Activation tagging for the identification of candidate genes responsible for high water-use efficiency and abiotic stress responses in *indica* rice

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for the award of Doctor of Philosophy

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"DECLARATION"

I, **Mazahar Moin** hereby declare that this thesis entitled "Activation tagging for the identification of candidate genes responsible for high water-use efficiency and abiotic stress responses in *indica* rice" submitted by me under the supervision of **Prof. P. B. Kirti,** is an original and independent research work. I also declare that it has not been submitted previously in part or in full for any degree or diploma of any other University or Institution.

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This is to certify that this thesis entitled **"Activation tagging for the identification of candidate genes responsible for high water-use efficiency and abiotic stress responses in *indica* rice"** is based on the results of the work done by **Mr. Mazahar Moin,** a research scholar for Ph.D. program in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, under my supervision. The work presented in this thesis is original and plagiarism free. No part of this thesis has been submitted for any degree or diploma of any other University or Institution.

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Publications from the Thesis

Moin M., Bakshi A., Saha A., Kumar M. U., Reddy A. R., Rao K. V., Siddiq E. A., Kirti P. B. (2016a) Activation tagging in indica rice identifies ribosomal proteins as potential targets for manipulation of water-use efficiency and abiotic stress tolerance in plants. **Plant Cell Environ.** doi: 10.1111/pce.12796 [Epub ahead of print].

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Original Article

Activation tagging in *indica* rice identifies ribosomal proteins as potential targets for manipulation of water-use efficiency and abiotic stress tolerance in plants

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ABSTRACT

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

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

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

INTRODUCTION

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

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

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

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

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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

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

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Dedicated to my

Parents

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Mazahar Moin

Abbreviations

µg Microgram

µl Microliter

µM Micro molar

ABA Abscisic Acid

ATP Adenosine triphosphate

BAP Benzyl amino purine

Bp Base pairs

CAMV Cauliflower mosaic virus

cDNA Complementary DNA

Cm Centimetre

CTAB Cetyl trimethylammonium bromide

d Day

DEPC Diethyl pyrocarbonate

DNA Deoxy ribonucleic acid

dNTPs Deoxy nucleotide triphosphates

EDTA Ethylenediaminetetraacetic acid

g Gram

h hours

*hpt*II Hygromycin phosphotransferase

IPTG Isopropyl-β-D-thiogalactoside

Kb Kilobases

KDa Kilodalton

LB Luria Bertani

M Molar

min Minutes

ml Milliliter

MS Murashige and Skoog

ng Nano gram

O.D Optical density

ORF Open reading frame

PCR Polymerase Chain Reaction

PPT phosphinothricin

pI Isoelectric point

RNA Ribo Nucleic Acid

Rpm Revolutions Per Minute

RPs Ribosomal proteins

RPL Ribosomal protein large subunit

TE Tris.EDTA

Tris Tris (hydroxymethyl) amino methane

U Units

UTR Untranslated regions

WT Wild type

WUE Water-use efficiency

Contents

Chapter 1:

Introduction and Review of Literature

1.1. Introduction

1.1.1 Rice, the world's staple cereal

The objective of any genome research is to identify all the genes contained in a genome and investigate their roles. Rice is the most appropriate model crop for generating a mutant resource for functional genomic studies because of its economic importance, availability of high-quality genome sequence and relatively small genome size. *Oryza sativa* or Asian rice, a staple cereal crop is consumed by more than 3.2 billion people globally feeding about 40% of the total world population and possibly providing more energy than any other food source to humanity. Although the green revolution and continuous breeding efforts have led to steady increase in rice productivity, >40% more yield still needs to be achieved (FAO, 2009) to keep pace with the world population, which is perpetually expanding from 7.3 billion in 2015 to a projected 9.5 billion by 2050. Of about 700 million tons of rice produced annually, ~640 million tons is grown in Asian countries like India, China, and Japan, constituting 90% of global production. Increased or sustainable productivity of rice demands more arable land, fertilizers and extensive irrigation facilities. The development of high-throughput genetic transformation technologies would facilitate better utilization of its genomic resources to produce transgenic rice rapidly for agronomically important traits such as high water-use efficiency, high nitrogen-use efficiency, tolerance to biotic/abiotic stresses and high nutritional value, which would otherwise take many years of conventional breeding to develop varieties with similar traits. Last 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.

1.1.2. Rice, a suitable monocot model crop

Among cereals and other crops, rice is perhaps the only crop that has made vast advances in the area of genomics and functional genomics within a decade's time, because unlike other cereal crops, rice is a diploid organism with the smallest genome size (Jung et al., 2008). Though *Arabidopsis* genome has provided a basis for comparative genomics in dicotyledons, this is not directly applicable to monocotyledons. Rice is an appropriate monocot model crop because of the availability of the high quality genome sequence (Goff et al., 2002, Yu et al., 2002) and its much smaller genome size (370 Mb) compared with related cereals such as

sorghum (700 Mb), maize (2800 Mb), barley (4900 Mb) and wheat (16000 Mb). Since rice has syntenic relationships with other cereals, functional characterization of genes in rice will provide key genetic insights that would lead to comparative genomics among closely related grass family members.

1.1.3. Rice genome, then and now

During 2000 to 2002, the genetic-blueprint of four important genomes were made publicly available. These included the genomes of the model plant *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), human (International Human Genome Sequencing Consortium, 2001) and two sub species of world's staple cereal, rice. Whole genome rice sequencing, initiated by Rice Genome Research Program (RGP), Japan, which originally started in 1991 continued through 1997 establishing the tools and techniques for genome analysis and leading to the foundation for unravelling the genetic makeup of rice. In 1998, RGP, through an international consortium of ten countries (including USA, UK, China, Korea, Thailand, France, Philippines, Brazil, Canada and India) started an International Rice Genome Sequencing Project (IRGSP) by initiating the cloning of large inserts of genomic DNA (contigs) in PAC/BAC vectors for sequencing chromosomal segments. Two years later, rough draft of genomes of *indica* and *japonica* were published online by Beijing Genomics Institute (Yu et al., 2002) and Syngenta's Torrey Mesa Research Institute (TMRI) (Goff et al., 2002) respectively through whole-genome shotgun sequencing analysis of small-insert clones (Tyagi et al., 2004). IRGSP (2005) finished the whole genome sequencing of Nipponbare rice and presented a detailed map of the genome covering the size of individual chromosomes, genetic homology, gene number and gene frequency, polymorphic sites (SNPs) that resulted in the divergence of *indica* and *japonica* rice varieties and endogenous transposon insertions.

According to recent release 7 of TIGR, a total of 3,473 BAC/PAC clones carrying inserts of 12 chromosomes have been generated; each pseudomolecule representing the nucleotide sequence of a chromosome is constructed by assembling the sequence of each clone based on order of previous clones mapped on the chromosome. During assembly, the overlapping sequences are removed and replaced by a stretch of Ns and each updated release of a database contained the sequence information of these non-overlapping segments or gaps.

Till date, about 56, 081 genes, of which 16, 979 are transposable element (TE) genes; 39, 102 Non-TE genes; with average gene size being 3,223 bp and 2,853 bp respectively were identified, of which 6,457 genes have been predicted to undergo alternative splicing to produce 10, 352 isoforms, which resulted in 56, 081 genes generating 66, 433 transcripts.

Chromosome-1 being the largest (43.3 Mb), has also the highest number of genes (TE: 6,542; Non-TE: 8,036), followed by chromosome-3 (36.4 Mb) (TE: 5,573; Non-TE: 7,027 genes), while chromosome-9 (23.01 Mb) is the smallest rice chromosome (Kawahara et al., 2013).
After the avalanche of complete genome sequence, databases were developed to provide access to the annotated data and to extend the annotations to other rice genomes. In addition to these, functional-genomic databases facilitate the identification of inserts or mutations in genes generated by random mutagenesis. Post-genome sequencing, more than 30 databases were made publicly available that enable the genetic manipulation (Table 1.1).
The two fundamentally different approaches, forward genetics (mutant phenotype to genotype) and reverse genetics (genotype to mutant phenotype) are used to establish the direct relation between gene function on the plant phenotype. Large scale random insertional mutagenesis with foreign DNA elements are used to identify and decipher the roles of the genes by disrupting or activating their functions. Over 447, 919 FSTs (Flanking Sequence Tags) have been isolated, of which, 336, 262 were precisely positioned on 12 chromosomes from about 426, 375 tagged transgenic lines generated through T-DNA, *Ac*/*Ds*, *Tos*17 and *En*/*Spm* elements (Wei et al., 2013). Of 336, 262 insertions, 32% (109, 407) of the total insertions were distributed between the genes, while 68% (226, 861) of them were intra-genic, precisely located within the genes (Wei et al., 2013). About 338, 744 T-DNA/transposon insertions are required to saturate the whole rice genome of 373 Mb with a knockout mutation at a probability of 99%. However, these numbers will change with the advancement of research and time.

1.1.4. Genetic transformation of rice

The success of *Agrobacterium* mediated transformation of rice started more than twenty years ago using embryogenic callus tissues as explants (Raineri et al., 1990, Hiei et al., 1994). Among the major factors that are known to determine the success of rice transformation, the nature of the explant is the most predominant one. Callus obtained from the scutellum tissue of mature seeds has been most commonly used for transformation. The ability of the transformed cells to regenerate into transgenic plants is crucial for an efficient transformation system. Another important factor is the genotype (Ge et al., 2006), *japonica* cultivars being more amenable to tissue culture manipulation than *indica* varieties (Abe and Futsuhara, 1986).

The low transformation efficiency of some of the cultivars, particularly of *indica* genotypes which comprise 80% of total cultivated rice, is insufficient to produce a large scale transgenic plants for functional genomics and only limited number of *indica* cultivars have so far been

successfully transformed through classical callus-mediated protocols. The genotypic barrier for susceptibility to *Agrobacterium* infection is due to differential expression of host genes involved in the transformation process starting from cellular attachment to cytoplasmic trafficking, nuclear entry and finally integration of the T-DNA into the recipient genome (Citovsky et al., 2007).

