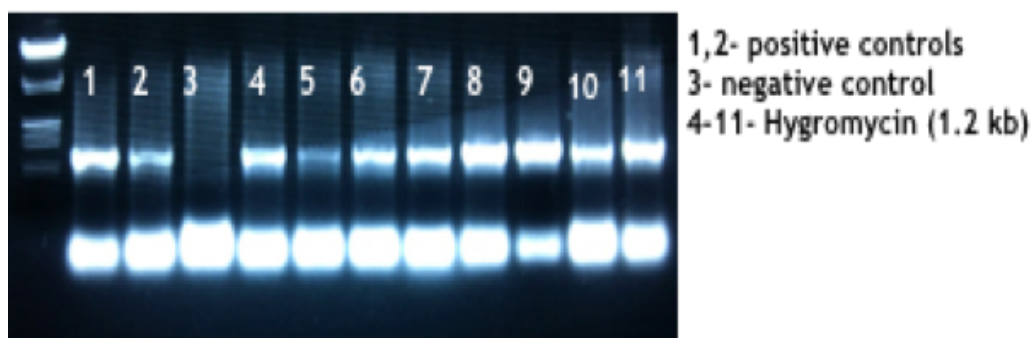


Figure 4.1: PCR with *hptII* specific primers for Screening of T₁ plants for putative positive lines.

The first two lanes were positive control, the third lane was negative control, and the 4th-11th lanes were positive with an expected 1.2 kb amplicon of *hptII* gene. The sizes labelled are according to λ *Eco*RI-*Hind*III marker.

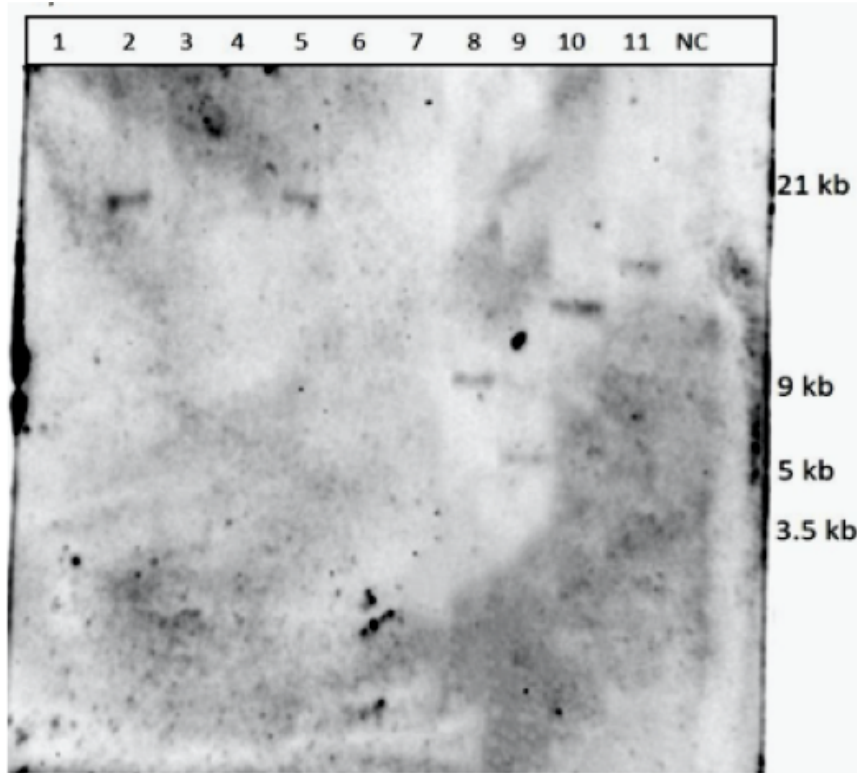


Figure 4.2: Southern-Blot Hybridization analysis of transgenic rice plants. Genomic DNA samples from T₂ generation transgenic plants (lanes 1–11) and NC were digested with the restriction enzyme *Bam*HI and hybridized with the DIG-dUTP labelled *hptII* gene probe. Transgenic samples in lanes 2, 5, 8, 9, 10 and 11 appeared positive with single-copy T-DNA insertion. NC, Non- transgenic Control; PC, Positive Control. The sizes labelled are according to λ *Eco*RI-*Hind*III marker.

4.3 Transcript analysis of *L6* gene in transgenic rice plants

The PCR positive lines were also checked for their transcript level of the *L6* gene with the help of Semi-Q and qRT-PCR analysis. The PCR positive plants of T₃ generation have been analyzed for their transcript level of the *L6* gene using semi-quantitative PCR with gene-specific primers along with a control set with housekeeping actin (*actII*) gene-specific primers. The observation of semi-Q analysis was verified with qRT-PCR data. The *RPL6* transcript levels in the transgenic lines were studied using qRT-PCR. Based on the transcript pattern, we got four high expression lines, and the rest have shown pale bands in semi-Q and lower fold change in qRT-PCR analysis. The transgenic lines with <2 fold of upregulation were considered low expression lines, and the ones with >20 folds were regarded as high expression lines. Based on the transcript level, *L6*-3, *L6*-5, *L6*-7 and *L6*-8 were considered as high expression lines with >20 and >5-fold change in root and shoot, respectively and *L6*-2, *L6*-4, *L6*-9 were considered as low expression lines. Among these, two lines *L6*-5 and *L6*-8, exhibited the highest transcript level of 37 and 32-fold in root and 9- and 5-fold upregulation in shoot tissue,

respectively. The semi-Q PCR analysis has been shown in Figure 4.3, and qRT-PCR data in form bar diagram has been demonstrated in Figure 4.4.

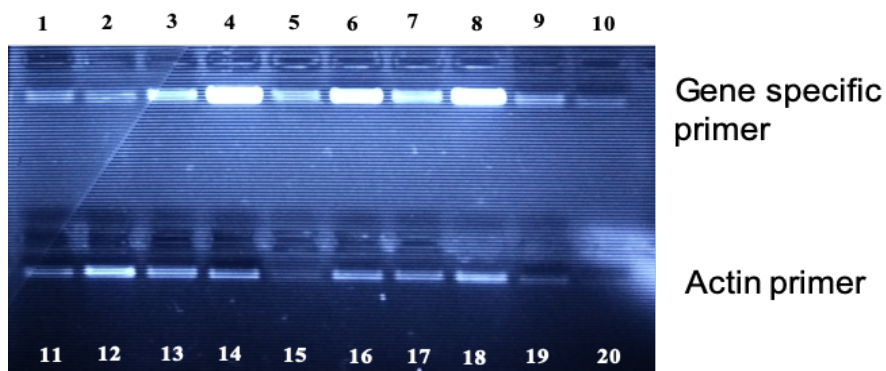


Figure 4.3: Transcript analysis using semi-Q PCR.

The PCR positive plants of T_3 generation have been analyzed for the transcript level of the *RPL6* gene using semi-quantitative PCR with *RPL6* gene-specific primers (upper lane marked 1-10) and along with a control set with *actII* gene-specific primers (lower lane marked 11-20). The plants showing broad bands with gene-specific primers were considered high expression lines, and subsequently, they were verified with qRT-PCR.

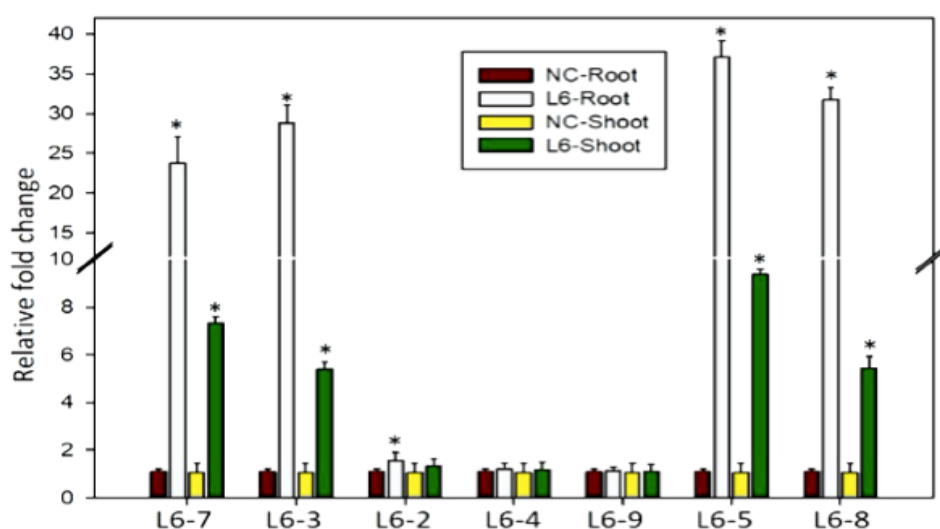


Figure 4.4: Transcript analysis using qRT-PCR.

The relative fold change of the *RPL6* gene in the selected PCR positive transgenic plants was studied using qRT-PCR by double normalization method with actin as a housekeeping gene. Based on transcript level, *L6-7*, *L6-3*, *L6-5*, *L6-8* were considered as high expression lines and *L6-2*, *L6-4*, *L6-9* were considered as low expression lines. The relative transcript levels were considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks in the bar diagram, which were prepared in SigmaPlot v.11

4.4 Evaluation of salt tolerance in *L6* transgenic seedlings

Seedlings from NC, high expression lines (*L6-3*, *L6-5*, *L6-7* and *L6-8*) and low expression lines (*L6-4* and *L6-9*) were exposed to moderate (150 mM) and high (200 mM) concentration of salt NaCl in MS liquid media for 3 and 5 days continuously. The seedlings from 150 mM stress did not show wilting or any significant phenotypic change after 3 days. Thus, for 150 mM stress, the result has been demonstrated after 5 days only. After 3 days of exposure to 150 mM stress, the NC has been observed to be wilted considerably, and slight wilting and stunted growth were observed in the low expression line *L6-4*, whereas the high expression lines *L6-5*, *L6-8* and *L6-3* have been observed to grow without much wilting.

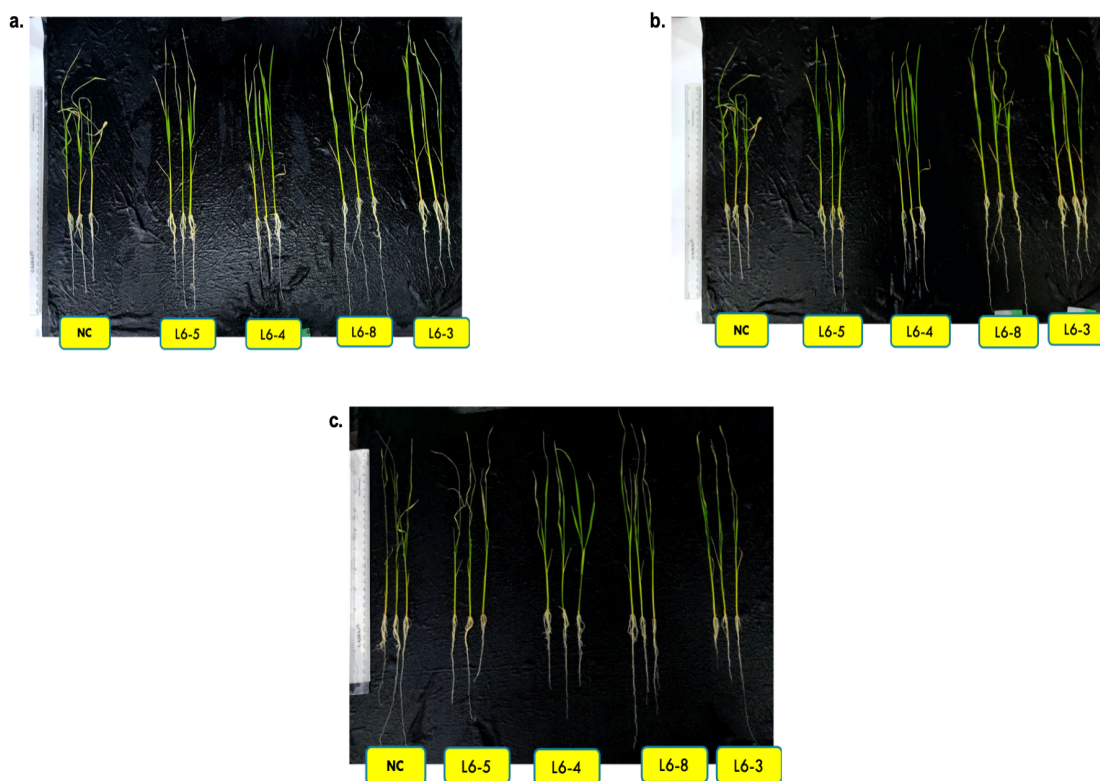


Figure 4.5: Transgenic seedlings post-salt stress

Two-week-old *RPL6* transgenic and Negative control (NC) seedlings were treated with 150 mM and 200 mM NaCl in liquid MS medium for 3 days and 5 days continuously in replicates of 3 seedlings. a) After treatment with 150 mM salt concentration for 3 days, negative control showed signs of wilting and drying. Not much difference was observed among high and low expression lines. b) After 3 days of exposure to 200 mM NaCl treatment, negative control showed wilting, yellowing and drying. Low expression line *L6-4* showed retarded growth compared to high expression lines *L6-5*, *L6-8* and *L6-3*. c) After 5 days of exposure to 200 mM NaCl treatment, the high expression lines *L6-5*, *L6-8* and *L6-3* seem to be significantly healthier than NC and low expression lines.

Under 200 mM of salt concentration, *L6-4* and NC showed stunted growth and considerable wilting after 3 days of exposure. After 5 days of exposure, wilting, leaf

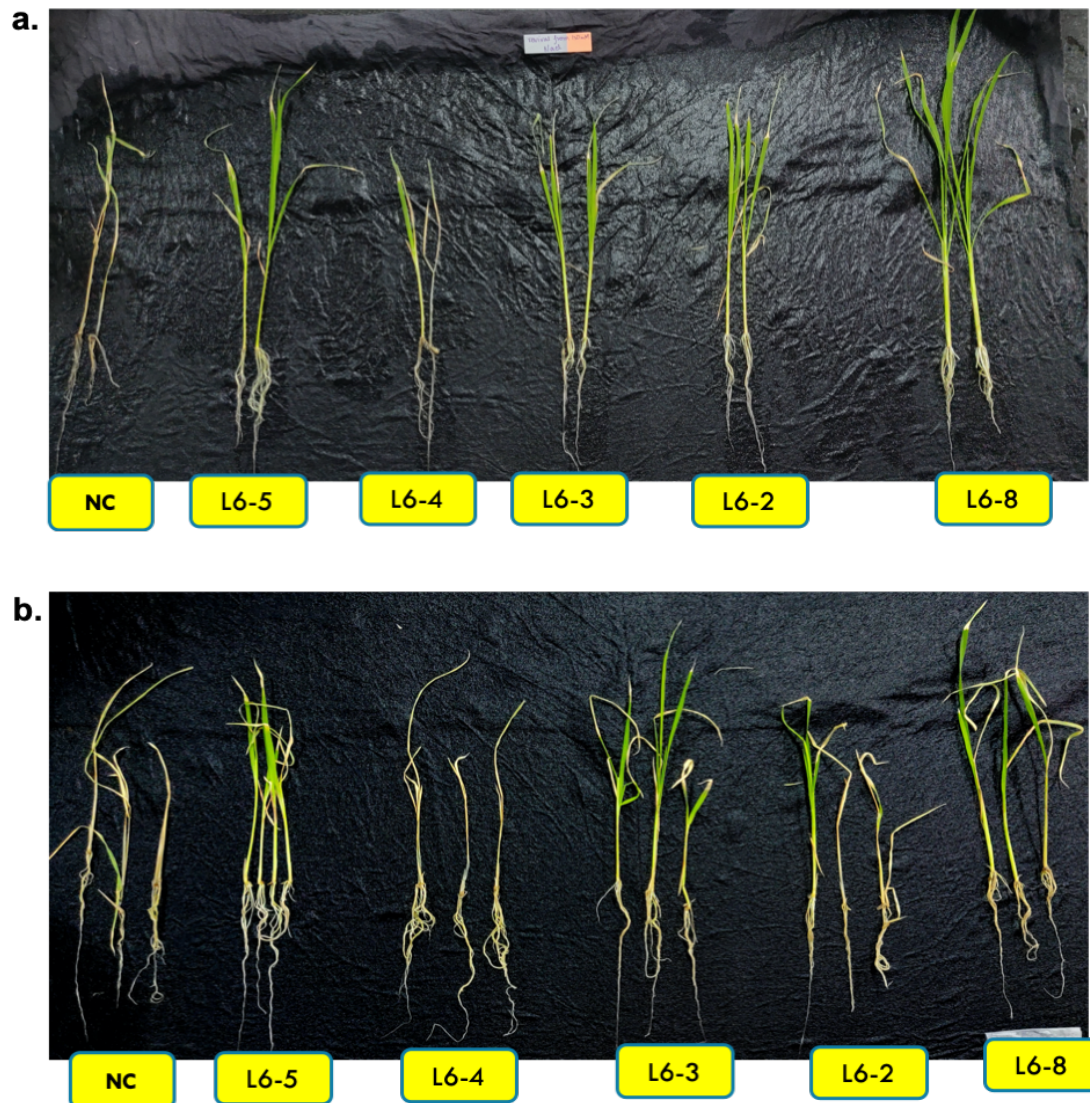


Figure 4.6: Transgenic seedlings after revival from salt stress

Two-week-old *RPL6* transgenic and Negative control (NC) seedlings after being treated with 150 mM and 200 mM NaCl in liquid MS medium for 5 days were continuously shifted to a salt-free MS medium allowed to recover. a) Transgenic lines and NC revived after 5 days exposure to 150 mM salt concentration- low expression lines *L6-2* and *L6-4* showed slight wilting, yellowing and stunted growth compared to the high expression lines. NC showed severe drying and wilting. b) Transgenic lines and NC revived after 5 days exposure to 200 mM salt concentration- low expression lines *L6-2* and *L6-4* and NC showed severe wilting, drying and yellowing compared to high expression lines *L6-5*, *L6-3*, *L6-8*, which seemed to be significantly healthier.

curling and yellowing were observed in the NC and low expression line *L6-4*, whereas the higher expression line appeared healthier. After NaCl treatments, all the seedlings were allowed to revive from the salt stress by shifting these seedlings to a salt-free medium. Readings were recorded when revived from both 150 mM and 200 mM salt stress. Under both the conditions, NC and low expression lines *L6-2* and NC appeared to be weak, wilted and curled, whereas high expression lines *L6-5*, *L6-3*, *L6-8* appeared

healthier. The low expression lines and NC further could not survive when transferred to pots under greenhouse conditions. The seedlings under 150 mM and 200 mM NaCl treatment for 3 and 5 days are demonstrated in Figure 4.5. The seedlings after revival from 150 mM and 200 mM salt stress after 5 days are shown in Figure 4.6. The phenotypic characteristics like Root length, shoot length, and fresh weight were measured and presented in bar diagrams by comparing the values with respective NC in Figure 4.7 and the parameters recorded after recovery from salt stress are represented in the form of bar diagrams in Figure 4.8.

Although much differentiation was not visible for shoot length in the high expression lines compared to NC and low expression lines, a significant difference was observed in root length and fresh weight between high and low expression lines along with NC. The readings were recorded under 150 mM and 200 mM salt stress and represented in bar diagrams in Figure 4.7. Similarly, distinct differences were observed in all parameters in the seedlings revived from 150 mM and 200 mM salt stress after 5 days with high expression (*L6-5*, *L6-8* and *L6-3*), showing better root length, shoot length and fresh weight measurements compared to low expression lines *L6-2* and *L6-4* and NC. Shoot length, root length and fresh weight were recorded for the transgenic lines and NC after revival from 150 mM and 200 mM salt post 5 days, and readings were represented in the form of bar diagrams in Figure 4.8.

4.5 Estimation of chlorophyll and proline contents in the transgenic lines

Chlorophyll content (Chlorophyll a, Chlorophyll b and Total chlorophyll) of NC, over-expression lines (*L6-5*, *L6-8*, *L6-3*) and low expression lines (*L6-2*, *L6-4*, *L6-9*) were measured under unstressed condition and represented in the form of Bar diagrams in Figure 4.9. Under the unstressed condition, chlorophyll contents were slightly higher in high expression lines than the NC and low expression lines. After revival from 200 mM salt stress, NC and the low expression lines did not survive except *L6-2*, and we have observed high chlorophyll content in high expression lines. Low expression line *L6-2* showed significantly less chlorophyll a, Chlorophyll b and total chlorophyll contents. Hence, it can be concluded that the chlorophyll content was significantly high in *L6-5*, moderately high in *L6-3* and *L6-8* and low in *L6-2* after recovery from salt stress. The high chlorophyll content is generally correlated with high quantum efficiency. Therefore, a better photosynthetic activity is integrally linked with sustainable yield in high expression lines.

Under normal unstressed conditions, the proline content in NC, high expression lines and low expression lines were 0.5, 0.9 and 0.3 $\mu\text{g}/\text{mg}$, respectively. After revival

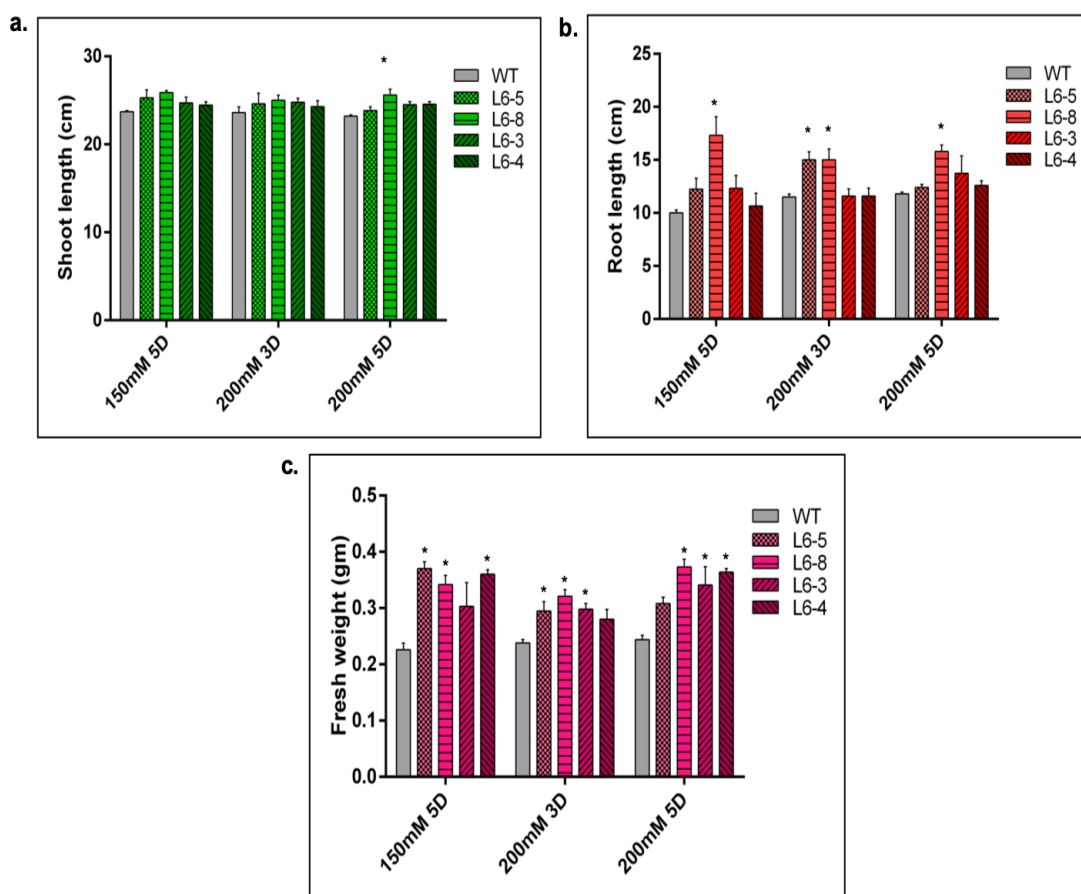


Figure 4.7: Phenotypic parameters of the seedlings under salt stress

The a) shoot, b) root and c) fresh weight of transgenic and NC seedlings were measured after 3 and 5 days of treatment with 150 mM and 200 mM NaCl concentration and compared with the negative control. The readings were recorded in replicates of three seedlings. A significant difference in root length and fresh weight is observed in transgenic lines post-exposure to stress. The asterisks in the bar diagrams represent statistical significance at $P < 0.05$ calculated through one-way ANOVA through SigmaPlot v.11.

from NaCl stress (200 mM and 150 mM), proline content in transgenic lines increased by several folds, with L6-5 having 1.6 $\mu\text{g}/\text{mg}$ and 2.3 $\mu\text{g}/\text{mg}$ proline after revival from 150 mM and 200 mM salt concentration, respectively. Hence, it can be seen that the proline content in the overexpression lines was considerably higher in the stressed samples. The proline content in unstressed and treated transgenic lines was represented in bar diagrams in Figure 4.10. To withstand salt stress, plants usually accumulate compatible solutes such as proline, whose enhanced concentration decreases the cytoplasmic osmotic potential facilitating water absorption and scavenging Reactive Oxygen Species (ROS).

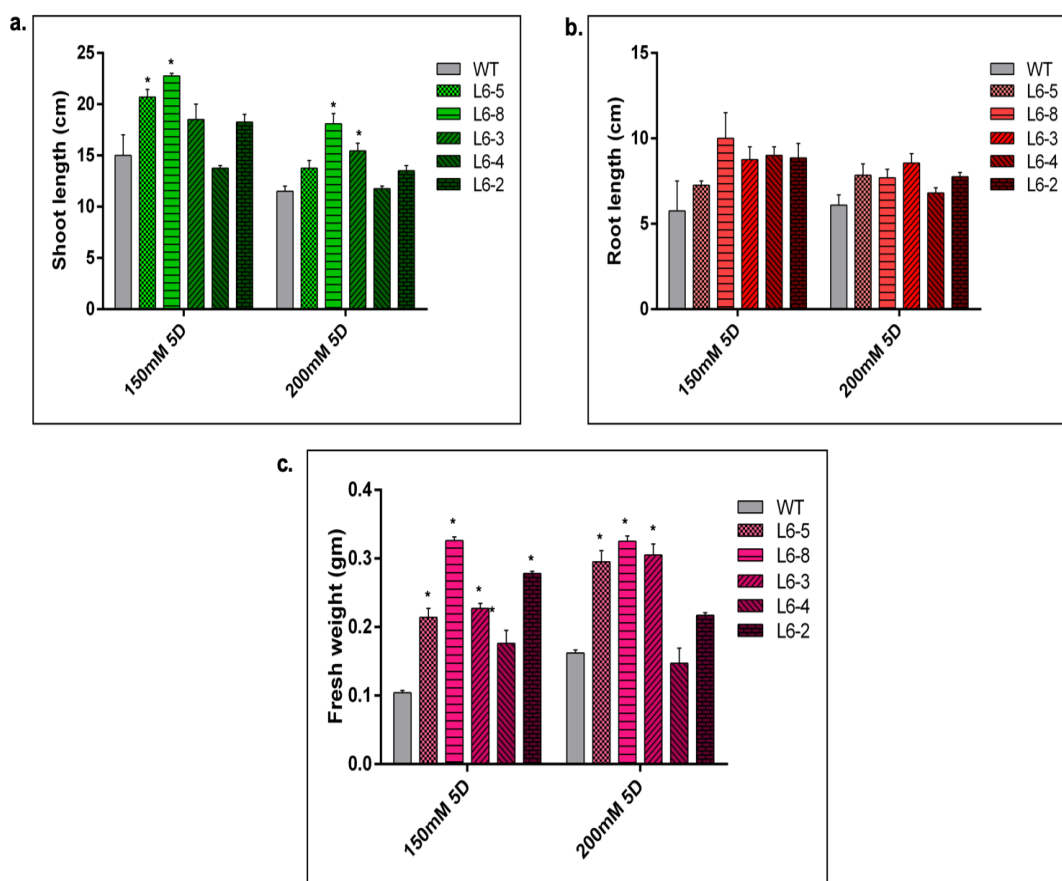


Figure 4.8: Phenotypic parameters of the seedlings post revival from salt stress

The a) shoot, b) root and c) fresh weight of transgenic seedlings and NC were measured after being revived from 150 mM and 200 mM salt treatment post 5 days. The readings were recorded in replicates of three seedlings. The high expression lines showed better root length, shoot length and fresh weight post recovery from salt stress than low expression lines and NC. The asterisks in the bar diagrams represent statistical significance at $P < 0.05$ calculated through one-way ANOVA through SigmaPlot v.11.

4.6 Photosynthetic efficiency of *RPL6* overexpression lines

The quantum efficiency of PSII in dark-adapted leaves of transgenic lines that were grown under normal circumstances without salt stress (untreated) and those that were recovered after the administration of 150 and 200 mM NaCl were assessed using MINI-PAM. The CF of WT rice was 0.76 in untreated conditions, but the CF of transgenic lines ranged from 0.70 (*L6-9*) to 0.78 (*L6-9*) under untreated conditions (*L6-5*). After 150 and 200 mM NaCl stress was applied, the quantum efficiency in a solely recovered low expression line, *L6-2*, was 0.62 and 0.52, respectively. After 5 d of NaCl treatment with 150 mM NaCl, the CF in three high expression lines, *L6-3*, *L6-5* and *L6-8*, was 0.68, 0.73 and 0.70, respectively, while the quantum efficiency in these three lines after 5 d of exposure to 200 mM was 0.63, 0.69 and 0.66, respectively. The readings were recorded and represented in the form of bar diagrams in Figure 4.11

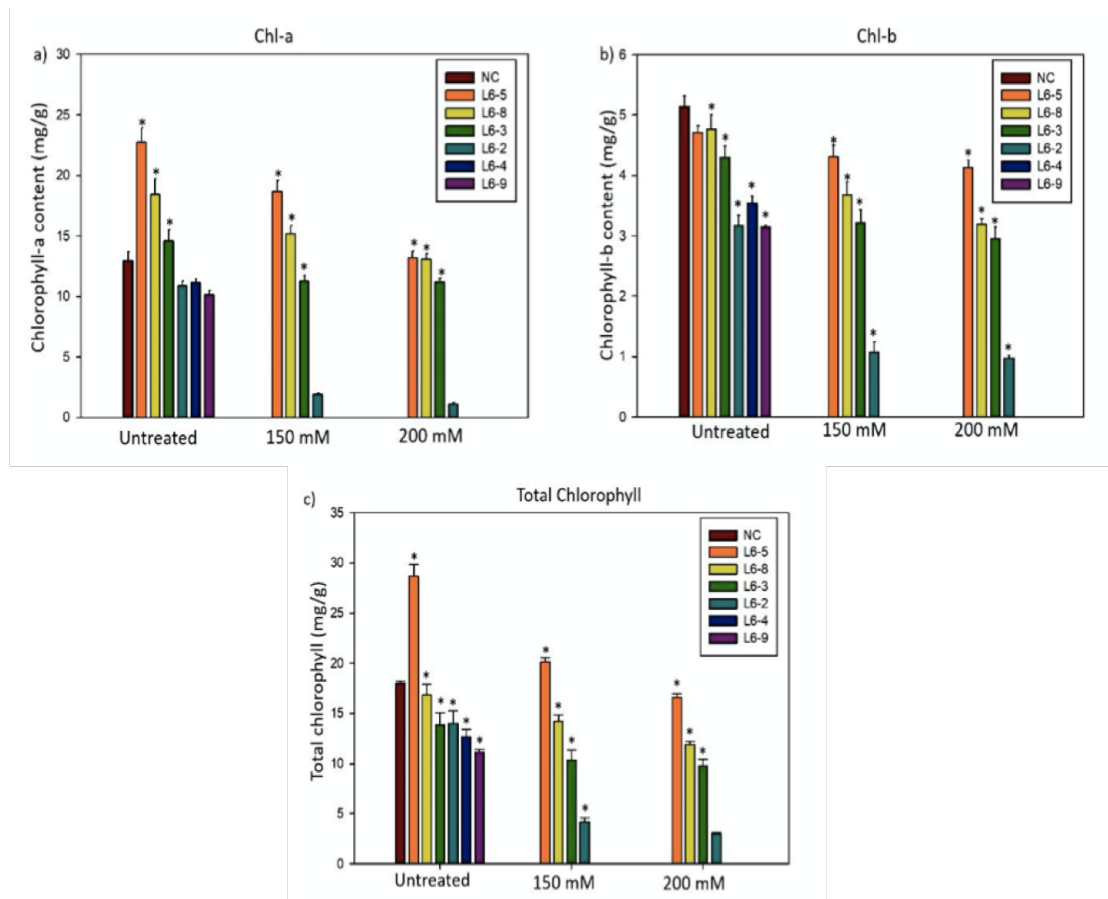


Figure 4.9: Estimation of Chlorophyll content

a) Chlorophyll a, b) Chlorophyll b, and c) total chlorophyll content of transgenic and NC plants were measured under normal conditions and after revival from 150 mM and 200 mM treatment with NaCl concentrations. The asterisks in the bar diagrams represent statistical significance at $P < 0.05$ calculated through one-way ANOVA through SigmaPlot v.11.

