Activation Tagging Identifies Helicases and GRAS Transcription Factors as Potential Candidate Genes for Orchestrating 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, **Mouboni Dutta**, hereby declare that this thesis entitled **"Activation Tagging Identifies Helicases and GRAS Transcription Factors as Potential Candidate Genes for Orchestrating 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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- **Dutta, M.**, Moin, M., Saha, A., Dutta, D., Bakshi, A., & Kirti, P. B. **(2021a)**. Gain-of-function mutagenesis through activation tagging identifies XPB2 and SEN1 helicase genes as potential targets for drought stress tolerance in rice. *Theoretical and Applied Genetics*, 1-20. (https://doi.org/10.1007/s00122-021-03823-0)
- **(Preprint) Dutta, M.**, Moin, M., Saha, A., Dutta, D., Bakshi, A., & Kirti, P. B. **(2021b)**. Genome-wide identification, expression and bioinformatic analyses of GRAS transcription factor genes in rice. (bioRxiv doi: https://doi.org/10.1101/2021.06.28.449579)

B. Presented in the following conferences:

- **4th Edition of Global Conference on "Plant Science and Molecular Biology" London, United Kingdom (September, 2019)**
  Identification of Two Helicases as Potential Targets for Mitigating Drought Stress in Rice ***(Poster)***
- **India-EMBO Symposium on "Sensing and signaling in plant stress response", New Delhi, India (April, 2019)**
  Activation Tagging Identifies SEN1 and XPB2 Helicase as Important Candidate Genes for Abiotic Stress Tolerance in Rice ***(Poster)***
- **The Third International Conference on Bioresource and Stress Management (ICBSM) State Institute of Agriculture Management, Jaipur, Rajasthan, India (November 2017)**
  Characterization of Helicase Genes in indica Rice Identified through Activation Tagging for Abiotic Stress Tolerance ***(Poster)***

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

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2. (Preprint) **Dutta, M.**, Moin, M., Saha, A., Dutta, D., Bakshi, A., & Kirti, P. B. **(2021b)**. Genome-wide identification, expression and bioinformatic analyses of GRAS transcription factor genes in rice. (bioRxiv doi: https://doi.org/10.1101/2021.06.28.449579)

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Prof. P.B. Kirti

Supervisor

Dedicated to my Dadu …

Acknowledgements

As they say, "We rise by lifting others", many people around me have directly and indirectly helped me reach this moment. I want to begin by acknowledging all of them who never gave up on me and whose assistance was a milestone in completing my research work. I would like to express my sincere gratitude to my supervisor **Prof. P. B. Kirti,** for his supervision and assistance at every stage of my work, insightful comments, suggestions and unwavering support and belief in me. He always allowed his students to work and think freely in the laboratory without burdening with any time-bound restrictions. His immense knowledge and great experience helped me in all phases of my research work. I am thankful for his timely and careful corrections of manuscripts. His constant encouragement has helped me gather my confidence in building international networks and it still motivates me to pursue higher research endeavours in my career.

I am grateful to my doctoral committee members, **Prof. G. Padmaja** and **Prof. J.S.S. Prakash** for their insightful comments and encouragement and for the questions which incented me to widen my research from various perspectives.

My gratitude extends towards **Prof. Ch. Venkata Ramana** (former head) and **Prof. G. Padmaja** (present head), Department of Plant Sciences, University of Hyderabad, to undertake my studies at the department. I would also like to thank the former Deans, Prof. P. Reddanna, Prof. M. N.V Prasad, Prof. N. ShivaKumar, Prof. Ramaiah and the present Dean, **Prof. S. Dayananda,** School of Life Sciences, who has helped me in completing my research. I would like to offer my special thanks to **Dr. Santosh Kanade**, who has been very patient and considerate in sharing his lab space till the completion of my study. My sincere thanks go to all teaching and non-teaching staff of the Department of Plant Sciences and School of Life Sciences who have directly or indirectly helped me in completing my work.

A huge thanks go to **Dr. Ulrike Bechtold** for hosting me in her lab at the University of Essex, UK, under the Newton-Bhabha PhD Placement Program. She has been very kind right from the beginning. Her warm welcome, constant guidance and feedback throughout my placement tenure have helped me feel at home even at a foreign university. I am grateful to the entire group, especially

***Osi, Sunitha** and **Wardah**. Their constant encouragement and enthusiasm have helped me ride through tough times, learn new techniques and finish my work over there peacefully and smoothly. I am thankful to them for tolerating my constant bickering when anything went wrong.*

I am thankful to all my teachers who have helped me inculcate a deep love in this field and have trained me with ethical and moral values.

*I would like to thank **UGC-JRF** and **SRF** and **UoH-BBL** for providing Research fellowship. A big thank you to **DBT, CSIR, DST-FIST, UGC-SAP** and **DBT-CREB** for funding in the form of projects to our lab and the Department. I am thankful to **British Council, UK** and **DBT, India** for funding my Newton Bhabha PhD placement award.*

*I want to express my appreciation to my 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 wonderful lab environment.*

*I extend my heartfelt appreciation to the previous and present lab attendants **Mahesh, Kishan Anna, Satish, Naresh, Manohar and Rajesh,** who have helped me in the lab and the field by taking care of the plants, and also providing things whenever necessary.*

As often said, "All work and no play makes Jack a dull boy" I would have led an otherwise boring and sad routine without friends to share the monotony. I am blessed to have so many good people in my life who have helped me get through this humdrum and turned it into fun and bright.

*Words fall short for expressing my love and gratitude towards **Anusree**. She has always been my Pole star who has guided and lifted me in my darkest of times. Without her, this ship would have never reached the shore. A very big thank you for putting up with all my tantrums, stresses and moans for all these years. Things were never easy for us, but you made me glide through every situation. I am blessed to share my entire career and room with you. I could not have asked for more. You are and always be my Avogadro's number.*

*I am grateful to share my journey with **Anirban,** who has always been by my side through thick and thin. You have been a fantastic friend, critic and brother.*

I thank you for the treasured memories that we got to make on this beautiful campus.

My heart goes out to my girl gang, Pina Colada ***(Anusree, Indu, Anusha, Divya and Smita)****, without whom I would have never made it through my PhD journey. We met at the most challenging times and successfully steered our way through it. They are the strongest and most amazing women I have ever met. I sincerely thank them for being there for me and wish them all the best in their future endeavours. I thank* ***Indu*** *for her constant support and encouragement. We came a long way and did not realize when our enmity flourished into a beautiful bond.* ***Anusha*** *and* ***Divya*** *are the apple of my eyes. I want to thank both of them for their positive influence in my life and also for random movie nights, impromptu dinner plans, Maggie plans and whatnot. Being in the same hostel, we had seen a lot happening around us, and every time I felt blessed to have my wolf-pack to share our anxieties.*

I am thankful to ***Tinni,*** *who has always had my back and encouraged me in every way possible. I would like to express my love and gratitude to all my batchmates, seniors and juniors from SLS, including* ***Prodosh, Supriyo, Saumashish, Neera, Payal, Swati, Rutuparna, Tamna, Haritha.***

I want to thank ***Sneha*** *for years of support and the belief you had in me. This campus would have never been more fun without* ***Arunabha and Argha*** *being around. I thank my friends outside UoH,* ***Adrija, Arpita, Sonali, Dibyendu, Sukanya,*** *who have always been kind, tolerant and supportive. Thank you for being the shoulder I can always rely on. A very special thanks go out to my UK housemates and friends, including* ***Manuel, Maria and Norah****. The four months spent there is possibly one of the best chapters in my life, all because of you. I am lucky to have met you; otherwise, it would have been very difficult and lonely in a foreign land. I am thankful to have met* ***Arpita, Chandrima, Sushobhan, Sudhashini and Nikhar*** *during my Newton Bhabha Placement and got to spend the most beautiful time with them.*

I am blessed to be born to such great parents who have given me complete freedom to pursue my dreams and never held me back. Thank you, ***Maa and Baba,*** *for being by my side throughout my life. I am so proud to be your daughter and will never let you down. My sincere gratitude to you for your endless love, encouragement, help and support. I am indebted to you for making me believe* *that I can and made me who I am today. I thank my little sister* ***Disha****, the youngest yet the smartest one in the family. It is bliss to see you mature into an incredible person day by day. I am thankful for all your help, especially your digital art. I am thankful to my grandparents,* ***Dadu, Amma, Dida and Puti,*** *who have always cheered me in my journey. My* ***dadu*** *has always been my source of strength and encouragement, and I can say undoubtedly that this would have never been possible without you. I hope you could be with us today to share my joy. I am sure you are there somewhere looking down at me, smiling, as always…*

~ Mouboni

Abbreviations

µg	Microgram
µl	Microliter
µM	Micro molar
ABA	Abscisic Acid
bp	Base pairs
CAMV	Cauliflower mosaic virus
cDNA	Complementary DNA
cm	Centimetre
d	Day
DAR	Days after revival
DAS	Days after stress
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	hours
*hpt*II	Hygromycin phosphotransferase
kb	Kilobases
KDa	KiloDalton
LB	Luria Bertani
M	Molar
min	minutes
ml	Millilitre
MS	Murashige and Skoog
ng	Nanogram
OD	Optical Density
PCR	Polymerase Chain Reaction
*p*I	Isoelectric point
PPT	phosphinothricin
RNA	Ribo Nucleic Acid
rpm	Revolutions Per Minute
s	seconds
TE	Tris EDTA
WT	Wild type
WUE	Water Use Efficiency

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CHAPTER 1

Introduction

INTRODUCTION

1.1.Rice production, its constraints and mitigation

Rice is a crop plant that loves to sustain itself in adequate levels of water on the field. About 1400 litres of water is required to produce 1Kg rice on average. 75% of the total water resource and 34-43% of total irrigation water worldwide is utilized solely for rice production (Laha et al., 2017). The green revolution caused a boom in rice production in many nations between 1940 and 1960 but continued population increase, and shortage of land and water resources imposed a challenge to its sustainability. For example, around 20% of rice cultivated area faces moderate to severe water stress during the cultivation period. Drought stress at any period of a rice life cycle, especially during the reproductive stage, can cause significant yield loss. Additionally, demand of rice is expected to increase by 70% in the next 30 years (Laha et al., 2017; Muthayya et al., 2014). Therefore, the development of varieties resistant to abiotic stresses like drought is an urgent requirement to meet snowballing demand of rice. Recent advances in the genome based research like the development of transgenic approaches, availability of high quality genome sequence, and rice transformation protocols provide ample opportunities for identifying and characterizing novel genes and integrate them for developing stress tolerant varieties of rice (Mottaleb et al., 2012).

1.2.Importance of rice in plant genome research

In order to identify essential traits and manipulate genetic sequences for attaining agronomic benefits, understanding the molecular and functional basis of plant physiology is of utmost importance. The genetic make-up of the plant guides such phenomena. Thus, having detailed information regarding gene sequences and their respective annotation is valuable for any genomics based research. The function of any model organism is to provide genomic information that can be shared with different biological systems. *Arabidopsis* was the first dicot plant to get its genome sequenced (in 2000), followed by rice (*Oryza sativa*). Rice is a diploid (2n=24) annual plant with a genome size of ~400Mb and a life span of 3-6 months (Izawa & Shimamoto, 1996; Rensink & Buell, 2004). It has been considered a model crop for cereals primarily because of its small genome size. Other cereals like maize, sorghum, and wheat possess a genome size of 3000 Mb, 750 Mb, and 16000 Mb, respectively, which automatically makes rice the crop with a higher gene density. It has been calculated that rice has one gene per 15 kb, while maize or wheat has one per 100 or 500 kb, respectively (Tyagi et al., 2004). Moreover, studies show that rice shares a syntenic relationship with other cereals like wheat and sorghum. So studies in rice can provide a direction in the functional characterization of genes in related cereal crops. The other reasons for rice to be considered a model crop are that it has lesser repetitive DNA and the availability of highly efficient transformation protocols with shorter regeneration time (Jackson, 2016; Tyagi et al., 2004). Rice has come a long way in the evolutionary trajectory that resulted in the introgression of characters from both wild and domesticated species. It has adapted itself to a wide range of ecological and environmental conditions leading to the incorporation of essential traits in its genome. Thus, the rice genome serves as a repertoire of critical agronomic characters that advanced genomics technologies can explore (Wing et al., 2018).

A total of four groups worked on sequencing the complete rice genome, which signifies the its importance in the arena of genomics research in crop plants. The initial sequencing efforts started in 1997 by the International Rice Genome Sequencing Project (IRGSP) comprised scientific teams from India, Japan, US, France, South Korea and China. Each group was responsible for sequencing specific chromosomes, e.g.: India was responsible for sequencing chromosome number 11, Japan for chromosomes 1, 6, 7 and 8, US for chromosomes 3 and 10 etc. Clone by clone (CBC) method was undertaken for this project. In 2000, the first draft genome was released by Monsanto. Alongside, two groups, i.e. Syngenta and Beijing Genomics Institute (BGI), initiated rice genome sequencing projects of their own. IRGSP, Monsanto and Syngenta focussed on sequencing the *japonica* cultivar Nipponbare, while BGI directed its work towards that of the *indica* cultivar 93-11. Later, data from Monsanto and Syngenta was merged with that of IRGSP, and the complete sequence was made available in the public domain in 2005 (Song et al., 2018; Vij et al., 2006). According to the latest release of IRGSP (release 7), a total of 55,986 gene loci were annotated. Thus the availability of high quality genome sequences paved the way for detailed studies in functional genomics, expression analyses, insights into evolutionary relationships, and comparative studies with other species.

1.3.Mutagenesis techniques

Mutagenesis study is the crucial technique to identify novel genes and develop tailor-made varieties of plants. The advancement in rice functional genomics and transformation protocols has enabled researchers to identify several candidate genes that can be manipulated for improving agronomic characteristics. We have discussed several mutagenesis techniques in the following section.

1.3.1. Loss of function mutagenesis

1.3.1.1.Random mutagenesis techniques

Traditional mutagenesis techniques involve random mutations via the application of physical and chemical mutagens or by inserting engineered DNA elements into the host plant genomes (insertional mutagenesis) (Viana et al., 2019). Bombardment of plant material, preferably seeds, with physical mutagens like X-rays, γ rays, and neutron bombardments cause mutations via deletions or DNA breakage. Fast neutron bombardment results in the deletion of large portions of DNA, thus proving an effective technique for functional genomics study in rice. Chemical mutagenesis involves the use of mutagens like ethyl methane sulphonate (EMS), methyl methane sulphonate (MMS), sodium azide, hydrogen fluoride etc. to induce point mutations (transitions or transversions) in the chromosomal sequences (Holme et al., 2019). Mutations via EMS has proved to be very efficient in crop improvement studies, but the main drawback to these techniques is that these are non-specific, time consuming and can hamper the viability of the plant (Chaudhary et al., 2019; Sikora et al., 2011). Insertional mutagenesis is another widely used approach in mutagenesis studies. In this approach, specific DNA elements like T-DNA or transposons are delivered into the plant genome via *Agrobacterium* mediated gene transformation. These elements, in turn, get lodged in the genic sequences of the genome, causing the loss of function of the respective gene. Availability of rice genome database and efficient transformation techniques have made this tool a very fruitful one in rice mutagenesis study (Chaudhary et al., 2019; Viana et al., 2019). Generation of a pool of mutants using T-DNA insertions takes a longer time, hence transposons (*Ac/Ds* or *En/Spm* system from maize) are preferred for developing large scale mutant populations in rice. Transposons, with their inherent excision-insertion capability in the genic regions can induce ten times more mutations in the genome as compared to the T-DNA technique. Thus, use of transposons become less labour intensive and more time efficient (Viana et al., 2019).

Targeting Induced Local Lesions IN Genome (TILLING) is a well-known reverse genetics approach involving traditional chemical mutagenesis techniques to induce random, high density point mutations in the genome. Mutations are followed by the identification of single nucleotide polymorphisms in the target sequences following a specific procedure. This tool can be used in any plant species regardless of its ploidy level and genome size. In rice, EMS is the widely used chemical mutagen for this technique (Kurowska et al., 2011).

1.3.1.2.Targeted mutagenesis techniques

1.3.1.2.1. Gene silencing

Gene silencing involves RNA interference (RNAi) technology, which can be either antisense induced, hairpin RNA (hpRNA) induced or virus induced. This technique is used to generate knockdown mutants in plants. RNAi using antisense technique involves either translational attenuation or degradation of mRNA via antisense RNA molecules. The hpRNA based RNAi technique, on the other hand, is associated with the introduction of both sense and antisense sequences of the target gene in a vector under the expression of a constitutive promoter. pHANNIBAL , pKANNIBAL, pHELLSGATE are some of the vectors used in this approach (Helliwell & Waterhouse, 2003; Kusaba, 2004). Virus induced gene silencing (VIGS) involves cloning of the target gene into a viral vector followed by plant transfection. Upon infection, suppression or degradation of the target mRNA occurs via RNAi mechanism. TMV, PVX, TGMV etc. are some of the viral vectors used in this process (Burch-Smith et al., 2004).

1.3.1.2.2. CRISPR/Cas9 technique

This technique is a targeted mutagenesis approach, which involves a site specific DNA endonuclease called Cas9. The enzyme forms a complex with a guide RNA that identifies and induces double strand breakage in the target DNA sequence, which is repaired either by non-homologous end joining (NHEJ) or by homologous recombination (HR) method. Both methods involve base pair substitutions, deletions or modifications which eventually leads to gene knockouts. In rice, the mutation frequency ranges from 85-100%. This budding method has proved to be cost-effective and less time consuming in functional genomics research (Belhaj et al., 2015; Bortesi & Fischer, 2015; Viana et al., 2019).

1.3.2. Gain-of-function mutagenesis techniques

Although many loss-of-mutation techniques have proven to be very efficient in mining novel genes, they have a major drawback that these tools cannot identify the function of the redundant and lethal genes. Eukaryotic organisms have efficient genetic machinery against null mutations in the genome. Multiple copies of genes exist, so that mutation in one copy is compensated by the other existing copies present in the genome. Thus it is difficult to identify such redundant genes as loss of a single copy fails to reciprocate any phenotypic changes in the plants. Such is the case for the genes responsible for the embryonic and gametophytic development of plants. Elimination of these genes can lead to lethality, thereby making it difficult to identify their function (Tani et al., 2004; Weigel et al., 2000). This limitation can be avoided by generating gain-of-function mutants through activation tagging (Qu et al., 2008). This forward genetics technique functions by activating the tagged gene above its normal level of expression. Such a gain-of-function approach is advantageous for identifying agronomically important traits in plants and analyzing dominant conditional mutants like stress responsive ones (Wan et al., 2009). Activation tagging in plants is mostly done either by introducing a strong promoter (CaMV35S promoter) or by increasing the endogenous gene expression level by introducing multiple CaMV35S enhancers in the genome. Mutants generated via promoters can only tag the genes, which are under its direct control, in contrast to those generated via enhancers can activate the flanking genes upto 10 kb upstream and downstream from the point of the integration of the multiple enhancer elements independent of its orientation (Jeong et al., 2002; Moin et al., 2017b; Weigel et al., 2000). The mechanism of activation tagging with promoters and enhancers is explained in figure 1.1(a, b).

***Ac/Ds* based activation tagging**

Maize *Ac/Ds* based transposon system has proved to be very efficient for generating a large scale mutant population. A typical transposon based activation tagging vector contains an *Ac* (*Activator*) element and a *Ds* (*Dissociator*) element. It relies on the conventional "cut-and-paste" method where *Ac* encodes a transposase enzyme that identifies the 11 bp inverted repeats present on either side in the *Ds* element, cuts and

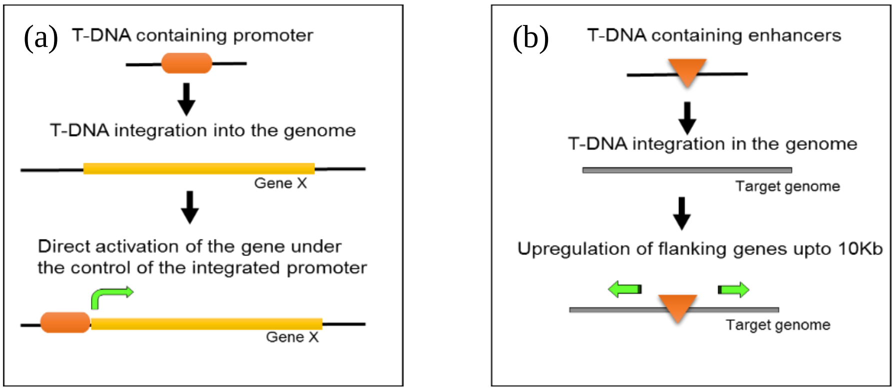


Fig. 1.1: Mechanism of Activation Tagging via (a) promoters and (b) enhancers

integrates it randomly into the genome. Here *Ac* is autonomous and does not depend on *Ds* for its transposition (McClintock, 1950). Therefore employing this transposon based activation tagging method, a large population of transgenic lines can be generated effectively (Tani et al., 2004).

Based on this concept, an *Ac/Ds* based *cis*-activation tagging vector (pSQ5) was developed by Qu et al. (2008). Our previous studies used this vector to generate a pool of mutant populations in the widely cultivated *indica* rice variety, BPT-5204. Several genes responsible for protein ubiquitination (*cullin4*) and ribosome biogenesis (*RPL6* and *RPL23A*), helicase activity (*SEN1* and *XPB2*) and transcription factors (*GRAS*, *WRKY 96* and NF-YC13) were identified from the tagged population (Dutta et al., 2021a; Manimaran et al., 2017; Moin et al., 2016).

1.4.Water use efficiency (WUE)

For crop scientists, water use efficiency is defined as the ratio of biomass production to the amount of water used. WUE is an essential parameter for both experimental and agricultural purposes to understand the productivity of a plant. A high water use efficient plant can produce more biomass or uphold sustainable yield even under limited water supply (Avila et al., 2015; Stanhill, 1986). Rice is the staple food consumed throughout the world. Compared to other crops like maize or sorghum, rice utilizes twice or thrice the amount of water. Usually, 5 cm flooding is maintained in rice fields, and about 30% of the total freshwater worldwide goes towards its cultivation (Karaba et al., 2007). Therefore, to make rice a water use efficient plant, we either need to sustain the water supply or develop certain varieties that can minimize their water utilization without compromising the yield. Environmental stress conditions like drought, salinity, increased population, competing interests of industries and agriculture have imposed serious water threats for rice cultivation. It is expected that such constraints are going to intensify in the upcoming years. Hence, developing tolerant varieties for efficient water utilization is required (Bhuiyan et al., 1995; de Avila et al., 2015).

Apart from reduced grain yield, several other secondary characters are important for determining the WUE of a plant during water stress. It includes early flowering, reduced chlorophyll content and a higher carbon isotope ratio. Other physiological parameters involve a reduction in plant growth and leaf area, leaf wilting percentage, regulation of stomata, extensive root proliferation and higher accumulation of osmolytes like proline, sugar etc. Efficiency can also be judged by studying the quantum efficiency of Photosystem II (PSII), which is directly associated with the photosynthetic capability of a plant. Such secondary characteristics can influence the WUE of a plant and can be used to identify tolerant varieties from the susceptible ones (Silva et al., 2013).

A pool of activation tagged mutants of *indica* rice (BPT5204) was developed (Moin et al., 2016), which were screened under limited water supply to gauge the WUE of the gain-of-function mutants. The above mentioned parameters were used to analyze the WUE, and those with high WUE were selected for further assessment. In the present work, we have identified and characterized the roles of two helicases (SEN1 and XPB2) to ameliorate drought stress tolerance in rice. We have also corroborated the function of GRAS transcription factors (identified previously by Moin et al., 2016 from the pool of mutants) in abiotic and biotic stress tolerance by their gene expression and bioinformatics analysis.

CHAPTER 2

Review of literature

REVIEW OF LITERATURE

2.1.Abiotic stress responses in plants

Environmental constraints like drought, flooding, salinity, extreme high and low temperature exert adverse effects on plant health, photosynthetic capability and yield. Such conditions elicit various molecular and biochemical changes within the cells that damage the cellular integrity, metabolism and osmotic balance (Hoang et al., 2017). Being sessile, plants cannot avoid any such adversities. Hence, they have developed strategies to combat them. These are mostly done either by stress avoidance or via stress tolerance mechanisms (Ahmad et al., 2014). The latter mode of plant response has proved to be an exciting strategy to scientists worldwide. Hence, with the help of modern technologies and easily available databases, the current focus is to develop stress-tolerant plants by tweaking the key players involved either in metabolic or in regulatory roles in a cell (Hoang et al., 2017; Joshi et al., 2016).

2.1.1. Drought stress

Among several abiotic stress factors, drought has imposed a maximum threat to food security. Studies indicate that more than 50% of crop losses occur due to drought, and this condition is likely to worsen in the upcoming days (Joshi et al., 2016). Plants respond to this stress via complicated physiological and molecular responses (Agarwal et al., 2006). It is either done via biosynthesis of osmolytes, heat shock proteins (HSP), ROS scavengers, transporters, LEA proteins or via modulating signaling cascades through hormones, kinases and TFs (Joshi et al., 2016). During water deficient conditions, plants tend to synthesize more osmolytes like proline, sugars, glycine betaine, etc. to maintain the osmolarity of the cells as well as to protect the cellular integrity. These osmoprotectants, in turn, act as ROS scavengers and protect the plants from oxidative stress. HSPs and LEA proteins help during stress by acting as protein chaperones and stabilizing the membranes, respectively (Ali et al., 2017). Stress meditation during drought can happen via ABA-dependant or independent pathways.

ABA is an important phytohormone, which can mediate drought stress via regulating the guard cells. *Arabidopsis* ABA mutants were found to be sensitive to drought (Ali et al., 2017). Several transcription factors are present that can either bind to the ABRE or DRE elements present in the promoter regions of the target genes, and thereby control the ABA dependant or independent pathways to follow, respectively (Ali et al., 2017; Joshi et al., 2016). Any kind of abiotic stress can be vulnerable to rice since more than 50% of the globally produced rice is rain-fed, with water scarcity and drought as the major constraints. It can affect the crop at any phase of its life cycle. The tillering, flowering and panicle formation stages are the most sensitive stages that can lead to massive crop loss (Kim et al., 2020; Venuprasad et al., 2007).

2.1.2. Salinity stress

About 20% of the irrigated area under cultivation is under salinity stress. It is another critical abiotic stress component that hampers plant productivity. Salt stress can be either irrigation mediated salinity or dryland salinity. In glycophytes, high salt concentration creates an osmotic imbalance leading to the cessation of water uptake. While the plant faces water stress initially, a high concentration of Na^+ and Cl^- ions in the cell cause salt toxicity leading to cell and ultimately plant death (Läuchli & Grattan, 2007). High salt stress can also cause nutritional disorders, reduce photosynthetic capability, and hamper plant lateral shoot formation. Plants deploy similar methods as mentioned earlier under drought stress like production of osmolytes, scavengers, LEA proteins, gene regulation etc., for the mitigation of the imposed stress (Sairam & Tyagi, 2015). Rice cultivation is also severely damaged due to salinity as it is often grown in saline marshy areas. Several mechanisms are currently being employed to overcome the yield losses in rice due to salt stress (Dionisio-Sese & Tobita, 1998).

2.2.Biotic stress responses in plants

Pests and pathogens are continuously threatening plants in both natural and cultivated environments. About 31-42% of the yearly agricultural yield is lost due to biotic stress factors (Pandey et al., 2020). Studying such aspects of plant health becomes difficult as it involves specific interaction between the host and the pathogen. Biotic stress responses in a plant can be divided into pathogen infection and diseases due to pest infestations (Diaz, 2018; Saraswathi et al., 2018). Plants avoid pathogen infections via excessive production of ROS and subsequent activation of signaling cascades, which restrict them from spreading. The downstream genes involve those encoding pathogen-related proteins (PR-proteins), kinases, TFs etc., involved in hormone and ROS signaling pathways. Apart from this, plants may put up morphological changes like cell lignification or stomatal closure, which prevent the entry of pathogens. Hormones like SA, JA, ABA and ethylene play essential roles in biotic stress tolerance (Madani et al., 2018; Rout & Das, 2013). Several QTLs have been identified for stress resistance in plants, including those for rice blast disease (Ballini et al., 2008), powdery mildew of barley (Aghnoum et al., 2010), blight diseases of potato (Danan et al., 2011), wheat (Buerstmayr et al., 2009) and barley (Massman et al., 2011) etc. Since each QTL contain multiples genes, it is often challenging to incorporate such traits into sensitive genotypes of the crop through conventional breeding. Hence, genetic engineering methods for introducing specific genes have proved to be more favourable (Kushalappa & Gunnaiah, 2013). Biotechnological approaches have exploited major pathways for achieving sustainable solutions to biotic stress responses in plants. The major breakthrough was the identification of *Bt* genes encoding *Cry* proteins identified from the bacterium, *Bacillus thuringiensis* that provided protection against lepidopteran insects in different crop plants. The first Bt crop was reported in tobacco (1985), which did not get commercialized. Later it was commercialized in corn, potato, cotton, brinjal and soybean (Abbas, 2018; Parmar et al., 2017). Other group of proteins that are being manipulated include protease inhibitors, trypsin inhibitor, cystatins, lectins etc. These have been reported to have important roles in defence mechanism of plants against insects, aphids and nematodes.

Bacterial, fungal and viral pathogens impose a more significant amount of stress on plants. Thus, genes encoding chinitase, glucanase, polyamines, defensins, PR proteins etc. were studied for their involvement in improving plant immunity (Parmar et al., 2017). Cell wall degrading enzymes like chitinase, glucanase etc. are the primary targets for improving plant stress tolerance against fungal pathogens (Ceasar & Ignacimuthu, 2012). Rice chitinase gene *RCC2* has been well exploited to develop tolerant varieties of chrysanthemum, grapevine and cucumber (Kishimoto et al., 2002; Takatsu et al., 1999; Yamamoto et al., 2000). Several PR proteins involved in systemic acquired resistance (SAR) are also being studied for improving disease resistance in plants. *AtNPR1* gene from *Arabidopsis* overexpressed in tomato has shown tolerance against tomato mosaic virus and bacterial wilt, bacterial spot and *Fusarium* wilt diseases (W. C. Lin et al., 2004).

2.2.1. Bacterial leaf blight (BLB) of rice

BLB was first reported in Japan way back in 1884-1885. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal organism responsible for causing this disease. This pathogen can cause a loss of 20-40% of rice yield at the tillering stage, while an infection at a younger vegetative stage can cause losses over 50%. This is a vascular disease, and a major infection can result in complete crop failure (Chukwu et al., 2019). The symptoms usually develop on leaf blades and sheath, sometimes on the grain. An infection can be observed as early as a month after transplantation of the seedlings. It appears as water soaked drops on leaf edges that gradually turn yellow and spread along the veins into the whole plant. Later, the spots turn white or greyish, leaving the plant to wither. "Kresek" is the final wilting stage of the infection. Stunted growth and discolouration are often associated with this disease (Mizukami & Wakimoto, 1969; Yasmin et al., 2017). A pale amber coloured bacterial ooze can be observed on the lesions, which primarily develop on the upper edges of the leaves with profuse water pores. These pores enable the bacteria to invade and spread more quickly. Usually, the tropical climate with a temperature of 25-34°C with 70% humidity favours the spread of the disease (Chukwu et al., 2019; Yasmin et al., 2017).

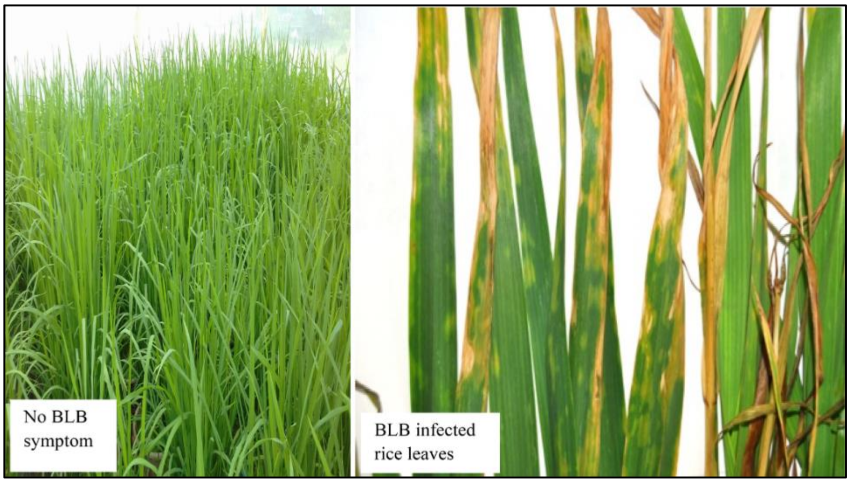


Fig. 2.1: Bacterial leaf blight infection in rice
The figure shows healthy rice plant without any symptom, while the other shows clear yellow lesion on the edges of leaf blades of rice (Chukwu et al., 2019).

Chemical control has not been very effective in controlling *Xoo* pathogens. Hence, the application of antibiotics like ampicillin, kanamycin, streptomycin etc. was tried. Reports suggest that Plant Growth Promoting Rhizobacteria (PGPR) is very useful in controlling the spread of the pathogen (Chithrashree et al., 2011). *Pseudomonas* sp. and *Bacillus* sp. have been particularly very useful. These PGPRs suppress the spread of the pathogen either by producing antibiotics, cell wall degrading enzymes, siderophores or by competing with the pathogen for nutrients (Bardin et al., 2015; Yasmin et al., 2017). Apart from biological control of *Xoo*, multiple strategies involving conventional breeding and molecular techniques are currently being employed to develop a sustainable solution to this infection (Chukwu et al., 2019).

2.2.2. Sheath blight (SB) of rice

SB was first observed in Japan in 1910. This fungal disease of rice is caused by the wide host range fungal pathogen, *Rhizoctonia solani*. It is a soil/ water borne fungus and can cause a yield loss of 45% depending on the severity of the infection. Favourable conditions can also lead to 100% yield loss (Kumar, 2020; Singh et al., 2019). To date, no resistant cultivars of rice have been identified. Hence, farmers have to rely on the use of fungicides to control this disease. The fungicides include carbendazim, carboxin, zineb etc. (Kumar, 2020). The high genetic variability in the pathogen and the broad range of host plants make it even more difficult to develop any control mechanism against this pathogen (Molla et al., 2020). *R. solani* can form sclerotia, which can spread via irrigation water to the entire field. It can also remain dormant over the cropping seasons (upto 2-3 years) and attack the plants when re-transplanted (Molla et al., 2020; Zhou et al., 2021). SB infection mostly occurs via appressorial penetration of the sclerotia and can spread from one plant to another via tillers and leaves. The initial symptoms originate on the margins of leaf sheaths and blades as water soaked brownish or greyish elongated spots. Such lesions show similarity with the snake’s skin because of which this disease is colloquially known as “snake skin disease” of rice. Eventually, such lesions interfere with grain filling, thereby hampering the yield. The use of semi-dwarf cultivars of rice, excessive usage of nitrogen fertilizers, humid conditions and high crop planting densities further aid in the spread of the pathogen (Molla et al., 2020; Singh et al., 2019). Recent advances have shown the use of PGPR (*Bacillus subtilis*), along with a reduced dosage of the fungicide Azoxystrobin can reduce *R. solani* infections (Zhou et al., 2021). Scientists are incorporating advanced molecular technologies to unravel the molecular mechanism governing the sheath blight disease and develop tolerance in rice.

Fig. 2.2: Tillers showing symptoms of sheath blight of rice (source: https://www.apsnet.org/edcenter/disandpath/fungalasco/pdlessons/Pages/RiceSheath.aspx)

2.3.Strategies for improving stress tolerance

Since stress tolerance is a complex multigenic trait, incorporating such traits in plant genotypes with the help of traditional breeding techniques becomes difficult and is met with limited success. This process is time consuming and involves tedious breeding techniques for several generations that can often result in mutations and somaclonal variations. With new-age functional genomics approaches like large scale expression data analysis through microarrays or omics techniques or mutant analysis (both loss and gain-of-function mutants), identification of novel stress tolerance genes has become a reality. Compared to conventional breeding techniques, introducing important candidate genes for the desired phenotype to target plants through genetic modifications has proved to be a much faster and reliable method (Ahmad et al., 2014; Cushman & Bohnert, 2000; Joshi et al., 2016). Genes that are being manipulated so far to improve stress tolerance in plants can be broadly classified into (a) genes involved in biosynthesis pathways, (b) genes responsible for water and ions transport and (c) genes involved in transcriptional control and cell signaling pathways (Cushman & Bohnert, 2000; Parmar et al., 2017). Out of these, the third category comprising TFs was very efficient in mediating stress tolerance in plants, as they can regulate multiple downstream genes by directly binding to their promoters (Joshi et al., 2016). A detailed list of few important genes for stress tolerance in plants is provided in Table 2.1.

Table 2.1: Classification of genes identified from various organisms for their roles in biotic and abiotic stress tolerance

Category		**Gene/ Gene family**	**Traits/ Induced by**	**Type of protein/function**	**Source organism**	**Target organism**	**References**
Genes involved in bio-synthetic pathways	Osmoprotectants	*P5CS*	Drought	Proline biosynthesis	*Vigna aconitifolia*	*Triticum aestivum*	(Vendruscolo et al., 2007)
		CodA	drought	Glycinebetaine biosynthesis	*Arthrobacter globiformis*	Potato	(Cheng et al., 2013)
		SoBADH	Salinity, oxidative stress, cold	Glycinebetaine biosynthesis	*Spinacia oleracea*	*Ipomoea batatas*	(Fan et al., 2012)
	Stress related proteins (chaperones, proteins for membrane stabilization)	*OsHSP18.6*	Heat, drought, salt, cold	Heat Shock Proteins	*Oryza sativa*	*Oryza sativa*	(Wang et al., 2015)
		AtHSFA1a	Heat, oxidative stress	Heat Shock Proteins	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Qian et al., 2014)
		CgHSP70	Heat, drought, salinity	Heat Shock Proteins	*Chrysanthemum morifolium*	*Arabidopsis thaliana*	(Song et al., 2014)
		OsLEA3-1	Drought, salt, ABA	Late embryogenesis abundant proteins	*Oryza sativa*	*Oryza sativa*	(Xiao et al., 2007)
		CaLEA	Drought, salt, heat	Late embryogenesis abundant proteins	*Capsicum annuum*	Chinese cabbage	(Park et al., 2003)
		SbDhn1/2	Oxidative stress	Dehydrins	*Sorghum bicolor*	*Nicotiana tabacum*	(Halder et al., 2018)
		AtLPK1	*Botrytis cinerea*, Salinity	Lectin	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Huang et al., 2013)
		OsEUL	Pathogen, insect infestation, Drought and salt stress	Lectin	*Oryza sativa*		(Lambin et al., 2020)
		Chickpea Lectin	*Alternaria brassicae*, Salt, drought	Lectin	Chickpea	*Brassica juncea*	(Kumar et al., 2015)
		RCC2	*Botrytis cinerea* (gray mould), *Uncinula necator* (powdery mildew)	Chitinase	*Oryza sativa*	Chrysanthemum, Grapevine, Cucumber	(Kishimoto et al., 2002; Takatsu et al., 1999;

							Yamamoto et al., 2000)
	Enzymes	*AtNCED3*	Drought	ABA biosynthesis	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Iuchi et al., 2001)
		OsGS	Cadmium stress	Glutamine synthetase	*Oryza sativa*	*Oryza sativa*	(Lee et al., 2013)
		AtNUDX2	Oxidative stress	Nudix hydrolase	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Ogawa et al., 2009)
	Membrane associated proteins	*TaEXPB23*	Oxidative stress	Expansins	*Triticum aestivum*	tobacco	(Han et al., 2015)
Genes responsible for water and ion transport	Water transport proteins	*AtPIP1;4* *AtPIP2;5*	Drought, cold	Aquaporins	*Arabidopsis thaliana*	*Arabidopsis thaliana* , *Nicotiana tabacum*	(Jang et al., 2007)
		TsTIP1;2	Drought, salt, oxidative stress	Aquaporins	*Thellungiella salsuginea*	*Arabidopsis thaliana*	(Wang et al., 2014)
		TaAQP8	Salt stress	Aquaporins	Wheat	Tobacco	(Hu et al., 2012)
		CfPIP2;1	Dehydration stress	Aquaporins	Cucumber	*Arabidopsis thaliana*	(Jang et al., 2007)
		MaPIP1;1	Drought, salt	Aquaporins	Banana	*Arabidopsis thaliana*	(Xu et al., 2014)
		MusaPIP1;2	Drought, salt, cold	Aquaporins	Banana	Banana	(Sreedharan et al., 2013)
		HvPIP2;5	Salt, osmotic stress	Aquaporins	*Hordeum vulgare*	*Arabidopsis thaliana*	(Alavilli et al., 2016)
	Ion transporters	*AtMPT*	Salt stress	Mitochondrial phosphate transporter	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Zhu et al., 2012)
		AtHKT1	Salt stress	Potassium transporter	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Ali et al., 2019)
		McHAK2/3	Salt stress	Potassium transporter	Common ice plant		(Su et al., 2002)
		AtSUC2/4	Salinity, osmotic stress, ABA treatment, low temperature	Sucrose transporter	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Gong et al., 2015)

		HtNHX1/2	Aluminium stress, soil acidity	Potassium transporter	*Helianthus tuberosus*	*Oryza sativa*	(Li et al., 2020)
		HtNHX1/2	Salinity	Potassium transporter	*Helianthus tuberosus*	*Oryza sativa*	(Zeng et al., 2018)
ROS scavengers		*CuZnSOD, APX and NDPK2*	Oxidative, high temperature stress	Superoxide-dismutase, ascorbate peroxidase and nucleoside diphosphate kinase 2	Potato	Potato	(Kim et al., 2010)
		miR529a	Oxidative stress	Micro-RNA encoding genes		*Oryza sativa*	(Yue et al., 2017)
Gene regulators	Kinases	SnRK2 Kinases	ABA signalling, drought stress	Serine/Threonine kinases	--	--	(Kulik et al., 2011)
		IbMPK3/6	Salt, SA, H_2O_2, ABA	MAP kinase	Sweet potato	--	(Kim et al., 2016)
		OsSAPK	*Xanthomonas oryzae*	Stress activated protein kinase	*Oryza sativa*	--	(Xu et al., 2013)
		CDPKs	Drought, salinity, oxidative, temperature stress, wounding and pathogen interactions	Calcium dependent protein kinases			(Singh et al., 2017)
		AtPIP5K	Drought, salt and ABA	Phosphatidylinositol-4-phosphate 5-kinase			(Mikami et al., 1998)
		At SRK2C	Drought	SnRK2 family	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Umezawa et al., 2004)
		AtNDPK2	Oxidative stress	NDP kinases	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Moon et al., 2003)
		AtMKK9	ABA and salt tolerance	MAP kinase kinase	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Alzwiy & Morris, 2007)
		ZmMPK17	ABA, H_2O_2, SA, JA, ethylene, cold and osmotic stress	MAP kinase	*Zea mays*	*Nicotiana tabacum*	(Pan et al., 2012)
	Protease inhibitors	BWI-1a	*Pseudomonas syringae Clavibacter michiganensis*	Serine proteinase inhibitor	Buckweed	Tobacco, Potato	(Khadeeva et al., 2009)

		NtKTI1	*Rhizoctonia solani* *Rhizopus nigricans* *Phytophthora parasitica*	Trypsin inhibitor	*Nicotiana tabacum*	*Nicotiana tabacum*	(Huang et al., 2010)
		TaMDC1	*Pseudomonas syringae* *Botrytis cinerea* *Alternaria alternate* *Leptinotarsa decemlineata*	Cystatin	*Triticum aestivum*	tomato	(Christova et al., 2018)
		FchCYS1	Wounding	Cystatin	*Fragaria chiloensis*		(Valenzuela et al., 2018)
	PR-protein	*AtNPR1*	Tomato Mosaic Virus Bacterial wilt, Bacterial spot, *Fusarium* wilt	PR-protein	*Arabidopsis thaliana*	Tomato	(W. C. Lin et al., 2004)

2.3.1. Helicases

Helicases are molecular ATPases that carry out a wide range of tasks by utilizing the energy released during ATP hydrolysis. A DNA or RNA helicase can be one of these motor proteins. DNA helicases are primarily involved in unwinding stable DNA duplexes so that genetic information can be replicated, transcribed, repaired, and recombined. On the other hand, RNA helicases are primarily involved in inducing conformational changes in RNA. A total of 217, 199, 215, and 248 helicase genes have been reported in *Arabidopsis*, rice, maize, and soybean, respectively (Passricha et al., 2018). All helicases consist of a three-dimensional conserved core region with two tandemly placed RecA domains (RecA1 and RecA2) connected via a flexible linker protein (Sloan & Bohnsack, 2018). The core domain includes 350-400 amino acids and 14 conserved motifs (Q, Ia, Ib, Ic, II, III, IIIa, IV, Iva, V, Va, Vb, VI from N terminal to C terminal, respectively). It serves as catalytic pockets for the helicases, where substrate interactions, unwinding activity coordination, and ATP binding and hydrolysis occur (Passricha et al., 2018; Umate et al., 2010). The diversity of functions within helicases may be attributed to differences in nucleic acid binding patterns or variations in the N and C terminal domains. Besides the core structure, some helicases may have additional N or C terminal extensions responsible for protein-protein interactions, nucleic-acid binding, oligomerization, and helicase specificity (Jankowsky & Fairman, 2007; Seraj et al., 2018). These are unidirectional proteins either translocating 3′ to 5′ or 5 ′to 3′ directions, with some being bipolar (Passricha et al., 2018).

Helicases are classified into six superfamilies based on their structural and functional properties (SF1 to SF6). Most DNA and RNA helicases belong to the SF1 and SF2 families, and they have monomeric architectures. The SF1 family of helicases has a well-defined structure and can be classified into various sub-groups, including UvrD/Rep, Pif 1-like, and Upf1-like. Upf1-like helicases are RNA helicases, while the others two are DNA helicases. SF2 comprises the largest helicase family capable of translocating on both single and double-stranded nucleic acids. It comprises of ten subgroups which are RecG-like, RecQ-like, Rad3/XPB, Ski2-like, T1R, Swi/Snf, RIG-I-like, DEAD box, DEAH/RNA and NS3/NPH. Out of these, Rec-G, Rec-Q, Rad3/XPB, T1R, Swi/Snf like helicases are DNA helicases, and the rest are RNA helicases (Passricha et al., 2018; Seraj et al., 2018).

2.3.1.1.Role of helicases in stress responses

Apart from the general housekeeping functions of helicases, several of them were found to be involved in mediating both abiotic and biotic stress conditions in plants. DNA helicases were mostly found to be expressed for repairing DNA damage. *Arabidopsis* Pif1-like DNA helicase has been reported to be induced by wounding, thereby indicating its role in biotic stress tolerance (Seraj et al., 2018). The most explored DNA helicase gene for salinity stress is *PDH45* isolated from pea and overexpressed in tobacco, rice, sugarcane, peanut, and chili (Passricha et al., 2018; Seraj et al., 2018; Singh et al., 2013). Another pea helicase (p68), a DEAD box RNA helicase was shown to provide salt tolerance in tobacco (Tuteja et al., 2014). This group of RNA helicases (DEAD box) has been well reported in stress responses. *Arabidopsis* RH8 helicase interacts with PP2C and modulates ABA signaling pathway. Heat tolerant DEAD-box helicase includes *TOGR1* and *AtRH7*, which are responsible for low temperature tolerance (Pandey et al., 2020). Some other DEAD-box helicases include *OsABP* of rice responsive to abiotic stress (Macovei et al., 2012), *SlDEAD31* of tomato responsive to salinity, and drought (Zhu et al., 2015), and rice *OsBIRH1* helicase responsive to pathogen infection and oxidative stress (Li et al., 2008). Apart from these, several *Arabidopsis RH* genes have been shown to have roles against several plant viruses (Kovalev et al., 2012; Kovalev & Nagy, 2014; Li et al., 2016) and tobacco *PINP1* against *Phytophthora* infections (Pandey et al., 2020; Qiao et al., 2015). RNA helicases like Mda-5 and RIG-I belonging to the DEAH family have been reported to detect and initiate antiviral responses in cells (Jankowsky & Fairman, 2007). Thus, helicases belonging to different families (not only DEAD-box) have significant roles in plant stress tolerance, and this shall be elaborated further in the subsequent sections.

2.3.1.2.XPB2 (Xeroderma Pigmentosa group B2)

Rice XPB2 (**X**eroderma **P**igmentosa group **B2**) is a DNA helicase (3′ to 5′ helicase) of the superfamily 2 group. *RAD25* (*SSL2*), *XPB2* and *XPB* (*ERCC3*) are the rice *XPB2* homologs in yeast, *Arabidopsis,* and humans, respectively (Bhatia et al., 1996; Umate et al., 2010). UV radiations and other chemical mutagens can distort DNA double helical structure or lead to defective replication and transcription (Guzder et al., 1995). Hence, the cellular machinery has developed an inherent DNA damage repair system in which a massive lesion is repaired by nucleotide excision repair (NER) and the damage is not transferred to the next generation (Morgante et al., 2005). NER involves several steps, including damage recognition, double incision around the lesion, unwinding of DNA and removal of damaged portion, gap filling, and sealing of the newly synthesized fragment with the existing one (Costa et al., 2001; Morgante et al., 2005).

XPB2 is a subunit of the eukaryotic transcription factor, TFIIH, associated with RNA polymerase II during transcription (Bhatia et al., 1996; Morgante et al., 2005). It functions as a DNA-dependent helicase that creates a DNA bubble during transcription initiation by RNA polymerase II. Besides, it also helps in NER by unwinding the DNA at the site of lesion (Raikwar et al., 2015; Richards et al., 2008). Altogether, these impart a dual role of TFIIH in eukaryotes *viz*. transcription initiation and DNA damage repair via nucleotide excision (Bhatia et al., 1996; Costa et al., 2001). TFIIH dissociates shortly after transcription elongation. When damage occurs, the RNA polymerase comes to a halt, and numerous coupling factors recruit the NER complex to the lesion site. This complex restores the damage and continues the elongation process (Bhatia et al., 1996). Defects in damage repair systems can lead to severe mutagenic effects, especially in humans (Guzder et al., 1995). Xeroderma pigmentosum is an autosomal recessive disorder, which arises due to the mutation of *XPB2*. These mutants are incapable of creating a double incision around the damaged portion of DNA, thereby making the individual much sensitive towards the light (Costa et al., 2001; Lehmann et al., 2018; Park et al., 1992). The mechanism of DNA repair is almost conserved in all prokaryotes as well as eukaryotes, but the proteins are not homologous (Morgante et al., 2005).

In yeast, a mutation in the walker A motif of RAD25 helicase was observed to be lethal. Hence, RAD25 is essential for the viability of the cells and RNA polymerase II mediated transcription (Guzder et al., 1995; Park et al., 1992). In contrast to both yeast and human, *XPB* mutation was not found to be lethal in *Arabidopsis*. In *Arabidopsis*, *XPB2* mutation caused delayed the developmental process, loss of seed viability, delayed germination, and sensitivity towards alkylating agents, although the mutants exhibited normal morphology. The reason behind such features might be the redundancy of the *XPB* genes present in *Arabidopsis*. The two homologs of *XPB* (*XPB1* and *XPB2*) were 95% similar (Costa et al., 2001; Morgante et al., 2005). Also, it was observed that the transcript level of *XPB2* was elevated during its early developmental stage (Morgante et al., 2005). Further, studies have shown that archaeal XPB protein isolated from *Sulfolobus solfataricus* also binds to its DNA around a lesion and exhibits DNA dependent ATPase activity (Richards et al., 2008). Recently, it has been reported that the promoter of rice *XPB2* gene is a multi-stress inducible and might play an essential role in orchestrating plant stress tolerance. The promoter has *cis*-elements that respond to various abiotic stresses (e.g., salt, dehydration, cold) and phytohormones (e.g., auxin, abscisic acid, methyl jasmonate; Raikwar et al., 2015). *OsXPB2* expression has also been influenced by gamma-irradiation and salinity stress (Macovei et al., 2014).

2.3.1.3.SEN1 (t-RNA splicing endonuclease)

Rice *SEN1* (t-RNA **s**plicing **en**donuclease) is an RNA helicase belonging to the Upf1-like subfamily under the superfamily 1B group. UPF1, SEN1, and SETX (*Senataxin*) are rice SEN1 homologs discovered in *Arabidopsis*, yeast, and humans, respectively (Martin-Tumasz & Brow, 2015; Umate et al., 2010). RecA1 and RecA2 are two conserved helicase cores that attach to the 3' and 5' ends of RNA, respectively and unwind it in a 5' to 3' orientation (Han et al., 2017; Leonaitė et al., 2017; Martin-Tumasz & Brow, 2015). Although the specific mechanism of rice SEN1 is unknown, the helicase domains are largely conserved among eukaryotes (Han et al., 2017; Leonaitė et al., 2017), suggesting that these proteins may work similarly. Non-coding aberrant RNAs are formed during pervasive transcription that interferes with the regular transcription of coding mRNAs (Han et al., 2017). SEN1 is a part of the NNS (NRD1-NAB3-SEN1) complex, which is responsible for transcription termination of yeast non-coding RNAs such as cryptic unstable transcripts (CUTs) and small nucleolar RNAs (sno RNAs; Leonaitė et al., 2017; Sariki et al., 2016). NRD1-NAB3 are RNA binding proteins, which form a heterodimer that identifies specific sequences on the nascent RNA, GUA(A/G) by NRD-1 and UCUU (G) by NAB3 (Han et al., 2017; Mischo et al., 2018). The serine residue (Ser5) belonging to the C-terminal heptapeptide (YSPTSPS) repeat of RNA polymerase II gets phosphorylated during transcription termination. The phosphorylated Serine residue interacts with NRD1 of the NNS complex (Mischo et al., 2011) and helps NRD1-NAB3 dimer in loading of SEN1 (having helicase properties) onto the nascent RNA, which dislodges the RNA polymerase II by its helicase activity (Mischo et al., 2018). After transcription is terminated, NRD1-NAB3 heterodimer recruits TRF4 of the TRAMP complex (TRF4/ AIR2/ MTR4 polyadenylation complex) to degrade the nascent transcripts. The complex polyadenylates the 3′ end of RNA and directs the exosomes to degrade it from 3′ to 5′ direction. These exosomes cause complete degradation of the CUTs and partial degradation of sno RNAs (Leonaitė et al., 2017; Mischo et al., 2011).

SEN1 is a rate limiting enzyme with low processivity in the NNS-mediated transcription termination. Reports have shown that SEN1 must be loaded within 20-40 nucleotides upstream of RNA polymerase II. Otherwise, it fails to terminate transcription (Han et al., 2017; Mischo et al., 2018). In a recent report, it has been observed that SEN1 levels are maintained low during the G1 phase by proteasomal degradation during the cell cycle (Martin-Tumasz & Brow, 2015), whereas its activity is increased during S and G2 phases. In the case of its human homolog SETX, the protein changes its localization during the cell cycle. Studies indicate that the abundance of SEN1 can cause excessive NNS-mediated transcription termination (Mischo et al., 2018). It plays an important role in several other functions like RNA processing, eliminating short protein coding genes, resolving R loop structures, and maintaining genomic stability (Mischo et al., 2011). In case of a double strand break, Sen1 has been reported to restrict the formation of DNA: RNA hybrids at the breakage site, thereby ensuring the appropriate repair of the break and maintenance of genomic stability (Rawal et al., 2020). Defects in SEN1 result in defective R loop resolution and increased frequency (Leonaitė et al., 2017; Martin-Tumasz & Brow, 2015; Mischo et al., 2018), abnormal nucleolar organization, genomic instability, and defects in replication (Mischo et al., 2018). *SEN1* mutation leads to an increased sensitivity of the cells towards DNA damaging agents and defects in cell regulation checkpoints (Sariki et al., 2016). Apart from regulating the expression of non-coding genes, SEN1 also co-ordinates the expression of small protein coding genes like *NRD1, HRP1, IMD2* and *CYC1* (Steinmetz et al., 2006). Mutant cells with N-terminal truncations of SEN1 had more sensitivity towards oxidative stress, loss of mitochondrial DNA, high ROS accumulation, resistance to rapamycin treatment, cell death and shortened life span. *Arabidopsis* homolog of SEN1, UPF1 plays a vital role in nonsense mediated decay (NMD) of abnormal RNA. It also prevents translation initiation of aberrant mRNA, thereby suppressing the production of truncated proteins. In *Arabidopsis*, *upf1* mutants exhibited abnormal floral and vegetative development (Arciga-Reyes et al., 2006). It also helps the plant in maintaining seed size (Yoine et al., 2006). In yeast, the *upf1* mutants were sensitive to oxidative stress (Rodríguez-Gabriel et al., 2006). Thus, it has a significant role in regulating both transcription and translation in most of the eukaryotes. SEN1 was also observed to have been associated with all three

RNA polymerases (Yuce & West, 2013). It was also found to be involved in transcription coupled repair mechanisms (Li et al., 2016). Hence, the primary function of SEN1 is likely to dissociate any stalled elongation complex on the nucleic acid during transcription.

2.3.2. Transcription factors

Regulation of gene expression is necessary for cellular activities like growth, differentiation, metabolism, signal transduction, and stress responses due to external and internal stimuli. Such regulation is mainly done at the transcriptional level via transcription factors (TFs) (Meshi & Iwabuchi, 1995). TFs are the proteins that influence gene expression by binding with several elements present in the promoter regions of their targets (Gonzalez, 2013). TFs are encoded by 5-7% of the coding sequences present in the plant genome (Hoang et al., 2017). Sequence analyses indicate that these belong to multigenic families and have evolved via genetic duplication events followed by their subsequent translocation. Such kind of evolution justifies the conserved structure and function of various TF families among different organisms. About 320k TFs from 165 different plant species have been reported. Some important transcription factors include WRKY, MYB, AP2/EREBP, bZIP, GRAS, and others (Shen et al., 2020; Sidhu et al., 2020; Zhang et al., 2018).

2.3.2.1.Domain organization of TFs

This group of proteins consists of a DNA binding site, a protein oligomerization region, a regulatory domain, and a nuclear localization signal (NLS) (Liu et al., 1999). DNA binding domain determines the specificity of the TFs and is mostly lined by basic amino acid residues. This enables them to interact with the major or minor groove of the DNA to either activate or repress the expression of their target genes (Gonzalez, 2013; Liu et al., 1999). In regulating gene expression, the interactions of the TFs with their partner proteins play essential roles. This is done either via hydrophobic interactions between the secondary structures of the oligomerization domain or hydrophilic interactions between the residues resulting in the formation of homo or heterodimers (Huang et al., 1996). Amino acid sequences are found to be conserved in this domain (Liu et al., 1999). Factors belonging to Leucine-zipper and helix-loop-helix families tend to function by forming dimers (Gonzalez, 2013). The regulatory domains of the TFs determine their ability to function as enhancers or repressors of gene expression. This activity is regulated by the subcellular localization of the factors. Thus, single or multiple NLS rich in basic amino acids like arginine and lysine can be observed in plant TFs. The number and composition of NLS vary among the families (Gonzalez, 2013; Liu et al., 1999).

2.3.2.2.Classification of TFs

Based on their DNA binding domains, TFs are divided into several families (Gonzalez, 2013). This includes the APETALA2/ethylene responsive element binding protein family (AP2/EREBP), the basic leucine zipper (bZIP) family, the MYB, MYC, WRKY, NAC, and GRAS transcription factor families (Agarwal et al., 2006; Hoang et al., 2017).

The DREB TFs belong to the AP2/EREBP family and have been reported to play crucial roles in responses against pathogen attacks as well as cold and drought stress. They mediate stress responses via ABA independent signaling pathway. The bZIP, MYB, and MYC factors are involved in ABA dependent signaling cascades (Agarwal et al., 2006; Singh et al., 2002). The bZIP factors or the master regulators bind to the ABA responsive elements (ABRE) of the promoter regions of the genes via their leucine zipper domain and regulate downstream genes responsible for multiple stress responses (Agarwal et al., 2006; Hoang et al., 2017; Joshi et al., 2016). MYB and MYC factors bind to their corresponding *cis*-acting elements via Leucine zipper or helix-loop-helix structure and control signaling cascades related to abiotic stress responses, especially drought, salinity, and extreme temperatures. WRKY class of TFs interact with DNA via specific WRKY domains and have been reported to have important roles against pathogen invasion and abiotic stress tolerance in plants. The NAC family of TFs work mostly in drought and salinity tolerance as well as auxin, ethylene, and ABA signaling pathways (Agarwal et al., 2006; Ahmad et al., 2014; Hoang et al., 2017; Joshi et al., 2016; Singh et al., 2002). Another family of TFs includes the GRAS family, which will be discussed in detail in the subsequent sections. The corresponding examples of each TF family is provided in the attached Table 2.2.

Table 2.2: List of few transcription factors along with their source and their roles in stress tolerance.

Gene	Traits/ induced by	Family	Source organism	Target organism	References
TSRF1	Drought, osmotic stress	ERF TF	Tomato	*Oryza sativa*	(Quan et al., 2010)
PsDREB2	Abiotic stress	ERF TF	*Paeonia suffruticosa*		(Liu et al., 2019)
OsABF2	Abiotic stress	bZIP TF	*Oryza sativa*	*Oryza sativa*	(Hossain et al., 2010)
OsAREB1	Drought, heat	bZIP TF	*Oryza sativa*	*Arabidopsis thaliana*	(Jin et al., 2010)
HAHB4	Wounding, *Spodoptera littoralis* *Spodoptera frugiperda*	HD-Zip TF	Sunflower	*Arabidopsis thaliana,* *Zea mays*	(Manavella et al., 2008)
SlICE1a	Cold, osmotic, salt stress	MYC TF	Tomato	Tobacco	(Feng et al., 2013)
AtMYC2	JA induced defence	MYC TF	*Arabidopsis thaliana*		(Lorenzo et al., 2004)
ZjICE1	Cold	MYC TF	*Zoysia japonica*	*Arabidopsis thaliana*	(Zuo et al., 2019)
OsMYB6	Drought, salinity	MYB TF	*Oryza sativa*	*Oryza sativa*	(Tang et al., 2019)
LeAN2	Oxidative, chilling stress	MYB TF	*Lycopersicon esculentum*	*Nicotiana tabacum*	(Meng et al., 2014)
OsWRKY45	Drought, disease resistance	WRKY TF	*Oryza sativa*	*Arabidopsis thaliana*	(Qiu & Yu, 2009)
AtWRKY30	Drought, heat	WRKY TF	*Arabidopsis thaliana*	*Triticum aestivum*	(Esawi et al., 2019)
OsNAC52	Drought	NAC TF	*Oryza sativa*	*Arabidopsis thaliana*	(Feng Gao et al., 2010)
RD26	Drought, salinity, ABA	NAC TF	*Arabidopsis thaliana*	*Arabidopsis thaliana*	(Fujita et al., 2004)
MbNAC25	Cold, salinity	NAC TF	*Malus baccata*	*Arabidopsis thaliana*	(Han et al., 2020)
GmNAC109	Abiotic stress	NAC TF	Soybean	*Arabidopsis thaliana*	(Yang et al., 2019)

2.3.2.3.Factors influencing TFs expression and their regulation

TFs can be expressed either constitutively or can be regulated spatially or temporally. These can be triggered by many environmental stressors such as drought, salinity, low temperature, nutrient-deficient conditions, light, heat, hypoxia, wounding, pathogens, and plant hormones like abscisic acid, gibberellins, auxin, ethylene, and salicylic acid (Hoang et al., 2017; Liu et al., 1999; Singh et al., 2002). Plant regulate these TFs at two levels; one at the time of their synthesis and another before their activity. The former level of regulation is done by controlling the synthesis of TFs in certain tissues. The *cis* and *trans*-acting elements of the gene promoter regions play an essential role. The latter is done via activation or repression of already existing TFs in the cell. One of the common methods of doing so is via phosphorylation or de-phosphorylation of the proteins (Latchman, 1997; Liu et al., 1999).

2.3.2.4.GRAS transcription factors

GRAS proteins are a group of plant-specific transcription factors first reported in bacteria and assigned to the Rossman fold methyl transferase superfamily (Zhang et al., 2012). Later, this group radiated towards the ancestors of bryophytes, lycophytes, and other higher plants via horizontal gene transfer (Cenci & Rouard, 2017; Zhang et al., 2018). Several GRAS genes have been identified in more than 30 different plant species, including *Arabidopsis*, rice, maize, *Populus trichocarpa*, and many others (Cenci & Rouard, 2017; Guo et al., 2017; Liu & Widmer, 2014; Tian et al., 2004). A list mentioning some of the GRAS genes identified to date is provided in Table 2.3.

Table 2.3: List of GRAS transcription factors identified from various plant species

Name of the organism	**Number of GRAS genes identified**	**References**
Arabidopsis thaliana	33	(Lee et al., 2008)
Oryza sativa	60	(Liu & Widmer, 2014)
Brassica rapa	48	(Song et al., 2014)
Pinus radiata	31	(Abarca et al., 2014)
Prunus mume	46	(Lu et al., 2004)
Populus trichocarpa	106	(Liu & Widmer, 2014)
Solanum lycopersicum	53	(Huang et al., 2015)
Vitis vinifera	43	(Xin Sun et al., 2016)
Phyllostachys edulis	59	(Zhao et al., 2016)
Ricinus communis	48	(Xu et al., 2016)
Nelumbo nucifera	38	(Wang et al., 2016)
Brachypodium distachyon	44	(Wu et al., 2014)
Glycine max	117	(Wang et al., 2020)
Gossypium hirsutum	150	(Zhang et al., 2018)
Hordeum vulgare	62	(To et al., 2020)
Cucumis sativus	37	(Lu et al., 2020)

GRAS TFs belongs to multigenic families and were divided into eight to upto thirteen subfamilies in *Arabidopsis*, rice, tomato, cotton, poplar, castors beans, and others (Huang et al., 2015; Liu & Widmer, 2014; Tian et al., 2004; Xu et al., 2016; Zhang et al., 2018). A higher number of genes in this gene family indicates that the expansion of this gene family might have happened via segmental and tandem duplication events followed by the retention of multiple copies post duplication events (Huang et al., 2015; Tian et al., 2004).

2.3.2.4.1. Domain organization of GRAS proteins

The nomenclature of GRAS proteins was derived from the first identified members of this family, i.e., Gibberellin-Acid Insensitive (GAI), Repressor of GAI (RGA), and Scarecrow (SCR) (Bolle et al., 2000; Pysh et al., 1999; Zhang et al., 2017). These proteins are 400-770 amino acids long and carry a variable N- terminal domain and a conserved C- terminal domain (GRAS domain), which transcriptionally regulate the downstream genes. The GRAS domain again comprises five motifs, which are (i) Leucine heptad repeat I (LHR I), (ii) VHIID motif, (iii) Leucine heptad repeat II (LHR II), (iv) PFYRE motif, and (v) the SAW motif respectively. (Pysh et al., 1999).

(i) Leucine heptad repeats

The LHR present in the GRAS domain indicates the ability of the protein to function in multimers. LHR I consists of two units LHR IA and LHR IB, separated by a proline residue. LHR IA consists of three to five repeats of heptad sequences, while IB has a couple of repeats. LHR II also has two to three repeat units in its sequence. This sequence is conserved in GRAS proteins (Pysh et al., 1999).

(ii) The VHIID motif

It consists of an identifiable VHIID motif with conserved P-N-H-D-Q-L residues in its sequence. This motif ends in the LRITG sequence towards the C- terminus. This motif is responsible for protein-DNA interactions (Pysh et al., 1999; Tian et al., 2004).

(iii) PFYRE motif

This motif can be divided into three parts, i.e., the P residue, the FY residue, and the RE residue. The sequences of this motif are not very conserved, but it shows similarity among the members belonging to the same family (Pysh et al., 1999).

(iv) SAW motif

This motif is situated at the C- terminal end of the GRAS domain, and the presence of W-W residues characterizes it. Other conserved regions in this motif are R-E and W-G residues.

The PFYRE and SAW motif function are not clearly known, but they are assumed to have important roles in regulation or maintaining the structural integrity of the GRAS domain's structural integrity (Pysh et al., 1999; Xiaolin Sun et al., 2012).

Fig. 2.3: Figure representing the motifs present in GRAS transcription factor

The GRAS transcription factor domain showing the sequential arrangement of five motifs from N- terminal to C- terminal ends (Dutta et al., 2021b)

2.3.2.4.2. Functions of GRAS genes

The majority of the GRAS proteins are localized in the nucleus, PAT1 being the exception is located in the cytoplasm (Tian et al., 2004). The conserved domain is important for gene regulation, while the variable N-terminus of GRAS proteins comprises of intrinsically disordered regions (IDRs), which aid in molecular recognition during developmental processes (Cenci & Rouard, 2017; Xiaolin Sun et al., 2012). This group of TFs identifies environmental and regulatory cues and plays crucial roles in plant growth and development. They are involved in various biological pathways like gibberellic acid signaling (GAI and RGA of DELLA subfamily and SLR1 of rice) (Pysh et al., 1999; Liu et al., 2014; Vinh et al., 2020), SHR and SCR genes in radial root patterning (Helaritutta et al., 2000), SCL3 in root elongation (Huang et al.,2015), HAM in shoot meristem formation (Stuurman et al., 2002), PAT genes in phytochrome signaling (Bolle et al., 2000), NSP1 and NSP2 in nodulation signaling pathway (Huang et al., 2015) and some others in abiotic and biotic stress responses (Sun et al., 2012; Zhang et al., 2018; Zeng et al., 2019). In many higher angiosperms, several GRAS genes like *ZmSCL7*, *AtRGA*, *AtGAI* were shown to have roles in salt stress tolerance in maize and *Arabidopsis* (Zeng et al., 2019). *PeSCL7* from *Populus* is associated with the modulation of drought and salt tolerance (Ma et al., 2010). *OsGRAS23* was shown to induce drought stress tolerance in rice (Xu et al., 2015). Other genes like *SlGRAS4* and

SLGRAS40 were shown to improve drought tolerance and phytohormone signaling in tomato (Liu et al., 2017, 2021). In *Arabidopsis* plants, several GRAS genes like *VaPAT1* of *Vitis amurensis* improved abiotic stress tolerance (Yuan et al., 1910), *BrLAS* of mustard improved drought tolerance (Li et al., 2018), and *HcSCL13* of *Halostachys caspica* (Zhang et al., 2020) exhibited roles in growth and salt tolerance.

CHAPTER 3

Screening of Activation Tagged Mutants and Identification of Helicases

SCREENING OF ACTIVATION TAGGED MUTANTS AND IDENTIFICATION OF HELICASES

3.1.Chemicals used

All the chemicals used in this study were obtained from Sigma-Aldrich Corporation, USA; Himedia Chemicals, Mumbai, India; SRL India, Invitrogen, USA; Clonetech, Takara Biotech, Japan; Fermentas, Germany.

3.2.Vectors used

3.2.1. TA cloning vector (pTZ57R/T, Fermentas, Germany)

pTZ57R/T vector was used to clone PCR amplified products with poly A overhangs followed by sequence confirmation. The positive clones were selected based on Ampicillin resistance marker and blue-white screening.