A further problem is that tissue-culture based protocols using *Agrobacterium* vectors involve multiple steps from callus induction through transformation to plant regeneration, a lengthy process, which also needs repeated rounds of subculturing during which cultures are vulnerable to contamination and somaclonal variation, which are prevalent during the prolonged exposure of explants to tissue-culture media. Furthermore, these callus-mediated protocols can only be used to produce a limited number of transgenic plants for expressing defined expression cassettes and cannot be used in functional genomic approaches, which necessitate the development of large populations of transgenic plants with independent T-DNA/transposon integrations. Among the *indica* genotypes, BPT-5204 (Samba Mahsuri) is one of the highly recalcitrant varieties to tissue-culture manipulations.

A significant advance in *Arabidopsis* research came with the development of simplified and reliable *in planta* transformation methods as a substitute for root transformation and regeneration. In its native form, it involves dipping of shoots bearing inflorescences in *Agrobacterium* suspension (Clough and Bent, 1998) followed by vacuum infiltration and sudden release of the vacuum (Bent, 2000; Weigel and Glazebrook, 2006). In rice, although attempts have been made to develop such protocols using 2 d old husked seeds (Supartana et al., 2005, Lin et al., 2009), not much emphasis has been given to improve the efficiency of this process. There are no reports on the development of an activation-tagged population in *indica* rice cultivars as they are more recalcitrant to tissue culture regeneration, which can only be used to produce a limited number of transgenic plants. However, functional genomic approaches necessitate the development of large populations of transgenic plants.

1.1.5. Water-Use Efficiency

To sustain under the diverse environmental and climatic changes plants, being sessile organisms have naturally evolved with armor in the form of genetic loci within their germplasm. However, identification of these genetic elements that contribute to plants survivability in challenging conditions is of paramount importance to further improve their productivity potential. The investigations into the mechanism of plant tolerance to the abiotic

two stresses assume significance worldwide. Development of high yielding rice varieties that utilize limited resources such as water and yet maintain sustained/improved productivity, an important agronomical trait called water-use efficiency could save a significant amount of irrigated water, which can be utilized to improve the productivity potential of other crops.

The most important abiotic factors that restrain rice plant performance and productivity are drought and salinity, both of which arise from reduced water availability. Rice and water are the world's two assets that are very closely associated. Rice requires extensive irrigation; 75% of rice productivity comes from irrigated land while <25% yield is achieved through rain and deep water systems (Hibberd et al., 2008) with an estimate of rice receiving 35% to 43% of total world's irrigation water. Rice is sensitive to water shortages, and if rice genetic system is engineered to use less water without compromising on its productivity, the saved water could be efficiently used to irrigate all other crops to enhance their productivity potential significantly. It is estimated that rice receives 35% to 43% of the total irrigation water in the world (Hibberd et al., 2008).

When crop plants like rice are subjected to water deficiency, they tend to develop a dehydration-avoidance or water-use efficient phenotype (Blum et al., 1988), which is characterized by early flowering, reduced plant height, leaf area, tillering and seed productivity; all of which are in sharp contrast to high yielding phenotypes (Blum et al., 2005). These drought-avoidance phenotypes are accompanied by stomatal closure to limit water loss, long root system to absorb deep soil moisture and low water potential. In the present study, we are trying to recombine water stress adaptation with high yield potential using a gain-of-function mutagenesis strategy. In the current study, we made attempts to recombine water stress adaptation with high yield potential using a gain-of-function mutagenesis strategy, the activation tagging.

The present work reports on the development of an activation-tagged population using an improved and efficient *in planta* transformation method. The transgenic plants generated were screened for WUE by growing them under limited water conditions in an *indica* rice, Samba Mahsuri (BPT-5204) which is a very widely cultivated, high-yielding variety with superior cooking quality. We here report for the first time on the tagging of ribosomal proteins in rice plants that performed well under water limiting conditions (with 100% field capacity) indicating their involvement in ameliorating abiotic stress including water limiting conditions. This was corroborated by the confirmation of their significant and immediate up-regulation in various stress treatments and these observations are reported here.

Table 1.1: Various publicly available rice databases

Database	Web link	Function	Source
BGI-Rice Information System (BIG-RIS)	http://rice.genomics.org.cn/rice/index2.jsp	A resource for rice genome	Beijing Genomics Institute (BIG)
TIGR, the Rice Genome Annotation Project Database (RGAP-DB)	http://rice.plantbiology.msu.edu/	Annotation for the rice genome	Michigan State University (MSU)
Rice Annotation Project Database (RAP-DB)	http://rapdb.dna.affrc.go.jp/	A hub for rice genomics	National Institute of Agrobiological Sciences (NIAS)
HapRice	http://qtaro.abr.affrc.go.jp/	SNP haplotype database	NIAS
RiceWiki	http://ricewiki.big.ac.cn/	Publicly editable open platform	BIG
Rice Annotation Database (RAD)	http://rad.dna.affrc.go.jp/	Map-based rice genomics	Ito et al., 2005
Rice-Map	http://www.ricemap.org/	Rice genome browser	Wang et al., 2012
RiceVarMap	http://ricevarmap.ncpgr.cn/	Database of rice genomic variations	National Centre of Plant Gene Research
Gramene	http://www.gramene.org/	Comparative Plant Genomics Resource	CSH, Oregon State University
GreenPhylDB v 4	http://www.greenphyl.org/cgi-bin/index.cgi	comparative and functional genomics in plants	CIRAD

IR64 genomic DNA deletion mutant browser	http://irfgc.irri.org/cgi-bin/gbrowse/IR64_deletion_mutants/	Detection of deletions in genes using Fast neutron γ-ray, DEB, EMS	Bruce et al., 2009
Rice Transposon Flanking Sequence Tag (FST) Database	http://sundarlab.ucdavis.edu/rice/blast/blast.html	Ds, dSpm, Gene trap insertion mutants	UC, Davis
RiceGE (*japonica* and *indica*)	http://signal.salk.edu/cgi-bin/RiceGE	T-DNA Enhancer trap FST database	Salk Institute Genome Analysis Laboratory
OryGenesDB	http://orygenesdb.cirad.fr/	Ac/Ds FST database	CIRAD
Rice TILLING	http://tilling.ucdavis.edu/index.php/Rice_results#Population	EMS	University of California, Bioinformatics Core
Taiwan Rice Insertional Mutant Database	http://trim.sinica.edu.tw/	T-DNA FST	Academia Sinica
Rice Functional Genomics Program (RFGP)	http://159.226.24.50/	cDNA Microarray Database	UC, Davis
Oryza Tag Line (OTL)	http://oryzatagline.cirad.fr/	T-DNA and Enhancer traps (ET)	CIRAD
Rice Mutant Database (RMD)	http://rmd.ncpgr.cn/	T-DNA and Enhancer traps (ET)	NCPGR
Rice Expression Database (RED)	http://cdna02.dna.affrc.go.jp/RED/	Expression profiling	National Institute of Agricultural Sciences
Oryzabase	http://www.shigen.nig.ac.jp/rice/oryzabase/about/oryzabase	TILLING	National Institute of Genetics Strains Research Centre

Knowledge-based Oryza Molecular biological Encyclopaedia (KOME)	http://cdna02.dna.affrc.go.jp/cDNA/	Full-length C-DNA clones	NIAS, RIKEN, BRAIN, FAIS
OryzaSNP Database	http://oryzasnp.org/iric-portal/	SNP genotyping	IRRI
Next Gen Seq Database	https://mpss.udel.edu/rice/mpss_index.php	Gene expression data base	Ohio State Univ.
The Database of Rice Transcription Factors (DRTF)	http://drtf.cbi.pku.edu.cn/	Rice Transcription factor database	Peking University
RiceSRTFDB	http://www.nipgr.res.in/RiceSRTFDB.html	Database of rice Transcription factors	NIPGR
UniVIO	http://univio.psc.riken.jp/	Transcriptome database	RIKEN
Oryza Map Alignment Project (OMAP)	http://www.omap.org/	Comparative genome physical maps of Oryza wild relatives	CSH, University of Arizona, Purdue University
Plant Reactome	http://plantreactome.gramene.org/ReactomeGWT/entrypoint.html	A Resource for Metabolic and Regulatory Pathways	The Plant Reactome team
RiceFOX	http://ricefox.psc.riken.jp/	*Arabidopsis* mutant lines overexpressing rice full-length cDNAs	RIKEN
Rice cDNA database (RICD)	http://www.ncgr.ac.cn/ricd	Full-Length cDNA (FLcDNA) resource	National Centre for Gene Research

Tos17 insertion mutant database	https://tos.nias.affrc.go.jp/	Tos17 FST	National Institute of Agrobiological Sciences

1.1. Review of literature

Although the sequencing of rice genome completed and well mapped, functional characterization of all the existing genes is yet to be achieved. Numerous strategies have been employed to investigate the functions of the annotated genes to capitalize on the information provided by the published genome sequences. Mutant populations are an indispensable tool for thoroughly investigating the gene functions (Hricova et al., 2010). Gene knockout methods, with the use of chemical mutagens such as ethyl methanesulfonate (EMS), physical agents like high energy radiations that include neutrons and gamma particles, post-transcriptional silencing approaches using double-stranded RNAs (siRNA, hpRNA), DNA elements like T-DNA and transposons were used for generating loss-of-function mutations in plants. In addition, to these methods gain-of-function mutagenesis approaches such as activation tagging, tissue specific tagging have been successfully established in rice for fucntional genomic studies.