4.7 Transcript analysis of salt stress-specific genes

To check if the salt tolerance induced by the L6 is in association with the upregulation of some salt-stress specific genes, the transcript levels of some important genes such as *OsALDH2a*, *OsAOX1a*, *OsLEA3-1*, *OsbZIP23*, *OsWRKY72*, *OsDREB2B* and *OsSOS1* were analyzed through qRT-PCR. In earlier studies, these genes have been reported to be involved in immediate defence after the onset of salt stress in plants. Transgenic rice plants were evaluated for their transcript levels before and after 5 days of exposure to 200 mM NaCl. The transcript levels of these genes in transgenic lines were adjusted with untreated NC before treatment. These transcripts in high expression lines were normalised with respect to a recovered low expression line since NC did not recover following treatment with 200 mM NaCl. Under the normal condition, slight variations in transcript levels were noticed between transgenic lines and NC, with *OsLEA3-1* showing considerable up-regulation. After treatment, *OsLEA3-1*, *OsDREB2B* and *OsSOS1* showed remarkable elevation with >10-fold transcript levels in all the high expression

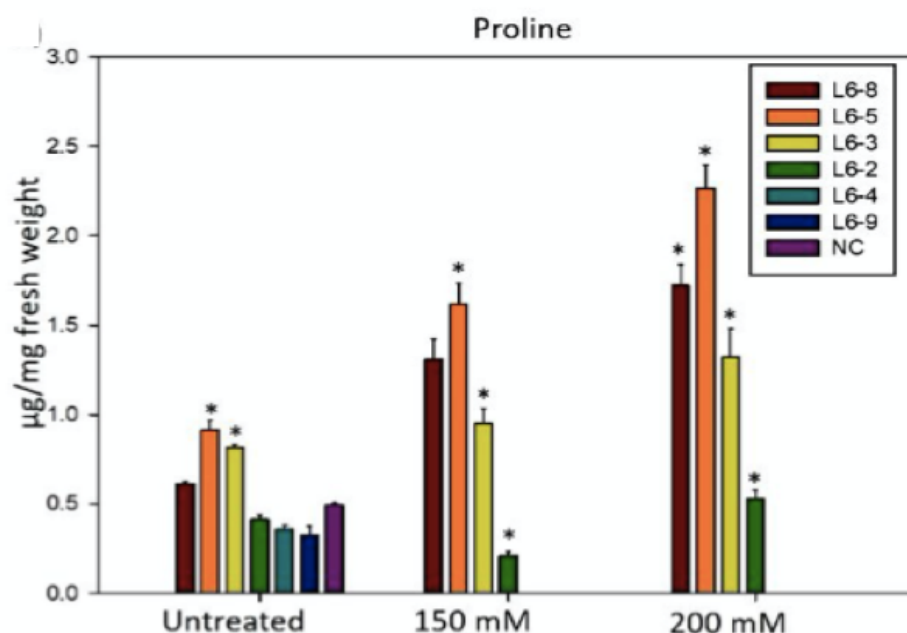


Figure 4.10: Estimation of proline content

Proline content of the transgenic and NC plants was measured under normal conditions and after revival from 150 mM and 200 mM treatments with corresponding NaCl concentrations. The asterisks in the bar diagrams represent statistical significance at $P < 0.05$ calculated through one-way ANOVA through SigmaPlot v.11.

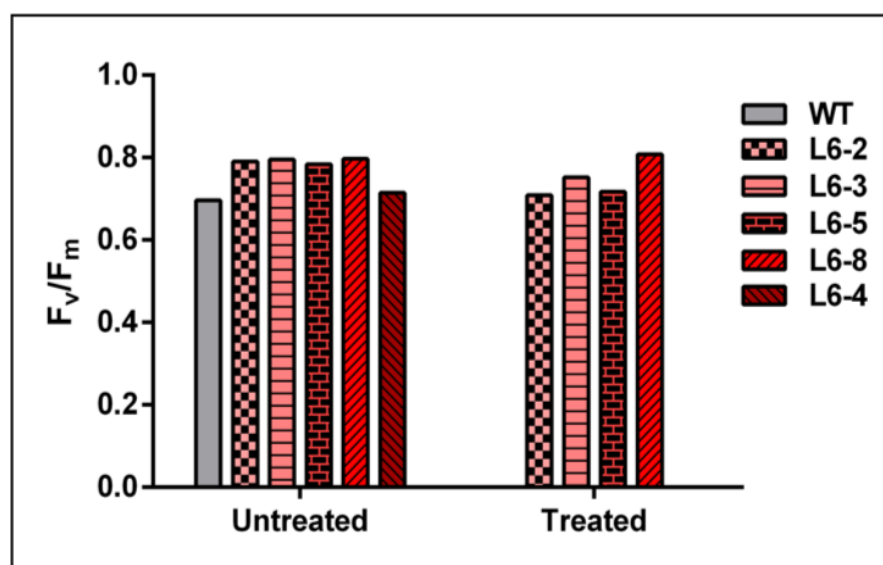


Figure 4.11: Chlorophyll Fluorescence (CF) using PAM fluorometer

Chlorophyll fluorescence (CF) was measured using a portable Pulse Amplitude Modulation (MINI-PAM). Measurements were recorded in triplicates in dark-adapted leaves of NC and transgenic lines under stressed conditions and after revival from 200 mM salt stress treatment for 5 days. Under the unstressed condition, CF of NC was 0.7, whereas the overexpression lines varied from 0.71-0.79. After revival from 200 mM salt stress, CF of the overexpression lines varied from 0.70-0.80.

lines. The transcript levels of *OsSOS1* was highest (50-fold) in L6-5. The SOS1 (salt overly sensitive 1) is a conserved Na^+/H^+ membrane antiporter and a Na^+ efflux protein. Ectopic expression of *OsSOS1* resulted in the complementation of extreme salt-sensitive defects of *sos-1 Arabidopsis* mutants indicating a significant role of SOS1 in imparting salt tolerance by possibly extruding the excess Na^+ ions at root epidermis and thereby maintaining Na^+ homeostasis (Martínez-Atienza et al., 2007; El Mahi et al., 2019). Transcriptional upregulation of *OsSOS1* and other genes in high expression lines probably maintains a low sodium concentration in the tissues (by Na^+ efflux). Because low salt concentration itself is not devastating to rice, it is the gradual build-up of internal sodium levels that causes cellular damage and growth defects (Yeo et al., 1991; Munns, 1993). Hence, maintaining a low internal sodium concentration might be a contributing factor for whole-plant salt tolerance. The transcript level of each of these genes in unstressed and revived transgenic and NC lines after 200 mM salt treatment for 5 days has been represented in the form of a bar diagram prepared using SigmaPlot v.11 (Figure 4.12).

4.8 Conclusion

Salinity has a tremendous impact on the growth and sustenance of several salt-sensitive plant species known as glycophytes, compared to salt-tolerant species known as halophytes. Since rice is a glycophyte, it is highly sensitive to salinity, and thus salt accumulation leads to a harsh response to rice cultivation. The transgenic rice plants overexpressing *RPL6* were also found to be tolerant to moderate (150 mM) and high (200 mM) salt concentration at the seedling stage when they were shifted to salt-free medium, and some of the seedlings from the high expression lines revived better than the negative control and the low expression lines. The salt-tolerant high expression lines also showed high chlorophyll contents, quantum efficiency, and proline accumulation and showed high transcript levels of several stress-responsive genes. This finding is in line with our previous finding where *RPL6* was one of the genes, which was significantly upregulated in the activation tagged mutant population of rice screened for water use efficiency (Moin et al., 2016b). Ribosomal proteins help maintain the structural integrity of ribosomes to ensure sustained and undisturbed protein synthesis even under stress. This can be hypothesized that upregulation of specific ribosomal protein genes is more beneficial in this aspect. This could be cardinal in inducing selective protein synthesis that triggers tolerance under adverse growth conditions, stress or continuous exposure to external stimuli. Our finding from this objective showed that higher expression of *RPL6* conferred salt tolerance on transgenic rice lines.

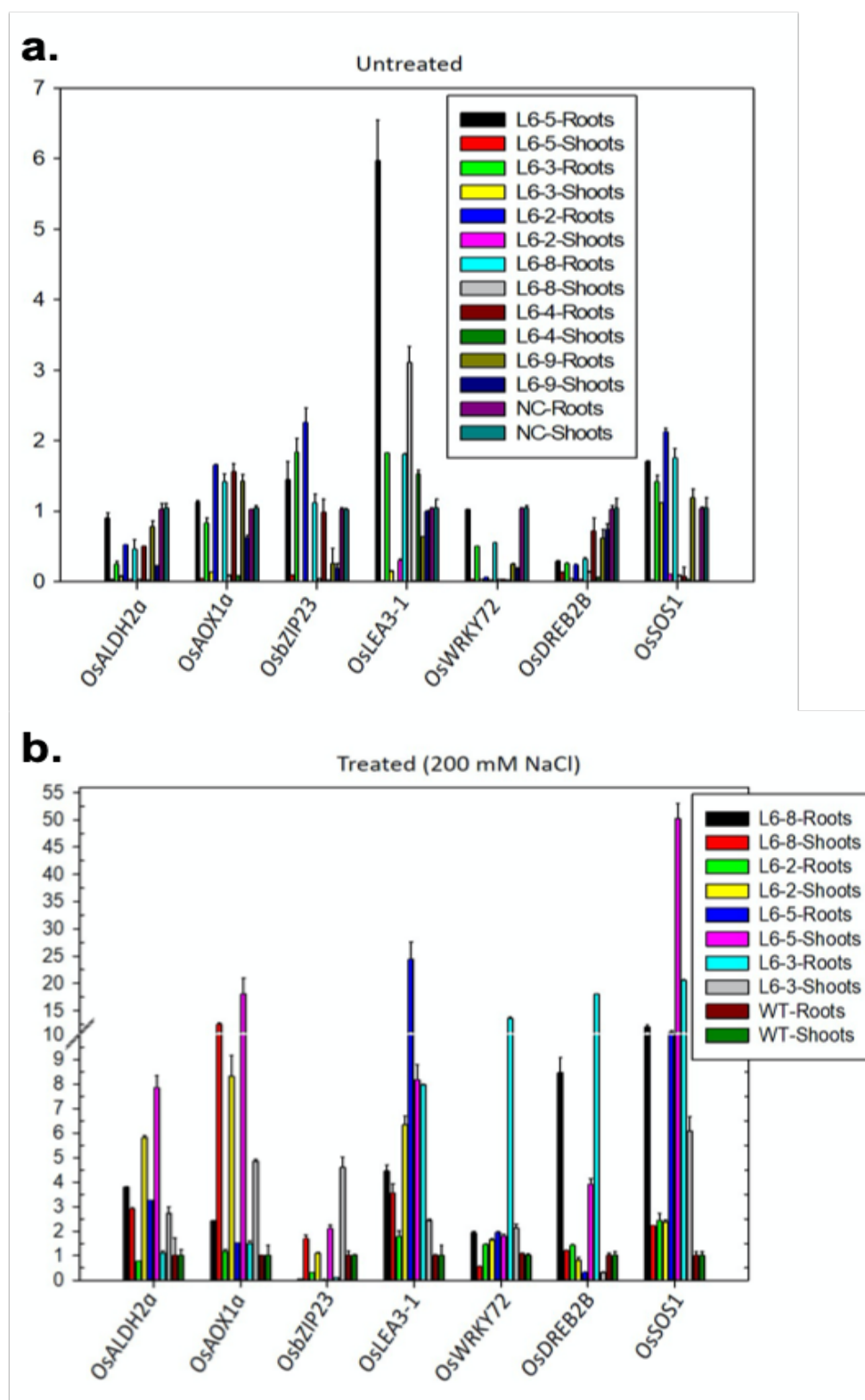


Figure 4.12: Expression analysis of stress-responsive genes

Transcript level of stress-responsive genes (*OsALDH2a*, *OsAOX1a*, *OsLEA3-1*, *OsZIP23*, *OsWRKY72*, *OsDREB2B* and *OsSOS1*) was determined in root and shoot samples of NC and transgenic lines under a) unstressed and b) stressed (200 mM NaCl stress for 5 days) samples using qRT-PCR analysis by double normalization method with actin as a housekeeping gene. The relative transcript levels were considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks in the bar diagram, which were prepared in SigmaPlot v.11

Chapter 5

Transcript analysis of RP gene expression in susceptible and resistant genotypes of *indica* rice challenged with insect pests, Brown Plant Hopper and Gall Midge

5.1 Introduction

Rice is a staple food crop consumed by about three billion people in Asia. Biotic stresses affecting the rice crop can impact growth, yield, grain quality and sustainability in a devastating fashion. The defence responses of rice under changing environmental conditions, including biotic stress, need to be elucidated to curtail the enormous global losses of crop yield. Biotic stress is brought about by pathogens such as fungi, bacteria, viruses and insects (Wang et al., 2011). The concept of susceptible and resistant plants depends on the specificity of diseases caused by these stressors. This specificity again depends on the method through which plants recognize the pathogens. The general perception about plant and pathogen interactions suggests that plants have the innate ability to resist fungal and bacterial pathogens. This is manifested in the form of the principles that directly and indirectly hinder the invasion of their impending attack. Infection promotes activation of metabolic pathways leading to the formation of substances that can interrupt the growth and survival of the pathogen. The defence responses are elicited in plants in response to stimuli from the pathogen, which are in the form of polysaccharides, small proteins or chemicals. These constitute the molecular patterns associated with herbivory (HAMP) and or damage (DAMP) (Abdul Malik et al., 2020). Plants recognize these molecules through receptors on the cell membrane and trigger an immune

response called pattern triggered immunity (PTI). In response, insects secrete specialized chemicals called effectors that can nullify PTI and allow the insect to feed and develop on the plants (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Tsuda and Katagiri, 2010; Boller and He, 2009). The second level of defence is induced by plants in response to the effectors and is termed effector-triggered immunity (ETI) (Tsuda and Katagiri, 2010). This defence is specific and involves both constitutive and induced responses with or without hypersensitive reaction (HR), oxidative burst, signal transduction, hormone regulation and systemic acquired resistance in the host (Cui et al., 2015; Hatsugai et al., 2017). Some of these involve secondary metabolism and the synthesis of defence chemicals that are not essential for the normal growth, development and reproduction of the plant but have the capacity to counter the attack. Thus, there is a compromise between primary and secondary metabolism when the pathogens and pests attack a plant (Hatsugai et al., 2017).

Insect resistance has long been proposed to consist of components like non-preference or antixenosis, antibiosis resulting in reduced fitness of the infesting insect and tolerance of the host to the insect attack (Cartier and Painter, 1956). While the first two components constitute the active plant defence system detailed above, the tolerance component accounts for the ability of the plant to compensate for the damage inflicted by the insect and maintain near normal growth, development and yield. This component has been least studied in detail. This primary metabolism-mediated insect resistance in rice may involve basic functions like photosynthesis and protein synthesis. In such a situation, the ribosomal proteins (RPs) might play a significant role as constituents of the protein synthesis apparatus. The present investigation aims to study this angle of insect resistance by monitoring a subset of RP coding genes.

RPs are considered to have a crucial function in the development of rRNA structure and the protein-synthesizing machinery in cells. These are also necessary for all organisms' growth and development. The formation of a ribosomal complex requires co-transcription, coordinated expression and assembly of hundreds of proteins (Moss et al., 2007). The ribosomal DNA (rDNA) exists as tandemly arrayed repeats separated by spacers at nucleolus organizing regions (NORs) on the chromosomes. The nucleolus is the site of transcription of rDNA, processing of primary transcripts into different rRNAs and assembly of pre-ribosomal subunits, which together form the linchpins of ribosome biogenesis (Tschochner and Hurt, 2003; Grummt, 2013; Sáez-Vásquez and Delseny, 2019). The rRNAs and RPs together maintain the structural stability of ribosomes that perform diverse cellular activities required for organismal growth and development (Ferreira-Cerca and Hurt, 2009). Thus, cells must normalize the ribosome biosynthesis process for their normal functioning to cope with the changing growth conditions and continuous exposure to external stimuli, which occur at different stages of the life cycle in an organism (Tschochner and Hurt, 2003; Kressler et al., 2010). Any

imbalance in ribosome biosynthesis or mutations in rRNA and RP-coding genes result in perturbed plant phenotypes, some of which might also be lethal to cells. Although there are no significant reports on the contribution of RP genes in conferring resistance to pests, we have identified potential Ribosomal Protein Large (RPL) subunit genes (*RPL23a* and *RPL6*) to be significantly upregulated in studies for enhanced water-use efficiency (WUE) in *indica* rice following a gain of function mutagenesis through the activation tagging approach (Moin et al., 2016b). Subsequently, we studied the regulatory roles of all the RPL and RPS genes under different external stimuli and observed the significant up-regulation of a considerable number of these genes under several stresses, including abiotic and biotic conditions in rice (Moin et al., 2016a; Saha et al., 2017). In the present investigation, we made a transcript analysis of selected RP genes (as selected in our previous studies) in susceptible (BPT-5204) and resistant (RPNF05) genotypes of *indica* rice challenged by two major insect pests, BPH and GM. We tried to identify the potential candidate RP genes specifically or commonly upregulated in one or both the above genotypes upon insect infestation.

5.2 Analysis of putative promoter regions of RP genes

For each of the selected RP genes, including RPL and RPS genes, the nucleotide sequences of 1.0 kb upstream regions from the transcription start sites have been retrieved and evaluated for the presence of *cis*-regulatory elements (CRE). Based on earlier reports, regulatory elements having an interrelation with wound signalling have been pointed out in this study. Most of these regulatory regions have been observed to possess elements responsive to phytohormones methyl jasmonate, salicylic acid and ethylene. In addition, motifs involved with wound signalling have also been detected. The list of the elements present in the putative promoter regions of each of these genes was presented in Table 5.1. Analysis of the putative promoter regions of all the RP genes suggested the presence of numerous regulatory elements. Fourteen RPL and twenty five RPS genes exhibited CGTCA motif; twenty one RPL and twenty six RPS genes carried TCACG motifs. These two motifs are associated with responsiveness to methyl jasmonate. Nine RPL and four RPS genes showed the W-Box motif, and one RPL and two RPS genes have the WUN motif. These two motifs are interconnected with wound signalling. Eleven RPL and fifteen RPS genes have exhibited the presence of one TCA element, which is associated with salicylic acid responsiveness. Three RPL and four RPS genes have one ERE element, which is an Ethylene-Responsive element. All these elements were present within the 1.0 kb region upstream to the transcription start site each gene analyzed.

Table 5.1: List of wound-responsive *cis*-elements present in the putative promoter regions of RP genes

<i>Cis</i>-elements	RPL genes	RPS genes
W-Box (wound-induced signalling)	RPL3, L4, L6, L8, L11, L18P, L23A, L30e, L34	RPS7, S9, S23, S25.
WUN- motif (wound-induced stress)	RPL23a	RPS10a, S24.
TCA element (SA responsive)	RPL3, L4, L10, L18a, L18P, L24a, L30e, L31, L32, L34, L35	RPS4, S4a, S7a, S9, S13, S13a, S17, S18, S23a, S24, S25a, S26, S27, S27a, S28
ERE (ethylene-responsive element)	RPL27.3, L26.1	RPS9, S13a, S27, S28
TGACG motif (MeJA responsive)	RPL6, L8, L10, L11, L13b, L14, L18a, L18P, L19.3, L21.2, L23a, L24, L26.1, L28, L31, L32, L34, L35, L36, L38, L44.	RPS3a, S4, S4a, S5, S6, S6a, S7, S9.2, S10, S10a, S13, S15, S15a, S18, S18a, S19, S23, S23a, S24, S25, S25a, S26, S27, S27a, S29, S30
CGTCA motif (MeJA responsive)	RPL10, L11, L14, L15, L18a, L21.2, L23a, L26.1, L30e, L31, L32, L36, L38, L44	RPS3a, S4, S4a, S5, S6, S6a, S7, S9.2, S10, S10a, S13, S15, S15a, S18, S18a, S19, S23, S23a, S24, S25, S25a, S26, S27, S27a, S29

5.3 Differential expression patterns of RPL genes in response to BPH and GM

The identification of ribosomal protein large and small subunit gene family members in rice has been described earlier. All the RP genes were included (except the homologous ones) to study their response to infestation by BPH and GM pests at two different time points. The genes that exhibited 2 fold transcript level were considered as upregulated. The original *P* values obtained from two-way ANOVA were adjusted with a Bonferroni correction. The term ‘significance’ means statistical significance at $P < 0.05$ from *P* adjusted values. Altogether, the transcript level of all the RPL genes in both genotypes of rice exhibits downregulation of more RPL genes post-infection with BPH and GM compared to their upregulation. Around 80% of RP genes were downregulated in RPNF05 than in BPT-5204 (Figure 5.1).

In RPNF05, infection with BPH at 6 hai showed *RPL15* and *RPL51* genes to be up-regulated with 15-fold and 4-fold transcript levels, respectively, whereas the rest of the thirty three RPL genes were downregulated. Eighteen of the downregulated genes have shown significant reduction in their transcript levels, including *RPL3*, *RPL4*, *RPL7*,

RPL11, *RPL12*, *RPL18a*, *RPL19.3*, *RPL21.2*, *RPL23A*, *RPL24b*, *RPL27.3*, *RPL29*, *RPL30e*, *RPL31*, *RPL32*, *RPL34*, *RPL36.2* and *RPL51*. At 120 hai, four genes have shown significant upregulation of *RPL15*, *RPL22*, *RPL26.1* and *RPL38* with 10-fold, 6-fold, 23-fold and 14-fold transcript levels. Two of the genes, *RPL7* and *RPL11*, have shown moderate (3-fold) upregulation. Comparing the similar data in BPT-5204 variety, we noticed that eleven RPL genes were upregulated and twenty four RPL genes were downregulated at 6 hai with BPH infestation. In contrast, about 85% of the RPL genes (30 genes) were upregulated, and only five genes remained downregulated or unaffected at 12 hai with BPH. At 6 hai in BPT-5204, *RPL4*, *RPL7*, *RPL10*, *RPL12*, *RPL15* and *RPL22* have been observed to have significant transcript levels of >10 fold with BPH infection. Further, among the genes that got downregulated, *RPL14*, *RPL19.3*, *RPL27.3*, *RPL31*, *RPL32*, *RPL34*, *RPL35a.3*, *RPL36.2* were significant. Now at 12 hai, fourteen out of thirty upregulated genes have >10 fold upregulation, and these genes were *RPL3*, *RPL7*, *RPL11*, *RPL13a*, *RPL13b*, *RPL18a*, *RPL19p*, *RPL19.3*, *RPL21.2*, *RPL22*, *RPL23a*, *RPL26.1*, *RPL27.3*, *RPL28*, *RPL29*, *RPL31*, *RPL32*, *RPL36.2* and *RPL38*. After BPH infection in BPT-5204, *RPL7*, *RPL12*, and *RPL22* had high upregulation at both the time points (6 and 12 hai). Among them, *RPL7* and *RPL22* were significantly induced. Interestingly, *RPL51* was significantly upregulated in RPNF05 and downregulated in BPT-5204 at 6 hai. Similarly, *RPL15* was significantly upregulated in RPNF05 but significantly downregulated in BPT-5204. Graphical representation of the differential transcription response of the individual RPL genes in response to BPH is shown in Figure 5.2.

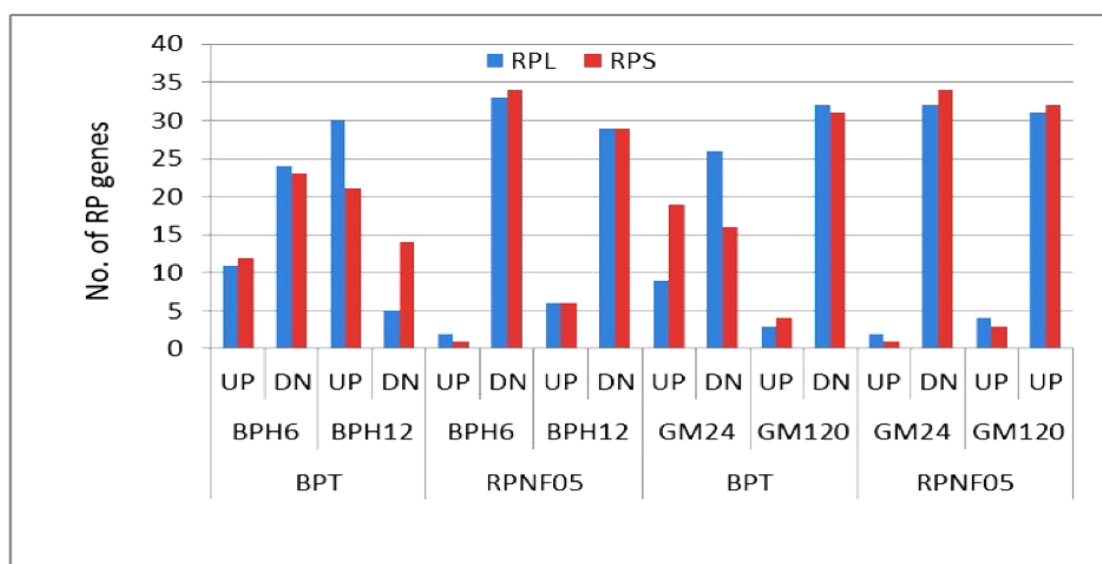


Figure 5.1: Transcript profiles of RPL and RPS genes

Overall, transcript profiles of 35 RPL and RPS genes in both the susceptible BPT-5204 and the resistant RPNF05 following infestation with either BPH or GM revealed a predominant number of genes to be downregulated in comparison to the number that was upregulated. UP, upregulated; DN, downregulated.



Figure 5.2: Graphical representation of the differential transcription response of the individual RPL genes upon infestation with BPH

To understand the transcript patterns of individual RPL genes upon infestation with BPH, bar diagrams were constructed for each RPL gene separately. The data on the y-axis is the relative fold change calculated using the $\Delta\Delta C_T$ method, whereas treatments are indicated on the x-axis. The asterisks in bar diagrams represent statistical significance at $P < 0.05$ obtained after adjusting probability values of two-way ANOVA with Bonferroni correction. Different coloured grids at the bottom of each figure correspond to the type of treatment. Bar diagrams were generated using statistical program R v.3.6.3, and SigmaPlot v.11 was used to draw the bar diagrams and calculate two way ANOVA, respectively. *UT, untreated; BPH, brown planthopper.

In BPT-5204 at 24 hai after GM infestation, seven genes became upregulated, and this included *RPL4*, *RPL6*, *RPL13b*, *RPL21.2*, *RPL22*, *RPL27.3* and *RPL30e*. Out of these, *RPL22* and *RPL4* exhibited 10 and 19-fold upregulation, respectively. Similarly, the downregulation of *RPL3*, *RPL5*, *RPL18p*, *RPL24a*, *RPL24b*, *RPL26.1*, *RPL36.2*, *RPL37* and *RPL44* genes was significant. At 120 hai, *RPL30e* exhibited the highest upregulation of 62-fold, whereas four genes (*RPL5*, *RPL29*, *RPL31* and *RPL34*) showed moderate upregulation. In RPNF-05, two genes (*RPL15* and *RPL18a*) were moderately upregulated at 24 hai following GM infestation. A significant downregulation was observed in transcript levels of *RPL8*, *RPL11*, *RPL18a*, *RPL18p*, *RPL21.2*, *RPL23a*, *RPL24a*, *RPL26.1*, *RPL27.3*, *RPL32*, *RPL35a.3*, *RPL38*, *RPL44* and *RPL51*. At 120 hai following GM infestation, sixteen genes were significantly downregulated. Among the upregulated ones, *RPL15*, *RPL36.2* and *RPL38* were significantly upregulated, and *RPL15* showed the highest upregulation (50-fold), but these genes were downregulated

in BPT-5204. The upregulation of *RPL18a* was specifically noticed in RPNF05, which was downregulated in BPT-5204. Graphical representation of the differential transcription response of the individual RPL genes in response to GM is shown in Figure 5.3. Also, *RPL15* was significantly induced in RPNF05 at 6 hai with BPH and 120 hai with GM but was downregulated at 120 hai with GM in BPT-5204. The transcripts of *RPL14*, *RPL23a*, *RPL24a* and *RPL24b* were commonly downregulated in response to BPH and GM infestations in BPT-5204 and RPNF05 genotypes. Of these, the downregulation of *RPL14* at 6 hai and *RPL24b* at 12 hai with BPH in BPT-5204 was significant, which was also significantly noticed at both the time points with GM infestation in RPNF05.

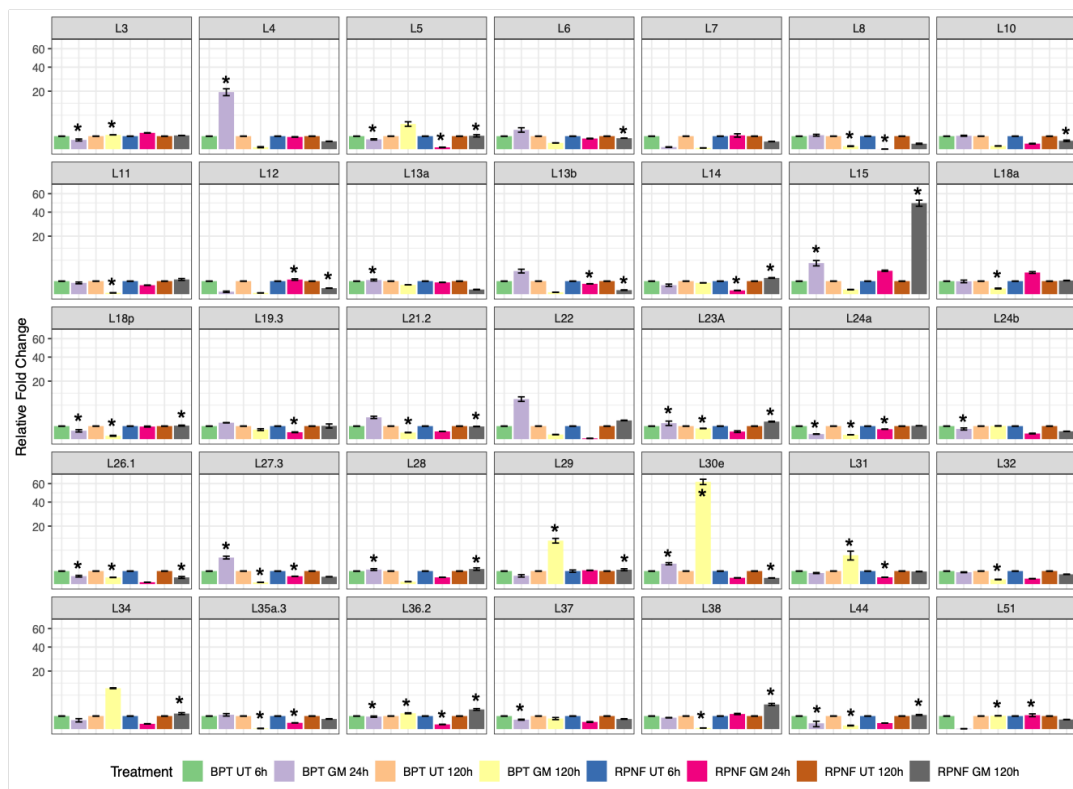


Figure 5.3: Graphical representation of the differential transcription response of the individual RPL genes upon infection with GM

To understand the transcript patterns of individual RPL genes upon infestation with GM, bar diagrams were constructed for each RPL gene separately. The data on the y-axis is the relative fold change calculated using the $\Delta\Delta C_T$ method, whereas treatments are indicated on the x-axis. The asterisks in bar diagrams represent statistical significance at $P < 0.05$ obtained after adjusting probability values of two-way ANOVA with Bonferroni correction. Different coloured grids at the bottom of each figure correspond to the type of treatment. Bar diagrams were generated using statistical program R v.3.6.3, and SigmaPlot v.11 was used to draw the bar diagrams and calculate two way ANOVA, respectively. *UT, untreated; GM, gall midge.

5.4 Differential expression pattern of RPS genes in response to BPH and GM

The overall response of RPS genes was similar to that noticed for RPL genes, with a majority of them being either downregulated or unaffected in response to either BPH or GM infestations in both susceptible BPT-5204 and resistant RPNF05 genotypes. Remarkable exceptions were at 24 hai with GM in BPT-5204, where a higher number of RPS genes were activated than the number of downregulated genes. This is in contrary to the RPL genes. However, like in RPL genes, many RPS genes were also upregulated in BPT-5204 at 6 hai following BPH infestations. Among the induced genes, more than 10-fold upregulation was noted in 12 genes (*RPS4a*, *RPS5*, *RPS6*, *RPS6a*, *RPS13*, *RPS13a*, *RPS18*, *RPS21*, *RPS23a*, *RPS24*, *RPS25a* and *RPS27a*) at 6 hai with BPH in BPT-5204 and a significant upregulation was observed for the genes *RPS5*, *RPS13*, *RPS18*, *RPS21*, *RPS23a*, *RPS25a* and *RPS27a*. At 12 hai, the transcripts of *RPS5*, *RPS10*, *RPS13a*, *RPS17*, *RPS23a*, and *RPS25* were significantly upregulated, whereas *RPS4*, *RPS4a*, *RPS5a*, *RPS6a*, *RPS7*, *RPS9-2*, *RPS18*, *RPS18a*, *RPS18b*, *RPS19*, *RPS21*, *RPS23*, *RPS27*, *RPS28* and *RPS30* were significantly downregulated. In RPNF05, only *RPS23a* became significantly upregulated (5-fold) at 6 hai, whereas six genes such as *RPS5*, *RPS5a*, *RPS6*, *RPS13*, *RPS23a* and *RPS25a* were upregulated at 12 hai. Among these, the highest induction was noticed in the transcripts of *RPS23a* (37-fold) and *RPS25a* (13-fold) and a significant upregulation were observed in *RPS5* and *RPS6*. Graphical representation of the differential transcription response of the individual RPS genes in response to BPH is shown in Figure 5.4. The transcript levels *RPS5a* (5-fold) were specifically upregulated at 12 hai with BPH in RPNF05 but was downregulated in BPT-5204 suggesting its possible role in resistance.