3.2.2. *Ac/Ds* based activation tagging vector (pSQ5) (Qu et al., 2008)

This vector works on the principle of *Activator*/*Dissociator* (*Ac*/*D*s) method of transposition as indicated by McClintock (1950). Based on this background, an *Ac/Ds* based activation tagging vector (pSQ5) was generated where tetrameric CaMV35S enhancers were introduced in the *Ds* element along with an Ubi::RFP tag as a reporter. The original inverted repeats were kept intact for the transposition events to occur. The *Ac* element was engineered to become stable and expressed under the control of CaMV35S constitutive promoter along with an Ubi::GFP tag in the T-DNA backbone. This was done so that the *Ds* element only with the inverted repeats containing the enhancers and the GFP tag can transpose during transposition. Plants containing only *Ac* or *Ds* element will be stable after segregation, and only those plants carrying both the elements will be able to have the process of transposition to randomly integrate *Ds* element further into newer locations in the genome. The tags were introduced for visual tracking of the elements. The vector map of pSQ5 and their transposition pattern is explained in figure 3.1. As transposons have the inherent ability to jump and get randomly integrated into the genome, many gain-of-function mutants were generated by *in-planta* method of plant transformation followed by repeated selfing (Moin et al., 2016).

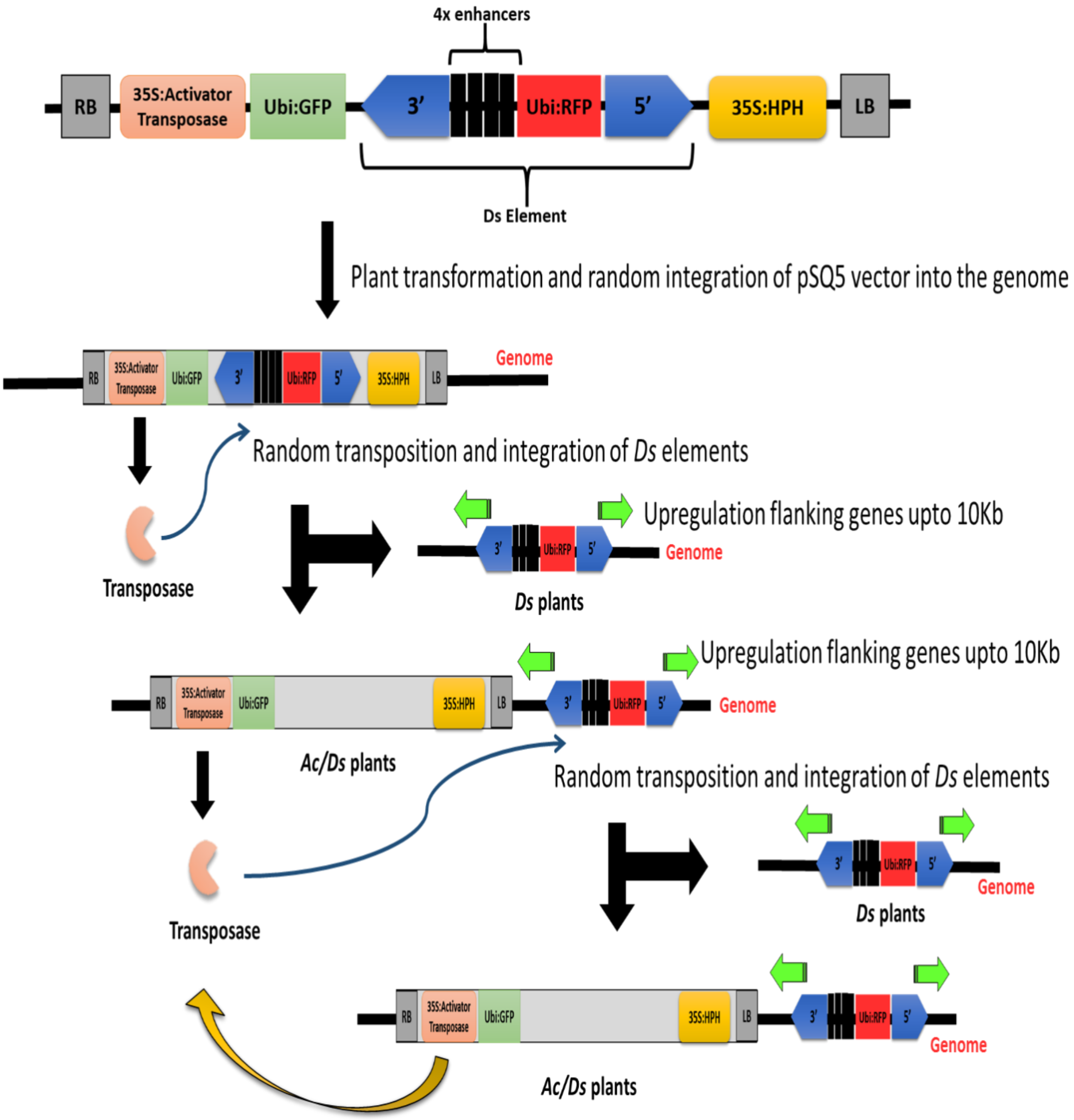


Fig. 3.1: pSQ5 vector with various elements and their integration patterns

The pSQ5 vector works on the principle of *Ac*/*Ds* transposition. It consists of an immobilized *Ac* (*Activator*) element that is stable and capable of encoding a transposase protein that identifies the inverted repeats of the *Ds* (*Dissociator*) element and integrates it randomly into a newer location in the genome. The modified *Ds* element consists of tetrameric CaMV35S enhancers that upregulate the expression of flanking genes present ~10 kb upstream and downstream from their integration point. Each transposition leads to the formation of *Ds* plants, *Ac/Ds* plants, and only *Ac* plants (not shown in the figure).

The *Ac/Ds* plants continue to transpose in subsequent generations due to both *Ac* and *Ds* elements and generate *Ac, Ds,* and *Ac/Ds* plants again. The *Ds* plants are stable and do not show further transposition due to the absence of the *Ac* element. Irrespective of the presence of the *Ac* element, the *Ds* element continues to upregulate the flanking genes when integrated into the genome.

3.3.Bacterial strain (DH5α)

For storage of pSQ5 vector and bacterial transformation *Escherichia coli* strain DH5α was used.

3.3.1. Preparation of *E. coli* competent cells

A single DH5α colony was chosen with a sterile tip, inoculated into 10 ml of LB broth, and cultured overnight at 37°C with steady agitation. About 200 µl of this primary culture was inoculated into 100 ml LB medium and grown further for 3-5 h until the OD_{600} reached 0.6. The secondary culture was kept on ice for 30 min and then centrifuged at 5000 rpm for 5 min at 4°C in Oakridge tubes. The supernatant was discarded, and the pellet was suspended in an equal volume of 0.1 M ice cold $CaCl_2$. The suspension was incubated on ice for 10-15 min and re-centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 3-5 ml of ice cold 0.1 M $CaCl_2$ and 15% (v/v) sterile glycerol solution. Aliquots of 200 µl of the suspension were stored in 1.5 ml Eppendorf tubes, freezed in liquid Nitrogen, and stored at -80°C.

3.3.2. *E. coli* transformation by heat shock method (Inoue et al., 1990)

The DH5α competent cells were thawed, and 20 µl of the ligation mixture was added and incubated on ice for 15 min. The cells were subjected to heat shock at 42°C in a water bath for 90 s and immediately transferred to ice. An aliquot of 1 ml of LB medium was added to the cells and incubated for 1 h at 37°C. The tubes were centrifuged at 3000 rpm for 5 min and the pellets were resuspended in LB medium and spread on specific antibiotic containing LA plates.

3.3.3. Bacterial growth conditions

The *E. coli* cells were incubated at 37°C in Luria Broth (LB) or Luria Agar (LA) media in an incubator shaker at 150-200 rpm. The glycerol stocks were maintained at -80°C in 50% glycerol (v/v).

3.3.4. Plasmid isolation

Plasmid from DH5α strains was isolated using both the kit method (Clonetech, Takara Biotech, Japan) and the conventional method (Birnboim & Doly, 1979).

For the conventional method, a single colony of DH5α strain was inoculated in 10 ml LB at 37°C and cultured overnight. The culture was taken and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded, and the step was repeated 4-5 times. The final pellet was dissolved gently in 200 µl of an ice cold solution I of 50 mM Glucose, 25 mM Tris- HCl pH 8.0, 10 mM EDTA, 100 µg RNase A. To this, 400 µl of freshly prepared solution II containing 200 mM NaOH and 1% (w/v) SDS was added and incubated for 3-5 min. Finally, 300 µl of solution III (3.0 M potassium acetate, pH 4.8) was added, mixed gently, and incubated on ice for 5 min. The tubes were then centrifuged at 12,000 rpm for 10 min at 4°C. An equal volume of ice cold isopropanol was added to the supernatant and incubated on ice for 20-30 min. The plasmid was pelleted down by centrifuging at 12,000 rpm for 10 min. The pellet was washed with 70% ethanol at 8000 rpm for 5 min, air-dried, and dissolved in 30-40 µl of nuclease free water.

3.4.Ligation

T4 DNA ligase was used in the ligation reaction (Fermentas, Germany). A mixture of 2 µl ligation buffer (10 x), 2U of Ligase, and a 1:3 to 1:5 ratio of plasmid to insert concentration was made up into a 20 µl ligation reaction mixture, which was incubated for 12-16 h at 16°C.

3.5.Plant material

The plant material used in this study was *Oryza sativa* ssp. *indica* cv. Samba Mahsuri (variety BPT-5204).

3.5.1. Seed sterilization and plant growth conditions

The seeds of BPT-5204 were dehusked and surface sterilized with 70% ethanol for 1 min. It was followed by a single wash with 4% aqueous sodium hypochlorite solution for 15 min. Finally the seeds were washed five times with sterile double distilled water for 2 min per wash. The blot dried seeds were inoculated on solid MS media (full or half strength depending on the experiment) under 16 h /8 h light/dark cycles. The acclimatized plants were shifted to pots containing black alluvial soil in the greenhouse with 30 ± 2°C, 16 h light/ 8 h dark photoperiod, and relative humidity of 55±5%.

3.5.2. Genomic DNA extraction (CTAB method)

Genomic DNA from rice leaves was isolated using CTAB buffer (CTAB: 2%, 1 M Tris base pH: 8; 0.5 M EDTA pH: 8; and 5 M NaCl). About 100 -150 mg leaf samples were crushed in a mortar and pestle using liquid Nitrogen and transferred to Eppendorf tubes containing 1 ml CTAB buffer. To this, 20 µl of β-mercaptoethanol was added, mixed, and incubated at 65°C for an hour. Post incubation, the tubes were centrifuged at 11000 rpm for 15 min at 4°C. The supernatant was collected and an equal volume of Phenol: Chloroform: Iso-amyl alcohol mixture (25:24:1) was added. The solution was mixed by inversion and was allowed to incubate at 4°C for 5 min. This was followed by centrifugation at 5000 rpm for 8 min. The upper aqueous layer was transferred into a fresh tube, and an equal volume of Chloroform: Iso-amyl alcohol (24:1) was added, incubated at 4°C for 15 min followed by centrifugation at 12000 rpm for 12 min. This step was repeated twice. The clear upper phase was taken, and an equal volume of ice cold isopropyl alcohol was added and mixed well. The mixture was incubated at -20°C for 8-12 h followed by centrifugation at 11000 rpm for 15 min. The supernatant was discarded, and the pellet was washed using 70% ethanol, air dried, and dissolved in nuclease free water. This resulted in high quality genomic DNA (2000 ng/µl) free from protein and salt contamination (260/280 = 1.8, 260/230= 2.1).

3.5.3. Isolation of total RNA using Tri-Reagent

The total RNA was isolated using the Tri-Reagent (Takara Bio, UK) as per the manufacturer's protocol. The RNA quality was checked on 1.2% agarose gel in TBE (Tris-Borate EDTA) buffer and quantified using a Nanodrop spectrophotometer. 2 µg/µl RNA was used to synthesize first strand cDNA using a reverse transcriptase enzyme (Takara Bio, UK).

3.5.4. Quantification of DNA and RNA

A Nano-drop spectrophotometer (ND-1000, USA) was used to analyze the quality and quantity of DNA and RNA. DNA with OD 260/280 and 260/230 values of 1.8 and 2.1, respectively, devoid of protein and salt contamination, was chosen. In the same way, values between 1.9 and 2.1 were chosen for RNA.

3.5.5. First strand cDNA synthesis

MMLV reverse transcriptase enzyme (Takara Bio, UK) was used to synthesize first strand cDNA from total RNA (2 µg concentration) as per the manufacturer's protocol. The cDNA was diluted seven to ten times depending on the experiment, and 2 µl of it was used in qRT-PCR analysis.

3.5.6. Quantitative Real-Time PCR (qRT-PCR)

Quantitative Real-Time experiments were done to check the expression of various genes in the mutants as well as the wild-type plants. The primers (IDT, USA) for all qRT-PCR analyses were prepared using OligoCalc and Primer3 online tools. All the primers had a working concentration of 10 µM. The qRT-PCR was performed using SYBR Green ® Premix (Takara Bio, USA) as per manufacturer's protocol with 2 µl of seven to ten times diluted cDNA as template in an Eppendorf Master Cycler Realplex^4 machine. The reaction conditions included an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, annealing temperature (depending on the primer) for 25 s, and 72°C for 30 s followed by a melting curve. The primer list is given in table 3.3.

3.6.Generation of activation tagged mutants

Our group has previously generated a large pool of gain-of-function mutant rice plants via the *Ac/Ds* method of activation tagging. The vector pSQ5 was mobilized into the *indica* rice genome via *Agrobacterium* mediated *in-planta* method of plant transformation. The transformed plants were initially screened on 50 mg/L Hygromycin containing solid MS medium followed by their molecular analysis through PCR and Southern-blot hybridization (Moin et al., 2016). The confirmed stable *Ds* plants were then examined for WUE traits by subjecting them to limited water conditions. Following their phenotypic and physiological analysis, these plants were further screened for the potential candidate genes that were tagged by the integrated enhancer elements of the pSQ5 vector.

3.7.Identification and confirmation of genes

3.7.1. Carbon isotope analysis

WUE in plants can be determined with a non-invasive method using carbon isotope discrimination ($\Delta^{13}C$). The negative association between the WUE of a plant and $\Delta^{13}C$ readings is a useful tool for identifying plants that perform better in low-water environments (Chen et al., 2011). In C_3 plants, the molar abundance ratio (R) of $^{13}C/^{12}C$ is less than that of the atmosphere as plants discriminate in the uptake of ^{13}C during photosynthesis. The carbon isotope composition ($\delta^{13}C$‰) is estimated using the formula $[(R_{sample}/R_{standard})-1]\times10^3$ and compared to a Pee Dee Belemnite (PDB) fossil carbonate standard. The carbon isotope discrimination ($\Delta^{13}C$‰) is measured by the formula $[(\delta^{13}C_a-\delta^{13}C_p)/(1+\delta^{13}C_p)\times10^3$ where $\delta^{13}C_a$ and $\delta^{13}C_p$ are the $\delta^{13}C$ values of atmosphere and plant, respectively (Farquhar et al., 1982, 1989; Gao et al., 2018). The mature leaf samples of 500 mg of the genotypes under study (control and the tagged lines) grown under limited water conditions were collected and dried at 65°C for 3 d to calculate the carbon isotope discrimination values. The samples were crushed, and the carbon isotope was detected using an Isotope Ratio Mass Spectrometer (IRMS).

3.7.2. Identification of flanking gene sequences

Genotypes with high WUE under limited water conditions were selected for flanking gene sequence analysis to identify the genes tagged by the integrated enhancers. They were subjected to thermal asymmetric interlaced PCR (TAIL PCR) technique (Liu et al., 1995). This tool utilizes one degenerate primer and three sets of specific or nested primers. The primary reaction involves the binding of a degenerate primer and the first nested primer that are relatively far apart in the genome. It is followed by secondary and tertiary reactions, which involve the same degenerate primer, but different sets of nested primers that bind relatively closer. After each reaction cycle, the PCR products were diluted and used as templates for the next reaction. It was done to obtain more specific products and reduce the number of non-specific PCR products.

In our experiment, we used three nested primers (NP) specific to the 5′ end of the integrated *Ds* element and one set of degenerate primers to locate the tag in the genome and identify the flanking gene sequences. NP1, NP2, and NP3 were respectively 1 kb, 500 bp, and 100 bp upstream to the *Ds* element. For the primary reaction, genomic DNA of rice was used as a template, while for secondary and tertiary reactions 1 µl of diluted products from the preceding cycles were used as templates. Multiple bands were observed at the end of the primary reaction, which gradually reduced in the subsequent reactions. The reaction cycle and the primer sequences are provided in table 3.1 and 3.2. The final amplicons were ligated in the TA cloning vector (pTZ57R/T) and subjected to Sanger sequencing commercially. The point of enhancer integration and the flanking gene sequences were identified by performing a BLAST search in the rice genome database (RGAP-DB, RAP-DB, and OrygenesDB). DEB. 36 (referred to as XM3) and En.124 (referred to as SM4) were the two potential mutants identified with significant WUE. Within the 20 kb spanning the region of the enhancer integration, three and five genes were present, respectively. The level of expression of these genes was analyzed via quantitative real-time PCR (qRT-PCR).

Table 3.1: The reaction protocol for TAIL-PCR

Reaction type	PCR Protocol	Number of cycles
Primary reaction (Primer concentration: 10 µM, NP1: degenerate primer =1:4)	95℃ 3 min	1
	94℃ 30 s*; 60℃ 30 s; 72℃ 2 min (annealing temperature is according to the NP)	3
	94℃ 30 s; 25℃ 2 min; 0.2 ℃/sec;72℃ 2 min	2
	94℃ 30 s; 60℃ 30 s; 72℃ 2 min 94℃ 30 s; 60℃ 30 s; 72℃ 2 min 94℃ 30 s; 35℃ 30 s; 72℃ 2 min	5-10
	72℃ 10 min.	1
Secondary reaction (Product from primary reaction was diluted to 100 µl and 1 µl was used; NP2 and degenerate primer added: 1 µl)	95℃, 3 min 94℃ 30 s; 35℃ 30 s; 72℃ 2 min 94℃ 30 s; 35℃ 30 s; 72℃ 2 min	1
	94℃ 30 s; 60℃ 30 s; 72℃ 2 min	30
	72℃ 5 min.	1
Tertiary reaction (Product from secondary reaction was diluted to 100 µl and 1 µl was used; NP3 and degenerate primer added: 1 µl)	95℃, 3 min	1
	94℃ 30 s; 60℃ 30 s; 72℃ 2 min	15-20
	72℃ 10 min.	1

* s means seconds

Table 3.2: List of primers used in TAIL PCR

Primer name	**Primer sequence (5'-3')**	**Purpose**
Degenerate primer 1	NGACGA(G/C)(A/T)GANA(A/T)GAA	TAIL-PCR
Degenerate primer 2	NGACGA(G/C)(A/T)GANA(A/T)GAC	TAIL-PCR
Ds.NP1	CTCACAGCACTTAGCAGTACAGCACGTCAGC	Nested primer in Primary TAIL PCR
Ds.NP2	GTGCGCGTGGGCATGGATGTGGC	Nested primer in Secondary TAIL PCR
Ds.NP3	ATAGTTTAGTTAAAGGTCAGTTGTGTC	Nested primer in Tertiary TAIL PCR

3.7.3. Expression analysis of flanking genes

The identified mutant lines XM3 (DEB.36) and SM4 (En.124) displaying normal or better growth and yield parameters under water limited conditions compared to their wild-type counterparts were subjected to qRT-PCR analysis. The transcript levels of all genes present in the 20 kb region on either side of the enhancer integration were analyzed using gene-specific primers in three biological and technical replicates. Total RNA was isolated from the leaves of 60 d old plant using Tri-Reagent (Takara Bio, UK) and 2 µg cDNA (Takara, Clonetech, USA) was prepared as per the manufacturer's protocol. The cDNA was diluted seven times, and a 2 µl aliquot of this was used for qRT-PCR. The expression level was normalized using rice *actin* (*act1*) as an internal reference gene, and the fold change was calculated using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001).

3.7.4. Differential transcript analysis of identified helicases

XPB2 and SEN1 helicases were identified from flanking sequence analysis that was responsible for the high WUE trait in the shortlisted mutants XM3 and SM4, respectively. In order to understand the involvement of these two helicases against various environmental stressors, differential expression patterns were evaluated. For this, 10 d old rice seedlings were treated with various phytohormone concentrations like 2 mM salicylic acid (SA), 100 µM methyl jasmonate (MJ) and 100 µM abscisic acid (ABA), and abiotic stress-inducing agents such as 15% polyethylene glycol (PEG 8000), 250 mM sodium chloride (NaCl) and heat treatment at 42°C. The phytohormone, salt, and dehydration stress (PEG) treatments were applied by growing the seedlings in the respective solutions. Seedlings were subjected to heat treatment by placing them in a hot air oven maintained at 42°C. The root and shoot samples were obtained separately at 0 h (collected at the beginning of stress treatment), 3 h, 6 h, 12 h, 24 h, and 48 h after the start of the stress treatments. Seedlings grown in a stress-free medium under identical growth conditions served as normalization controls. The primer sequences are provided in table 3.3.

Leaf samples of rice infected with *Xanthomonas oryzae* pv. oryzae (*Xoo*, which causes bacterial leaf blight of rice) and *Rhizoctonia solani* (which causes sheath blight of rice) were utilized for transcript analysis of these genes under biotic stress conditions. The stress treatments were carried out in controlled culture room conditions where bacterial suspension of *Xoo* was applied on the leaf blades, and *R. solani* containing agar blocks were placed on leaf sheaths of one months old rice plants. Samples were collected 20 and 25 d later for *Xoo* and *R. solani* treatment, respectively (Saha et al., 2017). The untreated leaf samples were considered as the controls for normalizing the gene expression levels. Rice *actin* was used as the internal reference gene, and the relative fold changes were calculated through the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001).

3.8.Analysis of identified lines

3.8.1. Physiological and biochemical analysis

3.8.1.1.Growth and phenotypic parameter measurements of tagged mutant lines under simulated stress

Seeds of XM3, SM4, and WT (BPT-5204) were grown on Murashige and Skoog medium for 25 d to study the behavioral tendencies of the two mutants under abiotic stress settings (Saha et al., 2017). They were then transferred to test tubes containing phytohormones and stress-inducing chemicals in a nutrient Yoshida liquid solution at half strength (Yoshida et al., 1976). As controls, seedlings were kept in a plain half-strength liquid Yoshida solution. Every seven days, the seedlings were subjected to fresh stress by changing the solutions. For revival experiments, the seedlings were transferred to full strength Yoshida solution twenty days after stress (DAS). Before shifting to revival solution, root and shoot samples were collected separately for transcript analysis, and entire seedling samples were collected for biochemical experiments. The samples were freeze-dried in liquid Nitrogen and stored at -80°C. During the plant recuperation, a similar pattern was followed. The seedlings were measured for root length (cm), shoot length (cm), fresh weight (g), percentage of leaf withering, and survival. For the leaf wilting percentage calculation, each leaf's length and wilted length were measured separately for each leaf. After the revival period, the seedlings were transferred to the greenhouse and grown till maturity. During their growth, several physiological and morphological parameters like plant height, number of total and productive tillers (panicles), boot leaf length v/s panicle length, photosynthetic efficiency, and yield related parameters like number of branches per panicle, number of seeds per panicle, and per plant and weight of 100 seeds were measured.

3.8.1.2.Chlorophyll fluorescence measurement

The chlorophyll present in the reaction centre of photosystem II (PSII) is responsible for the absorbance of light energy. A part of the absorbed energy, which is re-emitted as fluorescence, is utilized for photosynthesis. Hence, a measurement of chlorophyll fluorescence connotes the quantum yield of a plant. Pulse Amplitude Modulated fluorometer (PAM) gauges the photosynthetic performance of plants indirectly by testing the quantum efficiency of photosystem II (PSII). It involves the exposure of dark-adapted leaves to a strong pulse of light. The minimal level of fluorescence observed upon irradiation is considered F_o, while *Fm* shows the maximum fluorescence value. The difference between F_o and F_m is F_v or variable fluorescence. The ratio between variable fluorescence and the maximum fluorescence (F_v/F_m) indicates overall photosynthetic efficiency and the stress level experienced by a plant under unfavourable conditions. In a healthy unstressed plant, the F_v/F_m value ranges around ~0.83 (Murchie & Lawson, 2013), corresponding to the maximum photosynthetic yield in a plant. A significant reduction in this value indicates stress induction in the plant. We studied the quantum efficiency of selected mutants compared to their respective controls using a portable MINI-PAM (Walz, Effeltrich, Germany) by following the manufacturer's protocol. All of the plants were given a 30 min dark period before being exposed to a light pulse of 8000 μmol m^{-2} s^{-1}. A histogram of the F_v/F_m ratio was generated.

3.8.1.3.Chlorophyll and proline content estimation

Two sets of samples collected after application of stress followed by their revival under simulated conditions were used for chlorophyll estimation. About 100 mg of tissue was ground to a fine powder in liquid Nitrogen, and chlorophyll was extracted using di-methyl sulphoxide (DMSO). The corresponding absorbance was taken at 663 nm and 645 nm using a UV spectrophotometer, and chlorophyll a, b, and total chlorophyll contents were calculated (Zhang et al., 2009). The mean and standard errors were plotted in the form of bar graphs.

Similar samples were used to estimate the contents of an important osmolyte, proline (Bates et al., 1973). For this, 100 mg of plant tissue was homogenized in 5 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at 12,000 rpm for 15 min, and the supernatant was used for proline estimation. The supernatant was mixed with acid ninhydrin and glacial acetic acid (400 µl each) and was incubated at 100°C for 1 h.

The solution was immediately transferred to an ice bath, cooled, and 800 µl toluene was added, and the mixture was vortexed. Subsequently, the organic phase was pipetted out to a new tube, and the absorbance was recorded at 520 nm. The proline content was estimated using a standard curve, and the values from individual samples were indicated in a bar diagram.

3.8.1.4.Pot level water withholding experiments

For understanding the behavioural pattern of XPB2 and SEN1 helicases, fifteen days old mutants and the wild-type lines were first acclimatized under lab conditions, followed by their shifting into pots containing black alluvial soil. They were grown under similar greenhouse conditions as mentioned before. Three plants were transplanted in each 7.5 Kg pot, and triplicates were considered for further experiments. After growing them for thirty days under normal water conditions, the overlaying water was withdrawn, and the plants were subjected to water limiting conditions. This was continued consecutively for three and seven days, followed by their recovery.

Our previous study demonstrated that the Permanent Wilting Point (PWP) for BPT-5204 variety under our greenhouse conditions was 21 days (Moin et al., 2017a). Drought studies are often measured in percent field capacity (FC), which varies greatly depending on the soil type. The PWP for black alluvial soils found in South India, which were also used in this study, ranges from 10% to 18% FC (http://www.indiawaterportal.org). Therefore, the FC for three and seven days drought treatment would be ~60 and 40%, respectively, in this study. After the drought period was over, all three plants from each pot were uprooted, followed by the collection of their root and shoot samples to study the expression pattern of various drought responsive genes. The remaining pots were allowed to recover by gradual application of water (as required normally for rice cultivation) and were grown till maturity.

The setup was repeated three times, and a comparative investigation was carried out between the identified mutants with their treated and untreated control counterparts. The analysis was mostly done on yield related parameters to check if the overall yield of the mutants was reduced, maintained or improved under drought. Changes in 100 seed weight, seed length: breadth ratio, and amylose: amylopectin ratio were used to monitor the grain quality. The amylose content was measured spectrophotometrically using a standard amylose curve at 600 nm (Sowbhagya & Bhattacharya, 1971).

3.8.1.5.Seed germination assay

The seeds of the mutants and WT were germinated on half-strength MS media containing 50 µM and 75 µM ABA under 16/8 h light/dark cycles. Seeds germinated on half-strength MS media without ABA were used as untreated controls. The germination of the seeds was documented after five days.

3.8.2. Transcript analysis of stress-responsive genes

The transcript pattern of seven stress-regulated genes such as Trehalose Phosphate Phosphatase-1 (*OsTPP1*), Late Embryogenesis Abundant 3-1 protein (*OsLEA3-1*), type 2C Protein Phosphatase (*OsPP2C*), Dehydration Responsive Element Binding protein 2B (*OsDREB2B*), NAM-ATAF1-2-CUC2 proteins (*OsNAC1*, *OsNAC2*) and Ser/Thr protein Kinase -1 (*OsSIK1*) was examined in the root and shoot tissues of mutant and WT plants exposed to phytohormone (ABA), dehydration stress (PEG), and pot-level drought (for three and seven days). These genes play a role in ABA signaling as well as heat, cold, salt, and drought stress modulation. Normalization of the expression data in the mutant lines was done using the transcript levels of the said genes in the stress induced wild-type plants. qRT-PCR was used to examine the transcript patterns of the stress-related genes, and the relative fold change was estimated using the double-normalization approach. The primer sequences are provided in table 3.3.

Table 3.3: List of primers used in differential transcript analysis

Primer name	**Primer sequence (5'-3')**
OsSEN1 RT Fp	AATCATGGTGTGGGTTTCGT
OsSEN1 RT Rp	AGGAAGTCCTTGGGAATGGT
OsXPB2 RT Fp	TCAATGGGCATTTCAGTTCA
OsXPB2 RT Rp	ATGTGCAGGAACAACATGGA
OsLEA3-1Fp	GCGAGTGAGCAGGTGAAGA
OsLEA3-1Rp	GTGGCAGAGGTGTCCTTGTT
OsDREB2BFp	ATCCACAGGGTCCAAAGAAG
OsDREB2BRp	CACACCACGGAAGTCACAAC
OsNAC1Fp	AAATCCCTCACAACCCACAA
OsNAC11Rp	CTCATCCCCATCGCTTCTT
OsNAC22Fp	AGGGCGAGAAGACCAACTG
OsNAC2Rp	ACCCAATCATCCAACCTGAG

OsSIK1Fp	CTCGCATAATCCACAGAGATG
OsSIK1Rp	TGGCAGAGGGGACACATT
OsTPP1Fp	TTCTGCTTTGGCTTCCTTCA
OsTPP1Rp	TCATCCACAATAGGCGACAG
OsPP2CFp	GGAGGCACTTCTATGACACC
OsPP2CRp	AGAAGTTCAGAGTCCGTGCT
OsActin-1 Fp	CTCCCCCATGCTATCCTTCG
OsActin-1 Rp	TGAATGAGTAACCACGCTCCG

3.8.3. *In-silico* analysis

3.8.3.1. *In-silico* promoter analysis of *SEN1*

About 1 kb sequence upstream to the start codon of *SEN1* (LOC_Os10g02930) was retrieved from RGAP-DB and was subjected to an *in-silico* analysis for the presence of *cis*-acting elements using the PlantCARE (Lescot et al., 2002) online tool. Similar promoter analysis of the *XPB2* gene (LOC_Os01g49680) has been reported earlier (Raikwar et al., 2015).

3.8.3.2. Principal Component Analysis of the observed morpho-physiological and biochemical data

Principal component analysis (PCA) is a statistical tool for an efficient interpretation of highly correlated multivariate data. In this case, the dataset comprised of complex phenotypic and physiological traits of three genotypes (WT, XM3, and SM4) under four conditions (UT and three simulated drought conditions: PEG 50 µM, and 75 µM ABA). The main aim of PCA is to reduce the dimensionality of a dataset to two or three principal components while still capturing a majority of the variance of the variables (Wold et al., 1987; Yano et al., 2019). We performed PCA using the "R" programming language to identify the patterns in the dataset of the three genotypes under simulated conditions (Kassambara & Mundt, 2016; R Core Team, 2019). For analyzing the data, the cos2 values have been considered. A high cos2 value indicates a higher impact of that variable in the principal component. A cutoff of 0.5 cos2 value had been considered.

CHAPTER 4

Helicases as Potential Candidate Genes for Drought Stress Tolerance in Rice

HELICASES AS POTENTIAL CANDIDATE GENES FOR DROUGHT STRESS TOLERANCE IN RICE

4.1.Introduction

Generation of loss-of-function mutants is a traditional approach for dissecting the mechanisms of a genetic pathway. But this approach is difficult for characterization of redundant genes where the function of the mutant gene is masked by that of its existing isoforms. So is the case for the lethal genes, whose lack of function results in embryonic lethality. Therefore, the production of gain-of-function mutants serves as an alternative approach where the expression of the genes is upregulated by integrating either promoters or enhancers. This approach is known as activation tagging (Weigel et al., 2000).

Using a tetrameric 35S enhancer-based activation tagging strategy, we have previously generated a sizeable population of gain-of-function mutant lines in the extensively farmed *indica* rice variety BPT-5204 (Samba Mahsuri) (Moin et al., 2016). These mutants were screened based on their phenotypic and physiological performance under limited water availability. High WUE phenotypes were exhibited by some of the mutants, which indicated a probable role of gene activation by the integrated enhancers. High quantum efficiency and low carbon isotope discrimination values are considered to be proxies for high WUE. Based on this, Moin et al. (2016) and Manimaran et al. (2017) identified six mutants that showed the activation of GRAS, WRKY 96, and NF-YC13 transcription factors ubiquitination protein cullin4, and two ribosomal proteins (RPL6 and RPL23A).

We have analyzed the flanking sequences of the activation tags (tetrameric enhancers) in two other gain-of-function mutants, which led to the identification of a DNA helicase, XPB2, and an RNA helicase SEN1. In this chapter, we will investigate the experimental evidence on the roles of these helicases in drought stress mediation. Post identification of the helicases in the activation tagged lines, their differential expression patterns were studied under abiotic and biotic stress conditions. Based on the results, we further investigated the effects of these two genes in response to various stress situations, like phytohormone, dehydration, and drought stress treatments in *indica* rice. The performance of the mutant lines was studied with respect to the changes in their physiology and agricultural productivity, apart from WUE. Our findings imply that in addition to their fundamental cellular housekeeping functions, i.e., nucleic acid unwinding, the helicases encoded by these genes play a significant role in stress responses, possibly by protecting the genomic integrity of the plant when environmental stressors arise.

4.2.Identification of tagged genes

Two mutants, XM3 (DEB. 36) and SM4 (En. 124), were chosen for detailed analysis out of the selected activation tagged lines showing enhanced WUE through high photosynthetic performance and low carbon discrimination values. The function of these two genes has been well studied for nuclear activities, but their involvement in stress responses and WUE has not been elucidated till now.

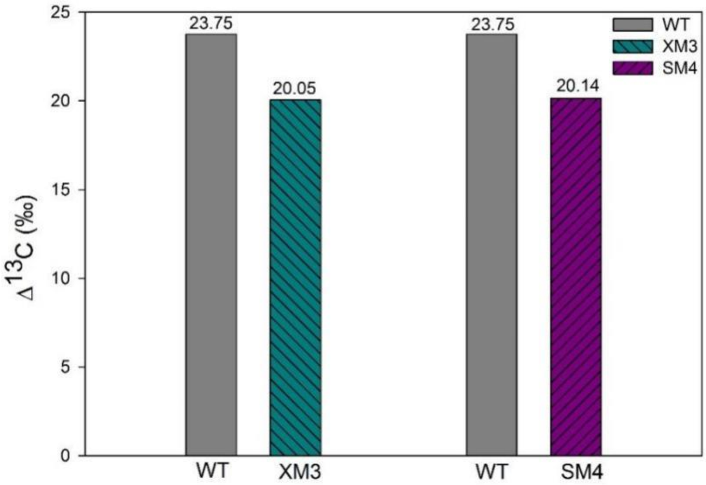


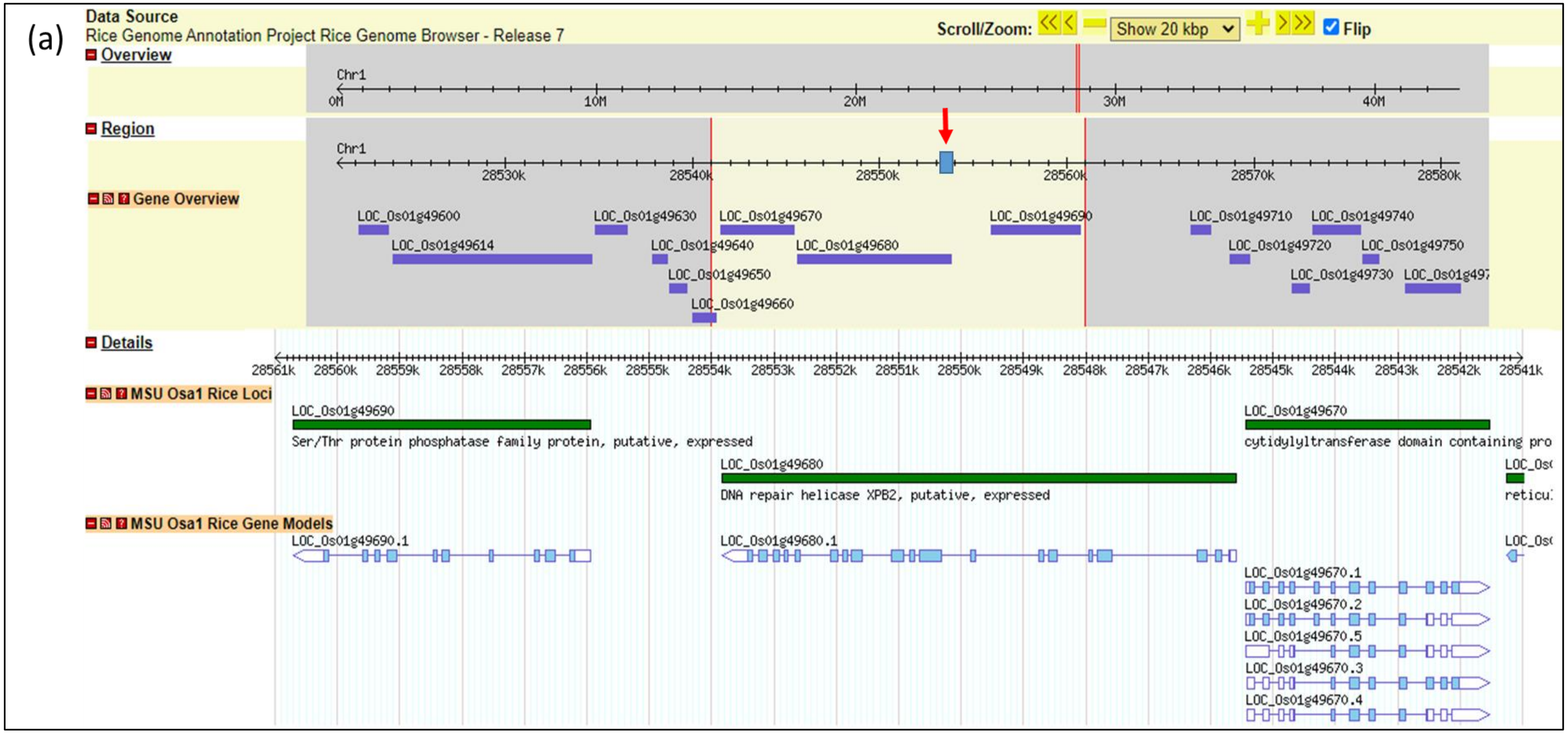
Fig. 4.1: Carbon isotope discrimination ($\Delta^{13}C$‰) as measured by Isotope Ratio Mass Spectrometer

Carbon isotope discrimination is an indirect method of identifying plants with higher WUE under water stress conditions. A high $\Delta^{13}C$ value indicates a lower WUE. Here the WT lines were observed to have 23.75‰ of $\Delta^{13}C$ under limited water conditions, which was higher than that of XM3 (20.05‰) and SM4 (20.14‰) lines.

Under limited water conditions, the WT lines were observed to have higher $\Delta^{13}C$ (23.75‰) values as compared to the selected mutant lines, XM3 (20.05‰) and SM4 (20.14‰) (Fig. 4.1). Since carbon discrimination values ($\Delta^{13}C$) are inversely related to WUE in plants (a lower value indicates higher WUE), these mutants were chosen for flanking gene sequence analysis. TAIL-PCR analysis was conducted on the chosen mutants, XM3 and SM4, to establish the transgenic nature of the plants, identify the genes in the immediate proximity of the enhancer integration, and designate the site of insertion of the tetrameric enhancers in their genomes. In order to map the exact point of integration of the tag, a BLAST search was performed by subjecting the sequence obtained from TAIL-PCR analysis in the rice genome database (Fig. 4.2). Thus, we identified the flanking genes present in the 20 kb span of the enhancer integration.

In the 20 kb span of the enhancer integration site, the tagged line XM3 had LOC_Os01g49670, LOC_Os01g49680, and LOC_Os01g49690 loci. LOC_Os01g49670 and LOC_Os01g49680, encoding for cytidylyl transferase domain containing protein and DNA repair helicase XPB2, respectively, were found 8 kb and 0.1 kb upstream of enhancers, respectively. The Ser/Thr protein phosphatase gene encoding loci, LOC_Os01g49690, was found 2 kb downstream of the enhancer integration. The enhancers in the SM4 mutants were flanked by the loci LOC_Os10g02890, LOC_Os10g02900, LOC_Os10g02910, LOC_Os10g02920, and LOC_Os10g02930. The first two genes situated 4 kb, and 1 kb upstream of the enhancers encoded unnamed putative proteins, but the following three genes situated 1 kb, 4 kb, and 7 kb downstream, encoded a transposon protein, cytochrome b561, and SEN1 helicase, respectively.

A qRT-PCR analysis showed a 16-fold activation of the genes XPB2 (LOC Os01g49680) and SEN1 (LOC Os10g02930) in the lines XM3 and SM4, respectively, in comparison to WT (Fig. 4.3 a to d), whereas no significant changes in the expression level of the other genes in the 20 kb regions of the selected mutants were observed.


(a)
Data Source
Rice Genome Annotation Project Rice Genome Browser - Release 7
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Overview
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LOC_Os01g49670.3
LOC_Os01g49670.4

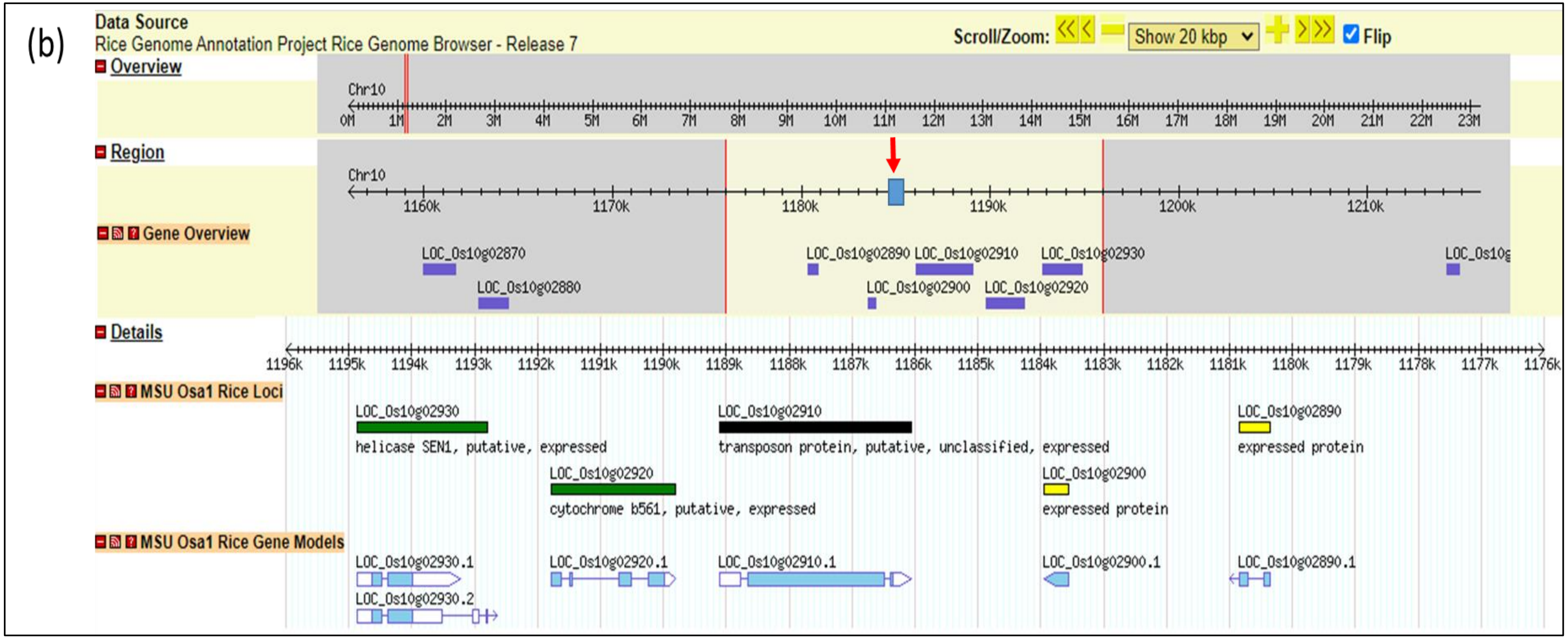


Fig. 4.2: Gene maps locating the point of integration of the 4X enhancer elements of activation-tagging vector and its 10 kb upstream and downstream genes as obtained from the rice genome database

The sequences obtained from TAIL-PCR of (a) XM3 (DEB.36) and (b) SM4 (En.124) were subjected to BLASTN analysis in the rice genome database. The blue rectangular box indicates the point of integration of the enhancer element and the selected region represents the surrounding 20 kb region of the enhancers. In the line XM3, 3 genes (LOC_Os01g49670, LOC_Os01g49680 and LOC_Os01g49690) in XM3 and 5 genes (LOC_Os10g02890, LOC_Os10g02900, LOC_Os10g02910, LOC_Os10g02920, LOC_Os10g02930) in SM4 were present in the selected span.

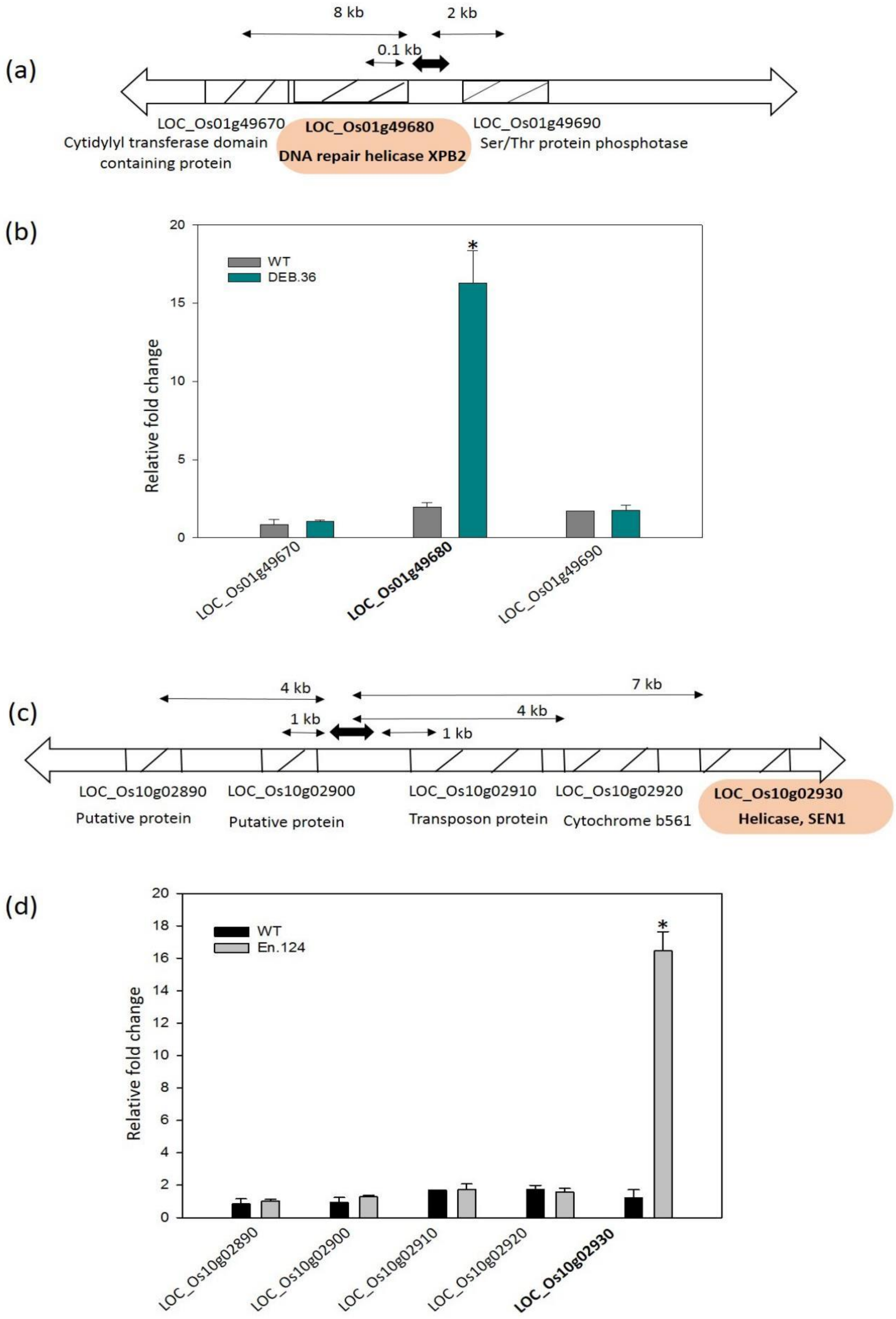


Fig. 4.3: Gene map and quantitative real-time PCR of the tagged genes

Pictorial representation of the point of integration of the tetrameric enhancer element of the activation tagging vector, the genes in the 20 kb span, and the subsequent quantitative real-time PCR analysis (a & c) The bold double-headed arrow represents the enhancer integration. In the line XM3, three genes were present (LOC_Os01g49670, LOC_Os01g49680, and LOC_Os01g49690), and in the line SM4 (En.124), five genes were present (LOC_Os10g02890, LOC_Os10g02900, LOC_Os10g02910, LOC_Os10g02920, LOC_Os10g02930) in the selected region. Quantitative real-time PCR (qRT-PCR) analyses showed up to 16 fold upregulation of two genes, i.e. (b) *XPB2* and (d) *SEN1,* compared to the WT in XM3 and SM4 lines, respectively. Other tagged genes showed an expression level similar to that of the WT. The data were normalized using rice *actin* as an internal reference gene. One way ANOVA was performed at a significance level $P < 0.05$ annotated by asterisks*.

4.3.Promoter analysis of *SEN1*

Many *cis*-acting elements were previously identified in the XPB2 promoter region, including one for early dehydration responsiveness (ABRELATERD1), a dehydration responsive element (CBFHV), and an MYBCORE element that responds to water stress (Raikwar et al., 2015). Several *cis*-acting stress-responsive elements were discovered in our *in-silico* promoter study of SEN1. Figure 4.4 and table 4.1 indicate the definite locations and the detailed list of the *cis*-regulatory elements. There were 12 MYB binding elements (CAACTG and CAACCA/CAACAG) and two ABRE motifs (CGTGG) relevant for drought responsiveness. The corresponding responsive elements in the putative promoter region can be attributed to the high transcriptional activation of these two genes in response to ABA and the dehydration stress-inducing agent, PEG.

Table 4.1: List of *cis*-acting elements and their functions found in the 1 kb upstream promoter region of SEN1 through PlantCARE database.

Element	Position	Strand	Sequence	Function
AE-Box	399	-	AGAAACTT	Light response module (Wei et al. 2013)
ARE	709	+	AAACCA	Element for anaerobic induction (Wei et al. 2013)
	780	-	AAACCA	
AuxRR Core	847	-	GGTCCAT	Element for auxin response (Sakai et al. 1996)
CAAT Box	176	+	CAAT	Element in promoter and enhancer region
	366	+	CAAT	
	365	+	CCAAT	
	647	-	CAAT	

	264	-	CCAAT	
	571	+	CCAAT	
	317	+	TGCCAAC	
	845	+	CAAT	
	251	+	CAAT	
	536	-	CAAT	
	306	-	CAAT	
	572	+	CAAT	
G-box	419	+	CACGAC	Light responsivenes element (Wei et al. 2013)
MBS	12	+	CAACTG	MYB binding site for drought inducibility (Ambawat et al. 2013)
	461	+	CAACTG	
	74	-	CAACTG	
	620	+	CAACTG	
MYB	4	+	CAACCA	
	491	+	CAACCA	
	239	-	CAACCA	
	860	+	CAACCA	
	87	-	CAACCA	
	558	-	CAACCA	
	101	+	CAACCA	
	874	-	CAACCA	
O2- site	575	-	GATGA(C/T)(A/G)TG(A/G)	Element involved in zein metabolism induction (Yunes et al. 1994)
TATA- box	705	-	TATA	Core promoter element
TCA-element	957	-	CCATCTTTTT	Element for salicylic acid responsiveness (Wei et al. 2013)
Unnamed_1	712	-	CGTGG	ABRE motif (Srivastav et al. 2010)
	778	+	CGTGG	
Unnamed_4	34	+	CTCC	Regulates gene expression for anther development (Zhou et al. 2017)
	525	+	CTCC	
	360	+	CTCC	
	913	-	CTCC	
	136	-	CTCC	
	760	-	CTCC	
	458	+	CTCC	

	659	+	CTCC	
	83	-	CTCC	
	867	+	CTCC	
	140	-	CTCC	
Box S	565	+	AGCCACC	Responsive to wounding and pathogen elicitation (Yin et al. 2017); Stress responsiveness (Ding et al. 2019)
Circadian	428	-	CAAAGATATC	Element for circadian control (Fu et al. 2014)
re2f-1	207	+	GCGGGAAA	E2F binding site (Chabouté et al. 2002); Responsible for cell cycle (Çakir et al. 2013)

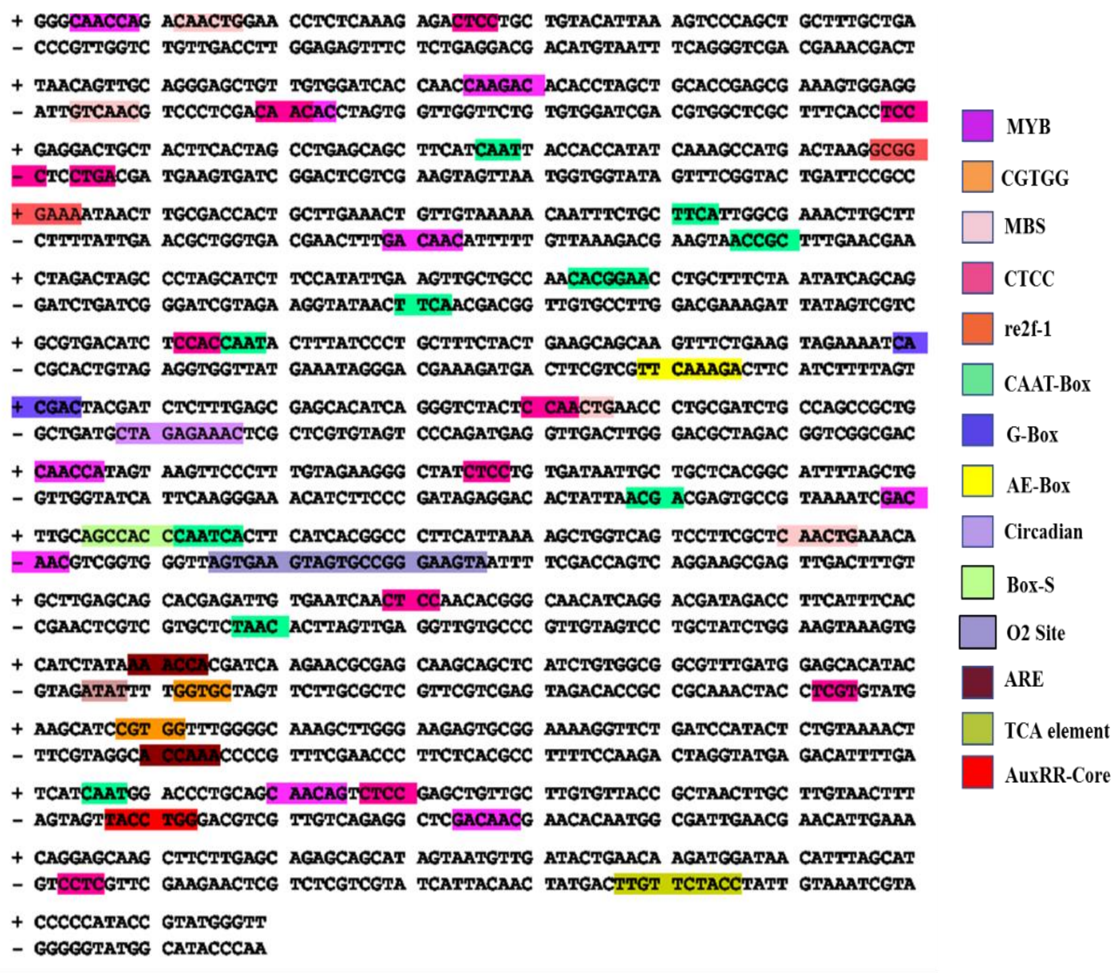


Fig. 4.4: ***In-silico* analysis of upstream promoter region of *SEN1*.**

The putative promoter sequence (1 kb) was retrieved from the rice genome database and subjected to PlantCARE online search tool to locate the *cis*-elements. Each element has been colour coded, and the index is provided along with the figure.

4.4.Transcript analysis of *XPB2* and *SEN1* under biotic and abiotic stresses

We investigated the responsiveness of the *SEN1* and *XPB2* genes to additional stressors after they were found to be associated with the enhancement of WUE in rice under low water conditions. Their transcript patterns were investigated in response to several phytohormones and abiotic stress-inducing stimuli. Both genes demonstrated significant overexpression, particularly in root tissues as compared to shoots. We classified the two gene expression patterns as early (expressing within 3 to 12 h of treatment) or late (expressing after 12 h of treatment).

As an early stress response, SA, NaCl, ABA, PEG, and heat stress (42°C) elevated the level of *XPB2* transcripts in the shoot by over threefold (Fig 4.5 a), except for PEG, which increased its expression up to six fold after 48 h. Within 3 to 12 h, the transcript level in roots was elevated by over five fold in all the six treatments illustrating the early responsiveness of the gene. High transcript levels were identified after 48 h in response to NaCl (11 fold), ABA (48 fold), and heat stress (14 fold) and the same in reaction to PEG (10 fold) were sustained after 12 h till the end of the treatment (Fig. 4.5 b).

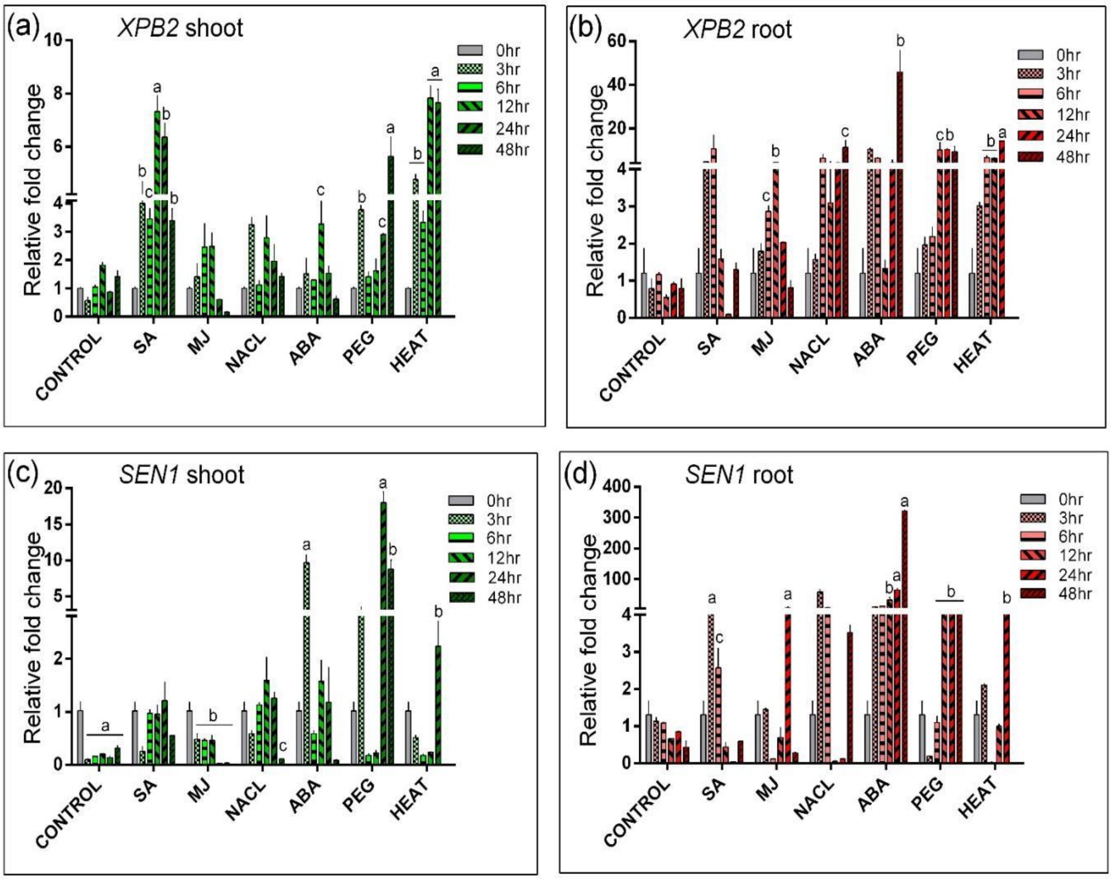


Fig. 4.5: Transcript analysis of *XPB2* and *SEN1* in response to phytohormones and stress inducing chemical agents

Quantitative real-time PCR (qRT-PCR) analyses of (a), (b) *XPB2* shoot and root (c), (d) *SEN1* shoot and root, respectively, in response to phytohormone and chemical treatments. Ten days old rice seedlings were subjected to SA, MJ, NaCl, ABA, PEG and heat treatments and root and shoot tissues were collected at various time points. Rice actin, *act1* was used as the internal reference gene. The fold change was calculated using the $\Delta\Delta C_T$ method. The mean and the standard error are plotted in vertical bar graphs. One way ANOVA was performed at a significance level $P < 0.001$ marked as a, $P < 0.025$ marked as b and $P < 0.05$ marked as c.

SEN1 transcript levels in shoots were induced immediately within 3 h of ABA (9 fold) and PEG (3 fold) treatments. Their levels in response to ABA dropped after the initial surge but that under PEG peaked by 18 fold after 48 h (Fig. 4.5 c). In roots, SA, NaCl, ABA and PEG exhibited an early response while the gene was induced late upon the application of MJ and heat stress treatments. From 12 to 48 h, the transcript level of SEN1 was maintained in response to PEG (5 fold), whereas ABA continued to enhance its upregulation, reaching 320 fold at the end of 48 h (Fig. 4.5 d).

The pathogens *Xoo* and *R. solani*, on the other hand, were unable to induce the expression of *SEN1* and *XPB2*. Both *SEN1* and *XPB2* were downregulated by 0.4 and 0.3 fold, respectively, following infection with *Xoo*. The transcript level of *SEN1* was similar to that of untreated samples in response to *R. solani*, but *XPB2* showed a 2.4-fold increase (Fig. 4.6).

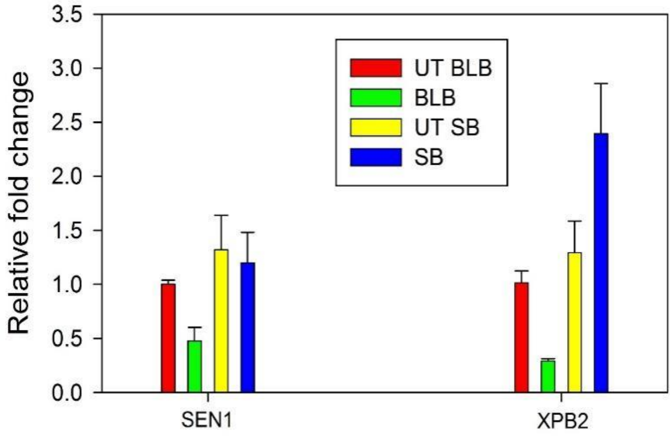
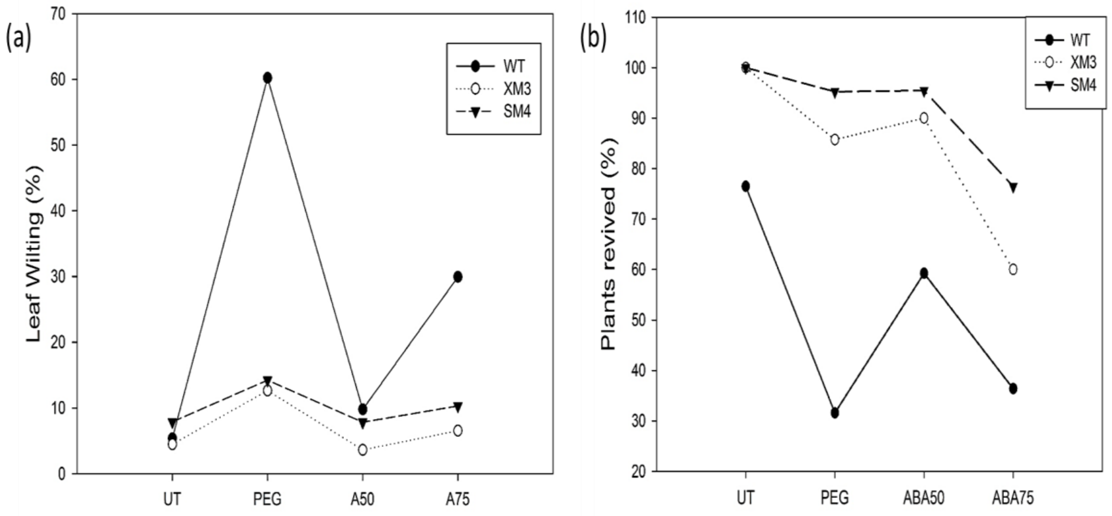


Fig 4.6: Transcript analysis of SEN1 and XPB2 in response to biotic stress

Quantitative real-time PCR (qRT-PCR) analyses of *SEN1* and *XPB2* genes in response to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Rhizoctonia solani*, which cause Bacterial Leaf Blight (BLB) and Sheath Blight (SB), respectively. The data were normalized using untreated (UT) plant samples grown under similar conditions. No significant upregulation was observed under biotic stress conditions. The fold change was calculated using the $\Delta\Delta C_T$ method.

4.5.Phenotypic and physiological analyses of the tagged mutants under PEG and ABA

Improved tolerance of the tagged mutants was observed in response to dehydration stress (10% PEG) and phytohormone (50 µM and 75 µM ABA) treatments 20 DAS. Under all three circumstances, the cumulative wilting of WT varied from 9% (50 µM ABA) to 60% (PEG) 20 DAS, with 30 % (PEG) to 60% (50 µM ABA) 20 DAR (Fig. 4.7 a to d). On the other hand, the XM3 and SM4 lines showed maximum wilting of 12 and 14 % under PEG and maximum recovery of 85 to 95% 20 DAR from 50 µM ABA and PEG, showing that the increased expression of both helicases enabled the plants more tolerant to water stress than the WT.



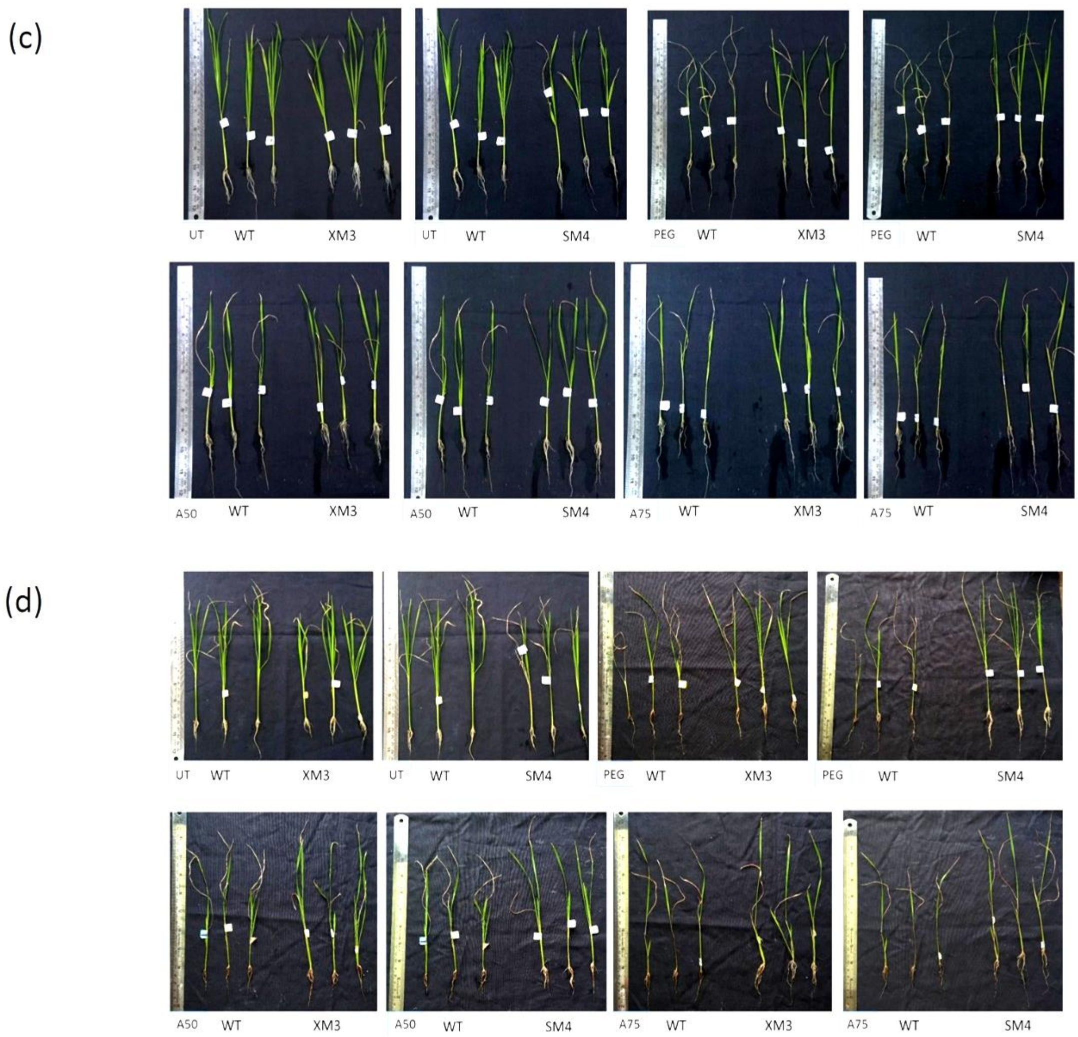
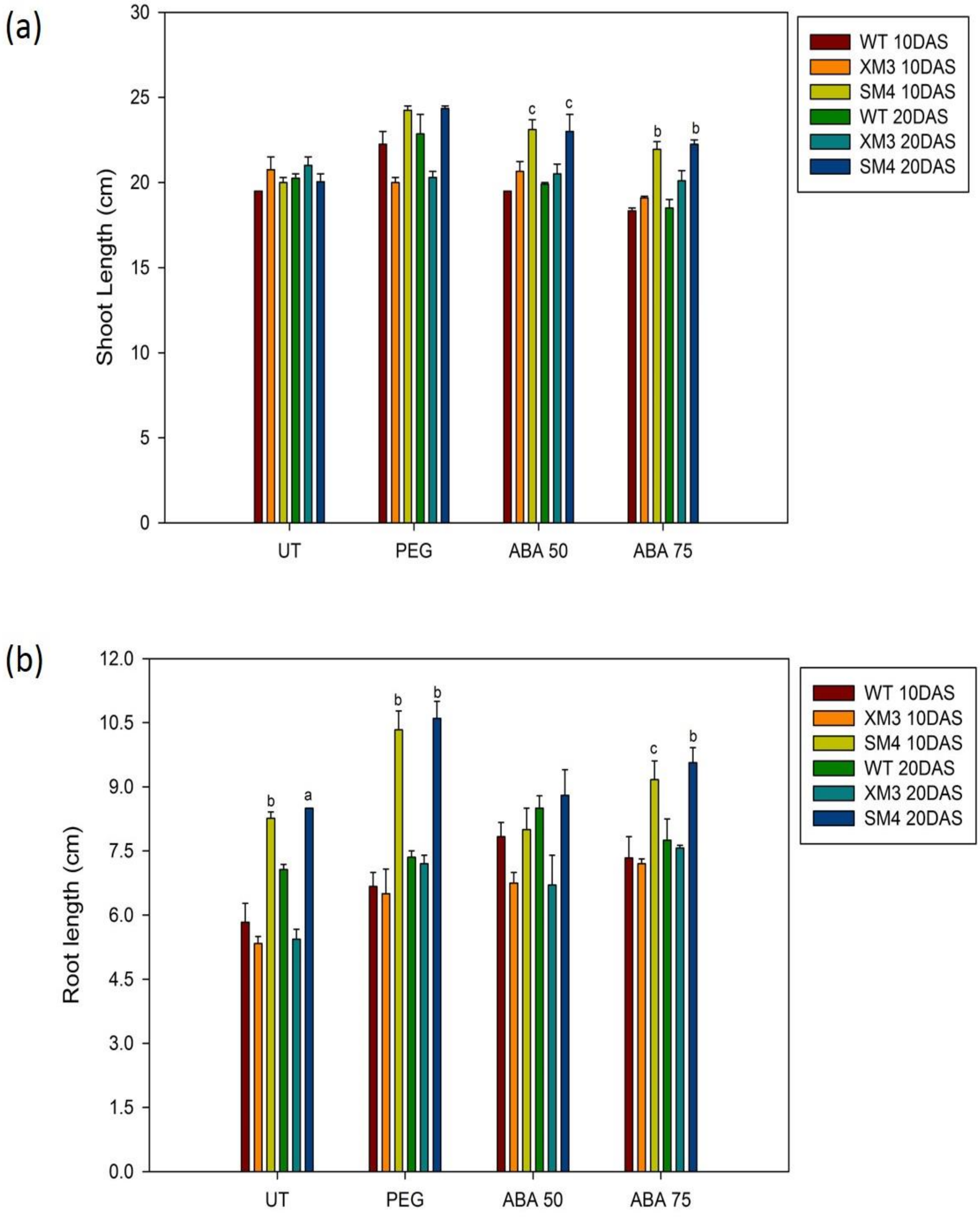


Fig. 4.7: Phenotypic comparisons of the mutants with the WT plants 20 DAS and 20 DAR

Figure depicting the phenotypes, wilting percentage and recovery of the plants WT, XM3 and SM4 lies 20DAS and 20 DAR. (a) shows the leaf wilting percentage 20 DAS and (b) depicting the percentage of the revival of plant 20 DAR; (c) and (d) showing the phenotypic differences of XM3 and SM4 20 DAS and 20 DAR, respectively in comparison to the wild type. The wild-type plants experienced a very high rate of wilting (60%) and low revival rate (30%) under PEG treatment compared to the tagged lines. XM3 and SM4 showed 12%-14% wilting under PEG, and more than 85% revival was observed under both PEG and 50 µM ABA. The stress conditions include 10% PEG, 50 µM ABA and 75 µM ABA.