1.2.1. Loss-of-function mutagenesis

1.2.1.1 TILLING

TILLING is a reverse genetics strategy in which seeds are exposed to varying concentrations of chemical agents such as EMS, sodium azide-methyl nitrosourea (MNU) and Diepoxybutane (Till et al., 2007). Chemical mutagenesis has the advantage that they are non-transgenic and high-throughput and induce large-scale random and irreversible mutations across the genome (Kim et al., 2006). PCR amplification of the genes with fluorescent-labelled primers is used to detect these mutations in the genome using an LI-COR analyzer (Till et al., 2007). The success of TILLING has accomplished only when high levels of mutations, in the coding sequences, are achieved which is particularly important in crops with longer genome size having high gene densities. For example, the mutation densities using 1.5% EMS or 1 mM sodium azide and 15 mM MNU were 1/294 kb and 1/265 kb, respectively (Till et al., 2007) in rice.

1.2.1.2. Physical mutagenesis

Ionizing particles such as gamma rays (Morita et al., 2009, Chun et al., 2012) and fast neutron bombardment generate random deletions and chromosomal rearrangements of various sizes in the genome. The currently used fast neutron technique in plants is Deleteagene (delete-a-gene), in which after exposure of seeds to high energy radiations, deletions are detected using PCR-based methods and microarrays. Since mutation sites do not physically associate with any

foreign molecule, it is hard to locate precisely the place of the mutation in the genome, which is effective only in organisms with smaller genome size and with sufficient sequence information to design the microarrays. Also, it is also difficult to monitor minor deletions or deletions in duplicated segments of chromosomes. Furthermore, the number of deletions occurring per exposure is experimentally unmanageable, and there is every possibility that more than one deletion can happen in a genome per exposure. In conditions, where the number of gene deletions is more, it is difficult precisely to identify the function of the corresponding gene responsible for the observed phenotype.

1.2.1.3. Post-transcriptional gene silencing (PTGS)

PTGS mechanisms include homology-dependent RNA degradation or sense/antisense gene silencing, double-stranded RNA-mediated silencing, co-suppression and virus-induced gene silencing (VIGS). Antisense vectors carrying coding sequences in reverse orientation under constitutive promoters for differential inhibition of the target genes is the first step in this direction. The degree of suppression of endogenous mRNA depends on the homology or sequence similarity between the endogenous mRNA and the antisense RNA generated in the plant by transformation of vectors carrying the sequence of target genes in a specific orientation. This technology has subsequently became popular as RNA interference (RNAi).

Gene silencing is also induced by cloning PCR-amplified gene fragments in sense and antisense orientations separated by a spacer in vectors like pHANNIBAL and pKANNIBAL. (Wesley et al., 2001). When transcribed, a hairpin RNA (hpRNA) is formed through RNA-dependent RNA-polymerase 6 that binds and degrades the target gene transcript (Harmoko et al., 2013).

The micro-RNAs (miRNAs) are another group of small RNA molecules that can inhibit gene expression (Tijsterman et al., 2004). These are single-stranded, stem-loop structures transcribed from non-protein coding sequences present within the genomes. The miRNAs negatively regulates the gene expression by degrading the target mRNAs in plants or by inhibiting the protein synthesis as in animals (Carrington et al., 2003). The miRNAs cause endogenous gene silencing within the organisms as a mechanism of defense against viruses, gene regulation during growth and development. The artificial miRNA (amiRNA) with sequence complementarity unique to the target genes can be generated to silence the endogenous genes (Warthmann et al., 2013).

Cosuppression is another mechanism of silencing wherein additional copies of the same gene (up to three) are cloned under strong promoters, to produce sufficient levels of sense RNAs that inhibit the expression of the target gene. When plants are infected with certain viruses

(RNA viruses like Tobacco Mosaic Tobamovirus, Tobacco rattle virus, Potato X Potexvirus, Tomato Nepovirus and DNA viruses like Geminivirus), initially they undergo severe localized symptoms followed by a mechanism of 'recovery' at the site of infection. These recovered tissues were found to be resistant to subsequent infections of same or similar viruses (Ratcliff et al., 1997). In VIGS, the recombinant viral vectors carry inserts homologous to the target genes in plants, so that the siRNAs corresponding to the inserted coding sequence guide the RNase complex to the endogenous target mRNA for degradation (Lu et al., 2003).

1.2.1.4. T-DNA Insertional mutagenesis

Post-genome sequencing, large-scale insertional mutagenesis has been undertaken to investigate the role of unknown genes in the rice genome. Generation of insertion mutant populations in rice greatly relied on elements like T-DNA, *Tos17*, *Ac/Ds* and En/Spm transposons. These generate unbiased, random loss-of-function mutations and provide unique signatures in the genome and the flanking sequences at the integration sites can be identified through appropriate genome walking methods.

T-DNA is a short segment of DNA transferred from the Ti plasmid of *Agrobacterium* to the plant genome upon infection by the soil pathogen *Agrobacterium tumefaciens.* T-DNA creates a unique signature when introduced into the plant genome, thereby allowing easy monitoring of its location in the genome by analyzing the flanking sequences at the site of insertion (Leehan and Feldmann 1997)**.** The classical approach of establishing the functions of genes is to disrupt their activity through random T-DNA insertional mutagenesis and analyzing the resultant consequences on the phenotype (Fig. 1.1). It was successfully carried out in rice generating loss-of-function mutants (Jeong *et al.,* 2002). However, loss-of-function screens have certain limitations; they do not uncover the functions of redundant genes whose loss-of-function is compensated by another member of the same gene family (Tani et al., 2004).

The genetic redundancy is predominant in plants as their genomes tend to undergo large-scale random, segmental duplications, a phenomenon called 'polyploidization' (Bowers et al., 2003). More than 80% of the *Arabidopsis* (Blanc et al., 2003) and 65% of the rice genomes (Yu et al., 2005), respectively constitute the duplicated segments. Knockout of any one of these genes present in the duplicated regions does not produce phenotypes distinct from their wild-type counterparts (Briggs et al., 2006). Loss-of-function mutations through T-DNA insertions in the individual *etr* genes (*etr-1* to *etr-4*) of ethylene response pathway in *Arabidopsis* did not induce any defects in ethylene-response. Rather, plants carrying knockout mutations in any one of these genes behave in a way similar to wild types (Hua and Meyerowitz, 1998). In cases of

partial redundancy, which is genetically more stable than complete redundancy (Briggs et al., 2006) and genes expressing only under given environmental conditions such as in response to abiotic or biotic stresses, the loss-of-function mutant phenotypes can be easily detected (Krishnan et al., 2009). Also, it is difficult to examine the function of the genes involved in early developmental stages of the life cycle, whose loss-of-function could result in embryonic lethality. In addition to all these factors, any given transgenic plant carries single or at most only a few copies of T-DNA insertions in the genome. Hence, large populations of insertion knockouts are needed to achieve the genome saturation.

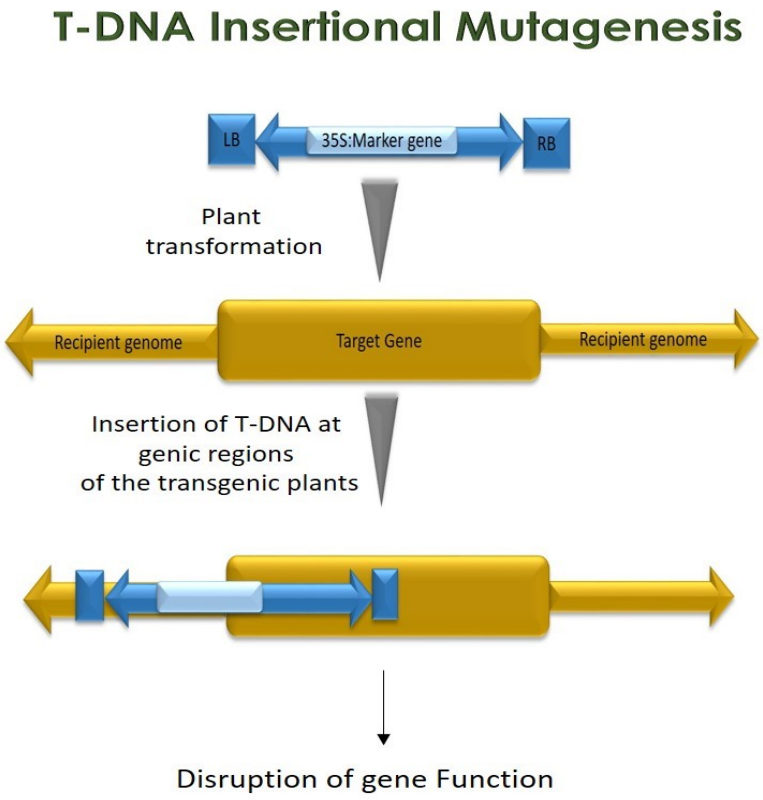


Fig. 1.1. Simplified representation of T-DNA insertional mutagenesis

T-DNA, derived from the part of Ti plasmid of *Agrobacterium tumefaciens* is transformed into the plant. If the T-DNA integrates at the genic regions, it causes the disruption of the target gene function.

1.2.1.5. Transposon tagging

Transposable elements (TEs) constitute 70% and 25% of total maize and rice genomes, respectively. Based on the nucleic acid intermediates through which they mobilize, (TE) are classified as class I and class II. Class I TEs are transposed through RNA-intermediates using

copy-paste mechanism while class II TEs use DNA intermediates for cut-paste transposition. The TEs, like *Ac/Ds*, *En-spm* and *mutator* transposons belong to class II type. Although class II TEs are endogenous and active in maize, they have been well characterized for generating a large-scale mutant resource for functional genomics in other organisms including rice.

The major advantage of transposon integrations over T-DNA insertions is that they tend to transpose in the genome in the presence of an active transposase, whereas T-DNA insertions are stably integrated and cannot transpose. Therefore, insertions generated by T-DNA are permanent in contrast to the insertions produced by the transposons that result in repetitive mutagenesis. The significance of this continued transposition is that a large number of events generated from a small number of primary transposon carrying transgenic plants.