Nineteen RPS genes got upregulated following GM infection at 24 hai in BPT-5204. Four of the upregulated genes have shown more than 10-fold upregulation, including *RPS4*, *RPS6*, *RPS13* and *RPS13a*. Overall significant upregulation was observed for ten genes viz., *RPS4*, *RPS6*, *RPS7a*, *RPS13a*, *RPS15*, *RPS18b*, *RPS21*, *RPS23a*, *RPS24* and *RPS25a*, whereas only four genes (*RPS7*, *RPS10a*, *RPS15* and *RPS18*) showed activation at 120 hai. Graphical representation of the differential transcription response of the individual RPS genes in response to GM is shown in Figure 5.5. *RPS15* was consistently upregulated at both time points. In RPNF05, the transcript level of *RPS25a* was upregulated at 24 hai, whereas *RPS5*, *RPS9.2* and *RPS25a* were upregulated at 120 hai, but were downregulated in BPT-5204. The up and downregulation of *RPS5* in RPNF05 and BPT-5204, respectively, were significant.

In summary, seven of the RP genes viz., *RPL51*, *RPL15*, *RPL18*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* were shortlisted for their possible involvement in insect resistance. These genes were selected because of their specific upregulation in resistant genotypes



Figure 5.4: Graphical representation of the differential transcription response of the individual RPS genes upon infection with BPH

To understand the transcript patterns of individual RPS genes upon infestation with BPH, bar diagrams were constructed for each RPS gene separately. The data on the y-axis is the relative fold change calculated using the $\Delta\Delta C_T$ method, whereas treatments are indicated on the x-axis. The asterisks in bar diagrams represent statistical significance at $P < 0.05$ obtained after adjusting probability values of two-way ANOVA with Bonferroni correction. Different coloured grids at the bottom of each figure correspond to the type of treatment. Bar diagrams were generated using statistical program R v.3.6.3, and SigmaPlot v.11 was used to draw the bar diagrams and calculate two way ANOVA, respectively. *UT, untreated; BPH, brown planthopper.

at a given time point in either of the two pests. The differential transcript levels of RPL and RPS genes were also depicted in the form of heatmaps (Figure 5.6). Each grid in the map corresponds to a particular RP gene, with the dark-coloured ones exhibiting higher transcript levels whereas, the pale-coloured grids represent weak or no induction. We have also shortlisted the genes that exhibited an overlap in transcript levels or those commonly up or downregulated. Venn diagrams were used to describe this overlap in transcript patterns (Figure 5.7).

5.5 Discussion

Under this objective, we have performed a differential transcript analysis of the genes encoding for Ribosomal proteins large and small subunit genes following infestation by two devastating insect pests BPH and GM, in resistant and susceptible genotypes of

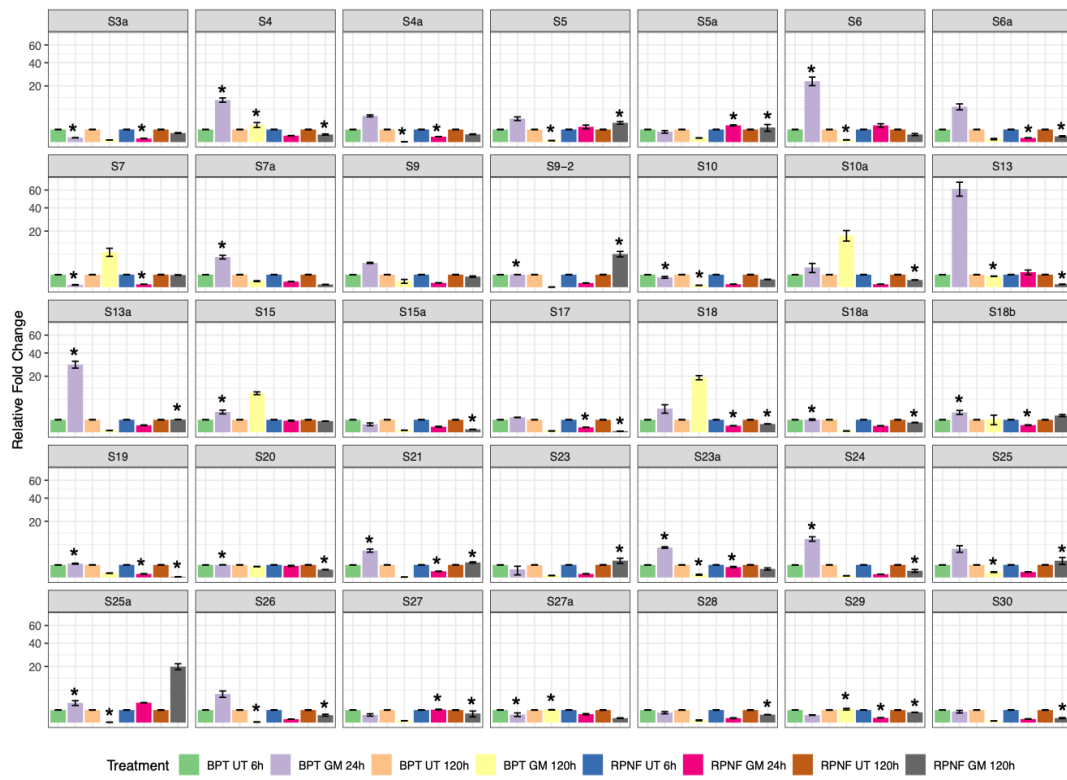


Figure 5.5: Graphical representation of the differential transcription response of the individual RPS genes upon infection with GM

To understand the transcript patterns of individual RPS genes upon infestation with GM, bar diagrams were constructed for each RPS gene separately. The data on the y-axis is the relative fold change calculated using the $\Delta\Delta C_T$ method, whereas treatments are indicated on the x-axis. The asterisks in bar diagrams represent statistical significance at $P < 0.05$ obtained after adjusting probability values of two-way ANOVA with Bonferroni correction. Different coloured grids at the bottom of each figure correspond to the type of treatment. Bar diagrams were generated using statistical program R v.3.6.3, and SigmaPlot v.11 was used to draw the bar diagrams and calculate two way ANOVA, respectively. *UT, untreated; GM, gall midge.

indica rice. We have shortlisted the upregulated genes in response to the insect infestation in each genotype. We have segregated these genes that were upregulated in both the genotypes and the ones that got induced specifically in the resistant genotype (RPNF05). The upregulation of many RPL genes was noticed in the susceptible genotype (BPT-5204) compared to the resistant genotype (RPNF05) against BPH. This indicates that these genes are unlikely to be associated with resistance against BPH. There are some RPL genes for which we have noticed an overlap in the transcript pattern in both BPT-5204 and RPNF05 when infected with GM. This suggests that there is no possible role for these genes in susceptibility or resistance. Some of the common genes like *RPL7*, *RPL12* or *RPS6*. *RPS13* and *RPS23a* against BPH or *RPS23a* against GM were activated in both rice genotypes, also indicating no possible role for them in plant-insect interactions. Some genes like *RPL15*, *RPL51*, *RPL18*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* were specifically activated in the resistant genotype RPNF05 but not in

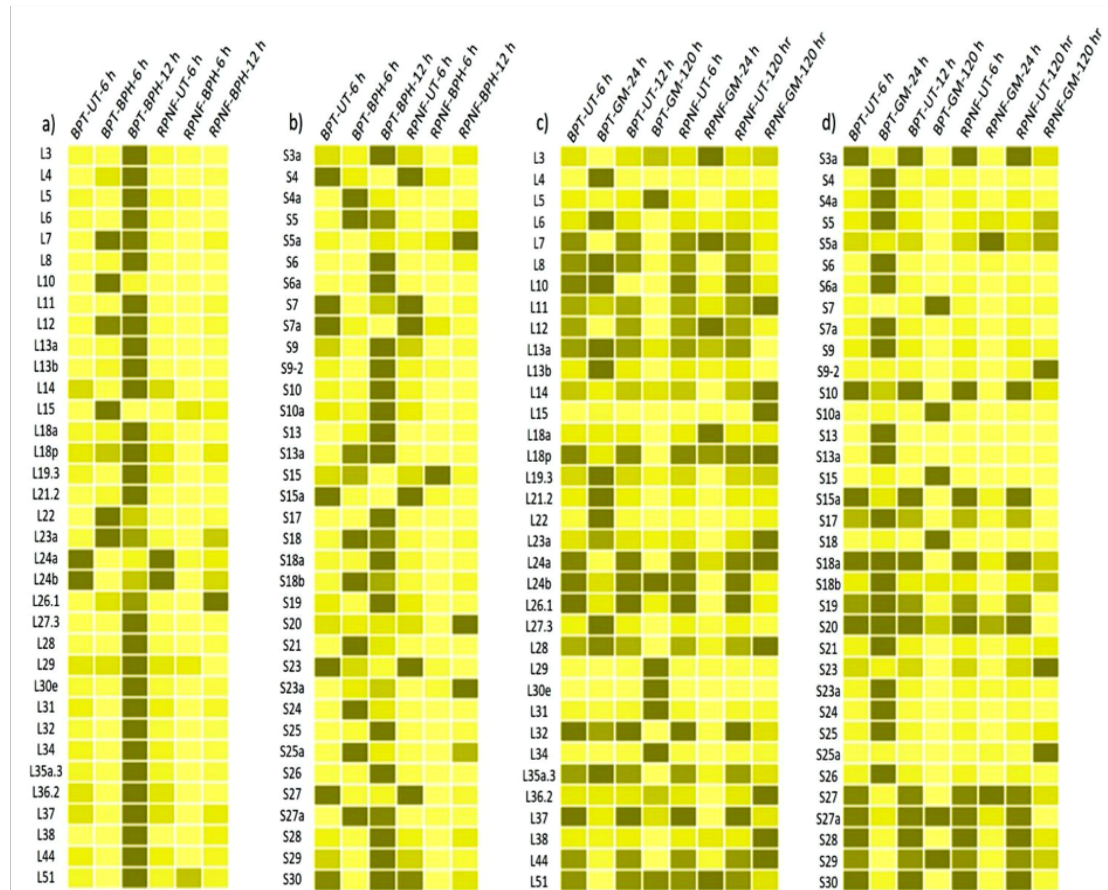


Figure 5.6: Heat map depiction of differential expression of RP genes upon infestation with BPH and GM

Heat maps were used to represent the transcript levels of RPL and RPS genes infested with (a, b) BPH and (c, d) GM pests. The transcript abundance was denoted with dark-coloured grids, whereas; the pale-coloured grids represent transcript inadequacy. Heat maps were generated using an online program, Morpheus. The asterisks in the grids represent statistical significance at $P < 0.05$ calculated through two-way ANOVA using SigmaPlot v.11 and adjusted with Bonferroni correction. . *UT, untreated; BPH, brown planthopper; GM, gall midge.

BPT-5204 in response to either BPH or GM. *RPS23* was highly induced in RPNF05 compared to BPT-5204 against BPH at 12 hai. This observation suggests a possible role for them in resistance against BPH and GM. *RPL15* was also found to be highly upregulated in response to bacterial blight (Moin et al., 2016a). *RPL15* carries a MeJa responsive element in its putative promoter region. Similarly, among the specifically upregulated genes in RPNF05, *RPL18a* has also been observed to have SA and MeJa responsive elements in their putative promoter regions like TCA, CGTCA and TGACG, which are related to wounding or mechanical injury inflicted by pests. Interestingly, this gene was also upregulated in abiotic stress treatments in our earlier study (Moin et al., 2016a). Further, *RPS5a* was upregulated in RPNF05 at 12 hai with BPH and *RPS5*, *RPS9.2* and *RPS25a* were upregulated at 120 hai with GM. Putative promoter regions of these three genes also exhibit both the Me-Ja responsive motifs (CGTCA and TGACG).

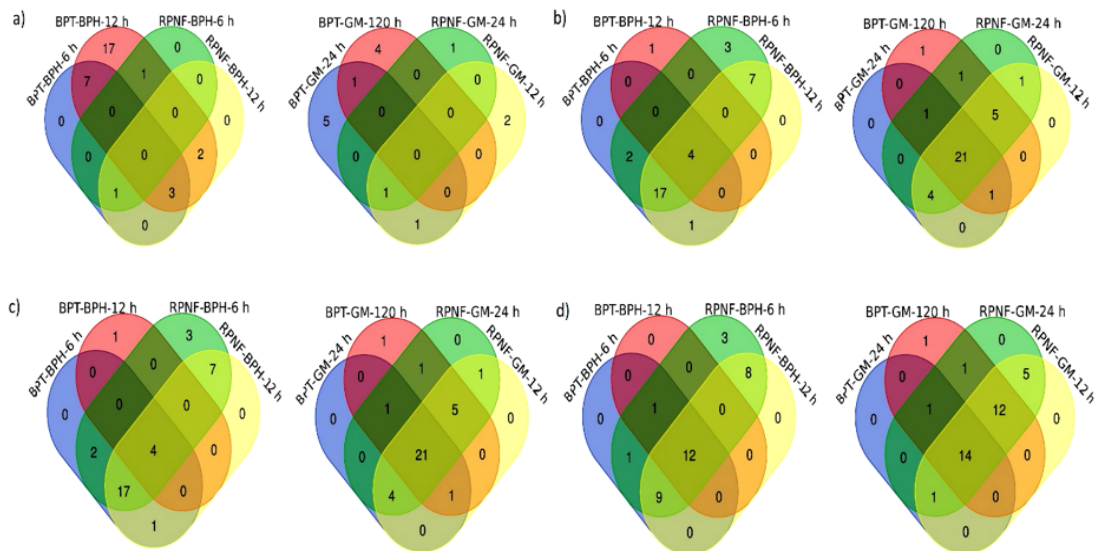


Figure 5.7: Venn diagrams representing the temporal up and downregulation of RP genes post infection with BPH and GM

The Venn diagrams were used to depict the total number of common (a) up and (b) downregulated RPL and (c) up and (d) downregulated RPS genes at 6 and 12 h after BPH and 24 and 120 h of GM infestation in BPT-5204 and RPNF05 genotypes of rice. *BPH, brown planthopper; GM, gall midge.

Ribosomal proteins interact with a network of other proteins to elicit defence-related signals under stressed conditions (Yang et al., 2019). Our observations demonstrating upregulation of specific RP genes in resistant genotype of rice when challenged with BPH and GM and the presence of *cis*-regulatory elements specific to stress responses also indicate the possible role of RP genes in providing resistance against these pests. Differential expression of specific RP genes under stress is a probable indication towards particular deviation from canonical ribosomal composition to facilitate the translation of stress-responsive proteins. The genes like *RPL15*, *RPL51*, *RPL18a*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* that were specifically upregulated in the resistant genotype might directly be involved in defence-responsiveness, and subsequent signal transduction pathways or they are probably associated with primary metabolism mediated tolerance component during pest infestation by modulating photosynthetic efficiency and increasing the vigour of the plant. It has been well reported that a tolerance component of resistance is vital in rice against BPH infestation (Panda and Heinrichs, 1983). The tolerance component is accompanied by the ability of a genotype to compensate for the loss inflicted by the insect due to feeding. This is generally achieved by inducing the photosynthetic rate and primary metabolism in rice (Panda and Heinrichs, 1983). The upregulation of RP genes solely in susceptible genotype (BPT-5204) in response to BPH can be explained in the context of the tolerance component. The RP genes induced in BPT-5204 under BPH attack are probably involved in enhancing protein synthesis to scale up the production and transport of primary metabolites to cope with the emergent

stress condition. *RPL22* showed more than 40-fold upregulation in BPT-5204 at both time points while it was not induced or poorly induced in RPNF05 at the corresponding time points. Besides, *RPS6a*, *RPS17*, *RPS21* among the RPS genes exhibited more than 25-fold upregulation in BPT-5204 with no such activation in RPNF05. Although comprehensive greenhouse studies are needed to confirm the defence and tolerance related roles of each of these RP genes, the differential induction of RP genes under pest attack with specific upregulation of a few genes in the resistant genotype strongly suggests their possible involvement in stress responsiveness.

Chapter 6

Discussion and summary of the work

6.1 Impact of stress on crop yield and identification of potential stress-responsive genes

For most crop plants, only 20% of the yield is achievable (Boyer, 1982). Factors influencing limitations in crop yield are abiotic stresses such as excess and scarcity of available water, extreme temperatures, salinity, heavy metal accumulation, and biotic stressors like infection by microbial, fungal and viral pathogens, weeds, and insects. Plants, being sessile, are constantly under the threat of exposure to the above-mentioned stress conditions. To counter these stresses, plants implement various physiological, metabolic, biochemical and molecular mechanisms (Todaka et al., 2012). Approximately 90% of all the arable land is vulnerable to at least one of the abiotic stressors mentioned above (Dos Reis et al., 2012). About 70% of the significant food crop yields are lost to the abiotic stress conditions (Mantri et al., 2012). According to estimates based on the combination of climate change and crop yield models, the productivity of important crops such as rice, wheat and maize would continue to decline, thereby posing a substantial threat to sustainability and food security (Tigchelaar et al., 2018). Yields are expected to decline by 7.4% for maize, 3.1% for soybean, 6% for wheat and 3.2% for rice for every degree increase in global mean temperature (Zhao et al., 2017). As the Earth temperature continues to warm up, meeting the global food demand for around 10 billion people will become increasingly complicated by the middle of the 21st century. The most catastrophic agricultural consequences will be felt in the tropics, which falls under the most food-insecure population belt in the world (Tigchelaar et al., 2018). In addition to the economic and nutritional values, rice is considered a significant model crop because of its smaller genome size. This characteristic feature of the rice genome has enabled the availability of the high-quality genome sequence and other essential resources in molecular biology studies (Jung et al., 2008; Goff et al., 2002; Yu et al., 2002). Complete genome sequences have facilitated genome-wide ex-

pression profiling and transcriptome analysis, which have helped in understanding the responses of various genes under abiotic stresses, relationships among several transcripts, and the role of *cis*-regulatory elements in stress responses. All these findings have pointed towards the existence of a complicated regulatory network influencing stress tolerance in plants (Hirayama and Shinozaki, 2010). Multiple stress signals regulate stress-inducible genes, and few of them are controlled by transcription factors (TFs) activated by these stress stimuli. Genes responsive to stress can be classified broadly into two groups. A subset of stress-inducible genes codes for specific proteins that are directly involved in stress tolerance. Other stress-inducible genes, such as signal transducers, encode regulatory proteins that regulate stress responses through positive and negative feedback loops (Hirayama and Shinozaki, 2010). A wide range of plants, including *Arabidopsis* and Rice, have been found to have a vast number of abiotic stress-sensitive genes. As discussed earlier, these stress-induced genes play a role in protecting cells from stress by producing key metabolic proteins and regulating genes, particularly transcription factors that are involved in signal transduction in stress responses. These transcription factors are responsible for mediating the expression of numerous downstream target genes under stress conditions. This kind of regulation is attained through *cis*-regulatory elements present in the promoter regions of the genes involved (Gao et al., 2007). Rice, among other crops, is subject to a variety of abiotic stress factors. Drought is a major environmental stress that causes a drop in agricultural productivity worldwide, and in order to meet demand, massive efforts are being made to boost crop yields. Drought impacts plant growth, development, membrane integrity, photosynthetic efficiency, osmotic adaptations and water content in various ways (Wani and Sah, 2014). After drought, salinity is the second most pervasive soil concern in rice-growing nations, and rice is severely impacted during the early seedling stage due to salinity finally limiting its productivity (Lutts et al., 1995; Todaka et al., 2012). One of the goals of investigating abiotic stress responses in plants is to improve stress tolerance through genetic manipulation. All of these abiotic stresses are complex and quantitative traits governed by multiple genes. Also, an important aspect of stress responses in plants is the interaction between abiotic and biotic stress responses. Moreover, plants in the fields are subjected to a variety of stress conditions simultaneously, and the constraints can last for a long time. Plant cells have been observed to undergo unanticipated physiological alteration due to combinations of abiotic and biotic stress conditions. Thus, rice cultivars that can withstand more than one stress condition at the same time while maintaining optimal yield levels constitute the need of the hour (Wani and Sah, 2014). Numerous breeding initiatives have been adopted to boost the production of rice under drought and salt stress. Traditional breeding methods such as hybridization, hybrid breeding, ideotype breeding have led to several salt and drought-resistant rice varieties in the Philippines, Bangladesh and India, but the success rate in

conventional breeding programmes is limited (Ismail et al., 2007). On the other hand, plant transformation techniques have allowed for the production of better crop plants by the introduction of cloned genes. Although gene transformation in *japonica* rice is a common practice, the constraints in genetic transformation in *indica* rice varieties are more challenging (Wani and Sah, 2014). The transformation protocol in *indica* rice developed by our research group during our earlier studies has totally eliminated the necessity for tissue culture, and the transgenic plants obtained may be compared to wild type in terms of genome constitution (Moin et al., 2016b). Under stress, plants adjust their level of expression of numerous genes in response to the damage inflicted by the stress. The transcriptional changes lead to lucrative advantages in adaptations that help the plant withstand and adjust to the impending stress. Genome expression profiling is becoming an indispensable tool to explore how each plant responds to environmental stress conditions. Expression profiling can differentiate between tolerant and sensitive responses in changing patterns of the expression of the genes involved. Following that, functional analysis of identified candidate genes through knockout/ overexpression is critical in confirming their role in abiotic stress responses (Hazen et al., 2003).

One such approach using activation tagging by our research team led to identifying potential candidate genes for water use efficiency. The transgenic plants carrying *Ds* stable element with tetrameric enhancers in the activation tagging approach were allowed to grow under the water-limited condition to screen for plants with enhanced water-use efficiency. We selected five plants out of 500 mutants generated based on quantum efficiency, low $\Delta^{13}\text{C}$ values, and revealing better growth and productivity. These mutants were used to study the expression of candidate genes situated within the 20 kb stretch on the rice genome on either side of the enhancer integration on the chromosome. It was found that only one of the three or four genes in the 20 kb region was up-regulated in four mutant lines, with two genes getting activated in one plant, demonstrating that the multiple enhancers can activate genes near their integration sites (Moin et al., 2016b). The activation of transcription factors (*WRKY96*, *GRAS*), protein engaged in ubiquitination (*cullin4*) and ribosomal biogenesis (*RPL6* and *RPL23A*) was discovered by analyzing the transcripts of the target genes investigated in these five mutants. This finding intrigued us as ribosomal protein genes have mostly been considered to have housekeeping roles. Subsequently, we studied the regulatory role of all the RPL genes under different external stimuli (Moin et al., 2016a). Significant upregulation of a considerable number of RPL genes under stress led us to explore the developmental and stress-regulated expression pattern of Ribosomal protein small subunit (RPS) genes (Saha et al., 2017). We functionally characterized one of the potential candidate genes (*RPL6*). Further, we performed a comprehensive transcript analysis of the entire RP gene family in susceptible and resistant rice genotypes challenged by two major insect pests, Brown Plant Hopper and Gall Midge. Our results from these investigations are

presented here.

6.2 Ribosomal Protein Small Subunit (RPS) genes are stress and developmentally regulated

The contribution of Ribosomal proteins in ribosome biogenesis and housekeeping functions was well validated previously in animal, plant and bacterial systems. Specific mutations in RPs have resulted in physiological and developmental defects in organisms. The physiological abnormalities resulting from RP knockouts imply the potential extra-ribosomal role of Ribosomal proteins (Lu et al., 2015). We have performed a comprehensive expression analysis of Ribosomal protein small subunit genes (RPS) in rice under multiple abiotic and biotic stresses and compared the expression patterns of Ribosomal Protein Small Subunit (RPS) genes with that of Ribosomal Protein Large (RPL) genes. We have short-listed the noteworthy RPS and RPL genes combinations of candidate genes that may effectively alter stress tolerance in rice based on the differential and native gene expression data. Our expression analysis has been backed up with *in-silico* analysis of the RPS protein properties and *cis*-regulatory elements present on the putative promoter regions of the studied genes. Combining our previous study related to RPL genes (Moin et al., 2016a), we have attempted to provide extensive information on the stress-responsive activation of all the ribosomal protein genes, including small and large subunits. Accessibility of a high-quality genome sequencing database has enabled us to retrieve genomic, CDS, protein sequences, protein structures and 5'-upstream regions of the genes we intended to study. A total of 56 RPS gene sequences were found from the database search, out of which 34 gene sequences were short-listed after removing the identical members. RPS genes seemed to be distributed evenly across the rice genome, with at least one RPS gene family member present on each of the 12 rice chromosomes. The majority of the amino acids in the RPs are positively charged, which is consistent with the theory that positively charged amino acids play a substantial role in electrostatic protein-RNA interactions inside the ribosomal complex (Law et al., 2006). The RPs are predicted to have comparable isoelectric pH ranges to aid their molecular interactions because they are primarily involved in interactivities that constitute the ribosomal complex and rRNAs. Proteins having higher isoelectric points interact with negatively charged nucleic acid molecules such as DNA, RNA, and other biomolecules, whereas proteins with lower isoelectric points avoid non-specific interactions with nucleic acids (Takakura et al., 2016). RPS6 has been shown to interact with a plant-specific histone deacetylase-2B and nucleosome assembly protein-1 in a recent study on controlling rDNA transcription in *Arabidopsis* (Son et al., 2015; Xiong and Sheen, 2015). RPS6 may benefit from having a low isoelectric point in order to reduce

non-specific interactions.

Native expression profiling of 13 different tissues revealed upregulation of RPS genes in mostly 45 d old mature leaves, shoots, 7 d shoot, plumules and root-shoot transition. Some RPS genes were also activated in tissues like panicle, endosperm, grain, roots and embryo. Interestingly, no RPS gene was activated in flowers. This could indicate that RPS gene expression regulates developmental stages mainly in vegetative tissues and not much in reproductive tissues. Promoter activity of *RPS18A* has been observed in *Arabidopsis* meristematic and embryonic stages, and mutation in their loci led to the development of pointed leaves (Van Lijsebettens et al., 1994). This observation can be correlated with induction of the genes *RPS18*, *RPS18a*, *RPS18b* in embryo, plumules, shoot, leaves and root-shoot transition in our study. Around 60% of the RPS genes in shoot and 20% of the genes in root were immediately up-regulated under all stress conditions (ABA, PEG, NaCl and H₂O₂). These genes are speculated to encode non-specific master regulators essential for plant core environmental stress response proteins, and their activation might be an instant reaction to the stress. The immediate-early response to particular stress may be non-specific at times, while responses that include persistent upregulation are deemed specific (Kilian et al., 2007). Furthermore, the genes that are up-regulated at about 1 h time period are thought to play a role in reassembling the protein synthesis machinery (Ouyang et al., 2007). As a result, we have looked for a pattern in the upregulation of immediate-early response RPS genes.

RPS13a, *RPS18a*, *RPS4* and *RPS4a* are only a few of the genes with their upregulation sustained throughout the stress. Following that, *RPS29* demonstrated a remarkably high-transcript level (≥ 100 fold) in roots when exposed to ABA, PEG, as well as a moderate level of expression (11-fold) when exposed to H₂O₂. *RPS28* was continuously expressed at a relatively high level in root tissues under all stress conditions. *RPL23A*, *RPL6*, *RPL8*, *RPL19.3*, *RPL38*, among the large subunit gene family members, have repeatedly demonstrated up-regulation in response to ABA, PEG and NaCl treatments. Under oxidative stress, *RPL18a*, *RPL24a*, *RPL24b*, *RPL30* and *RPL34* were substantially up-regulated (Moin et al., 2016a). Further, *RPL23A*, one of the candidate genes identified in water use efficiency screening using an activation tagging technique (Moin et al., 2016b), might have a role in altering abiotic stress tolerance in rice by co-ordinating the responses of other candidate genes from both subunits. *RPS3a*, *RPS6*, *RPS15a*, *RPS17*, *RPS18a*, *RPS 23a*, *RPS24*, *RPS25*, *RPS27a*, *RPS28* are among the RPS gene family members that are significantly up-regulated in shoot under at least two abiotic stress conditions.

Under pathogen attack, *RPL10* and *RPS4* were drastically up-regulated. *RPS18A* contains a few critical protein domains associated with it, and the same is up-regulated during all stress conditions. Therefore, short-listing genes to modify stress tolerance in

rice-producing transgenic plants overexpressing the genes of both subunits separately or in combination using the candidate genes up-regulated in small and large subunit categories identified in this study might be significant. Since the plant responses to a combination of stressors vary from individual stress responses, it is vital to investigate plant responses in the presence of diverse stress conditions in order to fully evaluate the resistance responses of candidate genes (Atkinson and Urwin, 2012). The genes that are predominantly up-regulated under all the stress situations are relevant in this perspective. *RPS23* and *RPS7a*, on the other hand, have maintained a low level of expression in the shoot in response to nearly all abiotic stress treatments, whilst *RPS19* has consistently shown downregulation in roots under all abiotic stress conditions. *RPS27a*, *RPS10*, *RPS6* and *RPS9* have shown either downregulation or effectively no change in expression under the remaining stress conditions, except for particular time points under ABA treatment. Regardless of the abiotic stress condition, *RPS18*, *RPS18a*, *RPS15* and *RPS13* showed low expression levels consistently. Barring the genes that got activated in response to stress, specific genes did not respond to the stress factors efficiently in the relevant tissues, suggesting that their involvement in stress tolerance is limited.

In bacterial leaf blight infected samples, *RPS4*, *RPS10a*, *RPS6a* were up-regulated, whereas *RPS4* and *RPS5* were up-regulated in sheath blight infected samples. Therefore, *RPS4* might be regarded as one of the key RPS genes with abiotic and biotic stress-responsive nature since it is up-regulated significantly under all the abiotic and biotic stress treatments. The *cis*- regulatory elements (CREs) in the putative promoter regions play important roles in the activation and repression of gene expression, leading to stress tolerance (Ibraheem et al., 2010; Hernandez-Garcia and Finer, 2014). Regulatory elements responding to abiotic and biotic treatments are found in numerous copies across the 5'-UTR region of essentially all RPS genes, explaining their increased expression under different stress treatments.

6.3 Constitutive expression of RPL6 regulates salt tolerance in transgenic rice plants

Salinity has a tremendous impact on the growth and sustenance of several salt-sensitive plant species known as glycophytes, compared to salt-tolerant species known as halophytes. Since rice is a glycophyte, it is susceptible to salinity, and thus, salt accumulation leads to a drastic response in rice cultivation. It is critical to understand the physiological mechanisms under salt stress to produce salt-tolerant rice by transgenic and other genome editing techniques or appropriate agronomic practices. When plants detect stress signals, a series of signalling events occur, resulting in physiological and metabolic changes that ensure plant survival under unfavourable conditions. In this

scenario, identifying novel candidate genes and alleles for improved salt tolerance and sustaining steady yields under stress is critical. Previously, we have identified the up-regulation of several RP genes through activation tagging and genome-wide transcript analyses. The findings from those approaches have prompted us to functionally characterize one of the RP genes to investigate its stress-responsive attributes. The presence of numerous RP paralogs suggests that Ribosomal proteins may have a functional specialization or are involved in extra-ribosomal activities such as stress response. The up-regulation of genes coding for ribosomal proteins in plants under stress circumstances contributed to an effective rearrangement of protein-synthesizing machinery in cells, which has been observed since salt stress may cause changes in protein synthesis (Wang et al., 2013; Sáez-Vásquez and Delseny, 2019). In this research objective, the detailed characterization of *RPL6* is done by overexpression of the gene under the constitutive 35S promoter in one of the most commonly grown and recalcitrant genotypes of *indica* rice (BPT-5204). We have reported the phenotypic and physiological responses of the overexpression lines to different degrees of salt stress.

The *RPL6* transcript levels in the *RPL6* overexpression lines were checked, and based on the transcript level, we have differentiated the lines into high expression and low expression lines. The seedlings of selected high and low expression lines were subjected to moderate (150 mM) and high (200 mM) salt concentration, and their growth parameters were compared with non-transformed (NC) controls. Wilting, leaf curling and yellowing were seen in the NC and low expression lines after 5 d exposure to mild and high salt concentration, but the high expression lines appeared healthy. When these were transferred to a salt-free medium, several seedlings of the high expression lines survived and showed growth and yield characteristics (root length, shoot length and fresh weight) that were close to NC/WT plants maintained without NaCl stress.

Chlorophyll pigment is essential for plant physiology, production and survival. A plant's photosynthetic capability is measured by the amount of chlorophyll per unit area. Nutrient availability and environmental stressors like as drought, salt, heat, and cold all influence the quantity of chlorophyll in leaf tissue. Therefore, chlorophyll provides the nutrient status of a plant. To estimate the effect of different stress factors (abiotic, biotic or xenobiotic) on the efficiency of photosynthesis and bio productivity, it is crucial to investigate the chlorophyll content of the plants (Barman et al., 2018; Palta, 1990). In our study, chlorophyll content in high expression lines was higher than NC and low expression lines under stress. After recovery from salt stress, the chlorophyll a, chlorophyll b and total chlorophyll content were significantly high in the high expression lines than NC and low expression lines. This indicated high quantum efficiency and better photosynthetic activity of the *RPL6* transgenic plants under salt stress.