4.5.1. Seedling shoot and root parameters

The fresh weight, shoot, and root lengths (Fig. 4.8 a to c) of the tagged genotypes were measured at 10 and 20 DAS. Because of increased branching, the XM3 line had a higher fresh weight 20 DAS (0.16 to 0.21 g) but identical shoot and root lengths to the WT line (0.11 to 0.17 g). SM4 line had a longer shoot and root lengths than the WT, and the fresh weight was found to be 0.21 to 0.25 g. The mean of these recordings was depicted as a bar graph.



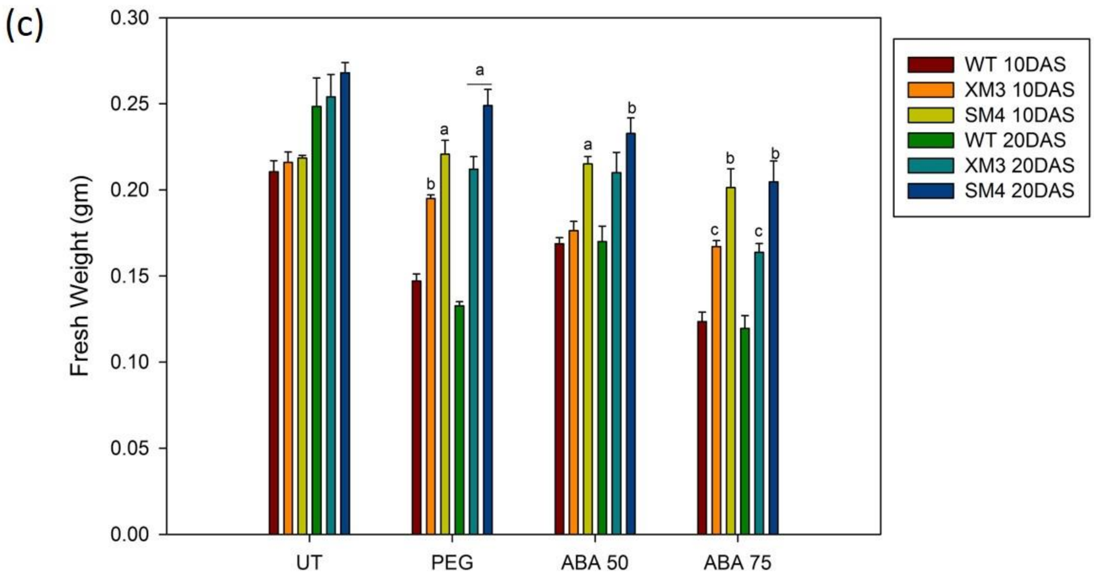


Fig. 4.8: Variation in growth parameters of the mutants and the wild type lines

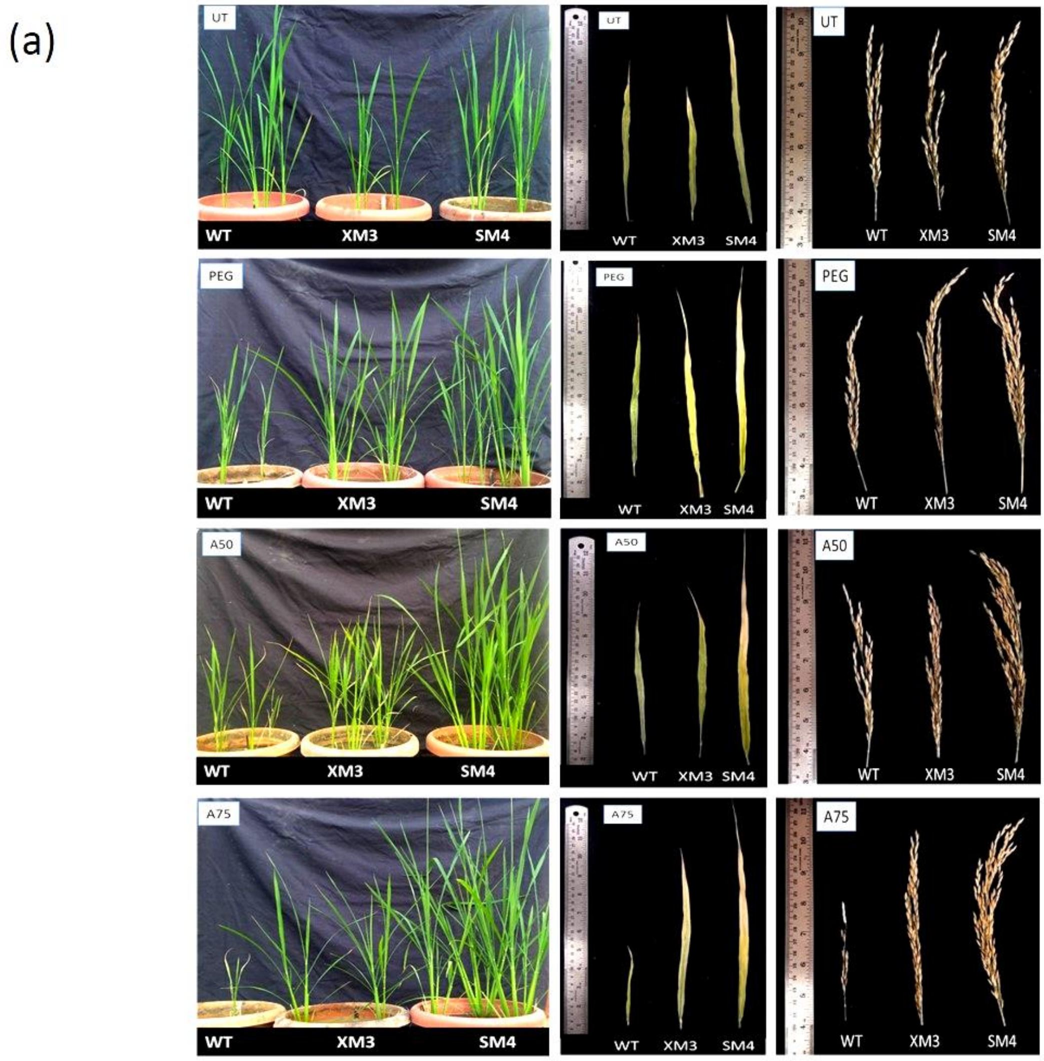
The mean readings of the growth parameters of the three genotypes were taken 10 and 20 DAS and were plotted in a bar chart. (a) Shows the variation in the fresh weight; (b) and (c) shows the variation in the shoot and root length, respectively. XM3 and SM4 maintained a higher fresh weight under stress conditions in contrary to the WT plants. The shoot length did not differ much between the tagged lines and the WT plants, but the root lengths of SM4 lines were significantly high under stress compared to the WT plants. The mean and the standard error is plotted in a vertical bar graph. One way ANOVA was performed at a significance level $P < 0.001$ marked as a, $P < 0.025$ marked as b and $P < 0.05$ marked as c.

4.5.2. Yield-related traits

Following greenhouse acclimation, the XM3 and SM4 lines showed enhanced phenotypic characteristics, including an increased number of tillers and panicles per plant, increased plant height, boot leaf, panicle length, number of seeds per plant, seed weight, and photosynthetic efficiency (Fig. 4.9 a &b). The parameters in the form of a histogram are provided in figure 4.10 and table 4.2 depicts their mean values with standard errors.

Following recovery from all three stress treatments, the WT lines displayed two to three tillers per plant with one to two bearing panicles, shorter plant height, boot leaf and decreased panicle length. The mutants, XM3 and SM4, respectively produced three to seven tillers and three to nine tillers per plant, and all were productive. These were larger than the WT, with longer boot leaves and panicles. The lengths of the boot leaf and the panicle were shown to be correlated.

A single WT plant produced only 11 seeds in response to 75 µM ABA. XM3 (~461 seeds) and SM4 (~557 seeds) produced 97 and 98% more seeds than the corresponding WT, respectively. Under 10% PEG, a variation in seed production of around 75 to 80% (XM3 and SM4, respectively) was also observed. Under untreated circumstances, the weight of 100 seeds in the WT and tagged lines were nearly identical. However, under PEG and 50 µM ABA stress conditions, a substantial difference was seen. These physiological characteristics suggested that SEN1 and XPB2 were less sensitive to high ABA concentrations.



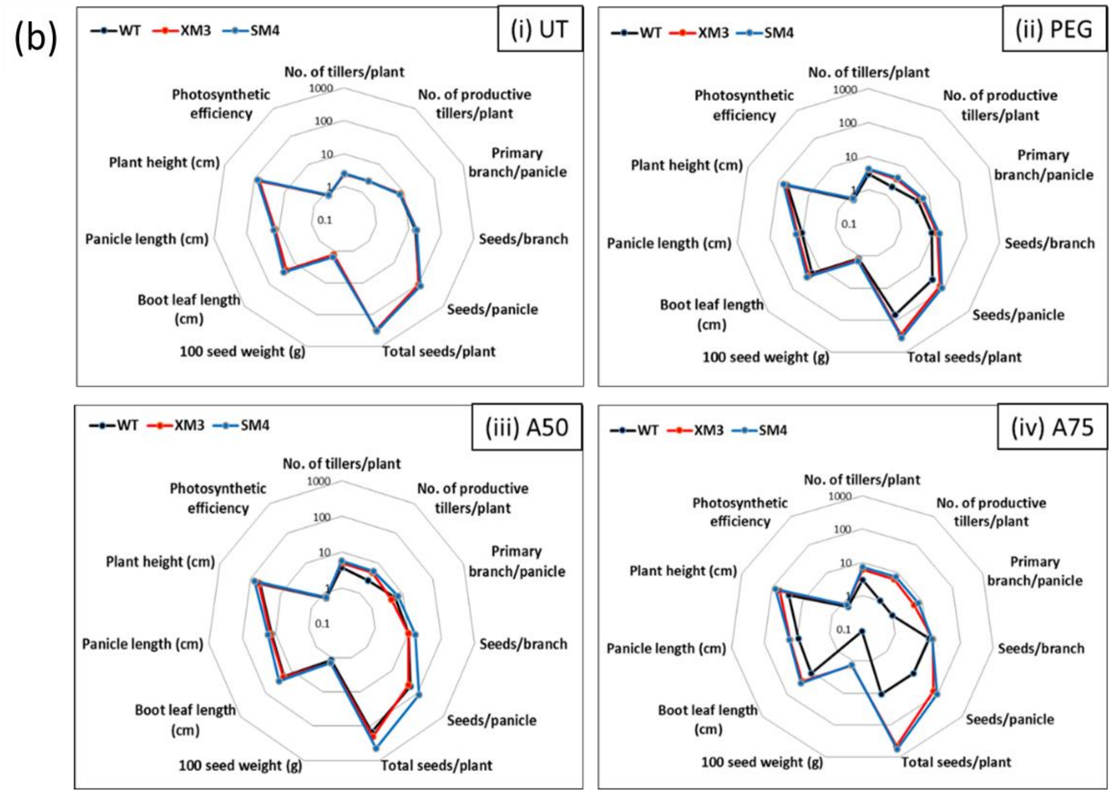


Fig. 4.9: Phenotypic and physiological analysis of tagged lines

Phenotypic and physiological observations were performed post acclimatization of the tagged lines (XM3 and SM4) compared to the WT plants. These included the differences in plant height, boot leaf, and panicle length (a) as observed phenotypically. (b) Represents the observed phenotypic features plotted as a radar graph. The parameters plotted were plant height (cm), number of tillers/plant, number of productive tillers/plant, primary branch/panicle, seeds/branch, seeds/panicle, total seeds/plant, 100 seed weight (g), boot leaf length (cm) and the panicle length (cm) and photosynthetic efficiency of the WT lines compared to XM3 and SM4 lines. (i), (ii), (iii) and (iv) represent different conditions such as untreated, 10% PEG, 50 µM and 75 µM ABA, respectively. The mean values have been plotted on a logarithmic ($\log_{10}$) scale. The tagged lines were observed to perform better under simulated stress conditions than the WT lines. The decrease in size of the black undecagon (WT) represents the same.

4.5.3. Photosynthetic efficiency

The quantum yield in untreated WT was 0.74, whereas it was 0.77 in both untreated mutants. When exposed to 10% PEG and 75 µM ABA, the efficiency of WT declined to 0.63 and 0.67, respectively. On the other hand, the tagged lines continued to have better quantum efficiency than the untreated controls. Even after PEG and ABA treatments, the F_v/F_m ratios in the XM3 and SM4 lines were in the range of 0.72 to 0.77.

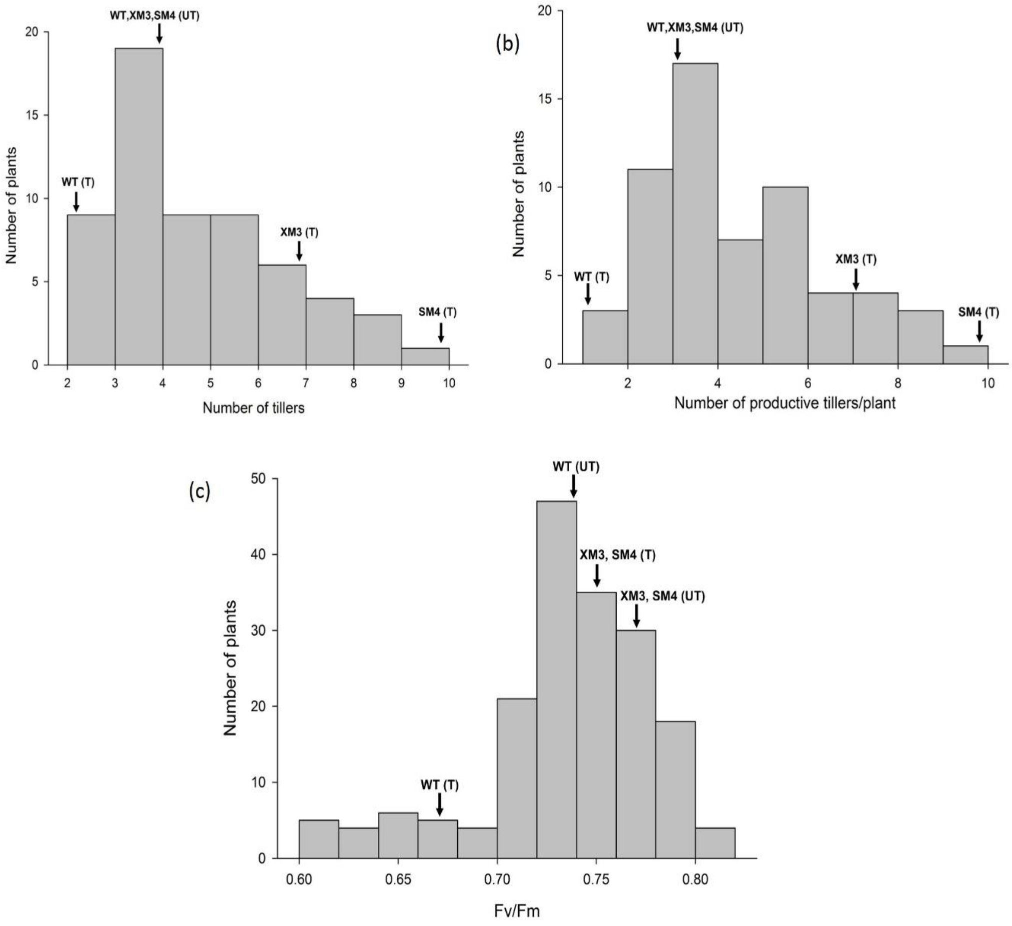


Fig. 4.10: Physiological analysis of the tagged lines

Figures (a) and (b) represent the number of tillers/plant and the number of productive tillers/plant, ranging from 3-9/plant, all of which were productive in the tagged lines. The WT had 2-3 tillers/plant, 1-2 of them was productive upon stress imposition (UT-untreated; T-treated). The readings are depicted in the form of a histogram. (c) Depicts the photosynthetic efficiency as measured by the mini PAM of the tagged lines compared to the wild-type plants. The F_v/F_m ratio was 0.72 to 0.77 in XM3 and SM4 even after the application of stress as compared to the wild type plants, whose efficiency dropped to 0.63 to 0.67. All the values have been plotted as a histogram.

Table 4.2: Phenotypic characteristics observed in the tagged lines and the WT plants post acclimatization in the greenhouse.

The observations included the number of primary branch/panicle, numbers of seeds/branch of the panicle, seeds/panicle, total seeds/plant, boot leaf length and the panicle length. The mean ± standard error is represented in the chart. One way ANOVA was performed at a significance level $P < 0.001$ marked as a, $P < 0.025$ marked as b and $P < 0.05$ marked as c.

Treatment	**Parameter**	**WT**	**XM3**	**SM4**
UT	Primary branch/panicle	7.33 ± 0.66	7.66 ± 0.33	7.33 ± 0.33
	Seeds/branch	15.55 ± 1.46	17.4667 ± 2.25	17.1538 ± 1.58
	Seeds/panicle	104.33 ± 5.2	102.66 ± 7.17	116.33 ± 9.24
	Total seeds/plant	328 ± 21.21	299.5 ± 8.5	324.5 ± 24.5
	100 seeds weight (g)	1.30 ± 0.02	1.28 ± 0.3	1.48 ± 0.01^{a}
	Boot leaf length (cm)	22.28 ± 1.04	21.62 ± 1.4	27.71 ± 1.13^{b}
	Panicle length (cm)	13.62 ± 0.66	13.42 ± 0.69	14.79 ± 0.43
	Plant height (cm)	73.25±1.06	68±1.15	78.4±0.97
PEG 10%	Primary branch/panicle	4.28±0.28	5.7±0.22^{b}	6.5±0.22^{a}
	Seeds/branch	8.62±.420	12.52±.442^{a}	14.16±.3^{a}
	Seeds/panicle	35.2±1.42	69.25±5.15^{a}	84.12±4.21^{a}
	Total seeds/plant	68.5 ± 0.5	277 ± 30^{b}	349.5 ± 31.5^{b}
	100 seeds weight (g)	1.24 ± 0.02	1.36 ± 0.00^{a}	1.45 ± 0.01^{a}
	Boot leaf length (cm)	18.7 ± 0.86	23.9 ± 0.8^{b}	28.11 ± 0.65^{a}
	Panicle length (cm)	10.92 ± 0.54	14.43 ± 0.5^{a}	15.99 ± 0.35^{a}
	Plant height (cm)	49.5±1.5	57.1±1.64	64.5±1.50^{a}
ABA 50µM	Primary branch/panicle	5.6±0.4	4.3±.21^{c}	7.1±.26^{b}
	Seeds/branch	10.3±.365	10.3±.579	16.64±.494^{a}
	Seeds/panicle	50.44±.365	45±4.88	111.62±4.72^{a}
	Total seeds/plant	151.33 ± 12.7	206.5 ± 18.5	446.5 ± 7.5^{a}
	100 seeds weight (g)	1.20 ± 0.04	1.39 ± 0.016^{b}	1.42 ± 0.03^{b}
	Boot leaf length (cm)	18.83 ± 0.83	20.32 ± 0.61	30.33 ± 0.75^{a}
	Panicle length (cm)	13.08 ± 0.57	14.36 ± 0.37	17.02 ± 0.35^{a}
	Plant height (cm)	47±1	52±1.15	72.25±1.18^{a}
ABA 75µM	Primary branch/panicle	1±0	5.33±.210^{b}	7.16±.477^{a}
	Seeds/branch	11±0	14.17±.565	13.83±.382
	Seeds/panicle	11±0	69±7.17^{b}	98.93±6.83^{b}
	Total seeds/plant	11±0	461 ± 16^{b}	557.5 ± 5.5^{b}
	100 seeds weight (g)	0.116 ± 0	1.35 ± 0.04^{a}	1.34 ± 0.03^{a}

	Boot leaf length (cm)	11 ± 0	25.21 ± 1.34^{b}	29.8 ± 1.25^{b}
	Panicle length (cm)	9 ± 0	16.29 ± 0.42^{a}	17.13 ± 0.38^{a}
	Plant height (cm)	28±0	56±3^{c}	78.5±0.5^{b}

4.5.4. Chlorophyll and proline estimation

In the WT and the tagged lines, the contents of chlorophyll a, b, and total chlorophyll were assessed post-stress (Fig. 4.11 a to c) and post-recovery (Fig. 4.12 a to c). Under PEG stress, the a, b, and total chlorophyll contents in XM3 were 17 µg, 10 µg, and 27 µg/50 mg fresh weight, respectively, compared to 10 µg, 8 µg, and 21 µg/50 mg fresh weight in WT. The chlorophyll content of XM3 was shown to be somewhat higher after recovery under PEG and 50 µM ABA.

The post-stress levels of a, b, and total chlorophyll in SM4 varied from 11 to 15 µg, 11 to 19 µg, and 23 to 34 µg/50 mg fresh weight, respectively, whereas those in the WT ranged from 8 to 11 µg, 5 to 8 µg, and 15 to 21 µg/50 mg fresh weight. Under all three stress situations, the chlorophyll content of SM4 was higher than that of WT following revival.

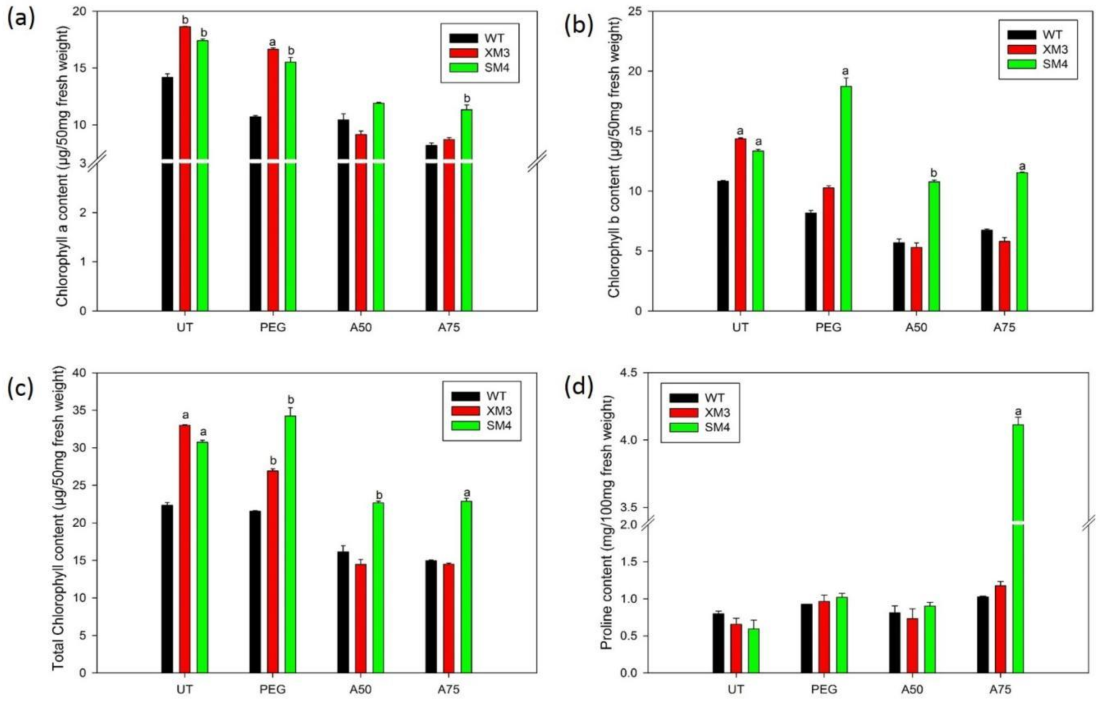


Fig. 4.11: Biochemical analysis of the tagged lines post stress

Graphical representation of the biochemical studies done on XM3 and SM4 compared to WT plants post 20 days of stress. (a), (b), (c), (d) depict chlorophyll a, chlorophyll b, total chlorophyll and proline contents post stress, respectively. One way ANOVA was performed at a significance level *P* < 0.001 marked as a, *P* < 0.025 marked as b, *P* < 0.005 marked as c.

WT, XM3, and SM4 exhibited similar proline levels under untreated circumstances, ranging from 0.6 to 0.7 mg/100 mg fresh weight. All of them had increased their proline content to 0.9 to 1.0 mg/100 mg fresh weight after being treated with 10% PEG and 50 µM ABA. In comparison to WT, SM4 acquired a remarkably higher proline level (4 mg/100 mg fresh weight) after treatment with ABA 75 µM (Fig. 4.11 d). The proline content of all of the treated lines decreased after revival, and they were nearly identical to their untreated counterparts (Fig. 4.12 d). Therefore, increased chlorophyll and proline levels appeared to be linked to better photosynthetic efficiency and stress tolerance in the tagged lines, resulting in sustainable production.

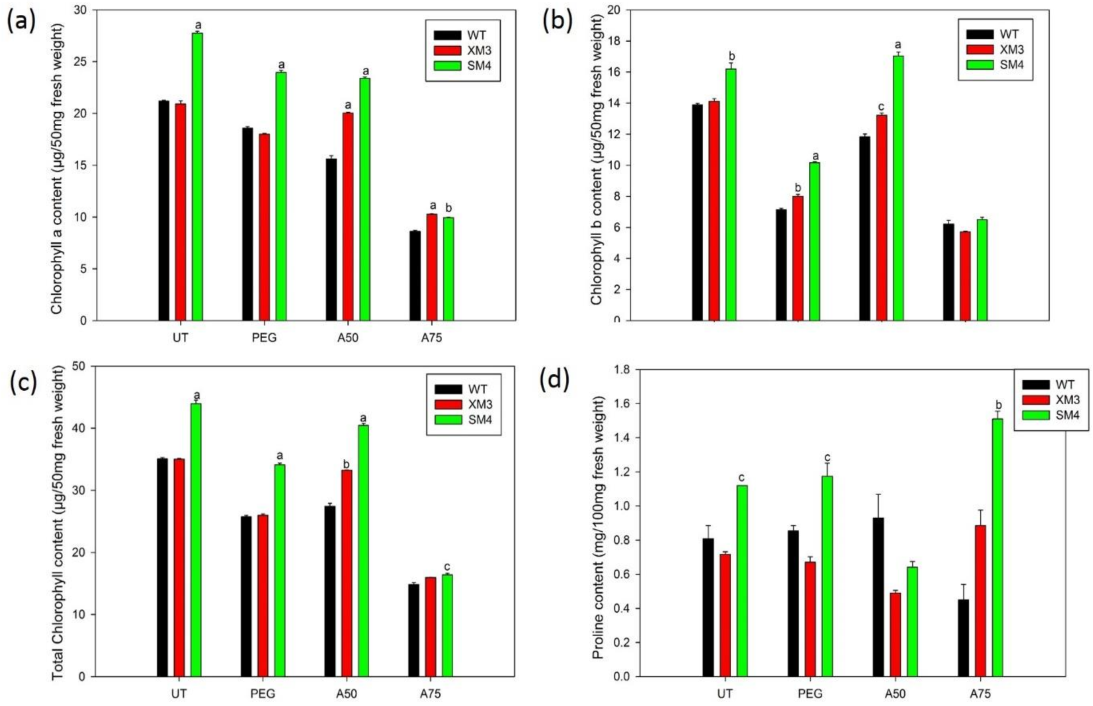


Fig. 4.12: Biochemical analysis of the tagged lines post revival

Graphical representation of the biochemical studies carried out on XM3 and SM4 in comparison to WT plants post revival. (a), (b), (c), (d) depicts chlorophyll a, chlorophyll b, total chlorophyll and proline content post revival, respectively. One way ANOVA was performed at a significance level *P* <0.001 marked as a, *P* <0.025 marked as b and *P* <0.05 marked as c.

The Principal Component Analysis of 24 morpho-physiological and biochemical characteristics revealed that the variables impacting the first two dimensions were responsible for 72.73% of the variation between genotypes (PC1 and PC2). The characters 20 DAS fresh weight, 20 DAS chlorophyll and proline contents, revival percentage, photosynthetic efficiency, plant height, tiller numbers and seven other yield-related metrics were among them (Fig. 4.13 a; Table 4.3, 4.4). The 2D plot indicated that the WT, XM3, and SM4 lines behaved the same under control conditions, but on the application of stress (PEG, ABA 50 µM and ABA 75 µM), they drifted farther away in different directions on the plot suggesting their differential behaviour (Fig. 4.13 b). SM4 and WT behaved differently in all three stress situations, whereas XM3 reacted differently to PEG and 75 µM ABA. Under 50 µM ABA, XM3 and WT had similar behavioural tendencies. These could be seen by altering the distance or proximity among the points. The overlapping region in the plot showed a similar behavioural pattern of XM3 and WT lines under PEG and ABA (50 µM) treatments, which are again depicted by parameters corresponding to higher PC. WT moved along PC1, representing increased leaf wilting, and SM4 moved towards PC2, having a higher tiller number and proline content (Fig. 4.14). XM3 remained intermediate between PC1 and PC2, correlating to intermediate changes in the parameters.

Table 4.3:Eigenvalue and the percentage of variance contributed by the parameters of each component

	Eigenvalue	**Percentage of variance**	**Cumulative percentage of variance**
Dim.1(PC1)	12.20	50.84	50.84
Dim.2(PC2)	5.25	21.89	72.73
Dim.3(PC3)	2.32	9.66	82.39
Dim.4(PC3)	1.45	6.04	88.43
Dim.5(PC5)	0.87	3.63	92.05
Dim.6(PC6)	0.72	2.99	95.05
Dim.7(PC7)	0.54	2.25	97.29
Dim.8(PC8)	0.34	1.40	98.69
Dim.9(PC9)	0.17	0.71	99.40
Dim.10(PC10)	0.09	0.36	99.77
Dim.11(PC11)	0.06	0.23	100.00

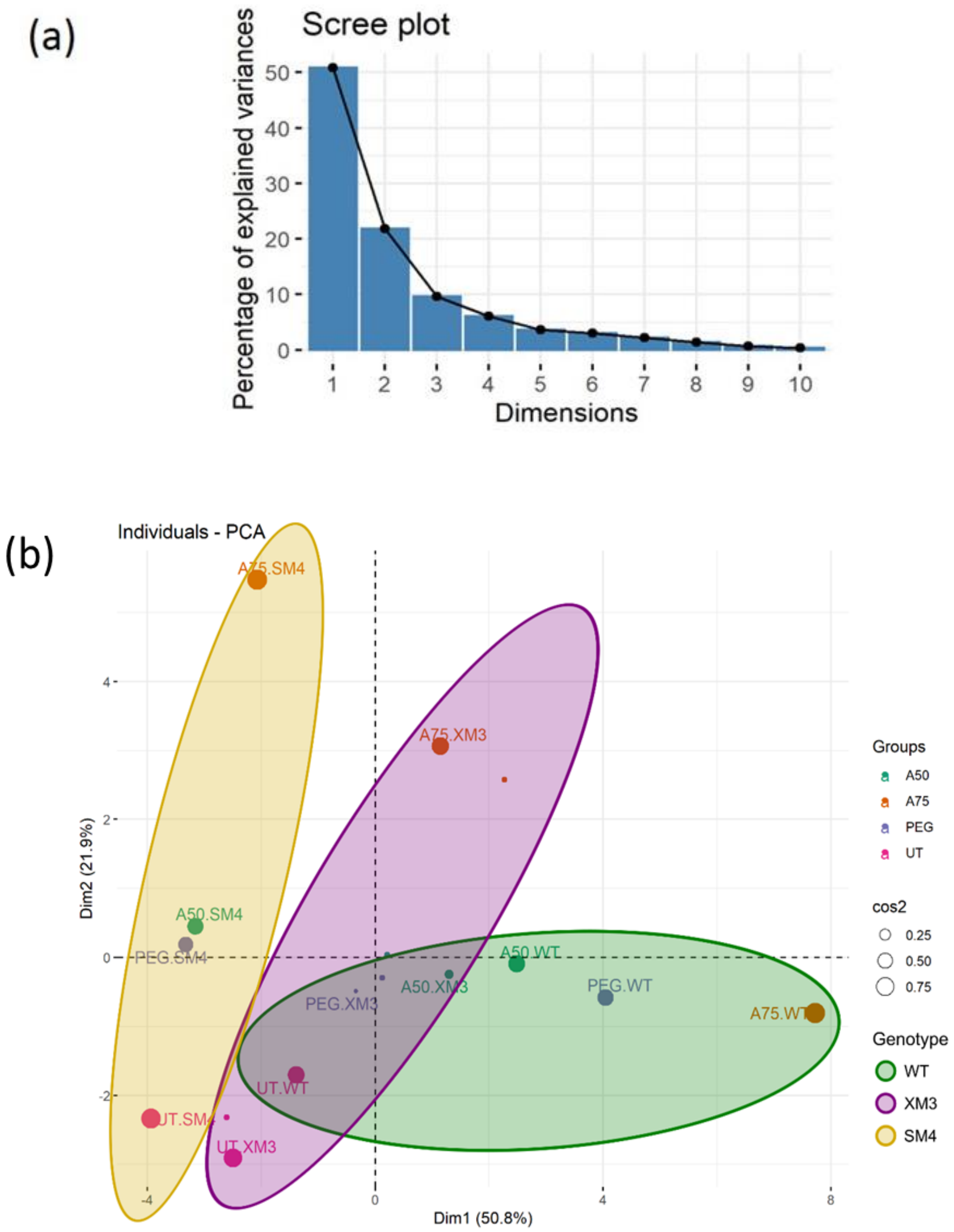


Fig. 4.13: PCA for plant morpho-physiological and biochemical parameters

Principal Component Analysis of the 24 observed phenotypic, physiological and biochemical parameters of the three genotypes (WT, XM3 and SM4) under simulated stress conditions (UT, PEG, ABA 50 and 75 µM). (a) Scree Plot for the observed variance under each dimension (or principal component). (b) Plot for PC1 and PC2, with the genotypes across simulated conditions plotted. Each oval encompasses the observed pattern of variance of each genotype across the simulated conditions under the first two principal components. The green, purple and yellow oval show the genotypes, i.e. WT, XM3 and SM4, respectively.

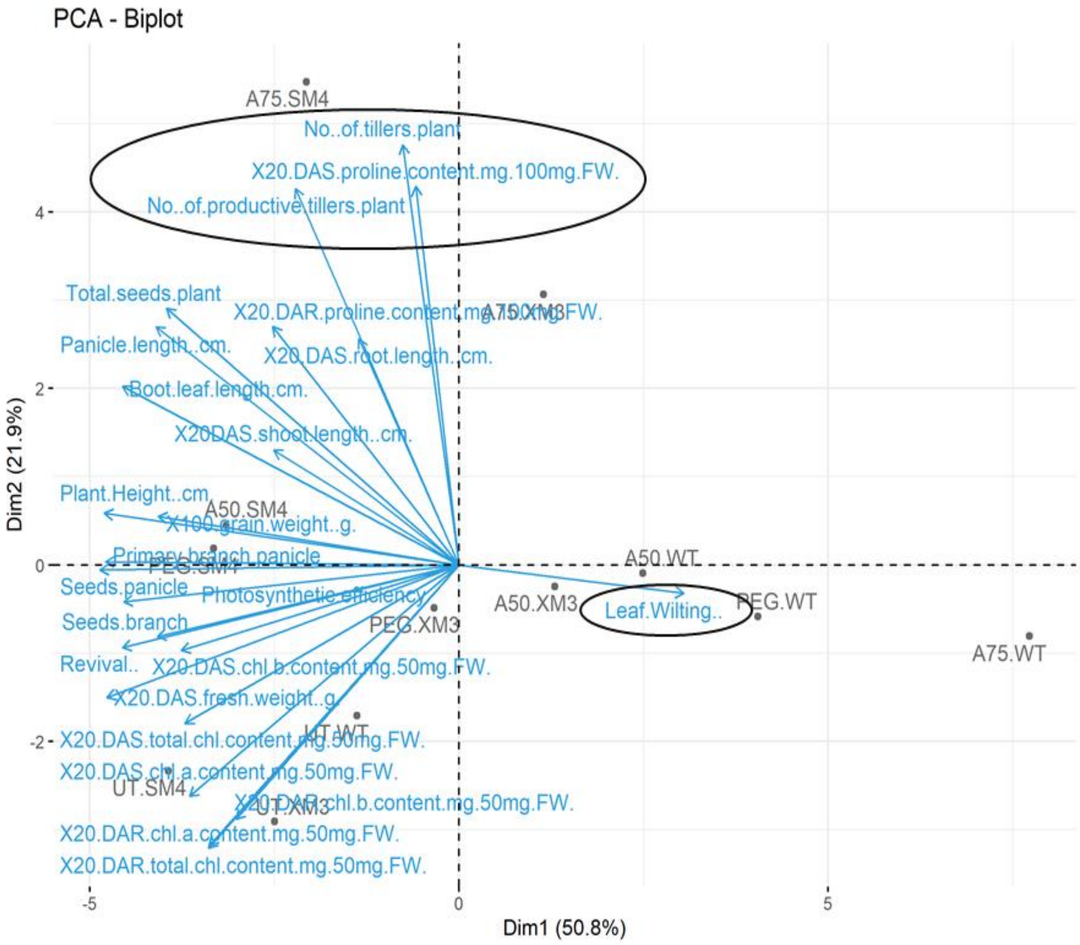


Fig. 4.14: BiPlot for morpho-physiological characters of the three genotypes

BiPlot showing the variance of 24 morpho-physiological and biochemical parameters of three genotypes under UT and three simulated stress conditions represented under Principal Component 1 and 2 (PC1 and PC2). The black ovals indicate the variance of the parameters along PC1 and PC2 based on which the WT and SM4 move under stress.

Table 4.4: cos2 values of each parameters in a principal component. The ones highlighted in green represent the parameters varying under PC1 and PC2

	Dim.1(PC1)	**Dim.2(PC2)**	**Dim.3(PC3)**	**Dim.4(PC3)**	**Dim.5(PC5)**	**Dim.6(PC6)**	**Dim.7(PC7)**	**Dim.8(PC8)**	**Dim.9(PC9)**	**Dim.10(PC10)**	**Dim.11(PC11)**	**Dim.12(PC12)**
Leaf.Wilting..	3.5E-01	3.9E-03	4.4E-01	4.7E-02	3.9E-02	4.1E-02	4.4E-02	1.4E-03	3.4E-02	1.0E-04	3.5E-04	9.9E-34
X20.DAS.fresh.weight.g.	8.6E-01	8.5E-02	1.0E-02	4.3E-03	3.9E-03	4.8E-03	4.3E-03	1.3E-02	1.4E-04	1.2E-02	4.2E-03	1.2E-33
X20DAS.shoot.length.cm.	2.4E-01	6.4E-02	3.3E-01	2.2E-01	6.9E-03	1.1E-02	8.8E-02	6.9E-03	3.2E-02	2.8E-04	1.2E-04	1.9E-32
X20.DAS.root.length..cm.	6.9E-02	2.5E-01	2.3E-01	5.5E-02	2.8E-01	2.1E-02	6.9E-02	1.6E-02	1.5E-03	2.7E-03	5.1E-03	1.3E-33
X20.DAS.chl.a.content.mg.50mg.FW.	5.0E-01	2.6E-01	5.6E-02	1.1E-01	3.9E-02	9.2E-03	2.7E-03	3.1E-05	2.2E-03	2.0E-02	1.4E-03	7.5E-32
X20.DAS.chl.b.content.mg.50mg.FW.	5.3E-01	3.5E-02	3.0E-01	3.9E-02	1.3E-02	5.4E-02	1.1E-02	6.0E-04	5.7E-03	6.5E-03	2.5E-05	6.6E-32
X20.DAS.total.chl.content.mg.50mg.FW.	5.2E-01	1.2E-01	2.6E-01	4.8E-02	7.9E-03	3.4E-02	3.0E-03	3.0E-04	2.3E-04	4.6E-03	3.5E-03	1.1E-32
X20.DAS.proline.content.mg.100mg.FW.	1.3E-02	6.9E-01	4.0E-02	9.2E-02	1.0E-03	1.8E-02	8.5E-05	1.3E-01	6.8E-03	2.1E-04	7.7E-04	1.5E-32
Revival..	7.8E-01	3.3E-02	6.0E-02	4.4E-06	1.4E-03	8.6E-02	2.0E-03	2.9E-02	1.4E-03	1.2E-04	3.5E-03	1.5E-31
X20.DAR.chl.a.content.mg.50mg.FW.	4.3E-01	3.8E-01	1.3E-02	1.5E-01	4.1E-04	3.4E-04	3.0E-03	3.0E-06	1.1E-02	3.6E-03	1.4E-07	2.1E-33
X20.DAR.chl.b.content.mg.50mg.FW.	3.4E-01	3.1E-01	9.8E-02	8.5E-02	8.6E-02	3.5E-02	1.1E-03	2.8E-02	6.3E-04	6.4E-03	2.5E-03	1.5E-31
X20.DAR.total.chl.content.mg.50mg.FW.	4.3E-01	3.9E-01	3.7E-03	1.4E-01	1.9E-02	8.1E-03	4.1E-04	5.1E-03	3.1E-03	1.4E-05	4.3E-04	1.3E-32
X20.DAR.proline.content.mg.100mg.FW.	2.4E-01	2.8E-01	2.4E-01	3.0E-02	2.0E-03	7.2E-02	1.2E-01	1.1E-04	3.2E-03	2.5E-03	1.7E-02	1.4E-32
Plant.Height..cm.	8.7E-01	1.3E-02	2.3E-04	6.5E-03	2.6E-04	8.6E-02	3.2E-03	4.2E-03	7.5E-03	5.9E-03	2.5E-03	2.6E-32

No..of.tillers.plant	2.2E-02	8.6E-01	5.0E-02	3.0E-02	4.1E-06	2.0E-02	8.6E-03	5.8E-03	2.2E-03	3.8E-03	1.6E-03	1.3E-33
No..of.productive.tillers.plant	1.9E-01	6.9E-01	6.8E-02	2.7E-02	7.7E-03	1.4E-02	7.6E-03	1.9E-03	6.8E-04	1.3E-04	7.5E-04	1.6E-32
Boot.leaf.length.cm.	7.8E-01	1.5E-01	1.8E-03	2.1E-02	2.2E-05	1.1E-04	1.9E-03	1.8E-02	1.7E-02	2.5E-03	4.1E-05	1.2E-32
Panicle.length..cm.	6.4E-01	2.7E-01	4.2E-02	2.4E-02	2.1E-04	1.1E-02	1.7E-04	1.2E-02	8.0E-05	4.7E-04	1.8E-05	1.6E-32
Photosynthetic.efficiency	7.8E-01	6.5E-03	1.0E-02	1.2E-02	1.1E-01	4.8E-02	2.7E-02	9.7E-04	6.7E-03	1.1E-03	2.0E-04	1.9E-31
Primary.branch.panicle	8.7E-01	4.4E-05	2.5E-04	4.5E-03	1.2E-02	7.3E-02	2.5E-04	2.3E-05	3.1E-02	1.1E-02	2.2E-03	1.0E-32
Seeds.branch	6.3E-01	2.5E-02	2.9E-02	1.3E-01	6.0E-02	3.1E-03	7.9E-02	3.6E-02	2.9E-04	5.6E-04	6.9E-03	1.7E-33
Seeds.panicle	8.9E-01	1.1E-04	5.2E-03	2.3E-02	9.0E-03	5.1E-02	1.3E-02	3.1E-03	4.3E-04	6.5E-04	1.9E-03	8.5E-33
Total.seeds.plant	5.9E-01	3.2E-01	3.2E-02	1.7E-02	2.0E-04	1.5E-03	1.9E-02	1.5E-02	4.5E-05	2.2E-03	7.3E-04	3.8E-33
X100.grain.weight..g.	6.3E-01	1.1E-02	4.2E-03	1.3E-01	1.7E-01	1.7E-02	3.1E-02	5.0E-03	2.7E-03	1.5E-04	1.9E-04	2.1E-33

4.6.Seed quality and yield-related observations on the tagged lines under pot-level drought conditions

The three genotypes under study (WT, XM3 and SM4) were subjected to periodic drought stresses for consecutive three (60% field capacity) and seven (40% field capacity) days. After seven days, higher leaf rolling and pale green phenotypes were observed in the case of WT lines compared to the other two mutants (Fig. 4.15), although no significant difference was observed in the phenotype after three days of drought stress. Seven days of continuous drought induction lead to a significant difference in yield among the three lines. WT lines yielded ~200 seeds per plant, where the mutants produced ~400 seeds per plant (Fig. 4.16). The grain quality was investigated by calculating 100 seed weight, their length: breadth ratio and amylose: amylopectin ratio (Table 4.5). Amylose: amylopectin ratio was observed to be 0.08 under both untreated and stress conditions in all three genotypes (WT, XM3 and SM4). Similarly, no changes in seed quality in terms of seed weight (1.3 to 1.4 g) and length: breadth ratio (2.7 to 2.9) were noticed. The mean and the standard errors of the data were provided in Table 4.5.

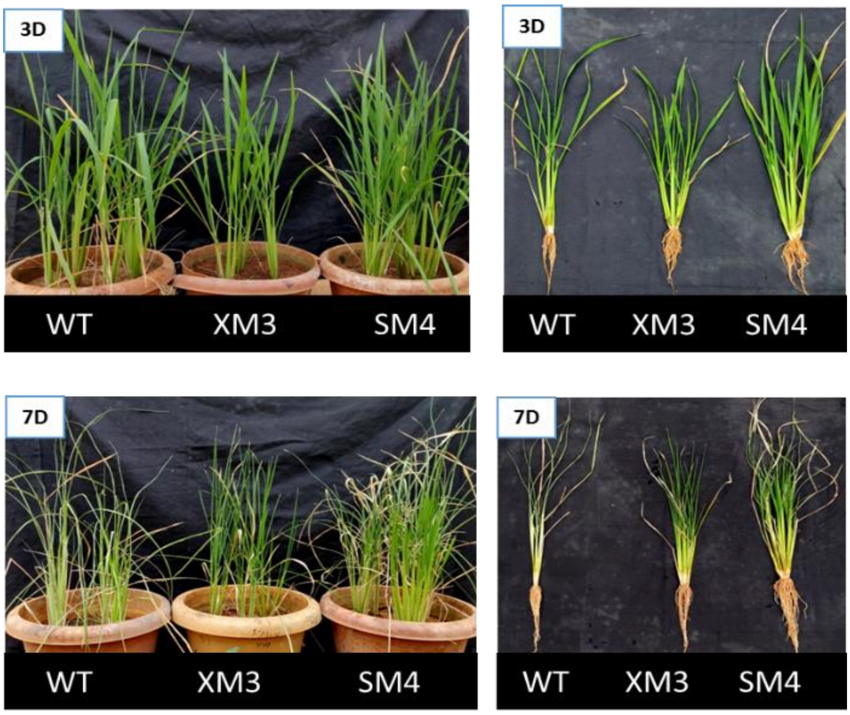


Fig. 4.15: Phenotypic observation of the WT, XM3 and SM4 plants after a periodic drought of 3 and 7 days

The WT lines showed less green phenotype with more leaf curling and wilting and poor root development after completion of stress treatment.

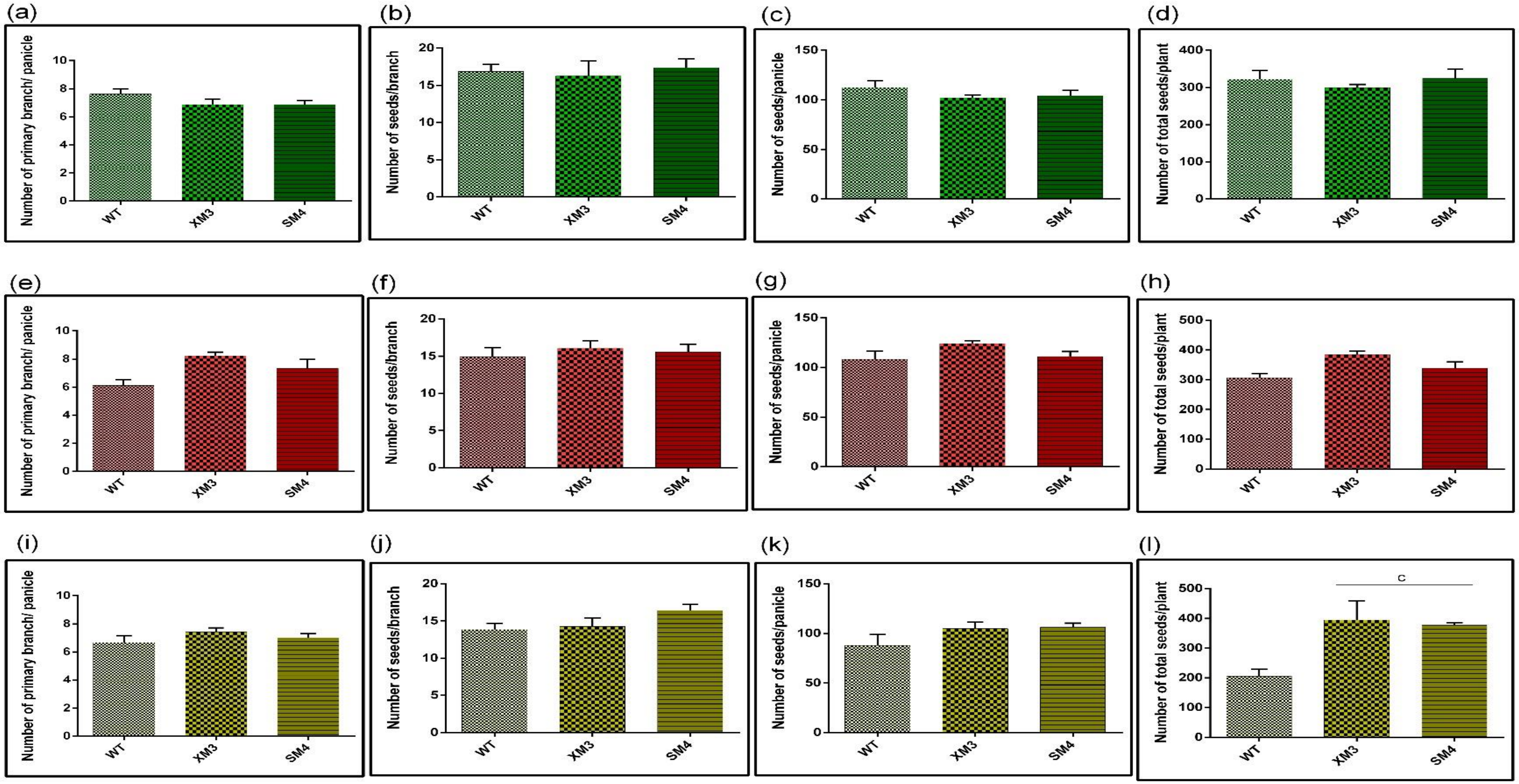


Fig. 4.16: Comparative analysis of yield related traits at pot-level drought conditions

Graphical representations of yield related traits were observed in the three genotypes (WT, XM3 and SM4) after imposing drought conditions. Periodic removal of water was done consecutively for three and seven days, followed by re-application of water until seed setting. For control setup all three genotypes were continuously watered normally throughout the experiment. (a,b,c,d) represents untreated condition and (e,f,g,h) and (i,j,k,l) represent consecutive three and seven day drought, respectively. A significant difference in the total seeds per plant was observed after seven days of drought. One-way ANOVA with a Bonferroni correction was performed at the significance level *P* < 0.05, marked as c in bar diagrams.

Table 4.5: Chart showing phenotypic characteristics observed in the tagged lines and the WT plants post 3 and 7 days of drought and revived till seed setting.

The observations included the weight of 100 seeds, seed length: breadth ratio (mm) and Amylose: Amylopectin ratio. The mean ± standard error is represented in the chart. One way ANOVA was performed at a significance level *P* <0.005 marked as c.

Treatment	**Parameter**	**WT**	**XM3**	**SM4**
UT	Primary branch/panicle	7.62 ± 0.37	6.88 ± 0.38	6.87 ± 0.29
	Seeds/branch	16.86 ± 0.99	16.29 ± 2.03	17.35 ± 1.23
	Seeds/panicle	112.4 ± 7.08	102 ± 3.08	104 ± 5.72
	Total seeds/plant	322 ± 23	299.5 ± 8.5	324.5 ± 24.5
	Weight of 100 seeds (g)	1.39 ± 0.01	1.41 ± 8.37E-03	1.43 ± 0.03
	Seed length: breadth ratio (mm)	2.9	2.9	3.0
	Amylose: Amylopectin ratio	.08	.08	.08
3D drought	Primary branch/panicle	6.11 ± 0.42	8.22 ± 0.27	7.33 ± 0.64
	Seeds/branch	14.92 ± 1.23	16.08 ± 0.96	15.55 ± 1.02
	Seeds/panicle	108.16 ± 8.29	124.14 ± 2.98	111 ± 5.19
	Total seeds/plant	306.66 ± 14.83	384.5 ± 11.5	338.5 ± 22.5
	Weight of 100 seeds (g)	1.29 ± 8.11E-03	1.45 ± 0.02	1.37 ± 0.01
	Seed length: breadth ratio (mm)	2.9	2.9	2.9
	Amylose: Amylopectin ratio	.08	.08	.08
7D drought	Primary branch/panicle	6.66 ± 0.49	7.42 ± 0.29	7 ± 0.32
	Seeds/branch	13.83 ± 0.85	14.27 ± 1.16	16.41 ± 0.84
	Seeds/panicle	87.83 ± 11.30	104.75 ± 6.73	106.14 ± 4.17

	Total seeds/plant	205 ± 22.94	394 ± 64.46^{c}	377.33 ± 8.45^{c}
	Weight of 100 seeds (g)	1.28 ± 0.01	1.34 ± 0.02	1.37 ± 4.91E-03
	Seed length: breadth ratio (mm)	2.7	2.9	2.9
	Amylose: Amylopectin ratio	.08	.08	.08

4.7.Seedling germination assay in ABA treatment

Retardation in seed germination was observed under 50 and 75 µM ABA containing medium after five days compared to the control set (Fig. 4.17). On ABA containing medium, seeds of the mutant lines XM3 and SM4 continued to germinate and sustain their growth. These seedlings had longer roots and showed signs of shoot emergence even at high ABA concentrations. On the contrary, the WT seeds exhibited mild germination under 50 µM ABA and completely failed to germinate at 75 µM. Hence, it can be concluded that higher *XPB2* and *SEN1* expression in the mutant lines rendered the plants less sensitive to ABA.

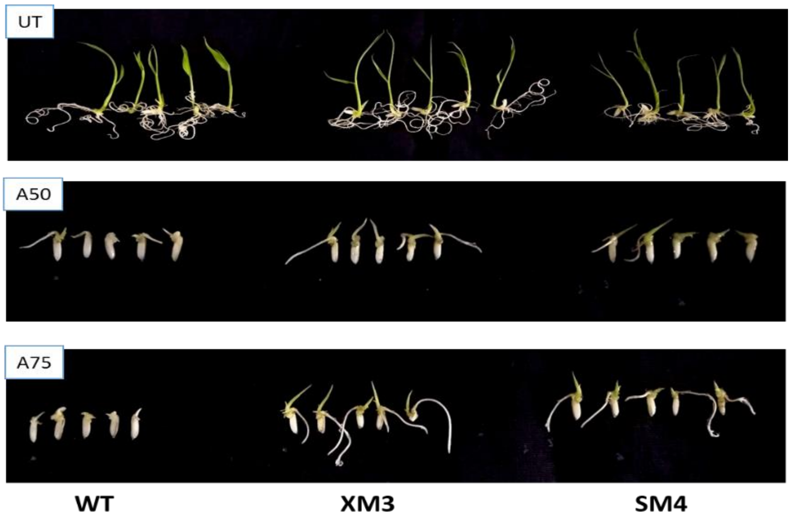


Fig. 4.17: Seedling germination assay on ABA containing medium

Figure depicting the seedling germination after 5 d. Growth retardation in the WT seedlings was observed under stress conditions (ABA 50 µM and ABA 75 µM), but the activation tagged lines continued to grow under high ABA concentration

4.8.Transcriptional analysis of stress-responsive genes in tagged lines

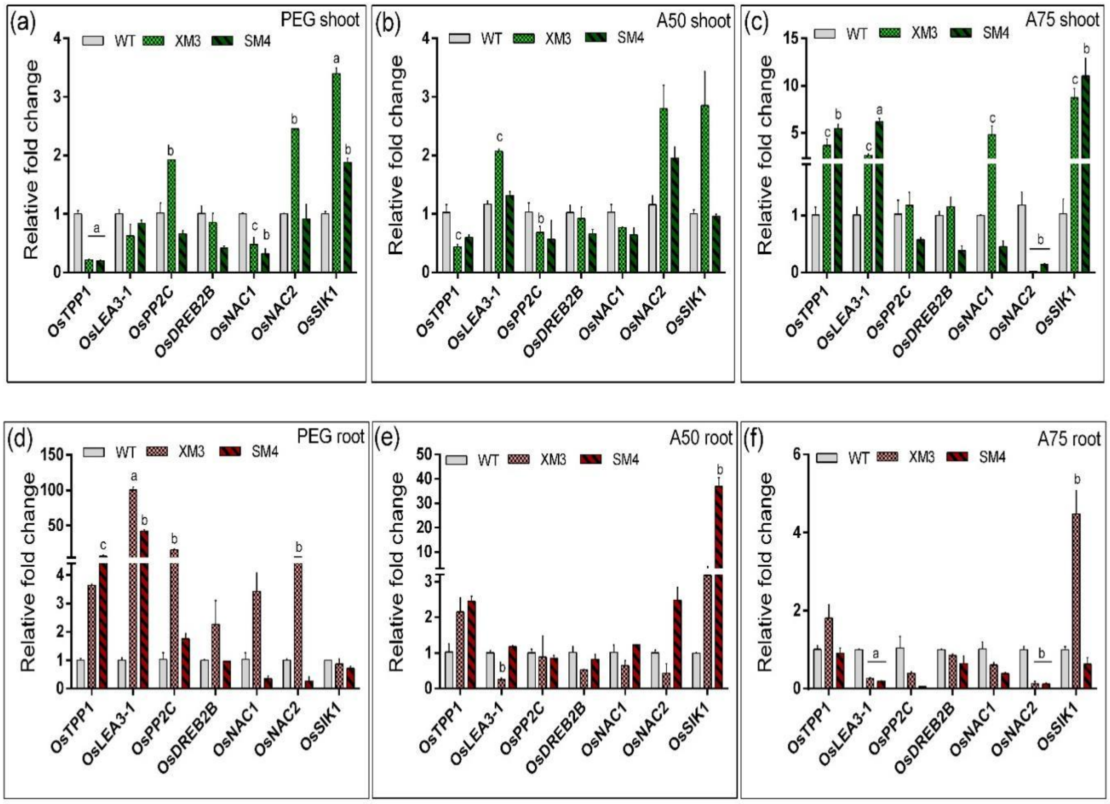
The expression levels of seven stress-regulated genes (*OsTPP1*, *OsLEA3-1*, *OsPP2C*, *OsDREB2B*, *OsNAC1*, *OsNAC2* and *OsSIK1)* were studied in the shoot (Fig. 4.18 a to c) and root tissues (Fig. 4.18 d to f) of the tagged and wild type lines under simulated stress conditions (10% PEG, 50 and 75 µM ABA). The majority of the genes were expressed in shoots under ABA treatment, while many were expressed in roots under PEG. Therefore, the transcript analysis indicated that these two helicases play an essential role in stress mediation by regulating the expression of various stress-responsive genes. In both tagged mutant lines, ABA-dependent gene *OsPP2C* and ABA independent gene *OsDREB2B* were either downregulated or exhibited an equal expression level as the WT under ABA. The expression was identical in both shoot and root tissues. Such expression pattern indicated that both XPB2 and SEN1 helicases are probably involved in ABA-dependent and ABA-independent pathways of stress tolerance.

In shoot tissues of XM3 lines, 50 µM ABA induced the expression of *OsNAC1*, and *OsTPP1* and *OsNAC1* were upregulated by 3 to 8 fold, respectively, under 75 µM ABA. *OsLEA3-1* and *OsSIK1* were induced (2 to 9 fold) under both concentrations of ABA. Under PEG treatment, *OsSIK1*, *OsNAC2* and *OsPP2C* showed 2-3 fold upregulation in shoots, whereas *OsTPP1*, *OsLEA3-1*, *OSPP2C*, *OsDREB2B*, *OsNAC1* and *OsNAC2* were upregulated moderately in roots. *OsLEA3-1* and *OsPP2C* exhibited the highest transcript levels up to 100 fold and 15 fold, respectively. In response to both 50 and 75 µM ABA, *OsTPP1* and *OsSIK1* showed 2-4 fold upregulation in XM3 lines.

SM4 shoots showed five to six fold upregulation of *OsTPP1*, *OsLEA3-1* under 75 µM ABA and a two fold upregulation of *OsNAC*2 under 50 µM ABA. PEG and ABA 75 µM treatments led to the induction of *OsSIK1* in shoots by eight fold and eleven fold, respectively. In root tissues, PEG upregulated the expression of *OsTPP1*, *OsLEA3-1* and *OSPP2C*, with *OsLEA3*-1 exhibiting the highest transcript level of forty-two fold. *OsTPP1* and *OsNAC2* were expressed by more than two fold and *OsSIK1* by thirty seven fold under 50 µM ABA treatment. Both 50 and 75 µM ABA induced the expression of *OsTPP1* and *OsSIK1*.

We have performed a similar comparative analysis on the tagged mutants by subjecting them to pot-level drought stress consecutively for three and seven days (Fig. 4.18 g). It was observed that the activation of these stress-responsive genes was more pronounced in root tissues than in shoots.

OsNAC2 and *OsSIK1* exhibited a moderate upregulation of two to three fold in shoot tissues after three days of drought induction. In roots, *OsNAC1* and *OsNAC2* were expressed by seven and two fold, respectively in the XM3 mutant. SM4 root demonstrated more than three fold upregulation in *OsSIK1*, whereas two to seven fold upregulation was noticed in *OsNAC1* and *OsNAC2* expression in root tissues. Therefore, three out of seven genes got expressed post three days of drought initiation. Prolonged drought for seven consecutive days led to the expression of six out of seven genes. *OsTPP1* exhibited a two fold upregulation in XM3 shoots, and XM3 roots detected a four fold upregulation in the *OsPP2C* gene. Likewise, a two fold upregulation of *OsTPP1* and *OsNAC2* was noticed in SM4 shoots, and about three fold upregulation was observed in the expression level of and *OsDREB2B, OsNAC1* and *OsSIK1* in root tissues.



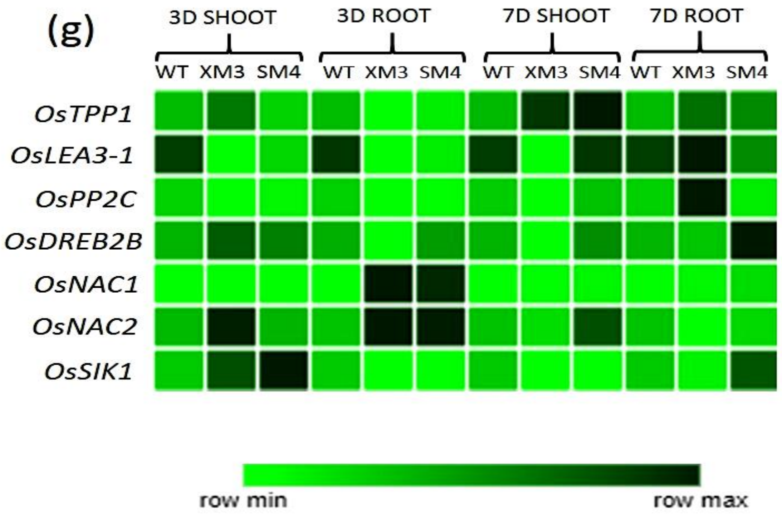


Fig. 4.18: Expression analysis of stress-responsive genes under simulated and pot-level drought conditions

Graph representing the transcript level of seven drought specific genes under imposed stress cues (10% PEG, 50 and 75 µM ABA) in shoots (a to c) and roots (d to f) tissues of the tagged lines with respect to the WT plants. Rice *actin* was used as the internal reference gene. The individual WT sample for each treatment was used to normalize the data. The fold change was calculated using the $\Delta\Delta C_T$ method. The mean and the standard error is plotted in a vertical bar graph. One-way ANOVA was performed at a significance level $P < 0.001$ marked as 'a', $P < 0.025$ marked as 'b' and $P < 0.05$ marked as 'c'. (g) Transcript analysis of seven drought specific genes in root and shoot tissues post 3days and 7 days drought. The data were normalized using rice *actin* as the internal reference gene. The corresponding WT samples for each drought treatment was used to normalize the data, and the fold change was calculated using the $\Delta\Delta C_T$ method. The results were depicted as heat maps generated by the MORPHEUS program.

4.9.Conclusion

Activation tagging is an effective approach to study the functions of redundant and lethal genes in plants (Weigel et al., 2000). With the help of pSQ5 activation tagging vector (Qu et al., 2008) our group generated gain-of-function mutant population in *indica* rice showing high WUE and photosynthetic efficiency (Moin et al., 2016). This study led to the identification of two important helicases, XPB2 and SEN1 having roles in stress tolerance in addition to their usual housekeeping functions.

- The identified helicases were induced significantly under ABA and PEG treatments rather than biotic stress, particularly in root tissues. Such preference indicated their probable role in drought stress tolerance. This was further corroborated by the presence of many stress-associated *cis*-acting elements in their promoter (Raikwar et al., 2015).

- An extensive analysis of morpho-physiological and biochemical data based on 24 different metrics under simulated stress conditions indicated the role of XPB2 and SEN1 in enhanced agricultural productivity under limited water conditions. The greenhouse based drought experiments further supported our simulated studies.

- Although ABA induced the expression of the helicases, the mutants were insensitive towards high ABA concentration, indicating the possibility of a negative feedback mechanism or crosstalk between ABA-dependent and ABA-independent machinery.

- The induction of various stress-responsive genes in the mutants suggests that the drought tolerance in the tagged lines is perhaps due to the coordinated expression of these helicases and stress-regulatory genes.

CHAPTER 5

Genome-Wide Identification of GRAS Transcription Factors in Rice

GENOME-WIDE IDENTIFICATION OF GRAS TRANSCRIPTION FACTORS IN RICE

5.1.Retrieval and nomenclature of *GRAS* sequences

Using *Ac/Ds* based activation tagging vector pSQ5, our lab has previously generated a pool of gain-of-function mutant lines and screened them for high WUE, low ΔC^{13} values and high photosynthetic activity. The integration of the tetrameric 35S enhancers in the genomic regions of some mutant lines led to the identification of important candidate genes, indicating their potential role in orchestrating stress tolerance in rice. Among them, we have identified one GRAS transcription factor gene (*ψOsGRAS4*) as a potential player for improved water use efficiency (Moin et al., 2016). In another report, Xu et al. (2015) suggested the role of *OsGRAS23* in improving drought stress tolerance in rice. Based on these cues, we performed a literature search and identified 57 *GRAS* genes already reported in rice (Tian et al., 2004). All 57 genes were subjected to a BLASTN search in the rice genome database (RGAP-DB, Orygenes DB), followed by the retrieval of the loci of 47 genes. We also performed a keyword search of GRAS, DELLA, Scarecrow, Monoculm, Chitin-inducible gibberellin-responsive protein, Gibberellin response modulator protein, Nodulation signalling pathway and Short Root in the database and matched the output results with those of the identified loci from the literature search. A total of 60 genes were identified and matched with the list of Liu & Widmer (2014) and followed their nomenclature pattern. We also performed a protein database search of GRAS domains in NCBI, SMART, Prosite and Pfam databases for further clarification. For our study, we shortlisted forty genes, one representing each paralogous group of the *GRAS* genes in rice.

5.2.Genomic distribution of *GRAS* genes

The identified coordinates of all 60 genes acquired from RGAP-DB were entered into NCBI Genome Decoration Page. The outputs were pooled, and the genes were marked for understanding the chromosomal location of the rice *GRAS* genes.