1.2.1.5.1. *Tos17* transposable elements

Generation of insertion mutant libraries in rice greatly relied on the use of foreign DNA elements like T-DNA, *Ac*/*Ds,* and *En*/*Spm*. *Tos17* is an endogenous retrotransposon, present in low copies in rice (2 copies in Nipponbare) and is usually inactive under normal conditions. However, stress such as that results from prolonged tissue culture conditions activates the *Tos17* copy present on chromosome 7. Plants regenerated from 3 to 5-month-old calli harbor 4 to 8 new copies of *Tos17* elements in the genome (Mieulet et al., 2013). *Tos17* TEs also prefers to integrate at the genic regions; this property has been exploited for generating a large-scale reverse-genetics mutant resource. According to the latest report, about 51,625 transgenic rice lines, carrying 5, 10,000 *Tos17* insertions are generated, which is the highest mutant resource among all other TEs in rice.

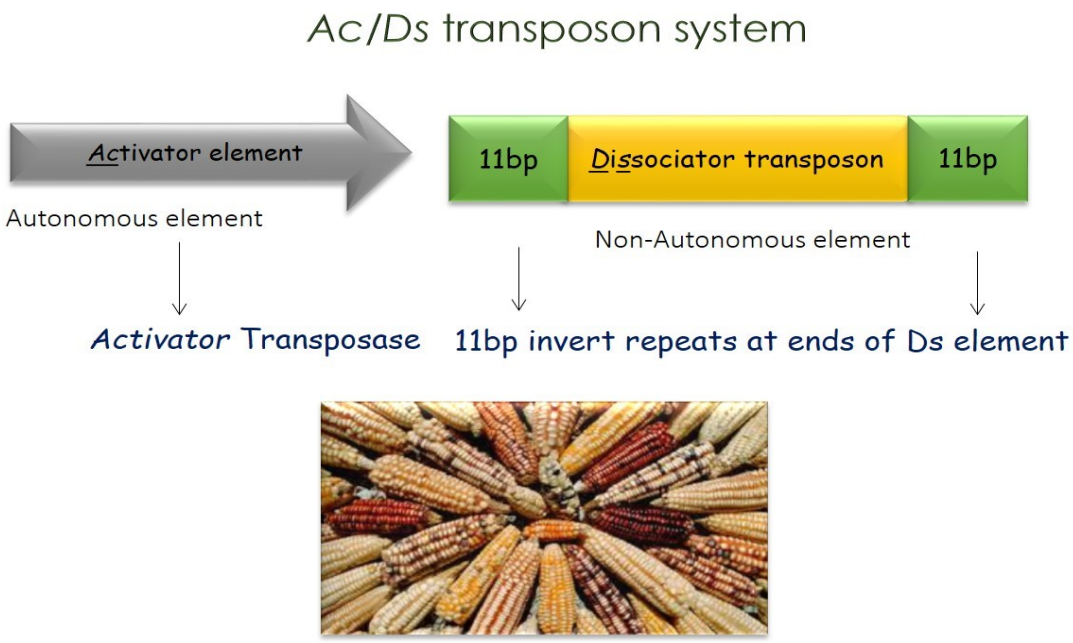
1.2.1.5.2. *En/Spm* transposons

The enhancer/suppressor-mutator (*En/Spm*) is a two component system of maize, which contains an autonomous 8.3 kb sequence, coding for *TnpA* and *TnpD* transposase. This transposase causes the mobilization its native sequence along with a non-autonomous inhibitor/defective *Spm* (*I*/*dSpm*) (Kumar et al., 2005). *En/Spm* elements carries a 13-bp terminal inverted repeats (TIR) and sub-terminal regions required for binding with *TnpA*, while *TnpD* stabilizes this dimeric complex, for endonucleolytic cleavage. The *I*/*dSpm* also carries TIR regions, but since they have internal deletions, they are incapable of producing a functional transposase. Thus, *I*/*Spm* alone cannot transpose (Krishnan et al., 2009). The T-DNA vectors used for *En/Spm* insertional mutagenesis carry a truncated *Spm*, coding for transposase, flanked by *dSpm* and a reporter gene in *cis*. The transposase mobilizes *dSpm,* but cannot excise its

sequence (Kumar et al., 2005). Maize *En/Spm* elements have been effectively used in studies on insertional mutagenesis in rice.

1.2.1.5.3. *Ac/Ds* transposable elements

Maize *Ac/Ds* transposons identified based on the varying color patterns of corn kernels (McClintick, 1950) are the widely and successfully used two-component system for rice insertional mutagenesis. The transposition mechanism of *Ac/Ds* is similar to *En/Spm* in that one of the two components codes for an active transposase while the other remains inactive in the absence of its partner (Fig. 1.2). However, unlike *En/Spm* only one protein is produced and required for mobilization. The *Ac*/*Ds* system comprised of an autonomous *Ac* element, coding for an *Ac* transposase while the truncated fragment of *Ac* constitutes the *Ds* or *Dissociator* element. During transposition, *Ac* transposase recognizes the inverted repeats common to both the elements, during which part of *Ac* transposase coding genomic region is eliminated, creating the *Ds* element (Skipper et al., 2013). During the meiotic segregation, *Ac* dissociates from the *Ds,* which cannot transpose further in the absence of an active transposase and hence, becomes stabilized in the plant genome. Thus, *Ds* artificial elements can be generated for transposon tagging, whose movement in the plant genome in subsequent generations are tracked by the activity of the marker or reporter gene associated with the element.



McClintock's discovery of *Ac/Ds* elements based on instability in colour phenotypes of Maize kernels

Fig. 1.2. The widely used two component transposon system for insertional mutagenesis in rice is *Ac*/*Ds,* in which *Ds* tends to transpose continuously as long as it is associated with *Ac* transposase and becomes stabilized after segregation. If the *Ds* is integrated in the genic regions, it causes the disruption of the target gene function.

1.2.2. Gain of function mutagenesis

Gain-of-function mutagenesis through forward genetics is an alternative approach to loss-of-function mutagenesis and in which the transcriptional enhancers are randomly inserted into the genome or the expression of genes under strong constitutive promoters (Walden et al., 1994). In this system, T-DNA carrying a strong promoter or enhancer elements becomes randomly inserted into the plant genome. While the introduced enhancer elements activate genes up to a 10 kb region and can function in either orientation, the use of strong promoters such as CaMV35S would result in direct activation of the gene that comes under its control (Wilmink et al., 1995, Weigel et al., 2000, Tani et al., 2004, Kondou et al., 2010). Gain-of-function mutant phenotypes overexpressing a member of a gene family having many isoforms can be visualized independently without the effect of other members of the same gene family. Thus, the roles of even redundant genes can be elucidated by dominant mutations.

The alternative approach to T-DNA insertional mutagenesis is to use gain-of-function mutagenesis through activation tagging based on the insertion of transcriptional CaMV35S enhancers into the genome. These enhancers cause endogenous over-expression of genes near the sites of integration, which could give rise to novel phenotypes (Weigel et al., 2000). Insertional mutagenesis approaches including activation tagging can only be successful when sufficiently large populations of tagged lines covering as many genes as possible in a genome are developed through genetic transformation. Rice has a genome of about 373 Mb with 56,081 genes. Of these, 16,979 are transposable element (TE) related genes and 39,102 are non-TE related genes (Kawahara et al., 2013). It has 44% of the genome that is transcriptionally active, with a minimum gene density of one gene/ 9.9 kb (International Rice Genome Sequencing Project, 2005). A substantial number of independent transgenic plants are needed to tag each of these 56,081 genes with activation-tags (4xEnhancers) to enhance their endogenous expression patterns. Once such a population is developed, it can be screened for all the possible phenotypes as desired and the population can be mined for novel genes controlling agronomically important characters.

Activation tagging utilizing enhancer sequences (Odell *et al.,* 1985) derived from the cauliflower mosaic virus CaMV35S promoter and was first successfully conducted a study that

identified a gene for auxin-independent growth (Hayashi et al., 1992). These enhancers cause the transcriptional activation of flanking genes, and since the activated genes are associated with T-DNA tags, this technique has become popularly known as activation tagging. With a strong enhancer, such as that derived from CaMV35S promoter, the endogenous pattern of gene expression can be activated more than the native levels (Neff et al., 1999). The individual CaMV35S promoters cause ectopic overexpression of the tagged genes whereas tetrameric enhancers do not lead to constitutive expression, rather they increase the endogenous expression levels of the target genes. Thus, the phenotype resulting from an improvement of the endogenous expression would more likely reflect the proper functioning of the activated gene (Weigel et al., 2000).

1.2.2.1. Activation tagging technology using *Ac/Ds* system

A typical *Ac/Ds* based activation tagging vector contains an *Ac* transposase in the T-DNA and a *Ds* element carrying tetrameric enhancers derived from CaMV35S promoter (Qu et al., 2008). In the presence of *Ac* transposase, *Ds* element tends to get transposed and integrated randomly across the genome, thus enhancing the expression of the genes at the sites of insertions (Fig. 1.3). Several factors determine the success of activation tagging, such as the activity of *Ac* transposase, the location of the transposon in the genome, type of enhancer elements used and the susceptibility of the target locus. The transposition of the *Ds* element depends on the promoter under which *Ac* transposase synthesized. When *Ac* expressed under a constitutive CaMV35S promoter, the *Ds* element excised early in developmental stages in tobacco, *Arabidopsis*, and rice (Keller et al., 1993, Smith et al., 1996, Greco et al., 2001). Such transposition events occurred at early developmental stages are carried through germ line and results in the same insertion patterns in the progenies of next generation (Kolesnik et al., 2004). The *Ds* element transposed late in barley during the developmental stages when the *Ac* element was expressed under the native promoter and gave rise to independent transpositions (Koprek et al., 2001).

The number of transposition events or transposition frequency of the *Ds* element was very less when *Ac* expressed under a constitutive as well as a native promoter. However, in *Arabidopsis*, when *Ac* synthesized under octopine synthase promoter, the number of transposition events increased drastically (Bancroft et al., 1993). However, detailed analysis by Kolesnik et al., (2004) indicated that the *Ds* element transposed late in rice development when the *Ac* was expressed under a CaMV35S promoter giving rise to several independent single copy *Ds*

transposition events within a family. This varied transposition patterns of the *Ds* element indicates that it is not only promoter-dependent but also species-specific.