As a consequence of osmotic stress, many plants and microorganisms accumulate proline intracellularly. It is considered to defend against damage caused by salinity and

drought because it is a suitable and compatible osmolyte and a scavenger of reactive oxygen species (ROS) (KAVI KISHOR and Sreenivasulu, 2014; Csonka and Hanson, 1991). A significant link has been found between proline accumulation and abiotic stress tolerance in several plants. According to earlier reports, proline also serves as a metal chelator and antioxidant defence molecule during stress despite its role as an osmolyte. In our study, proline content in transgenic lines has been seen to increase consistently after revival from NaCl stress (200 mM and 150 mM). We may infer that the overexpression line under stress accumulated much greater proline content, which is in line with the previous reports that demonstrated that proline accumulation could be correlated with abiotic stress tolerance in plants.

Chlorophyll fluorescence is a vital indicator of the overall photosynthetic activity of a plant and whether or not the plant is under stress. It also elucidates how photosystem II utilizes excitation energy. *RPL6* overexpression lines have shown chlorophyll fluorescence varying between 0.70-0.80. Hence, high chlorophyll and proline contents, better quantum efficiency and better growth parameters indicate that a higher expression of *RPL6* increased the photosynthetic activity, growth and vigour in transgenic plants even under salt stress conditions. After treatment with salt, the high expression lines exhibited high transcript levels of specific genes like *OsLEA3-1*, *OsDREB2B* and *OsSOS1*. These genes are known to be stress-responsive genes, which are usually up-regulated in response to abiotic stress, especially salinity stress. The high transcript level of these genes in the *RPL6* transgenic lines during salt-stressed conditions indicates that the gene *RPL6* has a significant role in regulating downstream protein synthesis, ultimately conferring salt stress tolerance.

6.4 Possible involvement of Ribosomal protein genes in rice for resistance against pests

Insect resistance in rice has been widely researched on morphological, anatomical, biochemical, physiological, and molecular levels. One method for this functional genomics-based study is the genetic characterisation of resistance (R) genes, their cloning, and determining the function of these cloned genes (Deng et al., 2006; Du et al., 2020). The other method has been to conduct a comparative investigation on a set of resistant vs susceptible cultivars and see how they respond to pest invasion (Liu et al., 2010; (Du et al., 2020). Antixenosis, antibiosis, and tolerance are the three main components of insect resistance, although these two methods have yet to combine and present a comprehensive understanding of these basic components (Cartier and Painter, 1956). Antixenosis or non-preference is attained through the production of volatiles like (Z) 3-hexanol, which attracts BPH (Wang et al., 2016) and other natural invaders through rice

hyperoxide lyase gene function (Tong et al., 2012). Reduced feeding owing to mechanical blockage, the presence of toxins, or a lack of critical nutrients in the plant might all contribute to antibiosis in terms of insect growth, survival, and fecundity (Pathak and Saxena, 2013). The tolerance component of planthopper resistance has not been well investigated. This might be because it is not a fundamental defence mechanism but symbolizes overall vitality and increased photosynthesis under stressful situations (Wei et al., 2009; Douglas, 2018).

We have performed a differential transcript analysis of RPL and RPS genes following infestation by two devastating insect pests BPH and GM, in resistant and susceptible genotypes of indica rice. The upregulation of many RPL genes was noticed in the susceptible genotype (BPT-5204) compared to the resistant genotype (RPNF05) against BPH. This indicates that these genes are unlikely to be associated with resistance against BPH. There are some RPL genes for which we have noticed an overlap in the transcript pattern in both BPT-5204 and RPNF05 when infected with GM. This suggests that there is no possible role for these genes in susceptibility or resistance. Some of the common genes like *RPL7*, *RPL12* or *RPS6*, *RPS13* and *RPS23a* against BPH or *RPS23a* against GM were activated in both the genotypes of rice, also indicating no possible role for them in plant-insect interactions. Some genes like *RPL15*, *RPL51*, *RPL18*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* were specifically activated in the resistant genotype RPNF05 but not in BPT-5204 in response to either BPH or GM. *RPS23* was highly induced in RPNF05 compared to BPT-5204 against BPH at 12 hai. This observation suggests a possible role for them in the resistance mechanism against BPH and GM. *RPL15* was also found to be highly up-regulated in response to bacterial blight (Moin et al., 2016a). *RPL15* carries a MeJa responsive element in its putative promoter region.

Similarly, among the specifically up-regulated genes in RPNF05, *RPL18a* has also been observed to have SA and MeJa responsive elements in its putative promoter region like TCA, CGTCA and TGACG, which are related to wounding or mechanical injury inflicted by pests. Interestingly, this gene also was up-regulated in abiotic stress treatments in our earlier study (Moin et al., 2016a). Further, *RPS5a* was up-regulated in RPNF05 at 12 hai with BPH and *RPS5*, *RPS9.2* and *RPS25a* were up-regulated at 120 hai with GM. Putative promoter regions of these three genes also exhibit both the Me-Ja responsive motifs (CGTCA and TGACG).

Ribosomal proteins interact with a network of other proteins to elicit defence-related signals under stressed conditions (Yang et al., 2019). Our observations demonstrating the upregulation of specific RP genes in resistant genotype of rice when challenged with BPH and GM and the presence of *cis*- regulatory elements in their putative promoter regions specific to stress responses also indicate the possible role of RP genes in providing resistance against these pests. Differential expression of specific RP genes under stress

is a probable indication towards particular deviation from canonical ribosomal composition to facilitate the translation of stress-responsive proteins. The genes like *RPL15*, *RPL51*, *RPL18a*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* that were specifically up-regulated in the resistant genotype might be involved directly in defence-responsiveness, and subsequent signal transduction pathways or they are probably associated with primary metabolism mediated tolerance component during pest infestation by modulating photosynthetic efficiency and increasing the vigour of the plant. It has been well documented that a tolerance component of resistance is essential in rice against BPH infestation (Panda and Heinrichs, 1983). The tolerance component is accompanied by the ability of a genotype to compensate for the loss inflicted by the insect due to feeding. This is generally achieved by inducing the photosynthetic rate and primary metabolism in rice (Panda and Heinrichs, 1983). The upregulation of RP genes solely in susceptible genotype (BPT-5204) in response to BPH can be explained in the context of the tolerance component. The RP genes induced in BPT-5204 under BPH attack are probably involved in enhancing protein synthesis to scale up the production and transport of primary metabolites to cope with the emergent stress condition. *RPL22* showed more than 40-fold upregulation in BPT-5204 at both the time points, while it was not induced or poorly induced in RPNF05 at the corresponding time points. Besides, *RPS6a*, *RPS17*, *RPS21* among the RPS genes exhibited more than 25-fold upregulation in BPT-5204 with no such activation in RPNF05.

6.5 Conclusion

Our study reports the extra-ribosomal attributes of Ribosomal Protein Small Subunit (RPS) genes through genome-wide expression analyses under several external stimuli. We have validated the salt-tolerant roles of one of the RP genes, *RPL6*, in transgenic rice lines. Our exploration in a comprehensive transcript analysis of the entire RP gene family in susceptible and resistant genotypes of rice under the attack of Brown Plant Hopper and Gall Midge underpins that several RP genes were up-regulated in the resistant genotype might compensate for the loss inflicted by the insects in the plants. We have noticed the upregulation of many RP genes in response to both abiotic and biotic stress factors. Some of these genes were immediately up-regulated at the onset of the stress. This rapid and coordinated activation of RP genes in response to stress might serve as an immediate defence. Moreover, the presence of a large number of RP gene paralogs in plants compared to other organisms indicates that a huge number of ribosomal protein combinations can be formed, leading to the formation of functionally dedicated ribosomes that respond to the effect of environmental stress. The spatial expression pattern is also relevant in deciphering the role of a particular ribosomal protein in a specific tissue under a given situation. Some of the RPL and RPS genes that

were remarkably up-regulated under different external stimuli can be considered major RP genes essential for maintaining the integrity of the ribosomal machinery, ensuring sustained and undisturbed protein synthesis even under stress. Further, the co-ordinated transcriptional upregulation of translation-related RP genes might be responsible for maintaining or ameliorating protein synthesis of ribosomal components and transcription factor or downstream signal transduction proteins under stress. As opposed to the general perception of the Ribosomal protein genes with their involvement in the growth and developmental functions, this study provides a strong basis on the possible involvement of RP genes in conferring abiotic and biotic stress tolerance in rice. However, the extent of tolerance conferred by each of these genes needs to be determined by their independent overexpression in transgenic rice plants.

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Appendix A

Publications



Genome-Wide Identification and Comprehensive Expression Profiling of Ribosomal Protein Small Subunit (RPS) Genes and their Comparative Analysis with the Large Subunit (RPL) Genes in Rice

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OPEN ACCESS

Edited by:

Andy Pereira,
University of Arkansas, United States

Reviewed by:

Ing-Feng Chang,
National Taiwan University, Taiwan
Els J. M. Van Damme,
Ghent University, Belgium

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Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 24 April 2017

Accepted: 25 August 2017

Published: 15 September 2017

Citation:

Saha A, Das S, Moin M, Dutta M, Bakshi A, Madhav MS and Kirti PB (2017) Genome-Wide Identification and Comprehensive Expression Profiling of Ribosomal Protein Small Subunit (RPS) Genes and their Comparative Analysis with the Large Subunit (RPL) Genes in Rice. *Front. Plant Sci.* 8:1553. doi: 10.3389/fpls.2017.01553

Ribosomal proteins (RPs) are indispensable in ribosome biogenesis and protein synthesis, and play a crucial role in diverse developmental processes. Our previous studies on Ribosomal Protein Large subunit (RPL) genes provided insights into their stress responsive roles in rice. In the present study, we have explored the developmental and stress regulated expression patterns of Ribosomal Protein Small (RPS) subunit genes for their differential expression in a spatiotemporal and stress dependent manner. We have also performed an *in silico* analysis of gene structure, *cis*-elements in upstream regulatory regions, protein properties and phylogeny. Expression studies of the 34 RPS genes in 13 different tissues of rice covering major growth and developmental stages revealed that their expression was substantially elevated, mostly in shoots and leaves indicating their possible involvement in the development of vegetative organs. The majority of the RPS genes have manifested significant expression under all abiotic stress treatments with ABA, PEG, NaCl, and H₂O₂. Infection with important rice pathogens, *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Rhizoctonia solani* also induced the up-regulation of several of the RPS genes. *RPS4*, *13a*, *18a*, and *4a* have shown higher transcript levels under all the abiotic stresses, whereas, *RPS4* is up-regulated in both the biotic stress treatments. The information obtained from the present investigation would be useful in appreciating the possible stress-regulatory attributes of the genes coding for rice ribosomal small subunit proteins apart from their functions as house-keeping proteins. A detailed functional analysis of independent genes is required to study their roles in stress tolerance and generating stress-tolerant crops.

Keywords: rice, ribosomal protein small subunit (RPS) genes, ribosomal proteins, stress responses, gene expression

INTRODUCTION

Ribosomal proteins (RPs) constitute the protein part of the ribosomes and have a significant role in ribosome biogenesis, protein synthesis, cell growth, development, and apoptosis (Ramakrishnan and White, 1998; Naora and Naora, 1999; Maguire and Zimmermann, 2001; Wang Z. et al., 2013). RPs have long been known primarily for their housekeeping functions. But, there are several reports on their extra-ribosomal functions in animal systems (Warner and McIntosh, 2009). The knockdown of individual RPs leads to P53 accumulation, cell death and certain developmental abnormalities in Zebra fish (Uechi et al., 2006; Danilova et al., 2008). Mutations in certain RPs were associated with Diamond-Blackfan Anemia (DBA) and increased risk of cancer (Gazda et al., 2008). On the contrary, specific RPs have been used as therapeutic targets in cancer treatment. Overexpression of RPS2 has been linked with the survival of prostate tumors, whereas suppression of RPS2 or RPS3a resulted in apoptosis (Wang et al., 2009).

The number of RPs varies within eukaryotes. In yeast 78 RPs (32 of the small subunit and 46 of large subunit) are encoded by 137 genes (Planta, 1997). Two hundred and forty nine genes encode the 80 RPs of *Arabidopsis*; 32 are small subunit, and 48 are large subunit proteins (Barakat et al., 2001; Wang J. et al., 2013). In rice, 34 large subunit proteins are encoded by 123 genes (Moin et al., 2016b) showing that multiple copies of genes encode an individual ribosomal protein in eukaryotes (McIntosh and Bonham-Smith, 2006). This can be correlated with the requirement of ample supply of RPs during rapid growth phase (Filipowicz and Hohn, 2012). Most of these genes are specifically expressed during a certain developmental stage or in a particular tissue, or they may be functionally redundant. The subsequent lack of sufficient quantity of individual RPs can lead to non-lethal phenotypic abnormalities and aberrant translational efficiency (Hulm et al., 2005; Komili et al., 2007; Degenhardt and Bonham-Smith, 2008).

Several studies have demonstrated important roles of RPs in development and physiology of organisms as their reduced level in the organisms resulted in detrimental effects. Semi-dominant mutation of cytoplasmic ribosomal protein RPL27aC resulted in multiple developmental defects such as changes in leaf patterning, inflorescence, floral meristem function and seed set (Szakonyi and Byrne, 2011). In *Arabidopsis*, PFL codes for the ribosomal protein S18 and a mutation at PFL locus resulted in developmental abnormalities involving reduced fresh weight, pointed first leaves and stunted growth indicating the importance of the corresponding protein in meristem activity (Van Lijsebettens et al., 1994). RPL18aB is presumed to have a critical role in male gametophyte development and embryo pattern formation in *Arabidopsis* with the *rpl18aB* mutants exhibiting defects in pollen growth (Yan et al., 2016). Genes coding for cytoplasmic ribosomal proteins, L10a, L9, and L5

play an important role in determining the adaxial and abaxial fate of leaf during development via their interaction with adaxial *HD-ZIPIII* gene, *REVOLUTA*, and abaxial *KANADI* gene (Pinon et al., 2008). Aberrant expression of ribosomal protein S6 in *Arabidopsis* leads to a deleterious effect on survival of cells indicating their role in a complex translational regulation. Insertion of ribosomal protein S6 gene in antisense orientation resulted in over-expression of antisense ribosomal protein S6 RNA, which led to a reduced level of *rps6* expression. This might be the reason for subdued expression of some other specific proteins, which is manifested in the form of complex phenotypic disorders including multiple leaves and flower formation in close vicinity (Morimoto et al., 2002). In *Saccharomyces cerevisiae*, RPs have been predicted to participate in several extra-ribosomal functions, which are related to ribosome assembly, replicative life span, DNA repair, adhesive growth and filament formation (Lu et al., 2015).

T-DNA insertional mutagenesis in one of the RPS27 genes (*AtRS27A*) led to strong growth inhibition and the formation of tumor-like structure in place of auxiliary roots in the presence of methyl methanesulfonate (MMS) in growth medium (Revenkova et al., 1999). In *Arabidopsis*, delayed cell division and complete developmental arrest was observed in RPS5 hetero- and homozygous mutants, respectively (Weijers et al., 2001). The mutation in a key translation initiation factor, RPL24 in *Arabidopsis* resulted in perturbed gynoecium development with diminished ovary and lengthened gynophore (Nishimura et al., 2004).

In addition to their crucial roles in developmental processes in plants, several reports also focused on their expression pattern in response to several external stimuli. The expression of several RP genes is also regulated by phytohormones, ABA and cytokinin. ABA negatively regulates the expression of *RPS14*, *RPS16*, *RPS13a*, and *RPL30*, whereas cytokinin induced the expression of these genes at both the transcript and protein levels (Cherepneva et al., 2003). In rice, *RPS4*, 7, 8, 9, 10, 19, 26, and *RPL2*, 5, 18, 44 were among the early responsive genes that were up-regulated under salt stress (Kawasaki et al., 2001). Nutrition deficiency also resulted in the differential expression pattern of RP genes in *Arabidopsis*. Comparison of differentially expressed RP genes under Fe and Pi deficiency in *Arabidopsis* roots revealed that both the RPS and Ribosomal Protein Large subunit (RPL) genes were differentially expressed under Fe deficiency, whereas only RPL genes were differentially expressed under Pi deficiency (Wang J. et al., 2013). Induction of soybean RPs, RPS13, RPS6, and RPL37 in response to cold treatment gave a clue to their probable role in secondary signaling under low-temperature conditions (Kim et al., 2004). These reports point toward the differential expression of RP genes in response to stress treatments leading to differential accumulation of RPs in the ribosomal apparatus, which might help ribosome remodeling and selective translation to cope up with nutrition deficiency or other unfavorable conditions (Wang J. et al., 2013).

Screening of a large-pool of activation tagged mutant population of rice for high water-use efficiency revealed the significant upregulation of RPL genes (6 and 23a) in our previous studies (Moin et al., 2016a). This subsequently persuaded us to

Abbreviations: RPL, Ribosomal Protein Large subunit; RPS, Ribosomal Protein Small subunit; RP, Ribosomal proteins; ABA, Absciscic Acid; PEG, Polyethylene Glycol; NaCl, Sodium chloride; H₂O₂, Hydrogen peroxide; BLB, Bacterial Leaf Blight; SB, Sheath Blight.

study the regulatory role of all the RPL genes under different external stimuli (Moin et al., 2016b). This study led another group to identify the insect resistance roles of *NlRPL5* in rice as an important responsive gene in resistance to the brown plant hopper, *Nilaparvata lugens* (Zhu et al., 2017). The objective of the present study is to ascertain whether RPS genes are stress responsive like the RPL genes (Moin et al., 2016b).

Hence, we have attempted to analyse the differential expression patterns of ribosomal protein small subunit genes (RPS genes, one from each family) for their spatiotemporal and stress induced regulation along with a comparative analysis with the large subunit counterparts and *in silico* studies on their promoter sequences, gene structures, protein properties and phylogeny. These observations are reported here.

MATERIALS AND METHODS

RPS Gene Sequence Retrieval

A list of all ribosomal protein family (both large and small subunit) was obtained using a keyword search, “ribosomal” in the RGAP-DB¹ and Phytozome v11² databases. From the list, a total of 56 ribosomal small subunit genes (40S) were shortlisted by their names starting with the letter “S” as a prefix (S for Small subunit). Nucleotide sequences of all the 56 genes were retrieved from RGAP-DB. These 56 sequences were further confirmed for their ribosomal origin through nucleotide and protein BLAST³ search in the NCBI⁴ and Hidden Markov Model (HMM) of Pfam⁵ databases, respectively. The availability of only 34 ribosomal small subunit proteins, which are coded by 56 genes indicated the existence of paralogs of the genes coding for more than one RPS protein. For the gene expression analysis, we selected 34 RPS genes, one from each group representing their corresponding paralogous groups. Identical gene copies were, thus, excluded from further analyses. We have listed the 34 genes along with their paralogs in the Supplementary Table 9. The sequences of RPS genes were retrieved from RGAP-DB (a Nipponbare database). Our analysis of gene expression is performed in an *indica* rice variety (Samba Mahsuri). Hence, we cross-checked the sequence similarity of the coding as well as the 5'- upstream regions of the genes selected for analysis in the present investigation in both *indica* and *japonica* sequence databases by doing a detailed BLAST analysis of the *japonica* sequences on *indica* genome databases, OryGeneDB⁶ and EnsemblPlants⁷. Further, we have looked for the similarity of rice (40S) RPS genes with RPS genes of *Arabidopsis* and performed a BLAST search of the Rice sequences in TAIR⁸ database and listed the similarity percentage in Table 1.

¹<http://rice.plantbiology.msu.edu/index.shtml>

²<https://phytozome.jgi.doe.gov/pz/portal.html>

³<https://blast.ncbi.nlm.nih.gov/Blast.cgi>

⁴<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>

⁵<http://pfam.xfam.org/>

⁶<http://orygenesdb.cirad.fr/tools.html>

⁷http://plants.ensembl.org/Oryza_indica/Tools/Blast?db=core;tl=dxXz9p4PIES2NtPR-17828772

⁸<https://www.arabidopsis.org/Blast/>

Chromosomal Location of RPS Genes

To obtain the genome-wide chromosomal distribution of all RPS genes on 12 chromosomes of rice, we had submitted the locus numbers (obtained from RGAP-DB) of 56 RPS genes as an input to the OrygenesDB. Based on the OrygenesDB outputs, all RPS gene locations were indicated on the chromosome maps, and the number of genes on each chromosome has also been shown. Genes, which were selected for expression analysis, have been shown in bold in Figure 1.

Structural Features of RPS Genes

To predict the gene structure features, such as intron and exon number, size, etc., we had submitted the genomic and corresponding cDNA sequences of the selected 34 genes to the Gene Structure Display Server (GSDSv2)⁹.

Putative Promoter Survey of 34 RPS Genes

Since all the 34 genes exhibited differential expression under multiple stress treatments, we checked for the presence of putative *cis*-elements in the putative promoter regions of the corresponding genes. To verify this, we have used an *in silico* approach, in which the nucleotide sequence of about ≤ 1 kb upstream of each of the 34 RPS genes was retrieved from RGAP-DB, and the sequences were cross-checked for their identity/ deviation with the *indica* genome in OrygeneDB and EnsemblPlants databases. Further, the sequences that exhibited the highest similarity with *indica* genome in the databases were submitted to PlantCARE (Cis-Acting Regulatory Elements) Database¹⁰ (Rombauts et al., 1999; Dhadi et al., 2009; Ding et al., 2011) for identifying the regulatory elements. We have compared the putative promoter regions of some of the significant genes in two other databases, such as PlantPAN 2.0¹¹ and the JASPAR¹² and found the functional similarity of the elements with respect to PlantCare database confirming the authenticity of our observations.

Ribosomal Protein Structural and Phylogenetic Properties

Predicted protein sequences available from the RGAP-DB were used for predicting the protein structure, molecular weight, isoelectric point (*pI*) prediction *in silico* by the online tool PSORT¹³ and ExPASyProtParam¹⁴. By submitting the protein sequences to SMART¹⁵ (Simple Modular Architecture Research Tool), we have identified various ribosomal domains and motifs. Sequence submission to ExPASyProtParam helped us in obtaining the GRAVY indices of RPSs, which indicated the probable hydrophobicity of the proteins by their negative values for the indices that are relevant for the hydrophobic proteins.

⁹<http://gsds.cbi.pku.edu.cn/>

¹⁰<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

¹¹<http://plantpan2.itps.ncku.edu.tw/promoter.php>

¹²http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=plants

¹³<https://psort.hgc.jp/form.html>

¹⁴<http://web.expasy.org/cgi-bin/protparam/protparam>

¹⁵<http://smart.embl-heidelberg.de/>

TABLE 1 | Similarity of Ribosomal protein small subunit (RPS) genes in rice with that in *Arabidopsis*.

S. No	Ribosomal Protein Small Subunit (RPS) genes in Rice	RPS genes in <i>Arabidopsis</i>	% similarity in amino acid sequence between Rice and <i>Arabidopsis</i>
1.	S3a LOC_Os02g18550	S3Ae AT4G34670.1	79
2.	S4 LOC_Os01g25610	Ribosomal S4 Family protein AT5G58420.1	83
3.	S4a LOC_Os05g30530	- Ribosomal S4 protein family AT5G58420.1 - Ribosomal protein S4 family (RPS4A) AT2G17360.1	82 81
4.	RPS5 LOC_Os01g01060	Ribosomal protein 5A AT3G11940.2	80
5.	RPS5A LOC_Os11g29190	- Ribosomal protein 5B AT2G37270 - Ribosomal protein 5A AT3G11940	78 80
6.	RPS6 LOC_Os01g12090	Ribosomal protein S6 family AT3G18760	89
7.	RPS6a LOC_Os03g27260	Ribosomal protein S6e RPS6B AT5G10360	82
8.	RPS7 LOC_Os02g21900	Ribosomal protein S7e family protein AT1G48830	85
9.	RPS7a LOC_Os03g18570	- Ribosomal protein S7e family protein AT1G48830 - Ribosomal protein S7e family protein AT3G02560	84 78
10.	RPS9 LOC_Os07g43510	Ribosomal protein S4 AT5G15200	81
11.	RPS9-2 LOC_Os03g05980	- Ribosomal protein S4 AT5G15200 - Ribosomal protein S4 AT5G39850	79 79
12.	RPS10 LOC_Os01g73160	S10 domain containing protein AT4G25740	92
13.	RPS10a LOC_Os02g34460	S10 domain containing protein AT5G52650	92
14.	RPS13 LOC_Os07g38540	Ribosomal protein 13a AAT4G00100	87
15.	RPS13a LOC_Os08g02400	- Ribosomal protein S13a AT4G00100 - Ribosomal protein S13 AT3G60770	84 82
16.	RPS15 LOC_Os07g08660	Cytosolic Ribosomal protein S15 AT1G04270	87
17.	RPS15a LOC_Os02g27760	Ribosomal protein S15A AT1G07770	81

(Continued)

TABLE 1 | Continued

S. No	Ribosomal Protein Small Subunit (RPS) genes in Rice	RPS genes in <i>Arabidopsis</i>	% similarity in amino acid sequence between Rice and <i>Arabidopsis</i>
18.	RPS17 LOC_Os03g01900	Ribosomal protein S17 family protein AT3G10610	83
19.	RPS18 LOC_Os03g58050	Ribosomal protein S18 family AT1G22780	83
20.	RPS18a LOC_Os07g07719	Ribosomal protein S18 protein family AT1G34030	81
21.	RPS18b LOC_Os07g07770	Ribosomal protein S18 (RPS18C) AT4G09800	82
22.	RPS19 LOC_Os03g31090	Ribosomal protein S19e family protein AT5G61170	80
23.	RPS2 LOC_Os03g46490	Ribosomal protein S21e AT3G53890	84
24.	RPS23 LOC_Os03g60400	Ribosomal protein S23family protein AT5G02960	83
25.	RPS23a LOC_Os10g20910	- Ribosomal protein S23 AT5602960 - Ribosomal protein S23 AT3G09680	81 82
26.	RPS24 LOC_Os01g52490	Ribosomal protein S24e family protein AT5G28060	82
27.	RPS25 LOC_Os08g44480	Ribosomal protein S25 family protein AT4G39200	85
28.	RPS25a LOC_Os11g05562	- Ribosomal protein S25 family protein AT4G39200 - Ribosomal protein S25 family protein AT2G21580	87 84
29.	RPS26 LOC_Os01g60790	Ribosomal protein S26e family protein AT2G40590	87
30.	RPS27 LOC_Os04g27860	Ribosomal protein S27 AT3G61110	83
31.	RPS27a LOC_Os01g22490	Ribosomal protein S27a AT1G23410	81
32.	RPS28 LOC_Os10g27174	Ribosomal protein S28 AT5G64140	95
33.	RPS29 LOC_Os03g56241	Ribosomal protein S29e family protein AT4G33865	85
34.	RPS30 LOC_Os02g56014	Ribosomal protein S30 family protein AT2G19750	83

Similarity percentage of rice ribosomal protein small subunit (RPS) genes in rice with the corresponding RPS gene in *Arabidopsis* has been mentioned after a BLAST analysis in TAIR database.

To predict the three-dimensional structure and to check for the presence of ligand binding sites on each RPS, we have used the Phyre2¹⁶ (Protein Homology/AnalogY Recognition Engine v2; Kelley et al., 2015) and 3DLigandSite¹⁷ programs (Wass et al., 2010), respectively. Results from these tools predicted the protein properties like percentage of α -helix, β -sheet, disordered protein fraction and ligands of each protein, ligand binding residues on the proteins and 3D model of the ligand bound protein. Multiple sequence alignment using ClustalW¹⁸ also deciphered the phylogenetic relationships among the 34 RPS proteins in MEGA6¹⁹ (Molecular Evolutionary Genetic Analysis) platform, which constructed an unrooted phylogenetic tree showing evolutionary sequence similarity among the proteins with bootstrap values of 100.

Plant Material, Growth Conditions and Stress Treatments

Oryza sativa L. ssp. *indica* var. Samba Mahsuri (BPT-5204) seeds were surface sterilized using 70% ethanol for 50 s followed by washing with 4% sodium hypochlorite for 15–20 min and 5–6 washes with sterile double-distilled water. After this, the seeds were dried on sterile blotting paper and were placed on solid MS media at $28 \pm 2^\circ\text{C}$ and 16 h light/8 h dark photoperiod cycle (Moin et al., 2016b).

For native expression studies, the surface sterilized seeds were allowed to germinate on solid MS medium. Some of the seeds were soaked for 16 h to collect the embryo and endosperm tissues using a sterile surgical blade. Shoot and root apical meristems of 3 d old seedlings and roots and shoots of 7 d old seedlings were collected for the expression studies. After transferring the seedlings from MS medium to soil in the pots; roots, shoots, leaves, root-shoot transition region, flowers, grains and panicles were collected (Moin et al., 2016b).

For abiotic stress treatments, 7 d old seedlings were exposed to ABA (100 μM), PEG (10%), NaCl (250 μM), and H_2O_2 (10 μM) by dipping them in the respective solutions. Around five seedlings in replicates were collected after 15 min, 3, 6, 12, 24, 48, and 60 h duration. Roots and shoots were collected separately for RNA isolation and cDNA synthesis. The untreated seedlings in water were used as control samples for normalization in qRT-PCR (Moin et al., 2016b).

To analyse the expression of the RPS genes under biotic stress, rice samples infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Rhizoctonia solani* were collected 20 and 25 d post infection, respectively. For the biotic stress experiments, 1-month-old rice plants were used for treatments. The untreated samples of the same age were used as corresponding controls. The suspension of *Xoo* bacterium was applied at the edge of the leaves using a sterile blade (Moin et al., 2016b) and agar blocks of *Rhizoctonia solani* were placed on the leaf sheaths (Park et al., 2008) as per the standard protocols (and maintained under controlled culture room conditions).

All the samples were collected as three biological and technical replicates, and qRT-PCR was performed with their respective control samples. Rice specific *act1* and β -*tub* genes were used as internal reference genes for normalization and the mean values of relative fold change were calculated as per $\Delta\Delta\text{C}_\text{T}$ method (Livak and Schmittgen, 2001). For native expression studies, the normalization was performed with only rice specific *act1* and β -*tub* genes (ΔC_T). Bar diagrams and heat maps were generated by Sigmaplotv11 and Morpheus²⁰ programs, respectively using the means of the fold change that were calculated from the qRT-PCR data.

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from the treated and untreated samples using Tri-Reagent (Takara Bio, UK) according to the manufacturer's protocol. The concentration of the isolated RNA was checked using a Nanodrop spectrophotometer, and 2 μg of RNA was used to synthesize first strand cDNA using reverse transcriptase (Takara Bio, UK). The synthesized cDNA was diluted ten times, and 2 μl of it was used to perform qRT-PCR and 10 μM of the primers for each gene was used per reaction. The primers specific to RPS genes were designed by using the on line tool Primer-3²¹ the sequences of forward and reverse primers are listed in Supplementary Table 5. The amplification protocol included an initial denaturation at 94°C for 2 min, with 40 cycles of 94°C for 30 s, primer specific annealing temperature for 25 s, an extension of 72°C for 30 s. This was followed by constructing a melt curve at the end to estimate the amplification efficiency of each gene. The fold change was calculated as per $\Delta\Delta\text{C}_\text{T}$ method, and the mean of these values was considered as the final fold change of the transcript levels (Moin et al., 2016b). Means of relative fold change were plotted in Sigmaplot v11 with standard error to produce the bar diagrams for all the genes in a specific stress.

RESULTS

Chromosomal Distribution

Rice genome has 12 chromosomes carrying 56 RPS genes. Based upon the OrygeneDB output, the RPS genes were found to be distributed throughout the rice genome covering all 12 chromosomes. Both the arms of chromosome randomly carried the RPS genes. Each chromosome carried at least one member of the RPS gene family. Most of the genes are located on chromosomes 3, 1, 7, and 11, with chromosome 3 bearing the highest number of eleven RPS genes. Both the chromosomes 1 and 7 carried a total of eight and nine genes, respectively. Chromosome 9 is the only example with only one RPS gene present near the telomere region of its long arm. The chromosome 10 and 1 carried three and eight RPS genes, respectively (Figure 1).