5.3.Phylogenetic relationship of rice *GRAS* genes

To comprehend the evolutionary relationships between the selected *GRAS* genes, we performed a multiple sequence alignment of the amino acid sequences in the MEGA7 software, then constructed an unrooted phylogenetic tree. The tree was constructed with a bootstrap value of 1000 using the Neighbour Joining method.

5.4.Motif arrangements and organization of *GRAS* genes

The amino acid sequences of the GRAS domains among the shortlisted genes were analyzed in the MEME suite to identify conserved motif residues. The scan was performed by setting the number of motifs to 10 and keeping the remaining parameters as default. Based on an earlier study by Pysh et al. (1999), the 10 MEME motifs were further identified as 5 GRAS motifs. Further, the structural organization of the genes was studied by submitting the genomic and coding sequences to the Gene Structure Display Server (GSDSv2). The number of exons, introns and untranslated regions (UTRs) were recorded.

5.5.*In-silico* analysis of the putative promoter region

The expression of a gene was modulated by the presence of different *cis*-acting elements in its promoter region, and the interaction of the different transcription factors and activators binding to these elements controls its expression at different levels. Therefore, an investigation of such regulatory elements helps associate the expression pattern of the genes with the genetic components. To identify important elements responsible for abiotic and biotic stress responses, we retrieved ≤1 kb upstream sequences of the selected *GRAS* genes and submitted them to PlantCARE (Cis-Acting Regulatory Elements) database. Later, we mapped them manually on the chromosomes.

5.6.Biochemical properties of GRAS proteins

With the help of ExPASyProtParam, the amino acid length, molecular weight and isoelectric point (*p*I) of the selected GRAS proteins were ascertained. 3DLigandSite software (Wass et al., 2010) was used to understand their three-dimensional structures and interacting ligands. In order to analyze the secondary structures of the protein, the generated three-dimensional structures were subjected to Phyre2 (Protein Homology/Analogy Recognition Engine v2; Kelley et al., 2015). Phyre2 online tool helps identify the percentage of α-helix, β-sheets and disordered regions present in a protein. The low complexity regions (LCRs) and the protein domains were analyzed using SMART (Simple Modular Architecture Research Tool). We also studied the hydropathicity of the proteins by investigating the GRAVY (Grand Average of Hydropathicity) indices obtained through ExpasyProtParam software. Further, the protein localization and existence of probable transmembrane helices were predicted using TargetP-2.0 and TMHMM software, respectively.

5.7.Preparation of Plant material for studying the gene expression under native and stress conditions

For simulated abiotic stress experiments, BPT-5204 (Samba Mahsuri) seeds were surface sterilized using 70% ethanol for 1 min followed by 4% aqueous sodium hypochlorite solution for 15 min and five washes with sterile double distilled water, each of two minutes duration. The sterile seeds were grown on Murashige and Skoog medium for 7 d under a 28 ± 2°C for 16 h/ 8 h photoperiodic cycle (Saha et al., 2017). The 7 d old seedlings were subjected to NaCl (250 µM), and ABA (100 µM) stress conditions for 60 h. Shoot, and root samples were collected periodically at 0 h, 15 min, 3 h, 12 h, 24 h and 60 h after the onset of stress. The untreated samples were taken as controls for normalization of gene expression.

We analyzed the native expression pattern of GRAS TFs in thirteen different developmental tissues (Saha et al., 2017), including embryo, endosperm from 16 h soaked seeds; plumule, radicle from 3 d old germinating seeds; shoot, root tissues from 7 d old seedlings; shoot, root, root-shoot transition region, flower, spikes and grain samples from mature 20 d old plants greenhouse transfer.

To analyze the expression pattern of *GRAS* genes in biotic stress conditions, the leaf samples of one month old rice plants infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo* that causes Bacterial Leaf Blight, BLB) and *Rhizoctonia solani* (that causes Sheath Blight, SB) were taken post 20 d and 25 d of infection, respectively. Leaf samples from plants of the same age without any pathogen infection were considered as controls. The protocol of infection has been stated previously in chapter 3.

5.8.cDNA preparation and Quantitative Real-Time PCR (qRT-PCR)

The plant material collected was used to isolate RNA using Tri-reagent following the manufacturer’s protocol (Takara Bio, UK) and cDNA was prepared using 2 µg total RNA samples (Takara Bio, UK). The cDNA samples were diluted ten times, and an aliquot of 2 µl of each sample per reaction was used for qRT-PCR. All the primers (IDT, USA) were designed using Primer3 software, and 10 µM primer concentration was used per reaction. The primer details are provided in Table 5.1. The PCR program included an initial denaturation step of 94°C for 2 min followed by 40 cycles of the second denaturation of 30 s, annealing for 25 s and extension at 72°C for 30 s. The samples for the current study were taken in biological and technical triplicates, and the fold changes were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Rice *actin* and *β-tubulin* genes were used as two housekeeping genes for internal normalization. For abiotic and biotic expression studies, housekeeping genes and individual control samples were used for double normalization. In contrast, single normalization was performed using the C_T value of housekeeping genes for native expression studies. The graphs were generated using the MORPHEUS program and GraphPad Prism software. One way ANOVA was performed using SigmaPlot software for discerning the significance of statistical differences between samples.

Table 5.1: List of primers

Name of primers	Sequence (5'-3')
OsGRAS1Fp OsGRAS1Rp	TTGCCGTCCACTTCACCA CATGAGGCTGAAGTCGATGAC
OsGRAS2 Fp OsGRAS2 Rp	GAGGGGAGTAGGTTCCTTGG CAAATCCCTCACAAGGTGGT
OsGRAS3 Fp OsGRAS3 Rp	CCCTTCTCAGAGGATTGCAG CACCTTTGCAGGCTTCAAGT
OsGRAS5 Fp OsGRAS5 Rp	CCGCCAACCAGTCGATAC GCTCTGCTTCTTTCGTGAGC
OsGRAS7 Fp OsGRAS7 Rp	CTTCCACCCCCTCATACTCC CGAGGACTCCTTCTCTCTCG
ΨOsGRAS2 Fp ΨOsGRAS2 Rp	CTGACCGTCGTGAACAACAC AGCTTCAGGGCAGACAAGAG
OsGRAS8 Fp OsGRAS8 Rp	TCACGAAGGTATCGACACGA GCGAGGTAGAGGTCATCTGG
OsGRAS10 Fp OsGRAS10 Rp	TCTGGTTTTGGCGGTATCTC CAGCTGCTTCTCCAGCTCTT
OsGRAS11 Fp OsGRAS11 Rp	AGGTAGCCAGTGGGCAACTA CATGACTAGCTGCTGGGACA
OsGRAS12 Fp OsGRAS12 Rp	GGCTCAAGGAGATGGTCTCA GTCCTGCAGCATCTGGAAC
OsSHR2 Fp OsSHR2 Rp	GAAGCTGGCCTCCTACTTCC CAGGAACGACTCGAGTATGG
ΨOsGRAS3 Fp ΨOsGRAS3 Rp	CGTGTGCCGACTCAGTCTC CTGAAGGCGTCGTTAGTGAG
ΨOsGRAS4 Fp	GCGCATTACATGAGGAAGCA

ΨOsGRAS4 Rp	GGCTCTGCTCAAACTAACACC
OsGRAS15 Fp	AAGTTGCTCAGCCAAAAGGA
OsGRAS15 Rp	AGCAATACGCCAATCTCTGG
OsSLR1 Fp	GTGCAGCAGGAGAACTTCG
OsSLR1 Rp	GGCAGGACTCGTAGAAGTGG
OsGRAS18 Fp	CAATACCCGTCTCCGTGACT
OsGRAS18 Rp	ATTTGGCAAGACGAAGCACT
OsGRAS19 Fp	CCAACCTACGGTTCGAGTTC
OsGRAS19 Rp	CCGTCGTGTTTCTCGATCA
ΨOsGRAS5 Fp	GGGTTGCTGGAGACTAGACC
ΨOsGRAS5 Rp	GGACGACGGCAATAAACATT
OsGRAS20 Fp	CCGCCTCCTACCTAAAGGAC
OsGRAS20 Rp	ATACAAGACGCGGTTGTGCT
OsGRAS22 Fp	CGCATGATGATTGCCTATTG
OsGRAS22 Rp	CCTTTCTTTCGGCCTCTCTT
OsGRAS23 Fp	CAGTTCCATCCGGTGCTATC
OsGRAS23 Rp	CATCGTATGCTGGCTGGAT
OsGRAS24 Fp	AGAGCTTCCTCAACCTGTGC
OsGRAS24 Rp	GCTCGTGGACGATGGTGA
OsGRAS25 Fp	CGCCTCGTCCACATTGTC
OsGRAS25 Rp	CGGCGTAGAACTCGAAAAAC
OsGRAS26 Fp	AGCTGGAGTTCAACGTCGTC
OsGRAS26 Rp	GGACCGTGTCGAGGAAGAG
OsGRAS28 Fp	ATCATCGCCTCCCCTCTCT
OsGRAS28 Rp	GTGGATGTGAAGGTGGTGAA
OsCIGR1 Fp	CGGGATAGATGACCCAGTGT
OsCIGR1 Rp	GGGTGTGGTGTAGCTGGAGT
OsGRAS32 Fp	CGTGCTGAGCTCCTACCTG
OsGRAS32 Rp	CCCTGCATGATGTCGAGGT
OsCIGR2 Fp	CGGTATCTGGTGAGCCACTT
OsCIGR2 Rp	ATAGCACCATTTGCCGACAT
OsSHR1 Fp	CTACTTCTTGCAGGGGCTGT
OsSHR1 Rp	CGACCTCCAGGAAGGACTC
OsGRAS35 Fp	ATCCTGTGGGTGCTCAACA
OsGRAS35 Rp	CGTGAGGTCGATGAAGCTG
OsSCR1 Fp	GGCTTGGGTGGATGGTATC
OsSCR1 Rp	GAGGGAGGAGAGCAGGATG
ΨOsGRAS8 Fp	GGCTTGGGTGGATGGTATC
ΨOsGRAS8 Rp	GAGGGAGGAGAGCAGGATG
OsGRAS39 Fp	GACACCATCCCCTCGTACAC
OsGRAS39 Rp	CATCAGTCCTCCCTGGGTTA
OsGRAS41 Fp	GAAAATGGAGCAGCCAGTTC
OsGRAS41 Rp	TCTAGGATGGCCTGATTGGT
ΨOsGRAS9 Fp	TGTTCTTTTTGCTGGGAACC
ΨOsGRAS9 Rp	TCGTCCGTCTCCTACTTTGC

OsGRAS43 Fp OsGRAS43 Rp	ACCACCAGAGCTACGTGCAT TAGGAGGAGGCACAGGAGAG
OsGRAS44 Fp OsGRAS44 Rp	GTGGATGAGCCCACGTTAAT CCCTTTCATGATCACCTCGT
OsGRAS47 Fp OsGRAS47 Rp	GCACGAGACATGCATCAAAG AAAGCGTGCTGCTTGATCTT
ΨOsGRAS10 Fp ΨOsGRAS10 Rp	GATTTCTCTGTCCCCAATGC AGGTCGTCAAGCCTCGACAT
OsGRAS53 Fp OsGRAS53 Rp	AGCTGAGGATTGCCATGAAG CGTGCTGCTTGATCTGCTTA

CHAPTER 6

GRAS Transcription Factors and their Spatio-Temporal Regulation

GRAS TRANSCRIPTION FACTORS AND THEIR SPATIO-TEMPORAL REGULATION

6.1.Introduction

Transcription factors are involved in almost all cellular activities, including growth, development, metabolism, signal transduction and resistance/ tolerance to abiotic and biotic stress factors. They control the gene expression by binding to DNA or protein sequences (Zhang et al., 2018). Therefore, the identification and analysis of TFs are critical in functional genomics research.

In our previous study (Moin et al., 2016), our group has generated a pool of gain-of-function mutants via activation tagging using tetrameric 35S enhancers and screening of some of these mutants for water use efficiency led to the identification of several genes that were associated with the target trait, the water use efficiency. These interesting gain of function mutants included RNA and DNA helicases (*SEN1* and *XPB2*) (Dutta et al., 2021a), and genes for ribosome biogenesis (*RPL6* and *RPL23A*), protein ubiquitination (*cullin4*) and transcription factors like *NF-YC13* (Manimaran et al., 2017), *WRKY 96* and *GRAS* (LOC_Os03g40080) (Moin et al., 2016). A *GRAS* gene was tagged in the mutant DEB.86 rice line, which showed a high quantum efficiency of 0.82 and a low $\Delta^{13}C$ value of 18.06‰. Since high photosynthetic efficiency and low carbon isotope ratio are proxies for high water use efficiency, DEB.86 was further analyzed for other phenotypic characters. The activation tagged line DEB.86 exhibited improved plant height with increased tillering and seed yield and had the *ΨOsGRAS4* gene tagged under the influence of the integrated 4X enhancers (Moin et al., 2016).

Several *GRAS* genes have already been identified in rice (Liu & Widmer, 2014), out of which, *OsGRAS23* has been reported to enhance tolerance to drought (Xu et al., 2015), and *ΨOsGRAS4* has been identified to be associated with enhanced photosynthetic efficiency and water use efficiency with enhanced agronomic features (Moin et al., 2016). These reports led us to the idea of studying the genome-wide expression analysis of this gene family. In this study, we have shortlisted forty genes, one gene representing each paralogous group and provided an experimental basis to identify the potential *GRAS* genes capable of imparting stress tolerance in rice. We have analyzed the genes selected in the GRAS family for their spatio-temporal and stress induced expression. The phylogenetic relationships among GRAS proteins, their genetic arrangements and structure, *in-silico* analysis of putative promoter elements and protein properties were also studied. This study helped identify important *GRAS* genes for stress tolerance, which can be further exploited for their functional characterization.

6.2.Chromosomal distribution of *GRAS* genes in the rice genome

A total of sixty *GRAS* genes were identified based on literature and keyword search in the rice genome database (RGAP-DB), which we matched with those of Liu & Widmer (2014). They were distributed on ten out of twelve chromosomes, with chromosomes 8 and 9 not bearing any *GRAS* genes. The number of genes on a single chromosome varied from a minimum of two on chromosome 10 to a maximum of 12 on chromosome 11. Among others, a total of nine genes were located on chromosome 3, while chromosomes 1, 7 and 12 carried six genes each, chromosome 2, 4 and 5 exhibited five genes each and chromosome 6 had four genes (Fig. 6.1). Out of the 60 genes located, we have shortlisted 40 genes for our study, with one representative from each paralogous group selected.

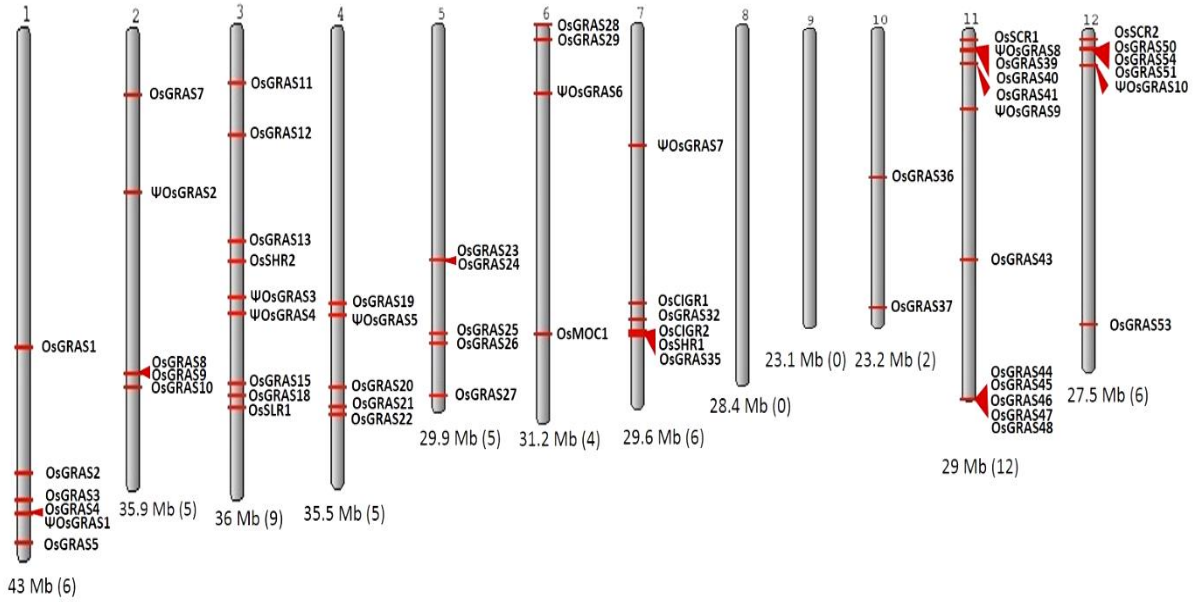


Fig. 6.1: Chromosomal distribution of *GRAS* genes in rice

Karyotypic representation of rice chromosomes was obtained from NCBI Genome Decoration Page. The Rice genome carries 60 *GRAS* genes, which are represented in the figure with red arrows indicating the position of each gene. The size of each chromosome and the number of genes present are provided below each in each bracket.

6.3. Analysis of evolutionary relationships of *OsGRAS* genes

In order to understand the evolutionary relationship among the rice GRAS family of genes, we subjected the retrieved sequences to the phylogenetic analysis (Fig. 6.2) using MEGA7 software. A total of 16 different clusters were observed. These clusters were divided into 14 subfamilies based on the previous report of Cenci & Rouard (2017). Members belonging to the same subfamily were found to cluster together except DLT and PAT subfamilies, where some genes belonging to different orthologous groups (according to Cenci & Rouard, (2017) formed separate clusters. Each cluster has been colour coded in the figure. The number of genes found in each subfamily included four in SCL3, three each in SCR, NSP2 and HAM, one in RAM, LS, SCL4/7and SCLA, two in DELLA, DLT, SHR and SCL32, six in PAT and nine in LISCL. LISCL was found to be the largest subfamily with the maximum member of genes getting clustered. *ΨOsGRAS4* and *ΨOsGRAS9* were placed close to the LISCL family since these sequences were still unclassified. The highly expressed genes under biotic and abiotic stress conditions belonged to SCL3, SHR, DELLA, HAM and PAT subfamilies.

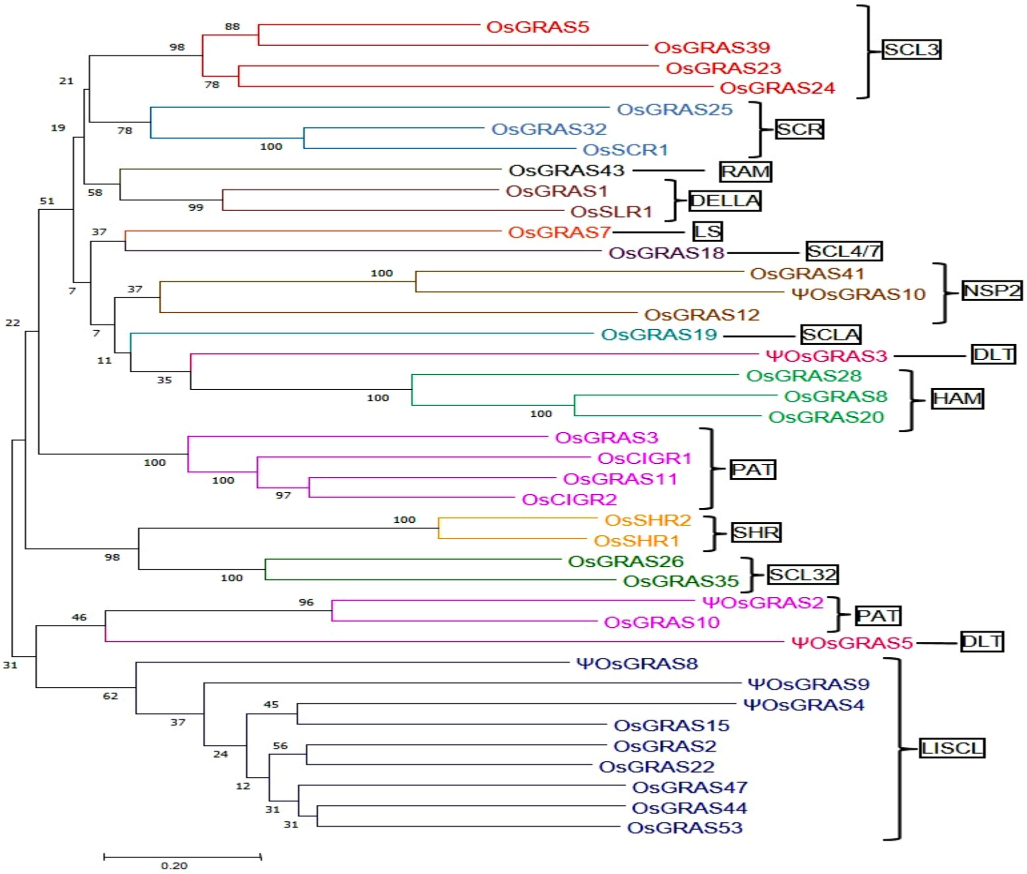


Fig. 6.2: Phylogenetic analysis of *OsGRAS* genes

An unrooted phylogenetic tree showing the evolutionary relationship among *OsGRAS* genes. The tree was constructed using the Neighbour Joining method in MEGA7 software with a bootstrap value of 1000. The number at each node represents the percentage bootstrap values. Based on the previous literature, the genes have been divided into 14 subfamilies (mentioned in boxes), and each subfamily has been colour coded.

6.4.Analysis of gene organization and GRAS motifs

The genomic and cDNA sequences of all the selected forty genes were subjected to the GSDS server to observe the organization of different *GRAS* genes selected from each paralogous group (Fig. 6.3). Based on the map generated by the server, it was observed that the genes varied in length and the distribution of exons, introns and untranslated regions (UTRs). The majority of genes (31 out of 40 genes studied) lacked introns in their gene structure and were only composed of exonic sequences and UTRs. *OsGRAS11* exon was flanked by a long stretch of UTR at its 5' and 3' ends. It completely lacked introns and was the longest gene in this study (6.7 kb). Ten genes were observed to contain only coding sequences in their structure without any introns and UTRs. Among them, *ΨOsGRAS3* had the smallest sequence of only 414 bp. Only nine genes carried introns in their structure and they were *OsGRAS3, OsGRAS39, OsGRAS41*, *OsGRAS43*, *OsSCR1*, *ΨOSGRAS4, ΨOsGRAS8, ΨOsGRAS9* and *ΨOsGRAS10.* The number of intronic sequences among the genes varied from one (*OsSCR1* and *ΨOsGRAS10*) to a maximum of seven (*ΨOsGRAS4*). All of them showed low (*OsGRAS43*), moderate (*OsGRAS3*, *ΨOSGRAS4* and *ΨOSGRAS8*) and very high (*OsGRAS39*, *OsGRAS41*, *OsSCR1*, *ΨOsGRAS9* and *ΨOsGRAS10*) expression levels under abiotic and biotic stress conditions. Six out of nine genes (*OsGRAS41*, *OsGRAS43*, *ΨOSGRAS4, ΨOsGRAS8, ΨOsGRAS9* and *ΨOsGRAS10)* did not exhibit any UTRs in their structure and were solely composed of introns and exons. The details of the genetic organization of rice *GRAS* genes have been provided in table 6.1.

Table 6.1*: List of genes and their organization details*

Locus id	**Gene name**	**Subfamily (according to Cenci and Rouard, 2017)**	**Chromosome number**	**Location**	**Gene size**	**Orientation**	**Splice forms**	**Introns**	**Exons**
LOC_Os01g45860	OsGRAS1	OG-DELLA-2	Chr1	26045843 - 26044166	1488	3'-5'	1	0	1
LOC_Os01g62460	OsGRAS2	OG-LISCL	Chr1	36158308 - 36161536	2463	5'-3'	2	0	1
LOC_Os01g65900	OsGRAS3	OG-PAT-3	Chr1	38265509 - 38261836	1662	3'-5'	1	2	3
LOC_Os01g71970	OsGRAS5	OG-SCL3	Chr1	41711881 - 41713811	1329	5'-3'	1	0	1
LOC_Os02g10360	OsGRAS7	OG-LS	Chr2	5453090 - 5451819	1272	3'-5'	1	0	1

LOC_Os02g21685	ΨOsGRAS2	OG-PAT-4	Chr2	12888147 - 12887704	444	3'-5'	1	0	1
LOC_Os02g44360	OsGRAS8	OG-HAM-II	Chr2	26841585 - 26844331	2130	5'-3'	1	0	1
LOC_Os02g45760	OsGRAS10	OG-PAT-4	Chr2	27856163 - 27853853	1857	3'-5'	1	0	1
LOC_Os03g09280	OsGRAS11	OG-PAT-1	Chr3	4847121 - 4853832	1608	5'-3'	3	0	1
LOC_Os03g15680	OsGRAS12	OG-NSP2-1	Chr3	8651497 - 8653224	1728	5'-3'	1	0	1
LOC_Os03g31880	OsSHR2	OG-SHR-1	Chr3	18240730 - 18238256	1812	3'-5'	1	0	1
LOC_Os03g37900	ΨOsGRAS3	OG-DLT	Chr3	21050297 - 21050710	414	5'-3'	1	0	1
LOC_Os03g40080	ΨOsGRAS4	close to LISCL(unclassified)	Chr3	22262511 - 22267163	2334	5'-3'	1	7	8
LOC_Os03g48450	OsGRAS15	OG-LISCL	Chr3	27592832 - 27588776	2196	3'-5'	4	0	1
LOC_Os03g49990	OsSLR1	OG-DELLA-1	Chr3	28512625 - 28515179	1878	5'-3'	1	0	1
LOC_Os03g51330	OsGRAS18	OG-SCL4/7	Chr3	29370719 - 29373265	1737	5'-3'	1	0	1
LOC_Os04g35250	OsGRAS19	OG-SCLA	Chr4	21425721 - 21424207	1515	3'-5'	1	0	1
LOC_Os04g37440	ΨOsGRAS5	OG-DLT	Chr4	22312712 - 22312254	459	3'-5'	1	0	1
LOC_Os04g46860	OsGRAS20	OG-HAM-II	Chr4	27764666 - 27767328	2136	5'-3'	1	0	1
LOC_Os04g50060	OsGRAS22	OG-LISCL	Chr4	29857542 - 29860559	1911	5'-3'	2	0	1
LOC_Os05g31380	OsGRAS23	OG-SCL3	Chr5	18234846 - 18236501	1656	5'-3'	1	0	1
LOC_Os05g31420	OsGRAS24	OG-SCL3	Chr5	18275763 - 18277445	1683	5'-3'	1	0	1
LOC_Os05g40710	OsGRAS25	OG-SCR-3	Chr5	23871516 - 23872997	1482	5'-3'	1	0	1
LOC_Os05g42130	OsGRAS26	OG-SCL32-2	Chr5	24631172 - 24632715	1278	5'-3'	1	0	1
LOC_Os06g01620	OsGRAS28	OG-HAM-II	Chr6	365301 - 367066	1443	5'-3'	1	0	1
LOC_Os07g36170	OsCIGR1	OG-PAT-2	Chr7	21616338 - 21620316	1716	5'-3'	2	0	1
LOC_Os07g38030	OsGRAS32	OG-SCR-2	Chr7	22807706 - 22809384	1374	5'-3'	1	0	1
LOC_Os07g39470	OsCIGR2	OG-PAT-1	Chr7	23650525 - 23654073	1635	5'-3'	1	0	1
LOC_Os07g39820	OsSHR1	OG-SHR-1	Chr7	23868417 - 23871145	1809	5'-3'	1	0	1
LOC_Os07g40020	OsGRAS35	OG-SCL32-1	Chr7	24014021 - 24018422	1422	5'-3'	1	0	1
LOC_Os11g03110	OsSCR1	OG-SCR-1	Chr11	1119742 - 1123350	1956	5'-3'	1	1	2
LOC_Os11g04400	ΨOsGRAS8	OG-LISCL	Chr11	1829394 - 1832069	1650	5'-3'	1	2	3
LOC_Os11g04570	OsGRAS39	OG-SCL3	Chr11	1939818 - 1933914	2565	5'-3'	1	3	4
LOC_Os11g06180	OsGRAS41	OG-NSP2-2	Chr11	2950162 - 2952223	1419	5'-3'	1	2	3
LOC_Os11g11600	ΨOsGRAS9	close to LISCL(unclassified)	Chr11	6452428 - 6453736	1038	5'-3'	1	4	5
LOC_Os11g31100	OsGRAS43	OG-RAM1	Chr11	18102879 - 18096831	2319	3'-5'	1	2	3
LOC_Os11g47870	OsGRAS44	OG-LISCL	Chr11	28870096 - 28872571	2079	5'-3'	1	0	1
LOC_Os11g47910	OsGRAS47	OG-LISCL	Chr11	28895187 - 28896974	1788	5'-3'	1	0	1
LOC_Os12g06540	ΨOsGRAS10	OG-NSP2-2	Chr12	3170547 - 3172219	1389	5'-3'	1	1	2
LOC_Os12g38490	OsGRAS53	OG-LISCL	Chr12	23634672 - 23637678	2217	5'-3'	1	0	1

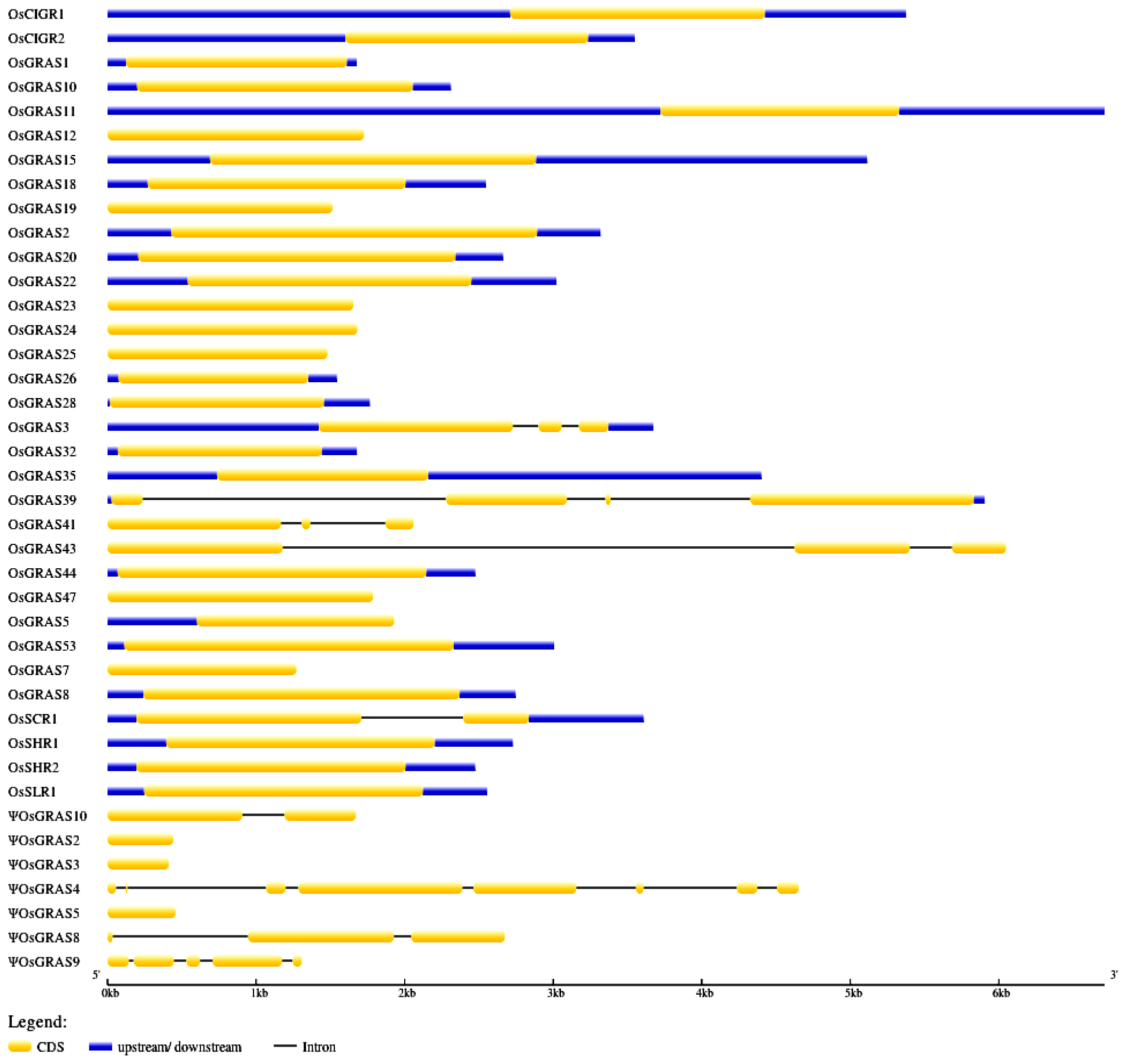


Fig. 6.3: Genetic organization of *OsGRAS* genes

Figure representing the rice *GRAS* gene organization (developed through Gene Structure Display Server (GSDSv2)). The yellow coded region indicates the coding sequence and the blue region indicates the untranslated regions. The black lines correspond to the intronic sequences.

The amino acid sequences of shortlisted genes were subjected to MEME analysis for identification of the conserved motifs in OsGRAS proteins. A total of ten motifs were identified, which corresponded to LHR I (motif 5, 9), VHIID (motif 2, 3, 10), LHR II (motif 8), PFYRE (motif 4, 7) and SAW (motif 1, 6) motifs (Fig. 6.4 and Fig 6.5). The C- terminal domain was found to contain the conserved GRAS domain, as reported earlier in literature. However, not all genes exhibited all the ten identified MEME-motifs. PAT and LISCL subfamilies carried all ten motifs, while others like SCR lacked motif 1. Proteins belonging to the same subfamily had similar motif composition.

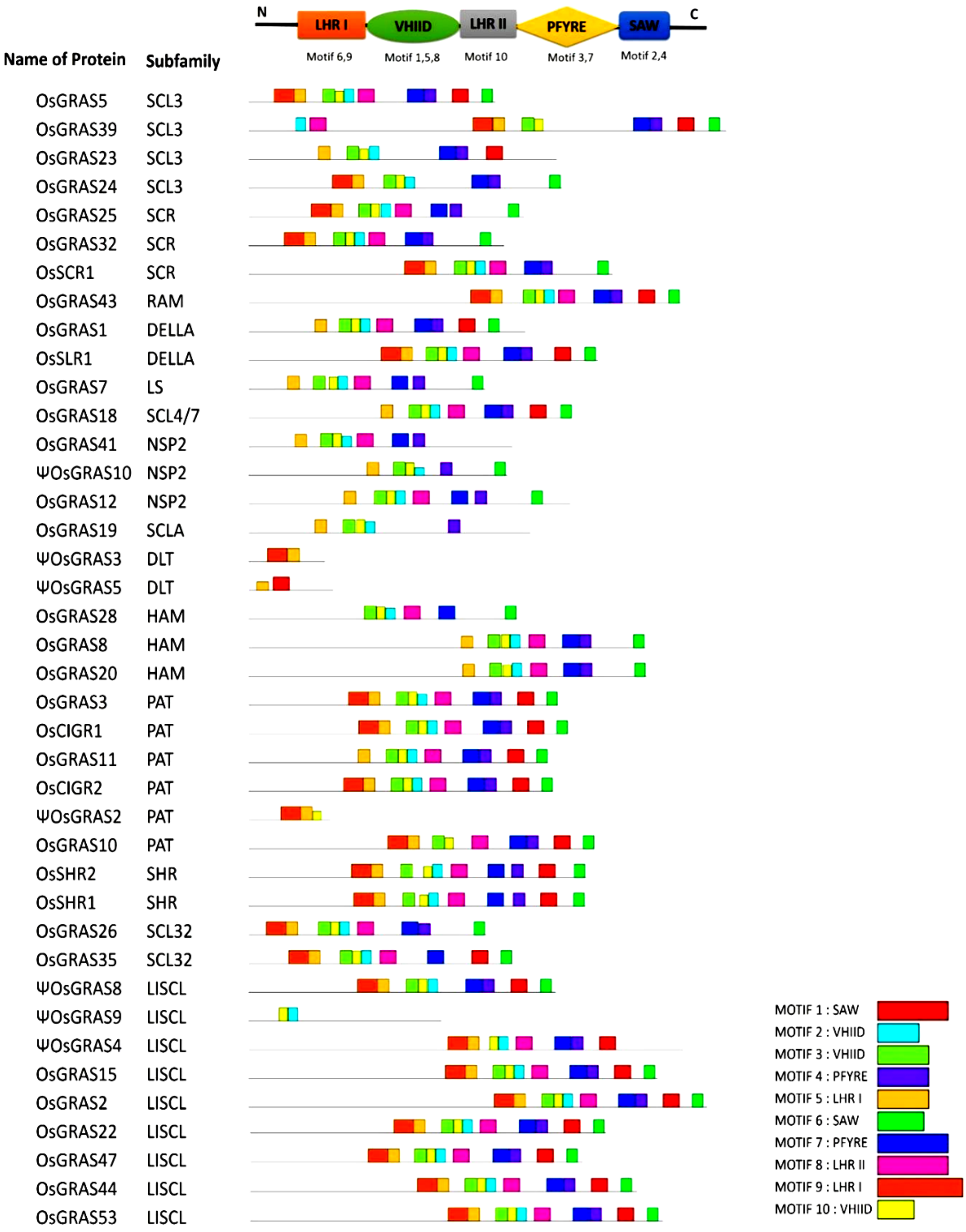


Figure 6.4: MEME-motif analysis of *OsGRAS* genes

Figure showing the identified MEME-motifs of *OsGRAS* genes. The conserved GRAS-motifs are provided at the top. A search for 10 MEME-motifs was done, and each of them has been assigned to the corresponding GRAS-motifs. Each coloured box represents one motif, and the legend has been provided below. The genes were organized based on their subfamilies.

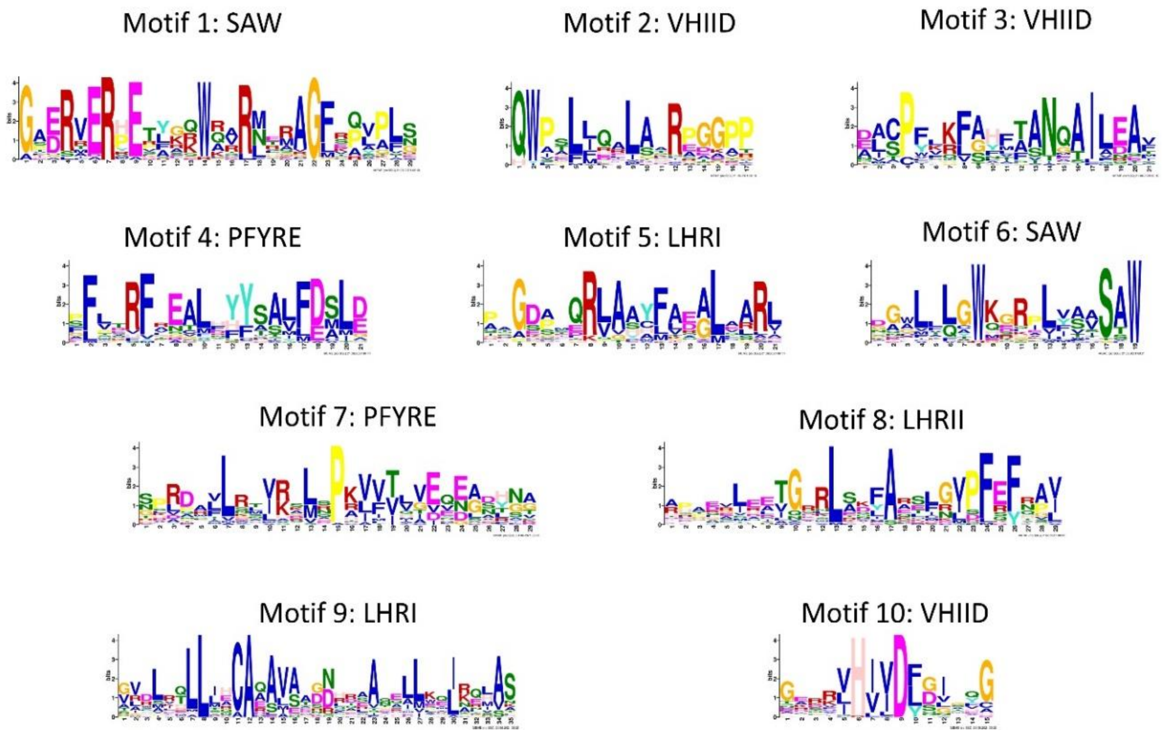

Motif 1: SAW
Motif 2: VHIID
Motif 3: VHIID
Motif 4: PFYRE
Motif 5: LHRI
Motif 6: SAW
Motif 7: PFYRE
Motif 8: LHRII
Motif 9: LHRI
Motif 10: VHIID

Fig. 6.5: Logos representing MEME motifs

The figure represents the logos of ten identified MEME motifs, which were classified according to the conserved motifs of the GRAS domain. Each logo represents the conserved sequence of the corresponding motif. The height of each amino acid in a logo indicates its frequency at that position.

6.5.Putative promoter analysis of *GRAS* genes and the search for *cis*-regulatory elements

A diverse expression pattern was observed for different *GRAS* genes under abiotic and biotic stress conditions and correlated the same with the regulatory elements present in their putative promoter regions. To identify the *cis*-acting elements, we retrieved ≤1 kb sequences from the 5' upstream region of each gene in the rice genome database and subjected them to an *in-silico* analysis. A total of eighteen stress-responsive elements were observed in the upstream putative promoter region of the *GRAS* genes. The function of each element has been provided in Table 6.2, and the physical mapping of the important stress-responsive elements on the putative promoter regions of the genes was provided in Figure 6.6. These included ABRE or ABA responsive elements, CCAAT box and MYB sites for binding of MYB transcription factors responsive to drought inducibility, the binding site for MYC transcription factors for defence responses, DRE or dehydration responsive elements, STRE or stress-responsive elements, TC-rich repeats for defence and stress responses, and the LTR or low temperature responsive element. Several phytohormones and wound responsive elements were also observed in their upstream regions, which included TCA-element for salicylic acid responses, CGTCA-motif or TGACG-motif as a methyl jasmonate responsive element, GARE-motif, TATC-box and P-box for gibberellin responses, ERE as ethylene responsive elements, TGA-element or AuxRR core or AuxRE for auxin responses, WUN-motif and WRE for responses against wounding, box-S for wounding and pathogen elicitation, and the W-box for binding of the WRKY class of transcription factors.

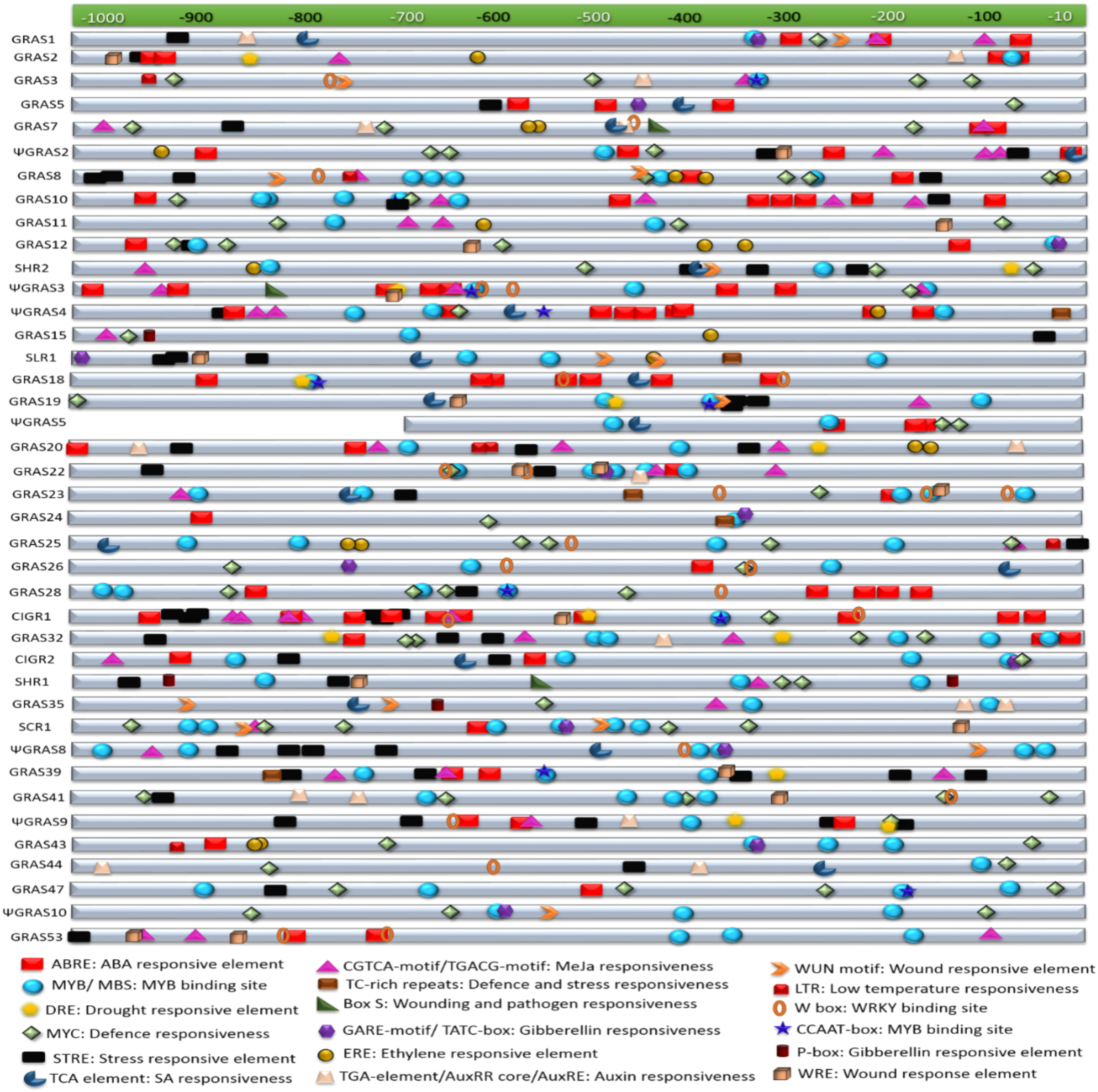


Fig. 6.6: *In-silico* analysis of putative promoter regions of *GRAS* genes

The selected *GRAS* genes were subjected to an *in-silico* analysis for *cis*-regulatory elements in their putative promoter regions (sequence retrieved from about ≤1 kb upstream region). This was performed using the PlantCARE database, and the figure was prepared by mapping the stress-regulatory elements in each of the sequences. The index for each element, along with its functions, is mentioned below the figure.

OsGRAS39, the highly expressive gene under both biotic and abiotic stress conditions in the present study, had three copies each of MYB binding factor sites and CGTCA-motif, five copies of STRE, two copies of ABRE and one copy each of DRE, TC-rich repeats and CCAAT-box justifying its expression under different stress treatments. Other responsive genes in both the stresses like *OsGRAS8*, *OsSHR1* and *OsSLR1* had combinations of MYB, STRE, ERE, WUN, TCA, CGTCA and MYC elements in their putative promoter regions. Apart from these, *OsGRAS8* exhibited ABRE, LTR and W-box elements, *OsSHR1* carried a DRE element, and *OsSLR1* had copies of TATC, WRE and TC- rich elements. *ΨOsGRAS5*, the only expressive gene in the shoot region, had two copies each of MYB and MYC binding elements and three copies of ABRE. Other important abiotic stress-responsive genes like *ΨOsGRAS2* and *OsSCR1* were observed to have multiple copies (up to six) of ABRE, MYB and MYC elements, STRE elements and ERE, CGTCA, GARE and WRE motifs in their 5' upstream regions. *OsCIGR1* was found to be highly induced under biotic stress conditions and carried ten copies of ABRE, seven copies of STRE, five copies of CGTCA element and one copy each of CCAAT-box, DRE, MYB, MYC and WRE. Other expressive genes under biotic stress conditions included *OsGRAS2*, *ΨOsGRAS3, OsGRAS19, OsGRAS20* and *OsGRAS23*, which had combinations of TCA-elements, W-box, WRE, ERE, AuxRE, CGTCA-box, box-S and WUN elements apart from other stress-responsive elements.

Table 6.2: List of *cis*-regulatory elements and their functions

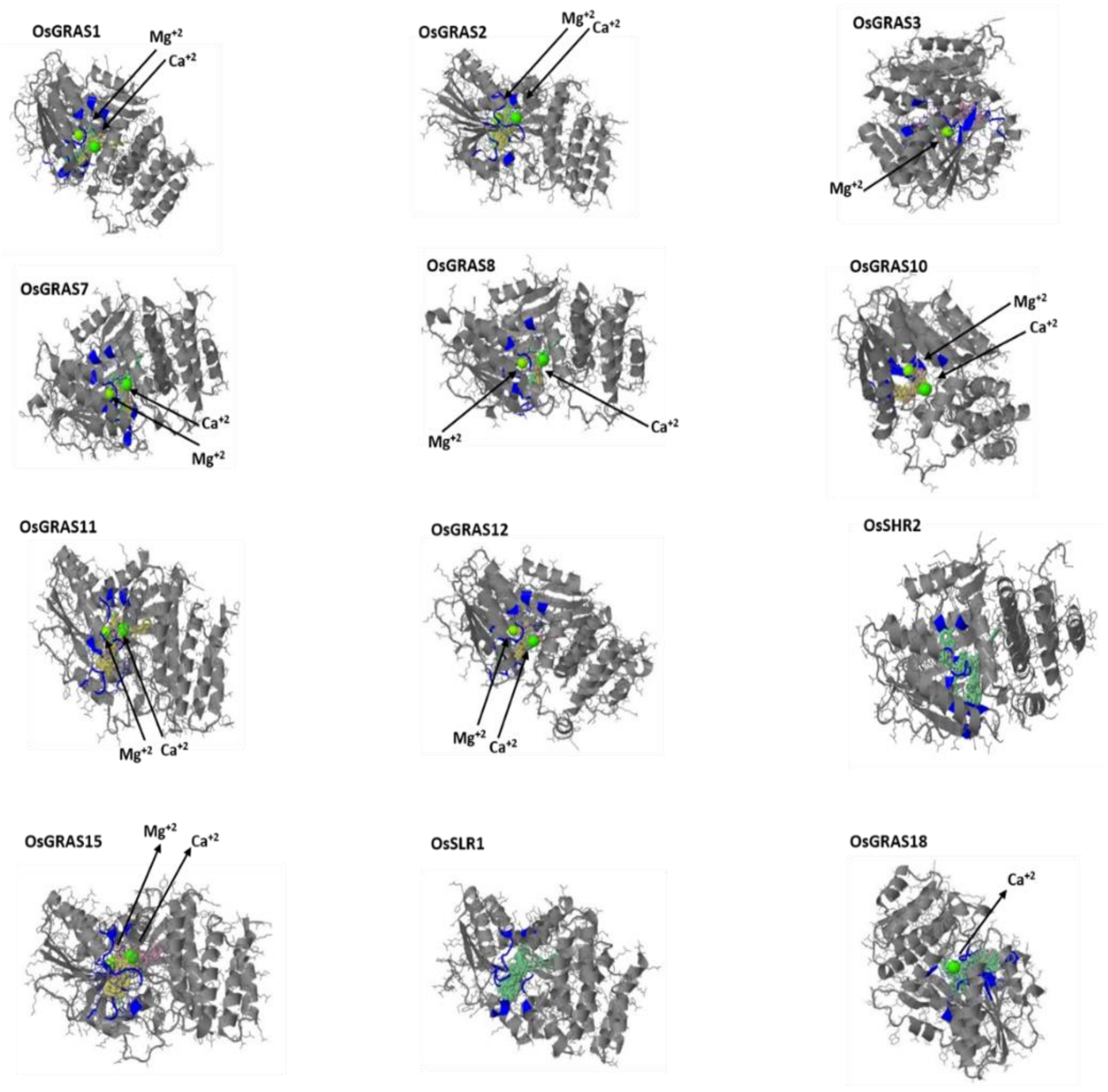
Name of *cis*-element	**Function**
ABRE	ABA responsive element (Choi et al., 2000)
MYB/MBS	MYB binding site for drought inducibility(Ambawat et al., 2013)
DRE	Dehydration responsive element (Narusaka et al., 2003)
MYC	Transcription factor for stress responses helps in dehydration induced expression of genes (Tran et al., 2004)
STRE	Stress-responsive element (Hwang et al., 2010)
TCA element	Element for salicylic acid responsiveness (Wei et al., 2013)
CGTCA-motif/TGACG-motif	Methyl-Jasmonate responsive element (Yin Wang et al., 2011)

TC-rich motifs	Responsible for defence and stress, transcription regulation (Bernard et al., 2010; Liu et al., 2017)
Box S	Responsive to wounding and pathogen elicitation (Yin et al., 2017); Stress responsiveness (Ding et al., 2019)
GARE-motif/TATC-box	Gibberellin responsive element (Bastian et al., 2010)
ERE	Element for ethylene responses (Oñate-Sánchez & Singh, 2002)
TGA-element/AuxRR core/ AuxRE	Element for auxin response (Sakai et al., 1996)
WUN motif	Wound responsive element for biotic stress (Xu et al., 2011)
LTR	Low temperature responsive element (Zhang et al., 2020)
W box	Binding sites for WRKY transcription factors (Dhatterwal et al., 2019)
CCAAT box	Binding site for MYB transcription factors
P-box	Gibberellin responsiveness (Zhang et al., 2020)
WRE	Wound responsive element (Whitbred & Schuler, 2000)

6.6.Properties of GRAS proteins, their ligand interactions and domain analysis

We studied the properties like amino acid length (aa), molecular weight (kDa) and theoretical *p*I of the shortlisted GRAS proteins through the ExPASyProtParam program. It was observed that the proteins had molecular weights ranging from 15 kDa (ΨOsGRAS3) to 94 kDa (OsGRAS39). ΨOsGRAS3 showed a minimum amino acid (aa) length of 137 aa, while OsGRAS39 had a maximum length of 854 aa. The *p*I of the proteins ranged from acidic to basic (4.5-10.1) with only eight proteins having a *p*I of more than 7. The majority of the proteins had a *p*I in the range of 4-7. Likewise, the remaining 32 proteins were found to be in the acidic range i.e. *p*I <7. This is because the majority of the proteins carried more negatively charged (acidic) amino acid residues like Aspartic acid and Glutamic acids in their composition as compared to basic amino acid residues. Only OsGRAS39 was found to have an equal number of acidic and basic residues in its composition with a *p*I around 7. According to TargetP-2.0 server, OsGRAS39 was predicted to be localized to the chloroplast while no signal peptides for chloroplast or mitochondria could be specified by the tool for the rest of the proteins.

We have also analyzed the proteins for their three-dimensional structures, and ligand binding residues in the 3DLigand site and the structures were submitted to the Phyre2 program to analyze their secondary structures like the percentage of disordered regions, α-helix and β-sheets. ΨOsGRAS3 showed a maximum of 71%, and OsGRAS8 had a minimum of 31% of the α-helical structure. Similarly, the maximum (14%) extent of β-sheets was noticed in the secondary structure of OsGRAS32. No β-sheets were present in ΨOsGRAS2 and ΨOsGRAS3. Several metallic and non-metallic ligands were also observed to be interacting with the GRAS proteins, which included Mg^{+2}, Ca^{+2}, SAM, SAH, NAP, NAD, ATP, Zn^{+2} and Ni^{+2}. The three-dimensional structures of the proteins along with their interacting ligands have been provided in figure 6.7.



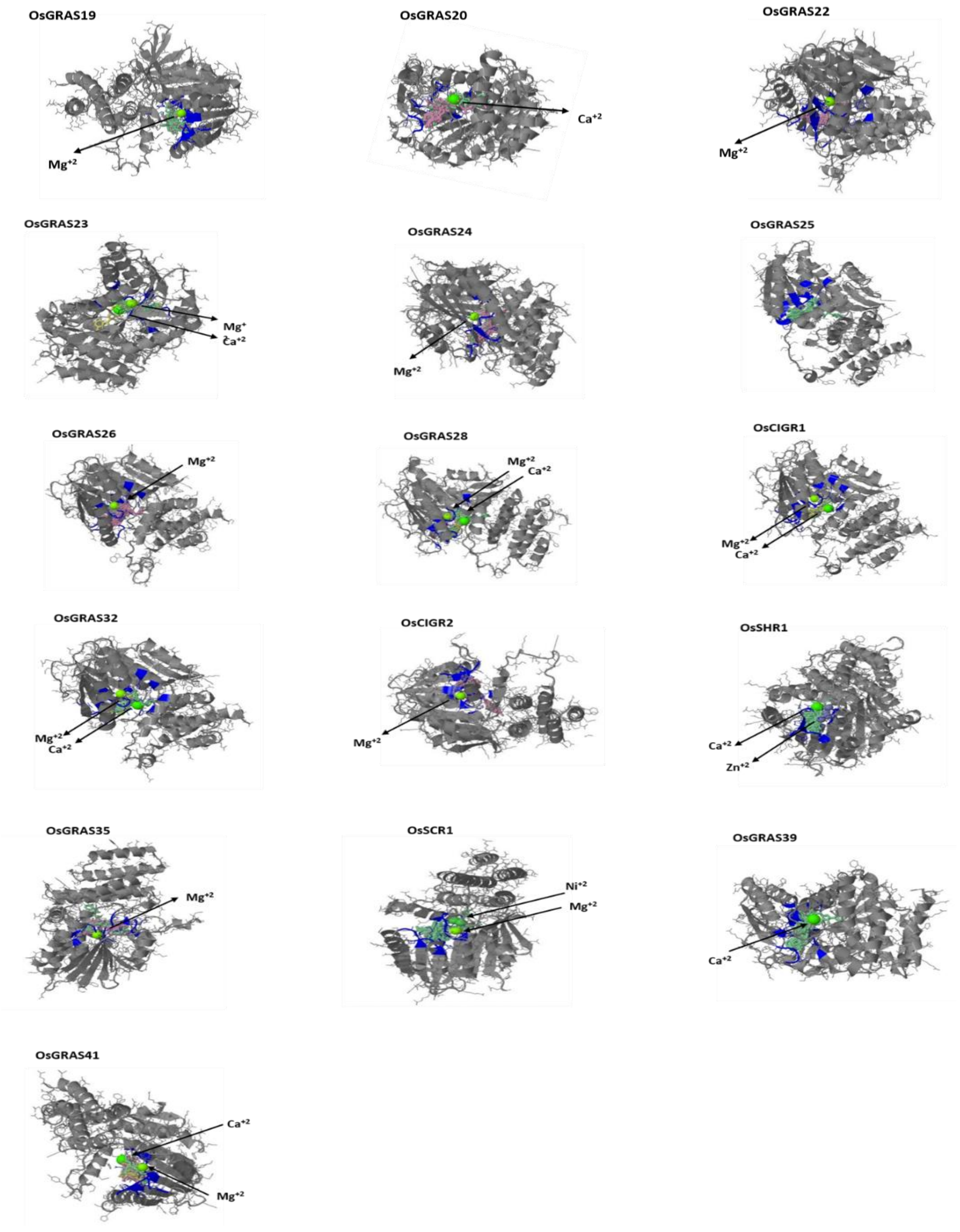


Fig. 6.7: Three-dimensional structure of GRAS proteins along with their interacting ligands

Three-dimensional structures of 28 GRAS proteins and their interacting ligands, as predicted by 3Dligand site and Phyre2 program. The blue labelled region indicates the point of interaction with the metallic and non-metallic ligands. The metallic ligands are labelled. These include Ca^{+2}, Mg^{+2}, Ni^{+2}, Zn^{+2}

Low complexity regions (LCR) are repetitive amino acid sequences found abundantly in the eukaryotic proteins. These play essential roles in protein-protein and protein-nucleic acid interactions (Toll-Riera et al., 2012). The number of LCRs in each protein varied from none to a maximum of eight in OsGRAS20 and OsGRAS43, respectively.

Grand average of hydropathicity index or GRAVY index indicates the hydrophobicity of a protein, considering its charge and size. Usually, GRAVY values range from -2 to +2 with more positive values indicating hydrophobicity and more negative values indicating hydrophilicity (Morel et al., 2006). Seven proteins had a positive GRAVY value while the rest 33 proteins had a value lesser than zero, which indicated that the majority of the GRAS proteins are hydrophilic. The list of all the observations has been provided in table 6.3.

In order to study the domains present in the genes, we utilized the SMART online tool and observed that all the proteins had at least one GRAS domain with ΨOsGRAS4, ΨOsGRAS8, OsGRAS39, ΨOsGRAS10 exhibiting two GRAS domains. Among them, ΨOsGRAS4 and ΨOsGRAS10 had two internal repeats designated as RPT1 along with two GRAS domains. One DELLA domain and one SCOP domain in addition to the GRAS domain were found in OsSLR1 and OsGRAS18, respectively. DELLA proteins are transcriptional regulators that function in gibberellic acid signaling by binding with GA receptor, GID1, followed by proteasomal degradation of the DELLA domain (Murase et al., 2008). OsGRAS41 had a transmembrane region; OsGRAS43 and OsGRAS53 had two RPT1 domains (internal repeats) along with their single GRAS domains. A detailed list of the domains and the LCRs with their sequences have been provided in table 6.4. The presence of the transmembrane domain in OsGRAS41 was further confirmed through TMHMM software.

Table 6.3: Chart depicting the protein properties of 40 GRAS genes under study. This includes their length, molecular weight, isoelectric points (*p*I), GRAVY indices, chelating ligands, low complexity region (LCR), localization and secondary structure details.

Name	**Length (aa)**	**MW (kDa)**	***p*I**	**GRAVY index**	**Ligand residues**	**Metallic & non-metallic ligands**	**LCR**	**TargetP-2.0 prediction**	**Disordered %**	**α- helix %**	**β-sheet %**
OsGRAS1	495	52067.35	5.06	-0.017	LEU168 HIS172 ASP193 PHE194 SER195 LEU196 MET197 GLN198 GLN201 ILE224 GLY225 PRO226 PRO229 SER262 LEU263 ASP264 VAL266 VAL284 GLN286 ARG289 LEU290	Mg^{+2}, SAM, Ca^{+2}, SAH	Three (26-38,41-82,452-486)	-	30	40	11
OsGRAS2	820	90671.06	5.7	-0.415	LYS530 HIS534 ASP555 TYR556 GLY557 ILE558 TYR559 TYR560 GLN563 ILE586 ASP587 THR588 PRO589 GLN590 GLY592 SER624 ARG625 PHE626 GLU627 VAL629 MET647 LYS649 ASN652	Mg^{+2}, SAM, Ca^{+2}, SAH	Four (85-97, 165-183,246-262,415-434)	-	45	39	7
OsGRAS3	553	61798.49	4.8	-0.358	MET260 PHE270 PHE274 ASP295 PHE296 ASP297 ILE298 ASN299 GLN300 GLN303 VAL326 ASP327 ASP328 ALA364 ASN365 ILE366 GLY367 VAL369 ALA387 GLN389 HIS392	Mg^{+2}, SAM, NAP, SAH	Two (91-107,183-553)	-	35	39	10
OsGRAS5	442	48023.77	6.15	-0.079	LEU139 ASP164 LEU165 GLY166 GLY167 ALA168 ASP169 GLN172 VAL195 HIS196 GLU197 ARG228 LEU229 ASP230 SER250 GLN252	Mg^{+2}, SAM, Ca^{+2}, SAH	Four (46-73,89-105,260-270,367-383)	-	24	45	12
OsGRAS7	423	44138.74	5.56	-0.113	LEU122 HIS126 ASP153 LEU154 ASP155 ALA156 ALA157 HIS158 GLN161	Mg^{+2}, SAM, Ca^{+2}, SAH	Two (21-29,39-420)	-	22	44	12

					ALA188 GLY189 THR190 LEU226 ALA227 VAL247 PHE249 LYS252						
ΨOsGRAS2	147	15305.11	9.18	-0.128			Three (38-62,81-91,131-144)	-	48	58	0
OsGRAS8	709	74248.23	5.65	-0.02	LEU434 ASP461 PHE462 ASP463 GLY465 VAL466 GLN469 PHE496 MET497 LEU532 ASP533 ALA534 PHE535 PRO554	Mg^{+2}, SAM, Ca^{+2}, SAH	Five (47-85,88-106,153-165,195-217,258-273)	-	51	31	8
OsGRAS10	618	64200.52	9.27	-0.115	PHE335 ASP360 PHE361 ASP362 VAL363 SER364 GLN367 VAL390 ALA391 ASP392 CYS430 ARG431 ALA432 PRO433 ILE435 ALA453 THR455 ARG458	SAM, Mg^{+2}, Ca^{+2},SAH	Six (23-35,40-50,62-72,98-116,143-160,162-179)	-	38	45	9
OsGRAS11	535	59647.91	5.86	-0.375	MET242 PHE252 TYR256 ASP277 PHE278 GLN279 ALA281 GLN282 ILE308 ASP309 ASP310 HIS315 ALA346 ALA347 SER348 HIS349 VAL351 ALA369 TYR370 GLN371 HIS374	Mg^{+2}, SAM, Ca^{+2}, SAH	One (80-93)	-	35	40	10
OsGRAS12	575	60848.2	5.04	-0.106	MET231 HIS235 ASP256 TYR257 ASP258 ILE259 ALA260 GLU261 GLN264 VAL289 SER290 ARG291 GLY294 GLY295 LEU325 ASP328 VAL349 LEU350 HIS351	Mg^{+2}, SAM, Ca^{+2}, SAH	Three (15-34,55-65,100-116)	-	35	43	9
OsSHR2	603	64247.77	5.93	-0.354	THR278 HIS282 LEU323 SER324 ASN325 THR326 PHE327 THR329 VAL354 VAL355 PRO356 THR357 HIS393 GLY395 ASP396 LEU397 VAL421 ASN422	SAM, SAH, NAD, ATP	Four (11-18,31-94,118-141,162-185)	-	41	36	9
ΨOsGRAS3	137	15192.7	7.87	-0.07			--	-	31	71	0
ΨOsGRAS4	777	88331.49	5.53	-0.486			--	-	40	34	9

OsGRAS15	731	82218.82	6.44	-0.483	LYS443 HIS447 ASP468 PHE469 GLY470 ILE471 TYR472 PHE473 ILE499 ASP500 VAL501 PRO502 GLN503 GLY505 PRO508 LYS538 TRP539 GLU540 ILE542 LEU560 ARG562 ASN565	Mg^{+2}, SAM, Ca^{+2}, SAH	--	-	40	39	8
OsSLR1	625	65406.24	5.14	-0.111	LEU323 HIS327 ASP348 PHE349 GLY350 ILE351 LYS352 GLN353 GLN356 VAL379 GLY380 PRO381 PRO382 GLN383 PRO384 ASP385 THR417 LEU418 ALA419 VAL446 PHE447 GLU448 ARG451	SAM, SAH	Four (9-17,128-140,185-207,209-232)	-	40	44	9
OsGRAS18	578	62477.53	5.63	-0.209	TYR283 HIS297 ASP318 PHE319 GLY320 ILE321 VAL322 VAL350 PRO351 SER352 PRO353 LEU354 LEU355 VAL390 MET411 LEU412 GLN413 TYR415 HIS416	SAM, Ca^{+2},SAH	One (144-206)	-	36	44	10
OsGRAS19	504	52579.87	8.67	-0.224	TYR169 TYR175 HIS179 ASP202 PHE203 ASP204 VAL205 SER206 TYR207 GLN210 PHE252 GLY253 ALA254 ASN285 ASN286 GLY287 SER288 THR290 VAL308	Mg^{+2}, SAM, SAH	One (10-42)	-	31	45	11
ΨOsGRAS5	152	16672.18	9.43	-0.472			Two (104-124,130-146)	-	38	38	14
OsGRAS20	711	74007.88	5.57	0.004	LEU437 ASP464 PHE465 ASP466 LEU467 GLY468 VAL469 GLN472 PHE499 VAL500 SER501 LEU535 ASP536 ALA537 PRO557 VAL558	SAM, NAP, Ca^{+2}, SAH	Eight (52-70,79-92,98-114,185-215,221-234,243-255,277-286,302-312)	-	52	32	8

OsGRAS22	636	71655.98	5.51	-0.424	PHE338 LYS348 ASP373 TYR374 GLY375 ILE376 GLN377 TYR378 VAL404 ASP405 LEU406 ARG412 ALA442 LYS443 TRP444 ASP445 ILE447 LEU467 ARG469 ASN472 MET473	Mg^{+2}, SAM, SAH	Three (2-15,56-76,237-251)	-	37	38	8
OsGRAS23	551	56280.97	5.41	0.197	VAL182 ASP208 LEU209 GLY210 GLY211 GLY212 VAL213 ASP214 GLN217 VAL241 ASN242 GLU243 SER274 ILE275 GLU276 ASN303 GLN305 ARG308	Mg^{+2}, SAM, Ca^{+2}, SAH	Three (6-27,51-59,513-541)	-	36	41	10
OsGRAS24	560	58183.56	7.85	0.182	LEU248 ASP273 LEU274 GLY275 GLY276 ILE277 HIS278 HIS281 VAL305 HIS306 GLU307 SER338 VAL339 GLU340 THR360 GLN362 ARG365	Mg^{+2},SAM, SAH	Four (5-20,32-52,55-83,89-141)	-	42	36	9
OsGRAS25	493	51644.41	5.75	0.008	TYR207 ASP228 LEU229 ASP230 VAL231 VAL232 PRO233 GLY234 GLN238 PHE261 GLY262 MET263 ARG294 PRO295 GLY296 ALA298 TRP317 LEU318 ARG319 HIS320	SAM, SAH	Two (13-36,98-111)	-	30	45	10
OsGRAS26	425	45430.82	5.81	0.017	HIS131 PHE135 ASP156 LEU157 SER158 VAL159 THR160 HIS161 GLN164 PRO187 SER188 VAL189 ARG190 PRO191 ALA192 SER226 ALA227 THR228 THR229 GLN266 SER267 TRP268	SAM, Mg^{+2},SAH	One (9-20)	-	21	50	12
OsGRAS28	480	51017.01	5.46	-0.058	VAL213 ASP238 PHE239 ASP240 VAL241 GLY242 PHE243 LEU273 VAL274 SER275 PRO276 GLY277	Mg^{+2}, SAM, Ca^{+2}, SAH	One (35-66)	-	36	45	11

					SER278 SER279 PHE309 THR334						
OsCIGR1	571	64602.19	5.87	-0.478	MET278 PHE288 TYR292 ASP313 PHE314 GLN315 ILE316 ALA317 GLN318 GLN321 ILE344 ASP345 ASP346 VAL382 TYR383 ALA384 THR385 VAL387 THR405 GLN407	Mg^{+2}, SAM, Ca^{+2}, SAH	--	-	40	36	9
OsGRAS32	457	49096.59	5.51	-0.127	PHE148 ASN152 VAL158 HIS162 ASP183 LEU184 ASP185 ILE186 MET187 GLN188 GLN191 LEU214 GLY215 ALA216 LYS247 ILE248 GLY249 VAL251 VAL269 TRP271 HIS274	Mg^{+2}, SAM, Ca^{+2}, SAH	Three (8-45,55-62,437-449)	-	27	47	14
OsCIGR2	544	60108.16	6	-0.324	MET251 PHE261 TYR265 ASP286 PHE287 HIS288 ILE289 SER290 GLN291 GLN294 ILE317 ASP318 ASP319 ILE355 SER356 GLY357 SER358 VAL360 THR378 GLU380 HIS383 ILE384	SAM, Mg^{+2}, SAH	--	-	35	40	10
OsSHR1	602	64709.33	5.61	-0.4	HIS286 LEU316 SER317 ASN318 THR319 PHE320 THR322 VAL347 VAL348 SER349 ALA350 HIS388 GLY390 ASP391 LEU392 VAL416 ASN417 MET527	SAM, Ca^{+2},SAH, Zn^{+2}	Three (11-40,46-80,122-147)	-	39	40	7
OsGRAS35	473	50596.45	5.31	0.022	TYR174 LEU196 SER197 THR198 THR199 HIS200 ALA226 ASP227 VAL228 ALA229 TYR241 THR266 SER274 LEU275 VAL276 HIS297 MET298 LEU299 THR302	Mg^{+2}, SAM, SAH	Two (9-25,34-54)	-	27	43	11
OsSCR1	651	69918.23	5.91	-0.221	PHE364 HIS378 ASP399 LEU400 ASP401 ILE402 MET403 GLN407 LEU430	Ni^{+2}, Mg^{+2}, SAM, NAP, SAH	Six (3-55,86-101,115-134,148-	-	48	37	9

					GLY431 ALA432 ASP462 LYS463 ALA464 LEU485 HIS487 LEU489 TYR490 ASP491 VAL492 THR493		157,188-229,235-279)				
ΨOsGRAS8	549	59950.36	4.98	-0.299			Four (29-40,108-122,140-147,177-193)	-	34	48	8
OsGRAS39	854	94267.73	7.45	-0.09	LEU495 TYR499 ASP520 PHE521 SER522 GLY523 PRO524 ALA525 ALA526 ASN527 GLN530 VAL553 HIS554 ASP555 ALA585 LYS586 LEU587 ASP588 VAL612 GLN614 ARG617	SAM, SAH, Ca^{+2}	One (370-391)	Chloroplast	23	45	12
OsGRAS41	472	52312.55	4.78	0.111	VAL135 HIS139 ASP160 LEU161 ASN162 ILE163 GLY164 GLU165 GLN168 ILE189 THR190 THR191 VAL225 HIS226 ASN227 GLU228 GLU230 THR248 THR249 SER250	Mg^{+2}, SAM, Ca^{+2}, SAH	--	-	23	53	7
ΨOsGRAS9	345	37286.47	10.13	-0.552			One (216-254)	-	35	49	8
OsGRAS43	772	81094.36	6.18	-0.156			Eight (2-13,165-177,183-201,223-230,280-288,293-306,326-341,355-364)	-	39	36	11
OsGRAS44	692	77442.34	5.01	-0.394			Four (14-27,82-111,209-218,279-299)	-	39	43	8
OsGRAS47	595	66666.1	5.99	-0.372			One (201-211)	-	33	48	10
ΨOsGRAS10	462	50587.65	4.53	-0.137			Two (22-30,47-60)	-	44	44	7
OsGRAS53	738	81365.04	5.17	-0.305			Nine (53-65,116-135,150-162,241-260,337-356,796-808,859-878,893-905,984-1003)	-	39	40	7

Table 6.4: List of domains observed in GRAS genes. The number, position and sequences of the observed domains and LCR are provided in the chart.