Activation-tagged mutant populations have so far been developed only in *japonica* varieties (Kolesnik et al., 2004, Qu et al., 2008, Droc et al., 2013, Yang et al., 2013, Lo et al., 2015). Similar efforts could not be made in any of the *indica* cultivars because of their recalcitrance to genetic manipulation. Table 1.2 lists the genes identified through activation tagging in various plant species.

Table 1.2: Genes identified through activation tagging in various plant species

Gene identified	Origin	Reference	Function
RPL6, RPL23A	*O. sativa*	Moin et al., 2016a	Water-Use Efficiency
Glutamate receptor-like	*O. sativa*	Lu et al., 2014	Drought tolerance
Rac1 (Rac Immunity1)	*O. sativa*	Kim et al., 2012	Resistance against rice blast fungus
Lesion mimic resembling ATPase	*O. sativa*	Fekih et al., 2015	Resistance against blast and blight
Nicotianamine synthase2	*O. sativa*	Lee et al., 2011	Zinc accumulation in seeds
Yellow stripe1-like16	*O. sativa*	Lee et al., 2012	Enhanced iron efficiency
OsAT1	*O. sativa*	Mori et al., 2007	Lesion mimic Spotted leaf mutant
edt1	*A. thaliana*	Yu et al., 2008	Drought tolerance with improved root system
Yucca	*A. thaliana*	Kim et al., 2007	Flavin Monooxygenase synthesis (Auxin)
OsAT1	*O. sativa*	Mori et al., 2007	Lesion mimic phenotype

Ga 2-ox	*O. sativa*	Hsing et al., 2006	Synthesis of gibberellin 2-oxidase
ces101	*A. thaliana*	Yasuo Niwa et al., 2006	Receptor-like kinase
asl1 (asymmetric leaves2 like1)	*A. thaliana*	Junior et al., 2005	Controls proximal-distal patterning in petals
asml2	*A. thaliana*	Masaki et al., 2005	Controls expression of sugar-inducible genes
Ctg	*A. thaliana*	Salaita et al., 2005	Cold temperature germinating mutants
dvl1	*A. thaliana*	Wen et al., 2004	Fruit development
cdr1-d	*A. thaliana*	Xia et al., 2004	Resistance to virulent *Pseudomonas syringae*
etc1 (enhancer of triptychon), cpc1(caprice)	*A. thaliana*	Kirik et al., 2004	Trichome and root hair cell patterning
asl5/lbd12	*A. thaliana*	Nakazawa et al., 2003	Epinastic leaves
ant1	*L. esculentum*	Mathews et al., 2003	Transcriptional regulator of anthocyanins
ga 2-oxidase	*Poplar*	Victor et al., 2003	Synthesis of gibberellins 2-oxidase.
orca3	*Catharanthus roseus*	Van der Fits et al., 2003	Regulates terpenoid indole alkaloid pathway
jaw-D	*A. thaliana*	Palatnik et al., 2003	Plant development
vas (vascular tissue size)	*A. thaliana*	Graaff et al., 2002	Increase in phloem cambial and pericycle cells
pga6 (or wuschel)	*A. thaliana*	Zuo et al., 2002	Role in embryogenesis
Sho	*Petunia hybrida*	Zubko et al., 2002	Cytokinin specific effects
bak1 (bri1 associated receptor kinase1-1 dominant)	*A. thaliana*	Li et al., 2002	Brassinosteroid signaling
Sturdy	*A. thaliana*	Huang et al., 2001	Controls inflorescence and branching

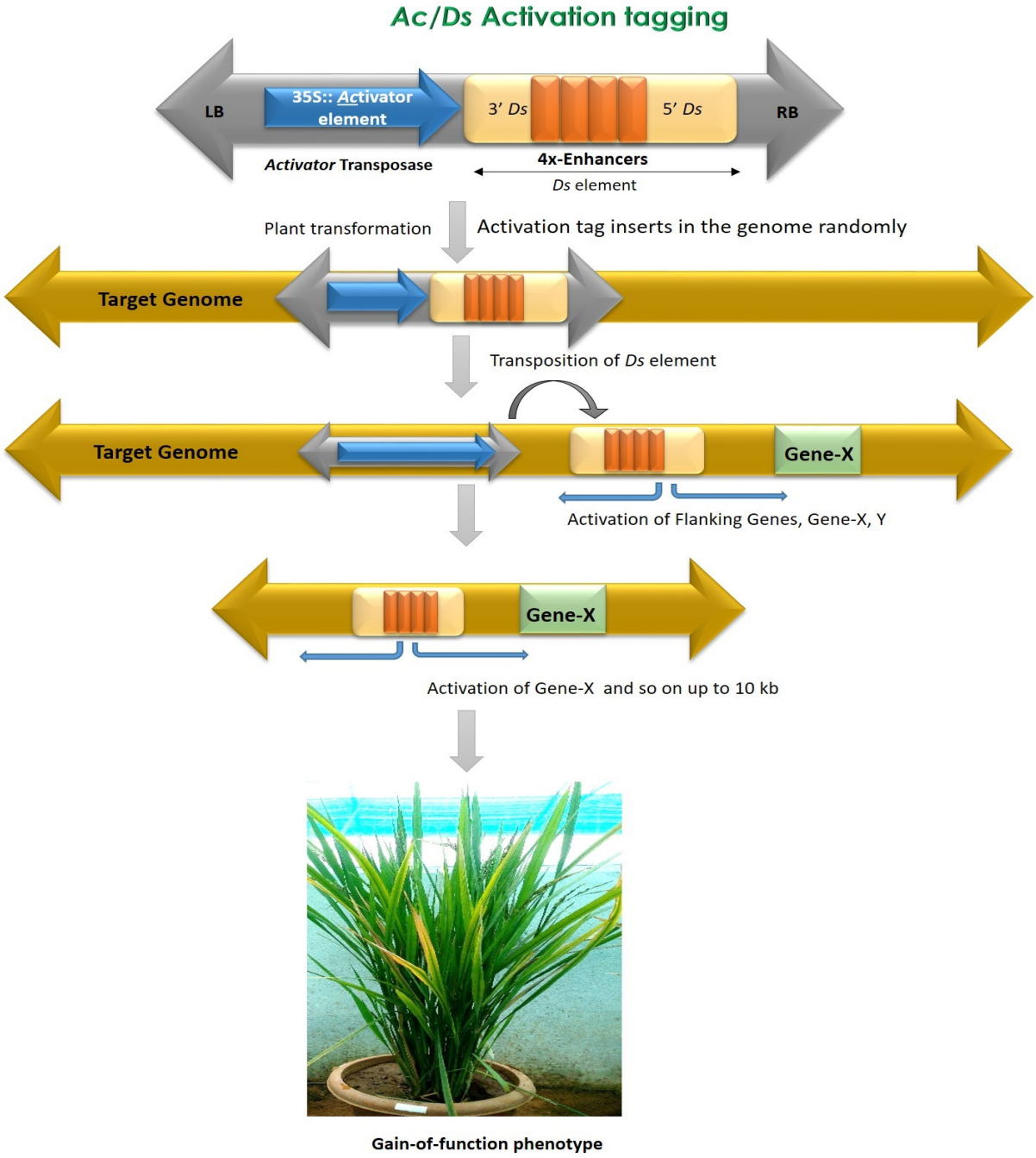
bri1	*A. thaliana*	Li et al., 2001	Receptor for brassinosteriods
lep (leafy petiole)	*A. thaliana*	Van der Graaff et al., 2000	Member of AP2/EREBP transcription factor
esc1D	*A. thaliana*	Weigel et al., 2000	Late flowering mutant
lhy (late elongated hypocotyl)	*A. thaliana*	Schaffer et al., 2001	Photoperiodic control of flowering
pap1-D	*A. thaliana*	Borevitz et al., 2000	Production of anthocyanin pigment
shi (short Internodes)	*A. thaliana*	Fridborg et al., 1999	Suppression of gibberellin response
Cdt1	*Craterostigma plantagineum*	Furini et al., 1997	Drought tolerance
ft (flowering locus)	*A. thaliana*	Kardailsky et al., 1999	Induce flowering
Axi	*A. thaliana*	Hayashi et al., 1992	Auxin independent plant growth

1.2.2.2. Significance of enhancer based activation tagging

Activation tagged populations have an important role in functional genomic studies. Though the sequencing of genomes provides sufficient raw data for sequence annotation, the data does not assume significance unless the functional characterization of the sequence is undertaken. Activation tagging identifies genes and their importance in a single step. The success of activation tagging depends on the level of saturation of the genome with the enhancer elements, thereby obtaining tags for as many coding sequences present in the genome. Once such a population developed, it can be screened for all the possible phenotypes as desired and mined for identifying novel genes for stress amelioration and agronomically important traits. After the first successful result (Hayashi et al., 1992), the activation tagging technique has been exploited in a wide range of plants including the model system, *Arabidopsis* (Weigel et al., 2000), cereal crops, rice, maize and barley and a tree Populus. Till date, there have been no reports of the development of an activation-tagged population in *O. sativa* ssp. *indica* because *indica* cultivars are more recalcitrant to tissue-culture regeneration than japonica varieties.

Gain-of-function mutagenesis approaches uncover the function of genes that remains unidentified by loss-of-function screens such as T-DNA or transposon tagging. It particularly applies to the genes exhibiting redundancy. The advantage of enhancer based activation tagging

over other methods of insertional mutagenesis that they cause endogenous activation of genes on either side of insertion throughout the plant genome. If an enhancer inserts within a gene, this intragenic insertion tends to inactivate the target gene and at the same time activate the neighboring gene, which may lead to a complex phenotype. Hence, intergenic insertions are preferred over intragenic insertions in the activation tagging. About 41.84% of T-DNA, 30.74% of *Ds* and 52% of *dSpm* mutations in mutant rice libraries are intergenic insertions (Wei et al., 2013). Hence, if enhancers introduced into the genome through T-DNA, *Ds* or particularly *dSpm* tags, a large number of genes can be activated through intergenic activation. Also, the number of insertions required to tag each gene with enhancers is less.