¹⁶<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>

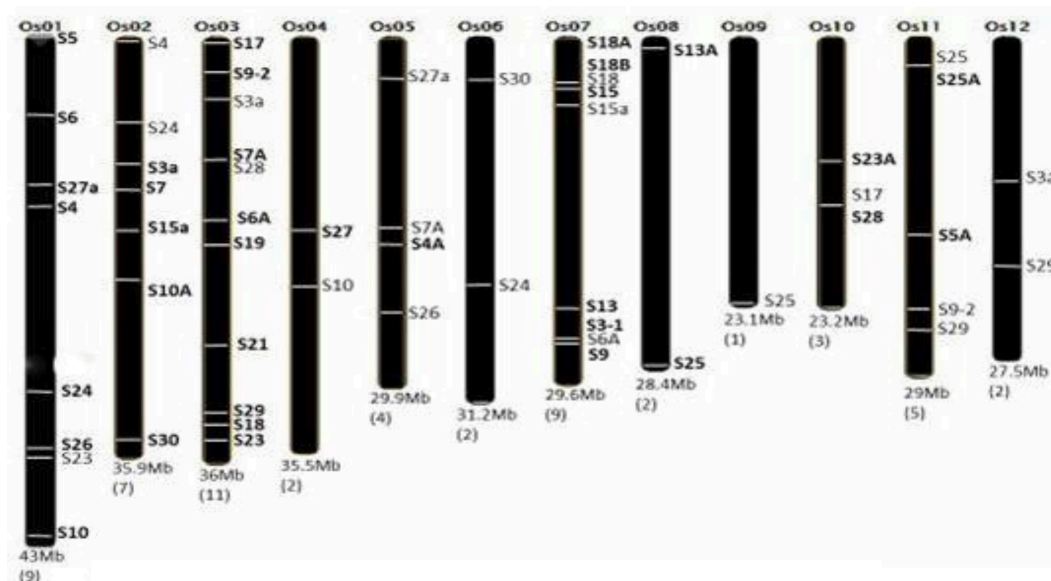
¹⁷<http://www.sbg.bio.ic.ac.uk/3dligandsite/>

¹⁸<http://www.genome.jp/tools/clustalw/>

¹⁹<http://www.megasoftware.net/>

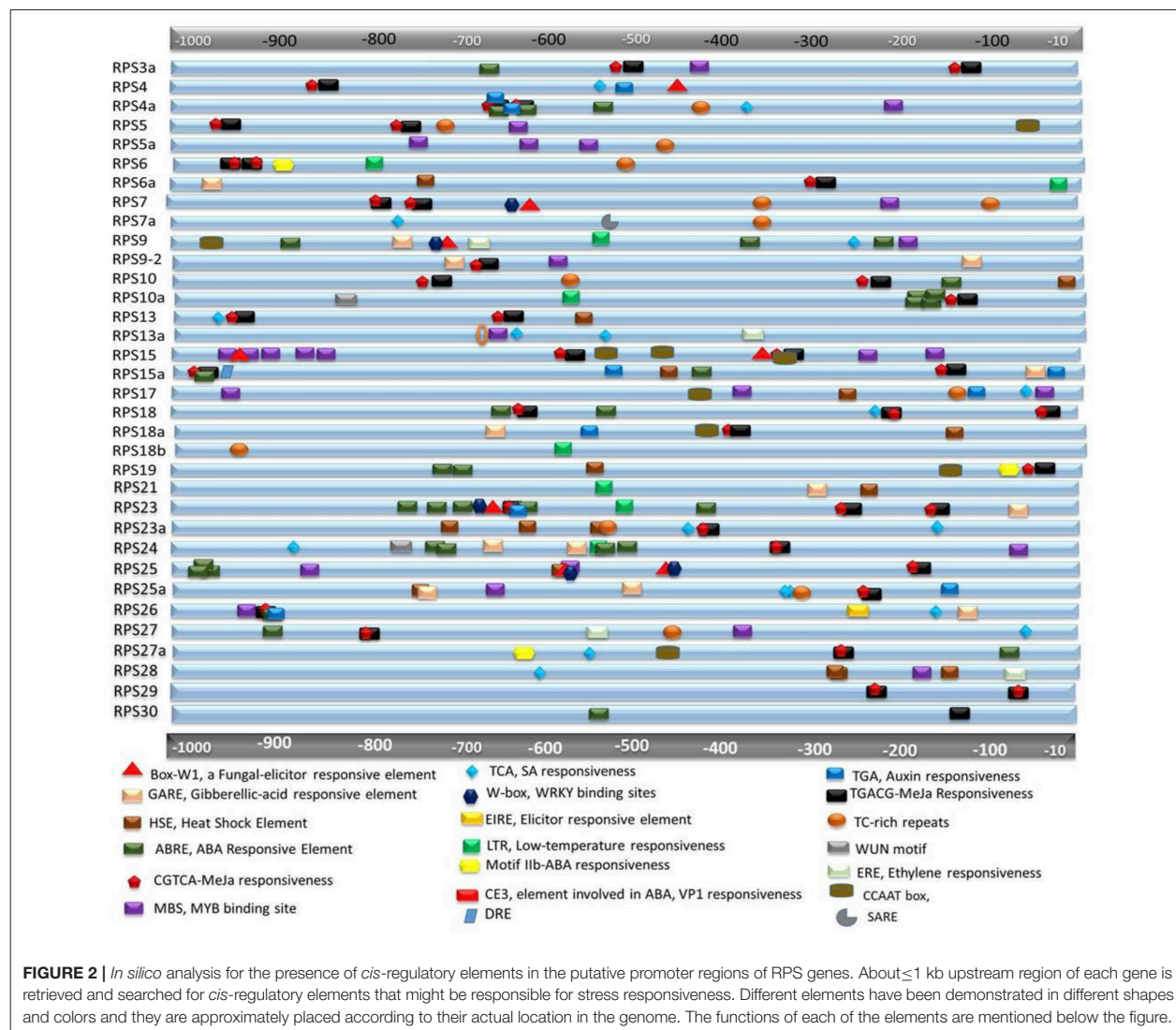
²⁰<https://software.broadinstitute.org/morpheus/>

²¹<http://bioinfo.ut.ee/primer3-0.4.0/>



Results from the GSDS suggested that most of the RPS genes consisted of both introns and exons (Supplementary Figure 1). *RPS25a* has a long stretch of intronic sequence covering most of its length. It is also the longest gene among the selected genes with a total length of approximately 6 kb. *RPS17* and *RPS30* lacked introns and possessed a single and small CDS flanked by UTRs. *RPS6*, *RPS19*, *RPS23a*, and *RPS7* lacked UTRs and are only composed of intronic and exonic sequences. *RPS23a* is the smallest gene with a total size of 0.5 kb. The number of exons also varied from a minimum of two in *RPS23a* and *RPS29* to a maximum of three exons in *RPS3a*. The details of properties related to the gene structure have been provided in Supplementary Table 2.

All the RPS genes have at least two stress responsive elements in their immediate upstream regions (up to 1 kb). *RPS30* has one copy of ABRE and TGACG, and *RPS18b* has one copy of LTR and TC rich repeat as their *cis*-acting regulatory elements (CAREs). Other than these two genes, putative promoter regions of the rest of the genes have more than two elements. SARE, EIRE, motif1b, DRE and ERE, which are responsive to SA, elicitor, ABA, dehydration and ethylene, respectively and are present in single copies in the putative promoter regions of some of the genes. *RPS23* showed five repeats of ABREs, three copies of TGACG motif, three copies of CGTCA motif and one copy each of TGA, Box W1, W box, and GARE elements. *RPS4a* exhibited three copies of ABREs, two repeats each of TGA, TGACG, and CGTCA elements. *RPS15* has two repeats of MBS. Similarly, the genes (*RPS4*, *10a*, *6a*, and *5*) that have exhibited remarkable up-regulation under biotic stress conditions have multiple MeJa responsive elements and some of them contain box W1 and TCA elements that are



related to responsiveness under pathogen attack. All the stress responsive *cis*-acting elements were pictorially represented by their physical mapping in **Figure 2** and **Table 2** mentioning the function of the corresponding motifs. The elements having roles in tissue specificity for each of the genes have been listed in Supplementary Table 7. The BLAST analysis for the 5'-upstream regions of the genes of both the varieties showed that 8 out of 34 genes had shown a slight variation in the sequences of upstream regions (97%) in the two varieties. The differences in regulatory elements due to the dissimilarity in their sequences have been compared and shown in the Supplementary Figure 7. The upstream regions could not be identified for three genes (*S15a*, *S18a*, and *S18b*) for which the promoter analysis has been performed using the *japonica* sequences only.

Protein Properties and Phylogeny

The submission of the protein sequences from RGAP-DB to the ExPasyProtParam server revealed many putative properties. Their molecular weights (MW) varied from approximately 7 to 30 kDa with RPS4 having the highest MW of 29.8 kDa and RPS30 with the lowest MW of 6.9 kDa. In accordance with the MW, the RPS4 is the largest protein with a sequence of 265 amino acids, while RPS30 is the smallest with 62 amino acids. *In silico* analysis using ExPasyProtParam server helped us to get the theoretical *pI*-values of each of the RPS. Most of them were in the range of 9 to 12 with very few exceptions out of this range. For example, RPS6 has a very low *pI* of 4.86. Most of the RPS proteins are rich in positively charged amino acids like Arg and Lys compared to negatively charged amino acids like Asp or Glu. The only exceptions are the RPS21, which has an equal number of both the

TABLE 2 | Functions of different stress responsive elements found in the promoter regions of the RPS genes.

Name of the elements	Function
ABRE (ABA responsive elements) DRE (dehydration responsive elements)	Osmotic stress-mediated transcriptional regulation (Kim et al., 2011).
HSE (heat stress element)	Tolerance to heat stress.
LTR (low temperature responsive)	Cold responsive.
MBS (MYB binding site)	Drought induced expression of genes involved in defense (Sazegari et al., 2015).
W-box- binding site of WRKY transcription factors	Pathogen induced response (Rushton et al., 2002).
Box-W1-fungal-elicitor responsive element	Involved in defense signaling (Hernandez-Garcia and Finer, 2014).
TCA and SARE	Responsive to salicylic acid.
TGACG and CGTCA	Responses related to methyl jasmonate.
ERE (ethylene responsive elements) TGA and AuxR	Crucial for stress signaling. Auxin responsive (Sutoh and Yamauchi, 2003; Bastian et al., 2010; Sazegari et al., 2015).
motif Ilb and CE3 (coupling element 3)	Involved in ABA mediated osmotic stress signaling (Hobo et al., 1999).
WUN motif (wound specific)	Involved in wound response (Pandey et al., 2015).

types and RPS6 having more of acidic amino acids. Percentage composition of secondary protein structures (disordered protein, alpha helix and beta sheet) has variations within the RPS family. RPS4 has the minimum value of 14% while the RPS30 has the maximum value of 76%, respectively; S10a exhibited 10%, and S29 and S13 have 64% α -helical content, respectively. The range on the percentage of β -sheet content also varied with RPS13, 13a having 0% and S27 showing 49% of the β -sheet content.

The Grand Average of Hydrophobicity (GRAVY) indices were below the value of zero for all 34 RPS proteins suggesting the hydrophobic nature of RPS proteins. We have also identified some of the interacting ligands of these RPS proteins from the 3DLigandSite. The metal and non-metal ligands include IF-1, FMN, FAD, Mg^{2+} , Zn^{2+} etc. and the specific residues involved in the ligand binding have been listed in the Supplementary Table 3. Most of the RPS proteins have Low Complexity Region (LCR) motifs, which are the stretches of biased amino acid sequences having a long repetition of single or few amino acids and they have been connected to many protein functions and interactions. LCRs that are located terminally in a protein have been considered to be translationally enriched and have stress responsive roles (Coletta et al., 2010). Three dimension protein structures from 3D ligand site have been included in Supplementary Figure 2. The ribosomal protein sequences were submitted to SMART database, and it was found that all of them exhibited ribosomal domains. Some of the proteins also contain KOW domain (for e.g., RPS4, RPS4a). RPS6 has a SPT2 domain; RPS5a has a HLH domain, a CARD domain and an ANTAR domain. RPS18a has a SANT domain, which is a MYB-related domain that binds to DNA and helps in transcriptional regulation (Feller et al., 2011) and it also

has a homeobox domain HALZ. All the domains associated with each of the RPS subunits are listed in Supplementary Table 8.

Protein sequence data was exploited to construct an unrooted phylogenetic tree of 34 RPS proteins in the MEGA6 software. Alignment with ClustalW employing a neighbor joining algorithm resulted in the generation of a tree that showed the evolutionary relationship between the RPS proteins. The tree suggested that a considerable number of cladogenesis events occurred during the course of evolution in the RPS gene family that was reflected in their protein sequence similarity and diversity. The earliest divergence separated the proteins into two major clades, of which one contains five proteins (RPS13, 13a, 3a, 19, and 21) and the other major clade carried all the other proteins, which has further diverged several times. These five RPS genes were checked for similarity with their paralogs and depicted as a phylogenetic tree in Supplementary Figure 3B. While RPS 19 and 21 have no paralogous members, RPS13 and RPS3a have two paralogous members, and they have shown similarity with their corresponding group. Nodes, from where some pairs of genes were diversified showed the bootstrap value of 100. Such high value indicates the high significance of the clade. The phylogenetic tree showing the evolutionary relationships among the RPSs is shown in Supplementary Figure 3.

Native Expression Profiling of the RPS Genes in Different Tissues

To gain insights into the expression patterns of RPS genes in rice development and also to provide a comparative analysis between RPs of both the large and small subunits, we performed qRT PCR of 34 RPS genes in 13 different tissues as described in Moin et al. (2016a) for the RPL genes. The mean values were utilized to construct heat maps to demonstrate clearly the native expression pattern of the RPS genes (Figure 3). The tissues used to study the native expression pattern included embryo and endosperm from 16 h old germinating seed, plumule and radicle from 3 d old seedlings; shoot and root of 7 d old seedling; shoot, root, flag leaves, panicle, flower, grains and root-shoot transition of mature 20 d old plants (after transfer to green house).

The complete tissue-specific up-regulation pattern of the RPS genes in native conditions has been summarized in Supplementary Table 4 and also in the form of heat maps (Figure 3). The up-regulation of RPS genes was significant in mature leaves (25 genes), shoots (21 genes), 7 d shoots (26 genes), plumules (21 genes), and root-shoot transition (23 genes). Apart from these four, seven and 15 genes were up-regulated in seedling radicles, 7 d old root and mature roots, respectively. An analogous pattern of gene expression was also observed under different stress conditions as well, where more number of RPS genes was found to be up-regulated in shoot than in the root tissues showing that the majority of the RPS genes were up-regulated in shoots, leaves and plumules compared to the other organs.

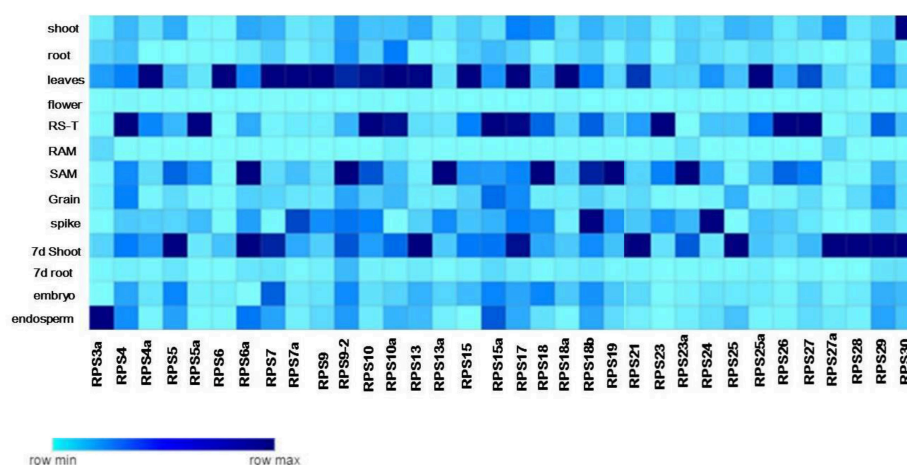


FIGURE 3 | Tissue specific expression of the RPS genes. The native expression of 34 RPS genes in 13 tissues is determined by qRT-PCR with single normalization with rice actin. The mean values of the fold change of the biological and technical replicates are represented in the form of a heat map.

About 17 RPS genes showed expression in rice grains and panicles indicating their involvement in inflorescence development and grain filling. None of the genes was expressed in florets implying that RPS proteins have probably no significant roles in the development of this meiotically active tissue. About 18 and 15 RPS genes were up-regulated in embryo and endosperm, respectively showing that these genes might have a role in seed development and maturity. *RPS23a*, *RPS27a*, *RPS10a*, and *RPS7* have exhibited no significant up-regulation in any of the tissues and *RPS23* is up-regulated only in root-shoot transition. A considerable up-regulation of *RPS28* and *RPS29* was observed in roots under different stresses. *RPS4*, *RPS5a*, *RPS7a*, *RPS9*, *RPS9-2*, *RPS13*, *RPS6*, *RPS17*, *RPS18b*, *RPS24*, and *RPS29* were up-regulated in most of the tissues, while *RPS13a*, *RPS18a*, *RPS4* were up-regulated in shoots throughout the duration of all the stress conditions. Similarly, these genes were also expressive in shoots, leaves, plumules and in native conditions as well.

Differential Expression Analysis of the RPS Genes in Response to Abiotic Stress Treatments

Our findings on the screening of a large-pool of activation tagged mutant population for high water-use efficiency revealed the significant up-regulation of RPL genes (*RPL6* and *RPL23A*) by the integrated enhancers in two of the mutant plants with sustained productivity under limited water conditions (Moin et al., 2016a). In the follow-up study, an extensive tissue specific and differential expression study of the entire RPL gene family was carried out, which showed significant up-regulation of certain RPL genes under abiotic and biotic stresses (Moin et al., 2016b). Results from these two studies prompted us to further investigate the expression of 40S Ribosomal Protein genes in response to abiotic and biotic

stress conditions and to arrive at proper combinations of RPS and RPL genes for subsequent genetic manipulation. For studying their differential expression, we have selected 34 RPS candidate genes from the respective paralogous groups in response to four abiotic stresses (ABA, PEG, NaCl, and H₂O₂) at seven time points and two biotic stresses (BLB and SB). ABA, PEG, NaCl, and H₂O₂ induce a stress similar to osmotic, drought, salinity and oxidative stress, respectively (Gao et al., 2007; Hamayun et al., 2010; Hossain et al., 2015; Sah et al., 2016). Treatment of the plants with these stress-inducing agents would mimic the corresponding stress situations and consequently activate numerous stress responsive pathways. Also, we also analyzed their native expression pattern in different tissues.

The genes that exhibited ≥ 3 fold transcript level on the log₂ scale were considered as up-regulated. The pattern of expression of the genes depended mostly on the type of stress signal and also the tissue in which the expression was studied. We have categorized the genes in terms of their timing and the intensity of response. Some of the genes were up-regulated as early as 15 min–3 h after the onset of the stress, while a few of them responded between 3 and 12 h after exposure while others were up-regulated 12 h after the application of the stress. We have differentiated them as Immediate Early (IE), Early (E), and Late (L) expressing genes, respectively.

Since the level of expression varied from 3 to several 100 folds, we have further categorized the up-regulated genes as highly expressive genes (≥ 30 fold), moderately expressive (between 10 and 30 fold) and low-expressive (between 3 and 10 fold). The expression of the RPS genes in the shoot samples has been observed to be higher compared to the corresponding root samples under almost all the abiotic stresses at different time intervals. Under some conditions, the up-regulation of the genes in the shoot samples has been detected to be several 100 folds, which is not the case for the corresponding root samples. In

the case of shoot samples, all the genes showed significant up-regulation for all the stress conditions for at least one time point, and they were predominantly IE type in their response, except under H_2O_2 treatment.

Although all the genes were up-regulated under all the stress conditions in shoots, their mode of response varied with the nature of the stress. For example, ABA induced the up-regulation of 26% of the genes (*RPS7*, *15a*, *18a*, *25a*, *4*, *7a*, *10*, *10a*, and *13a*) and these maintained a significant expression (≥ 3 -fold) until the last time point used (60 h). Of these, 50% were highly expressive (*RPS7*, *15a*, *S8a*, and *25a*) with >30 -fold transcript level throughout the stress treatment up to 60 h time point. *RPS7* and *RPS18a* showed up-regulation up to several 100 folds. PEG and NaCl also induced the up-regulation of a majority of genes in shoots until 60 h time point, except *RPS7a* and *RPS23* and *RPS5*, *RPS10a*, and *RPS23a* under PEG and NaCl treatments, respectively, which became down-regulated.

Some of the RPS genes maintained remarkably high transcript levels (≥ 100 fold). These include *RPS4a*, *RPS9*, *RPS9-2*, *RPS13*, *RPS17*, *RPS19*, *RPS27*, and *RPS30* under PEG treatment and *RPS4a*, *RPS7*, *RPS9-2*, *RPS18a*, and *RPS23a* under NaCl treatment. The up-regulation of *RPS4*, *RPS7*, *RPS9*, *RPS10*, *RPS19*, and *RPS26* under salt treatment is in agreement with the earlier studies (Kawasaki et al., 2001). Unlike in ABA, PEG, and NaCl treatments, several RPS genes were late responding in shoots under H_2O_2 treatment. Moreover, the level of up-regulation was also not as high as in other treatments. About 60% of the genes were up-regulated 15 min post treatment. About 30 genes have been found to maintain a considerable level of expression, and among these, *RPS4a*, *RPS9-2*, *RPS15a*, *RPS18*, and *RPS28* have exhibited ≥ 100 fold up-regulation. In roots, *RPS19* was down-regulated persistently in response to all the abiotic stresses at all the time intervals. *RPS10a*, *RPS18*, *RPS18a*, *RPS18b*, *RPS24*, *RPS27a*, and *RPS6a* were also observed to maintain a very low transcript level. Some of the genes that were initially up-regulated, subsequently, became down-regulated with the progression of the stress treatment. The expression pattern of all the genes under different stress treatments in the shoot and root tissues has been represented in the form of heat maps (Figures 4A,B).

There is an overlap in the expression of the genes that are up or down-regulated in response to the stresses at several time points, and this has been demonstrated in the form of Venn diagrams (Supplementary Figures 4, 5). Table 3 presents a detailed list of genes that were up/down-regulated in different stress conditions in the shoot and root tissues separately. The percentage of genes over-expressed at certain time point under the stress conditions in shoot and root has been represented in the form of Pie charts (Figures 5A,B), and the genes are tabulated in the tables that accompanied the figures. The genes that were commonly up-regulated under all the treatments, particularly during the early response may have significance in stress related signaling (Kilian et al., 2007) as the early responsive genes play a crucial role in defense against the environmental stresses (Mahajan and Tuteja, 2005; Ouyang et al., 2007).

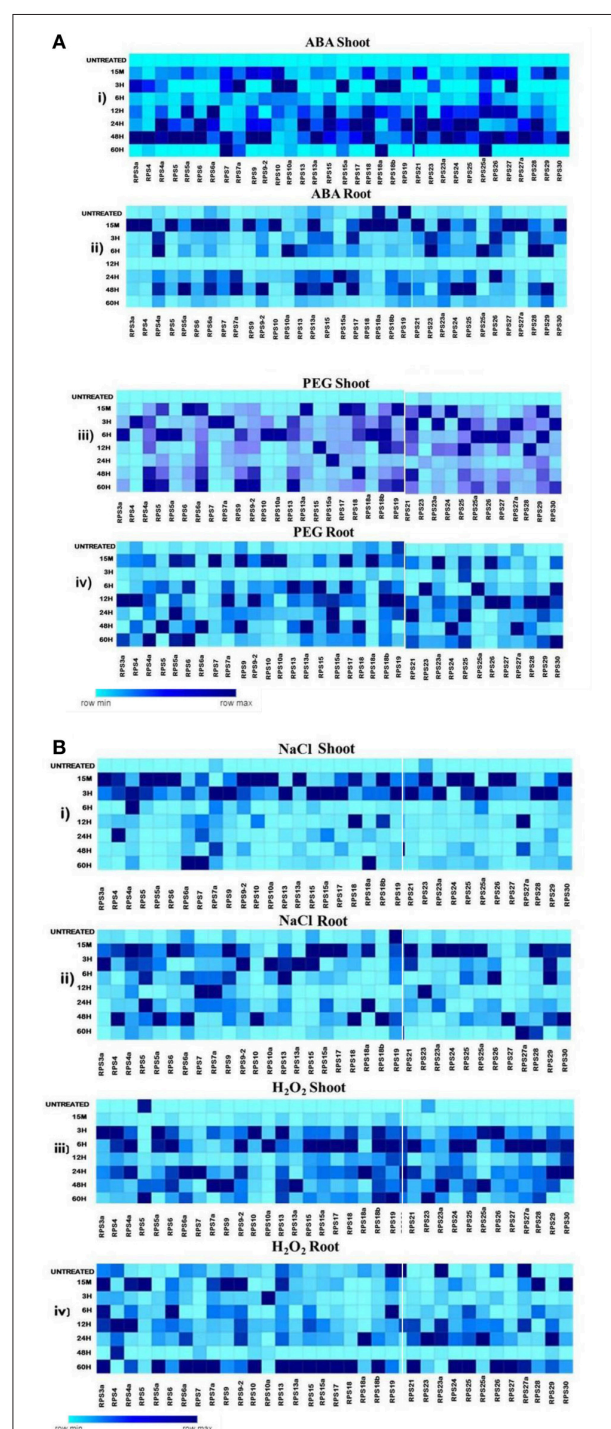


FIGURE 4 | Heat map representation of RPS genes in response to ABA, PEG, NaCl and H_2O_2 treatments. The 7d old seedlings were treated with ABA(100 μ M), PEG (10%), NaCl (250 μ M), and H_2O_2 (10 μ M) and qRT-PCR is used to determine the level of expression of 34 RPS genes in the shoot and the root tissues at the time points mentioned on the left side. The fold change has

(Continued)

FIGURE 4 | Continued

been normalized by $\Delta\Delta C_T$ method with respect to the unstressed seedlings maintained in water. Rice specific *act1* and β -*tub* genes were used as endogenous reference genes. The mean values of the fold changes of the biological and technical replicates were considered for preparing the heat maps. **A-** (i), (ii), (iii), and (iv) represent the expression of the genes in shoot and root tissues under ABA and PEG respectively and **B-** (i), (ii), (iii), and (iv) represent the expression of the genes in shoot and root tissues under NaCl and H₂O₂ respectively. The darkest color depicts the highly up-regulated genes whereas the lightest color represents the less up-regulated and down-regulated genes.

Differential Expression of RPS Genes upon Infection with *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Rhizoctonia solani*

Apart from the four abiotic stresses, the expression level of 34 RPS genes was also studied in response to the treatments with *Xoo* and *R. solani* pathogens that cause very serious Bacterial Leaf Blight (BLB) and Sheath Blight (SB) diseases, respectively in rice. In the BLB infected samples, 14 (40%) genes were down-regulated, and the rest of them were up-regulated (60%). *RPS6a* (26 fold), *RPS9* (18 fold), *RPS10a* (29 fold), and *RPS4* (13 fold) showed a high level of transcript upregulation when compared with the other up-regulated genes.

The qRT-PCR analysis of 34 RPS genes was performed from both the shoot and the leaf samples infected with *R. solani*. The up-regulation of genes was observed particularly in the shoot tissues for a majority of the RPS genes. Thirteen (37%) genes were down-regulated in response to this pathogen in the shoot region whereas the 22 RPS (63%) genes were up-regulated. Among the genes that were expressive, *RPS4* and *RPS5* have exhibited 22 and 14 fold up-regulation, respectively, which is the highest compared with the other genes. The expression pattern of all the genes under two biotic stresses has been depicted graphically in **Figures 6A,B**.

Comparative Expression Analysis of RPL and RPS Genes

Previously available information about the RPL genes (Moin et al., 2016b) has been utilized and subsequently combined with the information obtained from the current study on the expression patterns of RPS genes. The details regarding their stress responsiveness and tissue-specific expression pattern under native condition have been summarized in tabular form to select suitable combinations of RPS and RPL genes for stress alleviation in transgenic plants. All the RPL and RPS genes up-regulated in response to four individual stress treatments were depicted in Supplementary Table 6. Highly up-regulated RPL and RPS genes in each of the 13 different tissues under native conditions have been represented in **Figure 7**. Four heat maps combining the expression of RPL and RPS genes under four abiotic stresses have been represented separately (**Figures 8 A–D**) and Supplementary Figure 6 shows the comparative expression analysis of both RPL and RPS genes under 13 different tissues in the form of a heat map.

One of the intriguing observations from this comparative analysis is that majority of the RPL genes were up-regulated in both shoots and roots of the plant under native conditions. But under all abiotic stress conditions, the maximum up-regulation was observed primarily in roots than in shoots. On the contrary, under the native conditions, the expression of RPS genes was mostly confined to shoots and the leaves compared to roots. Taken together, the up-regulation of the RPS genes and RPL genes was predominantly noticed in shoots and roots, respectively at all the time intervals under all the stress conditions.

According to the previous studies, *RPL23A*, *L19.3*, and *L38* have shown to exhibit a high level of expression under ABA, PEG and NaCl treatments. *RPL6*, *12*, *23A*, *18A*, and *13A* have maintained high up-regulation under H₂O₂ treatment (Moin et al., 2016b). In the present study, *RPS13a*, *18a*, *4*, and *4a* have shown significant up-regulation in almost all the stresses in shoots, while *RPS29* and *RPS28* were highly expressive in roots. In the case of RPL genes, NaCl caused down-regulation of a majority of genes when compared to ABA and PEG. On the contrary, almost all the RPS genes showed up-regulation under salt stress in shoots. The H₂O₂ treatment caused down-regulation of 60 and 80% of RPL genes in shoots and roots, respectively when compared with MeJA, SA and cold treatments, which induced up-regulation of many of them. Most of the RPS genes were up-regulated under all the stresses in shoots, whereas in roots, 8, 22, 5, and 28% of the RPS genes were down-regulated under ABA, PEG, NaCl, and H₂O₂ treatments, respectively. The H₂O₂ treatment induced the down-regulation of many of them. *RPL30*, *44*, *22*, *14*, *29*, *13A*, and *15* were down-regulated under ABA, PEG, and NaCl treatments and *RPS27a*, *10*, *20*, *6*, and *9* were down-regulated in response to all the treatments (ABA, PEG, NaCl, and H₂O₂). *RPL10* was significantly activated upon infection with *Xoo* pathogen, whereas among the RPS genes, *RPS4* became commonly up-regulated under both *Xoo* and *R. solani* treatments.

DISCUSSION

Abiotic and nutrient stresses affect global agriculture by drastically reducing crop yield and productivity. Keeping the expanding world population in mind, stress management is one of the growing concerns for researchers worldwide. Rice is one of the most important staple food crops feeding a large portion of the world's population and is highly affected by some abiotic stresses, which include salinity, extreme temperatures, drought (Gao et al., 2007) and assailants like pests, fungi, virus and bacteria, etc. Being sessile, plants are highly vulnerable to these detrimental factors that drastically affect their growth and productivity. Hence, they have developed highly organized signaling responses and various other complex events to withstand and curtail the deleterious effects of these stress factors.

The role of RPs in ribosome assembly and house-keeping functions was very well recognized in several earlier studies on animal and plant systems. In addition to that, mutations in these RPs have led to physiological defects in several organisms.

TABLE 3 | Regulation of 35 RPS genes in shoots and roots under 4 abiotic stresses.