Name	Domain	Position	Sequence	LCR	Position	Sequence
OsGRAS1	GRAS	86-449	LVHLLMSCAGAIEAGDHALASAQLADSHAALAAVSAASGIGRVAVHFTTALSRRLFPSPVAPPTTDAEHAFLYHHFYEACPYLKFAHFTANQAILEAFHGCDHVHVIDFSLMQGLQWPALIQALALRPGGPPFLRITGIGPPSPTGRDELRDVGLRLADLARSVRVRFSFRGVAANSLDEVRPWMLQIAPGEAVAFNSVLQLHRLLGDPADQAPIDAVLDCVASVRPKIFTVIEQEADHNKTGFLDRFTEALFYYSAVFDSLDAASASGGAGNAMAEAYLQREICDIVCGEGAARRERHEPLSRWRDRLTRAGLSAVPLGSNALRQARMLVGLFSGEGHSVEEADGCLTLGWHGRPLFSASAWE	LCR1	26-38	PPPAAVAPDDGVG
				LCR2	41-82	DPPAGADVDAAALPEFAAAFPPCAPDAAAAVLAMRREEEEVA
				LCR3	452-486	GDGGGDNNNNSNSNVSGSSGSDSNNSGSSNGKSSG
OsGRAS2	GRAS	443-813	LETLLIHCAQSVATDDRRSATELLKQIRQHAHANGDGDQRLAHCFANGLEARLAGTGSQIYKNYTITRLPCTDVLKAYQLYLAACPFKKISHYFANQTILNAVEKAKKVHIVDYGIYYGFQWPCLIQRLSNRPGGPPKLRITGIDTPQPGFRPAERTEETGRYLSDYAQTFNVPFEFQAIASRFEAVRMEDLHIEEDEVLIVNCMFKFKNLMDESVVAESPRNMALKTIRKMNPHVFIHGVVNGSYNAPFFVTRFREALFHYSAIFDMLETNIPKDNEQRLLIESALFSREAINVISCEGLERMERPETYKQWQVRNQRVGFKQLPLNQDMMKRAREKVRCYHKDFIIDEDNRWLLQGWKGRILFALSTWK	LCR1	85-97	VSVSASAASSAAA
				LCR2	165-183	SSAANSCNSLSPCNCSSSS
				LCR3	246-262	SQSSSFASSNGSSVTFS
				LCR4	415-434	KHSGGGHGKGSSHGKGRGKK
OsGRAS3	GRAS	183-553	PKQLLFDCAMALSDYNVDEAQAIITDLRQMVSIQGDPSQRIAAYLVEGLAARIVASGKGIYKALSCKEPPTLYQLSAMQILFEICPCFRFGFMAANYAILEACKGEDRVHIIDFDINQGSQYITLIQFLKNNANKPRHLRITGVDDPETVQRTVGGLKVIGQRLEKLAEDCGISFEFRAVGANIGDVTPAMLDCCPGEALVVNFAFQLHHLPDESVSIMNERDQLLRMVKGLQPKLVTLVEQDANTNTAPFQTRFREVYDYYAALFDSLDATLPRESPDRMNVERQCLAREIVNILACEGPDRVERYEVAGKWRARMTMAGFTPCPFSSNVISGIRSLLKSYCDRYKFEEDHGGLHFGWGEKTLIVSSAWQ	LCR	91-107	SNVSQQNSQSISDNQSS
OsGRAS5	GRAS	50-437	LIHLLLNCAAAAAAGRLDAANAALEHIASLAAPDGDAMQRVAAAFAEALARRALRAWPGLCRALLLPRASPTPAEVAAARRHFLDLCPFLRLAGAAANQSILEAMESEKIVHVIDLGGADATQWLELLHLLAARPEGPPHLRLTSVHEHKELLTQTAMALTKEAERLDVPFQFNPVVSRLDALDVESLRVKTGEALAICSSLQLHCLLASDDDAAAVAGGDKERRSPESGLSPSTSRADAFLGALWGLSPKVMVVAEQEASHNAA			

			GLTERFVEALNYYAALFDCLEVGAARGSVERARVERWLLGEEIKNIVACDGGERRERHERLERWARRLEGAGFGRVPLSYYALLQARRVAQGLGCDGFKVREEKGNFFLCWQDRALFSVSAWR			
OsGRAS7	GRAS	39-420	ARGLVLACADLVHRGDLDGARRVAEAVLAAADPRGEAGDRLAHHFARALLALRGGGKGGHGGGGGVVPSSAAYLAYIKIAPFLRFAHLTANQAILEAAAADAGGAHRRVLHIVDLDAAHGVQWPPLLQAIADRADPAVGPPPEVRLTGAGTDRDVLLRTGDRLRAFSSSLNLPFRFHPLILPCTAELAADPTAALELHPDETLAVNCVLFLHKLGGDGELAAFLRWVKSMNPAVVTIAEREGVLGGDVDDDNVPDELPRRVAAAMDYYSSVFDALEATVPPASADRLAVEQEILSREIDAAVAAPGAGGGGRARDFDAWASAARAAGLAPRPLSAFAASQARLLLRLHYPSEGYKADDDGGRGACFLRWQQRPLMSVSSWQ	LCR	21-29	QPQPQPQPP
ΨOsGRAS2	GRAS	62-138	SRQLLSEAAAAIANGNHIVAASLLSALKLSVNPQGDAEQRLVAMMVAALSSCVGTSPSQHLADLYIGVGRRRWSEDR			
OsGRAS8	GRAS	349-708	LLDELAAAAKATEAGNSVGAREILARLNQQLPQLGKPFLRSASYLKEALLLALADSHHGSSGVTSPLDVALKLAAYKSFSDLSPVLQFTNFTATQALLDEIGGMATSCIHVIDFDLGVGGQWASFLQELAHRRGAGGMALPLLKLTAFMSTASHHPLELHLTQDNLSQFAAELRIPFEFNAVSLDAFNPAELISSSGDEVVAVSLPVGCSARAPPLPAILRLVKQLCPKVVVAIDHGGDRADLPFSQHFLNCFQSCVFLLDSLDAAGIDADSACKIERFLIQPRVEDAVIGRHKAQKAIAWRSVFAATGFKPVQLSNLAEAQADCLLKRVQVRGFHVEKRGAALTLYWQRGELVSISSWR	LCR1	47-85	SPSPPYSTSTLSSSLGGGSADSTGVAAVSESSTAAAGAT
				LCR2	88-106	GAPGEHGGGGKEEWGGGCE
				LCR3	153-165	QPGPPLPVLQQPL
				LCR4	195-217	SSSGAHTATGGGGKASLGFGLFS
				LCR5	258-273	PPNPAAALFMPLPPFP
OsGRAS10	GRAS	253-618	SRQLLSEAAAAVADGNHTAAASLLSALKLSANPRGDAEQRLVAMMVAALSSRVGTGPSQHLADLYSGEHRAACQLLQDVSPCFGLALHGANLAILDAVAGHRAIHLVDFDVSAAQHVALIKALADRRVPATSLKVTVVADPTSPFTPAMTQSLAATCERLKKLAQQAGIDFRFRAVSCRAPEIEASKLGCEPGEALAVNLAFTLSRVPDESVSPANPRDELLRRVRALGPRVVTLVEQELNTNTAPMAARFSDASAHYGAVLESLDATLGRDSADRTRAEAALASKVANAVGREGPDRVERCEVFGKWRARFGMAGFRAVAIGEDIGGRVRARLGPALPAFDVKLDNGRLGVGWMGRVVTVASAWR	LCR1	23-35	AALQAAARQQSQQ
				LCR2	40-50	GAGGAGVTGGV
				LCR3	62-72	QQQRQVAAQQA
				LCR4	98-116	GISGLSSGFGGISQQQPSS
				LCR5	143-160	TAQNQAVARAPAARPATA
				LCR6	162-179	ELVLLQELEKQLLGDDEE
OsGRAS11	GRAS	166-535	LKQVIAACGKAVDENSWYRDLLISELRNMVSISGEPMQRLGAYMLEGLVARLSSTGHALYKSLKCKEPTSFELMSYMHLLYEICPFFKFGYMSANGAIAEAVKGENFVHIIDFQIAQGSQWATMIQALAARPGGPPYLRITGIDDSNSAHARGGGLDIVGRRLFNIAQSCGLPFEFNAVPAASHEVMLEHLDIRSGEVIVVN	LCR	80-93	HSSTSSHISGSPIS

			FAYQLHHTPDESVGIENHRDRILRMVKGLSPRVVTLVEQEANTNTAPFFNRYLETLDYYTAMFEAIDVACPRDDKKRISTEQHCVARDIVNLIACEGAERVERHEPFGKWRARLSMAGFRPYPLSALVNNTIKKLLDSYHSYYKLEERDGALYLGWKNRKLVVSSAWR			
OsGRAS12	GRAS	132-526	LLHLLMAAAEALSGPHKSRELARVILVRLKEMVSHTASANAAASNMERLAAHFTDALQGLLDGSHPVGGSGRQAAAAASHHHAGDVLTAFQMLQDMSPYMKFGHFTANQAILEAVSGDRRVHIVDYDIAEGIQWASLMQAMTSRADGVPAPHLRITAVSRSGGGGARAVQEAGRRLSAFAASIGQPFSFGQCRLDSDERFRPATVRMVKGEALVANCVLHQAAATTTIRRPTGSVASFLSGMAALGAKLVTVVEEEGEAEKDDDGDSAGDAAAGGFVRQFMEELHRYSAVWDSLEAGFPTQSRVRGLVERVILAPNIAGAVSRAYRGVDGEGRCGWGQWMRGSGFTAVPLSCFNHSQARLLLGLFNDGYTVEETGPNKIVLGWKARRLMSASVWA	LCR1	15-34	SGCGSTTTTSSASSLDDGTG
				LCR2	55-65	DDDGGHDLHGL
				LCR3	100-116	GNGSNPSSTTTTNPGSP
OsSHR2	GRAS	188-502	AAQLLMECARAVAGRDSQRVQQLMWMLNELASPYGDVDQKLASYFLQGLFARLTTSGPRTLRTLATASDRNASFDSTRRTALKFQELSPWTPFGHVAANGAILESFLEAAAAGAAASSSSSSSSTPPTRLHILDLSNTFCTQWPTLLEALATRSSDDTPHLSITTVVPTAAPSAAAQRVMREIGQRLEKFARLMGVPFSFRAVHHSGDLADLDLAALDLREGGATAALAVNCVNALRGVARGRDAFVASLRRLEPRVVTVVEEEADLAAPEADASSEADTDAAFVKVFGEGLRFFSAYMDSLEESFPKTSNERLSLERAVGRAIVDLVSCPASQSAERRETAASWARRMRSAGFSPAAFSEDVADDVRSLLRRYKEGWSMRDAGGATDDAAGAAAAGAFLAWKEQPVVWASAWK	LCR1	11-18	HHHHHHQH
				LCR2	31-94	SYPSSRGSTSSPSSHHTHNHTYYHHSHSHYNNNSNTNYYYQGGGGGGGGYYYAEEQQPAAYLEE
				LCR3	118-141	SGTGAPSSAPVPPPPSATTSSAGG
				LCR4	162-185	GGSPAVPSSSGAGAGAGAAPSSSG
ΨOsGRAS3	GRAS	38-137	LVRMLTACADSVSAGNHEAAIYYLARLCEMASLAGPMPIHRVAAYFIEVLTLRVVRMWPHMFNISPPRELTNDAFSGDDDAMALRILNTITPILLLGKHS			
ΨOsGRAS4	GRAS	360-435	LRTLLINCAQAVSVSNHSLASDILKIIRHHASPTGDDSQRLALCLAYCLDVRLTGTGSQIYHKFITKRRNVKDILK			
	GRAS	428-672	RNVKDILKVHIIDFGICFGFQWPSLFEELAKIEDGPPKLRITGIELPESGFRPYARSNNIGLRLADYAKTFNIPFEYQHISSNKWEALSPEDFNIEKDEVLIVNCIYRIKDLGDETISINSARSRVLNTIRMMKPKVFVQGVLNGSYGVPFFLTRFKEVMYHYNSLFDMLDKNIPRDNETRMIIERDIYQYIMLNVIACEGPERIERPESYKKWKVRNLKAGLVQLPLNPAIVRETQDMSSDKAS			
	RPT1	730-756	VSDDFYHVSGDTREISCDTYQVLDDFY			
	RPT1	751-777	VLDDFYHVSGDTYEISDDTYRISGDSY			
OsGRAS15	GRAS	356-727	LRTLLIHCAQAVAADDRRTANELLKQIRQHAKPNGDGSQRLAYCFADGLEARLAGTGSQLYHKLVAKRTTASDMLKAYHLYLAACPFKRLSHFLSNQTILSLTKNASKVHIIDFGIYFGFQWPCLIRRLFKREG	LCR1	50-64	SSAASSTASRAAVSS

			GPPKLRITGIDVPQPGFRPTERIEETGQRLAEYAEKIGVPFEYQGIASKWETICVEDLNIKKDEVVIVNCLYRFRNLIDETVAIDSPRNRVLNTIRQVNPAIFIHGIVNGSYSVPFFITRFREALFHFSALFDMLETTVPRDDAQRALIERDLFGREALNVIACEGSDRVERPETYKQWQVRNLRAGFVQSPLNQDIVLKAKDKVKDIYHKDFVIDEDSEWLLQGWKGRIIYAISTWK			
				LCR2	133-148	PLDSPSESSTSSYPHS
OsSLR1	DELLA	39-120	DELLAALGYKVRSSDMADVAQKLEQLEMAMGMGGVSAPGAADDGFVSHLATDTVHYNPSDLSSWVESMLSELNAPLPPIPPA	LCR1	9-17	GGSSGGGSS
	GRAS	241-621	LVHALLACAEAVQQENFAAAEALVKQIPTLAASQGGAMRKVAAYFGEALARRVYRFRPADSTLLDAAFADLLHAHFYESCPYLKFAHFTANQAILEAFAGCHRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKLAQFAHTIRVDFQYRGLVAATLADLEPFMLQPEGEADANEEPEVIAVNSVFELHRLLAQPGALEKVLGTVHAVRPRIVTVVEQEANHNSGSFLDRFTESLHYYSTMFDSLEGGSSGQAELSPPAAGGGGGTDQVMSEVYLGRQICNVVACEGAERTERHETLGQWRNRLGRAGFEPVHLGSNAYKQASTLLALFAGGDGYRVEEKEGCLTLGWHTRPLIATSAWR	LCR2	128-140	STSSTVTGGGGSG
				LCR3	185-207	GGGSTSSSSSSSSLGGGASRGS
				LCR4	209-232	VEAAPPATQGAAAANAPAVPVVVV
OsGRAS18	SCOP	15-95	QQVIQQQQQQQQQQRHHHHHHLPPPPPPQSMAPHHHQQKHHHHHQQMPAMPQAPPSSHGQIPGQLAYGGGAAWPAGEHF	LCR1	144-206	TTPPPPVPSPPPTHAAATATATAATAAPRPEAAPALLPQPAAATPVACSSPSPSSADASCSAP
	GRAS	207-578	ILQSLLSCSRAAATDPGLAAAELASVRAAATDAGDPSERLAFYFADALSRRLACGTGAPPSAEPDARFASDELTLCYKTLNDACPYSKFAHLTANQAILEATGAATKIHIVDFGIVQGIQWAALLQALATRPEGKPTRIRITGVPSPLLGPQPAASLAATNTRLRDFAKLLGVDFEFVPLLRPVHELNKSDFLVEPDEAVAVNFMLQLYHLLGDSDELVRRVLRLAKSLSPAVVTLGEYEVSLNRAGFVDRFANALSYYRSLFESLDVAMTRDSPERVRVERWMFGERIQRAVGPEEGADRTERMAGSSEWQTLMEWCGFEPVPLSNYARSQADLLLWNYDSKYKYSLVELPPAFLSLAWEKRPLLTVSAWR			
OsGRAS19	GRAS	86-495	LVRLLLSAVAAGEAGDARAAAAALREVDRRASCRGGGDPAQRVAACYAAALAPRLAAGLRPARSSPAAPAAARAEQFLAYTMFYQASPFYQFAHFTANQAIVEAFESGGRRRLHVVDFDVSYGFQWPSLIQSLSDAAAAATSSSSHDDDDNGGGCGDGPVSLRITGFGASADELRETEARLRRFAAGCPNLRFEFEGILNNGSNTRHDCTRIDDDATVVVNLVFPASSREACAATRMAYINSLNPSMVFLIEKHDGGGGLTGGDNTTTGRSASLLPRFAANLRYFAAVFDSLHECLPADSAERLAIERDHLGREIADAVASLDHQHRRRHGGGGGGDHAAASWNWKAAMEGAGLDGVKLSSRTVSQAKLLLKMKSGCGGGGFRVVEGDGGMAMSLAWRDMALATATLWR	LCR	10-42	DGGGGGDAAAAVAKKSKVVGGGAVVVDGVGSSA
ΨOsGRAS5	GRAS	1-80	MHYLRYYDAAFDAVDAAGLLETRPARAKVEEMFAREIRNAVAFEGAERFERHESFAGRRRRMEDGGGLQWGSKAEEKCLL	LCR1	104-124	SLPPAVAAAPLVLPLPRASAA
				LCR2	130-146	APMPPTAAPLVLPPPLP
OsGRAS20	GRAS	352-710	LLDELAAAAKATEVGNSIGAREILARLNQQLPPIGKPFLRSASYLKDALLLALADGHHAATRLTSPLDVALKLTAYKSFSDLSPVLQFANFTVTQALLDEIASTTASCIRVIDFDLGVGGQWASFLQELAHRCGSG	LCR1	52-70	GSPSPPNSTSTLSSSHGSG

			GVSLPMLKLTAFVSAASHHPLELHLTQDNLSQFAADLGIPFEFNAINLDAFDPMELIAPTADEVVAVSLPVGCSARTPLPAMLQLVKQLAPKIVVAIDYGSDRSDLPFSQHFLNCLQSCLCLLESLDAAGTDADAVSKIERFLIQPRVEDAVLGRRRADKAIAWRTVLTSAGFAPQPLSNLAEAQADCLLKRVQVRGFHVEKRGAGLALYWQRGELVSVSAWR			
				LCR2	79-92	VAAVSESSAAAAEA
				LCR3	98-114	PGEHGGGGGGELPPIPG
				LCR4	185-215	SSPAALASDLSSSGGRSLTSSSGSNSKATSA
				LCR5	221-234	PEAALQPPPATTAP
				LCR6	243-255	PPLLGLPSPTLLL
				LCR7	277-286	QQQPLLQPPP
				LCR8	302-312	QPQPPPPAPAQ
OsGRAS22	GRAS	261-634	LTTLLIHCAQAAAIDDHRNSNELLKQIRQRSSAYGDAGQRLAHCFANALEARLAGTGSNIYRSLAAKRTSVYDILNAFKLYVTACPFKKISNFFSIEAILNASKGMTRLHIVDYGIQYGFQWPIFFQRISKRPGGPPSVRITGVDLPQPGFRPAQLIEATGRRLHDYARMFNVPFEYHAIAAKWDTIRVEDLKIDKDKDELLVVNCLFRMRNMMDEMVTDDSPRMQVLKTIRKMNPNLFIHGVVNGTYNAPFFVTRFKEALFYYSSLFDMLETTASRVDENRLLIERDLFGREALNVVACEGTERVERPETYKQWQVRNIRAGFKQLPLNQETVKKARYKVKKSYHRDFLVDEDNKWMLQGWKGRIIFALSAWE	LCR1	2-15	LDSGSYDDVDYGDL
				LCR2	56-76	STPSPTSTTTELENSEDLSES
				LCR3	237-251	KGSGNKRGRKKGKSG
OsGRAS23	GRAS	92-506	IAAFLADGTCQMQVNDGLSCVVDLAGGDADGGGVGEGRSAQRLASAFAEALALRFILPCDGVCRSLHLTRAPPPPAVSAARQGFRAMCPFVRLAAAAANLSIAEVMEAERAVVHVVDLGGGVDANQWVELVRLVAARPGGPPGLLRLTVVNESEDFLSAVAAYVAAEAQRLDLSLQFHPVLSSIEELSATATGSIGSRLVVIPGQPLAVVANLQIHRLLAFPDYVDGVASRRPAAEQSGSSQHTMTTATKTKADALLRAIRDLNPKLVVLTENEADHNVAELGARVWNALNYYAALFDALEASSTPPAAVPPHERACVERWVLGEEIKDIVVREGTGRRERHETLGRWAERMVAAGFSPVTAARALASTETLAQQMVAAGGGGAGAGVLRAAHGGGCFPVICWCDVPVFSVSTWT	LCR1	6-27	PRLALGGGGGGAGGERLPAAGE
				LCR2	51-59	AAMAAAAAA
				LCR3	513-541	PAPPLWPPAAAGGAGPSGSGYGGDGPSTA
OsGRAS24	GRAS	154-558	LHGHLRRCAEALAASRPADADAELASIARMASSDGDAVQRVAAAFAEAMARVVIRPWRGVSAALFPSDAGAAGDALTAWEAEFARQSFLNLCPLLHLAAVAVNEIILETTRNDKFIHIVDLGGIHHAHWVELLQGLATRRAAVRPCLRLTIVHEHKHFLGQAAQVLAAESDRHGVPLDLHIVESSVEALKLDALGVRSDHAVVIVSTLQLHRLVGAGILSTTAPPSPAAAAAASMITSPLPPANMSSKVDRLLRGFHLLSPRAIILTENEANHFVPSFTDRFASALPYYEQLFAAMEEAGAATVERKAAERYLLREEIKDVIACDHDGPRWARHETLGRWVVRMGAAGFALAPAITVVTAAGRVRAVAARLPGGGDERRYGVTEGGGWLILNREEKPMFCVSAWR	LCR1	5-20	AATAAATTTAAATTAA

				LCR2	32-52	MVPVPVASMATATAPAAVAAA
				LCR3	55-83	GGHGSSSASQNASGSGEGQGGSMSLSLQL
				LCR4	89-141	TPTAAVAVSVPPMAAAPMMAGPAAAAPAPAPPLATMAVAQNASLAAVASALAA
OsGRAS25	GRAS	116-484	MIALLMECAAAMSVGNLAGANGALLELSQMASPYAASCGERLVAYFARAMAARLVGSWVGVVAPMAPPPSCGAINAAFRALYNVAPFARLAYLACNQAILEAFHGKRLVHIVDLDVVPGGALQWLSLLPALAARPGGPPVIRVTGFGMSASVLHDTGNQLAGLARKLCMFFEFYAVAKRPGDADAVADMPGRRPGEAVAVHWLRHAMYDAAGDDGASMRLVRWLEPAAVTLVEQERAHGGGGGHGRFLDRFVSALHHYSAVFDAMGASRPDGEDASRHLAEHGVLGREIANVLAVGGPARSSGREGPGSWREVLARHGFAHAGGGGGGRAQLVAAACPGGLGYTVAGDHDGTVRLGWKGTPLYAVSAWT	LCR1	13-36	HHQYLYSSSSSNLPLQQPLLSHHH
				LCR2	98-111	ADVEQVAVEDEEEA
OsGRAS26	GRAS	36-423	IQQLLLHCAAALESNDVTLAQQAMWVLNNIASSQGDPSQRLTSWLLRALVARACRLCAAAPAGAAVEFLERGRAPPWGRAMSVTELADYVDLTPWHRFGFTASNAAILRAVAGASAVHVVDLSVTHCMQWPTLIDVLSKRPGGAPAIRITVPSVRPAVPPLLAVSSSELGARLAIFAKSKGVQLEFNVVESATTTSPKKTSTTLCQELASVLSDPPSLGLRDGEAVVVNCQSWLRHVAPDTRDLFLDTVRALNPCLLTVTDEDADLGSPSLASRMAGCFDFHWILLDALDMSAPKDSPRRLEQEAAVGRKIESVIGEEDGAERSEPGARLAERMSRKGFAGVVFDEEAAAEVRRLLSEHATGWGVKREDDMLVLTWKGHAAVFTGAWT	LCR	9-20	GGGVGAAAHGHG
OsGRAS28	GRAS	126-479	LVDDLLDAARLLDAGDSTSAREILARLNHRLPSLPSPPGHAHPPLLRAAALLRDALLPPTALPVSSTPLDVPLKLAAHKALADASPTVQFTTFTSTQAFLDALGSARRLHLLDFDVGFGAHWPPLMQELAHHWRRAAGPPPNLKVTALVSPGSSHPLELHLTNESLTRFAAELGIPFEFTALVFDPLSSASPPLGLSAAPDEAVAVHLTAGSGAFSPAPAHLRVVKELRPAVVVCVDHGCERGALNLLQSCAALLESLDAAGASPDVVSKVEQFVLRPRVERLAVGGGDKLPPPLQSMLASAGFAALQVSNAAEAQAECLLRRTASHGFHVEKRQAALALWWQRSELVSVSAWR	LCR	35-66	SSPSTSLGSCSSKPPEDPPPPIAADDDCDWDA
OsCIGR1	GRAS	201-571	VKQLLTRCAEALSEDRTEEFHKLVQEARGVVSINGEPIQRLGAYLLEGLVARHGNSGTNIYRALKCREPESKELLSYMRILYNICPYFKFGYMAANGAIAEALRTENNIHIIDFQIAQGTQWITLIQALAARPGGPPRVRITGIDDPVSEYARGEGLDIVGKMLKSMSEEFKIPLEFTPLSVYATQVTKEMLEIRPGEALSVNFTLQLHHTPDESVDVNNPRDGLLRMVKGLSPKVTTLVEQESHTNTTPFLMRFGETMEYYSAMFESIDANLPRDNKERISVEQHCLAKDIVNIIACEGKDRVERHELLGKWKSRLTMAGFRPYPLSSYVNSVIRKLLACYSDKYTLDEKDGAMLLGWRSRKLISASAWH			
OsGRAS32	GRAS	68-434	LLSLLLRCAEAVAMDQLPEARDLLPEIAELASPFGSSPERVAAYFGDALCARVLSSYLGAYSPLALRPLAAAQSRRISGAFQAYNALSPLVKFSHFTANQAIFQALDGEDRVHVIDLDIMQGLQWPGLFHILASRPTKPRSLRITGLGASLDVLEATGRRLADFAASLGLPFEFRPIEGKIGHVADAAALLGPRHHGEATVVHWMHHCLYDVTGSDAGTVRLLKSLRPKLITIVEQDLGHSGDFLGRFVEALHYYSALFDALGDGAGAAEEEAAERHAVERQLLGAEIRNIVAVGGPKRTGEVRVERWGDELRRAGFRPVTLAGSPAAQARLLLGMYPWKGYTLVEEDGCLKLGWKDLSLLTASSWE	LCR1	8-45	RAPGADAAAMKAKRAADDEEEGGERERARGKRLAAEGK

				LCR2	55-62	EEEEAAAE
				LCR3	437-449	DGDADADVAVAGD
OsCIGR2	GRAS	174-544	LKELLIACARAVEEKNSFAIDMMIPELRKIVSVSGEPLERLGAYMVEGLVARLASSGISIYKALKCKEPKSSDLLSYMHFLYEACPYFKFGYMSANGAIAEAVKGEDRIHIIDFHISQGAQWISLLQALAARPGGPPTVRITGIDDSVSAYARGGGLELVGRRLSHIASLCKVPFEFHPLAISGSKVEAAHLGVIPGEALAVNFTLELHHIPDESVSTANHRDRLLRMVKSLSPKVLTLVEMESNTNTAPFPQRFAETLDYYTAIFESIDLTLPRDDRERINMEQHCLAREIVNLIACEGEERAERYEPFGKWKARLTMAGFRPSPLSSLVNATIRTLLQSYSDNYKLAERDGALYLGWKSRPLVVSSAWH			
OsSHR1	GRAS	192-601	ASQLLLECARSVAARDSQRVQQLMWMLNELASPYGDVEQKLASYFLQGLFARLTASGPRTLRTLAAASDRNTSFDSTRRTALRFQELSPWSSFGHVAANGAILESFLEVAAAASSETQRFHILDLSNTFCTQWPTLLEALATRSADETPHLSITTVVSAAPSAPTAAVQRVMREIGQRMEKFARLMGVPFRFRAVHHSGDLAELDLDALDLREGGATTALAVNCVNSLRGVVPGRARRRDAFAASLRRLDPRVVTVVEEEADLVASDPDASSATEEGGDTEAAFLKVFGEGLRFFSAYMDSLEESFPKTSNERLALERGAGRAIVDLVSCPASESMERRETAASWARRMRSAGFSPVAFSEDVADDVRSLLRRYREGWSMREAGTDDSAAGAGVFLAWKEQPLVWASAWR	LCR1	11-40	QAASEQQQQQQSASYNSRSTTSSGSRSSS
				LCR2	46-80	SYSYYHHSSNSGGGGGGGGGYYYGGQQPPPSQYYY
				LCR3	122-147	PPASSTPTGTAPTPPLSTSSTAAGAG
OsGRAS35	GRAS	76-471	MEQLLVHCANAIEANDATLTQQILWVLNNIAPADGDSNQRLTAAFLCALVSRASRTGACKAVTAAVADAVESAALHVHRFTAVELASFIDLTPWHRFGYTAANAAIVEAVEGFPVVHIVDLSTTHCMQIPTLIDMLAGRAEGPPILRLTVADVAPSAPPPALDMPYEELGAKLVNFARSRNMSMDFRVVPTSPADALTSLVDQLRVQQLVSDGGEALVVNCHMLLHTVPDETAGSVSLTTAQPPVSLRTMLLKSLRALDPTLVVVVDEDADFTAGDVVGRLRAAFNFLWIPYDAVDTFLPKGSEQRRWYEAEVGWKVENVLAQEGVERVERQEDRTRWGQRMRAAGFRAAAFGEEAAGEVKAMLNDHAAGWGMKREDDDLVLTWKGHNVVFASAWA	LCR1	9-25	PPPPPLHPNGHGLGLGL
				LCR2	34-54	GGGGARPWSSSSSTTTLGGSG
OsSCR1	GRAS	283-644	LLTLLLQCAESVNADNLDEAHRALLEIAELATPFGTSTQRVAAYFAEAMSARLVSSCLGLYAPLPNPSPAAARLHGRVAAAFQVFNGISPFVKFSHFTANQAIQEAFEREERVHIIDLDIMQGLQWPGLFHILASRPGGPPRVRLTGLGASMEALEATGKRLSDFADTLGLPFEFCPVADKAGNLDPEKLGVTRREAVAVHWLRHSLYDVTGSDSNTLWLIQRLAPKVVTMVEQDLSHSGSFLARFVEAIHYYSALFDSLDASYSEDSPERHVVEQQLLSREIRNVLAVGGPARTGDVKFGSWREKLAQSGFRVSSLAGSAAAQAVLLLGMFPSDGYTLIEENGALKLGWKDLCLLTASAWR	LCR1	3-55	SSSLLLFPSSSSSATHSSYSPSSSSHAITSLLPPLPSDHHLLLYLDHQEQHHL
				LCR2	86-101	AAAAPSSASAQLPALP
				LCR3	115-134	AAPAPPPPQQQVAAGEGGPP
				LCR4	148-157	ASSGAAVSVA

				LCR5	188-229	SDPAPPPPPPPSHPALLPPDATAPPPPPTSVAALPPPPPPQP
				LCR6	235-279	EPQCQEQEPNQPQSPKPPTAEETAAAAAAAKERKEEQRRKQRDEE
ΨOsGRAS8	GRAS	199-356	LRELLMSCAQAVASGNRRSAGELLEQIKRHSSPTGDATERLAHYFADGLEARLAGAASLERRLVASAEERASAMELLEAYQVFMAACCFKWVAFTFANMAILRAAEGRNRLHIVDYGGQYHGLQWPSLLQRLAEREGGPPEFRAVAAARWETVTAEDV	LCR1	29-40	PAAPPSEAAAAA
	GRAS	339-542	EFRAVAAARWETVTAEDVVGVDPDDEAAVVVNDVLSLGTLMDESGVFDDPSPRDTVLGSIRDMRPAVFVQAVVNGAHGAPFFPTRFREALFFFSALFDMLGATTPEEGSHLRVVLERDVLRRAAVGVIAGEGAERVERPETYRRWQARNRRAGLRQAAVEGDVVEAVRRRVRRRHHEEFVIEEDAGWLLQGWKGRILYAHSAWV	LCR2	108-122	GSGNGRGRKGSKHGG
				LCR3	140-147	EEEEDDDD
				LCR4	177-193	AEKKCGKAARRRRRQAK
OsGRAS39	GRAS	67-365	RDVLVVHIVDLSCSAAHPWQWPKLLDDFHGRPGGAPELYLTVLHDDNDFLADMQSLLSKKAESLGVSFHFISVIGRLETLDFSNLRSTFQIKFGVAVAISCALQMHRLLLVDDNLSSTSIAQLQKMANFTQPKQMASSVCSPASTLNYLQTPSPRTPKLLARLLSAIRALKPNIMLIMEQDADHNTLLFRDRFNEVLNYYAALFDCFHAVAAANPGRTDERLRVDRMILREEIKNILVCEGVHRHERHERLDQWAMHMEESGFHNVQLSFSAIREAYVWQLKVQADNLRLCCTDRGMFQ	LCR	370-391	SSATSSPASSVYSPSPSPSNGS
	GRAS	405-843	LIGLLYQCAAEVSAGSFDRANLCLEHITQLASLDAPHALQRLAAVFADALARKLLNLILGLSRALLSSANSADAHLVPVARRHMFDVLPFLKLAYLTTNHAILEAMEGERFVHVVDFSGPAANPVQWIALFHAFRGRREGPPHLRITAVHDSKEFLANMAAVLSKEAEAFDIAFQFNAVEAKLDEMDFDALRHDLGVRSGEALAVSVVLQLHRLLAVDDGRRHAAAGCLTPVQIIARSSPRSFGELLERELNTRLQLSPDASVVSSLSPHSPAAATAAHPTTSTPKLGSFLSAVRSLSPKIMVMTEQEANHNGGAFQERFDEALNYYASLFDCLQRSAAAAAERARVERVLLGEEIRGVVACEGAERVERHERARQWAARMEAAGMERVGLSYSGAMEARKLLQSCGWAGPYEVRHDAGGHGFFFCWHKRPLYAVTAWR			
OsGRAS41	GRAS	4-339	LSDLLLAGAEAVEAGDSILASVAFSRLDDFLSGIPENGAASSFDRLAYHFDQGLRSRMSSASTGCYQPEPLPSGNMLVHQIIQELSPFVKFAHFTTNQAILDAIIGDMDVHVVDLNIGEGIQWSSLMSDLARCGGKSFRLTAITTYADCHASTHDTVVRLLSEFADSLELPFQYNSICVHNEDELHAFFEDCKGSVIVSCDTTSMYYKSLSTLQSLLLVCVKKLQPKLVVTIEEDLVRIGRGVSPSSASFVEFFFEALHHFTTVFESMASCFIGSSYEPCLRLVEMELLGPRIQDFVVKYGSVRVEANASEVLEGFMACELSACNIAQARMLVGLFNRVFGVVFKKISLLMVY			
	Transmemebrane region	354-376	VIWSSLAAGCGSHGIVVLAFYAA			

ΨOsGRAS9	GRAS	1-129	MSHLENTLEARLAGTGSQMYQSLVAKRTSTVDFLKAYKLFTAACCVKKTIYNAVAGKRKLHIVDYGLSYGFQWPALFFLLGTREGGPPEVRMTGIDVPQPGFRPADQIEETGRRLSICARAPVRCAIQV	LCR	216-254	SRSAPSSSPPRPSLHLHLHLRRRPPPSSSRHAADDAALH
OsGRAS43	RPT1	94-113	ASPRRDFMACSPKRDYMVTT	LCR1	2-13	AGGGAKLQQQQA
	RPT1	114-134	SSPKRDYMVTSSPKRDYMVSS	LCR2	165-177	HGGGGGGGHHLHH
	GRAS	401-771	LVHLLLACADLVSKGDHPAALRHLHLLRRVASPLGDSMQRVASHFADALAARLSLLSSPTSASPSPRAAAAAAPYPFPPSPETLKVYQILYQACPYIKFAHFTANQAIFEAFHGEDRVHVVDLDILQGYQWPAFLQALAARPGGPPTLRLTGVGHPPAAVRETGRHLASLAASLRVPFEFHAAAADRLERLRPAALHRRVGEALAVNAVNRLHRVPSSHLPPLLSMIRDQAPKIITLVEQEAAHNGPYFLGRFLEALHYYSAIFDSLDATFPAESTARMKVEQCLLAPEIRNVVACEGAERVARHERLERWRRLMEGRGFEAVPLSAAAVGQSQVLLGLYGAGDGYRLTEDSGCLLLGWQDRAIIAASAWR	LCR3	183-201	GGGMEGGGGGHGAQPQYGG
				LCR4	223-230	GGGSGGGG
				LCR5	280-288	GGVGGGGGG
				LCR6	293-306	SGASVSVVTAPASS
				LCR7	326-341	GGGDEAVAAAMAVAGE
				LCR8	355-364	GGGGEFGGEG
OsGRAS44	GRAS	303-683	LHTLLIHCAQAVATSDRRSATELLKQIKQNSSARGDATQRLACCFAEGLEARLAGTGSQVYKSLVAKCTSTVDFLKAYKLFAAACCIKKVSFIFSNKTILDAVAGKRKLHIVDYGLSYGFQWPGLFKCLSEREGGPPEVRITGIDFPQPGFRPADQIEETGRRLSNCARQFGVPFRFQAIAAKWETVRREDLHLDREEEEEEEEVLVVNCLHFLNALQDESVVVDSPSPRDMVLNNIRDMRPHVFVQCVVNGAYGAPFFLTRFRETLFFYSSQFDMLDATIPRDNDERLLIERDILGRWALNVIACEGADRVDRPETYKQWLVRNHRAGLTQLPLQPQVVELVRDKVKKLYHKDFVIDVDHNWLLQGWKGRILYAMSTWV	LCR1	14-27	LEPFSPSLFLDLPP
				LCR2	82-111	SDDTTTNSSDDSASATTNNTTNSAAAANAS
				LCR3	209-218	GRSGGSGRGR
				LCR4	279-299	AEKKARNGGGAGRRAARAKAA
OsGRAS47	GRAS	215-586	LRMLLIQCAQAMATDNQQSAGELLKKIKQHALATGDAMQRVAHYFAKGLEARLAGSGKHLYQNHVRMSLVEYLKVYKLYMAACCFKKVALMFAAMTIMQAVQGKKRLHIVDYGIRCGLHWPDLFRRLGSREDGPPEVRITIVDIPQPGFRPFQRIEAAGHCLSSCANEFRVPFRFQAVVAAKWETVGAEDLHIEPDEVLVVNDLWSFSALMDESIFCDGPNPRDVALRNISKMQPDVFIQGIINGGYGASFLSRFRGALLYYSALFDMLDATTPRESGLRLALEQNVLGPYALNAIACEGADLVERPEKYRQWQARNHRAGMQQLKLRPDIVDTIREEVNKYHHKDFLLGEDGQWLLQGWMGRVLFAHSAWV	LCR	201-211	KKKGKKGSSSK
ΨOsGRAS10	RPT1	61-73	FLDMMVIQESANE	LCR1	22-30	SSSSLLLWS
	RPT1	117-129	FLEMMAIQESAND	LCR2	47-60	DADHSHDQIHQDHQ
	GRAS	173-308	AGDLLLAGAMAVDAGDAVHASAIMSRLDDLLADIAGRRSCEATSPVDHLAYYFARGLKLRISGAATPASSPPPPAANWSSPAYRMLQELTPFVKFAHFTANQAILEATADDLDVHVVDFNVGEGVQWSSLMLKLLL			

	GRAS	305-460	KLLLLGTITILQPKLVILIEDELSRISKNPPSPSLAAPPPFPEFFSDAVAHFTAVMESTASCLVSYDDEAWLSLRRVGEEVVGPRVEDAVGRYGSLAGGAQMMEGLRAREVSGFSVAQGKMLAGLFGGGFGVVHQEKGRLALCWKSRPLISVSLWC			
OsGRAS53	RPT1	190-207	FLKGMEEANKFLPTENKL	LCR1	53-65	PPSPPPPTTTATT
	RPT1	219-236	YLRGLEEAKRFLPSDDKL	LCR2	116-135	LSDPSSNSRSSNSDDPRLSP
	GRAS	358-730	LRTLLIHCAQAVATDDRRSATELLKQIKQHAKPTGDATQRLAHCFAEGLQARIAGTGSLVHQSLVAKRTSAVDILQAYQLYMAAICFKKVSFIFSNQTIYNASLGKKKIHIVDYGIQYGFQWPCFLRRISQREGGPPEVRMTGIDLPQPGFRPTERIEETGHRLSKYAQEFGVPFKYNAIAAVKMESVRKEDLNIDPDEVLIVNCQYQFKNLMDESVVIDSPRDIVLSNIRKMQPHVFIHAIVNGSFSAPFFVTRFREALFFYSALFDVLDATTPRESEQRLLIEQNIFGRAALNVIACEGIDRVERPETYKQWQVRNQRAGFKQLPLNPEIVQVVRNKVKDCYHKDFVIDIDHQWLLQGWKGRILYAISTWT	LCR3	150-162	AAATATAVAAAAV
				LCR4	241-260	AAAAAPVVSVKKEAVDVVVA
				LCR5	337-356	GGKGGNGKVKGGRRGGRDVV

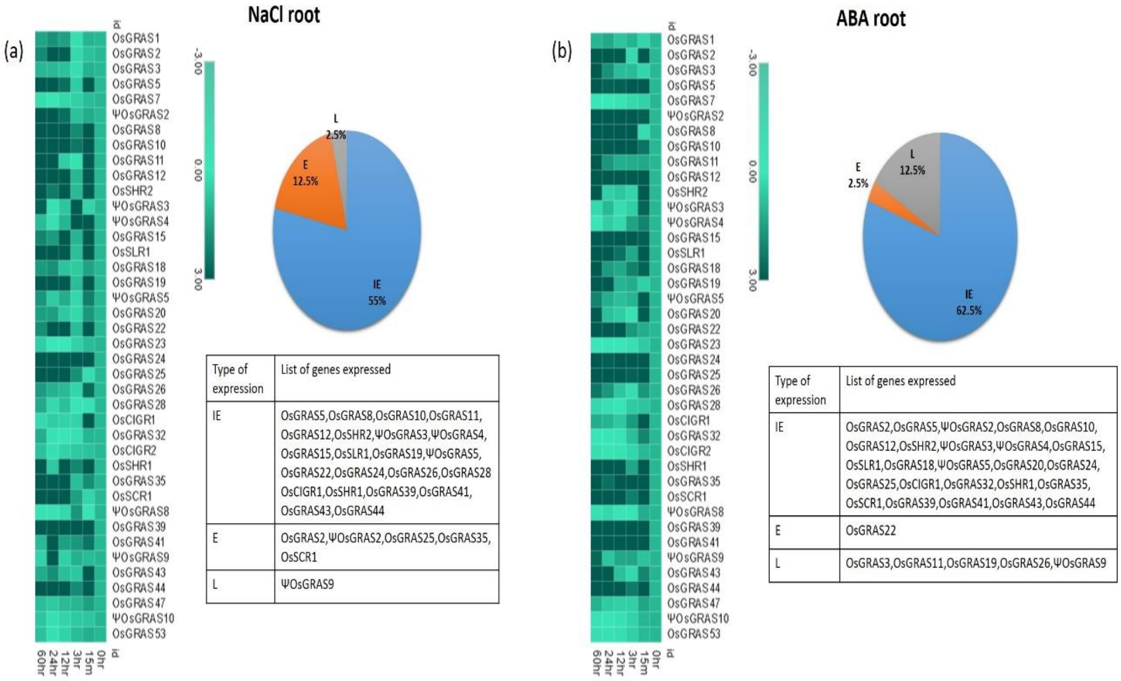
6.7.Expression analysis under simulated abiotic stress conditions

We identified a GRAS transcription factor (*ψOsGRAS4*) as a potential stress tolerance gene associated with enhanced water use efficiency by screening a pool of gain-of-function mutants in rice in our previous study (Moin et al., 2016). Another report by (Xu et al., 2015) suggested the role of *OsGRAS23* (reported as *OsGRAS22* in this study) in drought tolerance in rice. These observations have prompted us to analyze the differential expression pattern of the GRAS family of transcription factors under the influence of biotic and simulated abiotic stress conditions in the present study. We have analyzed the expression patterns of 40 selected genes separately in shoot and root tissues at six different time points for two abiotic (NaCl and ABA) and two biotic (BLB and SB) stresses. The native expression patterns of these genes in 13 different tissues were also studied. Based on the pattern of expression, we have divided the genes as immediate early (IE), early (E) and late (L) responsive genes. Some genes were expressed up to 100 folds after the incidence of stress. Thus, the genes were also categorized as expressive (2-10 fold), moderately expressive (10 -30 fold) and highly expressive (≥30 fold) types. Genes showing upregulation of ≥2 folds were considered as expressive.

The majority of the genes got upregulated in the root (Fig. 6.8 a, b) compared to the shoot (Fig. 6.8 c, d). As indicated in the pie chart, about 55-60% of the total genes showed IE type expression under both NaCl and ABA treatments. NaCl, however, induced more early (12.5%) responsive genes than late (2.5%), whereas ABA induced more late genes (12.5%) than early (2.5%). The list of the expressed genes has been provided in figure 6.8. More than half of IE genes continued their expression until 60 h of treatment, while others became downregulated or showed no expression later during the experimental timeline. Under ABA treatment, all highly upregulated genes, i.e. *ΨOsGRAS2, OsSHR1, OsSCR1* and *OsGRAS39,* were IE type and their expression persisted till the last time point of treatment, i.e. 60 h. Other IE type genes showed a split before increasing their expression at subsequent time points. Only *OsGRAS39* was highly expressive under both ABA and NaCl treatments (100 fold and 65 fold, respectively). *OsGRAS2*, *ΨOsGRAS2, OsGRAS25*, *OsGRAS35* and *OsSCR1* under NaCl and *OsGRAS22* under ABA were early (E) expressed genes, respectively, while *ΨOsGRAS9, OsGRAS3, OsGRAS11* and *OsGRAS26* under ABA and *ΨOsGRAS9* became upregulated under NaCl treatment with late (L) expression.

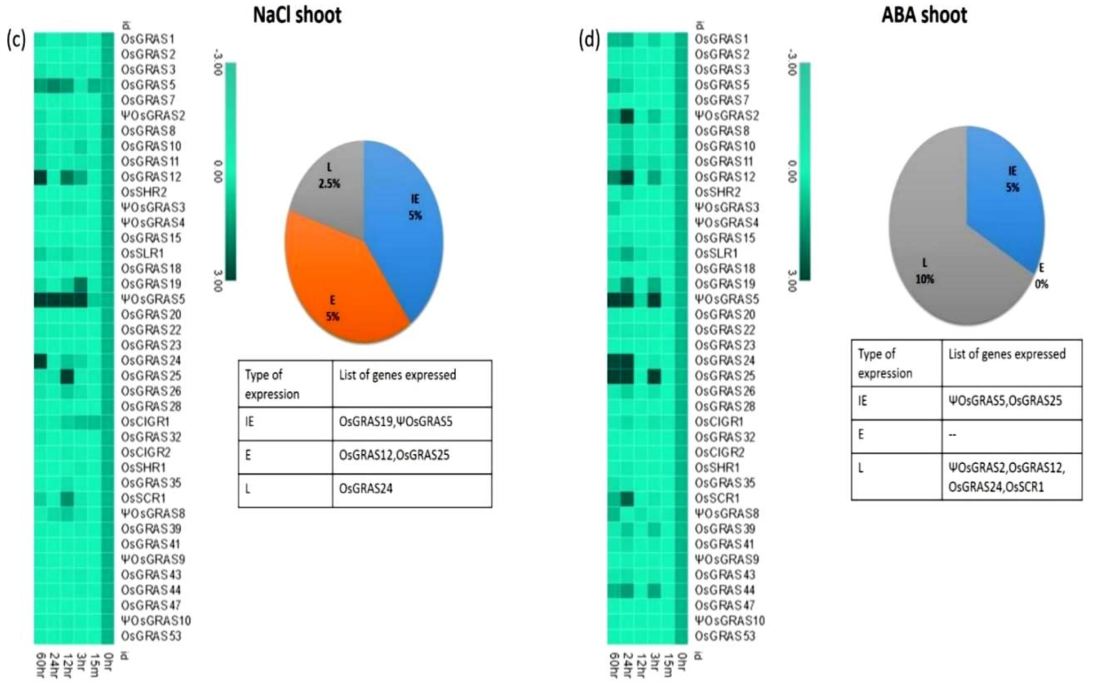
Twelve and thirteen genes (30 and 32%) were mild to moderately expressed, respectively, under the ABA treatment, whereas seven genes (17%) were moderately expressive under NaCl treatment and the rest 19 genes (47%) exhibited mild expression. Nine genes (22%) under ABA and thirteen (32%) genes under NaCl treatment were either downregulated or showed no change in the level of expression. Among them, *OsGRAS7, OsGRAS23, OsGRAS28* and *ΨOsGRAS8* were downregulated under both treatments. *ΨOsGRAS3, ΨOsGRAS4, OsCIGR1* and *OsGRAS32* under ABA treatment and *OsCIGR1* under NaCl treatment showed an immediate expression but were either downregulated or showed no expression at subsequent time points.

Very few genes were expressed in the shoot. *ΨOsGRAS5* was the only gene, which showed moderate expression (25-30 fold) under both ABA and NaCl treatments. This IE type gene maintained its expression till 60 h under NaCl but showed a split before reaching a peak under the ABA treatment. On the contrary, it showed low expression (2-3 fold) in root tissues under ABA and NaCl treatments. Among the other genes that were mildly expressive in both root and shoots were *ΨOsGRAS2, OsGRAS12, OsGRAS19, OsGRAS24, OsGRAS25* and *OsSCR1*. The rest of the genes were mainly downregulated or did not show any change in expression in shoot tissues under both the stress treatments. The expression level of all the genes studied has been provided in table 6.5.



Type of expression	List of genes expressed
IE	OsGRAS5,OsGRAS8,OsGRAS10,OsGRAS11, OsGRAS12,OsSHR2,ΨOsGRAS3,ΨOsGRAS4, OsGRAS15,OsSLR1,OsGRAS19,ΨOsGRAS5, OsGRAS22,OsGRAS24,OsGRAS26,OsGRAS28 OsCIGR1,OsSHR1,OsGRAS39,OsGRAS41, OsGRAS43,OsGRAS44
E	OsGRAS2,ΨOsGRAS2,OsGRAS25,OsGRAS35, OsSCR1
L	ΨOsGRAS9

Type of expression	List of genes expressed
IE	OsGRAS2,OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS10, OsGRAS12,OsSHR2,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15, OsSLR1,OsGRAS18,ΨOsGRAS5,OsGRAS20,OsGRAS24, OsGRAS25,OsCIGR1,OsGRAS32,OsSHR1,OsGRAS35, OsSCR1,OsGRAS39,OsGRAS41,OsGRAS43,OsGRAS44
E	OsGRAS22
L	OsGRAS3,OsGRAS11,OsGRAS19,OsGRAS26,ΨOsGRAS9



Type of expression	List of genes expressed
IE	OsGRAS19,ΨOsGRAS5
E	OsGRAS12,OsGRAS25
L	OsGRAS24

Type of expression	List of genes expressed
IE	ΨOsGRAS5,OsGRAS25
E	--
L	ΨOsGRAS2,OsGRAS12, OsGRAS24,OsSCR1

Fig. 6.8: Expression analysis of *GRAS* genes under abiotic stress

Heat map representation of temporal expression pattern of *GRAS* genes developed using MORPHEUS program. 7 d old seedlings were subjected to NaCl (250 µM) and ABA (100 µM) treatments, and the obtained quantitative real-time values were double normalized using rice *actin* and *tubulin* as the internal reference genes and that of the unstressed samples using the $\Delta\Delta C_T$ method. The experiment was conducted separately for root (a, b) and shoot (c, d) tissues. The percentage of genes upregulated under NaCl and ABA treatments are represented in the form of a pie chart beside their corresponding heat maps. The genes were separated based on their time point(s) of expression and annotated as immediate early (IE), early (E) and late (L) expressive genes. The names of the genes were provided in the list below.

Table 6.5: List of *GRAS* genes and their expression pattern under NaCl and ABA treatments

Gene name	Locus number	Regulation (up/down)	Type of Response	Max fold change	Regulation (up/down)	Type of Response	Max fold change
		Root			Shoot		
NaCl							
OsGRAS1	LOC_Os01g45860	DOWN	-	-	-	-	-
OsGRAS2	LOC_Os01g62460	UP	E(2.9)	3.3	-	-	-

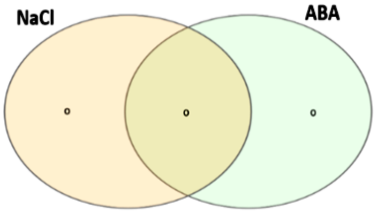
OsGRAS3	LOC_Os01g65900	DOWN	-	-	-	-	-
OsGRAS5	LOC_Os01g71970	UP	IE (5.5)	6.1	-	-	-
OsGRAS7	LOC_Os02g10360	DOWN	-		DOWN	-	-
ΨOsGRAS2	LOC_Os02g21685	UP	E(2.6)	18.8	DOWN	-	-
OsGRAS8	LOC_Os02g44360	UP	IE(5.6)	12	DOWN	-	-
OsGRAS10	LOC_Os02g45760	UP	IE(12.4)	12.4	DOWN	-	-
OsGRAS11	LOC_Os03g09280	UP	IE(3.8)	4.8	DOWN	-	-
OsGRAS12	LOC_Os03g15680	UP	IE(9.5)	9.5	UP	E(2.1)	6.8
OsSHR2	LOC_Os03g31880	UP	IE(3.6)	4.3	DOWN	-	-
ΨOsGRAS3	LOC_Os03g37900	UP	IE(3.7)	3.7	DOWN	-	-
ΨOsGRAS4	LOC_Os03g40080	UP	IE(3.0)	16.2	DOWN	-	-
OsGRAS15	LOC_Os03g48450	UP	IE(3.8)	3.8	DOWN	-	-
OsSLR1	LOC_Os03g49990	UP	IE(3.3)	11	DOWN	-	-
OsGRAS18	LOC_Os03g51330	-	-	-	DOWN	-	-
OsGRAS19	LOC_Os04g35250	UP	IE(4.2)	6.4	UP	IE(2.1)	2.1
ΨOsGRAS5	LOC_Os04g37440	UP	IE(2.2)	2.2	UP	IE(7.7)	25.2
OsGRAS20	LOC_Os04g46860	DOWN	-	-	DOWN	-	-
OsGRAS22	LOC_Os04g50060	UP	IE(3.2)	4.6	DOWN	-	-
OsGRAS23	LOC_Os05g31380	DOWN			DOWN	-	-
OsGRAS24	LOC_Os05g31420	UP	IE(4.0)	8.6	UP	L(4.6)	4.6
OsGRAS25	LOC_Os05g40710	UP	E(4.5)	4.5	UP	E(4.8)	4.8
OsGRAS26	LOC_Os05g42130	UP	IE(2.6)	2.6	DOWN	-	-
OsGRAS28	LOC_Os06g01620	DOWN	-	-	DOWN	-	-
OsCIGR1	LOC_Os07g36170	UP	IE(3.2)	3.2	DOWN	-	-
OsGRAS32	LOC_Os07g38030	DOWN	-	-	DOWN	-	-
OsCIGR2	LOC_Os07g39470	DOWN	-	-	DOWN	-	-
OsSHR1	LOC_Os07g39820	UP	IE(7.3)	11.1	DOWN	-	-
OsGRAS35	LOC_Os07g40020	UP	E(6.8)	17.4	DOWN	-	-
OsSCR1	LOC_Os11g03110	UP	E(3.8)	5.7	DOWN	-	-
ΨOsGRAS8	LOC_Os11g04400	DOWN			DOWN	-	-
OsGRAS39	LOC_Os11g04570	UP	IE(3.6)	65.3	DOWN	-	-
OsGRAS41	LOC_Os11g06180	UP	IE(2.4)	4.1	DOWN	-	-

ΨOsGRAS9	LOC_Os11g11600	UP	L(8.0)	8	DOWN	-	-
OsGRAS43	LOC_Os11g31100	UP	IE(3.5)	3.5	DOWN	-	-
OsGRAS44	LOC_Os11g47870	UP	IE(2.9)	5.8	DOWN	-	-
OsGRAS47	LOC_Os11g47910	-	-	-	DOWN	-	-
ΨOsGRAS10	LOC_Os12g06540	DOWN	-	-	DOWN	-	-
OsGRAS53	LOC_Os12g38490	DOWN	-	-	DOWN	-	-
ABA							
		Root			**Shoot**		
OsGRAS1	LOC_Os01g45860	-	-	-	DOWN	-	-
OsGRAS2	LOC_Os01g62460	UP	IE(3.3)	5.6	DOWN	-	-
OsGRAS3	LOC_Os01g65900	UP	L(3.5)	3.5	DOWN	-	-
OsGRAS5	LOC_Os01g71970	UP	IE(6.2)	12.2	DOWN	-	-
OsGRAS7	LOC_Os02g10360	DOWN	-	-	DOWN	-	-
ΨOsGRAS2	LOC_Os02g21685	UP	IE(2.8)	32.7	UP	L(3.5)	3.5
OsGRAS8	LOC_Os02g44360	UP	IE(5.5)	27.9	DOWN	-	-
OsGRAS10	LOC_Os02g45760	UP	IE(2.4)	16.7	DOWN	-	-
OsGRAS11	LOC_Os03g09280	UP	L(4.1)	4.1	DOWN	-	-
OsGRAS12	LOC_Os03g15680	UP	IE(8.2)	8.2	UP	L(3.8)	3.8
OsSHR2	LOC_Os03g31880	UP	IE(4.5)	14.3	DOWN	-	-
ΨOsGRAS3	LOC_Os03g37900	UP	IE(2.8)	2.8	DOWN	-	-
ΨOsGRAS4	LOC_Os03g40080	UP	IE(2.1)	2.1	DOWN	-	-
OsGRAS15	LOC_Os03g48450	UP	IE(2.9)	14.6	DOWN	-	-
OsSLR1	LOC_Os03g49990	UP	IE(7.9)	30.2	DOWN	-	-
OsGRAS18	LOC_Os03g51330	UP	IE(2.8)	3.4	DOWN	-	-
OsGRAS19	LOC_Os04g35250	UP	L(3.5)	11	DOWN	-	-
ΨOsGRAS5	LOC_Os04g37440	UP	IE(2.9)	2.9	UP	IE(8.2)	31.6
OsGRAS20	LOC_Os04g46860	UP	IE(2.9)	4.1	DOWN	-	-
OsGRAS22	LOC_Os04g50060	UP	E(5.5)	14.2	DOWN	-	-
OsGRAS23	LOC_Os05g31380	DOWN	-	-	DOWN	-	-
OsGRAS24	LOC_Os05g31420	UP	IE(15.3)	15.3	UP	L(6.9)	6.9
OsGRAS25	LOC_Os05g40710	UP	IE(6.2)	14.2	UP	IE(3.0)	10.5
OsGRAS26	LOC_Os05g42130	UP	L(2.4)	2.4	DOWN	-	-

OsGRAS28	LOC_Os06g01620	DOWN	-	-	DOWN	-	-
OsCIGR1	LOC_Os07g36170	UP	IE(7.3)		DOWN	-	-
OsGRAS32	LOC_Os07g38030	UP	IE(2.09)		DOWN	-	-
OsCIGR2	LOC_Os07g39470	DOWN			DOWN	-	-
OsSHR1	LOC_Os07g39820	UP	IE(14.1)	66.3	DOWN	-	-
OsGRAS35	LOC_Os07g40020	UP	IE(15.9)	15.9	DOWN	-	-
OsSCR1	LOC_Os11g03110	UP	IE(6.7)	34.3	UP	L(2.4)	2.4
ΨOsGRAS8	LOC_Os11g04400	DOWN	-	-	DOWN	-	-
OsGRAS39	LOC_Os11g04570	UP	IE(12)	101.4	DOWN	-	-
OsGRAS41	LOC_Os11g06180	UP	IE(5)	21.3	DOWN	-	-
ΨOsGRAS9	LOC_Os11g11600	UP	L(2.7)	2.7	DOWN	-	-
OsGRAS43	LOC_Os11g31100	UP	IE(2.07)	7.3	DOWN	-	-
OsGRAS44	LOC_Os11g47870	UP	IE(19.2)	19.2	DOWN	-	-
OsGRAS47	LOC_Os11g47910		-	-	DOWN	-	-
ΨOsGRAS10	LOC_Os12g06540	DOWN	-	-	DOWN	-	-
OsGRAS53	LOC_Os12g38490	DOWN	-	-	DOWN	-	-

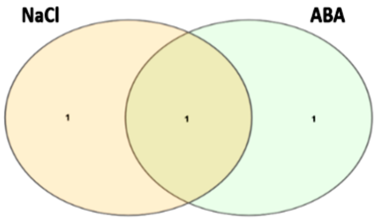
Among the genes studied, some genes were expressed only under NaCl or ABA treatments at certain time points, whereas some were found to be expressive under both treatments. Such overlaps have been depicted in the form of Venn diagrams in figure 6.9. The corresponding list of genes demonstrated that several genes were up/down-regulated simultaneously under both ABA and NaCl treatments at specific time points. In roots, the expression of 37.5% of the genes (IE type) overlapped under both stress treatments, while in shoots, only *ΨOsGRAS5* (IE) was expressive.