Gain-of-function phenotype

Fig. 1.3. Activation tagging using 4x-enhancers are widely used in forward genetics, in which enhancers activate the expression of genes up to as long as 10 kb on either side of insertion in the genome. The stable *Ds* insertion carrying plants can be obtained after transposition of the *Ds* element followed by segregation or carrying lines can also be generated directly by transforming vectors with 4x-enhancers in T-DNA/ *Ds*.

1.2.3. Candidate genes characterized in abiotic-stress tolerance in rice

Although more than 1000 genes have been identified as having a role in response to various abiotic-stresses such as salinity, dehydration, heat and cold, using RT-PCR, microarray, SAGE (Serial Analysis of Gene Expression) and MPPS (Massive Parallel Signature Sequencing) techniques in rice (Kawasaki et al., 2001, Rabbani et al., 2003, Yamaguchi et al., 2004, Yang et al., 2006, Fu et al., 2007, Gorantla et al., 2007, Silveira et al., 2015), about 600 (60%) of them have been characterized in detail by generating independent transgenic plants of each gene for corresponding stress-tolerance.

Proteins that most probably function in abiotic stress tolerance include heat shock proteins (*OsHsp17.0, OsHsp23.7*), group 3 LEA (*OsLEA3-1, OsLEA3-2*) proteins, transcription factors like bHLH (*OsHLHU8*), bZIP (*OsbZIP23/16/46/71)*, AP2/ERF (*OsDREB1A/2A/1B, OsERF1/4a/10a, AP37)*, MYB (*OsMYB2*), NAC (*SNAC1, OsNAC5/6/9/10*), Zinc fingure (*OsZFP182/245/252*), WRKY (*OsWRKY30)*, kinases (calcium dependent kinases, MAP kinases), osmoprotectants (*OsOAT*), water (Hong et al., 2004) and ion channels (Ahmed et al., 2015) and detoxifying enzymes. Overexpression of these endogenous genes in transgenic rice resulted in increased yield under drought and saline conditions. Members of bHLH (Seo et al., 2011), AP2/ERF (Wan et al., 2011), NAC (Jeong et al., 2013), MYB (Yang et al., 2012), bZIP (Tang et al., 2012) have been well characterized with their roles in regulation of plant defense and stress tolerance in rice system. *NAC*, the largest plant transcription factor family, is characterized by a highly conserved DNA binding domain at the N-terminal end.

Overexpression of stress responsive NACs like *NAC1*, (Zhang et al., 2004), *NAC2* (Hu et al 2008), *NAC6* (Nakashima et al 2007), root specific *NAC10* (Jeong et al., 2010), *NAC045* (Zheng et al., 2009) significantly enhanced drought and salt tolerance in transgenic rice. The *Arabidopsis* (HARDY) *HRD* gene coding for an AP2/ERF like transcription factor identified by a gain-of-function approach exhibited drought and salt tolerance. Overexpression of *Arabidopsis* HARDY in transgenic rice improved water-use efficiency by enhancing photosynthetic assimilation and reducing transpiration (Karaba et al., 2007). Also,

overexpression of *DREB* transcription factors in rice has been found to confer resistance to drought and salinity stress (Dubouzet et al., 2003).

Plants respond to the adverse environmental conditions by initiating a series of signaling processes that often involve protein kinases including calcineurin B like protein-interacting protein kinases (CIPKs). Transgenic rice overexpressing *CIPK12* and *CIPK15* showed improved tolerance to drought and salt stress respectively (Xiang et al., 2007). Using the expressed sequence tags generated from a cDNA library constructed from drought stressed leaf tissue of *indica* cultivar N22, nearly 125 stress responsive genes have been identified (Gorantla et al., 2007).

In addition to regulatory proteins like transcription factors and kinases, candidate genes have been characterized encoding Auxin efflux carrier (*OsPIN3t*) (Zhang et al., 2012), *myo*-inositol oxygenase (*OsMIOX*) (Duan et al., 2012), *Ski*-interacting protein (*OsSKIPa*) (Hou et al., 2009), His phosphotransfer protein (*OsAHP*) (Sun et al., 2014) for osmotic stress tolerance and Lipid transfer protein (*OsDIL*) (Guo et al., 2013), Ornithine δ-amino-transferase (*OsOAT*) (You et al., 2012), Trehalose-6-phosphate synthase (*OsTPS1*) (Li et al., 2011), Ring domain containing protein (*OsRDCP1*), β-catotene Hydroxylase (*DSM2*) (Bae et al., 2011), ABA receptor (*OsPYL/RCAR5*) (Kim et al., 2012), Deeper Rooting 1 (*OsDRO1*) (Uga et al., 2013) for drought stress tolerance in rice.

Chapter 2:

Development of Activation-tagged Population in an *indica* rice cultivar BPT-5204, Screening for High Water-Use Efficiency and Flanking Sequence Analysis

2.1. Chemicals

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

2.2. Bacterial strains

All the activation tagging binary vectors used in the study were stored as glycerol stock in *Escherichia coli*, DH5α strain. The *Agrobacterium tumefaciens* strain, EHA105 was used for rice transformation.

2.2.1. Bacterial growth conditions

The DH5α strain *E.coli* was grown at was grown at 37ºC in LB (Luria Broth) or LA (Luria Agar) medium, while *Agrobacterium* strain, EHA 105 was cultured at 28ºC. The LB and LA composition required for bacterial growth was obtained from (Himedia, Mumbai, India). The strains with vectors were maintained at -80ºC in 35-50% glycerol (v/v).

2.2.2. Preparation of *E.coli* competent cells and transformation

A single colony of DH5α strain was picked with a sterile tip, inoculated into 10 ml of LB broth and incubated at 37°C overnight under constant agitation. About 200 µl of this primary culture was further inoculated into 100 ml LB and allowed to grow for 3-5 hrs until the OD600 reaches 0.6. The culture was aliquoted into sterile Oakridge tubes and spun at 5000 rpm for 5 min at 4°C. The pellet was re-suspended in equal volume of ice cold 100 mM $CaCl_2$ and incubated for 10-15 min in ice. This was followed by centrifugation at 5000 rpm for 10 min and the the pellet was again dissolved in 3-5 ml of ice-cold 100 mM $CaCl_2$, 15% (v/v) sterile glycerol. The suspension was distributed into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80°C. For bacterial transformation 1-3 µl of plasmid or 20 µl of ligated product was added to the competent cells, incubated on ice for 10-15 min. The cells were subjected to heat shock at 42°C for 90 sec followed by immediate chilling in ice. About 1 ml of LB was added to the tube and incubated at 37°C for 1 h. The tube was centrifuged at 3000 rpm for 5 min and the pellet was re-suspended in LB medium and spread on the LA plates carrying appropriate antibodies.

2.2.3. Preparation of *Agrobacterium* competent cells and transformation

A single colony of *Agrobacterium* strain, EHA 105 was inoculated into 10 ml of LB broth and grown at 28°C overnight under constant agitation. About 500 µl of the primary culture was

inoculated into 100 ml of LB medium under shaking until the OD600 of the bacterium reaches 0.6. The culture was aliquoted into sterile tubes, centrifuged at 5000 rpm for 5 min. The pellet was re-suspended in equal volumes of ice cold $CaCl_2$ and spun at 5000 rpm for 5 min after an incubation for 10 min in ice. This step was repeated twice. The final *Agrobacterium* pellet was dissolved in 1 ml of ice cold $CaCl_2$ and 15% (v/v) glycerol and stored at -80°C in Eppendorf tubes.

The activation tagging binary vectors pSQ5, pDEB were mobilized into *Agrobacterium tumefaciens* strain EHA105 by freeze-thaw method. About 2-3 µg of plasmid DNA was added to the cells followed by immediate freezing in liquid nitrogen. The cells were then thawed at 37°C for 5-10 min followed by incubation at 28°C for 2-3 h under shaking after addition of 1 ml of LB medium. The cells were centrifuged at 5000 rpm for 10 min and the pellet was re-suspended in 200 µl of LB medium and spread on LA plate containing 50 mg l^{-1} of kanamycin and Rifampicin antibodies for 2-3 days until the growth of colonies were observed. The colonies were further confirmed by PCR based approaches after isolating plasmid.

2.3. Plasmid DNA vectors

All the vectors used in the study are detailed below:

2.3.1. pTZ57R (MBI Fermentas, Germany)

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

2.3.2. pBlueScript II SK (+) – Addgene Vector Database

The pBlueScript II was used as an intermediate vector for cloning *bar* gene before it was cloned in binary vector pCAMBIA2300.

2.3.3. pCAMBIA1300 and pCAMBIA2300 (CAMBIA, Australia)

The plant binary vectors, pCAMBIA1300 and pCAMBIA2300 were used in the study. pCAMBIA1300 has the *hpt*II gene as a plant selection marker whereas, pCAMBIA2300 contains *Kan*R as plant selection marker that induces resistance against antibiotic kanamycin. The *Kan*R was replaced with *bar* gene, a resistant marker for the herbicide phosphinothricin (PPT). The *bar* gene was isolated from pEGAD vector for selection of rice plants.

2.3.4. pRT100 (Addgene Vector Database)

pRT100 vector contains CaMV35 promoter and Poly-A tail. It was used for cloning of the genes identified for high water-use efficiency to release the expression cassette. The expression cassette carrying gene under CaMV35S promoter and Poly-A tail was further cloned in plant binary vector at appropriate cloning sites. The pRT100 contains AmpR for bacterial selection.