Locus Name	Protein	Regulation	Response	Maximum Fold Change	Regulation	Response	Maximum Fold Change	
				Shoot	Root			
ABA								
LOC_Os02g18550	RPS3a	UP	IE (47.23)	267.24	UP	IE (55.89)	55.89	
LOC_Os01g25610	RPS4	UP	IE (10.22)	280.43	UP	IE (19.29)	19.29	
LOC_Os05g30530	RPS4a	UP	IE (39.545)	197.20	UP	IE (6.06)	7.084	
LOC_Os01g01060	RPS5	UP	IE (25.13)	114.8852	UP	IE (45.03)	45.035	
LOC_Os11g29190	RPS5a	UP	IE (21.81)	55.41	UP	IE (7.235)	16.32	
LOC_Os01g12090	RPS6	UP	IE (29.16)	140.87	UP	IE (29.55)	29.55	
LOC_Os03g27260	RPS6a	UP	IE (31.25)	187.72	UP	IE (3.31)	3.315	
LOC_Os02g21900	RPS7	UP	IE (132.89)	245.67	UP	IE (7.805)	7.805	
LOC_Os03g18570	RPS7a	UP	IE (4.243)	12.568	UP	IE (6.48)	34.015	
LOC_Os07g43510	RPS9	UP	IE (39.066)	58.97	UP	IE (21.89)	21.89	
LOC_Os03g05980	RPS9-2	UP	IE (38.20)	73.30	UP	IE (4.59)	4.59	
LOC_Os01g73160	RPS10	UP	IE (54.88)	71.79	UP	IE (14.77)	14.77	
LOC_Os02g34460	RPS10a	UP	IE (5.646)	56.49	UP	E (9.458)	9.458	
LOC_Os07g38540	RPS13	UP	IE (3.57)	76.67	UP	E (4.612)	6.664	
LOC_Os08g02400	RPS13a	UP	IE (22.51)	90.80	DOWN	—	—	
LOC_Os07g08660	RPS15	UP	IE (5.99)	28.99	UP	L (4.159)	4.159	
LOC_Os02g27760	RPS15a	UP	IE (5.23)	224.545	UP	L (10.142)	10.142	
LOC_Os03g01900	RPS17	UP	IE (35.00)	186.78	UP	IE (11.17)	17.22	
LOC_Os03g58050	RPS18	UP	IE (44.34)	75.63	UP	IE (7.28)	7.28	
LOC_Os07g07719	RPS18a	UP	IE (34.18)	278.54	DOWN	—	—	
LOC_Os07g07770	RPS18b	UP	IE (54.40)	216.73	UP	IE (6.34)	6.34	
LOC_Os03g31090	RPS19	UP	IE (16.54)	153.23	DOWN	—	—	
LOC_Os03g46490	RPS21	UP	IE (53.26)	105.17	UP	IE (21.06)	21.06	
LOC_Os03g60400	RPS23	UP	IE (11.64)	35.78	UP	IE (6.63)	7.728	
LOC_Os10g20910	RPS23a	UP	IE (18.30)	114.56	UP	IE (5.55)	5.55	
LOC_Os01g52490	RPS24	UP	IE (34.23)	382.71	UP	IE (3.43)	8.76	
LOC_Os08g44480	RPS25	UP	IE (21.79)	106.15	UP	IE (14.5)	19.99	
LOC_Os11g05562	RPS25a	UP	IE (41.42)	56.23	UP	IE (4.34)	58.42	
LOC_Os01g60790	RPS26	UP	IE (16.23)	32.28	UP	L (3.121)	3.121	
LOC_Os04g27860	RPS27	UP	IE (25.71)	49.96	UP	IE (26.53)	26.53	
LOC_Os01g22490	RPS27a	UP	IE (6.57)	302.66	UP	IE (41.93)	41.93	
LOC_Os10g27174	RPS28	UP	IE (61.53)	167.88	UP	IE(5.5)	9.73	
LOC_Os03g56241	RPS29	UP	IE (128.60)	128.60	UP	IE (52.64)	213.33	
LOC_Os02g56014	RPS30	UP	IE (23.244)	113.63	UP	IE (57.34)	57.34	
PEG								
LOC_Os02g18550	RPS3a	UP	IE (26.5)	226.24	UP	IE (5.79)	13.56	
LOC_Os01g25610	RPS4	UP	IE (16.58)	467.00	DOWN	—	—	
LOC_Os05g30530	RPS4a	UP	IE (213.06)	641.1959	UP	IE (9.24)	15.29	
LOC_Os01g01060	RPS5	UP	IE (36.84)	46.53	UP	IE (4.62)	47.36	
LOC_Os11g29190	RPS5a	UP	IE (8.42)	140.56	UP	IE (5.07)	5.55	
LOC_Os01g12090	RPS6	UP	IE (182.15)	182.15	UP	IE (19.90)	24.11	
LOC_Os03g27260	RPS6a	UP	IE (61.71)	66.33	UP	L (12.40)	12.40	
LOC_Os02g21900	RPS7	UP	IE (19.53)	531.87	UP	IE (18.66)	18.66	
LOC_Os03g18570	RPS7a	UP	IE (3.14)	11.51	UP	IE (6.75)	36.64	
LOC_Os07g43510	RPS9	UP	IE (32.99)	100.11	DOWN	—	—	
LOC_Os03g05980	RPS9-2	UP	IE (364.83)	689.87	UP	IE (8.03)	14.98	

(Continued)

TABLE 3 | Continued

Locus Name	Protein	Regulation	Response	Maximum Fold Change	Regulation	Response	Maximum Fold Change	
				Shoot	Root			
LOC_Os01g73160	RPS10	UP	IE (14.67)	287.60	UP	IE (3.256)	3.256	
LOC_Os02g34460	RPS10a	UP	IE (4.94)	72.60	UP	IE (11.54)	11.54	
LOC_Os07g38540	RPS13	UP	IE (8.46)	96.86	UP	E (10.53)	10.53	
LOC_Os08g02400	RPS13a	UP	IE (91.59)	91.59	DOWN	–	–	
LOC_Os07g08660	RPS15	UP	IE (6.14)	142.01	UP	E (4.58)	4.58	
LOC_Os02g27760	RPS15a	UP	IE (21.99)	333.14	UP	E (7.59)	8.97	
LOC_Os03g01900	RPS17	UP	IE (413.73)	413.73	UP	IE (20.17)	20.17	
LOC_Os03g58050	RPS18	UP	IE (134.83)	144.28	UP	L (3.07)	3.07	
LOC_Os07g07719	RPS18a	UP	IE (34.17)	315.89	DOWN	–	–	
LOC_Os07g07770	RPS18b	UP	IE (49.35)	91.722	UP	E (3.81)	4.90	
LOC_Os03g31090	RPS19	UP	IE (202.27)	202.27	DOWN	–	–	
LOC_Os03g46490	RPS21	UP	IE (36.49)	58.40	UP	IE (15.15)	29.51	
LOC_Os03g60400	RPS23	UP	IE (6.05)	6.05	UP	IE (6.62)	33.39	
LOC_Os10g20910	RPS23a	UP	IE (71.23)	218.64	UP	IE (7.91)	7.91	
LOC_Os01g52490	RPS24	UP	IE (262.68)	262.68	UP	L (5.42)	5.42	
LOC_Os08g44480	RPS25	UP	IE (26.27)	110.35	UP	IE (7.62)	9.43	
LOC_Os11g05562	RPS25a	UP	IE (13.69)	41.51	UP	IE (26.88)	230.74	
LOC_Os01g60790	RPS26	UP	IE (7.09)	70.88	UP	IE (3.29)	3.40	
LOC_Os04g27860	RPS27	UP	IE (94.99)	131.19	UP	IE (9.57)	22.27	
LOC_Os01g22490	RPS27a	UP	IE (38.79)	251.68	DOWN	–	–	
LOC_Os10g27174	RPS28	UP	IE (110.33)	264.44	UP	IE (9.41)	32.11	
LOC_Os03g56241	RPS29	UP	IE (33.12)	33.56	UP	IE (5.32)	146.67	
LOC_Os02g56014	RPS30	UP	IE (33.43)	127.31	UP	IE (5.98)	9.07	
NaCl								
LOC_Os02g18550	RPS3a	UP	IE (394.95)	394.95	UP	IE (10.16)	17.009	
LOC_Os01g25610	RPS4	UP	IE (94.08)	107.34	UP	IE (4.21)	5.32	
LOC_Os05g30530	RPS4a	UP	IE (209.01)	754.56	UP	IE (14.40)	14.40	
LOC_Os01g01060	RPS5	UP	IE (64.99)	64.99	UP	IE (7.89)	8.65	
LOC_Os11g29190	RPS5a	UP	IE (233.41)	233.41	UP	IE (16.07)	36.43	
LOC_Os01g12090	RPS6	UP	IE (132.97)	132.97	UP	IE (5.44)	5.44	
LOC_Os03g27260	RPS6a	UP	IE (7.03)	47.95	DOWN	–	–	
LOC_Os02g21900	RPS7	UP	IE (87.15)	135.30	UP	IE (13.69)	32.01	
LOC_Os03g18570	RPS7a	UP	IE (5.44)	5.44	UP			
LOC_Os07g43510	RPS9	UP	IE (167.46)	325.91	UP	IE (3.75)	3.75	
LOC_Os03g05980	RPS9-2	UP	IE (1357.75)	1357.75	UP	IE (7.14)	11.27	
LOC_Os01g73160	RPS10	UP	IE (380.49)	380.49	UP	L (8.40)	8.40	
LOC_Os02g34460	RPS10a	UP	IE (129.63)	129.63	UP	IE (19.8)	19.8	
LOC_Os07g38540	RPS13	UP	IE (25.66)	364.99	UP	IE (4.61)	7.37	
LOC_Os08g02400	RPS13a	UP	IE (187.85)	187.85	UP	IE (3.52)	53.19	
LOC_Os07g08660	RPS15	UP	IE (10.02)	88.46	UP	IE (3.18)	3.18	
LOC_Os02g27760	RPS15a	UP	IE (103.02)	724.23	UP	IE (6.01)	6.01	
LOC_Os03g01900	RPS17	UP	IE (145.12)	252.99	UP	IE (18.42)	18.42	
LOC_Os03g58050	RPS18	UP	IE (819)	885.39	UP	IE (3.49)	9.76	
LOC_Os07g07719	RPS18a	UP	IE (266.91)	1068.83	UP	IE (3.62)	3.91	
LOC_Os07g07770	RPS18b	UP	IE (318.11)	318.11	UP	IE (4.55)	84.05	
LOC_Os03g31090	RPS19	UP	IE (166.16)	332.04	DOWN	–	–	
LOC_Os03g46490	RPS21	UP	IE (53.78)	96.45	UP	IE (13.65)	13.65	
LOC_Os03g60400	RPS23	UP	IE (3.37)	3.37	UP	IE (23.68)	164.02	

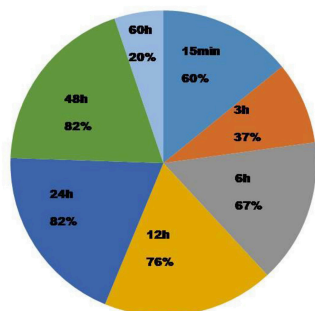
(Continued)

TABLE 3 | Continued

Locus Name	Protein	Regulation	Response	Maximum Fold Change	Regulation	Response	Maximum Fold Change
			Shoot	Root			
LOC_Os10g20910	RPS23a	UP	IE (58.99)	439.57	UP	IE (5.46)	5.46
LOC_Os01g52490	RPS24	UP	IE (238.23)	238.23	UP	IE (32.20)	32.20
LOC_Os08g44480	RPS25	UP	IE (129.19)	129.19	UP	IE (14.58)	14.58
LOC_Os11g05562	RPS25a	UP	IE (91.95)	91.95	UP	IE (34.99)	34.99
LOC_Os01g60790	RPS26	UP	IE (143.50)	143.50	UP	IE (3.25)	6.94
LOC_Os04g27860	RPS27	UP	IE (552.55)	552.55	UP	IE (22.45)	22.45
LOC_Os01g22490	RPS27a	UP	IE (216.54)	1420.60	UP	L (9.35)	9.35
LOC_Os10g27174	RPS28	UP	IE (26.19)	543.29	UP	IE (33.77)	33.77
LOC_Os03g56241	RPS29	UP	IE (24.27)	47.44	UP	IE (42.79)	50.48
LOC_Os02g56014	RPS30	UP	IE (65.93)	65.93	UP	IE (13.00)	15.40
H ₂ O ₂							
LOC_Os02g18550	RPS3a	UP	IE (8.8)	83.86	UP	IE (3.46)	3.46
LOC_Os01g25610	RPS4	UP	IE (29.68)	29.68	DOWN	–	–
LOC_Os05g30530	RPS4a	UP	IE (18.75)	487.002	UP	IE (9.86)	10.73
LOC_Os01g01060	RPS5	DOWN	–	–	UP	IE (22.29)	22.29
LOC_Os11g29190	RPS5a	UP	IE (33.87)	33.87	UP	E (6.49)	14.245
LOC_Os01g12090	RPS6	UP	IE (6.60)	53.10	UP	IE (8.03)	14.35
LOC_Os03g27260	RPS6a	UP	IE (12.95)	69.88	DOWN	–	–
LOC_Os02g21900	RPS7	UP	IE (16.64)	180.48	UP	L (3.00)	3.00
LOC_Os03g18570	RPS7a	UP	IE (10.30)	15.94	UP	IE (7.42)	9.804
LOC_Os07g43510	RPS9	UP	IE (4.76)	269.65	DOWN	–	–
LOC_Os03g05980	RPS9-2	UP	IE (33.16)	329.89	UP	IE (5.18)	5.18
LOC_Os01g73160	RPS10	UP	IE (10.29)	93.11	UP	L (5.65)	5.65
LOC_Os02g34460	RPS10a	UP	IE (16.59)	87.74	UP	IE (11.69)	11.69
LOC_Os07g38540	RPS13	UP	IE (11.59)	69.70	DOWN	–	–
LOC_Os08g02400	RPS13a	UP	IE (31.08)	137.24	DOWN	–	–
LOC_Os07g08660	RPS15	UP	IE (5.98)	51.09	UP	E (3.31)	6.49
LOC_Os02g27760	RPS15a	UP	IE (23.67)	298.48	UP	L (94.29)	4.29
LOC_Os03g01900	RPS17	UP	IE (4.18)	154.85	UP	L (7.78)	7.78
LOC_Os03g58050	RPS18	UP	IE (15.43)	187.73	UP	L (6.04)	6.04
LOC_Os07g07719	RPS18a	UP	IE (4.07)	491.19	DOWN	–	–
LOC_Os07g07770	RPS18b	UP	IE (6.82)	62.92	UP	L (4.56)	4.56
LOC_Os03g31090	RPS19	UP	IE (18.42)	71.76	DOWN	–	–
LOC_Os03g46490	RPS21	UP	IE (3.07)	81.90	UP	E (8.869)	11.50
LOC_Os03g60400	RPS23	UP	L (3.08)	3.08	UP	E (3.14)	14.99
LOC_Os10g20910	RPS23a	UP	IE (4.10)	164.71	DOWN	–	–
LOC_Os01g52490	RPS24	UP	IE (5.27)	66.26	UP	IE (10.69)	23.09
LOC_Os08g44480	RPS25	UP	IE (30.38)	58.89	UP	E (5.60)	7.79
LOC_Os11g05562	RPS25a	UP	IE (146.44)	155.55	UP	IE (4.65)	34.66
LOC_Os01g60790	RPS26	UP	IE (4.00)	47.17	DOWN	–	–
LOC_Os04g27860	RPS27	UP	IE (27.00)	66.18	UP	L (7.89)	23.34
LOC_Os01g22490	RPS27a	UP	IE (3.54)	146.72	DOWN	–	–
LOC_Os10g27174	RPS28	UP	IE (19.92)	137.66	UP	IE (78.78)	78.78
LOC_Os03g56241	RPS29	UP	IE (22.81)	37.30	UP	E (4.293)	16.65
LOC_Os02g56014	RPS30	UP	IE (3.12)	30.58	UP	IE (9.01)	9.01

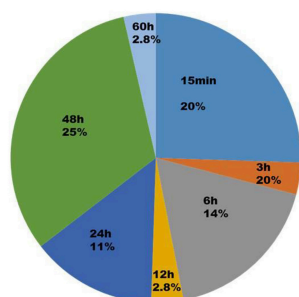
The regulation of all the 34 RPS genes in response to ABA, PEG, NaCl, and H₂O₂ in shoot and root are listed. Whether they are up-regulated or down-regulated, is mentioned separately. The genes having fold change ≥ 3 are considered to be up-regulated and the rest are considered to be down-regulated. Their extent of response is indicated as well with the fold change at that point. IE- immediate early genes (showing response within 15 min–3 h); E- early genes (showing response within 3 h– 12 h) and L- late responsive genes (showing response after 12 h post treatment). The overall maximum fold change for a gene under a specific treatment is also mentioned.

A



Time points	Genes up-regulated in shoot
15 min	S15a, S30, S13, S26, S23a, S27a, S9-2, S19, S3a, S24, S4a, S18b, S9, S10, S6, S18, S18a, S17, S15 and S21
3h	S15a, S13a, S3a, S25a, S4a, S27, S18b, S10a, S10, S7a, S18a, S4 and S7
6h	S13, S29, S13a, S23a, S27a, S9-2, S19, S3a, S25a, S24, S4a, S18b, S28, S9, S25, S6a, S5a, S6, S18, S18a, S17, S4 and S15
12h	S15a, S29, S26, S13a, S23a, S27a, S19, S3a, S24, S4a, S27, S18b, S28, S9, S10, S25, S6a, S5a, S6, S18, S18a, S17, S4, S15, S7 and S21
24h	S15a, S13, S29, S26, S13a, S23a, S27a, S9-2, S19, S3a, S25a, S24, S4a, S27, S18b, S28, S9, S10, S25, S6a, S5a, S6, S18, S18a, S17, S4, S15, S7 and S21
48h	S15a, S13, S29, S26, S13a, S23a, S27a, S9-2, S19, S3a, S25a, S24, S4a, S27, S18b, S28, S9, S10, S25, S6a, S5a, S6, S18, S18a, S17, S4, S7 and S21
60h	S15a, S13a, S25a, S10, S18a, S4 and S7

B



Time points	Genes up-regulated in root
15 min	S30, S9-2, S3a, S28, S7a, S5 and S6
3h	S28
6h	S28, S29, S23, S25a and S4a
12h	S25a
24h	S5a, S25a, S21 and S27
48h	S28, S25, S29, S5, S7a, S17, S24, S7 and S27
60h	S28

FIGURE 5 | Percentages of genes up-regulated in shoot and root under all the stresses at different time points. The percentage of genes that are upregulated in shoot (A) and root (B) at different time intervals after the application of the stress is represented in form of pie charts with the genes enlisted in the respective tables.

Effect on global protein synthesis due to these reduced RP functions could be one of the reasons behind developmental abnormalities. However, these physiological defects imply the potential extra-ribosomal and regulatory roles apart from the regular housekeeping functions for the RPs (Lu et al., 2015).

In this study, we have made comprehensive expression analyses of Ribosomal Protein Small subunit genes (RPS) in rice under multiple abiotic and biotic stresses and compared their expression with large subunit genes (RPL) genes (Moin et al., 2016b). Based on the differential and native expression data, we have short-listed the promising RPS and RPL combinations of candidate genes that might be useful in manipulating stress tolerance in rice. Also, we have presented an *in silico* analysis of the RPS protein properties and the putative promoter elements of the studied genes. With our previous study, which dealt with the extensive investigation on rice RPL genes (Moin et al., 2016b), we have tried to provide comprehensive information on the stress induced expression of all the ribosomal protein (RPS and RPL) genes in rice as a whole.

The Bacterial Leaf Blight (BLB) and Sheath Blight (SB) diseases are two of the major constraints in rice production with devastating influence on rice yield and its productivity (Zheng et al., 2013; Khan et al., 2014).

Availability of high-quality rice genome database helped us retrieve the genomic, CDS and protein sequences along with the 5' upstream regions of the selected genes. A total of 56 RPS gene sequences were retrieved from the search in the databases, and finally, 34 genes were short listed excluding the identical members. The distribution of RPS genes appeared to be throughout the rice genome with each of the 12 chromosomes carrying at least one RPS gene family member with chromosome 3 exhibiting a maximum of 11 RPS genes. The RPs are mostly rich in positively charged amino acids, which can be correlated with the hypothesis that positively charged amino acids might play a significant role in electrostatic protein-RNA interactions (Law et al., 2006) within the ribosomal complex. Since the RPs are involved mostly in interactions that make up the ribosome complex along with rRNAs, the RPs are expected to have similar isoelectric pH range to facilitate their molecular interactions. Proteins with high isoelectric points tend to interact with DNA, RNA and other biomolecules that are negatively charged whereas the proteins with low isoelectric points prevent non-specific interaction with nucleic acids (Takakura et al., 2016). In a recent study, RPS6 has been reported to interact with a plant specific histone-deacetylase-2B and also with nucleosome assembly protein-1 thereby regulating the transcription of r-DNA in *Arabidopsis* (Son et al., 2015; Xiong and Sheen, 2015). Having a low isoelectric point might, therefore, be beneficial for RPS6 to minimize non-specific interactions. The RPs also have several domains associated with them like KOW, SPT2, HLH, CARD, ANTAR, HALZ, SANT domains etc. These domains have different functions including interaction with other proteins, transcription regulation, protein dimerization, apoptosis, binding to RNA and so on (Kypides et al., 1996; Bouchier-Hayes and Martin, 2002; Shu and Zhulin, 2002; Jones, 2004; Novoseler et al., 2005; Feller et al., 2011).

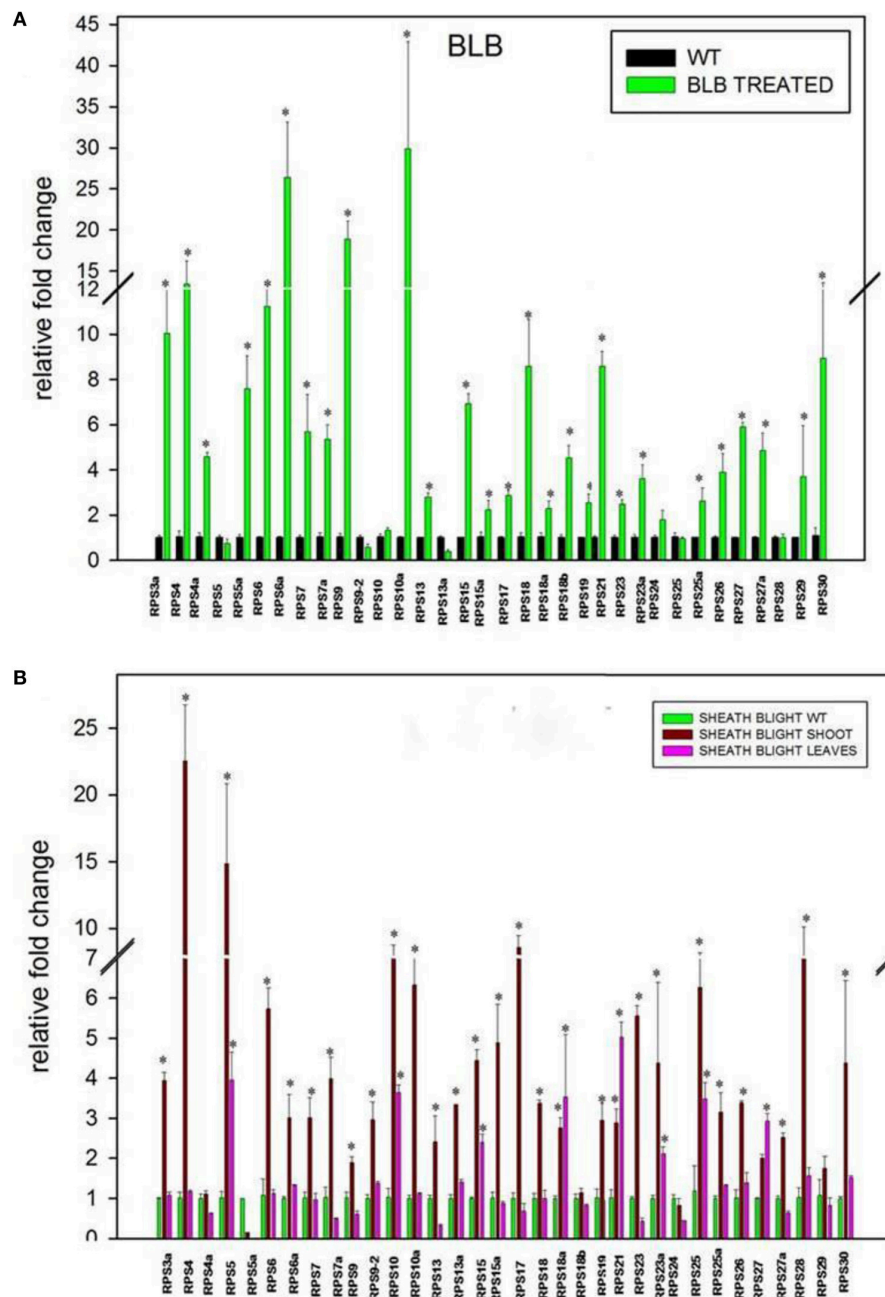
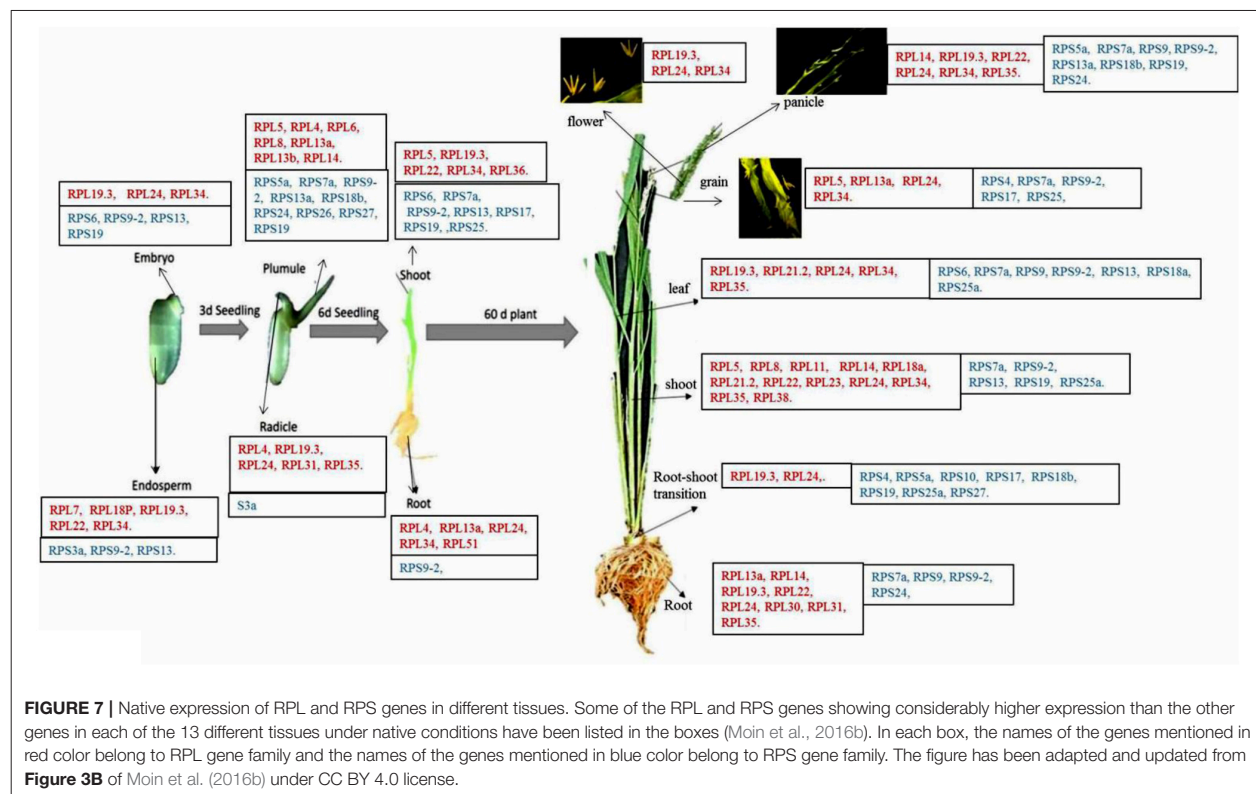


FIGURE 6 | Differential expression of RPS genes in response to *Xanthomonas oryzae* pv. *oryzae* (Xoo) (A) and *Rhizoctonia solani* (B), which cause Bacterial Leaf Blight (BLB) and Sheath Blight (SB) respectively. The expression of 34 RPS genes was determined in BLB and SB infected leaf samples after 20 and 25 d respectively after infection. The expression was normalized with corresponding untreated samples in the identical conditions. The statistical significance of expression analysis has been represented with asterisks * $P < 0.05$.

Under native conditions in 13 different tissues, the RPS genes are prevalently up-regulated in 45 d old mature leaves, shoots and root-shoot transition and 7 d shoots and plumules and some of

them were up-regulated in roots, panicles, embryo, endosperm and grains. But, none of them was found to be expressive in flowers. The role of RPS genes might be presumed to be involved



in modulating developmental stages in vegetative tissues, mainly shoots and not to a greater extent in reproductive tissues. The activity of *RPS18A* promoter of *Arabidopsis* was seen in meristematic tissues and embryonic stage and mutations in these loci resulted in the formation of pointed leaves (Van Lijsebettens et al., 1994). This can be correlated with the transcriptional up-regulation of *RPS18*, *RPS18a*, and *RPS18b* in embryo, plumules, shoots, leaves, and root-shoot transition in the present study.

Around 60 and 20% of the genes were up-regulated immediately after treatment in shoots and roots, respectively under all the stress conditions. Up-regulation of these genes might fall under immediate response to the stress, and these genes are presumed to encode non-specific master regulators needed for plant core environmental stress response proteins. The immediate-early response to a certain stress might sometimes be non-specific, and responses with continuous up-regulation are considered to be specific ones (Kilian et al., 2007). And, the genes that are up-regulated at around 1 h time points can be assumed to have a pivotal role in early defense signaling and might have a function in reconstructing the proteins synthesis apparatus (Ouyang et al., 2007). This has led us to look for continuity in the up-regulation of immediate-early responsive RPS genes.

There are a considerable number of genes for which the up-regulation is maintained throughout the duration of the stress for example, *RPS13a*, *18a*, *4* and *4a*. Subsequently, *RPS29* has shown a remarkably high transcript level (≥ 100 fold) in roots under ABA and PEG treatments and also showed a moderate

level of expression (11-fold) under H_2O_2 treatment. *RPS28* has consistently displayed a moderately high level of expression in all the stress conditions in root tissues.

On the other hand, *RPL23A*, *6*, *8*, *L19.3*, and *L38* among the members of large subunit genes have consistently shown up-regulation under ABA, PEG, NaCl treatments. *RPL18a*, *L24a*, *L24b*, *L30*, and *L34* were significantly overexpressed under oxidative stress. Moreover, *RPL23A*, one of the candidate genes that were identified using an activation tagging approach in water use efficiency (Moin et al., 2016a) might have a potential role to be utilized in modifying abiotic stress tolerance in rice orchestrating the responses of other candidate genes from both the subunits. Among the RPS genes, *RPS3a*, *RPS6*, *S15a*, *S17*, *S18a*, *S23a*, *S24*, *S25*, *S27a*, and *S28* are substantially up-regulated in shoots under a minimum of two abiotic stress conditions. *RPL10* and *RPS4* are significantly up-regulated under pathogen treatments. *RPS18a* has few significant protein domains associated with it and *RPS18a* has shown upregulation throughout all the stress treatments. Hence, the candidate genes overexpressed in small and large subunit categories listed in this study might be crucial in shortlisting genes to manipulate stress tolerance in rice by generating transgenic plants overexpressing the genes of both the subunits either individually or in combination.

It is crucial to look into the response of plants in the presence of multiple stress factors in order to completely evaluate the resistance responses of the candidate genes as the plant responses to a combination of stresses differ from individual

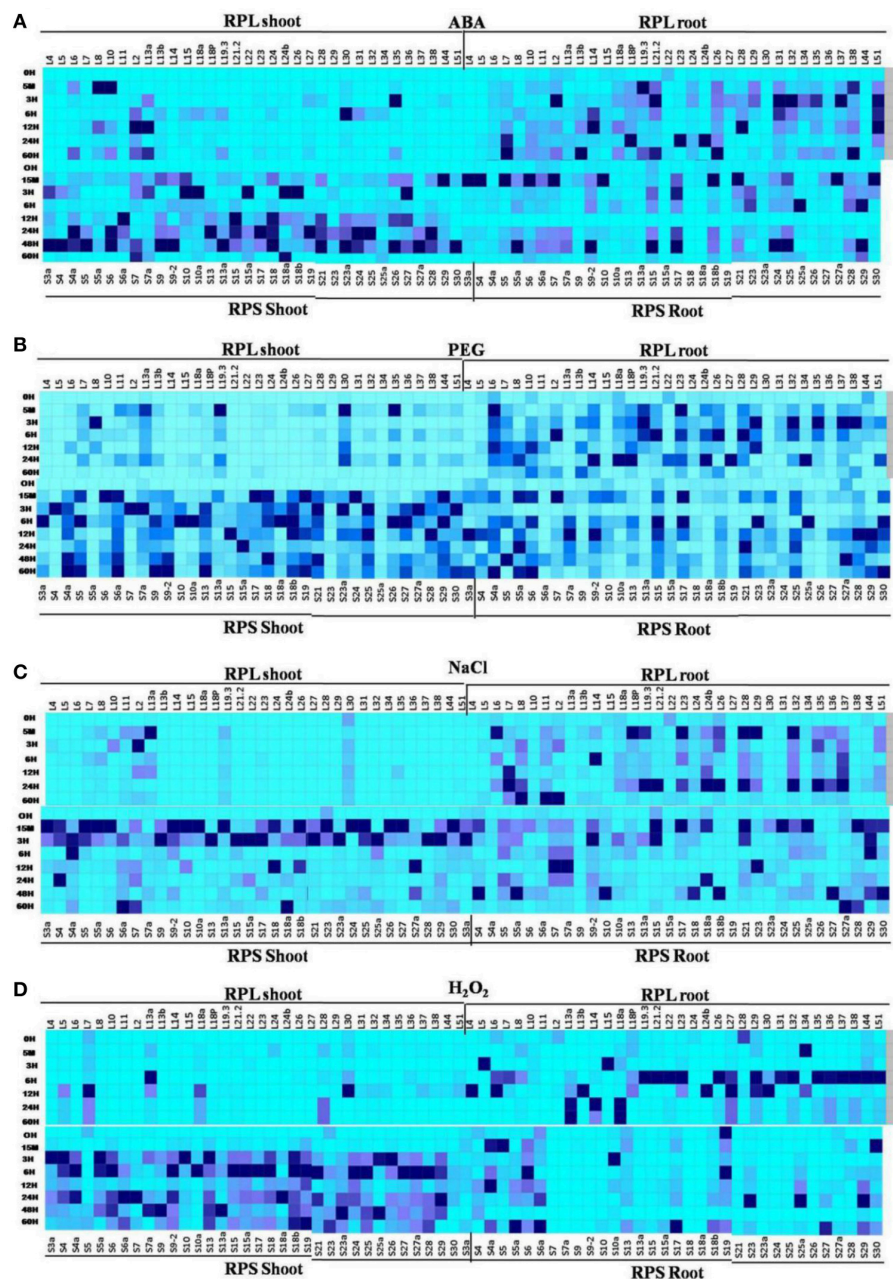


FIGURE 8 | combined heat maps depicting differential expression of RPL and RPS genes under 4 abiotic stresses in shoot and root tissues. Differential expression of all the RPL and RPS genes under 4 abiotic stress is depicted together in 4 heat maps (**A**- ABA, **B**- PEG, **C**- NaCl, and **D**- H_2O_2). The mean of the fold change values of the biological and technical replicates are represented in the form of heat maps.

stress responses (Atkinson and Urwin, 2012). In this context, the genes that are mostly up-regulated under all the stress conditions are significant. On the contrary, *RPS23* and *RPS7a* have maintained a low level of expression at all the time points under almost all the abiotic stresses in shoots, while *RPS19* has consistently exhibited down-regulation in roots under all the

abiotic stress conditions. Except at certain time points under ABA treatment, *RPS27a*, 10, 6, and 9 have shown either down-regulation or no change in expression under rest of the stress conditions. *RPS18*, 18a, 15, and 13 have consistently displayed a low level of expression irrespective of the abiotic stress condition.