I.a) 15min shoot (upregulated genes)



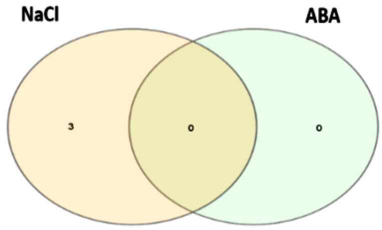
Treatment	Number of genes	Name of genes
NaCl	0	-
ABA	0	-
NaCl+ABA	0	-

I.b) 3h shoot (upregulated genes)



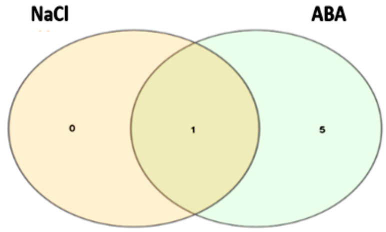
Treatment	Number of genes	Name of genes
NaCl	1	OsGRAS19
ABA	1	OsGRAS25
NaCl+ABA	1	ΨOsGRAS5

I.c) 12h shoot (upregulated genes)



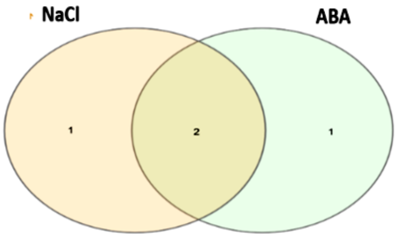
Treatment	Number of genes	Name of genes
NaCl	3	OsGRAS12,ΨOsGRAS5, OsGRAS25
ABA	0	-
NaCl+ABA	0	-

I.d) 24h shoot (upregulated genes)



Treatment	Number of genes	Name of genes
NaCl	0	
ABA	5	ΨOsGRAS2,OsGRAS12 OsGRAS24,OsGRAS25 OsSCR1
NaCl+ABA	1	ΨOsGRAS5

I.e) 60h shoot (upregulated genes)

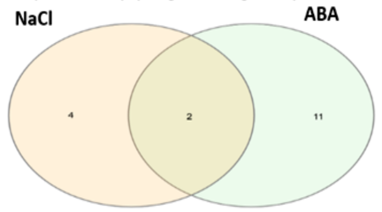


Treatment	Number of genes	Name of genes
NaCl	1	OsGRAS12
ABA	1	OsGRAS25
NaCl+ABA	2	ΨOsGRAS5 OsGRAS24

II.a) 15min root (upregulated genes)

Treatment	Number of genes	Name of genes
NaCl	5	OsGRAS8,OsGRAS11,OsGRAS19,OsGRAS22,OsGRAS26
ABA	9	OsGRAS2,ΨOsGRAS2,ΨOsGRAS3,OsGRAS18,OsGRAS20,OsGRAS25,OsGRAS32,OsGRAS35,OsSCR1
NaCl+ABA	15	OsGRAS5,OsGRAS10,OsGRAS12,OsSHR2,ΨOsGRAS4,OsGRAS15,OsSLR1,ΨOsGRAS5,OsGRAS24,OsCIGR1,OsSHR1,OsGRAS39,OsGRAS41,OsGRAS43,OsGRAS44

II.b) 3h root (upregulated genes)

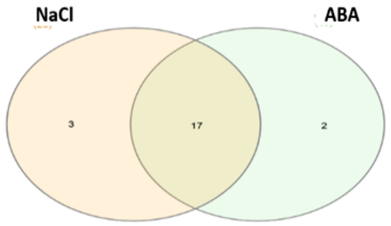


Treatment	Number of genes	Name of genes
NaCl	4	OsGRAS10,ΨOsGRAS3,ΨOsGRAS4,OsSHR1
ABA	11	OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS12,OsGRAS15,OsGRAS18,OsGRAS25,OsGRAS35,OsSCR1,OsGRAS41,OsGRAS44
NaCl+ABA	2	OsGRAS24,OsGRAS39

II.c) 12h root (upregulated genes)

Treatment	Number of genes	Name of genes
NaCl	2	OsSHR2,OsGRAS19
ABA	1	OsGRAS41
NaCl+ABA	16	OsGRAS2,OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS10,OsGRAS12,OsGRAS15,OsSLR1,OsGRAS22,OsGRAS24,OsGRAS25,OsSHR1,OsGRAS35,OsSCR1,OsGRAS39,OsGRAS44

II.d) 24h root (upregulated genes)



Treatment	Number of genes	Name of genes
NaCl	3	OsGRAS11,OsSHR2,ΨOsGRAS9
ABA	2	OsGRAS15,OsSHR1
NaCl+ABA	17	OsGRAS2,OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS10,OsGRAS12,OsSLR1,OsGRAS19,OsGRAS22,OsGRAS24,OsGRAS25,OsGRAS35,OsSCR1,OsGRAS39,OsGRAS41,OsGRAS43,OsGRAS44

II.e) 60h root (upregulated genes)

Treatment	Number of genes	Name of genes
NaCl	1	ΨOsGRAS3
ABA	8	OsGRAS2,OsGRAS3,OsGRAS18,OsGRAS20,OsGRAS22,OsGRAS41,ΨOsGRAS9,OsGRAS43
NaCl+ABA	19	OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS10,OsGRAS11,OsGRAS12,OsSHR2,OsGRAS15,OsSLR1,OsGRAS19,ΨOsGRAS5,OsGRAS24,OsGRAS25,OsGRAS26,OsSHR1,OsGRAS35,OsSCR1,OsGRAS39,OsGRAS44

III.a) 15min shoot (downregulated genes)

Treatment	Number of genes	Name of genes
NaCl	1	ΨOsGRAS2
ABA	2	ΨOsGRAS5,OsCIGR1
NaCl+ABA	32	OsGRAS8,OsGRAS10,OsGRAS11,OsGRAS12,OsSHR2,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15,OsSLR1,OsGRAS18,OsGRAS19,OsGRAS20,OsGRAS22,OsGRAS23,OsGRAS24,OsGRAS25,OsGRAS26,OsGRAS28,OsGRAS32,OsCIGR2,OsSHR1,OsGRAS35,OsSCR1,ΨOsGRAS8,OsGRAS39,OsGRAS41,ΨOsGRAS9,OsGRAS43,OsGRAS44,OsGRAS47 ΨOsGRAS10,OsGRAS53

III.b) 3h shoot (downregulated genes)

Treatment	Number of genes	Name of genes
NaCl	3	OsGRAS25,OsGRAS39,OsGRAS44
ABA	17	OsGRAS2,OsGRAS3,OsGRAS5,OsGRAS7,OsGRAS8,OsGRAS10,OsGRAS11,OsSHR2,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15,OsSLR1,OsGRAS18,OsGRAS20,OsGRAS22,OsGRAS24,OsCIGR1
NaCl+ABA	15	OsGRAS23,OsGRAS26,OsGRAS28,OsGRAS32,OsCIGR2,OsSHR1,OsGRAS35,OsSCR1,ΨOsGRAS8,OsGRAS41,ΨOsGRAS9,OsGRAS43,OsGRAS47,ΨOsGRAS10,OsGRAS53

III.c) 12h shoot (downregulated genes)

Treatment	Number of genes	Name of genes
NaCl	0	
ABA	13	OsGRAS1,OsGRAS2,OsGRAS3,OsGRAS5,OsGRAS12,OsGRAS19,ΨOsGRAS5,OsGRAS24,OsGRAS25,OsGRAS26,OsCIGR1,OsSCR1,ΨOsGRAS8
NaCl+ABA	27	OsGRAS7,ΨOsGRAS2,OsGRAS8,OsGRAS10,OsGRAS11,OsSHR2,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15,OsSLR1,OsGRAS18,OsGRAS20,OsGRAS22,OsGRAS23,OsGRAS28,OsGRAS32,OsCIGR2,OsSHR1,OsGRAS35,OsGRAS39,OsGRAS41,ΨOsGRAS9,OsGRAS43,OsGRAS44,OsGRAS47,ΨOsGRAS10,OsGRAS53

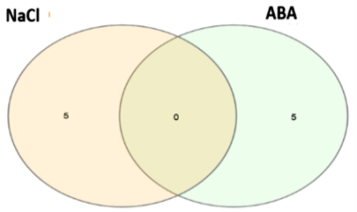
III.d) 24h shoot (downregulated genes)

Treatment	Number of genes	Name of genes
NaCl	14	ΨOsGRAS2,OsGRAS8,OsGRAS10,OsGRAS11,OsGRAS12,OsSHR2,OsSLR1,OsGRAS19,OsGRAS24,OsGRAS25,OsGRAS26,OsSCR1,OsGRAS39,OsGRAS44
ABA	3	OsGRAS2,OsGRAS3,OsGRAS5
NaCl+ABA	21	OsGRAS7,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15,OsGRAS18,OsGRAS20,OsGRAS22,OsGRAS23,OsGRAS28,OsCIGR1,OsGRAS32,OsCIGR2,OsSHR1,OsGRAS35,ΨOsGRAS8,OsGRAS41,ΨOsGRAS9,OsGRAS43,OsGRAS47,ΨOsGRAS10,OsGRAS53

III.e) 60h shoot (downregulated genes)

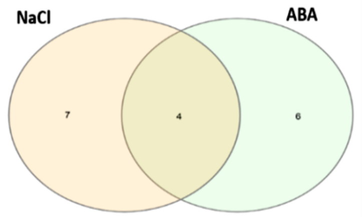
Treatment	Number of genes	Name of genes
NaCl	5	ΨOsGRAS2,ΨOsGRAS3,OsGRAS25,ΨOsGRAS8,OsGRAS44
ABA	4	OsGRAS2,OsGRAS3,OsGRAS10,OsSLR1
NaCl+ABA	25	OsGRAS7,OsGRAS8,OsGRAS11,OsSHR2,ΨOsGRAS4,OsGRAS15,OsGRAS18,OsGRAS19,OsGRAS20,OsGRAS22,OsGRAS23,OsGRAS26,OsGRAS28,OsCIGR1,OsGRAS32,OsCIGR2,OsSHR1,OsGRAS35,OsGRAS39,OsGRAS41,ΨOsGRAS9,OsGRAS43,OsGRAS47,ΨOsGRAS10,OsGRAS53

IV.a) 15min root (downregulated genes)



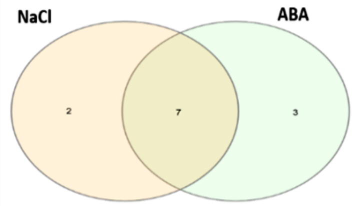
Treatment	Number of genes	Name of genes
NaCl	5	ΨOsGRAS3,OsGRAS25,OsGRAS28 OsSCR1,ΨOsGRAS8
ABA	5	OsGRAS1,OsGRAS3,OsGRAS7 OsGRAS8,OsGRAS19
NaCl+ABA	0	

IV.b) 3h root (downregulated genes)



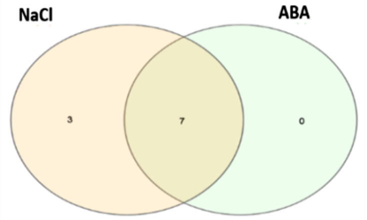
Treatment	Number of genes	Name of genes
NaCl	7	OsGRAS1,OsGRAS3,OsGRAS11,ΨOsGRAS5, OsGRAS28,OsCIGR1,OsGRAS32
ABA	6	OsGRAS7,OsSHR2,OsCIGR2,ΨOsGRAS8, OsGRAS43,ΨOsGRAS10
NaCl+ABA	4	OsGRAS2,OsGRAS20,OsGRAS23,OsGRAS26

IV.c) 12h root (downregulated genes)



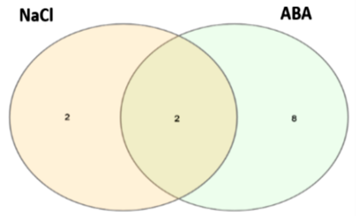
Treatment	Number of genes	Name of genes
NaCl	2	OsCIGR1,ΨOsGRAS9
ABA	3	ΨOsGRAS3,ΨOsGRAS4,OsGRAS28
NaCl+ABA	7	OsGRAS7,OsGRAS23,OsGRAS32,OsCIGR2,Ψ OsGRAS8,ΨOsGRAS10,OsGRAS53

IV.d) 24h root (downregulated genes)



Treatment	Number of genes	Name of genes
NaCl	3	OsGRAS7,ΨOsGRAS3,ΨOsGRAS4
ABA	0	--
NaCl+ABA	7	OsGRAS23,OsGRAS28,OsGRAS32,OsCIGR2, ΨOsGRAS8,ΨOsGRAS10,OsGRAS53

IV.d) 60h root (downregulated genes)



Treatment	Number of genes	Name of genes
NaCl	2	OsCIGR1,ΨOsGRAS9
ABA	8	ΨOsGRAS3,ΨOsGRAS4,OsGRAS23,OsGRAS28, OsGRAS32,OsCIGR2,ΨOsGRAS10,OsGRAS53
NaCl+ABA	2	OsGRAS7,ΨOsGRAS8

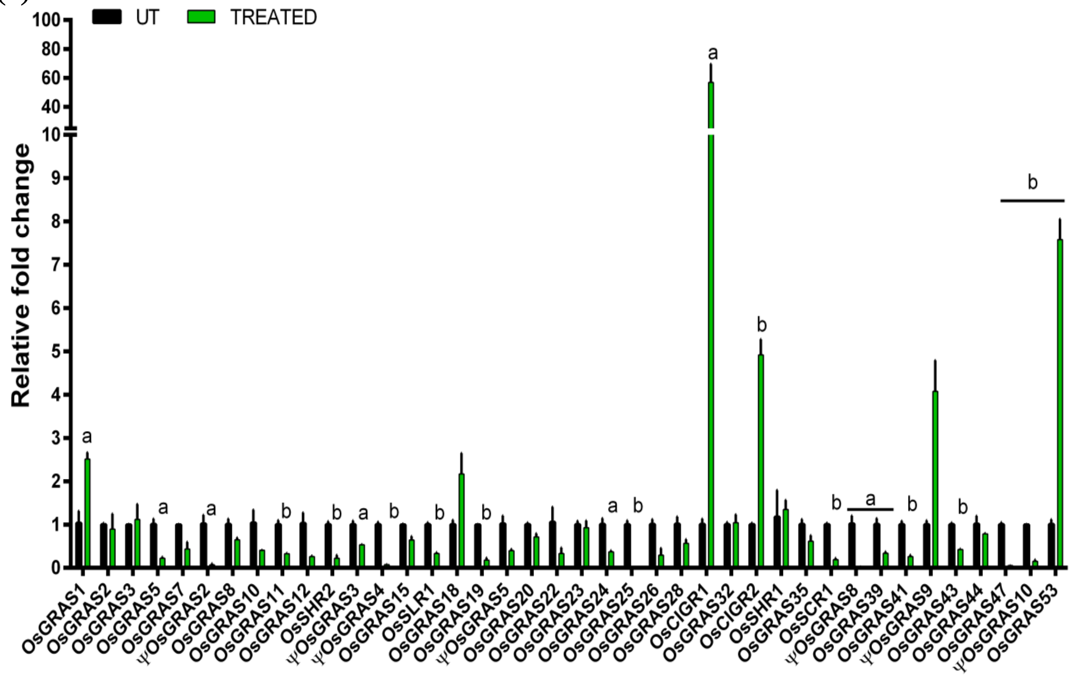
Fig. 6.9: Venn diagrams showing the expression pattern of *GRAS* genes under abiotic stress

Figure showing the number of genes upregulated in the shoot (I.a-e) and root (II.a-e) or downregulated in the shoot (III.a-e) and root (IV.a-e) during treatment with NaCl and ABA. The corresponding number and list of genes under each treatment and in combination are mentioned in the figure.

6.8.Differential expression analysis of *GRAS* genes under biotic stress

We have studied the expression patterns of the selected GRAS transcription factors in the leaf samples of rice infected with *Xoo* and *R. solani* pathogens that cause Bacterial Leaf Blight (BLB) and Sheath Blight (SB) diseases, respectively (Fig. 6.10). Six genes were upregulated in BLB, of which five (*OsGRAS1, OsGRAS18, OsCIGR2, ΨOsGRAS9, OsGRAS53*) showed low expression while one gene (*OsCIGR1*) was highly upregulated upto 57 folds. More genes were upregulated in SB infected leaves compared to the BLB treated ones. Out of the thirty expressed genes in SB infected leaves, twelve showed very high expression levels, while the rest of the genes exhibited low to moderate expression. *OsGRAS2, ΨOsGRAS3, OsGRAS19, OsGRAS20, OsGRAS23* and *OsSHR1* were expressed by ≥100 folds under the SB treatment. A total of 22 genes in BLB and three in SB treated samples were downregulated. Those that were downregulated in SB treated samples (*OsSHR2*, *OsGRAS24* and *OsGRAS43*) were also downregulated in BLB treated leaves. Twelve genes under BLB and seven under SB showed no changes in their expression levels.

(a)



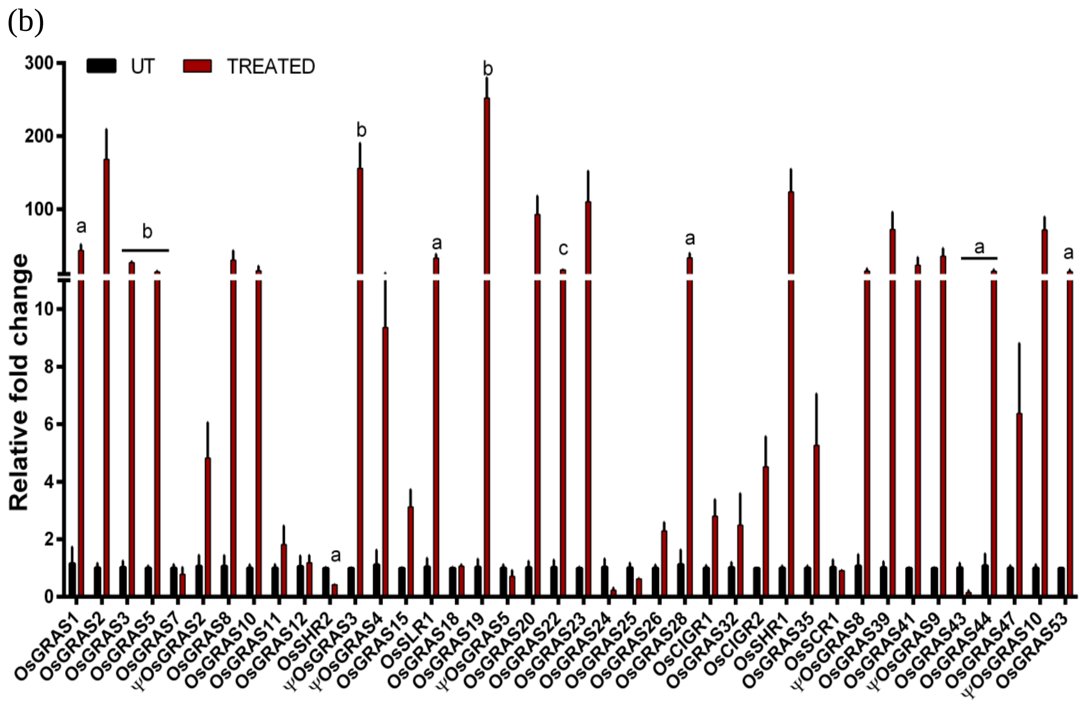


Fig. 6.10: Quantitative real-time expression analysis of *GRAS* genes under biotic stress treatments

Expression analysis of *GRAS* genes under the infection of *Xanthomonas oryzae* pv. *oryzae* causing bacterial leaf blight (6a) and *Rhizoctonia solani* causing sheath blight (6b) were studied. The genes were double normalized using rice *actin* and *tubulin* as internal reference genes, and the C_T values untreated samples by $\Delta\Delta C_T$ method. One way ANOVA was performed on the data, and a represents $P < 0.05$, b represents $P < 0.025$, and c represents $P < 0.001$.

6.9.Native expression analysis of GRAS genes in various tissues at specific developmental stages in rice

In order to study the native expression patterns of GRAS transcription factor genes in different developmental stages of the rice plant, we performed qRT-PCR analysis of thirteen different tissues, including shoot, root, root-shoot transition, flag leaves, flower, spikes and grain of mature 20 d old plants (after shifting to greenhouse), shoot and root of 7 d old seedlings, 3 d old plumule and radicle, embryo and endosperm of 16 h germinating seeds (Fig. 6.11). The mean values were used to plot a heat map (Fig. 6.12). Expression analysis showed a conspicuous downregulation of all genes in most tissues, particularly in plumule, radicle, embryo, and the endosperm. Out of forty selected genes, only seven were expressed in mature vegetative and reproductive tissues. *OsGRAS2* and

OsGRAS3 were upregulated only in mature leaves, *OsGRAS28* in 20 d root, flower and spike, *OsCIGR1* and *OsCIGR2* in root-shoot transition and leaves, *OsGRAS39* in 20 d root and *OsGRAS47* in 20 d root and flower. Out of these seven genes, five were upregulated either in the roots or in the root-shoot transition region indicating the preference of GRAS genes towards expression in the root tissue. It is also in accordance with the expression analysis under abiotic stress conditions, where the genes were highly expressive in roots rather than in the shoot tissue.

Three out of seven mildly expressive genes were upregulated in flower and spike of 20 d old plants with none of them expressing in the grain. *OsGRAS39*, which was upregulated in root tissues under native conditions, is highly expressive in roots under abiotic stress conditions and responded immediately after applying stress treatment. This might indicate its tissue specificity and its potential as a stress-tolerant transcription factor gene.

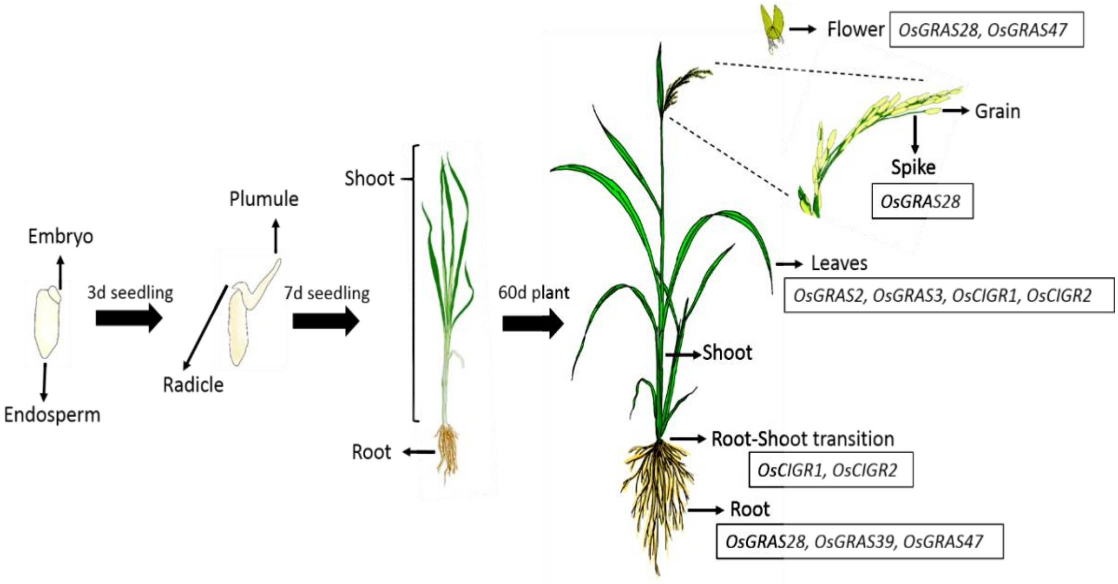


Fig. 6.11: Spatial regulation of *OsGRAS* genes

The native expression pattern of *GRAS* genes as was studied in thirteen different developmental tissues of rice plants. The majority of them were downregulated, with some getting expressed in mature vegetative and reproductive tissues. The list of the genes expressed in each tissue is mentioned in the boxes beside them. The figure has been adopted from Saha et al. (2017).

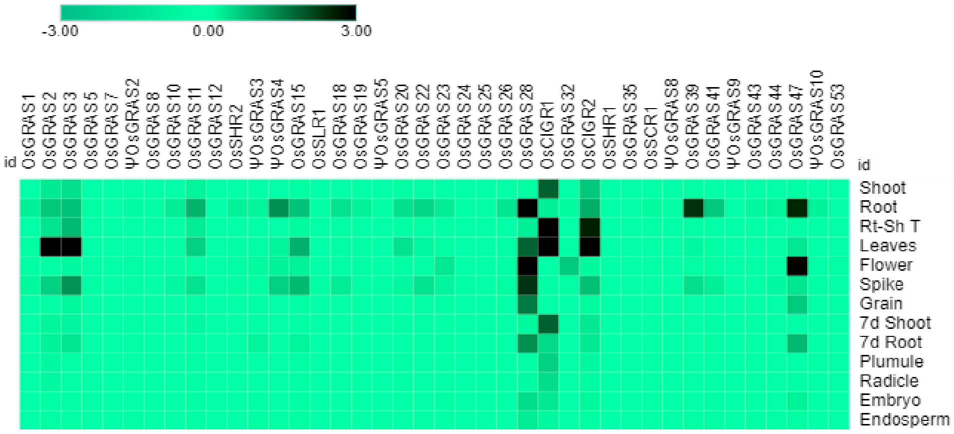


Fig. 6.12: Native expression analysis of *GRAS* genes

Heat map representing the spatial expression pattern of GRAS genes under thirteen different developmental stages of rice. The map was generated using the MORPHEUS program. The data was single normalized using rice *actin* as the internal reference gene.

6.10. Conclusions

We have 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., 2016). Recent studies indicated their role in biotic and abiotic responses, in addition to their diverse roles in plant growth and development. Although this family of TFs received significant attention, not many genes were explicitly identified for their roles in mediating stress tolerance in rice. In the present study, we aimed to understand the spatio-temporal regulation of the rice GRAS transcription factors under simulated abiotic (NaCl and ABA treatments) and biotic (bacterial leaf blight and sheath blight) stress conditions, as well as under various developmental stages. The expression analysis was further correlated with *in-silico* studies involving genetic organizations, protein properties, interacting partners and others. The major findings of this study are discussed below.

- We identified 60 *GRAS* genes distributed throughout the rice genome with chromosome 11 bearing the highest gene density and shortlisted 40, one representing each paralogous group. These were further classified into 14 subfamilies, out of which LISCL had the maximum number of genes. Genes belonging to the same subfamilies formed separate clades and had similar MEME motif arrangements compared to those belonging to different subfamilies. The majority of the genes were intronless probably because they originated in bacteria and expanded into higher eukaryotes via horizontal gene transfer and gene duplication events (Huang et al., 2015).

- These proteins have been predicted to interact with several metallic and non-metallic ligands for their activity, and the majority of them were acidic and hydrophilic in nature. Such properties make the interactions of the GRAS proteins specific and justify their role in cell signaling pathways.
- Under simulated abiotic stress conditions, GRAS genes were mostly induced in plant roots, with 55-60% of them being immediate early responsive type. The expression of such IE type genes protects the plant from the initial stress. *OsGRAS39* was highly expressed under both NaCl and ABA stress treatments, and *ΨOsGRAS2***,** *OsSHR1* and *OsSCR1* were highly expressed under ABA. These continued their expression till 60 h indicating their probable role in stress mediation. *ΨOsGRAS5* (IE type) was moderately expressive in shoot under both stress conditions.
- Only seven genes were induced in mature vegetative and reproductive tissues out of thirteen different developmental tissues, in which the genes were highly expressive in roots rather than in the shoot tissue, while a majority of them were downregulated in the germinating seeds.
- *OsGRAS39*, *OsGRAS8*, *OsSHR1* and *OsSLR1* might be considered to be quite important as they were expressive under both biotic and abiotic stress conditions. Our earlier functional characterization (Moin et al., 2016) followed by the genome-wide characterization of the GRAS gene family members in the present study clearly shows that they are highly relevant candidate genes for manipulating stress tolerance in rice and other crop plants.

CHAPTER 7

Discussion

DISCUSSION

Sustainable crop production is constantly under threat from environmental constraints like drought, salinity, temperature changes, rapid population growth, scarcity of land and water resources (Mottaleb et al., 2012; Muthayya et al., 2014). Being sessile, plants cannot escape from such environmental adversities, including their interactions with pathogenic microorganisms, which, altogether, impose a threat to their productivity (Lin et al., 2017). Environmental constraints lead to a decrease in crop production by 50-100% per year under extreme situations, which is worsened by the rapid population growth. It is expected that a 70% hike in food production is required to feed the burgeoning population. Therefore, adopting new strategies for developing improved crop varieties is necessary to address the issue of food security throughout the world (Food Security Information Network, 2020).

Rice is the staple food for 3.5 billion people in the world. Currently, its production is facing two major challenges, like providing adequate food to meet the yield gap and maintaining sustainable production under severe challenges imposed by environmental factors (Hussain et al., 2020). More than 90% of the world’s total rice production and consumption occurs in Asian countries where drought imposes a significant constraint, particularly in India, Bangladesh, Thailand, and Myanmar. The land under water stress is expected to double by 2050. Moreover, the majority of the rice production occurs in the deltaic regions of South, East and South-East Asian countries, which is getting affected severely by climatic changes. Such a drastic effect on rice production has its consequences in the international market of rice (Wassmann et al., 2009). Apart from abiotic stresses, pathogen interactions also play an important role in sustainable production of rice. Rice has been a host to innumerable bacterial, viral and fungal pathogens as it has been cultivated for over a thousand years. Major diseases include fungal and bacterial blight, rot, spot and blast diseases, to name a few (Papademetriou et al., 2000). All these adversities impose threats to sustainable rice production. Therefore, understanding the rice genome properties is very important to obtain proper genetic enhancement towards developing suitable varieties, meeting the current demand of rice and mitigating the yield gap. This way, we can develop tailor-made rice varieties of desirable agronomic importance with the help of advanced functional genomics strategies and improved transformation protocols.

The availability of high quality genome sequences and efficient transformation protocols make it easier for identifying and manipulating novel genes for stress tolerance in rice. Several mutagenesis techniques like using physical, chemical or DNA elements as mutagens for generating loss-of-function mutants are effective in several instances but can be null in case of redundant genes. Genes having multiple copies can be difficult to detect in knockout lines as the loss of function of one member can be compensated by the other existing copies in the genome. This holds true for genes responsible for embryonic and gametophytic development. In such cases, loss-of-function of the genes can lead to lethality. Therefore the concept of enhanced gene expression via generation of gain-of-function mutants came forward to overcome this problem (Moin et al., 2017b; Tani et al., 2004). Generation of gain-of-function mutants via activation tagging gained importance in functional genomics, which uses either promoters or enhancers to upregulate the expression of the genes. The promoter (CaMV35S) can cause an ectopic overexpression of genes in transcriptional fusion with them, which can sometimes lead to pleiotropism in transgenic lines. On the contrary, enhancers containing partial components of the 35S promoter cause an endogenous upregulation of the genes present in their vicinity instead of a constitutive expression. Therefore transgenic plants developed via enhancer based activation tagging are more likely to reflect the original function of the tagged genes (Dutta et al., 2021a). About 31% of enhancer integration via *Ac/Ds* based activation tagging vector was observed to be intergenic, which is why this technique is preferred for generating a large number of the tagged line for mining novel uncharacterized genes (Moin et al., 2017b).

Using pSQ5 *Ac/Ds* based activation tagging vector, our group has previously generated a population of gain-of-function mutant lines in *indica* rice variety. Since the T-DNA of the transformed vector contained both the immobilized *Ac* and mobile *Ds* elements, the transposition events occurred during meiotic segregation. The progeny plants carrying only *Ds* elements exhibited stable expression of the tagged lines, which were identified from the population and subjected to further analysis. These lines were screened for high WUE and high photosynthetic efficiency under limited water conditions.

Plants can react to water stress either by drought avoidance or drought tolerance mechanisms. While drought avoidance involves water conservation by plants via WUE, it can also lead to stomatal closure, reduced carbon assimilation and compromised yield. On the other hand, drought tolerance mobilizes the stem reserves without hampering the net yield of the plant (Moin et al., 2016). A higher expression of WUE genes in transgenic plants can lead to improved yield even under water stress conditions. Therefore, the analysis of tagged lines under water stress conditions helped us identify some important candidate genes that can be further manipulated for developing stress-tolerant plants. The previous analysis led to the identification of ribosome biosynthesis genes (*RPL6* and *RPL23A*), protein ubiquitination genes (*Cullins*), transcription factor genes (*GRAS*, *NF-YC13*) (Manimaran et al., 2017; Moin et al., 2016). This work has been taken forward in this study, and two helicases (SEN1 and XPB2) were further identified as potential drought-tolerant genes (Dutta et al., 2021a). We have analyzed the probable roles of these helicases in the light of plant productivity and also identified important members of the GRAS transcription factor family and reflected on their spatio-temporal regulation. We will be discussing the findings of the two studies in the next part of this chapter.

7.1.Helicases as potential targets for stress tolerance in rice

This study selected two mutants (XM3 and SM4) containing *Ds* elements and exhibiting stable expression of the tagged genes (XPB2 and SEN1 helicases, respectively). Flanking gene sequence analysis showed intergenic integration of the enhancers in the rice genome, thereby eliminating the chances of functional disruption of other endogenous genes due to intragenic insertional inactivation. The WUE and drought tolerance phenotypes of the two chosen mutants were possibly due to the stable, intergenic integration of the enhancer elements and enhanced expression of the tagged gene(s). These lines were chosen based on their quantum efficiency and $\Delta^{13}C$ values. Plants restrict their stomatal opening in response to water stress, thereby lowering the intercellular CO_2 concentrations (Ci). The Ci has a direct effect on $\Delta^{13}C$, and thus its decrease during stress increases carbon discrimination and decreases the WUE (Chen et al., 2011; Martin & Thorstenson, 1988). The higher quantum efficiency and lower $\Delta^{13}C$ of mutants (XM3 and SM4) reported in this study implied better photosynthetic performance and WUE, respectively, resulting in higher yield under water withdrawal circumstances. XPB2 DNA helicase, located closer to the enhancer, was tagged in the XM3 line, but other genes in the 10 kb upstream and downstream flanking region exhibited a similar expression level as the WT. Additionally, the SEN1 RNA helicase proximal to tetrameric enhancers was upregulated in the SM4 line.

The administration of stress inducible phytohormones, ABA and PEG, increased the expression of SEN1 and XPB2 helicases, notably in root tissues. Because roots are the primary organs that perceive stress signals, an upregulation in gene expression in root tissues demonstrated their function in regulating a signaling cascade for water stress responses (Janiak et al., 2016). Unlike abiotic stress treatments, pathogens like *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia. solani* did not significantly upregulate the transcript levels of SEN1 and XPB2.

7.2.XPB2 and SEN1 helicases for enhanced productivity in rice under limited water conditions

The gain-of-function mutants were allowed to grow in PEG and two concentrations of ABA for simulated water stress studies. These lines were further subjected to drought stress conditions by the periodic withdrawal of water. Both genotypes showed superior phenotypes compared to the WT lines under all imposed stress conditions. The pattern of phenotypic expression hidden in the multivariate, high-dimensional dataset of the morpho-physiological and biochemical dataset was rapidly and intuitively identified with the help of PCA. We successfully illustrated that the untreated responses in the test genotypes were similar, and so was the tolerance of the tagged mutants under simulated stress conditions. The tagged lines localized in different positions on the PCA biplot from the WT, indicating a distinct phenotypic response. Such occurrences were explained based on the difference in the capacity of these genotypes to respond to stress driven by the gain-of-function mutations.

Root length is a crucial characteristic for plant productivity when there is a water shortage. Thus, the fact that SM4 lines had longer roots under simulated water stress conditions implied that they were more tolerant than the control (Janiak et al., 2016; Sharp & LeNoble, 2002). Similarly, the tagged lines displayed robust phenotypes following three and seven days of drought treatment in pots compared to the WT. Drought disrupts the water balance in the plant, causing growth retardation and early leaf senescence (Anjum et al., 2011; Sreenivasulu et al., 2012). Under simulated stress circumstances, we observed similar phenomena in the WT, which had reduced biomass, a greater wilting percentage, and a lower plant revival percentage than the tagged mutant counterparts. As a result, SEN1 and XPB2 appeared to be involved in supplying the plant with rigidity or vigor during and after the treatment with the stressors. An overabundance of ABA inhibits shoot development, reduces carbon assimilation, and in turn, the yield (Blum, 2005; Zhang et al., 2006). Such stress avoidance mechanism was observed in the WT, which had reduced plant height and yield after revival. On the contrary, the physiology and the yield of the gain-of-function mutants were not altered on exposure to exogenous stress stimuli. Prolonged drought causes the stomata to shut, resulting in inefficient gas exchange and decreased plant photosynthetic capacity (Sreenivasulu et al., 2012). The difference in the photosynthetic performance of mutant genotypes versus the WT lines demonstrated the same. PAM indirectly measures the photosynthetic capacity of plants by measuring the quantum efficiency of photosystem II (PSII). The F_v/F_m ratio obtained through this technique also indicated the degree of stress experienced by the plants (Murchie & Lawson, 2013; Osmolovskaya et al., 2018). The healthy untreated WT and mutant plants had quantum efficiency ranging from 74 to 77%. On exposure to stress, the efficiency of the WT decreased to 63-67%, while that of the tagged lines remained unaltered (72-77%). This indicated that the external stress cues did not affect the ability of the tagged lines to photosynthesize efficiently under stress. Because both chlorophyll a and b are drought-susceptible, their higher levels in the tagged lines indicated that the mutants had improved photosynthetic competence and, as a result, yield (Anjum et al., 2011; Osmolovskaya et al., 2018; Zhang et al., 2018). SM4 lines also acquired a significant amount of proline after ABA treatment. Plants retain a turgor pressure during drought stress by assimilating ions and organic solutes, and proline is one of them. A higher accumulation of osmoprotectants like proline corresponds to improved drought tolerance and osmotic potential adjustments (Anjum et al., 2011; Yang et al., 2018; Zhang et al., 2018).

In rice plants, boot leaves are recognized as a principal source of metabolites, contributing to plant productivity. As observed in previous studies, their length was positively correlated with the panicle length and plant productivity (Rahman et al., 2014). Also, excessive ABA buildup in reproductive tissues causes pod abortion and shortens the grain filling time (Sreenivasulu et al., 2012). The sensitivity of the WT plants to drought is indicated by a reduction in the boot leaf size and panicle length when stressed. Under simulated conditions, the tagged lines had a 26-98 % higher yield per plant than the WT, and the tagged lines had a 9-50 % higher yield per plant under pot-level drought experiments. This implied that the SEN1 and XPB2 helicases are not only involved in maintaining a sustainable yield, but also responsible for improved yield in gain-of-function mutants even under severe stress conditions. These findings were supported by our statistical analyses as well. WT lines showed a significant reduction in their photosynthetic performance and yield after seven days of periodic drought, whereas no remarkable changes were observed in the mutant lines. Under drought conditions, there was a substantial variation in yield across genotypes. These findings add to the existing evidence that helicases have a role in increasing drought/dehydration stress tolerance. Seed weight, size, and endosperm chemical composition are usually used to determine the seed quality of rice (Chang & Somrith, 1979). The linear chain amylose and the branching chain amylopectin are the two components of starch, which form the major component of the endosperm. The cooking and feeding quality of rice are determined by the amylose-amylopectin ratio, which impacts its swelling and disintegration characteristics (Juliano, 1979). Therefore, this ratio in the seed is critical in establishing its quality. It was observed that the increased helicase expression improved yield without affecting the grain quality.

Previous literature indicated the role of several DEAD/H box helicases in stress mediation when overexpressed in plants. However, those tagged in the mutants understudy did not belong to this group. XPB2 belongs to subgroup SF2, and SEN1 belongs to Upf-1 like subfamily under SF1B (SEN1) group of helicases (Martin-Tumasz & Brow, 2015; Raikwar et al., 2015). Both have been extensively studied in yeast and mammalian systems, but their role in maintaining genomic stability under drought stress in plants remains unexplored. Other than a few *in-silico* and knockout studies on XPB helicase (Costa et al., 2001; Raikwar et al., 2015), no studies on drought stress responses have been reported so far. *OsSUV3* is a known DEAD/H box helicase from rice, whose overexpression in the IR64 rice variety has led to improved drought tolerance (Tuteja et al., 2013). OsRH58, a rice RNA helicase, exhibited drought-tolerant responses in *Arabidopsis* (Nawaz & Kang, 2019). Two pea DNA helicases, PDH45 and PDH47, also enhanced similar tolerance when overexpressed under a constitutive promoter in groundnut (Manjulatha et al., 2014), chilli (Shivakumara et al., 2017), and rice (Singha et al., 2017). SlDEAD31, an RNA helicase, imparted drought tolerance when expressed constitutively in tomato (Zhu et al., 2015). RH5, RH8 and RH25 are some of the

Arabidopsis helicases known for drought stress tolerance in plants (Baek et al., 2018; Kant et al., 2007).

The seeds of the present mutant genotypes germinated and exhibited normal growth under 75 μM ABA concentration, while WT lines failed to do so. Such a high concentration of ABA impairs seed germination and seedling establishment resulting in cell dehydration, wilting and death (Zhang et al., 2018). The ability of tagged lines to germinate on ABA containing medium suggests that SEN1 and XPB2 might play a role in the negative control of ABA-mediated seed dormancy. A previous report on such negative regulation was observed in the *FtMYB10* gene from Tartary buckwheat, which was ABA induced, but its overexpression lines were less sensitive to it (Gao et al., 2016).

7.3.Probable existence of a crosstalk between the helicases with other stress regulatory pathways results in drought tolerance of rice

We assessed the expression level of seven stress-specific genes in the three genotypes under simulated and pot-level drought conditions to understand the correlation between the helicases and the stress-regulatory pathways. Seven genes were upregulated in XM3 and five in SM4 under simulated conditions, while under pot conditions, four genes in XM3 and all seven in SM4 were upregulated. This indicated that the helicases probably interact with other stress-regulatory genes, as a result of which the tagged lines were drought-tolerant and were also able to uphold their productivity under adverse conditions. However, the interaction between the stress-regulatory genes and the pathways they operate requires further investigation.

Altogether we can conclude that the mutant lines with higher expression of SEN1 and XPB2 had an advantage over the WT lines in combating drought and dehydration stress. This can be further explored by raising independent transgenic lines for these genes, followed by their functional characterization to understand the underlying mechanism of stress tolerance. Preliminary studies indicated that the expression of both helicases is modulated by ABA, PEG and other stress factors, but there is no clear evidence that they work in an ABA-dependent or independent manner. *In-silico* analysis revealed the presence of ABRE, DRE and MYB *cis*-acting elements in the putative promoter region of the XPB2 gene (Raikwar et al., 2015) and ABRE and MYB elements in that of SEN1 genes (Dutta et al., 2021a). The occurrence of both DRE and ABRE elements in the putative promoter region of the helicases imply that both ABA-dependent and independent gene regulation may exist simultaneously (Roychoudhury et al., 2013; Yoshida et al., 2014). A similar observation was made in the *rd29A* gene promoter in *Arabidopsis* (Narusaka et al., 2003). MYB transcription factors usually work in an ABA-dependent manner during stress; however other MYB factors, such as OsMYB3R-2, were less susceptible to ABA (Dai et al., 2007). According to our findings, XM3 and SM4 gain-of-function mutant lines exhibited lower sensitivity to ABA, even though their expression is induced by it. Therefore it can be predicted that SEN1 and XPB2 might play a transitory role between ABA-dependent and independent pathways. *OsPP2C* and *OsDREB2B*, two potential ABA-dependent and independent genes respectively, either exhibited an equivalent expression like the control or got downregulated in both root and shoot tissues in response to external stress cues. Such expression pattern of both ABA-dependent and independent genes during the expression analysis under ABA treatment is most likely due to the interaction between the two pathways or the presence of a negative feedback mechanism. Such regulation has been previously reported in *OsNAC2,* where this gene negatively regulates the expression of stress marker genes despite itself getting induced by ABA (Shen et al., 2017).

Based on the preceding discussion, it can be assumed that both genes regulate stress tolerance mechanisms either by triggering DNA repair pathways to overcome DNA damage caused by stress, or by efficiently aborting pervasive transcription, or by resolving unwanted DNA: RNA or RNA: RNA hybrids formed during stress and upregulating the expression of other important stress regulators (Han et al., 2017; Mischo et al., 2011, 2018; Raikwar et al., 2015; Richards et al., 2008). These genes appear to have a beneficial effect on rice plant stress resistance.

7.4.Rice GRAS transcription factor family genes in stress responses: their phylogenetic relationships, gene organization and protein properties

Identification of novel genes and their characterization is a crucial step in any functional genomics study. In this context, transcription factor genes are crucial as they directly regulate the genes involved in several signaling pathways. Manipulating such TF genes or Master genes would render the plant more accommodating towards the particular stress under consideration. In our previous analyses of activation tagged lines, we have identified a GRAS transcription factor gene (*ΨOsGRAS4)* with a probable role in maintaining high WUE and photosynthetic efficiency under limited water conditions

(Moin et al., 2016). Plants employ various acclimatization and adaptation strategies to deal with the impending stress, which is primarily controlled by hormones and regulators (Lin et al., 2017). Understanding the expression patterns of the GRAS gene family, which play a major role in gibberellin signaling and their spatiotemporal control, might help us find potential targets for strengthening endogenous plant defences, particularly rice in this case. In this investigation, we identified key GRAS genes involved in abiotic and biotic stress tolerance. We have also looked at their *in-silico* characteristics and compared them to our expression data.

According to existing literature (Liu & Widmer, 2014), the rice genome contains 60 GRAS genes scattered throughout all the twelve chromosomes except chromosomes 8 and 9; and chromosome 11 bearing maximum gene density. For our investigation, we chose 40 genes, with one member belonging to each paralogous group. We were able to gain insights into the phylogenetic, genomic, and protein characteristics of the GRAS genes because of the availability of high-quality genomic sequences. We divided the genes into 14 subfamilies (Cenci & Rouard, 2017), with LISCL having the largest number of genes. The SCL3, SHR1, DELLA, HAM, and PAT subfamilies, on the other hand, consisted of the most expressive genes. The conserved five GRAS motifs were identified with the ten MEME identified motifs. Similar motif configurations were found in genes belonging to the same subfamily, although this differed among subfamilies, which might be attributed to the different biological roles of GRAS genes. This protein family is claimed to have evolved from bacteria, which then migrated into eukaryotic genomes by horizontal gene transfer and recurrent duplication events, with the possibility of retroposition of intronless genes (Huang et al., 2015a). Our genomic organization analysis indicated 31 out of 40 *OsGRAS* genes to be intronless, which is consistent with prior findings.

The presence of many interacting metallic and non-metallic ligands coupled to GRAS genes, as well as their hydrophilic character (as shown by the GRAVY index), suggested the role of these proteins in cell signaling, catalysis, and protein-protein interactions (Jing et al., 2017; Ulucan et al., 2014). Most of the genes had *p*I less than seven, rich in negatively charged amino acid residues such as glutamic acid and aspartic acid. Since proteins with low *p*I avoid non-specific interactions with other proteins and nucleic acids, such composition makes the GRAS proteins interactions extremely selective (Takakura et al., 2015). All GRAS genes contained at least one GRAS domain, while some had two or a DELLA domain associated with it, which is known to play a critical role in the gibberellic acid signaling pathway (Urbanova & Leubner-Metzger, 2018).

7.5. Differential expression patterns of *OsGRAS* genes and their spatio-temporal regulation

Spatio-temporal regulation of gene expression is necessary to control the abundance of certain transcripts and proteins in cells for proper response to external environmental stimuli. The majority of GRAS genes were expressed in roots, with 55-60% of them displaying IE gene expression. Stress reactions in plants may be classified into two categories, early and late. Within minutes of stress application, early response genes get activated, providing protection and repair from the initial stress. This reaction “alarms” the plant, causing it to prepare for additional stress tolerance or avoidance. On the other hand, late responsive genes are mostly engaged in protein synthesis, which affects downstream genes, thereby reacting to the “adaptation” component of stress regulation (Bahrami & Drabløs, 2016; Lin et al., 2017). In root, *ΨOsGRAS9* was found to be late expressing in both treatments indicating that it might have a role in successive stages of stress alleviation. Surprisingly, 50-60% of IE-expressed genes maintained their expression until the treatment was completed. Among them, OsGRAS39 was the only gene substantially expressed in roots when exposed to both NaCl and ABA. Besides that, *ΨOsGRAS2, OsSHR1* and *OsSCR1* continued to express till 60 h following ABA treatment. These genes are probably crucial and are required by the plant continuously during the stress period. Few other genes like *ΨOsGRAS3, ΨOsGRAS4, OsCIGR1* and *OsGRAS32* under ABA and *OsCIGR1* under NaCl induced initially (IE type) either stopped expressing or got downregulated at later time points. Such genes are most likely necessary for early stress reactions, after which their function is taken up by other downstream genes in the signaling cascade. Under both stress situations, *ΨOsGRAS5* (IE type) was the sole moderately expressed gene in the shoot. Because the root is the first organ to detect a stress signal, it sets in motion a signaling cascade that progresses towards the shoot. The preferential expression of GRAS genes in roots over shoot indicated that they play critical roles in stress responses (Janiak et al., 2016). Furthermore, earlier reports have suggested that the function of these genes in pattern formation and signal transduction allows them to be more expressive in roots (Pysh et al., 1999).

BLB and SB infections have a significant impact on rice productivity. BLB infection at the tillering stage can lower crop productivity by 50%, whereas it can reduce yields by 20-40% if infected at a younger stage. SB diseases can reduce rice yields by up to 45% (Chukwu et al., 2019; Singh et al., 2019). Thus, identifying essential genes and analyzing their expression patterns are critical for generating disease-tolerant rice cultivars. Only six genes (15%) were upregulated during BLB treatment, whereas thirty genes (75%) were expressed during SB infection. Several genes were expressed during SB infection and both abiotic stress conditions, among which the upregulation of *OsGRAS39*, *OsGRAS8*, *OsSHR1* and *OsSLR1* were noteworthy. Therefore, these genes can be deemed significant since they are also expressed under abiotic stress conditions with key roles in disease resistance. In ABA treated roots and SB infection, the majority of the genes were significantly expressed. Our expression data was further validated by numerous stress-responsive elements in the putative promoter regions of *OsGRAS* genes, indicating that they might play a role in strengthening plant defence against biotic and abiotic stress.

Out of thirteen different developmental tissues, only seven genes were expressed in mature vegetative and reproductive tissues. Bioactive gibberellic acid induces proteasomal degradation of proteins belonging to the DELLA subfamily for gibberellin signaling to occur as proteins of such category negatively regulate seed germination (Urbanova & Leubner-Metzger, 2018). This clarifies why all the GRAS genes were downregulated in the plumule, radicle, embryo, and endosperm. Thus, it can be inferred that *OsGRAS* genes are probably involved in regulating developmental patterns in mature rice plants.

The induction of *OsGRAS28*, belonging to HAM subfamily, and *OsGRAS47*, belonging to LISCL subfamily, in reproductive tissues indicates their role in floral development. It can be correlated with the expression of *PhHAM* genes of *Petunia hybrida* (Stuurman et al., 2002), *AtSCL6* and *AtSCL27* genes of *Arabidopsis* (Fan et al., 2017) and *PtGRAS67* of *Populus* (Liu & Widmer, 2014) flowers. All of these HAM subfamily genes were found to be involved in floral differentiation. The LISCL subfamily gene from lily plants had also been linked to microsporogenesis (Bolle, 2004). *OsGRAS39* expression was observed in roots under both native and abiotic stress conditions suggesting that it is tissue specific. This gene is a member of the SCL3 subfamily, which modulates GA signaling in roots through protein-protein interactions (Weng et al., 2020). Such increased *OsGRAS39* expression in roots under all stress situations might be investigated further for its possible involvement in stress tolerance. The GRAS gene family has been widely researched in many plant species, but we have effectively established a foundation for future research on rice *GRAS* genes. The distinct expression profiles of these genes imply their relevance in stress alleviation. Our findings shed light on the role of GRAS genes in stress tolerance and their spatiotemporal regulation. This analysis will make it easy to determine essential genes that may be further exploited to produce stress-tolerant rice and other related crops.

7.6.Conclusion

Our study has utilized one of the most important tools of functional genomics viz., activation tagging for mining novel genes responsible for high WUE in rice. Previous analyses identified several essential transcription factors, ubiquitination related proteins and ribosomal proteins (Manimaran et al., 2017; Moin et al., 2016). In this investigation, we have added two more genes, one encoding a DNA helicase, XPB2 and the other coding for an RNA helicase, SEN1, to the list. We have analyzed the tagged mutants in the light of agricultural productivity and morpho-physiological responses. We have also performed a genome-wide analysis of the GRAS transcription factor family of genes, which was also tagged in one of the mutant populations analyzed by our group earlier (Moin et al., 2016). Their expression analyses and spatio-temporal regulation were explored in various developmental stages and by using simulated and biotic stress cues.

We screened the population of activation tagged *indica* rice lines based on physiological parameters followed by flanking gene analyses to identify the two helicases. Our findings suggested that the expression of XPB2 and SEN1 helicases were significantly upregulated in root tissues on the application of external stress cues. These were also less sensitive to ABA during seed germination, although they were induced by it. We analyzed the performance of the tagged lines in comparison to the wild type control under three different situations mimicking dehydration (PEG) and phytohormone (ABA 50 and 75 µM) stress. Both the tagged mutants were superior to the control setup, indicating their potential in ameliorating drought stress in rice. *In-silico* studies and pot-level drought experiments further corroborated such observations. Both XM3 and SM4 mutants had higher yield and photosynthetic performance than the WT lines as observed under simulated conditions. Activation of these two helicases further induced the expression of several stress-specific genes, which indicated their probable role in crosstalk with other regulatory pathways. The exact mechanism of action needs further exploration.

Our previous analyses of tagged lines identified a GRAS transcription factor for its probable role in high WUE. Hence, we explored the GRAS family and studied their genomic distribution, protein structure and properties, phylogenetic relationships, motif organization and ligand interactions. The complete annotation of the rice genome enabled us to perform these studies. We analyzed the expression patterns of these genes under simulated abiotic (NaCl and ABA) and biotic (bacterial leaf blight and sheath blight infected samples) stress conditions and in different developmental stages of rice. Our *in-silico* studies further corroborated the outcome. The majority of the genes were expressive in root tissues, particularly under ABA treatment. *OsGRAS39* was highly expressive under both biotic, abiotic stress conditions, which can be further exploited for its role in stress tolerance. Other promising genes that need further exploration include *OsGRAS8*, *OsSHR1* and *OsSLR1*. Therefore, this study provides a promising background based on which future analysis on rice stress tolerance can be explored.

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PUBLICATIONS

Theoretical and Applied Genetics
https://doi.org/10.1007/s00122-021-03823-0

ORIGINAL ARTICLE

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

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Received: 17 May 2020 / Accepted: 23 March 2021

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	Abscisic acid

Communicated by Matthias Wissuwa.

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Introduction

Stresses like drought, salinity, extreme temperatures and biotic stresses (fungal, viral or bacterial infections) are severe threats to sustainable agricultural productivity. Hence, the identification of genes responsible for orchestrating plant tolerance to different stresses is a continuous process and an indispensable step in developing tailored crop varieties, which can withstand such challenging and destructive environmental conditions.

In an earlier investigation, we have generated a large population of gain-of-function mutant lines in a widely cultivated *indica* rice variety BPT-5204 (Samba Mahsuri) using a tetrameric 35S enhancer-based activation tagging system (Moin et al. 2016a). Upon screening of these mutants under limited water conditions along with associated phenotypic and physiological studies, some of them exhibited high-WUE phenotypes suggesting that the genes which became activated in them through the integrated enhancers might have roles in improving WUE in rice. We initially identified five mutant lines with high quantum efficiency and low Δ^{13}C, which are the proxies for WUE, showing the activation of transcription factors (GRAS and WRKY 96) and proteins involved in protein ubiquitination (cullin4) and ribosome biogenesis (RPL6 and RPL23A) (Moin et al. 2016a). Subsequent analysis of sequences flanking the activation tags (4X enhancers) in two other WUE mutants resulted in the identification of plant DNA and RNA helicases, *XPB2* and *SEN1*, respectively.

Helicases are molecular ATPases, which utilize the energy released during ATP hydrolysis to carry out a wide range of functions either on DNA (known as DNA helicases) or on RNA (identified as RNA helicases). Besides their housekeeping functions associated with the induction of conformational changes in DNA or RNA, they are also reported to be involved in combating biotic and abiotic stress conditions in plants (Tuteja 2003; Linder and Owttrim 2009). A majority of the helicases have a three-dimensional conserved core region consisting of two tandemly placed RecA domains (RecA1 and RecA2) connected via a flexible linker region (Sloan and Bohnsack 2018). The core domain comprises of 350–400 amino acids and 14 conserved motifs serving as their catalytic pockets (Umate et al. 2010; Passricha et al. 2018). The functional diversity of helicases may originate from differential binding patterns of the nucleic acids or the variations in their N- or C-terminal domains (Jankowsky and Fairman 2007; Seraj et al. 2018). Based on the structural and functional attributes, helicases are divided into six superfamilies (SF1 to SF6). Of them, a majority of the DNA and RNA helicases fall under SF1 and SF2 categories. While the SF2 family of helicases is considered to be the largest family of helicases, the SF1 family is well characterized (Seraj et al. 2018; Passricha et al. 2018).

Rice *XPB2* (**X**eroderma **P**igmentosa group **B2**) is a DNA helicase (3′–to 5′ helicase) belonging to the superfamily 2. The homologs of rice *XPB2* in yeast, Arabidopsis and humans are known as *RAD25* (*SSL2*), *XPB2* and *XPB* (*ERCC3*), respectively (Bhatia et al. 1996; Umate et al. 2010). Any distortions in the DNA are repaired via nucleotide excision repair (NER) mechanism so that the damage is not advanced to the next generation (Guzder et al. 1995; Morgante et al. 2005). XPB2 is a subunit of the eukaryotic transcription factor, TFIIH that opens up a DNA bubble during RNA polymerase II-mediated transcription initiation (Bhatia et al. 1996; Morgante et al. 2005). It acts as a DNA-dependent helicase, which also helps in NER by unwinding the DNA at the site of the lesion (Richards et al. 2008; Raikwar et al. 2015). Ergo, TFIIH in eukaryotes has a dual role viz., transcription initiation and DNA damage repair via nucleotide excision (Bhatia et al. 1996; Costa et al. 2001). Defects in XPB2 have been linked with an autosomal recessive disease Xeroderma Pigmentosum in humans. These mutants are more sensitive in response to photoperiod (Park et al. 1992; Costa et al. 2001). Recently, it has been reported that the promoter of the rice *XPB2* gene is a multi-stress inducible one playing an essential role in orchestrating plant stress tolerance (Raikwar et al. 2015).

Rice *SEN1* (t-RNA **s**plicing **en**donuclease) is an RNA helicase belonging to the Upf1-like subfamily under the superfamily 1B, which unwinds the RNA in 5′–3′ direction. The homologs of rice SEN1 found in Arabidopsis, yeast and human are UPF1, SEN1 and SETX (*Senataxin*), respectively (Umate et al. 2010; Martin-Tumasz and Brow 2015). The exact mechanism of rice SEN1 activity is elusive, but since the helicase domains are highly conserved among the eukaryotes (Han et al. 2017; Leonaite et al. 2017), these proteins might also function like other similar proteins reported in eukaryotes.

In yeast, transcription termination of non-coding RNAs occurs via the NNS (NRD1-NAB3-SEN1) complex (Sariki et al. 2016; Leonaite et al. 2017), where the NRD1-NAB3 heterodimer interacts with specific sequences on the nascent RNA (Han et al. 2017; Mischo et al. 2018) and with the C-terminal domain of the RNA polymerase II during termination (Mischo et al. 2011). This interaction helps in recruiting SEN1 onto the nascent RNA, which dislodges the RNA polymerase II by its helicase activity (Mischo et al. 2018). After the termination of transcription, the RNA is degraded by the combined activity of the TRAMP (TRF4/ AIR2/ MTR4 polyadenylation complex) complex and the exosomes (Leonaite et al. 2017; Mischo et al. 2018). SEN1 also plays a vital role in RNA processing, elimination of short protein-coding sequences, resolving R loop structures and maintaining genomic stability (Mischo et al. 2011). Defects in SEN1 result in defective R loop resolution and an increase in its frequency (Martin-Tumasz and Brow 2015; Leonaite et al. 2017; Mischo et al., 2018), genomic instability and errors in replication (Mischo et al. 2018). Apart from regulating the expression of non-coding genes, SEN1 also coordinates the expression of small protein-coding genes like *NRD1*, *HRP1*, *IMD2* and *CYC1* (Steinmetz et al. 2006). Yeast cells with N-terminal truncation of SEN1 had higher cell death and shortened life span. Arabidopsis homolog of SEN1, UPF1 plays an important role in nonsense-mediated decay (NMD) of abnormal RNA. It helps the plant in maintaining proper seed size (Yoine et al. 2006), floral and vegetative development (Arciga-Reyes et al. 2006). Thus, it has significant roles in regulating both transcription and translation in most eukaryotes. SEN1 is also involved in transcription-coupled repair mechanisms (Li et al. 2016). Hence, the primary function of SEN1 is to alienate any stalled elongation complex on the nucleic acid during transcription.

In the present study, we characterized the roles of these two genes in response to various stress conditions, particularly water stress, with an emphasis on seed yield and productivity apart from WUE in *indica* rice. Our findings suggested that the helicases encoded by these genes also have roles in stress responses by possibly preserving the genomic integrity of the plant upon the onset of environmental stresses besides their basic cellular housekeeping activities (nucleic acid unwinding).

Materials and Methods

Identification of *SEN1* and *XPB2* helicases in activation-tagged mutants

An *Ac/Ds*-based activation tagging vector, pSQ5 (Qu et al. 2008) was used to generate gain-of-function mutant lines of *indica* rice cultivar, Samba Mahsuri (variety BPT-5204). The *Ds* element of the vector carries tetrameric repeats of CaMV35S enhancers (4X enhancers), which upregulate the genes 10 kb upstream or downstream from the point of integration in the plant genome. This vector was transformed into rice using an *in planta* mediated rice transformation protocol and the transformed plants were confirmed by molecular analysis (PCR and Southern-blot hybridization) and screening on a solid MS medium containing 50 mg/L Hygromycin (Moin et al. 2016a). After selection of the stable *Ds* mutants, they were further examined for WUE trait by growing under limited water conditions followed by phenotypic and physiological studies. Carbon isotope discrimination (Δ^{13}C) is a noninvasive way of determining WUE in plants. The negative relationship between the WUE of a plant and Δ^{13}C values is an effective way of identifying plants with an improved efficiency under limited water conditions (Chen et al. 2011). The carbon isotope composition (δ^{13}C‰) of plants is calculated by the formula $[(R_{\text{sample}}/R_{\text{standard}})\text{-}1] \times 10^3$ and is compared with the standard of Pee Dee Belemnite (PDB) fossil carbonate (Farquhar et al. 1982, 1989; Gao et al. 2018). For calculating the carbon isotope discrimination values, 500 mg of mature leaf samples of WT, XM3 and SM4 lines grown under limited water conditions were collected and dried at 65 °C for 3 days (d). The samples were powdered and the carbon isotope was measured using an Isotope Ratio Mass Spectrometer (IRMS).

Mutants having high WUE under limited water conditions were subjected to flanking sequence analysis via TAIL-PCR (thermal asymmetric interlaced PCR) using one degenerate and three nested primers. The protocols for the TAIL-PCR and the related analyses were followed as detailed out in Moin et al. (2016a). The final amplicons were cloned in pTZ57R/T vector and subjected to Sanger sequencing. The enhancer integration sites and flanking genes were identified by a BLAST search in rice genome databases (RGAP-DB, RAP-DB and OryGenesDB). Two potential mutants (DEB. 36 and En. 124) with high WUE were found to have three and five genes, respectively, within the 20 kb region of enhancer integration. The activation tagging of these genes was analyzed via qRT-PCR using appropriate primers.

Quantitative reverse transcription PCR (qRT-PCR) analysis of flanking genes

The two mutant lines viz. DEB.36 (referred to as XM3) and En.124 (designated as SM4), which displayed normal growth and yield-related parameters under limited water conditions with respect to WT were selected for quantitative PCR analysis (qRT-PCR analysis). The transcript levels of all the genes present in 10 kb upstream and downstream regions from the site of enhancer integration were studied in DEB.36 and En.124 by qRT-PCR using gene-specific primers in three biological and technical replicates. The total RNA was isolated from the leaves of 60-d-old plants using Tri-Reagent (Takara Bio, UK) and 2 µg cDNA (Takara, Clontech, USA) was prepared as per the manufacturer's protocol. The cDNA was diluted seven times, and a 2 µl aliquot of this was used for qRT-PCR. The expression level was normalized using rice *actin* (*act1*) as an internal reference gene and the fold change was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

In silico promoter analysis of *SEN1*

About 1 kb sequence upstream to the start codon of *SEN1* (LOC_Os10g02930) was retrieved from RGAP-DB and was subjected to an in silico analysis for the presence of cis-acting elements using the PlantCARE (Lescot et al. 2002) online tool. Similar promoter analysis of the *XPB2* gene

(LOC_Os01g49680) has been reported earlier (Raikwar et al. 2015).

Differential transcript analysis of *XPB2* and *SEN1* helicases

To understand the involvement of *XPB2* and *SEN1* genes in response to biotic and abiotic stresses, their differential transcription patterns were evaluated in 10 d old seedlings of WT BPT-5204 rice. For this, various phytohormones like 2 mM salicylic acid (SA), 100 µM methyl jasmonate (MJ) and 100 µM abscisic acid (ABA) and abiotic stress-inducing agents such as 15% polyethylene glycol (PEG 8000), 250 mM sodium chloride (NaCl) and heat treatment at 42 °C were applied to the seedlings. Salt, dehydration stress (PEG) and phytohormone treatments were given by dipping the seedlings in their respective solutions, while the heat treatment was induced by exposing the seedlings in a hot air oven maintained at 42 °C. The root and shoot samples were collected immediately after the onset of the stress treatments denoted as the 0 h, followed by 3 h, 6 h, 12 h, 24 h and 48 h after treatments. Seedlings maintained in the stress-free medium under similar growth conditions were used as the controls for normalization.

For the transcript analysis of these genes under biotic stress conditions, leaf samples of rice infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo* that causes bacterial leaf blight) and *Rhizoctonia solani* (that causes sheath blight) were used. The infection process of these pathogens on rice plants was followed as described earlier (Moin et al. 2016b; Saha et al. 2017). Leaf samples from untreated plants were used as controls to normalize gene expression levels. Rice *actin* was used as the internal reference gene. The relative fold changes were calculated through the $\Delta\Delta C_T$ method.

Measurement of growth and phenotypic parameters of the activation-tagged mutant lines

To examine the behavioral patterns of the two mutants under abiotic stress conditions, seeds of XM3, SM4 and WT (BPT-5204) were germinated on Murashige and Skoog medium for 25 days (Saha et al. 2017). Subsequently, they were transferred to test tubes containing phytohormones and stress-inducing agents in half-strength liquid nutrient Yoshida solution (Yoshida et al. 1976). Seedlings kept in plain half-strength liquid Yoshida solution were served as controls. Fresh stress was applied after every 7 days by changing the solutions. The seedlings were transferred to full strength Yoshida solution 20 days after stress (DAS) for revival experiments. Before shifting, root and shoot samples were collected separately for transcript analysis and the whole seedling samples were collected for the biochemical experiments. A similar pattern was followed during the recovery of the plants. The root length (cm), shoot length (cm), fresh weight (g), percentage of leaf wilting and survival of the seedlings were recorded. To understand the leaf wilting percentage, we measured the length of the whole leaf and the wilted region separately and determined the percentage of wilting of each leaf. The seedlings were transferred to the greenhouse and maintained under controlled conditions. During their growth, various parameters were measured, which included plant height, number of total and productive tillers (panicles), boot leaf length versus panicle length, photosynthetic efficiency and yield-related parameters like number of branches per panicle, number of seeds per panicle and per plant and overall weight of 100 seeds.

Measurement of chlorophyll fluorescence

The Pulse Amplitude Modulated fluorometer (PAM) gauges the photosynthetic performance of plants indirectly by testing the quantum efficiency of photosystem II (PSII). It involves the exposure of dark-adapted leaves to a strong pulse of light. The minimal level of fluorescence observed upon irradiation is considered as F_o, while F_m shows the maximum value of fluorescence. The difference between F_o and F_m is F_v or variable fluorescence. The ratio between variable fluorescence and the maximum fluorescence (F_v/F_m) indicates overall photosynthetic efficiency and also the level of stress experienced by a plant under unfavorable conditions. In a healthy unstressed plant, the F_v/F_m value ranges around ~ 0.83 (Murchie and Lawson 2013), which corresponds to the maximum photosynthetic yield in a plant. A significant reduction in this value shows stress induction in the plant. In our experiments, we have employed a portable MINI-PAM and followed the manufacturer's protocol (Walz, Effeltrich, Germany) to study the quantum efficiency of selected mutants with respect to their corresponding controls. All the plants were initially incubated in dark for 30 min and were then subjected to a short pulse of 8000 µmol m^{-2} s^{-1} light. The F_v/F_m ratio was plotted as a histogram.

Estimation of chlorophyll and proline contents

For chlorophyll estimation, two sets of samples collected after application of stress and their revival were used. The chlorophyll pigments were extracted from 100 mg plant tissues using di-methyl sulfoxide (DMSO). The corresponding absorbance obtained at 663 nm and 645 nm wavelengths using a UV spectrophotometer was used to calculate chlorophyll a, b and total chlorophyll contents (Zhang et al. 2009). The proline content was assessed spectrophotometrically at 520 nm from 100 mg plant tissue according to Bates et al. (1973), using the proline standard curve.

Principal component analysis of the observed morpho-physiological and biochemical data

Principal component analysis (PCA) is a statistical tool for efficient interpretation of highly correlated multivariate data. Here, the dataset comprised complex phenotypic and physiological traits of three genotypes (WT, XM3 and SM4) under four conditions (UT, PEG, 50 µM and 75 µM ABA). The main purpose of PCA is to reduce the dimensionality of a dataset to two or three principal components while still capturing most of the variance of the variables (Wold et al. 1987; Yano et al. 2019). We performed PCA using the "R" program to establish the patterns in the dataset of the three genotypes under simulated conditions (Kassambara and Mundt 2016; R core team 2019). For analyzing the data, the cos2 values have been considered. A high cos2 value indicates a higher impact of the corresponding variable in the principal component. A cutoff of 0.5 cos2 value has been considered.

Pot-level water withholding treatments

To study the response of *SEN1* and *XPB2* tagged mutants under pot-level drought conditions, 15-day-old mutants and WT seedlings were shifted to the pots containing black alluvial soil provided with ample water and maintained under greenhouse conditions (30 ± 2 °C, 16 h light/ 8 h dark photoperiod). Three plants per 7.5 kg pot were transplanted and triplicates for each condition were considered for further experiments. After 30 days, the overlaying water from each pot was withdrawn, and all the plants were exposed to drought conditions for three and seven consecutive days. In our previous study, we noticed that the Permanent Wilting Point (PWP) for BPT-5204 in our greenhouse conditions was 21 days (Moin et al. 2017). The drought experiments are usually defined in Percent Field Capacity (FC), which mainly depends on the type of soil. For black alluvial soils available in South India, which was also used in this study, the PWP occurs between an FC of 10–18% (http://www.indiawaterportal.org). Accordingly, the FC on 3- and 7-day drought treatments would be ~60 and 40%, respectively, in this investigation. After completion of drought treatments, all three plants from one pot were uprooted, and their root and shoot samples were collected independently for studying the transcript patterns of potential drought-responsive genes under stress conditions. The remaining pots were then allowed to recover after drought treatments by gradually supplying a required level of water for normal rice cultivation and were grown till plant maturity. The setup was repeated thrice and comparative yield-related studies were carried out between treated mutant plants with corresponding treated and untreated control counterparts to study whether the total yield of mutants was decreased, sustained or improved under drought. The grain quality was monitored by recording the changes in 100 seed weight, seed length:breadth ratio and its amylose:amylopectin ratio. The amylose content was determined spectrophotometrically using a standard amylose curve at 600 nm (Sowbhagya and Bhattacharya, 1971).

Transcript analysis of stress-responsive genes

The root and shoot tissues of mutant and WT plants exposed to phytohormone (ABA), dehydration stress (PEG) and pot-level drought (for 3 and 7 days) were used to check the transcript pattern of seven stress-regulated genes such as Trehalose Phosphate Phosphatase-1 (*OsTPP1*), Late Embryogenesis Abundant 3–1 protein (*OsLEA3-1*), type 2C Protein Phosphatase (*OsPP2C*), Dehydration Responsive Element Binding protein 2B (*OsDREB2B*), NAM-ATAF1-2-CUC2 proteins (*OsNAC1*, *OsNAC2*) and Ser/Thr protein Kinase-1 (*OsSIK1*). These genes are involved in ABA signaling and in modulating heat, cold, salt and drought stresses. The transcript levels of these genes in tagged lines were normalized with those of the corresponding stress-induced WT plants. The transcript patterns of stress-related genes were analyzed by qRT-PCR and relative fold change was calculated by the double-normalization method.

Seed germination assay with ABA treatment

The seeds of the mutants and WT were germinated on half-strength MS media containing 50 µM and 75 µM ABA under 16/8 h light/dark cycles. Seeds germinated on half-strength MS media without ABA were used as untreated controls. The germination of the seeds was documented after 5 days.

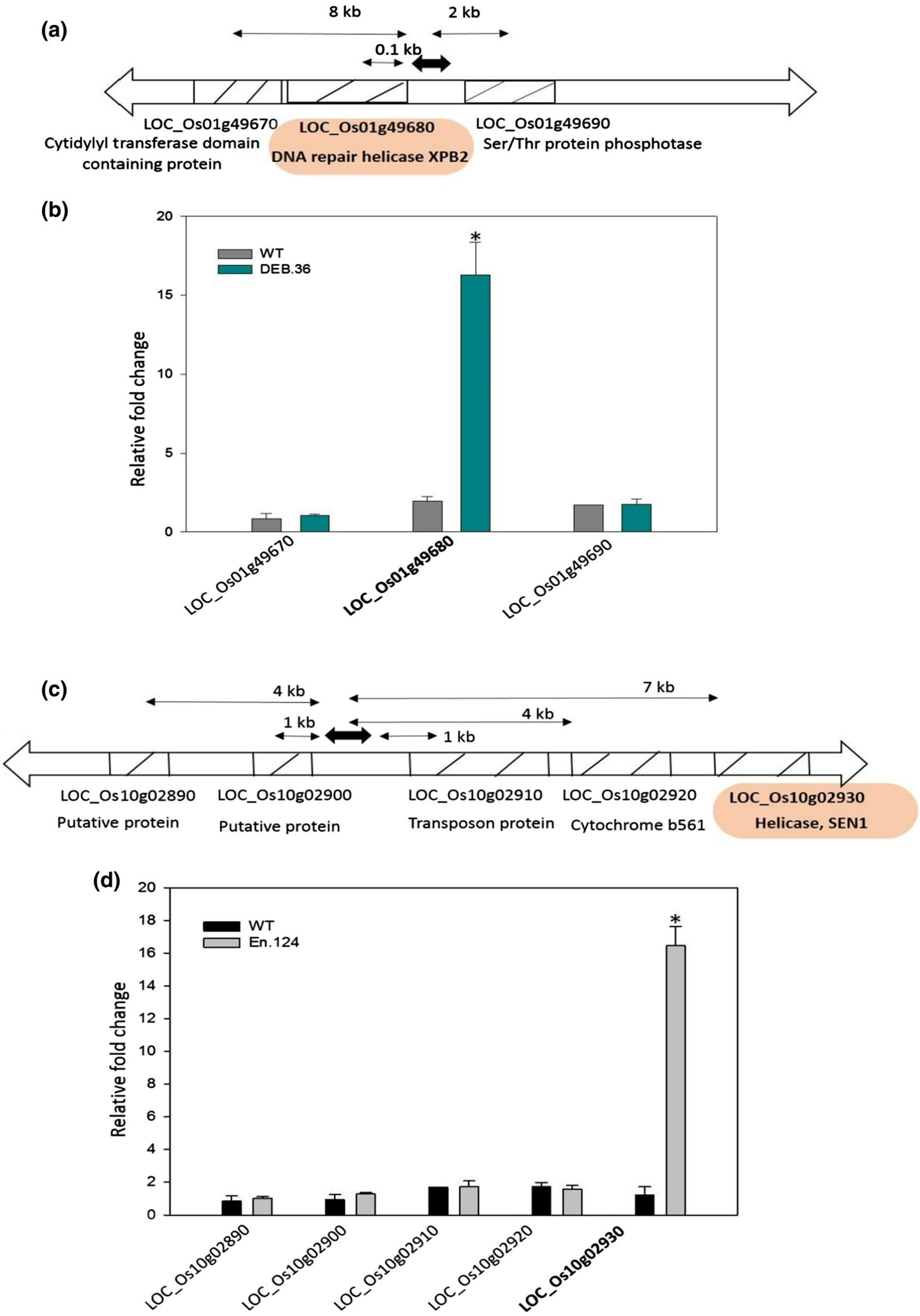
Statistical analysis

All the qRT-PCR experiments were conducted in three biological and technical replicates, whereas phenotypic and physiological experiments were performed in replicates of three plants. Statistical analysis of the mean values has been calculated using one-way ANOVA in SigmaPlotv11 at significance level $P < 0.001$ marked as "a", $P < 0.025$ marked as "b" and $P < 0.05$ marked as "c" in bar diagrams.