2.3.5. Isolation of plasmid DNA by conventional method (Bimboim and Doly, 1979)

The plasmid was isolated using both convention and kit method (Clontech, Takara Biotech, Japan). For conventional isolation, the bacterial culture (*E.coli*) was allowed to grow at 37°C for overnight in a 10 ml LB medium containing appropriate antibodies. The bacterial suspension was taken in Eppendorf tubes and centrifuged at 12,000 rpm for 1min. This step was repeated for 3-4 times. The pellet was dissolved in ice-cold solution containing 50 mM Glucose, 25 mM Tris- HCl pH 8.0, 10 mM EDTA, 100 µg RNase A. The pellet was dissolved by gently mixing with the pipette. To this 200 µl freshly prepared solution-II (200 mM NaOH, 1% (w/v) SDS) was added and incubated for 3-5min. This was followed by the addition of 150 µl of solution-III (3.0 M potassium acetate, pH 4.8) and incubated on ice for 5 min. After incubation, the tubes were spun at 12,000 rpm for 10 min, to the supernatant ice-cold isopropanol was added and incubated in ice for 20-30 min. The plasmid DNA was pelleted down at 12,000 rpm for 10 min and the plasmid DNA was washed with 70% ethanol at 8,000 rpm for 5 min. The pellet was dissolved in nuclease free water and stored at -20°C.

2.4. Restriction endonuclease treatments

Various restriction enzymes were employed in the study for cloning and southern-blot hybridization analysis. For plasmid digestion, 20 µl reaction mixture carrying 10x buffer, template and 5 U of restriction enzyme/µg of DNA was incubated at 37°C for 2-3 h. For digestion of genomic DNA, incubation was carried for 24 h. The double digestion reactions were carried either separately or using compatible buffers in the same reaction mix.

2.5. Ligation

The ligation reaction was carried using T4 DNA ligase (Fermentas, Germany). The 20 µl ligation reaction mix contains 2 µl ligation buffer (10 x), 2U of Ligase and 1:3 to 1:5 ratio of plasmid to insert concentration. The reaction mixture was incubated for 12 to 16 h at 16°C for sticky-end ligation and 22°C for blunt-end ligation.

2.6. Plant materials

Oryza sativa L. sp. *indica* var. Samba Mahsuri (BPT-5204) were employed in the study.

2.6.1. Plant growth condition

The seeds of *Oryza sativa* L. sp. *indica* var. Samba Mahsuri (BPT-5204) maintained in greenhouse conditions were surface sterilized with 70% ethanol for 50-60 sec followed by 4% sodium hypochlorite for 20 min. Seeds were then washed thrice with sterile double-distilled water, blot dried and cultured on solid MS medium at 28 ± 2°C and 16 h light/8 h dark photoperiods.

2.7. Isolation and quantification of nucleic acids

The plant genomic DNA and total RNA was isolated using CTAB and TriZol method, respectively. Both the nucleic acids were quantified using Nano-drop spectrophotometer.

2.7.1. Extraction of plant genomic DNA

Genomic DNA was isolated from transgenic plants using the CTAB method with certain modifications to the standard protocols to obtain high quality and high yield DNA. After grinding of leaf samples (150 mg), 1 ml of CTAB buffer + 20 µl β-mercapto-ethanol were added and the macerated tissue was incubated at 65°C. After one hour, samples were spun at 11,000 rpm for 15 min. The supernatant was transferred to a fresh vial, an equal volume of Phenol: Chloroform: Iso-amyl alcohol (25:24:1) was added and the mixture incubated at 4°C for 5 min followed by centrifugation at 5,000 rpm for 8 min. Incubation at 4°C (instead of the usual incubation at room temperature) resulted in better separation of proteins and nucleic-acids. An equal volume of Chloroform: Iso-amyl alcohol (24:1) was then added to the supernatant, which was further incubated at 4°C for 15 min with gentle shaking followed by centrifugation at 12,000 rpm for 12 min. This step was repeated twice. The clear upper phase was taken and an equal volume of Iso-propanol was added and incubated for 8-12 h at -20°C. Genomic DNA was pelleted down, washed with 70% ethanol, air dried and dissolved in 100 µl nuclease free water. This resulted in the extraction of high-yield (2000 ng µl-1) and good quality genomic DNA, which was free from protein and salt contamination (260/280=1.8, 260/230=2.1).

2.7.2. Extraction of total RNA

Total RNA was isolated from the tissue samples using TriReagent (Takara Bio, UK) following the manufacturer's protocol. The quality of extracted RNA was checked on 1.2% agarose gel prepared in TBE (Tris-borate-EDTA) buffer and quantified using Nanodrop. Total RNA (2 µg) was used to synthesize the first strand cDNA using reverse transcriptase (Takara Bio, UK).

2.7.3. Quantification of DNA and RNA

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

2.8. First strand cDNA synthesis

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

2.9. Activation tagging vectors

2.9.1. Activation tagging vector, pSQ5 (Qu et al., 2008)

The T-DNA of the *Ac*/*Ds* activation tagging vector, pSQ5 (Qu et al., 2008) contains an *Activator (Ac)* transposase and a *Dissociator (Ds)* element carrying tetrameric enhancer sequences derived from the CaMV35S promoter. Since the *Ds* element tends to jump in the presence of an *Ac* transposase, a large number of events can be generated from a small number of primary transposon-carrying transgenic plants by the repeated and controlled selfing. Hence, this vector was transformed into rice through a callus-transformation protocol to develop transgenic plants. However, the excision frequency of the *Ds* element in BPT-5204 was very low and it took three to four generations to obtain plants carrying stable *Ds* element by controlled selfing and plant maintenance. Therefore, the *Ds*-Red from pSQ5 was replaced with *Ds-bar* and cloned in a binary vector pCAMBIA 2300 to select stable *Ds* plants directly and efficiently. The T-DNA of the pDEB vector carries tetrameric repeats of CaMV35S enhancers cloned within the *Ds* element along with the *bar* gene, a resistant marker for the herbicide

phosphinothricin (PPT). Fig.1 represents the T-DNA maps of all the activation tagging vectors used in the study.

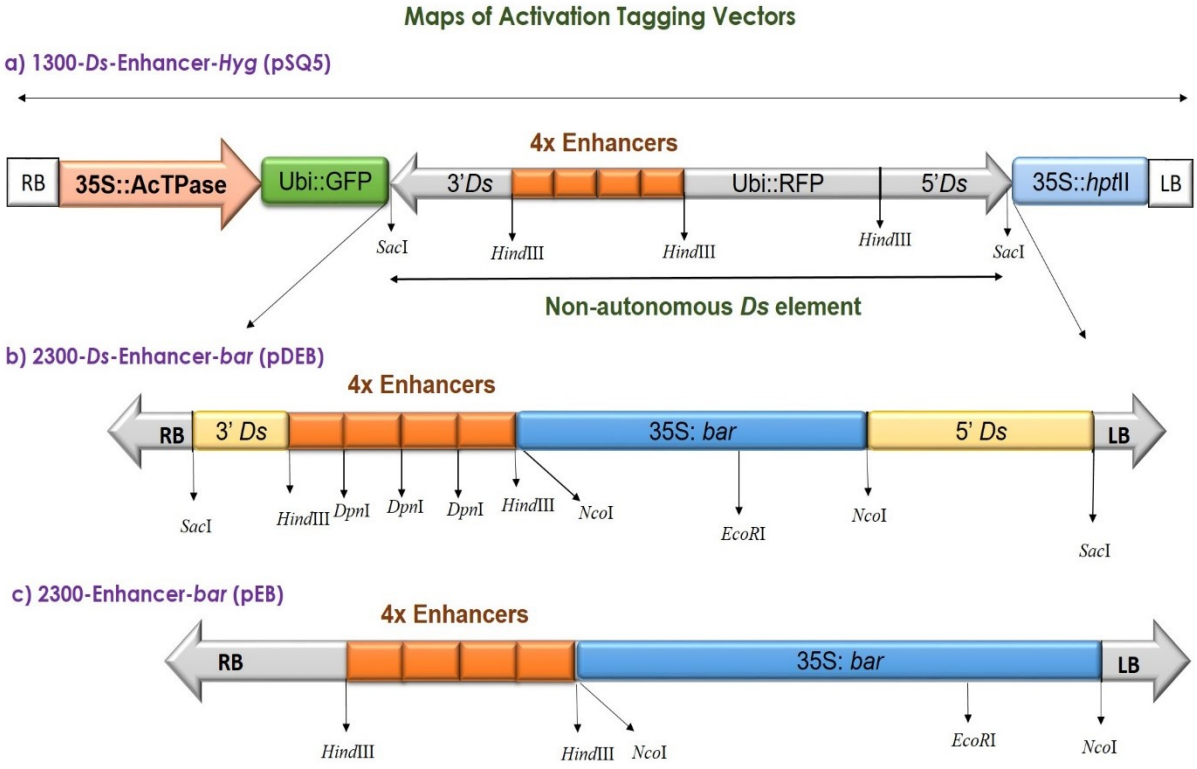


Fig. 2.1 T-DNA of the activation tagging vectors, pSQ5, pDEB and pEB

a) Restriction map of T-DNA of the activation tagging vector pSQ5 (b) pDEB and (c) pEB. RB and LB, Right and Left borders of the T-DNA; 35S: AcTPase, activator transposase; 4X enhancers, tetrameric repeats of CaMV35S promoter; Ubi:GFP, Ubi:RFP, reporter genes for green and red fluorescent proteins respectively; CaMV35S:*hpt*II and CaMV35S:*bar*, are plant selection marker genes for hygromycin and phosphinothricin selection, respectively.