Apart from the genes that have been up-regulated in response to stress, there are also certain genes (mentioned above) that did not respond to the stress factors effectively in the respective tissues, probably indicating the limitations of their roles in stress tolerance. Under biotic stress, *RPS4*, *10a*, and *6a* were up-regulated in BLB infected leaf samples, and *RPS4* and *5* were up-regulated in sheath blight infected leaf samples. Since *RPS4* has been up-regulated remarkably under all the abiotic and biotic stress conditions; it can be considered as one of the important RPS genes having both abiotic and biotic stress responsive roles.

The *cis*-regulatory elements (CREs) in the 5' UTR regions play significant roles in activation and suppression of gene expression that can confer tolerance to stresses (Ibraheem et al., 2010; Hernandez-Garcia and Finer, 2014). Throughout the putative promoter regions of almost all the RPS genes, regulatory elements that respond to abiotic and biotic stress treatments are distributed in multiple copies justifying their enhanced expression under various stress treatments.

The alarming change in climatic conditions will have a drastic consequence on global agriculture providing impetus to the already existing abiotic and biotic stresses. Temperature increase is more likely to be accompanied by recurrent flood, drought, enhanced salinity and prevalent occurrence of pathogen attacks (Atkinson and Urwin, 2012). This propels the researchers to produce transgenic plants with better yielding traits, which can withstand the altered environmental conditions. Ribosomal protein genes are important in this context because ribosome constitutes the protein synthesis machinery in a cell and under unfavorable conditions, the composition of the ribosome has been thought to change in order to assist biased protein translation (Wang J. et al., 2013). Moreover, some of the Genome-wide RNA sequencing technology and microarray analyses of other crop plants like Sorghum and alfalfa also revealed ribosomal protein genes to be important in response to abiotic stress (Fracasso et al., 2016; Liu et al., 2017).

Peptide mass spectrometry provides information that RPs of both prokaryotes and eukaryotes undergo a high degree of post-translational modifications (Arragain et al., 2010). Six RPs of *Escherichia coli* are methylated, three are acetylated, and one of them is methyl-thiolated. Although these modifications might have specific regulatory functions, the actual significance of these modifications is unclear (Nesterchuk et al., 2011). In plants, RPs are mostly methylated and methylation appears to be important for ribosome biogenesis and perhaps, its function (Friso and van Wijk, 2015). Apart from methylation, phosphorylation and glycosylation also play critical roles in post-translational modification of ribosomal proteins. Modulation of diurnal protein synthesis in plants has been predicted to have some link

with differential phosphorylation of some ribosomal proteins. In higher plants, phosphorylation status of ribosomal protein S6 contributes to rapid adjustments of growth under environmental changes. Auxin enhances S6 protein phosphorylation in maize, which selectively increases ribosomal protein synthesis (Turkina et al., 2011). In terms of glycosylation, N glycosylation of Asn165 residue of Rps3, which is secreted in cancer cell lines, regulates the migration and invasive phenotype of the cancer cells (Kim et al., 2016). Overall the post-translational modification of RPs is important for the accuracy of the decoding machinery (Arragain et al., 2010) and it might also establish an additional RNA-protein contact which might be beneficial (Polikanov et al., 2015).

Differential expression of RPL and RPS genes under abiotic and biotic stresses, as we have described in the present study, indicates their role in stress responsiveness and might also modulate stress tolerance. The RPL and RPS genes that are remarkably up-regulated in response to almost all the stresses in comparison to the other genes can be considered as the cardinal RP genes that are essential to maintain the integrity of ribosomal units as well as ensuring sustained and undisturbed protein synthesis even under stress. This study provides an insight into the changes in the expression of RP genes in response to the environmental condition. Thus, the stress responsive increase in expression of several ribosomal protein genes can be considered for further exploitation as a resource in crop improvement programs.

AUTHOR CONTRIBUTIONS

AS and SD performed the experiments. AS, MM, and PK designed the experiments and prepared the manuscript. AB and MD helped in qRT-PCR experiments and analysis. MSM performed the *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani* infection on rice plants. PK supervised the work. AS, MM, and PK edited the manuscript.

ACKNOWLEDGMENTS

The funding for the current work has been obtained through a grant sponsored by DBT, GOI to PK with grant number BT/PR13105/AGR/02/684/2009. AS, MM are grateful to DBT for proving Research Fellowships. AB is also thankful to DBT for Project Fellowships.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2017.01553/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH ARTICLE

Study on Transcriptional Responses and Identification of Ribosomal Protein Genes for Potential Resistance against Brown Planthopper and Gall Midge Pests in Rice

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Abstract: Background: Our previous studies have revealed the roles of ribosomal protein (RP) genes in the abiotic stress responses of rice.

Methods: In the current investigation, we examine the possible involvement of these genes in insect stress responses. We have characterized the RP genes that included both *Ribosomal Protein Large (RPL)* and *Ribosomal Protein Small (RPS)* subunit genes in response to infestation by two economically important insect pests, the brown planthopper (BPH) and the Asian rice gall midge (GM) in rice. Differential transcript patterns of seventy selected RP genes were studied in a susceptible and a resistant genotype of *indica* rice: BPT5204 and RPNF05, respectively. An *in silico* analyses of the upstream regions of these genes also revealed the presence of *cis*-elements that are associated with wound signaling.

Results: We identified the genes that were up or downregulated in either one of the genotypes, or both of them after pest infestation. The transcript patterns of a majority of the genes were found to be temporally-regulated by both the pests. In the resistant RPNF05, BPH infestation activated *RPL15*, *L51* and *RPS5a* genes while GM infestation induced *RPL15*, *L18a*, *L22*, *L36.2*, *L38*, *RPS5*, *S9.2* and *S25a* at a certain point of time. These genes that were particularly upregulated in the resistant genotype, RPNF05, but not in BPT5204 suggest their potential involvement in plant resistance against either of the two pests studied.

Conclusion: Taken together, *RPL15*, *L51*, *L18a*, *RPS5*, *S5a*, *S9.2*, and *S25a* appear to be the genes with possible roles in insect resistance in rice.

Keywords: Ribosomal protein genes, brown planthopper, rice, gall midge pests, transcriptional response.

1. INTRODUCTION

The global population is growing exponentially and is expected to exceed nine billion by 2050 [1]. One of the important ways of ensuring food security and feeding the burgeoning population is through enhanced and sustained productivity of staple crops. Rice is a principal source of nutrition and a staple cereal for more than two billion population in Asia and thus, accounts for more than 40% of the calorie consumption. Increasing demand necessitates a proportionate increase in production of rice, which is alarmingly affected by biotic and abiotic stresses that result in unstable productivity.

Biotic stress comprises attacks by insect pests, bacterial, fungal and viral pathogens, and weed competition, while abiotic stresses such as salinity, drought and extreme climates cause a huge impediment towards sustainable rice production across the world [2]. The major biotic stresses include bacterial leaf blight (BLB), fungal blast diseases and attack by insect pests like BPH, gall midge and stem borers that together account for a major share of losses across the globe [3]. Out of this, approximately 21% of the losses are solely attributed to insect pests. Among the three major groups of insect pests listed above, BPH represents sucking pests that mainly feed on phloem sap depriving the plant of nutrients, which results in plant mortality called "hopper burn".

Gall midges are unique gall formers that do not kill the plant but hijack its normal developmental processes by inducing the formation of galls and sterility. Though these two pests are managed by the farmers through the application of insecticides, these are also amenable for management through host-plant resistance. Extensive research has identi-

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fied scores of rice germplasm lines that are resistant to the attack by these pests [4]. Intensive studies have unveiled genetics of resistance leading to tagging and mapping plant resistance (R) genes and the development of linked molecular markers that are being used in marker-assisted selection (MAS). MAS has made breeding rice for insect resistance more precise with rapid pyramiding of genes for multiple and durable resistance. Concomitant research over decades has tried to understand insect resistance in rice at genetic, anatomical, physiological, biochemical and molecular levels [5]. These studies, in summary, highlight a two-tier defence system in rice against insects as in the case of pathogen attack. The first line of defence is an innate immune response of general nature triggered by molecular patterns associated with herbivory (HAMP) and or damage (DAMP) [6]. Plants recognize these molecules through receptors on the cell membrane and trigger an immune response called pattern triggered immunity (PTI). This defence is race non-specific. In response, insects secrete specialized chemicals called effectors that nullify PTI and allow the insect to feed and develop on the plants [7-10].

The second level of defence is induced by plants in response to the effectors and is termed as effector-triggered immunity (ETI) [10]. This defence is race-specific and involves both constitutive and induced responses with or without hypersensitive reaction (HR), oxidative burst, signal transduction, hormone regulation and systemic acquired resistance [11, 12]. Some of these involve secondary metabolism of synthesis of defence chemicals that are not essential for the regular growth, development and reproduction of plant. Thus, there is a compromise between primary and secondary metabolism [12].

Insect resistance has long been proposed to consist of components like nonpreference or antixenosis, antibiosis resulting in reduced fitness of the infesting insect and tolerance [13]. While the first two components constitute the active plant defence system detailed above, the tolerance component accounts for the ability of the plant to compensate for the damage inflicted by the insect and maintain near normal growth, development and yield. This component has been least studied in greater detail. This primary metabolism-mediated insect resistance in rice may involve basic functions like photosynthesis and protein synthesis. In such a situation, the ribosomal proteins (RPs) might play a role here. The present investigation is aimed at this angle of insect resistance through monitoring a subset of RP coding genes.

RPs are known to play an integral role in rRNA structure and formation of protein synthesizing machinery in cells. These are also crucial for the growth and development of all the organisms. The formation of a ribosomal complex is a compendium process requiring co-transcription, coordinated expression and assembly of hundreds of proteins [14]. The ribosomal DNA (rDNA) exists as tandemly arrayed repeats separated by spacers at nucleolus organizing regions (NORs). The nucleolus is the site of transcription of rDNA, processing of primary transcripts into different rRNAs and assembly of preribosomal subunits, which together form the linc-

pins of ribosome biogenesis [15-17]. The rRNAs and RPs together maintain the structural stability of ribosomes that perform diverse cellular activities required for organismal growth and development [18]. Thus, to cope up with the changing growth conditions and continuous exposure to external stimuli, which occur at different stages of the life cycle in an organism, cells must normalize the ribosome biosynthesis for its normal functioning [15, 19]. Any imbalance in ribosome biosynthesis or mutations in rRNA and RP-coding genes results in perturbed plant phenotypes, which might also be lethal to cells. Mutations in different RP genes caused varying consequences from morphological changes [20-25] to embryonic lethality [26]. For example, *RPL23aA* exists as two paralogs, *RPL23aA* and *aB*. While the knockout of *aA* resulted in an overall reduction in ribosome biogenesis producing phenotypes characterized by growth retardation and reduced fertility, the knockout of *aB* showed no obvious phenotypic consequences [27]. Also, *RPL23aB* was found to be the only paralog that did not show any phenotypic change upon knockout [28]. However, these variations in *aA* and *aB* knockout mutations have been attributed to their native expression levels, pointing to the fact that these two paralogs are functionally equivalent [29].

It has been observed that upregulation of RP genes in plants under abiotic stress conditions might suggest a differential reconstruction of protein-synthesizing machinery by the incorporation of different paralogous members of RPs in different situations [30-36]. This heterogeneity of ribosomes formed by different combinations of RP paralogs is controlled by specific cell types, developmental stages and environmental factors [34, 37]. The high ribosomal heterogeneity, a characteristic feature of sessile plants has been attributed to their potential to adapt under different environmental signals [30, 35]. Although there are no significant reports on the contribution of RP genes in conferring resistance to pests, we have identified potential Ribosomal Protein Large (RPL) subunit genes (*RPL23a* and *RPL6*) to be significantly upregulated in studies for enhanced water-use efficiency (WUE) in *indica* rice following gain of function mutagenesis through the activation tagging approach [38]. Subsequently, we studied the regulatory roles of all the *RPL* and *RPS* genes under different external stimuli and observed the significant up-regulation of a considerable number of these genes under several stresses including abiotic and biotic conditions in rice [39, 40]. In the present investigation, we made a transcript analysis of selected 70 out of 255 RP genes in susceptible (BPT5204) and resistant (RPNF05) genotypes of *indica* rice challenged by two major insect pests, BPH and GM. We have also tried to identify the potential candidate RP genes that are specifically or commonly upregulated in one or both the above genotypes.

2. MATERIALS AND METHODS

2.1. Nucleotide Sequence Retrieval of RP Genes

The nucleotide sequences of all the RP genes were retrieved from MSU Rice Genome Annotation Project data base (RGAP-DB)¹ under putative function search tool using

a keyword 'ribosomal'. This search provided a list of a total of 428 genes. Because plants also contain chloroplast ribosomes, the 50S and 30S *RP* genes were excluded and only cytosolic *RP* genes were selected. The large and small subunit genes were differentiated based on their prefixes. The genes starting with a prefix 60S or 'L' (for large) were considered as *RPL* genes and those starting with 40S or 'S' (for small) were categorized as *RPS* genes. According to the latest update of RGAP-DB, the cytosolic ribosomal proteins of rice are encoded by at least 70 genes out of a family of 255 *RP* genes that include paralogs [38-40]. In the current study, these 70 *RP* candidate genes (35 *RPL* and 35 *RPS*) excluding the paralogs were selected for transcript analysis. After sequence retrieval, the 70 genes were validated by a BLAST search in other databases like NCBI², RAP-DB³ and Phytozome⁴.

2.2. In silico Putative Promoter Analysis of *RP* Genes

To determine the presence of *cis*-elements that respond to wound signaling resulting from insect attack, about 1 kb nucleotide sequence upstream from each of 70 *RP* genes was retrieved from RGAP-DB and submitted to the PlantCARE⁵ database. The complete list of *cis*-elements in promoter regions of *RP* genes has been reported in the published literature [39, 40] and those concerning insect attack responses have been emphasized here.

2.3. Plant Growth Conditions and Infestation with Insect Pests

Two rice genotypes, BPT5204 and RPNF05 were used in this study. BPT5204 is susceptible to both pests and is obtained from the Indian Institute of Rice Research, Hyderabad, India. RPNF05, which has been shown to exhibit moderate to a high level of resistance against BPH and GM, respectively is collected from the Agri Biotech Foundation, Hyderabad, India [41, 42] and is used as a positive control. It is a derivative of BPT5204 obtained by systematic introgression of bacterial blight (*Xa21*, *xa13* and *xa5*), fungal blast (*Pi54*) and GM resistance genes (*Gm1*, *Gm3*, *Gm4* and *Gm8*) [42]. Hence, the background of both the genotypes used in this study is similar except for the introgression of the desirable genes governing pest stress tolerance in RPNF05.

The growth of BPT5204 and RPNF05 genotypes and their infestation with gall midge (GM) and brown planthopper (BPH) were performed as mentioned earlier [42]. In brief, rice seeds of both the genotypes were sown in plastic trays (60 X 40 X 7 cm) and plants were raised under green

house conditions (30 ± 2° C, 16 hour (h) light/ 8 h dark photoperiods, Supplementary Fig. (1a). Three-week-old plants were challenged with gall flies belonging to gall midge biotype 1 (GMB1) for 24-48 h, following which the plants were shifted to a humidity chamber for the next 48 h to facilitate hatching of eggs. On the fifth day (120 h), some of the plants were observed for the presence of maggots at the apical meristem region through plant dissection to confirm the infestation. Stem samples of 2 cm length above the soil level were cut at 24 and 120 hours after infestation (HAI) and stored in liquid nitrogen for RNA isolation. Samples collected from uninfested plants of similar age groups grown under similar conditions were used as corresponding controls for double normalization. All the samples were collected in biological (independent experiments) and technical triplicates for transcript analysis. Likewise, three-week-old test plants were infested with BPH nymphs, and lower leaf sheath samples were collected at 6 and 12 HAI (Supplementary Fig. 1b).

2.4. Total RNA Extraction, cDNA Synthesis and Quantitative-PCR (qRT-PCR)

Total RNA was isolated from shoot samples of infested and uninfested BPT5204 and RPNF05 plants using the Trizol (Sigma-Aldrich, US) method. About 2 µg of total RNA was used to synthesize the first-strand cDNA with reverse transcriptase (Takara Bio, Clontech, USA). The cDNA was diluted in 1:7 ratio with sterile Milli-Q water and 2 µl of this diluted cDNA was used for analyzing the transcript levels of *RPL* and *RPS* genes in the two genotypes. The rice-specific *RPL* and *RPS* primers, designed through the primer-3⁶ tool was used in qRT-PCR. Primer sequences used in the study are listed in Supplementary Table 2. The qRT-PCR was performed using SYBR Green R Premix (Takara Bio, USA). The qRT-PCR cyclic conditions included an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, an annealing temperature specific to each *RP* gene for 25 s and an amplicon extension step at 72 °C for 30 s. This was followed by a melting curve step to analyze the specificity of each amplicon. The qRT-PCR was performed as three technical and biological repeats and the fold change was calculated using the standard $\Delta\Delta C_T$ double normalization method [43].

2.5. Statistical Analysis

The fold change in each BPH and GM infested sample was normalized with the corresponding uninfested samples using two reference genes, *act-1* and *β-tub* that are specific to rice. The mean fold change of each replicate obtained after normalization with these two genes was considered as the final fold change. In this study, genotype and the infestation time were the major experimental factors. A two-way analysis of variance (ANOVA) was employed for each gene on all replicates to look for effects of genotype and infestation time and their interactions. Tukey's pairwise comparisons were used to calculate the statistical significance. Probability of $P < 0.05$ was considered as statistically significant. SigmaPlot v.11 and R program v.3.6.3 were used for statisti-

¹<http://rice.plantbiology.msu.edu/>

²<https://blast.ncbi.nlm.nih.gov/Blast.cgi>

³<https://rapdb.dna.affrc.go.jp/index.html>

⁴<https://phytozome.jgi.doe.gov/pz/portal.html>

⁵<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

⁶<http://bioinfo.ut.ee/primer3-0.4.0/>

⁷<https://www.statisticssolutions.com/bonferroni-correction/>

⁸<https://software.broadinstitute.org/morpheus/>

cal analyses. The *P* values obtained from two-way ANOVA were adjusted with a Bonferroni correction in Microsoft excel v.2013 following the instructions available on web tool⁷. The transcript patterns of *RP* genes were also represented in the form of heatmaps constructed using the mean of fold change values. These maps were generated with the online program, Morpheus⁸.

3. RESULTS

3.1. *In silico* Analysis of *RP* Gene Upstream Promoter Sequences

The upstream nucleotide sequences up to 1 kb from the transcription start site of each of 70 genes were assessed for the presence of *cis*-elements that were shown to be associated with wound signaling in earlier reports. A majority of the promoter sequences were found to have elements that responded to phytohormones such as salicylic acid (TCA-motif), ethylene (Ethylene-responsive element) and methyl jasmonate (TGACG and CGTCA motifs). In addition, the motifs associated with wound signalling like W-box and WUN-motif are also present. Nine *RPL* and four *RPS* genes have W-box motif; one *RPL* and two *RPS* genes have WUN-motif, 11 *RPL* and 15 *RPS* genes have one TCA element, three *RPL* and four *RPS* genes have one ERE-element, 21 *RPL* and 26 *RPS* genes have the TGACG motif, 14 *RPL* and 25 *RPS* genes exhibited the CGTCA motif in their upstream re-

gions. The list of these *cis*-elements in the promoter regions of *RP* genes has been summarized in Supplementary Table 2.

3.2. Differential Transcriptional Regulation of *RPL* Genes in Response to BPH and GM

The cytoplasmic RPs of rice are encoded by at least 70 genes out of a family of 255 *RP* genes including paralogs. These 70 genes (35 *RPL* and 35 *RPS*) excluding the paralogs were selected in the present study to investigate their response to infestation by the BPH and GM pests at two different time points. Those genes that exhibited ≥ 2 fold transcript levels were considered as upregulated. The original *P* values obtained from two-way ANOVA were adjusted with a Bonferroni correction. The term significance hereafter refers to statistical significance at $P < 0.05$ from *P* adjusted values. Overall, transcript profiles of 35 *RPL* genes in both the susceptible BPT5204 and the resistant RPNF05 following infestation by either BPH or GM revealed that a predominant number of genes were downregulated in comparison to the number that was upregulated. These pests induced the downregulation of a large number of *RP* genes ($>80\%$) in RPNF05 than in BPT5204 (Fig. 1). In BPT5204, BPH infestation at 6 HAI resulted in 11 upregulated *RPL* genes, while 24 were downregulated. At 12 hai with BPH, 30 of the 35 *RPL* genes were upregulated as against the remaining five genes that were either downregulated or unaffected.

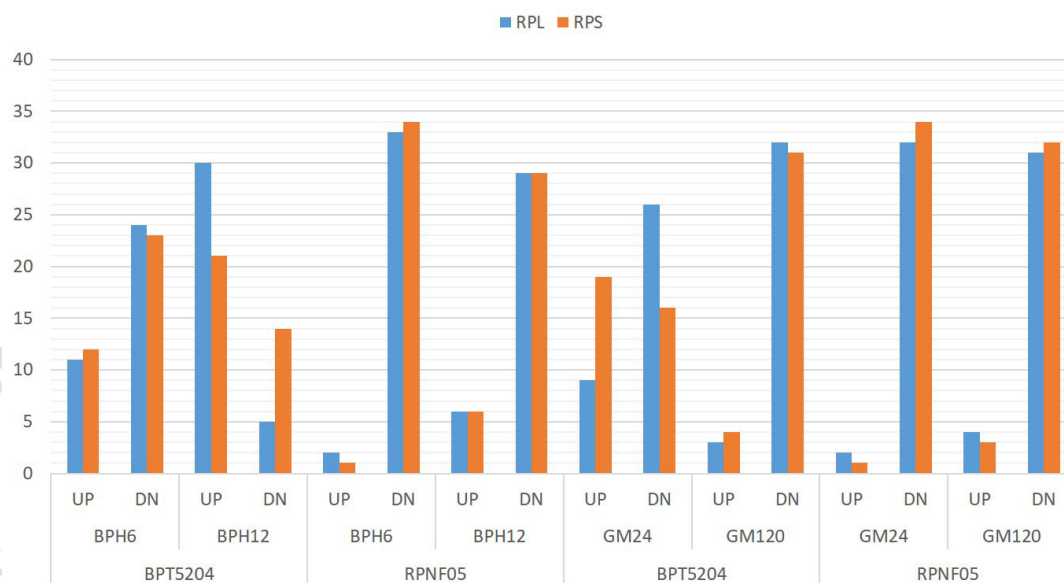


Fig. (1). Transcript profiles of *RPL* and *RPS* genes.

Overall, transcript profiles of 35 *RPL* and *RPS* genes in both the susceptible BPT5204 and the resistant RPNF05 following infestation with either BPH or GM revealed a predominant number of genes to be downregulated in comparison to the number that was upregulated. The numbers on the y-axis correspond to the mean of the total number of *RP* genes that were up or downregulated after technical and biological triplicate experiments. The blue bars are *RPL* and the orange bars indicate *RPS* genes. *UP, upregulated; DN, downregulated BPH6, brown planthopper 6 HAI; BPH12, brown planthopper 12 HAI; GM24, gall midge 24 HAI; GM120, gall midge 120 HAI.

Further, six genes viz., *RPL4*, *L7*, *L10*, *L12*, *L15* and *L22* among the 11 upregulated *RPL* genes registered significant transcript levels of >10-fold at 6 hai with BPH in BPT5204. Among those that were downregulated, *RPL14*, *L19.3*, *L27.3*, *L31*, *L32*, *L34*, *L35a.3* and *L36.2* were significant. At 12 hai, 30 genes were upregulated, of which 14 genes (*RPL4*, *L6*, *L7*, *L8*, *L11*, *L12*, *L13a*, *L13b*, *L21.2*, *L22*, *L26.1*, *L27.3*, *L28* and *L38*) were of >10-fold magnitude and upregulation of 19 genes (*RPL3*, *L7*, *L11*, *L13a*, *L13b*, *L18a*, *L19p*, *L19.3*, *L21.2*, *L22*, *L23a*, *L26.1*, *L27.3*, *L28*, *L29*, *L31*, *L32*, *L36.2*, *L38*) was significant. At both, the time points, three genes (*RPL7*, *L12* and *L22*) had higher transcript levels, with the upregulation of *RPL7* and *L22* being significant. In RPNF05, only *RPL15* (15-fold) and *L51* (4-fold) were significantly upregulated at 6 hai and transcripts of the remaining 33 genes were downregulated. Among these, expression of 18 genes (*RPL3*, *L4*, *L7*, *L11*, *L12*, *L18a*, *L19.3*, *L21.2*, *L23a*, *L24b*, *L27.3*, *L29*, *L30e*, *L31*, *L32*, *L34*, *L36.2* and *L51*) became significantly declined. At 120 hai, four genes, *RPL15* (10-fold), *L22* (6-fold), *L26.1* (23-fold) and *L38* (14-fold) showed high upregulation, whereas *RPL7* and *L11* were moderately induced (3-fold) (Fig. 2a). The upregulation of all these transcripts except *RPL26.1* was significant. Interestingly, *RPL51* was significantly upregulated in RPNF05 and downregulated in BPT5204 at 6 hai. Likewise, *RPL15* was significantly upregulated in RPNF05 but significantly downregulated in BPT5204 at 12 HAI.

Of the seven upregulated genes (*RPL4*, *L6*, *L13b*, *L21.2*, *L22*, *L27.3* and *L30e*) in BPT5204 at 24 hai following GM infestation, *L22* and *L4* exhibited 10 and 19-fold induction in transcript levels, respectively. The upregulation of *RPL4*, *L27.3* and *L30e* and the downregulation of *RPL3*, *L5*, *L18p*, *L24a*, *L24b*, *L26.1*, *L36.2*, *L37* and *L44* were significant. At 120 hai, *L30e* recorded highest and significant (62-fold) upregulation and the transcripts of *RPL5*, *L29*, *L31* and *L34* were moderately induced. In RPNF05, only *RPL15* and *L18a* were moderately upregulated at 24 hai with GM. The upregulation of *L18a* was specifically noticed in RPNF05, which was downregulated in BPT5204. A significant downregulation was observed in transcripts of *RPL8*, *L11*, *L18a*, *L18p*, *L21.2*, *L23a*, *L24a*, *L26.1*, *L27.3*, *L32*, *L35a.3*, *L38*, *L44* and *L51*. At 120 hai following GM infestation, 16 genes were significantly downregulated. Among the upregulated ones, *RPL15*, *L36.2* and *L38* were significantly induced with the transcript level of *L15* showing the highest upregulation (50-fold), but these were downregulated in BPT5204 (Fig. 2b). Also, *RPL15* was significantly and highly activated in RPNF05 at 6 hai with BPH and 120 hai with GM but was downregulated at 120 hai with GM in BPT5204. The transcripts of *RPL14*, *L23a*, *L24a* and *L24b* were commonly downregulated in response to BPH and GM infestations in both BPT5204 and RPNF05 genotypes. Of these, the downregulation of *RPL14* at 6 hai and *L24b* at 12 hai with BPH in BPT5204 was significant, which was also significantly noticed at both the time points with GM infestation in RPNF05.

3.3. Differential Transcriptional Regulation of *RPS* Genes

The overall response of *RPS* genes was similar to that noted for *RPL* genes with the majority of them being either downregulated or unaltered in response to either BPH or GM infestation in both susceptible BPT5204 and resistant RPNF05 genotypes. Notable exceptions were at 24 hai with GM in BPT5204 wherein a slightly higher number of *RPS* genes were upregulated than those downregulated, in contrast to *RPL* genes. But, as in the case of *RPL* genes, a higher number of *RPS* genes were also upregulated in BPT5204 at 6 hai following BPH infestation.

Among the induced genes, more than 10-fold upregulation was noted in 12 genes (*RPS4a*, *S5*, *S6*, *S6a*, *S13*, *S13a*, *S18*, *S21*, *S23a*, *S24*, *S25a* and *S27a*) at 6 hai with BPH in BPT5204 and a significant upregulation was observed in *RPS5*, *S13*, *S18*, *S21*, *S23a*, *S25a* and *S27a*. At 12 hai, the transcripts of *RPS5*, *S10*, *S13a*, *S17*, *S23a*, and *S25* were significantly upregulated, whereas *RPS4*, *S4a*, *S5a*, *S6a*, *S7*, *S9-2*, *S18*, *S18a*, *S18b*, *S19*, *S21*, *S23*, *S27*, *S28* and *S30* were significantly downregulated. In RPNF05, only *RPS23a* became significantly upregulated (5-fold) at 6 hai, whereas at 12 hai, six genes such as *RPS5*, *S5a*, *S6*, *S13*, *S23a* and *S25a* were upregulated. Among these, the highest induction was noticed in the transcripts of *S23a* (37-fold) and *RPS25a* (13-fold) and a significant upregulation was observed in *RPS5* and *S6* (Fig. 3a). The transcript levels of *RPS5a* (5-fold) were specifically upregulated at 12 hai with BPH in RPNF05 but was downregulated in BPT5204 suggesting its possible role in resistance.

In response to GM at 24 HAI in BPT5204, a total of 19 genes were induced. Among them, high upregulation (more than 10-fold) was noted in four genes (*RPS4*, *S6*, *S13* and *S13a*) and a significant upregulation was observed in 10 genes (*RPS4*, *S6*, *SS7a*, *S13a*, *S15*, *S18b*, *S21*, *SS23a*, *S24* and *S25a*). At 120 HAI, only *RPS7*, *S10a*, *S15* and *S18* were upregulated (Fig. 3b). *RPS15* was consistently upregulated at both the time points. In RPNF05, the transcript level of only *RPS25a* was induced at 24 hai, whereas three genes (*RPS5*, *S9.2* and *S25a*) were upregulated specifically at 120 hai but were downregulated in BPT5204. The up and downregulation of *RPS5* in RPNF05 and BPT5204, respectively was significant.

In summary, seven of the *RP* genes viz., *RPL51*, *RPL15*, *RPL18*, *RPS5*, *RPS5a*, *RPS9.2* and *RPS25a* were shortlisted for their possible involvement in insect resistance. These genes were selected because of their specific upregulation in resistant genotype at a given time point in either of the two pests. The differential transcript levels of *RPL* and *RPS* genes were also depicted in the form of heatmaps (Fig. 4). Each grid in the map corresponds to a particular *RP* gene, with the dark-colored ones exhibiting higher transcript levels whereas, the pale-colored grids represent weak or no induction. We have also shortlisted the genes that exhibited an overlap in transcript levels or those that were commonly up



Fig. (2). Graphical representation of the differential transcriptional response of individual *RPL* genes.

To understand the transcript patterns of individual *RPL* genes upon infestation with (a) BPH and (b) GM pests, bar diagrams were constructed for each *RPL* gene separately. The data on the y-axis is the relative fold change calculated using the $\Delta\Delta C_T$ method, whereas treatments are indicated on the x-axis. The asterisks in bar diagrams represent statistical significance at $P < 0.05$ obtained after adjusting probability values of two-way ANOVA with Bonferroni correction. Different colored grids at the bottom of each figure correspond to the type of treatment. Bar diagrams were generated using statistical program R v.3.6.3, two-way ANOVA was calculated in SigmaPlot v.11 and p values were adjusted in Microsoft excel v.2013. *UT, untreated; BPH, brown planthopper; GM, gall midge.



Fig. (3). Differential transcript patterns of individual *RPS* genes.

The bar diagrams were constructed separately for each *RPS* gene upon infestation with (a) BPH and (b) GM pests. These were prepared using the statistical program 'R' v.3.6. The statistical significance at $P < 0.05$ obtained after adjusting the original p values of two-way ANOVA with Bonferroni correction was represented with asterisks in bar diagrams. The data on x and y-axis corresponds to the treatment and the relative fold change, respectively. Statistical program R v.3.6.3 and SigmaPlot v.11 were used to draw the bar diagrams and calculate two-way ANOVA, respectively. *UT, untreated; BPH, brown planthopper; GM, gall midge.