Results

Identification of tagged genes

Out of the selected activation-tagged mutant lines identified for enhanced WUE through studies on photosynthetic performance and Δ^{13}C analysis, two lines XM3 and SM4 were chosen for a detailed analysis. Although the roles of these two gene encoding proteins have been well studied


(a)
8 kb
2 kb
0.1 kb
LOC_Os01g49670
Cytidylyl transferase domain containing protein
LOC_Os01g49680
DNA repair helicase XPB2
LOC_Os01g49690
Ser/Thr protein phosphotase
(b)
Relative fold change
0
5
10
15
20
WT
DEB.36
*
LOC_Os01g49670
LOC_Os01g49680
LOC_Os01g49690
(c)
4 kb
7 kb
1 kb
4 kb
1 kb
LOC_Os10g02890
Putative protein
LOC_Os10g02900
Putative protein
LOC_Os10g02910
Transposon protein
LOC_Os10g02920
Cytochrome b561
LOC_Os10g02930
Helicase, SEN1
(d)
Relative fold change
0
2
4
6
8
10
12
14
16
18
20
WT
En.124
*
LOC_Os10g02890
LOC_Os10g02900
LOC_Os10g02910
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◂**Fig. 1** Gene map and quantitative real-time PCR of the tagged genes. Pictorial representation of the point of integration of the tetrameric enhancer element of the activation tagging vector, the genes in the 20 kb span and the subsequent quantitative real-time PCR analysis. (**a**, **c**) The bold double-headed arrow represents the enhancer integration. In the line XM3, three genes were present (LOC_Os01g49670, LOC_Os01g49680 and LOC_Os01g49690) and in the line, SM4 (En.124), five genes were present (LOC_Os10g02890, LOC_Os10g02900, LOC_Os10g02910, LOC_Os10g02920, LOC_Os10g02930) in the selected region. Quantitative real-time PCR (qRT-PCR) analyses showed up to 16-fold upregulation of two genes, i.e., **b** *XPB2* and **d** *SEN1* compared to the WT in XM3 and SM4 lines, respectively. Other tagged genes showed an expression level similar to that of the WT. The data were normalized using rice actin as an internal reference gene. One-way ANOVA was performed at a significance level $P < 0.05$ annotated by asterisks*

in nuclear activities, their involvement in stress responses or WUE has not been emphasized so far. It was observed that under limited water conditions, WT had higher $\Delta^{13}C$ (23.75‰) values compared to both the selected mutant lines, XM3 (20.05‰) and SM4 (20.14‰), respectively (Fig. S1). Since lower carbon discrimination values indicate higher WUE ($\Delta^{13}C$ is inversely related to WUE), these lines were carried further for flanking gene analysis. TAIL-PCR analysis was performed on the selected mutants, SM4 (En.124) and XM3 (DEB.36) to support the transgenic nature of the selected plants, specify the site of integration of the tetrameric 35S enhancers in their genomes and identify the genes in their immediate vicinity. The list of TAIL-PCR primers is provided in Supplementary Table S6. The sequences obtained from TAIL-PCR were subjected to a nucleotide BLAST search in the rice genome database to map the exact location of integration of the tetrameric enhancer elements (Fig. S2a, b). This led us to identify the genes on either side of the 4X enhancers in the two mutants.

In the tagged line, XM3, LOC_Os01g49670, LOC_Os01g49680 and LOC_Os01g49690 were present in a 20 kb region. Among these, LOC_Os01g49670 and LOC_Os01g49680 were situated 8 kb and 0.1 kb upstream of enhancers, encoding cytidylyltransferase domain containing protein and the DNA repair helicase XPB2, respectively. LOC_Os01g49690 was located 2 kb downstream from the enhancer integration and encodes a Ser/Thr protein phosphatase. In the SM4 mutant, the enhancers were flanked by LOC_Os10g02890, LOC_Os10g02900, LOC_Os10g02910, LOC_Os10g02920 and LOC_Os10g02930 loci in a 20 kb region. The first two genes that were located 4 kb and 1 kb upstream of the enhancers encode unidentified putative proteins, while the remaining three that were located 1 kb, 4 kb and 7 kb downstream, and encode a transposon protein, cytochrome B561 and SEN1 helicase, respectively.

A qRT-PCR analysis revealed a 16-fold upregulation of the genes, *XPB2* (LOC_Os01g49680) and *SEN1* (LOC_Os10g02930) in the lines XM3 and SM4, respectively (Fig. 1a–d) with respect to WT, while there was no considerable change in the level of expression of the other genes in the 20 kb regions of the selected mutants.

Promoter analysis of *SEN1*

The *XPB2* promoter region was earlier reported to have many *cis*-acting elements including the one for early responsiveness toward dehydration (ABRELATERD1), and dehydration responsive element (CBFHV) and MYBCORE element that respond to water stress (Raikwar et al. 2015). Our in silico promoter analysis of *SEN1* also revealed several cis-acting stress-responsive elements. The definite location and the detailed list of the cis-regulatory elements are provided in Supplementary Figure S3 and Table S2. These included 12 MYB binding elements (CAACTG and CAACCA/CAACAG) and two ABRE motifs (CGTGG) responsible for drought responsiveness among the others. The high transcriptional upregulation of these two genes in response to ABA and dehydration stress-inducing agent, PEG can be correlated with the presence of the corresponding responsive elements in the putative promoter region.

Transcript analysis of *XPB2* and *SEN1* under biotic and abiotic stresses

Since the *SEN1* and *XPB2* genes were suggested to be involved in improving the WUE of rice under limited water conditions, we found out the responsiveness of these two genes to other stresses as well. Their transcript patterns were studied in response to various phytohormones and abiotic stress-inducing factors. Both the genes showed high upregulation, notably in the root tissues compared to the shoots. We have categorized the expression patterns of the two genes as early (expressing within 3–12 h of treatment) and late (expressing after 12 h of treatment) responsive.

In the shoot, the level of *XPB2* transcripts was induced over threefold by SA, NaCl, ABA, PEG and heat stress (42 °C) (Fig. 2a) as an early stress response, which reached the peak during this period except for PEG, which increased up to sixfold after 48 h. The transcript level in roots was upregulated over fivefold under all six conditions within 3 to 12 h indicating the early responsiveness of the gene. In response to PEG, the high transcript levels (tenfold) were maintained after 12 h, while under NaCl (11-fold), ABA (48-fold) and heat stress (14-fold) treatments, high transcript levels were detected after 48 h also (Fig. 2b). In shoots, *SEN1* transcript level was induced as an immediate response under ABA (ninefold) and PEG (threefold) treatments within 3 h. Later, their levels decreased under ABA, while in response to PEG, the levels achieved a peak of 18-fold after 48 h (Fig. 2c). Early response of *SEN1* was observed under SA, NaCl, ABA and PEG in roots, while a late response was

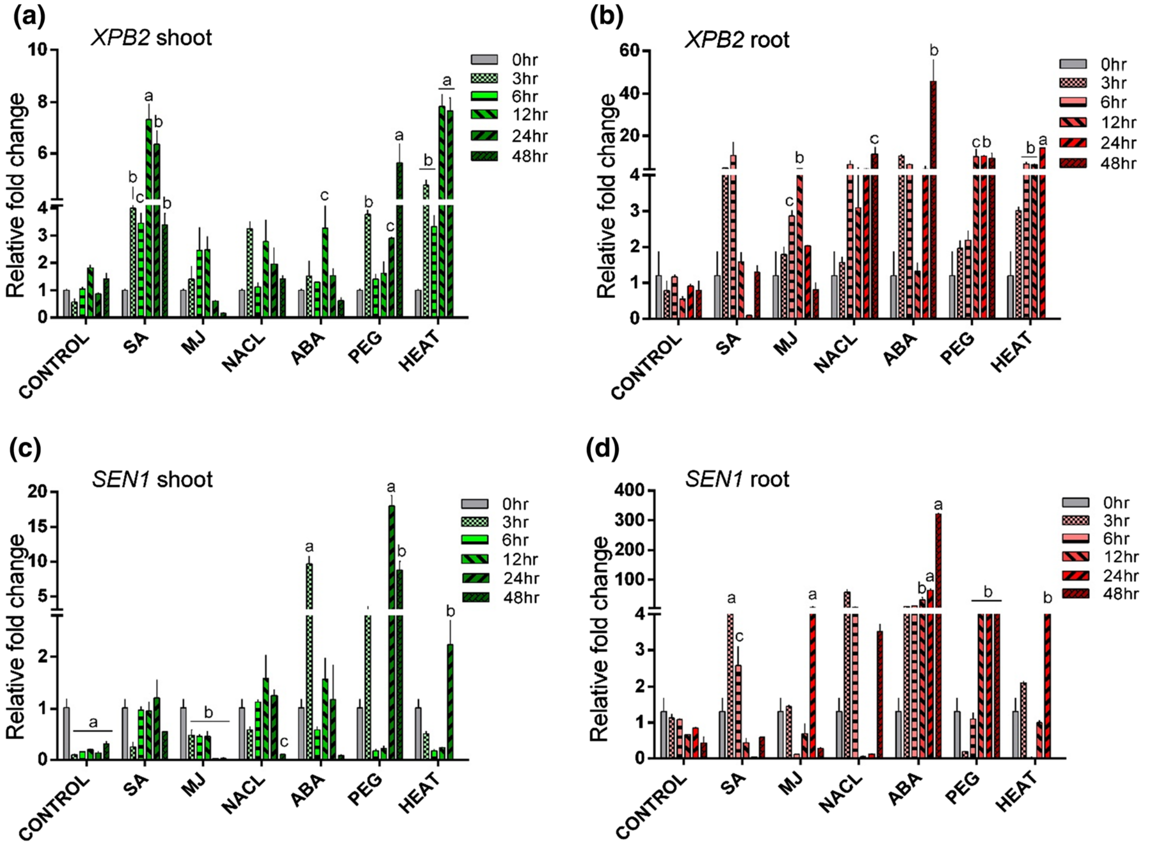


Fig. 2 Transcript analysis of *XPB2* and *SEN1*. Quantitative real-time PCR (qRT-PCR) analyses of **a**, **b** XPB2 shoot and root and **c**, **d** SEN1 shoot and root, respectively, in response to phytohormone and chemical treatments. Ten-day-old rice seedlings were subjected to SA, MJ, NaCl, ABA, PEG and heat treatments and root and shoot tissues were collected at various time points. Rice actin, *act1*, was used as the internal reference gene. The fold change was calculated using the $\Delta\Delta C_T$ method. The mean and the standard error are plotted in vertical bar graphs. One-way ANOVA was performed at a significance level $P<0.001$ marked as a, $P<0.025$ marked as b and $P<0.05$ marked as c

noted upon exposure to MJ and heat stress. The transcript level was maintained in response to PEG (fivefold) from 12 to 48 h, while ABA continued to increase its upregulation with the response reaching up to 320-fold at the end of 48 h (Fig. 2d).

On the contrary, *Xoo* and *R. solani* pathogens failed to induce the transcript levels of *SEN1* and *XPB2*. Upon infection with *Xoo*, both *SEN1* and *XPB2* were downregulated by 0.4-fold and 0.3-fold, respectively. In response to *R. solani*, the transcript level of *SEN1* was like that of untreated samples whereas, *XPB2* showed an elevation of around 2.4-fold (Fig. S4).

Phenotypic and physiological analyses of the tagged mutants under PEG and ABA

The two tagged mutants exhibited improved tolerance in response to dehydration stress (10% PEG) and phytohormone (50 µM and 75 µM ABA) treatments 20 DAS. The cumulative wilting of WT ranged between 9% (50 µM ABA) to 60% (PEG) under all three conditions and 30% (PEG) to 60% (50 µM ABA) 20 DAR (Fig. S6a to d). XM3 and SM4 lines, on the contrary, exhibited maximum wilting of 12 and 14% under PEG and maximum recovery of 85 to 95% 20 DAR from 50 µM ABA and PEG indicating that the enhanced expression of both the helicases rendered the plants more tolerant to water stress compared to the WT.

Seedling shoot and root parameters

The fresh weight, shoot and root lengths (Fig. S7a–c) of the tagged lines were recorded 10 and 20 DAS. The XM3 line exhibited a higher fresh weight 20 DAS (0.16–0.21 g) because of increased branching but showed similar shoot and root lengths related to the corresponding WT (0.11–0.17 g). In the SM4 line, the fresh weight was observed to be 0.21–0.25 g because of longer shoot and root lengths than the WT. The mean of these recordings for the WT and mutant plants 10 and 20 DAS was plotted as bar graphs.

Yield-related traits

Post-acclimatization in the greenhouse, the XM3 and SM4 lines were noticed to have better phenotypic parameters with an increased number of tillers and panicles per plant, improved plant height, boot leaf, panicle length, number of seeds per plant, seed weight and photosynthetic efficiency (Fig. 3a, b). The histogram depiction of the phenotypic parameters is provided in Figure S8(a–c), and the mean values along with their standard errors are provided in Supplementary Table S5.

The WT plants after revival from all the three stress treatments had two to three tillers per plant with one to two bearing panicles, shorter plant height, boot leaf and decreased panicle length. XM3 and SM4 lines had three to seven tillers and three to nine tillers per plant, respectively, all being productive. These were bigger, had longer boot leaf and panicle lengths than the WT. The boot leaf and panicle lengths were observed to be codependent.

In response to 75 µM ABA, a single WT plant survived bearing only 11 seeds. The total seed yield of XM3 (~461 seeds) and SM4 (~557 seeds) was 97 and 98% more than the corresponding WT, respectively. About 75 to 80% (XM3 and SM4, respectively) difference in seed yield was also observed under 10% PEG. Under untreated conditions, the weight of 100 seeds was almost similar in WT and the tagged lines. However, a significant difference was noted under PEG and 50 µM ABA stress conditions. These physiological parameters indicated decreased sensitivity of SEN1 and XPB2 toward high ABA concentrations.

Photosynthetic efficiency

In untreated WT, the quantum yield was found to be 0.74, whereas in both the untreated tagged mutants the efficiency was 0.77. Under 10% PEG, 50 and 75 µM ABA, the quantum yield of the WT decreased to 0.63 and 0.67, respectively. But the tagged lines continued to maintain higher quantum efficiency, which was almost similar to the untreated controls. The F_v/F_m ratio ranged from 0.72 to 0.77 in XM3 and SM4 lines, respectively, even after PEG and ABA treatments.

Chlorophyll and proline estimation

The contents of a, b and total chlorophyll were measured in the WT, and the tagged lines post-stress (Fig. 4a–c) and post-recovery (Fig. S9a–c). In XM3, the a, b and total chlorophyll contents were 17 µg, 10 µg and 27 µg/50 mg fresh weight, respectively, compared with WT, which had 10 µg, 8 µg and 21 µg/50 mg fresh weight, respectively, under PEG stress. The chlorophyll contents of XM3 post-recovery were observed to be slightly high under PEG and 50 µM ABA.

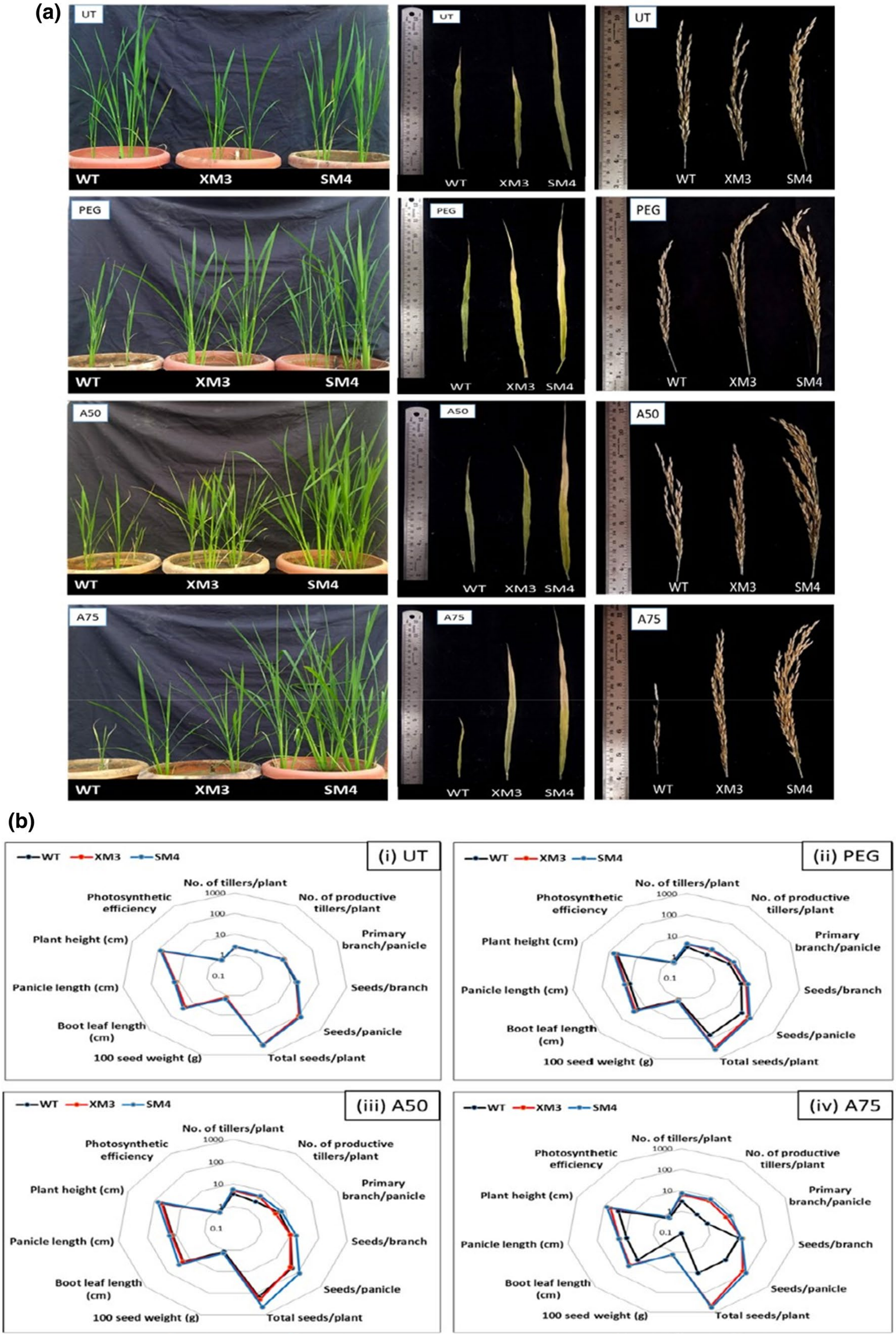
In SM4, the a, b and total chlorophyll contents post-stress ranged from 11 to 15 µg, 11 to 19 µg and 23 to 34 µg/50 mg fresh weight, while those of the WT ranged from 8 to 11 µg, 5 to 8 µg and 15 to 21 µg/50 mg fresh weight, respectively. Post-revival, the chlorophyll contents of SM4 were greater than the WT under all three stress conditions.

Under untreated conditions, WT, XM3 and SM4 had similar proline content, which ranged from 0.6 to 0.7 mg/100 mg fresh weight. Treatments with 10% PEG and 50 µM ABA had induced the proline content to rise to 0.9–1.0 mg/100 mg fresh weight in all of them. Under ABA 75 µM treatment, SM4 accumulated a remarkably high proline level (4 mg/100 mg fresh weight) compared with WT (Fig. 4d). After revival, the proline content of all the treated lines dropped and was almost similar to their corresponding untreated controls (Fig. S9d). Thus, higher chlorophyll and proline contents appeared to be related to improved photosynthetic efficiency and stress tolerance of the tagged lines leading to sustainable productivity.

The PCA of 24 morpho-physiological and biochemical properties showed that 72.73% of the variance among the genotypes was because of the factors influencing the first two dimensions (PC1 and PC2). These included 20 DAS fresh weight, 20 DAS chlorophyll and proline contents, revival percentage, photosynthetic efficiency, plant height, tiller numbers and seven other yield-related parameters (Fig. 5a and Tables S3 and S4). It is apparently noticed in the 2D plot (Fig. 5b) that WT, XM3 and SM4 behaved similarly under untreated conditions, but after the application of stress (PEG, ABA 50 µM and ABA 75 µM), they drifted farther away on the plot in different directions indicating their differential behavior. SM4 and WT responded differently under all three stress conditions whereas, XM3 behaved differently under PEG and 75 µM ABA conditions. Under 50 µM ABA, XM3 and WT showed similar behavioral patterns. These are observed by setting the distance or the closeness among the points. The overlapping region of XM3 and WT under PEG and ABA (50 µM) treatments showed similar behavioral pattern under these conditions, which are depicted by the parameters belonging to higher PC. WT moves along PC1 representing increased leaf wilting and SM4 moves toward PC2 showing higher tiller number and proline content (Fig. S5). XM3 remained intermediate between PC1 and PC2 corresponding to intermediate changes in the parameters.

Seed quality and yield-related observations on the tagged lines under pot-level drought conditions

The tagged lines and the WT plants were exposed to periodic drought conditions in pots for 3 (60% FC) and 7 (40% FC) days. The WT showed a pale green phenotype and higher leaf


(a)
UT
WT
XM3
SM4
PEG
A50
A75
(b)
WT
XM3
SM4
(i) UT
(ii) PEG
(iii) A50
(iv) A75
No. of tillers/plant
No. of productive tillers/plant
Primary branch/panicle
Seeds/branch
Seeds/panicle
Total seeds/plant
100 seed weight (g)
Boot leaf length (cm)
Panicle length (cm)
Plant height (cm)
Photosynthetic efficiency
1000
100
10
1
0.1

◀**Fig. 3** Physiological analysis of tagged lines. Phenotypic and physiological observations were performed post-acclimatization of the tagged lines (XM3 and SM4) in comparison with the WT plants. These include the difference in plant height, boot leaf and panicle length **a** as observed phenotypically. **b** represents the observed phenotypic features plotted as a radar graph. The parameters plotted are plant height (cm), number of tillers/plant, number of productive tillers/plant, primary branch/panicle, seeds/branch, seeds/panicle, total seeds/plant, 100 seed weight (g), boot leaf length (cm) and the panicle length (cm) and photosynthetic efficiency of the WT lines compared to XM3 and SM4 lines. (i), (ii), (iii) and (iv) represent different conditions such as untreated, 10% PEG, 50 µM and 75 µM ABA, respectively. The mean values have been plotted in a logarithmic ($\log_{10}$) scale. The tagged lines were observed to perform better under simulated stress conditions than the WT lines. The decrease in size of the black undecagon (WT) represents the same

rolling compared to XM3 and SM4 after 7 days (Fig. S10). No substantial difference in yield and phenotypic parameters were observed post 3 days of drought treatment. After induction of successive drought for 7 days, WT yielded ~ 200 seeds per plant and each of two mutants produced ~ 400 seeds per plant (Fig. 6). We studied a few important physiochemical properties of the seeds, such as the weight of 100 seeds, their length:breadth ratio and amylose:amylopectin contents (Table S6). Amylose:amylopectin ratio was observed to be 0.08 under both untreated and stress conditions in all the three genotypes (WT, XM3 and SM4). Likewise, no changes in hundred-seed weight (1.3–1.4 g) and length:breadth ratio (2.7–2.9) were noticed. The mean and the standard errors of the data are provided in Table S6.

Seedling germination assay in ABA treatment

The seed germination in the presence of 50 and 75 µM ABA showed germination retardation after 5 days compared with the untreated control (Fig. S11). The seeds of the tagged mutant lines continued to germinate and sustained on ABA-containing medium. Both XM3 and SM4 had longer roots with emerging shoots even at higher concentrations of ABA whereas the WT seeds showed mild germination under 50 µM concentration and failed to germinate at 75 µM. These results suggest that higher expression of *SEN1* and *XPB2* renders the plant less sensitive to ABA.

Transcriptional analysis of stress-responsive genes in tagged lines

We checked the transcript levels of seven stress-responsive genes (*OsTPP1*, *OsLEA3-1*, *OsPP2C*, *OsDREB2B*, *OsNAC1*, *OsNAC2* and *OsSIK1*) in the shoot (Fig. 7a–c) and root (Fig. 7d–f) tissues of tagged lines under treated conditions (PEG, 50 and 75 µM ABA). Most of these genes in shoots were upregulated under the influence of ABA treatment, while several of them were upregulated under PEG in roots. This showed that the helicases might affect the expression of several stress-responsive genes resulting in stress tolerance. Two genes, *OsPP2C* (ABA-dependent) and *OsDREB2B* (ABA-independent), were observed to be downregulated or have an equal level of expression as the WT in shoot and root tissues under ABA. The observation was the same in both XM3 and SM4 tagged lines. This suggests that XPB2 and SEN1 are implicated in both ABA-dependent and independent pathways of stress tolerance.

In shoot tissues of XM3 lines, 50 µM ABA induced the expression of *OsNAC1*, and *OsTPP1* and *OsNAC1* were upregulated by threefold to eightfold, respectively, under 75 µM ABA. *OsLEA3-1* and *OsSIK1* were induced (twofold to ninefold) under both concentrations of ABA. Under PEG treatment, *OsSIK1, OsNAC2* and *OsPP2C* showed twofold–threefold upregulation in shoots, whereas *OsTPP1*, *OsLEA3-1, OSPP2C, OsDREB2B, OsNAC1* and *OsNAC2* were upregulated moderately in roots. *OsLEA3-1* and *OsPP2C* exhibited the highest transcript levels up to 100-fold and 15-fold, respectively. In response to both 50 and 75 µM ABA, *OsTPP1* and *OsSIK1* showed twofold–fourfold upregulation in XM3 lines.

The shoots of SM4 showed fivefold to sixfold upregulation of *OsTPP1*, *OsLEA3-1* under 75 µM ABA and twofold upregulation of *OsNAC*2 under 50 µM ABA. *OsSIK1* was highly upregulated by eightfold and 11-fold under PEG and ABA 75 µM treatments, respectively. In root tissues, *OsTPP1*, *OsLEA3-1* and *OSPP2C* were upregulated under PEG treatment and *OsLEA3-1* exhibited the highest transcript level up to 42-fold. Under 50 µM ABA treatment, *OsTPP1* and *OsNAC2* were expressed by 2.5-fold and *OsSIK1* was upregulated by 37-fold. *OsTPP1* and *OsSIK1* were upregulated under both the concentrations of ABA.

We have likewise performed a comparable analysis on the tagged lines after exposing them to pot-level drought stress for 3 and 7 days (Fig. 7g). It was realized that the upregulation of these genes was more prominent in the root tissues compared with the shoots. *OsNAC2* and *OsSIK1* showed a moderate upregulation of twofold–threefold in shoots after 3 days, whereas in roots, *OsNAC1* and *OsNAC2* were upregulated to sevenfold and twofold, respectively, in the XM3 line. In the SM4 line, *OsSIK1* showed 3.5-fold upregulation in shoots and *OsNAC1* and *OsNAC2* showed sevenfold and twofold upregulation in roots. Thus, three out of seven genes were upregulated after 3 days of treatment. After 7 days of prolonged drought treatment, six out of seven genes became upregulated. A twofold upregulation of *OsTPP1* was detected in XM3 shoots, whereas *OsPP2C* showed fourfold upregulation in roots. In SM4 shoots, *OsTPP1* and *OsNAC2* genes showed a twofold upregulation, and *OsDREB2B*, *OsNAC1* and *OsSIK1* were upregulated by 2.6-fold in SM4 roots.

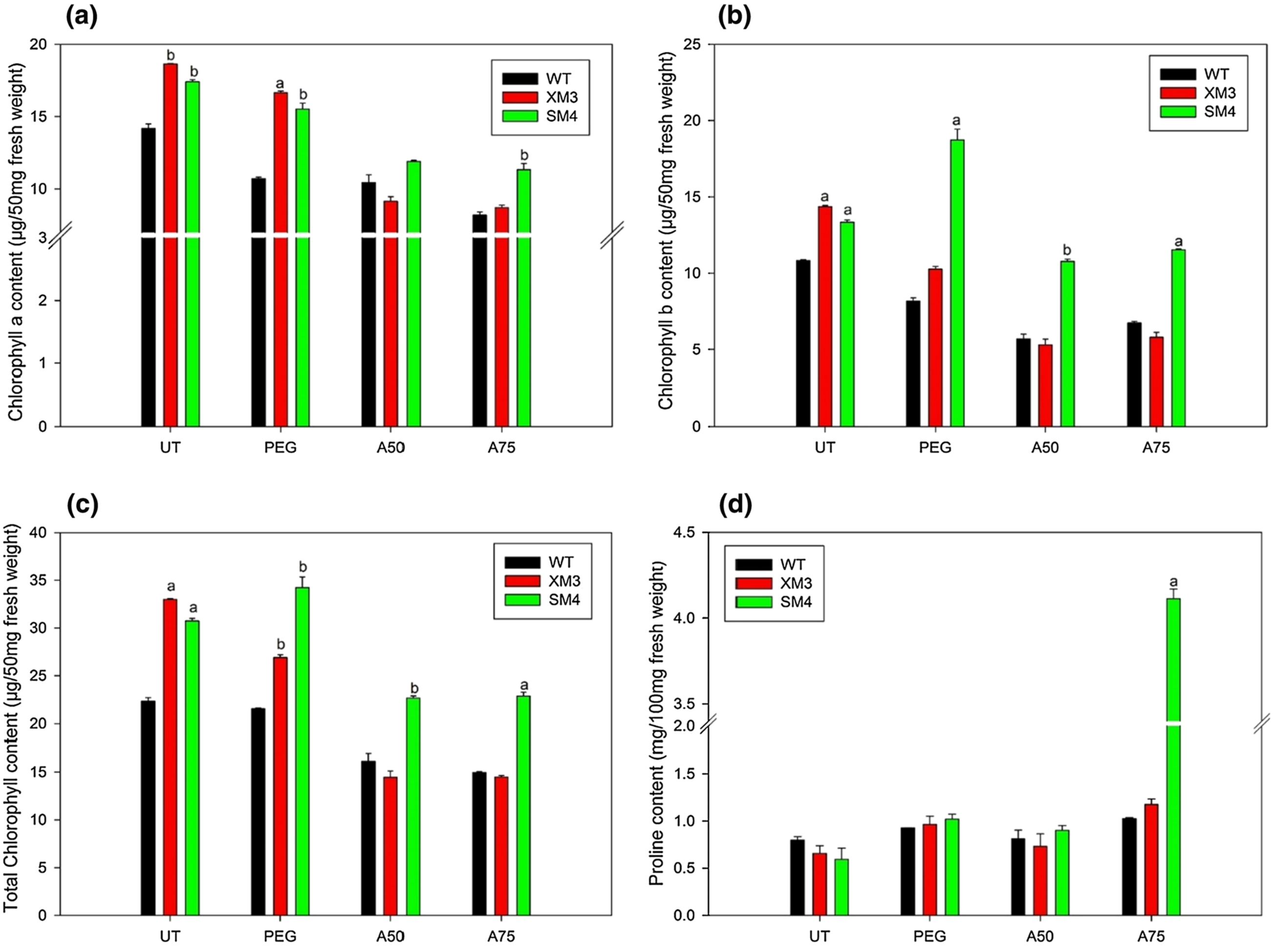


Fig. 4 Biochemical analysis of the tagged lines. Graphical representation of the biochemical studies done on XM3 and SM4 in comparison with WT plants post 20 days of stress. **a**–**d** depict chlorophyll a, chlorophyll b, total chlorophyll and proline contents post-stress, respectively. One-way ANOVA was performed at a significance level $P < 0.001$ marked as a, $P < 0.025$ marked as b, $P < 0.005$ marked as **c**

Discussion

The functions of redundant and embryonic lethal genes in plants can be effectively studied by integrating tetrameric 35S enhancers that activate the adjacent genes in either orientation in the plant genome by a mechanism called activation tagging (Weigel et al. 2000; Jeong et al. 2002; Wan et al. 2009). Some activation-tagged lines in an *indica* rice variety generated earlier by our group showed high WUE-related physiological parameters and yield under limited water conditions (Moin et al. 2016a). The individual CaMV35S promoters cause ectopic overexpression of gene(s) that are in transcriptional fusion with them, and this occasionally leads to pleiotropic effects in the transgenic plants. However, the enhancers, which are only one of the components in the complete 35S promoter, do not lead to constitutive expression but upregulate the target genes more than their endogenous levels. Thus, the plant phenotype resulting from enhancer-mediated activation would more likely reflect the original function of the tagged gene (Weigel et al. 2000; Jeong et al. 2002; Tani et al. 2004).

XPB2 and *SEN1* helicases are induced in rice roots under chemical and hormonal treatments

The two mutant lines employed in this study were stable *Ds* lines devoid of the *Ac* element. Also, the enhancer integration was intergenic and hence, the function of other endogenous genes might not have been disrupted, which would have otherwise occurred because of intragenic inactivation. Owing to stable and intergenic integration, the WUE and drought-tolerant phenotypes of the two selected mutants have resulted more likely by enhanced activation of their respective genes. The mutant lines were shortlisted based on their quantum efficiency and $\Delta^{13}C$ values. During water stress, plants close their stomatal aperture, reducing their

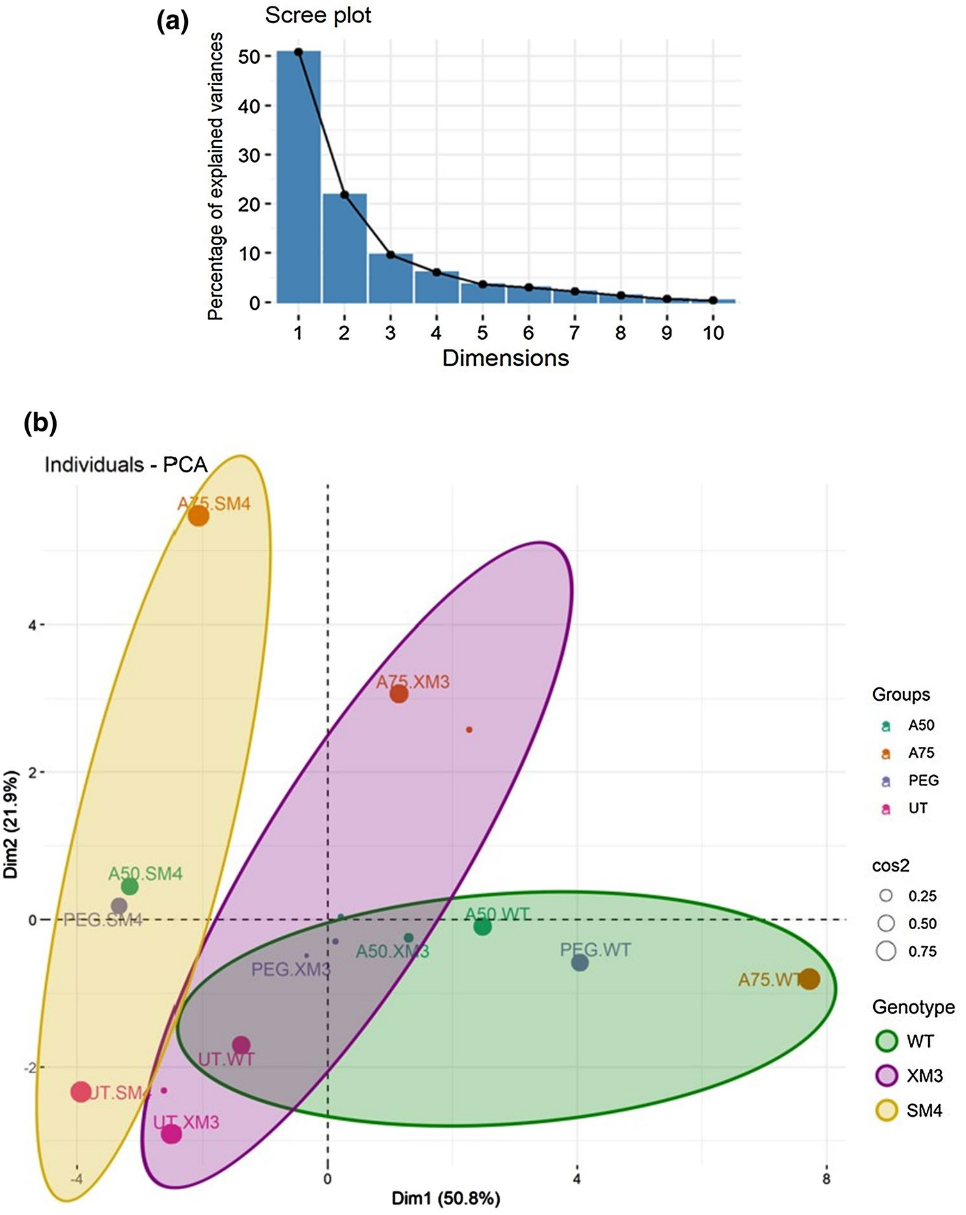


Fig. 5 PCA for plant morpho-physiological and biochemical parameters. Principal component analysis of the 24 observed phenotypic, physiological and biochemical parameters of the three genotypes (WT, XM3 and SM4) under simulated stress conditions (UT, PEG, ABA 50 and 75 µM). **a** Scree Plot for the observed variance under each dimension (or principal component). **b** Plot for PC1 and PC2, with the genotypes across simulated conditions plotted. Each oval encompasses the observed pattern of variance of each genotype across the simulated conditions under the first two principal components. The green, purple and yellow oval show the genotypes, i.e., WT, XM3 and SM4, respectively

intercellular CO_2 concentration (C_i). Δ^{13}C is directly influenced by the C_i and hence, its decline during stress enhances carbon discrimination and lowers the WUE (Martin and Thorstenson 1988; Chen et al. 2011). The higher quantum efficiency and low Δ^{13}C of mutants as observed in this study indicate high photosynthetic performance and WUE, respectively, thereby culminating in increased yield under water withdrawal conditions. In the XM3 line, *XPB2* DNA helicase, near the enhancer was upregulated as compared to other genes present in the neighborhood, which showed no upregulation as in the WT. Also, *SEN1* RNA helicase proximal to tetrameric enhancers became upregulated in SM4.

The expression of *SEN1* and *XPB2* helicases was induced particularly in root tissues by application of stress-inducible phytohormone, ABA and PEG. Since roots are the primary organs that perceive the stress signals, an upregulation of the genes in root tissues shows their role in regulating a signaling cascade for water stress responses (Janiak et al. 2016). Unlike abiotic stress treatments, the transcript levels of *SEN1* and *XPB2* were not significantly activated by the pathogens, *Xoo* and *R. solani.*

XPB2 and *SEN1* help in enhancing productivity in rice under limited water conditions

The *SEN1* and *XPB2* gain-of-function mutants were subjected to simulated water stress conditions by allowing them to grow in PEG and two concentrations of ABA, and drought by subjecting them to periodic water withdrawal conditions

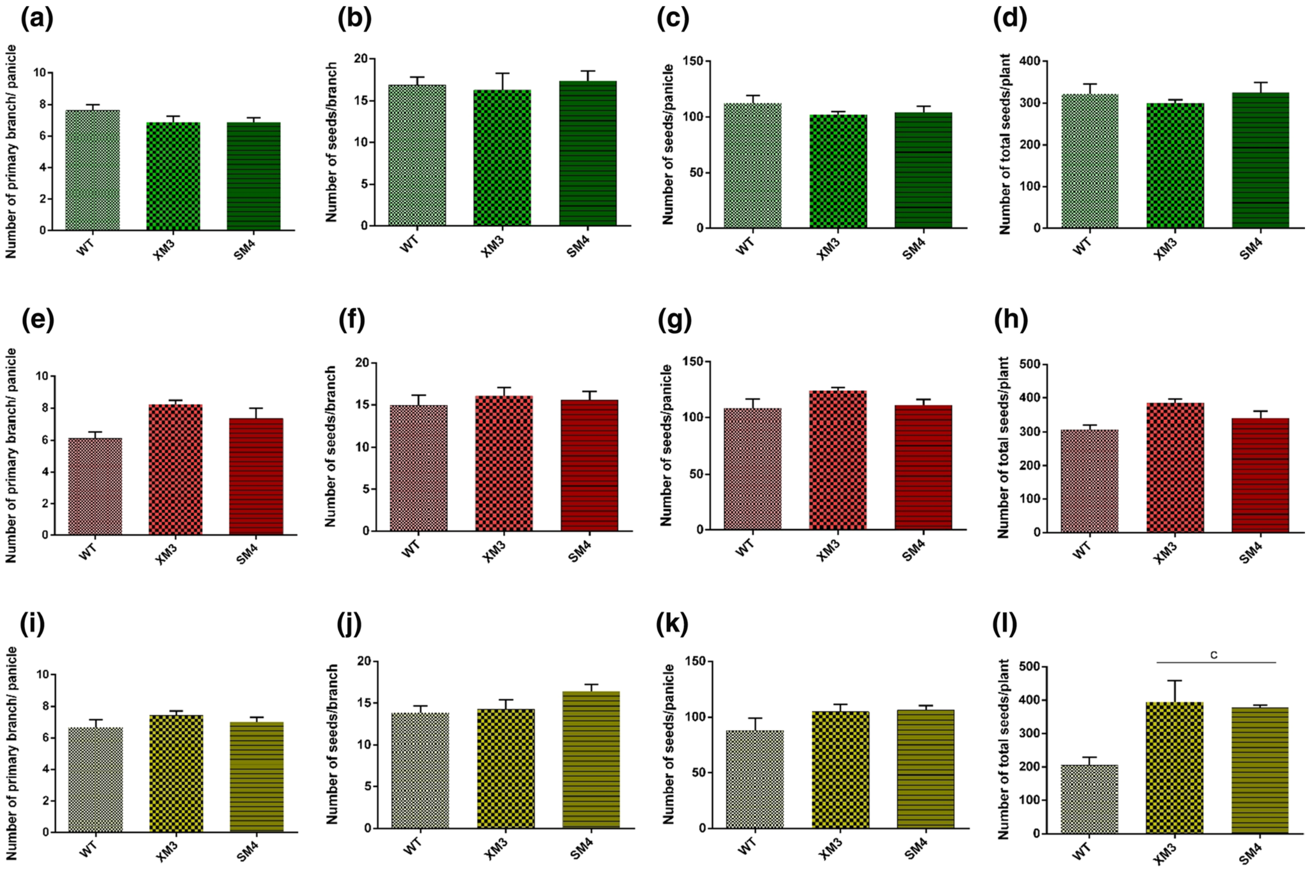


Fig. 6 Comparative analysis of yield-related traits at pot-level drought conditions. Graphical representation of yield-related traits was observed in the three genotypes (WT, XM3 and SM4) after imposing drought conditions. Periodic removal of water was done consecutively for 3 and 7 days followed by re-application of water until seed setting. For the control setup, all three genotypes were continuously watered normally throughout the experiment. **a–d** represents the untreated condition and **e–h** and **i–l** represent consecutive 3- and 7-day drought, respectively. A significant difference in the total seeds per plant was observed after 7 days of drought. One-way ANOVA with a Bonferroni correction was performed at the significance level $P < 0.05$ which are marked as c in bar diagrams

in pots. The performance of tagged lines, XM3 and SM4 was superior under all the stress conditions.

PCA allowed us to quickly and intuitively identify the phenotypic pattern hidden in the high-dimensional multivariate dataset of morpho-physiological and biochemical parameters. We could demonstrate the similar phenotypic responses of the genotypes untreated conditions, and the tolerance of the gain-of-function mutants, XM3 and SM4 under simulated stress conditions. On the PCA plot, they remain in different locations from the WT indicating a distinctive phenotypic response, which could be because of a difference in their ability to respond to the stress as a response driven by the gain-of-function mutations in these genotypes.

Root length under water deficiency is an important trait for plant productivity. Thus, comparatively longer roots in SM4 lines under simulated water stress indicate their enhanced tolerance (Sharp and LeNoble 2002; Janiak et al. 2016). Similarly, the tagged lines showed vigorous phenotypes after 3 and 7 days of drought treatment in pots with respect to the WT. Drought hampers the water balance of the plant that causes growth retardation and premature senescence of leaves (Anjum et al. 2011; Sreenivasulu et al. 2012). We observed similar phenomena in the WT, which had lower biomass, higher wilting percentage and smaller plant revival percentage compared with the tagged mutant counterparts under simulated stress conditions. Therefore, SEN1 and XPB2 appeared to be engaged in providing rigidity or vigor to the plant during and after stresses.

An excessive level of ABA leads to the inhibition of shoot growth, reduced carbon accumulation and yield (Zhang et al. 2006; Blum 2005). Such stress avoidance mechanism was found in the WT, which showed decreased plant height and yield post-revival. The exogenous stress cues had little impact on the physiology and yield of the gain-of-function mutant lines. Prolonged drought closes the stomata, causing ineffective gaseous exchange and a decline in the photosynthetic capability of the plant (Sreenivasulu et al. 2012). This is clear from the photosynthetic performance

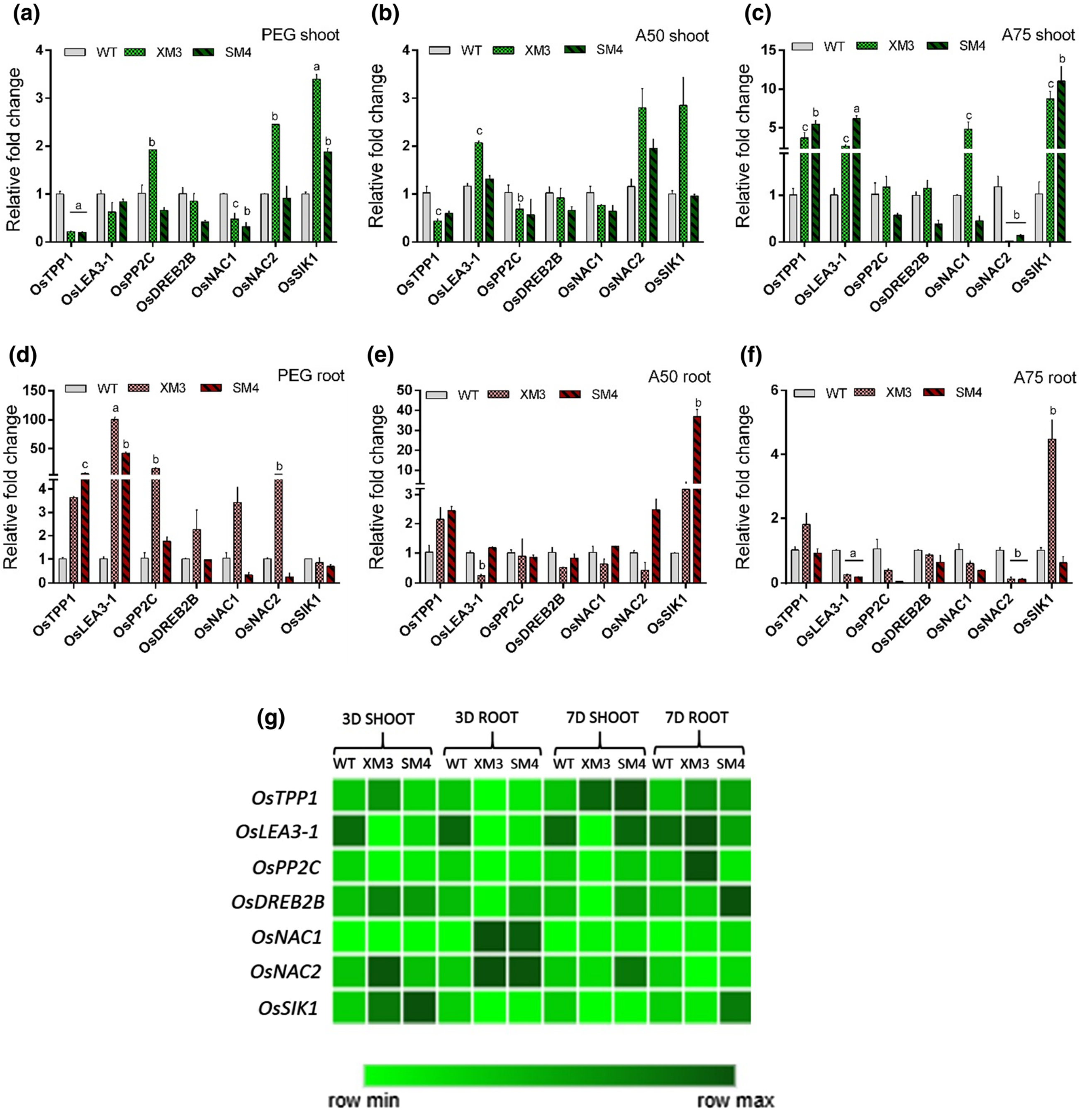


Fig. 7 Expression analysis of stress-responsive genes under simulated and pot-level drought conditions. Graph representing the transcript level of seven drought specific genes under imposed stress cues (10% PEG, 50 and 75 µM ABA) in shoots (**a–c**) and roots (**d–f**) tissues of the tagged lines with respect to the WT plants. Rice *actin* was used as the internal reference gene. The individual WT sample for each treatment was used to normalize the data. The fold change was calculated using the $\Delta\Delta C_T$ method. The mean and the standard error are plotted in a vertical bar graph. One-way ANOVA was performed at a significance level $P < 0.001$ marked as "a", $P < 0.025$ marked as "b" and $P < 0.05$ marked as "c". **g** Transcript analysis of seven drought specific genes in root and shoot tissues post 3 days and 7 days drought. The data were normalized using rice *actin* as the internal reference gene. The corresponding WT samples for each drought treatment were used to normalize the data, and the fold change was calculated using the $\Delta\Delta C_T$ method. The results were depicted as heatmaps generated by the MORPHEUS program

of the WT compared to XM3 and SM4 lines. The F_v/F_m ratio indicates the photochemical efficiency or the degree of stress experienced by the plant (Murchie and Lawson 2013; Osmolovskaya et al. 2018). The healthy untreated WT and mutant plants had a quantum efficiency ranging from 74 to 77%. When exposed to stress, the efficiency of the WT decreased to a range of 63–67%, while that of the tagged lines remained nearly unaltered (72–77%). This points to the fact that the photosynthetic ability of the tagged lines was not deterred by the stress. Since both chlorophyll a and b are susceptible to drought stress, their higher contents in the tagged lines corroborate better photosynthetic competence of XM3 and SM4 and, in turn, their yield (Anjum et al. 2011; Osmolovskaya et al. 2018; Zhang et al. 2018). Similarly, SM4 lines also accumulated a high level of proline under ABA treatment. During drought stress, plants maintain turgor pressure by accumulating ions and organic solutes, and proline is one of these compatible solutes. A higher level of this constitutes a known osmotic adjustment in cells. Greater accumulation of osmoprotectants indicates better drought tolerance and regulation of osmotic potential (Anjum et al. 2011; Yang et al. 2018; Zhang et al. 2018).

The boot leaves are regarded as an important source of metabolites in rice plants that contribute to plant productivity. Their length has a positive correlation with the panicle length and yield (Rahman et al. 2014). Also, high ABA accumulation in reproductive tissues can lead to the abortion of pods and reduce the grain filling period (Sreenivasulu et al. 2012). Therefore, a decrease in the boot leaf and panicle length in the WT under stress indicates its susceptibility toward drought. A 26–98% higher yield per plant in the tagged lines over the WT under simulated conditions and 9–50% more yield per tagged line under pot-level drought experiments imply that the SEN1 and XPB2 helicases are not only involved in maintaining a sustainable yield but also responsible for improved yield in the gain-of-function mutants even under severe stress conditions. Our statistical analyses also support these findings. The phenotypic performance and yield of WT significantly declined after 7 days of persistent drought (at $P < 0.05$), while the mutant lines showed no significant change in yield pre- and post-stress conditions. A significant difference in yield was also noticed within the genotypes under the drought. These investigations further reinforce the role of helicases in promoting tolerance toward drought/dehydration stress. While improving the yield, the enhanced expression of helicases did not affect grain quality. It has been determined by seed weight, dimensions and chemical composition of the endosperm (Chang and Somrith 1979). Starch is made of two components, one is linear chain amylose and the other being branched chain amylopectin. The amylose-amylopectin ratio determines the cooking and feeding quality of rice, which affects swelling and disintegration properties (Juliano 1979). Hence, this composition is crucial for determining the seed quality.

The helicases identified in this study do not belong to the DEAD/H box helicases, which are well known for inducing drought tolerance when overexpressed in plants. XPB2 falls under subgroup, SF2, while SEN1 belongs to Upf-1-like subfamily under SF1B (SEN1) group of helicases (Raikwar et al. 2015; Martin-Tumasz and Brow 2015). Both the genes have been surveyed extensively in yeast and humans for their role in maintaining genomic stability, but have not been explored for drought stress tolerance in plants. Apart from a few in silico and *AtXPB1* knockout studies (Costa et al. 2001; Raikwar et al, 2015), no reports on these helicases are available in drought stress responses. *OsSUV3* is a known DEAD/H box helicase from rice, whose overexpression in IR64 rice variety has led to improved drought tolerance (Tuteja et al. 2013). Another rice RNA helicase, OsRH58 exhibited drought-tolerant activity in Arabidopsis (Nawax and Kang 2019). PDH45 and PDH47 are pea DNA helicases, which have similar tolerance when expressed under the constitutive promoter in groundnut (Manjulatha et al. 2014), chili (Shivakumara et al. 2017) and rice (Singha et al. 2017). SIDEAD31, an RNA helicase from Arabidopsis imparted drought tolerance when expressed constitutively in tomato (Zhu et al. 2015). Other helicases from Arabidopsis include RH5, RH8 and RH25, which had been reported for enhancing drought tolerance in plants (Kant et al. 2007; Baek et al. 2018).

The WT seeds inoculated on 75 µM concentration of ABA failed to germinate after 5 days, but XM3 and SM4 tagged lines germinated normally and continued to grow. Usually, high ABA concentration compromises the germination rate and seedling establishment causing cell dehydration, wilting and death. (Zhang et al. 2018). The germination ability of tagged lines shows the probable role of SEN1 and XPB2 in the negative regulation of ABA-mediated seed dormancy.

Existence of a probable cross-talk between *SEN1* and *XPB2* with other stress regulatory pathways resulting in drought tolerance of rice

The expression level of seven stress-specific genes was studied in the tagged lines under PEG, ABA and pot-level drought after the complete withdrawal of water. There was an upregulation of all seven genes in XM3 and five genes in SM4 under simulated stress conditions. Under pot-level drought, four genes in XM3 and seven genes in SM4 became upregulated. This implies a possible cross-talk between *SEN1*/*XPB2* and other stress-tolerant genes, which together not only induced stress-tolerance in the tagged lines but also improved their productivity under stress. However, the interaction between the stress regulatory genes and the pathways they operate requires further investigation.

In conclusion, the gain-of-function mutant lines of *SEN1* and *XPB2* genes had the advantage over the WT in overcoming drought and dehydration stress. Currently, efforts are underway to independently overexpress these two genes for comprehensive functional characterization and likewise to understand the underlying mechanism of stress tolerance.

The expression of these genes is regulated by ABA, PEG and other stress factors, but there is no convincing evidence that these genes work in an ABA-dependant or an independent manner. The promoter analysis of *XPB2* showed the existence of ABRE, DRE and MYB cis-acting elements and that of *SEN1* showed MYB and ABRE motifs. The presence of both DRE and ABRE elements suggests the simultaneous existence of ABA-dependent and independent gene regulation (Roychoudhury et al. 2013; Yoshida et al. 2014). Such phenomenon had been seen in Arabidopsis *rd29A* gene promoter (Narusaka et al. 2003). Also, MYB transcription factors are known to function in an ABA-dependent manner during stress, but certain MYB factor like OsMYB3R-2 was less sensitive to ABA (Dai et al. 2007). From our observations, the XM3 and SM4 gain-of-function mutant lines have decreased sensitivity toward ABA, although their expression is induced by it. Thus, SEN1 and XPB2 helicases might play a transitional role in ABA-dependent and independent pathways. *OsPP2C* is a very potent ABA-dependent gene, and *OsDREB2B* is another ABA-independent gene that either shows an equal level of expression as the control or gets downregulated both in root and shoot tissues. The downregulation or consistent expression of *OsPP2C* (an ABA-dependent gene) and *OsDREB2B* (an ABA-independent gene) during the expression analysis under ABA treatment is probably an outcome of cross-talk between the two pathways or the existence of a negative feedback mechanism. Reports suggest the existence of such negative regulation of stress marker genes by *OsNAC2* despite the gene itself getting induced by ABA (Shen et al. 2017). Based on the previous considerations, it can also be presumed that both genes regulate stress-tolerance mechanism either by inducing DNA repair pathways to overcome DNA damage emanating from stress or by effectively terminating pervasive transcription or by resolving unwanted DNA: RNA or RNA: RNA hybrids formed during stress (Mischo et al. 2011, 2018; Richards et al. 2008; Raikwar et al. 2015; Han et al. 2017) thereby, upregulating the transcription of other important stress regulatory genes. These genes appear to have a positive regulation toward stress tolerance of rice plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00122-021-03823-0.

Acknowledgments MD is grateful to UoH BBL and UGC for the research fellowship and to DBT for funding the rice activation tagging project. MM is thankful to DST for the fellowship and research grant in the form of the INSPIRE-faculty program. AS and AB are grateful to DBT, and DD to DST-INSPIRE for proving Research Fellowships. MD is grateful to Dr. M.S. Madhav, Department of Biotechnology, Indian Institute of Rice Research, Hyderabad, India, for providing the infected rice samples and wild-type BPT-5204 seeds. PBK is grateful to the National Academy of Sciences-India for the NASI-Platinum Jubilee Senior Scientist award. The authors are grateful to Prof. M. Udaya Kumar, Bengaluru, India, for his help in IRMS analysis.

Author contribution statement PBK, MM and MD designed the experiments and prepared the manuscript. MD performed the experiments. MM and AB generated and maintained the activation-tagged mutant lines. MM performed the flanking sequence analysis through TAIL-PCR and identified the SEN1 and XPB2 genes. AS helped in the qRT-PCR experiments, analysis and seed counting. DD helped in PCA of the dataset. PBK supervised the work. MD, MM and PBK prepared the manuscript.

Funding The activation-tagged rice population was developed in a DBT funded Project (BT/PR13105/Agr/02/684/2009) sanctioned to PBK while he was working at the University of Hyderabad. A part of the funding for this work is also obtained from the DST-INSPIRE faculty project (IFA17-LSPA67) sponsored to MM.

Declarations

Conflict of interest The authors declare that the research has been performed without any financial and commercial conflict of interest.

Ethics approval Not applicable.

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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 correlated them with *in-silico* studies. Fully annotated genomic sequences available in rice database have enabled us to study the protein properties, ligand interactions, domain analysis and presence of *cis*-regulatory elements in a bioinformatics analysis. Most of the genes were induced immediately after the onset of stress particularly in the roots of ABA treated plants. *OsGRAS39* was found to be very highly expressive gene under sheath blight infection and both abiotic stress treatments while *OsGRAS8*, *OsSHR1* and *OsSLR1* were also responsive. Our earlier functional characterization (Moin et al., 2016a) followed by the genome wide characterization of the GRAS gene family members in the present study clearly show that they are highly appropriate candidate genes for manipulating stress tolerance in rice and other crop plants.

Keywords: GRAS genes, rice, stress tolerance, genome-wide analysis

1. Introduction:

Identification and analysis of transcription factors (TFs) is an essential aspect of functional genomics research. TFs bind to DNA or protein sequences and regulate gene expression (Zhang et al., 2018). They play important roles in almost all cellular functions like growth, development, metabolism, signal transduction, resistance/ tolerance to abiotic and biotic stress factors among others. About 320k TFs from 165 different plant species have been reported. Among them, some important transcription factors include WRKY, MBS, MADS, ARF, AP2/EREBP, HB, SBP, bZIP, GRAS etc. (Zhang et al., 2018; Zijie et al. 2019; Navjot et al., 2020)

GRAS group of transcription factors are plant specific proteins, first observed in bacteria and assigned to the Rossman fold methyl transferase superfamily (Zhang et al. 2012). Later, this group radiated towards the ancestors of bryophytes, lycophytes and other higher plants. (Cenci et al., 2017; Zhang et al., 2018). A large number of GRAS genes have been identified in various plant species including 34 in Arabidopsis, 60 in rice, 86 in maize, 106 in *Populus trichocarpa* and many others (Tian et al., 2004; Liu et al., 2014; Guo et al., 2017). The higher number of genes in this gene family indicates that the expansion of the gene family might have happened via segmental and tandem duplication events in evolution and retention of multiple copies post duplication events (Tian et al., 2004; Huang et al., 2015). Till date, the GRAS family of TFs have been studied in 30 different plant species including Arabidopsis, rice, mustard, lotus, tomato, castor bean, poplar, pine, grapevine and others (Cenci et al., 2017). This gene family has been divided into eight subfamilies in Arabidopsis and rice, while the number varied from eight to thirteen in tomato, poplar and castor beans (Tian et al., 2004; Liu et al., 2014; Huang et al., 2015; Wei et al., 2016).

GRAS proteins consist of 400-770 amino acid residues and derive the name from the first three identified members of this family viz. Gibberellin-Acid Insensitive (GAI), Repressor of GAI (RGA) and Scarecrow (SCR) (Pysh et al., 1999; Bolle et al., 2004; Zhang et al., 2017). These genes have a conserved C- terminal region, which forms the GRAS domain and a variable N- terminal region. The conserved region or the GRAS domain comprises five motifs in the following order; leucine heptad repeat I (LHR I), VHIID motif, leucine heptad repeat II (LHR II), PFYRE motif and the SAW motif. (Pysh et al., 1999). The conserved C- terminal domain is responsible for the transcriptional regulation of the genes that exist under their control. The LHR region is required for protein dimerization and the VHIID is necessary for protein-DNA interactions. PFYRE and SAW are the other important regulatory domains that are present in GRAS TFs. Mostly GRAS genes are nuclear localized except PAT1, which is found in the cytoplasm. (Pysh et al., 1999; Tian et al., 2004). The variable N- terminal region consists of intrinsically disordered regions (IDRs,) which are important for molecular recognition during plant development. Due to these IDRs, the GRAS transcription factors are functionally polymorphic (Sun et al., 2012). This gene family integrates environmental and growth regulatory cues and play significant roles in plant development. This family of genes is responsible for a variety of biological functions including gibberellic acid signaling (GAI and RGA of DELLA subfamily and SLR1 of rice) (Pysh et al., 1999; Liu et al., 2014; Vinh et al., 2020), SHR and SCR genes for radial root patterning (Helariteutta et al., 2000), SCL3 for root elongation (Huang et al.,2015), HAM for shoot meristem formation (Stuurman et al., 2002), PAT genes for phytochrome signaling (Bolle et al., 2000), NSP1 and NSP2 for nodulation signaling pathway (Huang et al., 2015) and some others for abiotic and biotic stress responses (Sun et al., 2012; Zhang et al., 2018; Zeng et al., 2019). In many higher angiosperms, several GRAS genes like *ZmSCL7, AtRGA*, *AtGAI* were shown to have roles in salt stress tolerance in maize and Arabidopsis (Zeng et al., 2019). *PeSCL7* from *Populus* is associated with the modulation of drought and salt tolerance (Ma et al., 2010). *OsGRAS23* (here named as *OsGRAS22*) was shown to induce drought stress tolerance in rice (Xu et al., 2015).

In our previous study (Moin et al., 2016a), we have generated a pool of gain-of-function mutants via activation tagging using tetrameric 35S enhancers and screening of some of these mutants for water use efficiency led to the identification of several genes that were associated with the target trait, the water use efficiency. These interesting gain of function mutants included RNA and DNA helicases (*SEN1* and *XPB2*) (Dutta et al., 2021), and genes for ribosome biogenesis (*RPL6* and *RPL23A*), protein ubiquitination (*cullin4*) and transcription factors like *WRKY 96* and *GRAS* (LOC_Os03g40080) (Moin et al, 2016a). A *GRAS* gene was tagged in the mutant DEB.86 rice line, which showed a high quantum efficiency of 0.82 and a low $\Delta^{13}C$ value of 18.06‰. Since high photosynthetic efficiency and low carbon isotope ratio are proxies for high water use efficiency, DEB.86 was further analyzed for other phenotypic characters. The activation tagged line DEB.86 exhibited improved plant height with increased tillering and seed yield and had the *ΨOsGRAS4* gene tagged for activation tagging (Moin et al., 2016a).

A total of 60 *GRAS* genes have already been identified in rice (Liu et al., 2014), out of which, *OsGRAS23* has been reported to enhance tolerance to drought (Xu et al., 2015) and *ΨOsGRAS4* has been identified to be associated with enhanced photosynthetic efficiency and water use efficiency with enhanced agronomic features (Moin et al., 2016a). These reports led us to the idea of studying the genome-wide expression analysis of this gene family.

In this study, we have shortlisted forty genes, one gene representing each paralogous group, and provided an experimental basis to identify the potential GRAS genes capable of imparting stress tolerance in rice. We have analyzed the genes selected in the GRAS family for their spatio-temporal and stress induced expression. The phylogenetic relationship among GRAS proteins, their genetic arrangements and structure, *in-silico* analysis of putative promoter elements and protein properties were also studied. This study helps in the identification of important GRAS genes for stress tolerance, which aids in their further functional characterization.

2. Materials and methods:

2.1.Retrieval and nomenclature of GRAS sequences:

Our previous work on gain of function mutants generated through activation tagging technology using the tetrameric 35S elements identified a GRAS gene as a potential player in enhancing water use efficiency in rice (Moin et al., 2016a). Also, Xu et al. (2015) suggested that *OsGRAS23* is involved in inducing drought stress responses in rice. This has led us to undertake literature search in the present study and we observed that Tian et al. (2004) have identified 57 GRAS genes in rice. We searched the accession numbers of all 57 genes in NCBI and did a BLASTN search in rice genome database (RGAP-DB, Orygenes DB), and retrieved the locus numbers of 47 genes. Simultaneously, we did a key word search of GRAS, DELLA, Scarecrow, Monoculm, Chitin-inducible gibberellin-responsive protein, Gibberellin response modulator protein, Nodulation signaling pathway and Short Root, and combined the search results with the 47 genes retrieved from the literature search. We matched our list of 60 genes with that of Cenci and Rouard (2017) and followed the same nomenclature. For more clarity, we had performed a protein database search for the GRAS domain in NCBI, SMART, Prosite and Pfam databases. We had selected 40 genes, one each from all the paralogous groups for our analyses.

2.2.Genomic distribution of GRAS genes

The coordinates of all 60 GRAS genes were obtained from RGAB-DB and were fed in the NCBI Genome Decoration Page. The outputs were combined and the genes were marked for understanding the genomic distribution of *OsGRAS* genes.

2.3.Phylogenetic relationship of rice GRAS genes

In order to understand the evolutionary relationships between the rice GRAS genes, we aligned the amino acid sequences in MEGA7 software followed by the construction of an unrooted phylogenetic tree. The tree was constructed using the Neighbour Joining method with a bootstrap value of 1000.

2.4.Motif arrangements and organization of GRAS genes

All the 40 GRAS genes were subjected to MEME suite for conserved motif analysis using default parameters. The number of motif scan was set to 10. Based on the previous article of Pysh et al. (1999), the MEME-motifs were further classified into conserved GRAS motifs. The gene organization was studied by subjecting the genomic and coding sequences in the Gene Structure Display Server (GSDSv2). The number of exons, introns, untranslated regions (UTRs) etc. were noted.

2.5.*In-silico* analysis of the putative promoter region

The *cis*- acting elements in the promoter regions play a major role in the coordinated expression of the genes. Hence, it is crucial to identify these regulatory elements in order to correlate the expression data with the genetic components. We retrieved ≤1kb upstream sequences of all 40 selected GRAS genes under study from the rice genome database and identified important elements responsible for biotic and abiotic stress responses in them. The identification of the elements was performed by subjecting the sequences in PlantCARE (Cis-Acting Regulatory Elements) database and manually mapping them on the chromosomes.

2.6.Biochemical properties of GRAS proteins

The sequences of forty GRAS genes that were shortlisted were subjected to ExPASyProtParam tool to gauge their encoded proteins with amino acid length, molecular weight and theoretical isoelectric points (*p*I). The three-dimensional structures of the proteins and their ligand interactions were studied using 3DLigandSite software (Wass et al., 2010). The structures were then subjected to Phyre2 (Protein Homology/Analogy Recognition Engine v2; Kelley et al., 2015) program for analysis of the protein secondary structure composition. This tool gives an idea of the percentage of secondary structures in a protein i.e. the percentage of α-helix, β-sheets and disordered regions in proteins. The SMART (Simple Modular Architecture Research Tool) online tool was used to analyse the protein domains and their low complexity regions (LCRs). ExpasyProtParam tool also indicated the GRAVY indices of the proteins, which provide information regarding the hydrophobicity of proteins. The localization and existence of transmembrane helices of the genes were predicted using TargetP-2.0 and TMHMM software, respectively.

2.7.Preparation of Plant material for studying the gene expression under native and stress conditions

For simulated abiotic stress experiments, BPT-5204 (Samba Mahsuri) rice seeds were surface sterilized using 70% ethanol for 50 sec followed by 4% aqueous sodium hypochlorite solution for 15 min and five washes with sterile double distilled water, each of one minute duration. The sterile seeds were grown on Murashige and Skoog medium for 7 d under a 28 ± 2°C for 16 h/ 8 h photoperiodic cycle (Saha et al., 2017). The seedlings were then subjected to NaCl (250 µM) and ABA (100 µM) stress conditions for 60 h. Shoot and root samples were collected periodically at 0 h, 15 min, 3 h, 12 h, 24 h and 60 h after the onset of stress. The untreated samples were taken as controls for normalization of gene expression.

For studying the native expression patterns of the GRAS genes, tissue samples from thirteen regions in rice seedlings were collected (Moin et al., 2016b, Saha et al., 2017). These included embryo and endosperm from 16 h soaked seeds, plumule and radicle from 3 d old germinating seeds, shoot and root tissues from 7 d old seedlings and shoot, root, root-shoot transition region, flower, spikes and grain samples from mature 20 d old plants post-transfer to the greenhouse.

In order to study the expression of GRAS genes under biotic stress conditions, leaf samples of one month old rice plants infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo* that causes Bacterial Leaf Blight, BLB) and *Rhizoctonia solani* (that causes Sheath Blight, SB) were taken post 20 d and 25 d of infection, respectively. Samples from plants of the same age without the pathogen treatment were taken as the controls. The infection protocol was followed as per Saha et al. (2017).

2.8.c-DNA preparation and Quantitative Real-Time PCR (qRT-PCR)

The plant material collected was used to isolate of RNA using Tri-reagent following manufacturer’s protocol (Takara Bio, UK) and c-DNA was prepared using 2 µg total RNA samples (Takara Bio, UK). The c-DNA samples were diluted ten times and an aliquot of 2 µl of each sample per reaction was used for qRT-PCR. All the primers were designed using

Primer3 software and 10 µM primer concentration was used per reaction. The PCR program included an initial denaturation step of 94°C for 2 min followed by 40 cycles of second denaturation of 30 sec, annealing for 25 sec and extension at 72°C for 30 sec. The samples for the current study were taken in biological and technical triplicates and the fold changes were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Rice *actin* and *β-tubulin* genes were used as two housekeeping genes for internal normalization. For abiotic and biotic expression studies, housekeeping genes and individual control samples were used for double normalization. In contrast, single normalization was performed using the C_T value of housekeeping genes for native expression studies. The graphs were generated using MORPHEUS program and GraphPad Prism software. One way ANOVA was performed using SigmaPlot software for discerning the significance of statistical differences between samples.

3. Results

3.1. Chromosomal distribution of GRAS genes in rice genome

Liu and Widmer (2014) showed that there are 60 GRAS genes in the genome that are distributed on 10 out of 12 chromosomes of rice. Based on the literature and database search, we observed that chromosome 8 and 9 did not carry any GRAS genes. The number of genes on a single chromosome ranged from a minimum of two on chromosome 10 to a maximum of twelve on chromosome 11. Among the rest, a total of nine genes were located on chromosome 3, while chromosome 1, 7 and 12 carried six genes each, chromosome 2, 4 and 5 exhibited five genes each and chromosome 6 had four genes (Fig. 1). Out of the 60 genes located, we have shortlisted 40 genes for our study with one representative from each paralogous group selected.