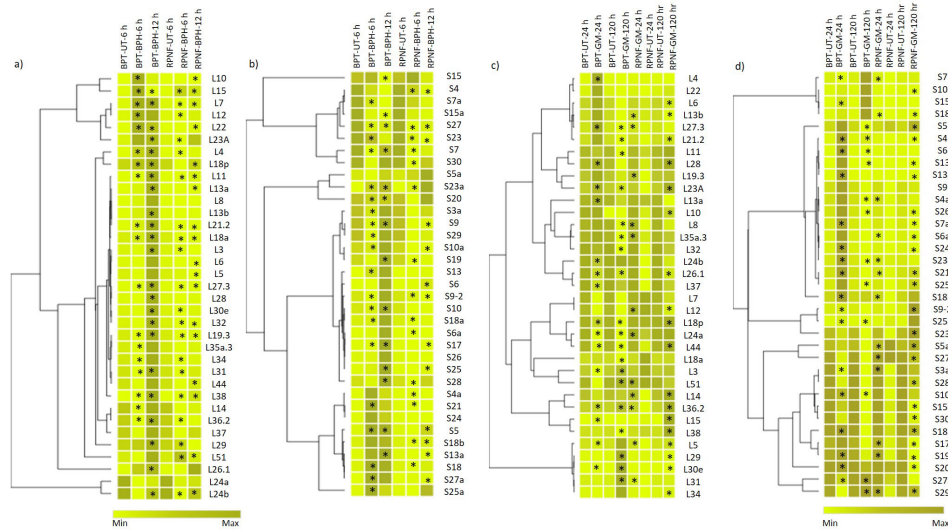


Fig. (4). Heatmap depiction of differential transcriptional regulation of *RP* genes.

Heatmaps were used to represent the transcript levels of *RPL* and *RPS* genes infested with (a, b) BPH and (c, d) GM pests. These were generated by incorporating the mean of fold change values obtained from three biological and three technical replicates normalized by the $\Delta\Delta C_T$ method. The hierarchical clustering of genes with one minus Pearson correlation and average linkage method was used to group the genes with similar transcript patterns. A color scale is provided at the left and right bottom of the figure that denotes an increase in transcript levels from a gradient of light to dark color. The light-colored grids indicate a decrease in transcripts and the dark-colored grids correspond to an increase in transcript levels. The asterisks in the grids represent statistical significance at $P < 0.05$ calculated through two-way ANOVA using SigmaPlot v.11 and adjusted with Bonferroni correction. Heatmaps were developed using an online program, Morpheus. *UT, untreated; BPH, brown planthopper; GM, gall midge.

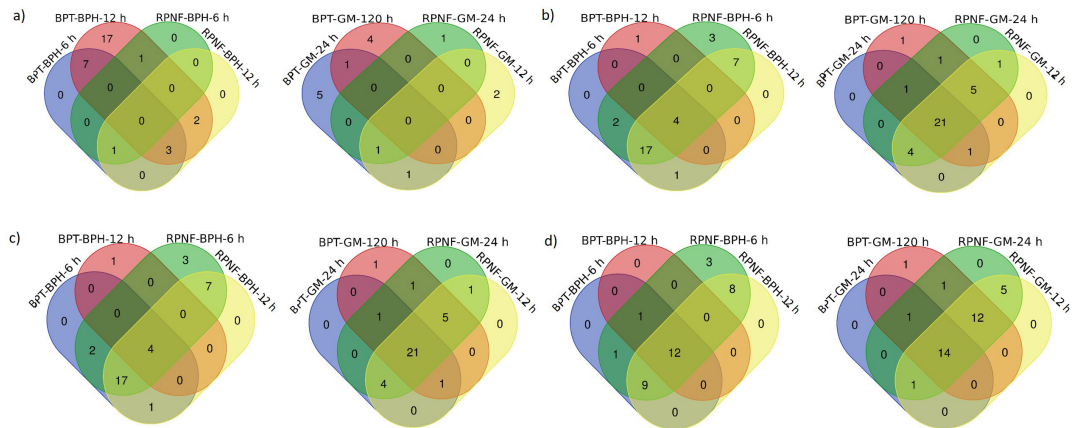


Fig. (5). Venn diagrams representing the inducible up and downregulation of *RP* genes.

The Venn diagrams were used to depict the total number of commonly (a) up and (b) downregulated *RPL* and (c) up and (d) downregulated *RPS* genes at 6 and 12 h after BPH and 24 and 120 h of GM infestation in BPT5204 and RPNF05 genotypes of rice. *BPH, brown planthopper; GM, gall midge.

or downregulated. Venn diagrams were used to describe this overlap in transcript patterns (Fig. 5) and the list of these genes was provided in Supplementary Table 3. The *P* values of each gene under a given insect treatment calculated through two-way ANOVA were adjusted with a Bonferroni correction and the statistical significance of *RPL* and *RPS* genes was represented in Supplementary Tables. 4 and 5, respectively.

4. DISCUSSION

Plants, being sessile, respond to changing environmental conditions by maintaining an equilibrium between their growth and development and stress adaptation mechanisms [44]. One of the important cellular changes that occur during the onset of stress is the regulation of protein turnover starting from transcription through translation to post-translational modifications including protein ubiquitination [45, 46]. Phytohormones are also the critical regulators of tolerance to biotic and abiotic stresses besides their role in growth and development [47, 48]. While abiotic stresses induce the modulation of ABA, auxin, methyl Jasmonate (MeJa), and salicylic acid (SA) responsive genes, the biotic stresses also involve genes responsive to the same phytohormones along with cytokinin and ethylene [49, 50]. MeJa and/or SA are found to be positive regulators of resistance to certain insect pests or pathogen attacks whereas, ABA generally acts as a negative regulator [51, 52].

There have been extensive studies on understanding the morphological, anatomical, biochemical, physiological and molecular basis of insect resistance in rice [5]. One approach for these studies is based on functional genomics with the genetic characterization of resistance (*R*) genes, their cloning and understanding the function of these cloned genes [53] while the other approach has been through the studies on a set of resistant versus susceptible varieties and following their responses to insect infestation [54, 55]. These two approaches are yet to merge and provide a vivid understanding of the three basic components of insect resistance viz., antixenosis, antibiosis and tolerance [13]. Our current understanding of antixenosis or non-preference is through green leaf volatiles like (Z)3-hexanol that attracts BPH [56] and/or natural enemies of the pest through rice hydroperoxide lyase (*OsHPL3*) gene function [57]. Antibiosis component in terms of insect development, survival and fecundity could be the result of reduced feeding due to mechanical obstruction or due to the presence of toxins or lack of essential nutrients in the plant [58]. The tolerance component of planthopper resistance is not well studied. This may be because it is not a true defence pathway, but represents general vigor and enhanced photosynthesis under stress conditions [59, 60]. The underlying genes may be mostly constitutively expressed and difficult to capture by over-expression or omics studies.

Ribosomes, being the essential cellular moieties, respond to the environmental cues likely by the co-ordinated transcriptional upregulation of a few selected or a group of *RP* genes [39, 40]. The upregulation of a majority of *RP*

genes was also observed in response to macro element deficiency in Arabidopsis [61]. This transcriptional induction of genes involved in ribosome biogenesis is a strategy adapted under stresses [17, 62]. The induction might also help in maintaining or improving the synthesis of not only of its own proteins involved in inducing structural stability but also of the other transcription factors or signal transduction proteins under the conditions of stress [63]. In response to both abiotic stresses and insect pests, we noticed the upregulation of a large number of *RP* genes. The transcripts of some of these genes responded by immediate upregulation after the onset of stress and some were elevated to high fold levels. This co-ordinated high and instantaneous activation of *RP* genes in response to invaders might function as an immediate defence. In rice, the RPs are encoded by at least 70 genes, of which, the proteins of large and small subunits are encoded by 35 genes each. A majority of these *RP* genes further exist as 2-3 paralogs in the rice genome taking the total number of *RP*-encoding genes to 255. Thus, the same *RP* is encoded by several paralogous genes. The presence of a large number of *RP* genes suggests that the RPs in a ribosomal complex are heterogeneous. The *RP* synthesized from a member of a paralogous group is incorporated into a given ribosome in a particular tissue or under a given condition [64]. The existence of a large number of *RP* gene paralogs in plants compared to other biological systems means that some thousands of *RP* combinations and hence, the formation of functionally dedicated ribosomes are possible [33-37, 65]. Environmental stress plays a major role in modifying ribosomal composition by differential expression of *RP* genes [66, 67]. The likely existence of dedicated ribosomes and multiple divergent paralogs provides a platform for mining individual *RP* gene functions, particularly in stress responses. Therefore, the identification of specific *RP* genes that respond to environmental signals is the first step in this direction.

Our studies of activation tagged gain-of-function mutants revealed the activation of two *RPL* genes, *RPL6* and *RPL23a*, for enhanced water use efficiency (WUE) trait in rice, indicating their involvement in extra-ribosomal activities apart from their basic cellular functions [38]. Subsequent transcript analysis of the entire *RP* gene family indicated that they were differentially regulated under various abiotic stresses [39, 40]. Further, functional validation of one candidate *RPL* gene, *RPL23a*, under multiple abiotic stress treatments highlighted the role of *RP* genes in the amelioration of abiotic stresses in rice [68]. However, the response of *RP* genes under pest attack is less investigated and no direct report is hitherto available to suggest their role in insect resistance.

In the current study, we have conducted a differential transcript analysis of *RP* genes in response to infestation by two divergent insect pests representing gall-forming ones and phloem feeders. Here, we have also shortlisted the genes that were highly induced under a given insect challenge in each genotype. These included the genes that were common to both the genotypes and also the genes that were specifically upregulated in the resistant genotype, RPNF05. Taken to-

gether, we noticed the upregulation of a large number of *RPL* genes in the susceptible genotype, BPT5204, compared to RPNF05 against BPH. Therefore, it is unlikely that such genes are involved in resistance against BPH but instead contribute to susceptibility in BPT5204. Modulation or overlap in the upregulation of a few *RPL* genes in both BPT5204 and RPNF05 under infestation with GM suggests that they have no possible role either in susceptibility or resistance. Unlike *RPL*, the *RPS* genes, in general, were low responding in numbers against either BPH or GM infestation in either of the genotypes. When the response of individual gene(s) was considered, some of the common genes like *RPL7*, *L12* or *RPS6*, *S13* and *S23a* against BPH or *RPS23a* against GM were activated independently of genotype or stress indicating that they might not have a specific role in insect-plant interactions. In contrast, the genes, including *RPL15*, *L51*, *L18a*, *RPS5*, *S5a*, *S9.2* and *S25a* which were specifically upregulated in the resistant genotype, RPNF05, but not in BPT5204 either against BPH or GM suggest their possible role in plant resistance against the corresponding pest. *RPL15* was also found to be highly upregulated in response to bacterial blight [39]. *RPL15* also contains a MeJa-responsive motif (CGTCA) in its promoter region. The upstream sequence of *RPL18a* was found to have SA (TCA-element) and MeJa-responsive (CGTCA and TGACG) *cis*-elements that responded to wounding or mechanical damage induced by pests. This gene was also highly upregulated under abiotic stress treatments [39]. Among the *RPS* genes, the transcript levels of *RPS5a* were specifically upregulated at 12 hai with BPH whereas *RPS5*, *S9.2* and *S25a* were upregulated at 120 hai with GM in RPNF05. The promoter regions of these three genes carry both MeJa-responsive motifs (CGTCA and TGACG). In our previous study, several *RPL* genes including *RPL6*, *L10*, *L11*, *L15* and *L24a* also showed considerable upregulation in rice plants infected with bacterial blight causing *Xanthomonas oryzae* pv. *oryzae* [39]. *RPL6* and *L10* were also activated in samples treated with MeJA and SA, the key hormones involved in plant defence mechanisms against insects and pathogens [39]. The proteins encoded by wound-responsive genes are involved in either direct repairing of damage site, inhibiting growth of the pest, eliciting the defence signaling cascade or modulating the plant metabolism to compensate the nutritional loss [69].

In addition to forming dedicated ribosomes to translate specific mRNAs [34-37, 70], the involvement of RPs in defence-related signals might also occur by individually interacting with a network of other proteins to form a functional circuit [71]. In humans, *RPL6*, *L8* and *RPS14* were found to be important members of repair signaling cascade that are recruited at sites of DNA damage induced by environmental stresses [71]. The temporal and resistant genotype-specific upregulation of these *RP* genes upon challenge with BPH or GM along with the presence of related stress-responsive elements and their repeats on their respective putative promoter regions further provides a strong basis for their possible involvement in providing resistance to these pests. The differential regulation of *RP* transcripts in response to infestation by two pests at two different time points might have oc-

curred co-ordinately to change the canonical ribosomal composition to translate specific stress-related mRNAs. In addition to the exchange of paralogs, ribosome heterogeneity also includes sequence variation of rRNAs, absence of specific RPs, posttranscriptional or posttranslational modifications of rRNA or RPs [36]. For instance, the up and downregulation, respectively of 11 and 24 *RPL* genes at 6 hai with BPH; 30 and five genes at 12 hai in BPT5204 in the current study can be correlated with ribosome heterogeneity for adaptation under different stress levels. Alternatively, the up-regulated genes might also have specialized functions in inducing resistance [72-74]. This should be investigated in future studies. The genes that were specifically upregulated (*RPL15*, *L51*, *L18a*, *RPS5*, *S5a*, *S9.2* and *S25a*) in resistant genotype might directly participate in defence-related signal transduction pathways or probably involved in primary metabolism-mediated tolerance component of insect resistance such as enhancing overall photosynthesis and vigor of the plant under stress.

The tolerance component of resistance is important in rice against BPH [75]. It refers to the genetic ability of the genotype that can compensate for the loss inflicted by the insect through feeding. The physiological basis of tolerance covers an increased rate of photosynthesis and primary metabolism as studied in rice varieties like Triveni, Utri Rajapana and Kenchana [75]. Increased activities of the majority of both *RPL* and *RPS* genes in BPT5204, but not in RPNF05 at 6 and 12 hai with BPH strongly suggest enhanced protein synthesis to gear up production and transport of primary metabolites, especially, sugars. Among these, *RPL22* displayed more than 40-fold upregulation in BPT5204 at both the time points while it was either not induced or poorly induced in RPNF05 at the corresponding time points. Likewise, *RPS6a*, *RPS17* and *RPS21* among *RPS* genes registered more than 25-fold upregulation in BPT5204 with little or no induction in RPNF05. While induction of these genes suggests a strong component of tolerance in BPT5204 against BPH, more greenhouse studies are needed to confirm this observation. Interestingly, one of the *RPS* genes, *RPS23* was highly induced in RPNF05 compared to BPT5204 against BPH at 12 hai. The observations from this study on differential induction of *RP* genes under pest treatments with specific upregulation of a few of them in resistant genotype clearly point to their involvement in extra-ribosomal functions like stress responses and possibly in inducing resistance.

CONCLUSION

The information provided in this study on the differential transcriptional analyses of selected *RP* genes in response to two economically important pests in rice needs to be exploited further by independent functional characterization of the selected genes. In particular, investigations on the *RPL* and *RPS* genes that are specifically upregulated in the resistant genotype is of importance and such genes require further focus in the direction of developing transgenic crop plants resistant to the respective pests.

AUTHORS' CONTRIBUTIONS

Mazahar Moin, Kirti PB and Madhav MS designed the experiments. Mazahar Moin performed all the experiments. Anusree Saha and Achala Bakshi helped in performing a few qRT-PCR experiments. Divya D. performed the insect infestations on rice. Mazahar Moin and Kirti P.B. prepared the manuscript. All the authors read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

The funding for the current work has been obtained through a grant sponsored by the Department of Science and Technology (DST), Government of India, in the form of DST-INSPIRE faculty award to Mazahar Moin. The grant number is IFA17-LSPA67.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Mazahar Moin is grateful to the Department of Science and Technology (DST), Government of India for providing research grants and fellowship in the form of INSPIRE-faculty award. Achala Bakshi is thankful to the Department of Biotechnology for Research Associate (DBT-RA) fellowship. Authors also acknowledge Dr J S Bentur for helping in conducting pest treatments on rice plants. Mazahar Moin acknowledges Pritam Saha for help in 'R' statistical program. Kirti PB acknowledges the Senior Scientist position awarded by the National Academy of Sciences-India (NASI). Authors acknowledge the facilities obtained from the Biotechnology Division, ICAR-Indian Institute of Rice Research, Hyderabad, India.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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Genome-wide identification, expression and bioinformatic analyses of GRAS transcription factor genes in rice

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Abstract:

Our group has previously identified the activation tagging of a GRAS transcription factor (TF) gene in the gain-of-function mutant population of rice (*indica* rice variety BPT 5204) screened for water use efficiency (Moin et al, 2016a). This family of GRAS transcription factors has been well known for their diverse roles in gibberellin signaling, light responses, root development, gametogenesis etc. Recent studies indicated their role in biotic and abiotic responses as well. Although this family of TFs received significant attention, not many genes were identified specifically for their roles in mediating stress tolerance in rice. Only *OsGRAS23* (here named as *OsGRAS22*) was reported to code for a TF that induces drought tolerance in rice. In the present study, we have analyzed the expression patterns of rice GRAS TF genes under abiotic (NaCl and ABA treatments) and biotic (leaf samples infected with pathogens, *Xanthomonas oryzae* pv. *oryzae* that causes bacterial leaf blight and *Rhizoctonia solani* that causes sheath blight) stress conditions. In addition, their expression patterns were also analyzed in thirteen different developmental stages. We studied their spatio-temporal regulation and



Gain-of-function mutagenesis through activation tagging identifies *XPB2* and *SEN1* helicase genes as potential targets for drought stress tolerance in rice

Mouboni Dutta¹ · Mazahar Moin² · Anusree Saha¹ · Dibyendu Dutta³ · Achala Bakshi² · P. B. Kirti⁴

Received: 17 May 2020 / Accepted: 23 March 2021 / Published online: 5 April 2021
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Abstract

Key message *XPB2* and *SEN1* helicases were identified through activation tagging as potential candidate genes in rice for inducing high water-use efficiency (WUE) and maintaining sustainable yield under drought stress.

Abstract As a follow-up on the high-water-use-efficiency screening and physiological analyses of the activation-tagged gain-of-function mutant lines that were developed in an *indica* rice variety, BPT-5204 (Moin et al. in Plant Cell Environ 39:2440–2459, 2016a, <https://doi.org/10.1111/pce.12796>), we have identified two gain-of-function mutant lines (XM3 and SM4), which evidenced the activation of two helicases, ATP-dependent DNA helicase (*XPB2*) and RNA helicase (*SEN1*), respectively. We performed the transcript profiling of *XPB2* and *SEN1* upon exposure to various stress conditions and found their significant upregulation, particularly in ABA and PEG treatments. Extensive morpho-physiological and biochemical analyses based on 24 metrics were performed under dehydration stress (PEG) and phytohormone (ABA) treatments for the wild-type and the two mutant lines. Principal component analysis (PCA) performed on the dataset captured 72.73% of the cumulative variance using the parameters influencing the first two principal components. The tagged mutants exhibited reduced leaf wilting, improved revival efficiency, constant amylose:amylopectin ratio, high chlorophyll and proline contents, profuse tillering, high quantum efficiency and yield-related traits with respect to their controls. These observations were further validated under greenhouse conditions by the periodic withdrawal of water at the pot level. Germination of the seeds of these mutant lines indicated their insensitivity to high ABA concentration. The associated upregulation of stress-specific genes further suggests that their drought tolerance might be because of the coordinated expression of several stress-responsive genes in these two mutants. Altogether, our results provided a firm basis for *SEN1* and *XPB2* as potential candidates for manipulation of drought tolerance and improving rice performance and yield under limited water conditions.

Abbreviations

XPB2 Xeroderma pigmentosa group B2
SEN1 T-RNA splicing endonuclease

WUE Water-use efficiency
PEG Polyethylene glycol
ABA Absciscic acid

Communicated by Matthias Wissuwa.

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Research paper



Constitutive expression of Ribosomal Protein *L6* modulates salt tolerance in rice transgenic plants

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ARTICLE INFO

Keywords:

Rice
RPL6
Ribosomal Protein genes
Salt tolerance
iTRAQ
Regulatory networks

ABSTRACT

We have functionally characterized the *RPL6*, a Ribosomal Protein Large subunit gene for salt stress tolerance in rice. The overexpression of *RPL6* resulted in tolerance to moderate (150 mM) to high (200 mM) levels of salt (NaCl). The transgenic rice plants expressing *RPL6* constitutively showed better phenotypic and physiological responses with high quantum efficiency, accumulation of higher chlorophyll and proline contents, and an overall increase in seed yield compared with the wild type in salt stress treatments. An iTRAQ-based comparative proteomic analysis revealed the high expression of about 333 proteins among the 4378 DAPs in a selected overexpression line of *RPL6* treated with 200 mM of NaCl. The functional analysis showed that these highly accumulated proteins (HAPs) are involved in photosynthesis, ribosome and chloroplast biogenesis, ion transportation, transcription and translation regulation, phytohormone and secondary metabolite signal transduction. An *in silico* network analysis of HAPs predicted that RPL6 binds with translation-related proteins and helicases, which coordinately affect the activities of a comprehensive signaling network, thereby inducing tolerance and promoting growth and productivity in response to salt stress. Our overall findings identified a novel candidate, RPL6, whose characterization contributed to the existing knowledge on the complexity of salt tolerance mechanism in plants.

1. Introduction

Among several abiotic stresses, soil salinity is emerging as one of the crucial factors having a detrimental impact on crop production, and is particularly known to affect photosynthesis by disrupting chloroplast function and stomatal conductance. It also affects metabolism, protein synthesis, and severe stress can even threaten the very survival of the plants. Salt stress refers to the presence of excess Na^+ and Cl^- ion contents in the soil or the medium in which the plant is growing. The toxicity of salt is more deleterious than any other agent for a glycophyte like rice as it induces both osmotic stress and ion toxicity (Hu et al., 2006; James et al., 2011). Because of the accumulation of high sodium ion content during salt stress, water absorption by roots is reduced and the rate of transpiration through stomata is increased resulting in membrane damage, impairment of redox detoxification and an overall decrease in photosynthetic activity (Munns, 2005). Thus, an initial

growth suppression of plants occurs due to hyperosmotic effects, and subsequent growth arrest can be attributed to the toxic levels of ions.

Salt stress also induces enzyme inhibition and ROS accumulation, and the latter is known to cause DNA damage (Saha et al., 2015). The mechanism of salt stress tolerance in plants is complex and has been shown to occur at three levels involving osmotic tolerance (reduction in shoot growth), ion-exclusion (transporter-mediated exclusion of ions by roots) and tissue tolerance (inter and intracellular ionic compartmentalization) (Tester and Davenport, 2003; Roy et al., 2014). Each of these processes occurs either mutually or in isolation, and is regulated by a network of candidate genes (Munns et al., 2012). Manipulation of the expression of these genes has resulted in transgenic plants with improved tolerance to salt stresses (Park et al., 2001; Mukhopadhyay and Vij, 2004; Hu et al., 2006; Chen et al., 2014; Lee et al., 2017; Jiang et al., 2019).

Ribosomal proteins play intrinsic roles in the generation of rRNA

Abbreviations: RP, Ribosomal Protein; RPL, Ribosomal Protein Large subunit; RPS, Ribosomal Protein Small subunit; DEPs, differentially expressed proteins; HEPs, highly expressed proteins.

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<https://doi.org/10.1016/j.gene.2021.145670>

Received 30 December 2020; Received in revised form 14 March 2021; Accepted 15 April 2021

Available online 20 April 2021

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Gain-of-function mutagenesis approaches in rice for functional genomics and improvement of crop productivity

Mazahar Moin, Achala Bakshi, Anusree Saha, Mouboni Dutta, and P. B. Kirti*

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Abstract

The epitome of any genome research is to identify all the existing genes in a genome and investigate their roles. Various techniques have been applied to unveil the functions either by silencing or over-expressing the genes by targeted expression or random mutagenesis. Rice is the most appropriate model crop for generating a mutant resource for functional genomic studies because of the availability of high-quality genome sequence and relatively smaller genome size. Rice has syntenic relationships with members of other cereals. Hence, characterization of functionally unknown genes in rice will possibly provide key genetic insights and can lead to comparative genomics involving other cereals. The current review attempts to discuss the available gain-of-function mutagenesis techniques for functional genomics, emphasizing the contemporary approach, activation tagging and alterations to this method for the enhancement of yield and productivity of rice.

Key words: activation tagging; functional genomics; gain-of-function mutagenesis; rice; tissue-specific tagging; water-use efficiency (WUE)

Introduction

About 25 species of rice are found globally, of which Asian rice or *Oryza sativa* is widely cultivated and consumed. Rice, considered as the poor's staple cereal, is consumed by >3.2 billion people across the globe, feeding about 40% of the world population. Sustained or increased productivity of rice demands more arable land, irrigation facilities and manpower. Therefore, a better understanding of its genome function can facilitate the development of tailor-made varieties of agricultural importance. The past decade has been the *decennium mirabilis* in the rice genome research with (1) the avalanche of complete genome sequence, (2) development of tools and techniques for functional

genomic studies and (3) identification and characterization of relevant, candidate genes for agronomical traits in transgenic rice plants. Mutant populations are the indispensable tools for mining the functions of plant genes. Mutants generated by the use of chemical agents, high-energy radiations and T-DNA/transposable elements disrupt the function of genes in the target genome. These gene-disruption technologies have the limitations that they induce recessive loss-of-function mutations and are unable to produce a distinct mutant phenotype of genetically redundant genes.

As an alternative, several gain-of-function mutagenesis strategies have been developed that use multiple enhancers or

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Mouboni Dutta is a Research Fellow in the Department of Plant Sciences, University of Hyderabad. She is working on the validation of genes identified through activation tagging in abiotic stress tolerance in rice.

P B Kirti is a professor in the Department of Plant Sciences, University of Hyderabad. His research interests are on crop genetic manipulation for yield enhancement and stresstolerance.



Rice Ribosomal Protein Large Subunit Genes and Their Spatio-temporal and Stress Regulation

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Specialty section:

This article was submitted to
Plant Biotechnology,
a section of the journal
Frontiers in Plant Science

Received: 09 June 2016

Accepted: 11 August 2016

Published: 24 August 2016

Citation:

Moin M, Bakshi A, Saha A, Dutta M,
Madhav SM and Kirti PB (2016) Rice
Ribosomal Protein Large Subunit
Genes and Their Spatio-temporal
and Stress Regulation.
Front. Plant Sci. 7:1284.
doi: 10.3389/fpls.2016.01284

Ribosomal proteins (RPs) are well-known for their role in mediating protein synthesis and maintaining the stability of the ribosomal complex, which includes small and large subunits. In the present investigation, in a genome-wide survey, we predicted that the large subunit of rice ribosomes is encoded by at least 123 genes including individual gene copies, distributed throughout the 12 chromosomes. We selected 34 candidate genes, each having 2–3 identical copies, for a detailed characterization of their gene structures, protein properties, *cis*-regulatory elements and comprehensive expression analysis. RPL proteins appear to be involved in interactions with other RP and non-RP proteins and their encoded RNAs have a higher content of alpha-helices in their predicted secondary structures. The majority of RPs have binding sites for metal and non-metal ligands. Native expression profiling of 34 ribosomal protein large (RPL) subunit genes in tissues covering the major stages of rice growth shows that they are predominantly expressed in vegetative tissues and seedlings followed by meiotically active tissues like flowers. The putative promoter regions of these genes also carry *cis*-elements that respond specifically to stress and signaling molecules. All the 34 genes responded differentially to the abiotic stress treatments. Phytohormone and cold treatments induced significant up-regulation of several RPL genes, while heat and H₂O₂ treatments down-regulated a majority of them. Furthermore, infection with a bacterial pathogen, *Xanthomonas oryzae*, which causes leaf blight also induced the expression of 80% of the RPL genes in leaves. Although the expression of RPL genes was detected in all the tissues studied, they are highly responsive to stress and signaling molecules indicating that their encoded proteins appear to have roles in stress amelioration besides house-keeping. This shows that the RPL gene family is a valuable resource for manipulation of stress tolerance in rice and other crops, which may be achieved by overexpressing and raising independent transgenic plants carrying the genes that became up-regulated significantly and instantaneously.

Keywords: ribosomal proteins, abiotic stress, biotic stress, gene expression, rice

Abbreviations: H₂O₂, hydrogen peroxide; MeJa, methyl jasmonate; RP, ribosomal protein; RPL, ribosomal protein large subunit; SA, salicylic acid.

Original Article

Activation tagging in *indica* rice identifies ribosomal proteins as potential targets for manipulation of water-use efficiency and abiotic stress tolerance in plantsMazahar Moin¹, Achala Bakshi¹, Anusree Saha¹, M. Udaya Kumar², Attipalli R. Reddy¹, K. V. Rao³, E. A. Siddiq⁴ & P. B. Kirti¹¹Department of Plant Sciences, University of Hyderabad, Hyderabad, 500046, India, ²Department of Crop Physiology, University of Agricultural Sciences – GKVK, Hebbal, Bangalore, India, ³Center for Plant Molecular Biology, Osmania University, Hyderabad, 500007, India and ⁴Institute of Agricultural Biotechnology, PJTSA Agricultural University, Rajendranagar, Hyderabad, 500030, India

ABSTRACT

We have generated 3900 enhancer-based activation-tagged plants, in addition to 1030 stable *Dissociator*-enhancer plants in a widely cultivated *indica* rice variety, BPT-5204. Of them, 3000 were screened for water-use efficiency (WUE) by analysing photosynthetic quantum efficiency and yield-related attributes under water-limiting conditions that identified 200 activation-tagged mutants, which were analysed for flanking sequences at the site of enhancer integration in the genome. We have further selected five plants with low $\Delta^{13}\text{C}$, high quantum efficiency and increased plant yield compared with wild type for a detailed investigation. Expression studies of 18 genes in these mutants revealed that in four plants one of the three to four tagged genes became activated, while two genes were concurrently up-regulated in the fifth plant. Two genes coding for proteins involved in 60S ribosomal assembly, RPL6 and RPL23A, were among those that became activated by enhancers. Quantitative expression analysis of these two genes also corroborated the results on activating-tagging. The high up-regulation of RPL6 and RPL23A in various stress treatments and the presence of significant *cis*-regulatory elements in their promoter regions along with the high up-regulation of several of RPL genes in various stress treatments indicate that they are potential targets for manipulating WUE/abiotic stress tolerance.

Key-words: functional genomics; rice; Samba Mahsuri (BPT-5204).

Abbreviations: Ac, activator; Ds, dissociator; PPT, phosphinothricin; RPs, ribosomal proteins; RPL, ribosomal protein large subunit; WUE, water-use efficiency

INTRODUCTION

The objective of any genome research is to identify all the genes contained in a genome and investigate their roles. Rice is the most appropriate model crop for generating a mutant resource for functional genomic studies because of its

economic importance, availability of high-quality genome sequence and relatively small genome size. *Oryza sativa* or Asian rice, a staple cereal crop, is consumed by more than 3.2 billion people globally feeding about 40% of the total world population and possibly providing more energy than any other food source to humanity. Although the green revolution and continuous breeding efforts have led to a continuous increase in rice productivity, 60% more yield still needs to be achieved (FAO 2009) to keep pace with the world population, which is perpetually expanding from 7.3 billion in 2015 to a projected 9.5 billion by 2050. Increased or sustainable productivity of rice demands more arable land, fertilizers and extensive irrigation facilities. The development of high-throughput genetic transformation technologies would facilitate better utilization of its genomic resources to produce transgenic rice rapidly for agronomically important traits such as high water-use efficiency (WUE), high nitrogen-use efficiency, tolerance to biotic/abiotic stresses and high nutritional value, which would otherwise take many years of conventional breeding to develop varieties with similar traits.

The most important abiotic factors that restrain rice plant performance and productivity are drought and salinity, both of which arise from reduced water availability. It is estimated that rice receives 35% to 43% of the total irrigation water in the world (Hibberd *et al.* 2008). When crop plants such as rice are subjected to water deficiency, they tend to develop a dehydration-avoidance or water-use-efficient phenotype (Blum 1988), which is characterized by reduced plant biomass, tillering and seed productivity, all of which are in sharp contrast to high-yielding phenotypes (Blum 2005). In the present study, we made attempts to recombine water stress adaptation with high-yield potential using a gain-of-function mutagenesis strategy.

A significant advance in *Arabidopsis* research came with the development of simplified and reliable *in planta* transformation methods as a substitute for root transformation and regeneration. In its native form, it involves dipping of shoots bearing inflorescences in *Agrobacterium* suspension (Clough & Bent 1998) followed by vacuum infiltration and sudden release of the vacuum (Bent 2000; Weigel & Glazebrook 2006). In rice, although attempts have been made to develop such protocols using 2-day-old husked seeds (Supartana *et al.* 2005; Lin *et al.* 2009), not much emphasis has been given to improve the

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Appendix B

Plagiarism Report

Ribosomal Protein Genes As Potential Targets for Manipulating Stress Tolerance in Rice

by Anusree Saha

Submission date: 03-Aug-2021 04:35PM (UTC+0530)

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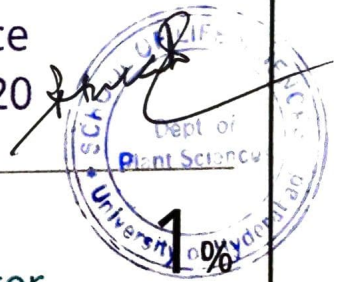
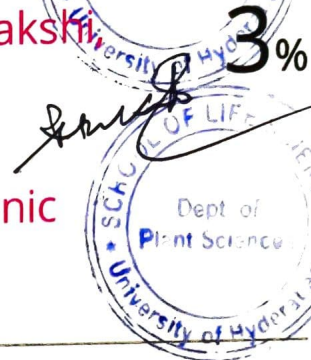
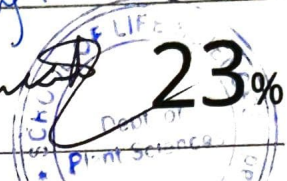
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