3.2. Analysis of evolutionary relationship of *OsGRAS* genes

In order to understand the evolutionary relationship among the rice GRAS family of genes, we subjected the retrieved sequences to a phylogenetic analysis (Fig. 2) in MEGA7 software. A total of 16 different clusters were observed. These clusters were divided into 14 subfamilies based on a previous report of Cenci and Rouard (2017). Members belonging to the same subfamily were found to cluster together except DLT and PAT subfamilies where some genes belonging to different orthologous groups (according to Cenci and Rouard, 2017) formed separate clusters. Each cluster has been colour coded in the figure. The number of genes found in each subfamily included four in SCL3, three each in SCR, NSP2 and HAM, one in RAM, LS, SCL4/7and SCLA, two in DELLA, DLT, SHR and SCL32, six in PAT and nine in LISCL. LISCL was found to be the largest subfamily with maximum member of genes getting clustered. *ΨOsGRAS4* and *ΨOsGRAS9* were placed close to LISCL family since these sequences were still unclassified. The highly expressed genes under biotic and abiotic stress conditions belonged to SCL3, SHR, DELLA, HAM and PAT subfamilies.

3.3.Analysis of GRAS motifs and gene organization

The amino acid sequences of selected 40 genes were subjected to MEME analysis for identifying the conserved motifs in rice GRAS gene encoded proteins. A total of ten motifs were identified, which corresponded to LHR I (motif 5, 9), VHIID (motif 2, 3, 10), LHR II (motif 8), PFYRE (motif 4, 7) and SAW (motif 1, 6) motifs (Fig. 3 and Fig S1). The C- terminal domain was found to contain the conserved GRAS motifs as reported earlier in literature. However, not all genes exhibited all the ten MEME-motifs. PAT and LISCL subfamilies carried all the ten domains, while others like SCR lacked motif 1. Proteins belonging to same subfamily had similar motif composition.

The genomic and cDNA sequences of all the selected 40 genes were subjected to GSDS server to observe the organization of different GRAS genes selected from each of the paralogous groups (Fig S2). Based on the map that was generated by the server, it was observed that the genes varied in length and the distribution of exons, introns and untranslated regions (UTRs).

The majority of genes (31 out of 40 genes studied) lacked introns in their gene structure and were only composed of exonic sequences and UTRs. *OsGRAS11* exon is flanked by a long stretch of UTR at its 5' and 3' ends. It completely lacked introns and is the longest gene in this study (6.7Kb). Ten genes were observed to contain only coding sequences in their structure without any introns and UTRs. Among them, *ΨOsGRAS3* had the smallest sequence of only 414 bp. Nine genes carrying introns only in their structure were *OsGRAS3, OsGRAS39, OsGRAS41*, *OsGRAS43*, *OsSCR1*, *ΨOSGRAS4, ΨOsGRAS8, ΨOsGRAS9* and *ΨOsGRAS10.* The number of intronic sequences among the genes varied between one (*OsSCR1* and *ΨOsGRAS10*) to a maximum of seven (*ΨOsGRAS4*). All of them showed low (*OsGRAS43*), moderate (*OsGRAS3*, *ΨOSGRAS4* and *ΨOSGRAS8*) and very high (*OsGRAS39*, *OsGRAS41*, *OsSCR1*, *ΨOsGRAS9* and *ΨOsGRAS10*) expression levels under abiotic and biotic stress conditions. Six out of nine genes (*OsGRAS41, OsGRAS43, ΨOSGRAS4, ΨOsGRAS8, ΨOsGRAS9* and *ΨOsGRAS10)* did not exhibit any UTRs in their structure and were solely composed of introns and exons. The details of genetic organization of rice GRAS genes have been provided in the Supplementary Table S1.

3.4.**Putative promoter analysis of GRAS genes and the search for *cis*-regulatory elements**

Since diverse expression patterns were observed for diffenernet GRAS genes under abiotic and biotic stress conditions, we tried to correlate their expression patterns with the putative regulatory sequences observed in their upstream regions. In order to achieve this correlation, we retrieved 1 Kb sequences from 5' upstream region of each gene under study from the rice genome database and subjected them to an *in-silico* analysis for the identification of the *cis*-putative regulatory elements observed in them. A total of eighteen stress responsive elements were observed in the upstream putative promoter region of the GRAS genes. These included ABRE or ABA responsive elements, CCAAT box and MYB sites for binding of MYB transcription factors responsive to drought inducibility, binding site for MYC transcription factors for defence responses, DRE or dehydration responsive elements, STRE or stress responsive elements, TC-rich repeats for defence and stress responses, and the LTR or low temperature responsive element. Several phytohormones and wound responsive elements were also observed in their upstream regions, which included TCA-element for salicylic acid responses, CGTCA-motif or TGACG-motif as a methyl jasmonate responsive element, GARE-motif, TATC-box and P-box for gibberellin responses, ERE as ethylene responsive elements, TGA-element or AuxRR core or AuxRE for auxin responses, WUN-motif and WRE for responses against wounding, box-S for wounding and pathogen elicitation, and the W-box for binding of WRKY transcription factors.

OsGRAS39, the highly expressive gene under both biotic and abiotic stress conditions in the present study had three copies each of MYB binding factor sites and CGTCA-motif, five copies of STRE, two copies of ABRE and one copy each of DRE, TC-rich repeats and CCAAT-box justifying its expression under different stress treatments. Other responsive genes in both the stresses like *OsGRAS8*, *OsSHR1* and *OsSLR1* had combinations of MYB, STRE, ERE, WUN, TCA, CGTCA and MYC elements in their putative promoter regions. Apart from these, *OsGRAS8* exhibited ABRE, LTR and W-box elements, *OsSHR1* carried a DRE element and *OsSLR1* had copies of TATC, WRE and TC- rich elements. *ΨOsGRAS5*, only expressive gene in the shoot region had two copies each of MYB and MYC binding elements and three copies of ABRE. Other important abiotic stress responsive genes like *ΨOsGRAS2* and *OsSCR1* were observed to have multiple copies (upto six) of ABRE, MYB and MYC elements, STRE elements and ERE, CGTCA, GARE and WRE motifs in their 5' upstream regions. *OsCIGR1* that was found to be highly induced under biotic stress conditions carried ten copies of ABRE, seven copies of STRE, five copies of CGTCA element and one copy each of CCAAT-box, DRE, MYB, MYC and WRE. Other expressive genes under biotic stress conditions included

OsGRAS2, ΨOsGRAS3, OsGRAS19, OsGRAS20 and *OsGRAS23*, which had combinations of TCA-elements, W-box, WRE, ERE, AuxRE, CGTCA-box, box-S and WUN elements apart from other stress responsive elements. The function of each elements has been provided in Table 1 and the physical mapping of the important stress responsive elements on the putative promoter regions of the genes was provided in Fig 4.

3.5.Properties of GRAS proteins, their ligand interactions and domain analysis

We studied the properties of 40 shortlisted GRAS proteins like amino acid length (aa), molecular weight (KDa) and theoretical *p*I through ExPASyProtParam program. It was observed that the proteins had a molecular weight ranging from 15 kDa (ΨOsGRAS3) to 94 kDa (OsGRAS39). ΨOsGRAS3 showed a minimum amino acid (aa) length of 137 aa while OsGRAS39 had a maximum length of 854 aa. The *p*I of the proteins ranged from acidic to basic (4.5-10.1) with only eight proteins having a *p*I of more than 7. The majority of the proteins fall under the *p*I range of 4-7. Likewise, the remaining 32 proteins were found to be in the acidic range i.e. *p*I <7. This is because of the observation that the proteins carried more negatively charged (acidic) amino acid residues like Aspartic acid and Glutamic acids in their composition as compared to basic amino acid residues. Only OsGRAS39 was found to have an equal number of acidic and basic residues in its composition. According to TargetP-2.0 server, OsGRAS39 was predicted to be localized to the chloroplast while no signal peptides for chloroplast or mitochondria could be specified by the tool for the rest of the proteins.

We have also analyzed the proteins for their three dimensional structures and ligand binding residues in the 3DLigand site and the structures were submitted to the Phyre2 program to analyse their secondary structures like the percentage of disordered regions, α-helix and β-sheets. ΨOsGRAS3 showed a maximum of 71% and OsGRAS8 had a minimum of 31% of α-helical structure. Similarly, maximum (14%) extent of β-sheets were noticed in the secondary structure of OsGRAS32. No β-sheets were present in ΨOsGRAS2 and ΨOsGRAS3. Several metallic and non-metallic ligands were also observed to be interacting with the GRAS proteins, which included Mg^{+2}, Ca^{+2}, SAM, SAH, NAP, NAD, ATP, Zn^{+2} and Ni^{+2}. The three dimensional structures of the proteins along with their interacting ligands have been provided in the Fig. S3.

Low complexity region (LCR) are repetitive amino acid sequences found abundantly in the eukaryotic proteins. These play essential roles in protein-protein and protein-nucleic acid interactions (Toll-Riera et al., 2012). It was noted that the number of LCRs in each of the proteins varied from none to a maximum of eight in OsGRAS20 and OsGRAS43, respectively.

Grand average of hydropathicity index or GRAVY index indicates the hydrophobicity of a protein taking into consideration its charge and the size. Usually GRAVY values range from -2 to +2 with more positive values indicating hydrophobicity and more negative values indicating hydrophilicity (Morel et al., 2006). Seven proteins had a positive GRAVY value while the rest 33 proteins had a values lesser than zero, which indicated that the majority of the GRAS proteins are hydrophilic. The list of all the observations have been provided in the supplementary table S2.

In order to study the domains present in the genes, we utilized the SMART online tool and observed that all the proteins had at least one GRAS domain with ΨOsGRAS4, ΨOsGRAS8, OsGRAS39, ΨOsGRAS10 exhibiting two GRAS domains. Among them, ΨOsGRAS4 and ΨOsGRAS10 had two internal repeats designated as RPT1 along with two GRAS domains. One DELLA domain and one SCOP domain in addition to the GRAS domain were found in OsSLR1 and OsGRAS18, respectively. DELLA proteins are transcriptional regulators, which function in gibberellic acid signaling by binding with GA receptor, GID1 followed by proteasomal degradation of DELLA domain (Murse et al., 2008). OsGRAS41 had a transmembrane region, OsGRAS43 and OsGRAS53 had two RPT1 domains (internal repeats) along with their single GRAS domains. The detailed list of the domains and the LCRs with their sequences have been provided in the Table S3. The presence of transmembrane domain in OsGRAS41 was further confirmed through TMHMM software.

3.6.Expression analysis under simulated abiotic stress conditions

We have identified a GRAS transcription factor as a potential stress tolerance gene by screening a pool of gain-of-function mutants in rice in our previous study (Moin et al., 2016a). Another report by Xu et al. (2015) suggested the role of *OsGRAS23* (reported as *OsGRAS22* in this study) in drought tolerance in rice. These observations have prompted us to analyse the differential expression pattern of GRAS family of transcription factors under the influence of biotic and simulated abiotic stress conditions in the present study. We have analyzed the expression patterns of 40 selected genes separately in shoot and root tissues at six different time points for two abiotic (NaCl and ABA) and two biotic (BLB and SB) stresses. The native expression patterns of these genes in 13 different tissues were also studied.

Based on the pattern of expression, we have divided the genes as immediate early (IE), early (E) and late (L) responsive genes. Some genes were expressed up to 100 folds after the incidence of stress. Thus, the genes were also categorized as expressive (2-10 fold), moderately expressive (10 -30 fold) and highly expressive (≥30 fold) types. Genes showing an upregulation of ≥2 folds were considered as expressive.

The majority of the genes got upregulated in the root (Fig. 5a, b) compared to the shoot (Fig. 5c, d). As indicated in the pie chart, about 55-60% of the total genes showed IE type expression under both NaCl and ABA treatments. NaCl, however, induced more early (12.5%) responsive genes than late (2.5%) whereas, ABA induced more late genes (12.5%) than early (2.5%). The list of the expressed genes has been provided in Fig. 5. More than half of IE genes continued their expression till 60 h of treatment, while some others became downregulated or showed no expression at all later during the experimental timeline. Under ABA treatment, all highly upregulated genes i.e. *ΨOsGRAS2, OsSHR1, OsSCR1* and *OsGRAS39* were IE type and their expression persisted till the last time point of treatment i.e. 60 h. Other IE type genes showed a split before increasing their expression at subsequent time points. Only *OsGRAS39* was highly expressive under both ABA and NaCl treatments (100 fold and 65 fold, respectively). *OsGRAS2*, *ΨOsGRAS2, OsGRAS25, OsGRAS35* and *OsSCR1* under NaCl and *OsGRAS22* under ABA were early (E) expressed genes respectively, while *ΨOsGRAS9, OsGRAS3, OsGRAS11* and *OsGRAS26* under ABA and *ΨOsGRAS9* became upregulated under NaCl treatment with late (L) expression.

Twelve and thirteen genes (30 and 32%) were mild to moderately expressed, respectively under the ABA treatment, whereas seven genes (17%) were moderately expressive under NaCl treatment and rest 19 genes (47%) exhibited mild expression. Nine genes (22%) under ABA and thirteen (32%) genes under NaCl treatment were either downregulated or showed no change in the level of expression. Among them, *OsGRAS7, OsGRAS23, OsGRAS28* and *ΨOsGRAS8* were downregulated under both treatments. *ΨOsGRAS3, ΨOsGRAS4, OsCIGR1* and *OsGRAS32* under ABA treatment and *OsCIGR1* under NaCl treatment showed an immediate expression, but was either downregulated or showed no expression at subsequent time points

Not many genes were expressed in the shoot. However, *ΨOsGRAS5* is the only gene, which showed moderate expression (25-30 fold) under both ABA and NaCl treatments. This gene was IE type maintaining its expression till 60 h under NaCl, but showed a split before reaching a peak under the ABA treatment. On the contrary, it showed low expression (2-3 fold) in root tissues under ABA and NaCl treatments. Among the other genes that were mildly expressive in both root and shoots were *ΨOsGRAS2, OsGRAS12, OsGRAS19, OsGRAS24, OsGRAS25* and *OsSCR1*. The rest of the genes were mainly downregulated or did not show any change in expression in shoot tissues under both the stress treatments. The expression level of all the genes studied has been provided in Table 2.

Among the genes studied, some genes were observed to be expressed only under NaCl or ABA treatments at certain time points, whereas some were found to be expressive under both treatments. Such overlaps has been depicted in the form of Venn diagrams in Fig. S4. The corresponding list of genes clearly demonstrates that several genes were up/down-regulated simultaneously under both ABA and NaCl treatments at certain time points. In roots, the expression of 37.5% of the genes (IE type) overlapped under both stress treatments, while in shoots only *ΨOsGRAS5* (IE) was expressive.

3.7.Differential expression analysis of GRAS genes under biotic stress

We have studied the expression of the selected GRAS transcription factors in the leaf samples of rice infected with *Xoo* and *R. solani* pathogens that cause Bacterial Leaf Blight (BLB) and Sheath Blight (SB) diseases, respectively (Fig. 6). Six genes were upregulated in BLB of which five (*OsGRAS1, OsGRAS18, OsCIGR2, ΨOsGRAS9, OsGRAS53*) showed low expression while one gene (*OsCIGR1*) was highly upregulated upto 57 folds. More genes were upregulated in SB infected leaves compared to the BLB treated ones. Out of the thirty expressed genes in SB infected leaves, only twelve showed very high expression levels while the rest of the genes exhibited low to moderate expression. *OsGRAS2, ΨOsGRAS3, OsGRAS19, OsGRAS20, OsGRAS23* and *OsSHR1* were expressed by ≥100 folds under the SB treatment. A total of 22 genes in BLB and three in SB treated samples were downregulated. Those that were downregulated in SB treated samples (*OsSHR2*, *OsGRAS24* and *OsGRAS43*) were also downregulated in BLB treated leaves. Twelve genes under BLB and seven under SB showed no changes in their expression levels.

3.8.Native expression analysis of GRAS genes in various tissues at specific developmental stages in rice

In order to study the native expression patterns of GRAS transcription factors in different tissues of the rice plant, we performed qRT-PCR analysis of 13 different tissues, which included shoot, root, root-shoot transition, flag leaves, flower, spikes and grain of mature 20 d old plants (after shifting to greenhouse), shoot and root of 7 d old seedlings, 3 d old plumule and radicle, embryo and endosperm of 16 h germinating seeds. The mean values were used to plot a heat map (Fig. 7).

Expression analysis showed a conspicuous downregulation of all genes in most of the tissues particularly in plumule, radicle, embryo and the endosperm. The number of downregulated genes in other tissues are: 39 genes in 7 d shoot, 38 genes in 20 d shoot, grain and 7d root, and 37genes in root-shoot transition and flower followed by 36 genes in spikes, 34 genes in leaves and 30 genes in 20 d root. Out of 40 selected genes, only seven were expressed in certain tissues. *OsGRAS2* and *OsGRAS3* were upregulated only in mature leaves, *OsGRAS28* in 20 d root, flower and spike, *OsCIGR1* in root-shoot transition and leaves, *OsGRAS39* in 20 d root and *OsGRAS47* in 20 d root and flower. Out of these seven genes, five were upregulated either in the roots or in the root-shoot transition region indicating the preference of GRAS genes towards expression in the root tissue. This is also in accordance with the expression analysis under abiotic stress condition, where the genes were highly expressive in roots rather than in the shoot tissue. Three out of seven mildly expressive genes were upregulated in flower and spike of 20 d old plants with none of them expressing in the grain. *OsGRAS39*, which was upregulated in root tissues under native conditions, is highly expressive in roots under abiotic stress conditions also responding immediately after the application of stress treatment. This might be an indication of its tissue specificity and its potential as a stress tolerance transcription factor gene.

4. Discussion

Being sessile, plants cannot escape the onslaught from environmental stresses like cold, heat, drought etc. , nor can they avoid harmful interactions with microorganisms like fungi and bacteria (Lin et al., 2017). Such adversities impose a threat to agricultural productivity and sustainability. In order to support the burgeoning global population, the development of stress tolerant crops is of utmost importance (Cushman and Bohnert, 2000). Characterization of insertional mutants is an important functional genomics based method of identifying novel genes responsible for inducing stress tolerance in crop plants (Cushman and Bohnert, 2000). TF genes are of particular importance in this context as they act upstream in the pathway(s) and control the expression of several genes working under their control. Because of this, the manipulation undertaken using TF genes as ‘Master’ genes would render the plant more accommodative towards the particular stress under consideration. Our previous studies have identified several key players for stress tolerance in *indica* rice variety via enhancer based activation tagging method. A GRAS transcription factor gene, *ΨOsGRAS4* was one of the important genes that was identified in the study along with others for enhanced water use efficiency associated with enhanced photosynthetic efficiency (Moin et al., 2016a).

4.1.Evolutionary relationships, gene organization and protein properties of rice GRAS genes

Plants use certain acclimation and adaptive measures to cope up with the impending stress, which is mostly modulated through the action of hormones and regulators (Lin et al., 2017). Thus understanding the expression patterns of GRAS family of genes, which play a key role in gibberellin signaling and their spatio-temporal regulation help us identify candidate genes for improving the endogenous defence ability of plants, particularly rice in the present context. In this study, we have shortlisted important GRAS genes responsible for abiotic and biotic stress tolerance. We have also studied the *in-silico* properties of these genes and have correlated them with our expression data.

According to the published evidence that is available (Liu and Widmer, 2014), 60 GRAS genes were reported in the rice genome, which are distributed on all the twelve chromosomes except chromosome numbers 8 and 9. Highest number of gene density was observed on chromosome 11. We have selected 40 genes for our study, drawing one member representing each paralogous group. The availability of high quality genomic sequences enabled us to get an insight into the phylogenetic, genomic and protein properties of the GRAS genes. In our analysis, we have classified the genes into 14 subfamilies (Cenci and Rouard, 2017) of which LISCL constituted the maximum number of genes. However, most expressive genes belonged to SCL3, SHR1, DELLA, HAM and PAT subfamilies. The ten MEME-identified motifs were categorized into five conserved C-terminal GRAS motifs. Genes belonging to the same subfamily exhibited similar motif arrangements, but this varied within the subfamilies, which might be due to the diverse biological functions of GRAS genes. This group of proteins were reported to have originated in bacteria, which later expanded into eukaryotic genomes via horizontal gene transfer and repeated duplication events with the possible retroposition of intronless genes (Huang et al., 2015). Our genomic organization study revealed 31 *OsGRAS* genes out of 40 to be intronless and this observation is in line with previous studies. Several interacting metallic and non-metallic ligands associated with GRAS genes along with their hydrophilic nature (as indicated by the GRAVY index) indicated their involvement in cell signaling, catalysis and protein-protein interactions (Ulucan et al., 2014; Jing et al., 2017). The majority of the genes were observed to have a *p*I less than seven and were found to be rich in negatively charged amino acids like glutamic acid and aspartic acid. This makes the interactions of GRAS proteins very specific as proteins with low *p*I tend to minimize the chances of non-specific interactions with nucleic acids and other acidic proteins (Takakura et al., 2016). All GRAS genes have at least one GRAS domain, but some were found to have two or possess a DELLA domain, which is known to have important role in gibberellic acid signaling (Urbanova and Metzger, 2018).

4.2.Differential expression patterns of *OsGRAS* genes and their spatio-temporal regulation

Based on external cues, spatio-temporal regulation of gene transcription is required to control the concentration of particular transcripts and proteins in the cells for their adjustment to the environmental changes. Most of the GRAS genes were observed to be upregulated in roots with 55- 60% of them showing IE type of gene expression. In plants, stress responses can be divided broadly into early and late response types. Early responsive genes are expressed within minutes of stress induction and this provides protection and repair from the initial stress. Such response “alarms” the plant to prepare for further stress tolerance or avoidance. On the other hand, late responsive genes are mostly involved in protein synthesis that regulates downstream genes, thereby responding to the “adaptation” part of stress mediation (Bahrami and Drablos, 2016; Lin et al., 2017). *ΨOsGRAS9* was observed to be late expressive under both treatments in root, indicating that it might have an important role in subsequent steps of stress amelioration. Interestingly, 50-60% of IE expressed genes continued to express till the end point of the treatment. Among them, *OsGRAS39* was the only gene that was observed to be highly expressive under both NaCl and ABA treatments in roots. Apart from this, *ΨOsGRAS2, OsSHR1* and *OsSCR1* continued their expression till 60 h under ABA treatment. This probably indicates that these constitute an important set of genes required by the plant throughout for stress remediation. Others like *ΨOsGRAS3, ΨOsGRAS4, OsCIGR1* and *OsGRAS32* under ABA and *OsCIGR1* under NaCl were the genes induced initially (IE type), which either stopped expressing or got downregulated at subsequent time points. These genes are probably required for initial stress responses whose function is later on taken up by other downstream genes in the signaling cascade. *ΨOsGRAS5* (IE type) was found to be the only moderately expressive gene in shoot under both stress conditions. Since root is the first organ to perceive the stress signal, it induces a signaling cascade that extends towards shoot. Such preferential expression of *GRAS* genes in roots over shoots indicates their important role in stress tolerance (Janiak et al., 2016). Also, previous studies indicated that probably the role of these genes in pattern formation and signal transduction enables them to be more expressive in roots (Pysh et al., 1999).

Rice productivity is severely hampered by BLB and SB diseases. BLB infection during tillering stage can cause a yield decline of 20-40%, while it can reduce crop productivity by 50% at a younger stage. Upto 45% yield losses in rice are caused by SB infections (Chukwu et al., 2019; Singh et al., 2019). Thus, identification of key genes and understanding their expression patterns are important for developing tolerant varieties of rice for these diseases. Under BLB treatment, only six genes (15%) were expressive compared to thirty (75%) expressive genes under SB infection. Quite a number of genes were expressive under SB infection and both abiotic stress conditions. Noteworthy among them are *OsGRAS39*, *OsGRAS8*, *OsSHR1* and *OsSLR1*. Thus, these genes can be considered as to be quite important as they are expressive under both stress conditions with important roles in disease resistance. Majority of the genes were highly expressive in ABA treated roots and SB infection. The presence of multiple stress responsive elements in their putative promoter regions are corroborated by our expression data and these observations indicate their probable roles in improving plant defence against biotic and abiotic stress.

Proteins belonging to DELLA subfamily of GRAS transcription factors are known to be negative regulators of seed germination as bioactive gibberellic acid causes proteasomal degradation of such proteins for gibberellin signaling to occur (Urbanova and Metzger, 2018). This explains the downregulation of all genes in plumule, radicle, embryo and endosperm.

OsGRAS39 was expressive in roots under native as well as abiotic stress conditions indicating its tissue specificity. This gene belongs to SCL3 subfamily, which is known for modulating GA signaling in roots via protein-protein interactions (Weng et al., 2020). Hence, high expression of *OsGRAS39* in roots under all stress conditions can be further exploited for its potential role in stress tolerance.

GRAS gene family has been studied extensively in many plant species, but we have successfully provided a backdrop based on which future exploration on rice GRAS genes can be done. The differential expression patterns of these genes indicates their importance in stress remediation. Our study provides an insight into the role of GRAS genes in stress tolerance along with their spatio-temporal regulation. Based on this report, it would be possible to pick up important genes that can be further manipulated to develop stress tolerant varieties of rice and other related crops.

Author contribution statement

PBK and MD designed the experiments and prepared the manuscript. MD performed the experiments. AS helped in the qRT-PCR experiments and analysis. MM identified GRAS gene from activation tagged lines and conceived the initial idea. PBK supervised the work.

Acknowledgements

MD is grateful to UoH BBL and UGC for research fellowship and contingency and to DBT for funding the rice activation tagging project. She is also grateful to Prof. S. Dayananda, Dean School of Life Sciences, UoH for his help. MD and AS are grateful to DBT for providing research fellowship and contingency. MD is grateful to Dr. M.S. Madhav, Department of Biotechnology, Indian Institute of Rice Research, Hyderabad, India, for providing the infected rice samples and wild type BPT-5204 seeds. PBK acknowledges the National Academy of Sciences-India for the award of NASI-Platinum Jubilee Senior Scientist position.

Conflict of interest

The authors hereby declare that the research has been performed without any financial and commercial conflict of interest.

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Table 1: List of *cis*- regulatory elements and their functions

Name of *cis*-element	**Function**
ABRE	ABA responsive element (Choi et al. 2000)
MYB/MBS	MYB binding site for drought inducibility (Ambawat et al. 2013)
DRE	Dehydration responsive element (Narusaka et al. 2003)
MYC	Transcription factor for stress responses, helps in dehydration induced expression of genes (Tran et al. 2004)
STRE	Stress responsive element (Hwang et al. 2010)
TCA element	Element for salicylic acid responsiveness (Wei et al. 2013)
CGTCA-motif/TGACG-motif	Methyl-Jasmonate responsive element (Wang et al. 2011)
TC-rich motifs	Responsible for defense and stress, transcription regulation (Bernard et al. 2010; Liu et al. 2017)
Box S	Responsive to wounding and pathogen elicitation (Yin et al. 2017); Stress responsiveness (Ding et al. 2019)
GARE-motif/TATC-box	Gibberellin responsive element (Bastian et al. 2010)
ERE	Element for ethylene responses (Sanchez and Singh 2002)
TGA-element/AuxRR core/ AuxRE	Element for auxin response (Sakai et al. 1996)
WUN motif	Wound responsive element for biotic stress (Xu et al. 2011)
LTR	Low temperature responsive element (Zhang et al. 2020)
W box	Binding sites for WRKY transcription factors (Dhatterwal et al. 2019)
CCAAT box	Binding site for MYB transcription factors
P-box	Gibberellin responsiveness (Zhang et al. 2020)
WRE	Wound responsive element (Whitbred and Schuler 2000)

Table 2: List of GRAS genes and their expression pattern under NaCl and ABA treatments

Gene name	Locus number	Regulation (up/down)	Type of Response	Maximum fold change	Regulation (up/down)	Type of Response	Maximum fold change
		Root			**Shoot**		
NaCl							
OsGRAS1	LOC_Os01g45860	DOWN	-	-	-	-	-
OsGRAS2	LOC_Os01g62460	UP	E(2.9)	3.3	-	-	-
OsGRAS3	LOC_Os01g65900	DOWN	-	-	-	-	-
OsGRAS5	LOC_Os01g71970	UP	IE (5.5)	6.1	-	-	-
OsGRAS7	LOC_Os02g10360	DOWN	-		DOWN	-	-
ΨOsGRAS2	LOC_Os02g21685	UP	E(2.6)	18.8	DOWN	-	-
OsGRAS8	LOC_Os02g44360	UP	IE(5.6)	12	DOWN	-	-
OsGRAS10	LOC_Os02g45760	UP	IE(12.4)	12.4	DOWN	-	-
OsGRAS11	LOC_Os03g09280	UP	IE(3.8)	4.8	DOWN	-	-
OsGRAS12	LOC_Os03g15680	UP	IE(9.5)	9.5	UP	E(2.1)	6.8
OsSHR2	LOC_Os03g31880	UP	IE(3.6)	4.3	DOWN	-	-
ΨOsGRAS3	LOC_Os03g37900	UP	IE(3.7)	3.7	DOWN	-	-
ΨOsGRAS4	LOC_Os03g40080	UP	IE(3.0)	16.2	DOWN	-	-
OsGRAS15	LOC_Os03g48450	UP	IE(3.8)	3.8	DOWN	-	-
OsSLR1	LOC_Os03g49990	UP	IE(3.3)	11	DOWN	-	-
OsGRAS18	LOC_Os03g51330	-	-	-	DOWN	-	-
OsGRAS19	LOC_Os04g35250	UP	IE(4.2)	6.4	UP	IE(2.1)	2.1
ΨOsGRAS5	LOC_Os04g37440	UP	IE(2.2)	2.2	UP	IE(7.7)	25.2
OsGRAS20	LOC_Os04g46860	DOWN	-	-	DOWN	-	-
OsGRAS22	LOC_Os04g50060	UP	IE(3.2)	4.6	DOWN	-	-
OsGRAS23	LOC_Os05g31380	DOWN			DOWN	-	-
OsGRAS24	LOC_Os05g31420	UP	IE(4.0)	8.6	UP	L(4.6)	4.6
OsGRAS25	LOC_Os05g40710	UP	E(4.5)	4.5	UP	E(4.8)	4.8
OsGRAS26	LOC_Os05g42130	UP	IE(2.6)	2.6	DOWN	-	-
OsGRAS28	LOC_Os06g01620	DOWN	-	-	DOWN	-	-
OsCIGR1	LOC_Os07g36170	UP	IE(3.2)	3.2	DOWN	-	-
OsGRAS32	LOC_Os07g38030	DOWN	-	-	DOWN	-	-
OsCIGR2	LOC_Os07g39470	DOWN	-	-	DOWN	-	-
OsSHR1	LOC_Os07g39820	UP	IE(7.3)	11.1	DOWN	-	-
OsGRAS35	LOC_Os07g40020	UP	E(6.8)	17.4	DOWN	-	-
OsSCR1	LOC_Os11g03110	UP	E(3.8)	5.7	DOWN	-	-
ΨOsGRAS8	LOC_Os11g04400	DOWN			DOWN	-	-
OsGRAS39	LOC_Os11g04570	UP	IE(3.6)	65.3	DOWN	-	-
OsGRAS41	LOC_Os11g06180	UP	IE(2.4)	4.1	DOWN	-	-
ΨOsGRAS9	LOC_Os11g11600	UP	L(8.0)	8	DOWN	-	-
OsGRAS43	LOC_Os11g31100	UP	IE(3.5)	3.5	DOWN	-	-
OsGRAS44	LOC_Os11g47870	UP	IE(2.9)	5.8	DOWN	-	-
OsGRAS47	LOC_Os11g47910	-	-	-	DOWN	-	-
ΨOsGRAS10	LOC_Os12g06540	DOWN	-	-	DOWN	-	-

OsGRAS53	LOC_Os12g38490	DOWN	-	-	DOWN	-	-

Gene name	Locus number	Regulation (up/down)	Type of Response	Maximum fold change	Regulation (up/down)	Type of Response	Maximum fold change
		Root			**Shoot**		
ABA							
OsGRAS1	LOC_Os01g45860	-	-	-	DOWN	-	-
OsGRAS2	LOC_Os01g62460	UP	IE(3.3)	5.6	DOWN	-	-
OsGRAS3	LOC_Os01g65900	UP	L(3.5)	3.5	DOWN	-	-
OsGRAS5	LOC_Os01g71970	UP	IE(6.2)	12.2	DOWN	-	-
OsGRAS7	LOC_Os02g10360	DOWN	-	-	DOWN	-	-
ΨOsGRAS2	LOC_Os02g21685	UP	IE(2.8)	32.7	UP	L(3.5)	3.5
OsGRAS8	LOC_Os02g44360	UP	IE(5.5)	27.9	DOWN	-	-
OsGRAS10	LOC_Os02g45760	UP	IE(2.4)	16.7	DOWN	-	-
OsGRAS11	LOC_Os03g09280	UP	L(4.1)	4.1	DOWN	-	-
OsGRAS12	LOC_Os03g15680	UP	IE(8.2)	8.2	UP	L(3.8)	3.8
OsSHR2	LOC_Os03g31880	UP	IE(4.5)	14.3	DOWN	-	-
ΨOsGRAS3	LOC_Os03g37900	UP	IE(2.8)	2.8	DOWN	-	-
ΨOsGRAS4	LOC_Os03g40080	UP	IE(2.1)	2.1	DOWN	-	-
OsGRAS15	LOC_Os03g48450	UP	IE(2.9)	14.6	DOWN	-	-
OsSLR1	LOC_Os03g49990	UP	IE(7.9)	30.2	DOWN	-	-
OsGRAS18	LOC_Os03g51330	UP	IE(2.8)	3.4	DOWN	-	-
OsGRAS19	LOC_Os04g35250	UP	L(3.5)	11	DOWN	-	-
ΨOsGRAS5	LOC_Os04g37440	UP	IE(2.9)	2.9	UP	IE(8.2)	31.6
OsGRAS20	LOC_Os04g46860	UP	IE(2.9)	4.1	DOWN	-	-
OsGRAS22	LOC_Os04g50060	UP	E(5.5)	14.2	DOWN	-	-
OsGRAS23	LOC_Os05g31380	DOWN	-	-	DOWN	-	-
OsGRAS24	LOC_Os05g31420	UP	IE(15.3)	15.3	UP	L(6.9)	6.9
OsGRAS25	LOC_Os05g40710	UP	IE(6.2)	14.2	UP	IE(3.0)	10.5
OsGRAS26	LOC_Os05g42130	UP	L(2.4)	2.4	DOWN	-	-
OsGRAS28	LOC_Os06g01620	DOWN	-	-	DOWN	-	-
OsCIGR1	LOC_Os07g36170	UP	IE(7.3)		DOWN	-	-
OsGRAS32	LOC_Os07g38030	UP	IE(2.09)		DOWN	-	-
OsCIGR2	LOC_Os07g39470	DOWN			DOWN	-	-
OsSHR1	LOC_Os07g39820	UP	IE(14.1)	66.3	DOWN	-	-
OsGRAS35	LOC_Os07g40020	UP	IE(15.9)	15.9	DOWN	-	-
OsSCR1	LOC_Os11g03110	UP	IE(6.7)	34.3	UP	L(2.4)	2.4
ΨOsGRAS8	LOC_Os11g04400	DOWN	-	-	DOWN	-	-
OsGRAS39	LOC_Os11g04570	UP	IE(12)	101.4	DOWN	-	-
OsGRAS41	LOC_Os11g06180	UP	IE(5)	21.3	DOWN	-	-
ΨOsGRAS9	LOC_Os11g11600	UP	L(2.7)	2.7	DOWN	-	-
OsGRAS43	LOC_Os11g31100	UP	IE(2.07)	7.3	DOWN	-	-
OsGRAS44	LOC_Os11g47870	UP	IE(19.2)	19.2	DOWN	-	-
OsGRAS47	LOC_Os11g47910		-	-	DOWN	-	-
ΨOsGRAS10	LOC_Os12g06540	DOWN	-	-	DOWN	-	-
OsGRAS53	LOC_Os12g38490	DOWN	-	-	DOWN	-	-

Figure 1: Chromosomal distribution of GRAS genes in rice

Karyotypic representation of rice chromosomes obtained from NCBI Genome Decoration Page. Rice genome carries 60 GRAS genes, which are represented in the figure with red arrows indicating the position of each gene. The size of each chromosome and the number of genes present are provided below each in each bracket.

Figure 2: Phylogenetic analysis of *OsGRAS* genes

An unrooted phylogenetic tree showing the evolutionary relationship of *OsGRAS* genes. The tree was constructed using the Neighbour Joining method in MEGA7 software with a bootstrap value of 1000. The number at each node represents the percentage bootstrap values. Based on the previous literature, the genes have been divided into 14 subfamilies (mentioned in boxes) and each subfamily has been colour coded.

Figure 3: MEME-motif analysis of *OsGRAS* genes

Figure showing the identified MEME-motifs of *OsGRAS* genes. The conserved GRAS-motifs are provided at the top. A search for 10 MEME-motifs was done and each of them has been assigned to the corresponding GRAS-motifs. Each coloured box represents one motif and the legend has been provided below. The genes were organized based on their subfamilies.

Figure 4: *In-silico* analysis of putative promoter regions of GRAS genes

The selected GRAS genes were subjected to *in silico* analysis for *cis*- regulatory elements in their putative promoter regions (sequence retrieved from about ≤1kb upstream region). This was performed in PlantCARE database and the figure was prepared by mapping the stress regulatory elements in the each of the sequences. The index for each element along with its functions are mentioned below the figure.

Figure 5: Expression analysis of GRAS genes under abiotic stress

Heat map representation of temporal expression pattern of GRAS genes developed using MORPHEUS program. 7 d old seedlings were subjected to NaCl (250µM) and ABA (100µM) treatments and the obtained quantitative real-time values were double normalized using rice *actin* and *tubulin* as the internal reference genes and that of the unstressed samples using the $\Delta\Delta C_T$ method. The experiment was conducted separately for root (4a, b) and shoot (4c, d) tissues. Percentage of genes upregulated under NaCl and ABA treatments is represented in the form of a pie chart beside their corresponding heat maps. The genes were separated based on their time point(s) of expression and annotated as immediate early (IE), early (E) and late (L) expressive genes. The name of the genes is provided in the list below.

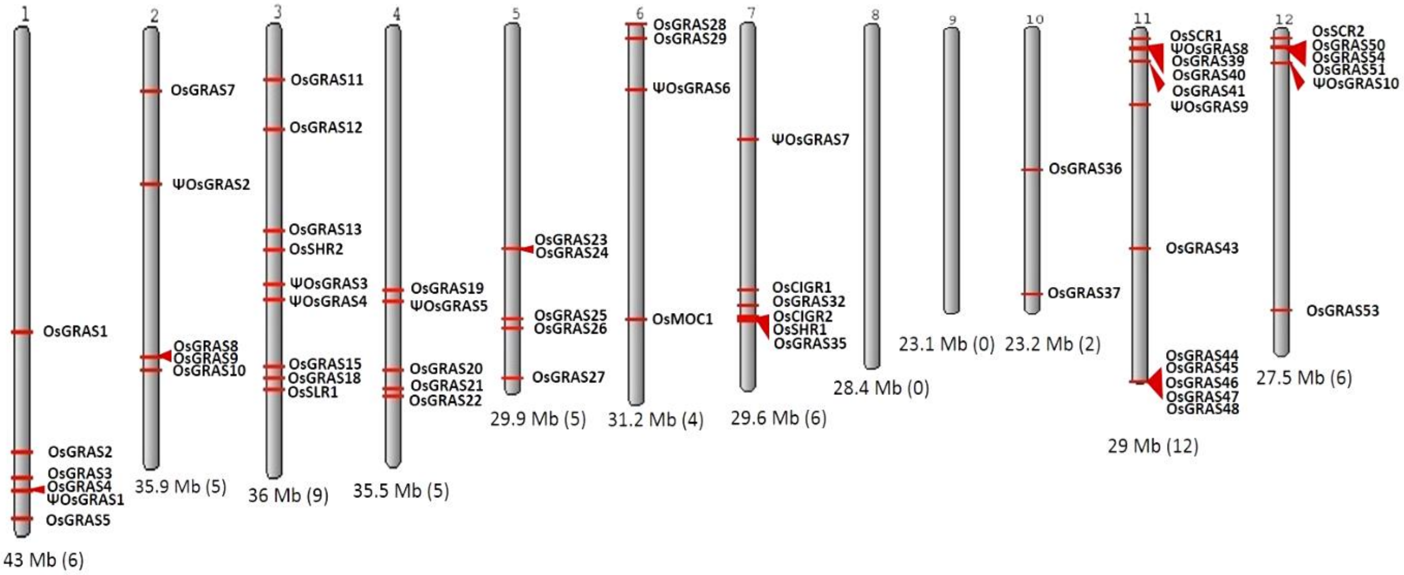
Figure 6: Quantitative real-time expression analysis of GRAS genes under biotic stress

Expression analysis of GRAS genes under the infection of *Xanthomonas oryzae* pv. *oryzae* causing bacterial leaf blight (6a) and *Rhizoctonia solani* causing sheath blight (6b) were studied. The genes were double normalized using rice *actin* and *tubulin* as internal reference genes and the C_T values untreated samples by $\Delta\Delta C_T$ method. One way ANOVA was performed on the data and a represents $P<0.05$, b represents $P<0.025$ and c represents $P<0.001$

Figure 7: Native expression analysis of GRAS genes

Heat map representing the spatial expression pattern of GRAS genes under 13 different developmental stages of rice. The map was generated using MORPHEUS program. The data was single normalized using rice *actin* as the internal reference gene.

Figure 1


1
OsGRAS1
OsGRAS2
OsGRAS3
OsGRAS4
ΨOsGRAS1
OsGRAS5
43 Mb (6)
2
OsGRAS7
ΨOsGRAS2
OsGRAS8
OsGRAS9
OsGRAS10
35.9 Mb (5)
3
OsGRAS11
OsGRAS12
OsGRAS13
OsSHR2
ΨOsGRAS3
ΨOsGRAS4
OsGRAS15
OsGRAS18
OsSLR1
36 Mb (9)
4
OsGRAS19
ΨOsGRAS5
OsGRAS20
OsGRAS21
OsGRAS22
35.5 Mb (5)
5
OsGRAS23
OsGRAS24
OsGRAS25
OsGRAS26
OsGRAS27
29.9 Mb (5)
6
OsGRAS28
OsGRAS29
ΨOsGRAS6
OsMOC1
31.2 Mb (4)
7
ΨOsGRAS7
OsCIGR1
OsGRAS32
OsCIGR2
OsSHR1
OsGRAS35
29.6 Mb (6)
8
28.4 Mb (0)
9
23.1 Mb (0)
10
OsGRAS36
OsGRAS37
23.2 Mb (2)
11
OsSCR1
ΨOsGRAS8
OsGRAS39
OsGRAS40
OsGRAS41
ΨOsGRAS9
OsGRAS43
OsGRAS44
OsGRAS45
OsGRAS46
OsGRAS47
OsGRAS48
29 Mb (12)
12
OsSCR2
OsGRAS50
OsGRAS54
OsGRAS51
ΨOsGRAS10
OsGRAS53
27.5 Mb (6)

811 **Figure 2**

812

813

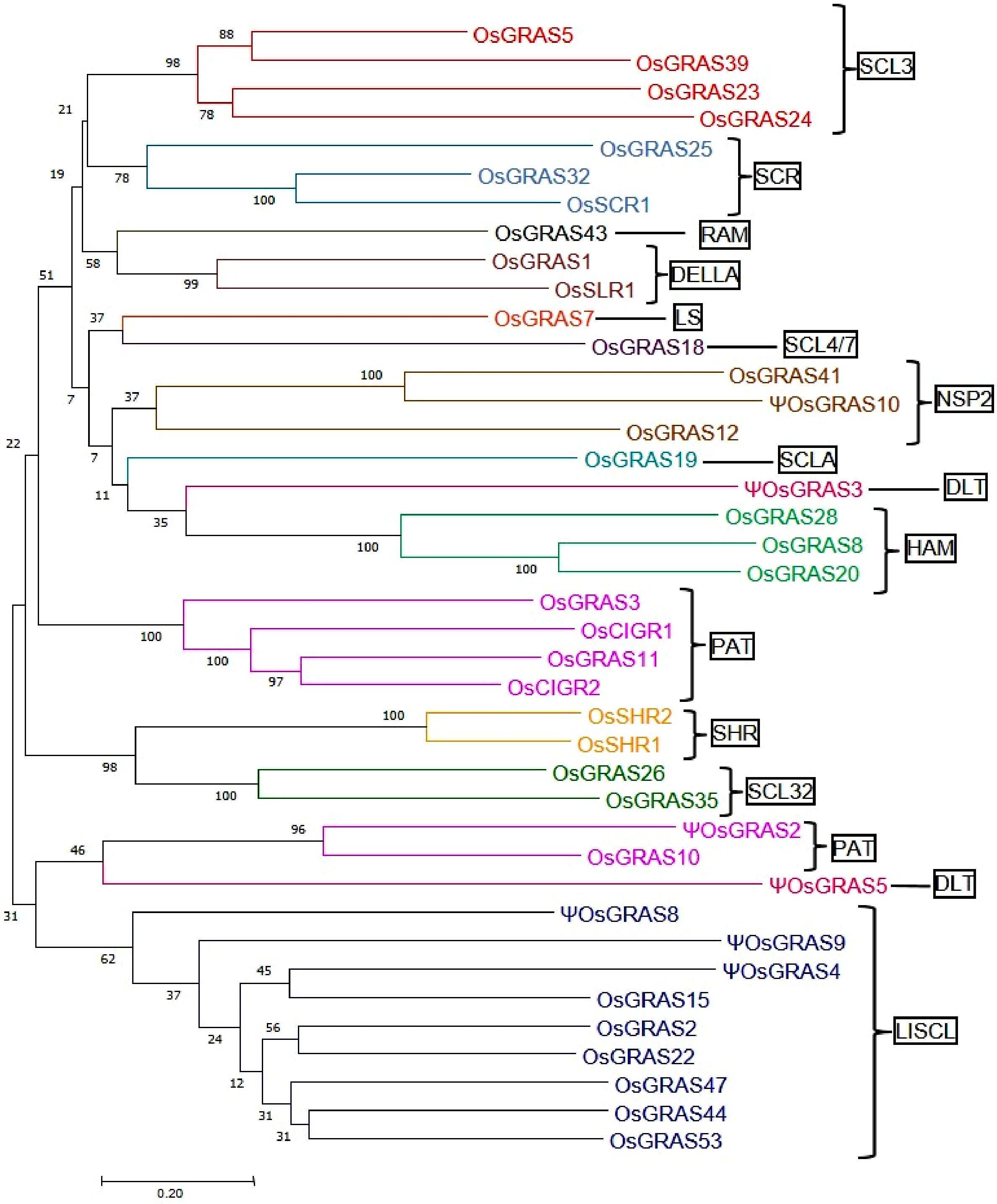


Figure 3

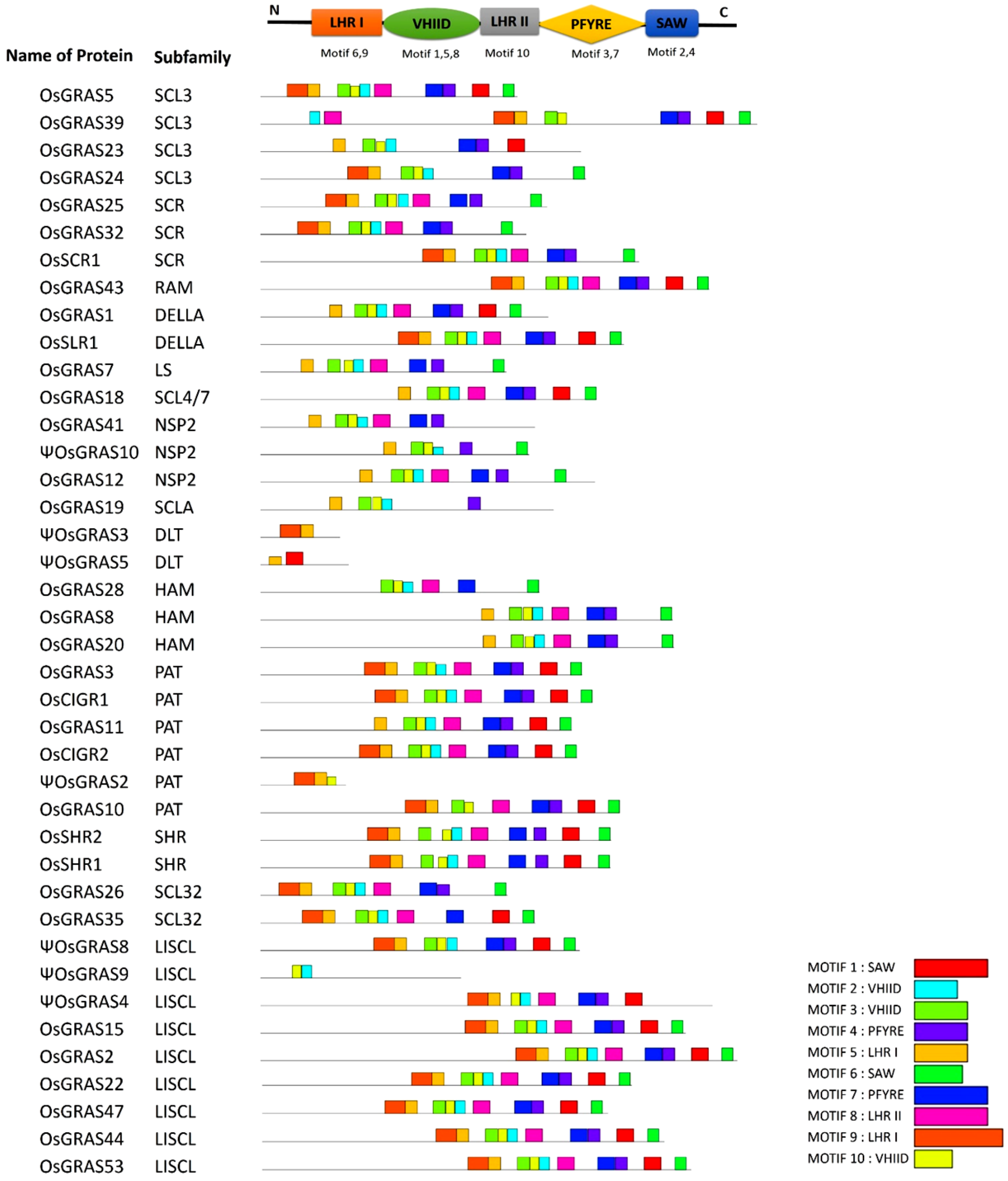

N
LHR I
VHIID
LHR II
PFYRE
SAW
C
Motif 6,9
Motif 1,5,8
Motif 10
Motif 3,7
Motif 2,4
Name of Protein
Subfamily
OsGRAS5 SCL3
OsGRAS39 SCL3
OsGRAS23 SCL3
OsGRAS24 SCL3
OsGRAS25 SCR
OsGRAS32 SCR
OsSCR1 SCR
OsGRAS43 RAM
OsGRAS1 DELLA
OsSLR1 DELLA
OsGRAS7 LS
OsGRAS18 SCL4/7
OsGRAS41 NSP2
ΨOsGRAS10 NSP2
OsGRAS12 NSP2
OsGRAS19 SCLA
ΨOsGRAS3 DLT
ΨOsGRAS5 DLT
OsGRAS28 HAM
OsGRAS8 HAM
OsGRAS20 HAM
OsGRAS3 PAT
OsCIGR1 PAT
OsGRAS11 PAT
OsCIGR2 PAT
ΨOsGRAS2 PAT
OsGRAS10 PAT
OsSHR2 SHR
OsSHR1 SHR
OsGRAS26 SCL32
OsGRAS35 SCL32
ΨOsGRAS8 LISCL
ΨOsGRAS9 LISCL
ΨOsGRAS4 LISCL
OsGRAS15 LISCL
OsGRAS2 LISCL
OsGRAS22 LISCL
OsGRAS47 LISCL
OsGRAS44 LISCL
OsGRAS53 LISCL
MOTIF 1 : SAW
MOTIF 2 : VHIID
MOTIF 3 : VHIID
MOTIF 4 : PFYRE
MOTIF 5 : LHR I
MOTIF 6 : SAW
MOTIF 7 : PFYRE
MOTIF 8 : LHR II
MOTIF 9 : LHR I
MOTIF 10 : VHIID

Figure 4

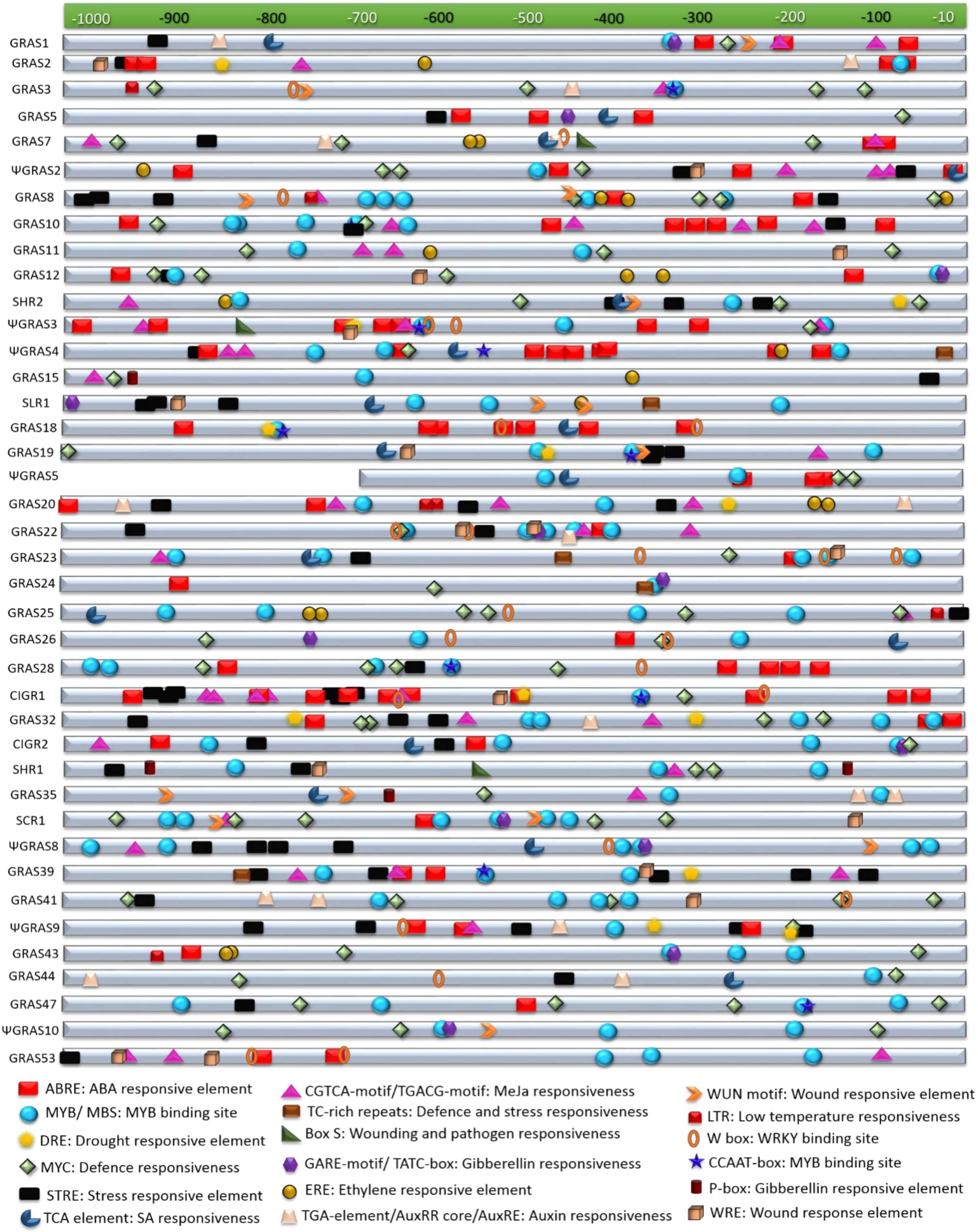

-1000
-900
-800
-700
-600
-500
-400
-300
-200
-100
-10
GRAS1
GRAS2
GRAS3
GRAS5
GRAS7
ΨGRAS2
GRAS8
GRAS10
GRAS11
GRAS12
SHR2
ΨGRAS3
ΨGRAS4
GRAS15
SLR1
GRAS18
GRAS19
ΨGRAS5
GRAS20
GRAS22
GRAS23
GRAS24
GRAS25
GRAS26
GRAS28
CIGR1
GRAS32
CIGR2
SHR1
GRAS35
SCR1
ΨGRAS8
GRAS39
GRAS41
ΨGRAS9
GRAS43
GRAS44
GRAS47
ΨGRAS10
GRAS53
ABRE: ABA responsive element
MYB/ MBS: MYB binding site
DRE: Drought responsive element
MYC: Defence responsiveness
STRE: Stress responsive element
TCA element: SA responsiveness
CGTCA-motif/TGACG-motif: MeJa responsiveness
TC-rich repeats: Defence and stress responsiveness
Box S: Wounding and pathogen responsiveness
GARE-motif/ TATC-box: Gibberellin responsiveness
ERE: Ethylene responsive element
TGA-element/AuxRR core/AuxRE: Auxin responsiveness
WUN motif: Wound responsive element
LTR: Low temperature responsiveness
W box: WRKY binding site
CCAAT-box: MYB binding site
P-box: Gibberellin responsive element
WRE: Wound response element

Figure 5

(a) NaCl root

Type of expression	List of genes expressed
IE	OsGRAS5,OsGRAS8,OsGRAS10,OsGRAS11, OsGRAS12,OsSHR2,ΨOsGRAS3,ΨOsGRAS4, OsGRAS15,OsSLR1,OsGRAS19,ΨOsGRAS5, OsGRAS22,OsGRAS24,OsGRAS26,OsGRAS28 OsCIGR1,OsSHR1,OsGRAS39,OsGRAS41, OsGRAS43,OsGRAS44
E	OsGRAS2,ΨOsGRAS2,OsGRAS25,OsGRAS35, OsSCR1
L	ΨOsGRAS9

(b) ABA root

Type of expression	List of genes expressed
IE	OsGRAS2,OsGRAS5,ΨOsGRAS2,OsGRAS8,OsGRAS10, OsGRAS12,OsSHR2,ΨOsGRAS3,ΨOsGRAS4,OsGRAS15, OsSLR1,OsGRAS18,ΨOsGRAS5,OsGRAS20,OsGRAS24, OsGRAS25,OsCIGR1,OsGRAS32,OsSHR1,OsGRAS35, OsSCR1,OsGRAS39,OsGRAS41,OsGRAS43,OsGRAS44
E	OsGRAS22
L	OsGRAS3,OsGRAS11,OsGRAS19,OsGRAS26,ΨOsGRAS9

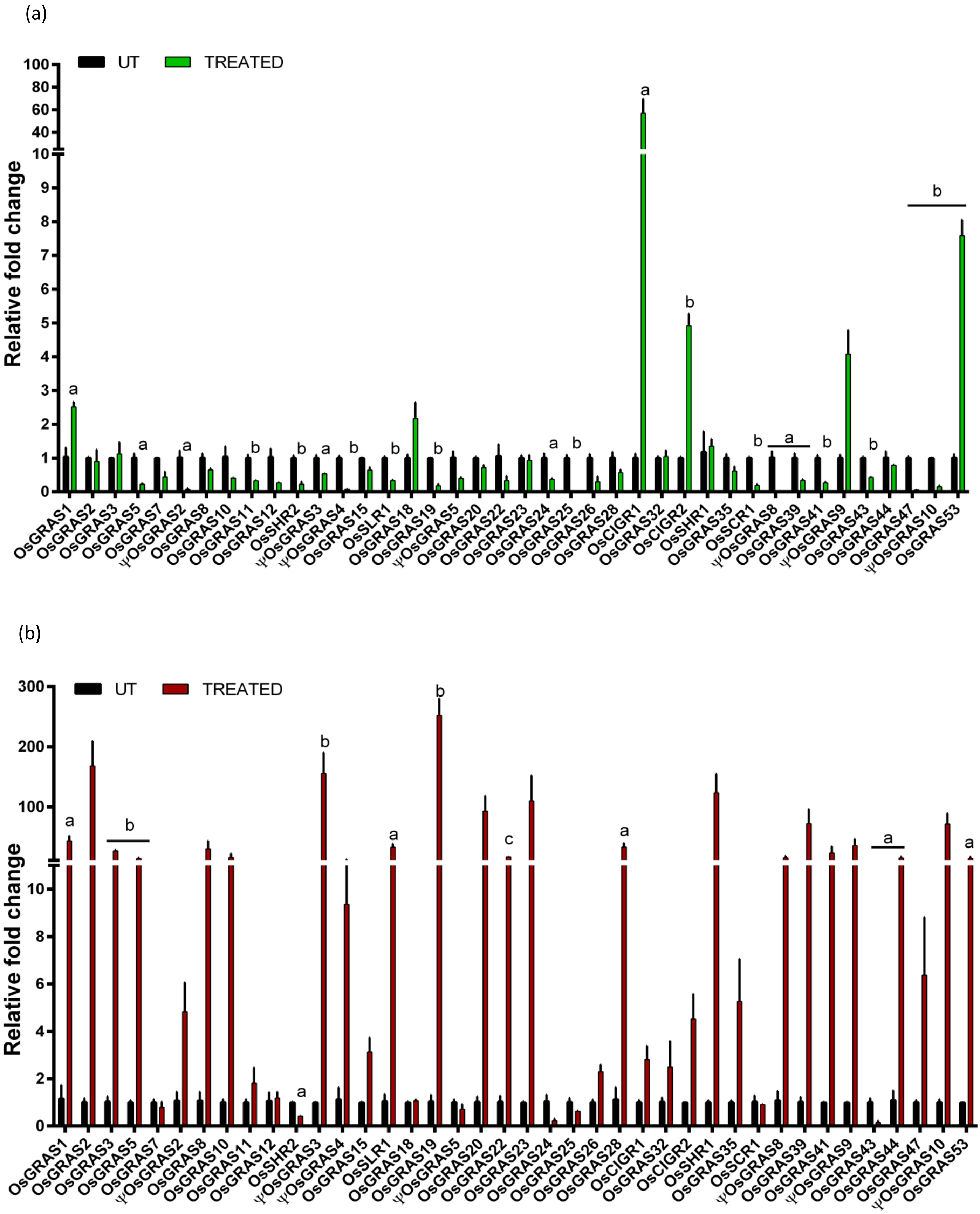
(c) NaCl shoot

Type of expression	List of genes expressed
IE	OsGRAS19,ΨOsGRAS5
E	OsGRAS12,OsGRAS25
L	OsGRAS24

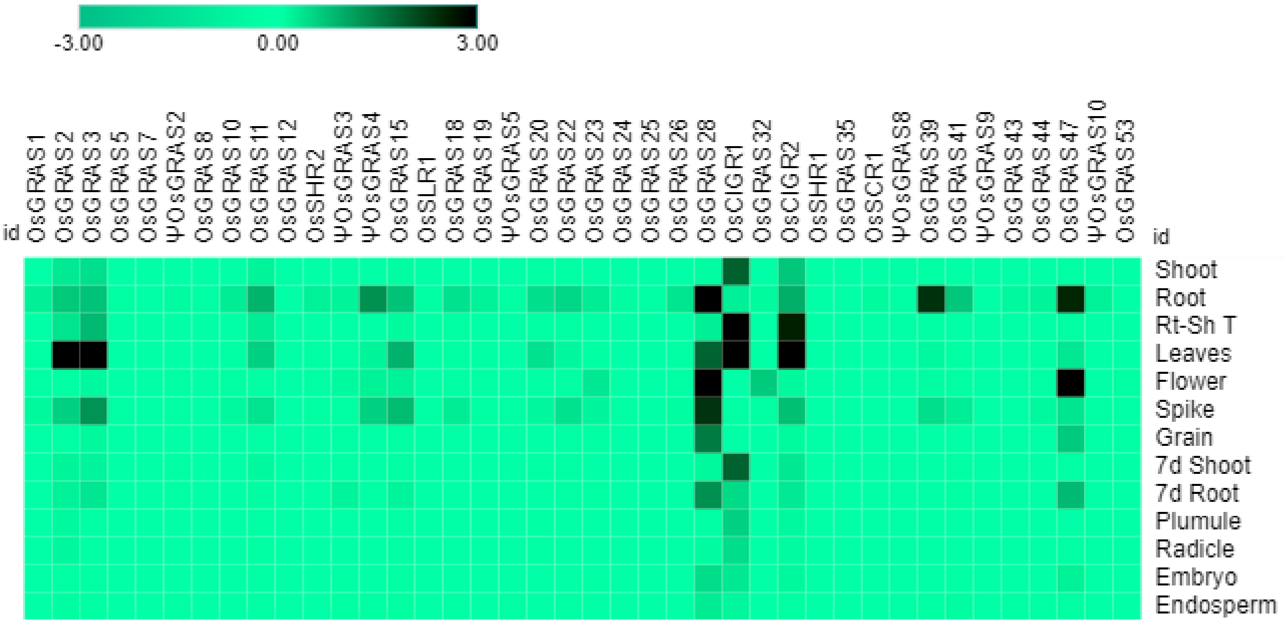
(d) ABA shoot

Type of expression	List of genes expressed
IE	ΨOsGRAS5,OsGRAS25
E	--
L	ΨOsGRAS2,OsGRAS12, OsGRAS24,OsSCR1

Figure 6


(a)
UT
TREATED
Relative fold change
100
80
60
40
10
9
8
7
6
5
4
3
2
1
0
a
a
a
b
b
a
b
b
b
a
b
a
b
b
a
b
b
b
OsGRAS1
OsGRAS2
OsGRAS3
OsGRAS5
OsGRAS7
ψOsGRAS2
OsGRAS8
OsGRAS10
OsGRAS11
OsGRAS12
OsSHR2
ψOsGRAS3
ψOsGRAS4
OsGRAS15
OsSLR1
OsGRAS18
OsGRAS19
ψOsGRAS5
OsGRAS20
OsGRAS22
OsGRAS23
OsGRAS24
OsGRAS25
OsGRAS26
OsGRAS28
OsCIGR1
OsGRAS32
OsCIGR2
OsSHR1
OsGRAS35
OsSCR1
ψOsGRAS8
OsGRAS39
OsGRAS41
ψOsGRAS9
OsGRAS43
OsGRAS44
OsGRAS47
ψOsGRAS10
OsGRAS53
(b)
UT
TREATED
Relative fold change
300
200
100
10
8
6
4
2
0
a
b
b
a
b
c
a
a
a
a
OsGRAS1
OsGRAS2
OsGRAS3
OsGRAS5
OsGRAS7
ψOsGRAS2
OsGRAS8
OsGRAS10
OsGRAS11
OsGRAS12
OsSHR2
ψOsGRAS3
ψOsGRAS4
OsGRAS15
OsSLR1
OsGRAS18
OsGRAS19
ψOsGRAS5
OsGRAS20
OsGRAS22
OsGRAS23
OsGRAS24
OsGRAS25
OsGRAS26
OsGRAS28
OsCIGR1
OsGRAS32
OsCIGR2
OsSHR1
OsGRAS35
OsSCR1
ψOsGRAS8
OsGRAS39
OsGRAS41
ψOsGRAS9
OsGRAS43
OsGRAS44
OsGRAS47
ψOsGRAS10
OsGRAS53

862 **Figure 7**


-3.00
0.00
3.00
id
OsGRAS1
OsGRAS2
OsGRAS3
OsGRAS5
OsGRAS7
ΨOsGRAS2
OsGRAS8
OsGRAS10
OsGRAS11
OsGRAS12
OsSHR2
ΨOsGRAS3
ΨOsGRAS4
OsGRAS15
OsSLR1
OsGRAS18
OsGRAS19
ΨOsGRAS5
OsGRAS20
OsGRAS22
OsGRAS23
OsGRAS24
OsGRAS25
OsGRAS26
OsGRAS28
OsCIGR1
OsGRAS32
OsCIGR2
OsSHR1
OsGRAS35
OsSCR1
ΨOsGRAS8
OsGRAS39
OsGRAS41
ΨOsGRAS9
OsGRAS43
OsGRAS44
OsGRAS47
ΨOsGRAS10
OsGRAS53
id
Shoot
Root
Rt-Sh T
Leaves
Flower
Spike
Grain
7d Shoot
7d Root
Plumule
Radicle
Embryo
Endosperm

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 September 2017
doi: 10.3389/fpls.2017.01553

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

Anusree Saha[1], *Shubhajit Das*[1], *Mazahar Moin*[1], *Mouboni Dutta*[1], *Achala Bakshi*[1], *M. S. Madhav*[2] *and P. B. Kirti*[1]*

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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 H_2O_2. 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

Briefings in Functional Genomics, 16(4), 2017, 238–247

doi: 10.1093/bfgp/elw041
Advance Access Publication Date: 30 January 2017
Review paper

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*

*Corresponding author: P. B. Kirti, Department of plant Sciences, University of Hyderabad, Hyderabad, 500046, India. Tel.: +040-23134545; E-mail: pbkirti@yahoo.com

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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Mazahar Moin is a Research Fellow in the Department of plant Sciences, University of Hyderabad. His research program is on activation tagging in rice for identification of the candidate genes responsible for water-use-efficiency and the characterization of novel genes.
Achala Bakshi is a Research Fellow in the Department of Plant Sciences, University of Hyderabad. She is working on activation tagging in rice and stress-responsive roles of novel candidate genes in rice.
Anusree Saha is a Research Fellow in the Department of Plant Sciences, University of Hyderabad. She is working on seed-specific tagging of genes for yield improvement in rice.
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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 August 2016
doi: 10.3389/fpls.2016.01284

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

Mazahar Moin[1], Achala Bakshi[1], Anusree Saha[1], Mouboni Dutta[1], Sheshu M. Madhav[2] and P. B. Kirti[1]*

[1] Department of Plant Sciences, University of Hyderabad, Hyderabad, India, [2] Department of Biotechnology, Indian Institute of Rice Research, Hyderabad, India

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 H_2O_2 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

Edited by:
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Biswapriya Biswavas Misra,
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University of California, Davis, USA

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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

Abbreviations: H_2O_2, hydrogen peroxide; MeJa, methyl jasmonate; RP, ribosomal protein; RPL, ribosomal protein large subunit; SA, salicylic acid.

PLAGIARISM REPORT

Activation Tagging Identifies Helicases and GRAS Transcription Factors as Potential Candidate Genes for Orchestrating Stress Tolerance in Rice

by Mouboni Dutta

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Submission ID: 1634763611
File name: Mouboni_thesis_for_plagiarism_check.docx (310.97K)
Word count: 25118
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ORIGINALITY REPORT

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2 Mouboni Dutta, Mazahar Moin, Anusree Saha, Dibyendu Dutta, Achala Bakshi, P. B. Kirti. "Gain-of-function mutagenesis through activation tagging identifies XPB2 and SEN1 helicase genes as potential targets for drought stress tolerance in rice", Theoretical and Applied Genetics, 2021 — 12%
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