2.9.2. Construction of the activation tagging vector, pDEB

To construct pDEB, a restriction map of the *Ds* element of pSQ5 was developed by digesting it with various restriction enzymes for subsequent cloning (Fig. 2.1). Because the *Ds* element of pSQ5 contains multiple sites for most of the commonly used enzymes, a multi-step strategy was followed in vector development. The plasmid pBlueScript (pBS) was double digested with *Xba*I-*Xho*I restriction enzymes followed by end filling using T4 polymerase to create blunt ends for self-ligation. This step removed the *Hin*dIII restriction site from the multiple cloning site of pBS for subsequent cloning. In the next step, the *Ds* element, released as a *Sac*I fragment from pSQ5, was cloned at the *Sac*I site of pBS. The pBS-*Ds* was then digested with *Hin*dIII

enzyme to release the RFP cassette, which also released the 4X enhancer element. The enhancer was cloned back at the same location in the *Ds* element at the *Hin*dIII site. This step removed the expression cassette of the RFP gene from the *Ds* element. The expression cassette of the *bar* gene was amplified from the pEGAD vector (GenBank accession: AF218816.1) using primers with *Nco*I sites on either ends, and cloned into the pTZ57RT vector. After sequence confirmation, it was subsequently cloned into the pBS-*Ds*-En vector using the *Nco*I site. Finally, the expression cassette of the *bar* gene along with 4x enhancers flanked by *Ds* element was cloned into the binary vector pCAMBIA 2300 using *Sac*I enzyme which was subsequently mobilized into *Agrobacterium tumefaciens* for plant transformation (Fig. 2.2) (Moin et al., 2016a).

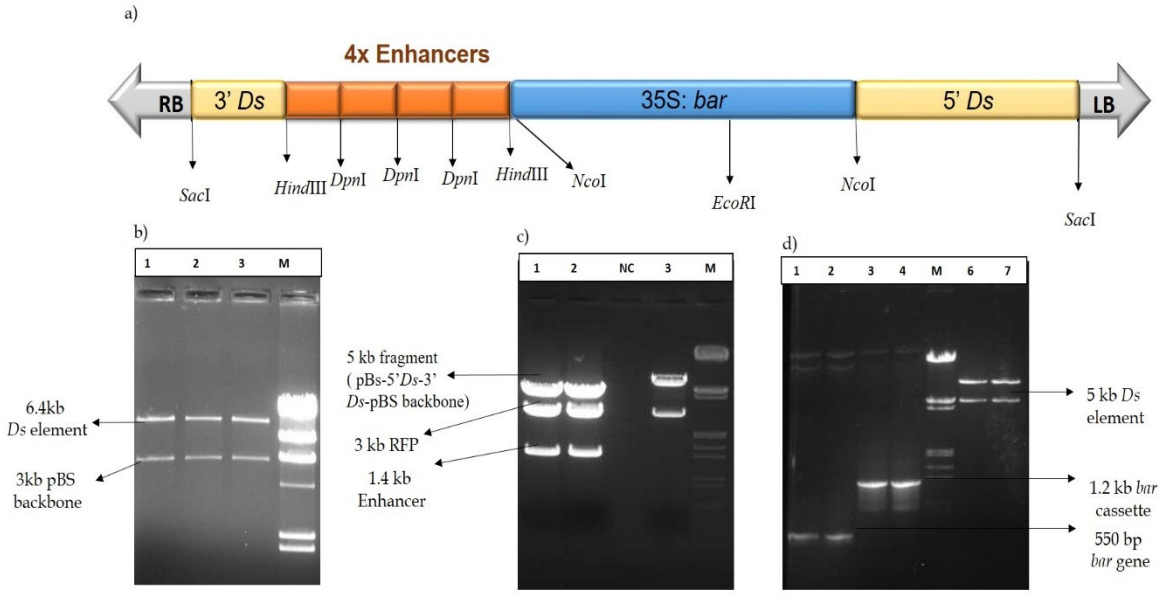


Fig. a) T-DNA of the activation tagging vector pDEB. b) Confirmation of *Ds* insert in pBlueScript by restriction digestion with *Sac*I. c) Restriction digestion of pBlueScript to release 3kb RFP cassette, and 1.4 Kb Enhancer. d) (1-2) PCR confirmation of *bar* gene (550 bp) and (3-4) *bar* expression cassette (1.2 kb) (6-7) *Sac*I digestion to release 5 kb *Ds* element

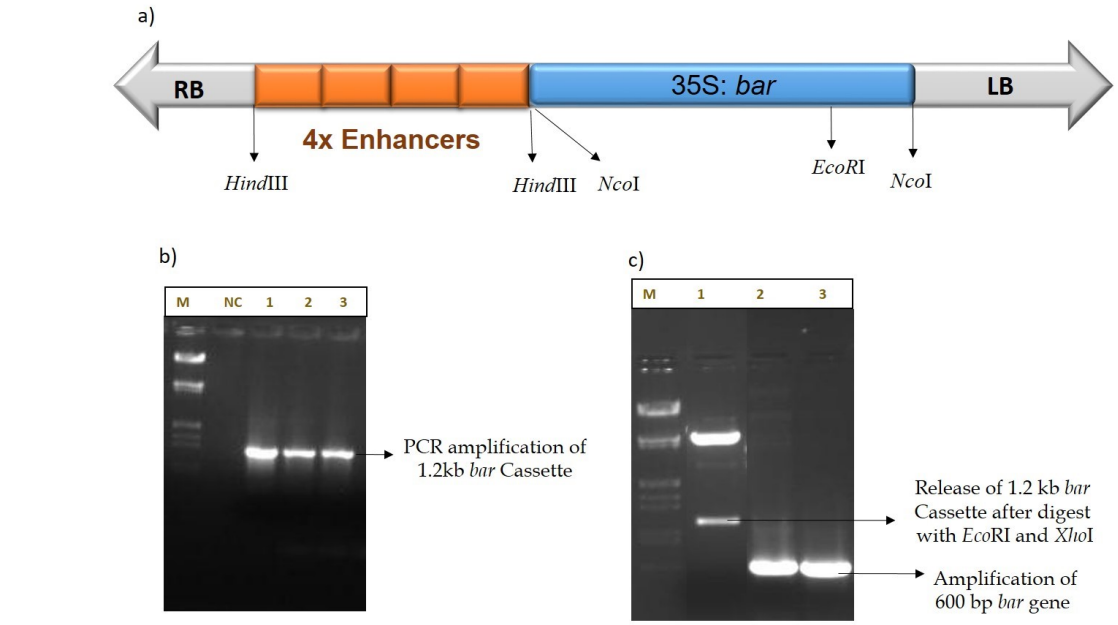
Fig. 2.2. Representation of activation tagging vector, pDEB construction

a) T-DNA of the activation tagging vector pDEB. b) Confirmation of *Ds* insert in pBlueScript SK (+) by restriction digestion with *Sac*I. c) Restriction digestion of pBlueScript to release 3 kb RFP cassette, and 1.4 kb Enhancer. d) (1-2) PCR confirmation of *bar* gene (550 bp) and (3-4) *bar* expression cassette (1.2 kb) (6-7) *Sac*I digestion to release 5 kb *Ds* element.

2.9.3. Construction of the activation tagging vector, pEB

To construct the pEB vector, the binary vector, pCAMBIA2300 was initially double digested with *EcoR*I-*Xho*I to remove the *kan*R resistance gene. The expression cassette of the *bar* gene,

that confers resistance to the herbicide phosphinothricin was cloned in the place of *kan*R gene using the *Eco*RI-*Xho*I sites (Fig. 2.3). The tetrameric CaMV enhancers were released using *Hind*III restriction enzyme from pSQ5 and cloned in the *Hind*III site at multiple-cloning site of pCAMBIA2300. This construct was mobilized into the strain EHA105 for plant transformation.



a) T-DNA of pCAMBIA 2300 carrying 35S:*bar* and caMV35S Enhancers b) PCR Confirmations of the vector with *bar* cassette c) Restriction digestion to release *bar* cassette

Fig. 2.3. Construction of activation tagging vector, pEB

a) T-DNA of pCAMBIA2300 carrying 35S:*bar* and caMV35S Enhancers b) PCR Confirmations of the vector with expression cassette of *bar* gene c) Restriction digestion to release *bar* cassette

2.10. Transformation of Rice

The transgenic rice plants were produced using two approaches such as callus-mediated and *in planta* transformations.

2.10.1. Callus-mediated transformation

The mature BPT-5204 seeds of rice were surface sterilized using 75% ethanol for one min and 4% Hypo twice for 10 min followed by five washes with sterile double distilled water. The seeds were cultured on callus induction medium (solid MS with 2.5 mg l^{-1} 2, 4 dichlorophenoxyacetic acid; 300 mg l^{-1} kinetin; pH 5.6) at 26°C. After emergence of the callus

within 3-4 d, it was sub-cultured further onto the same medium. The *Agrobacterium* culture carrying the binary vector pSQ5 was grown overnight at 28°C in 100 mL Luria Bertani (LB) medium, the culture was centrifuged at 5000 rpm for 5 min and the pellet was re-suspended in an equal volume of *Agrobacterium* suspension (MS basal salts supplemented with 30 g l^{-1} sucrose, 100 µM Acetosyringone, pH 5.4). Two weeks after callus induction, they were suspended in suspension solution at 28°C for 15-20 min in a rotary shaker. The infected calli were blot dried and incubated in co-cultivation medium (MS salts containing 30 g l^{-1} sucrose, 1.0 g l^{-1} proline, 100 µM Acetosyringone and 7 g l^{-1} agar, pH 5.4) in dark for three days at 28°C seeds. The callus tissues were washed thrice each with carbenicillin and cefotaxime (250 mg l^{-1}), blot dried and shifted to selection medium carrying MS salts with 2.5 mg l^{-1} 2, 4 D and 50 mg l^{-1} Hygromycin.

After four weeks of selection, the actively dividing calli were shifted to shoot-induction medium carrying MS salts with 3 mg l^{-1} Benzyl amino purine and 1.5 mg l^{-1} Naphthalene acetic acid, pH 5.6 at 16/8 h light/dark photoperiod. Callus starts to regenerate into shoot after 2-3 weeks of transfer in light (Fig. 2.4). The regenerating tissue was sub-cultured until the development of plantlets which were transferred to the green house.

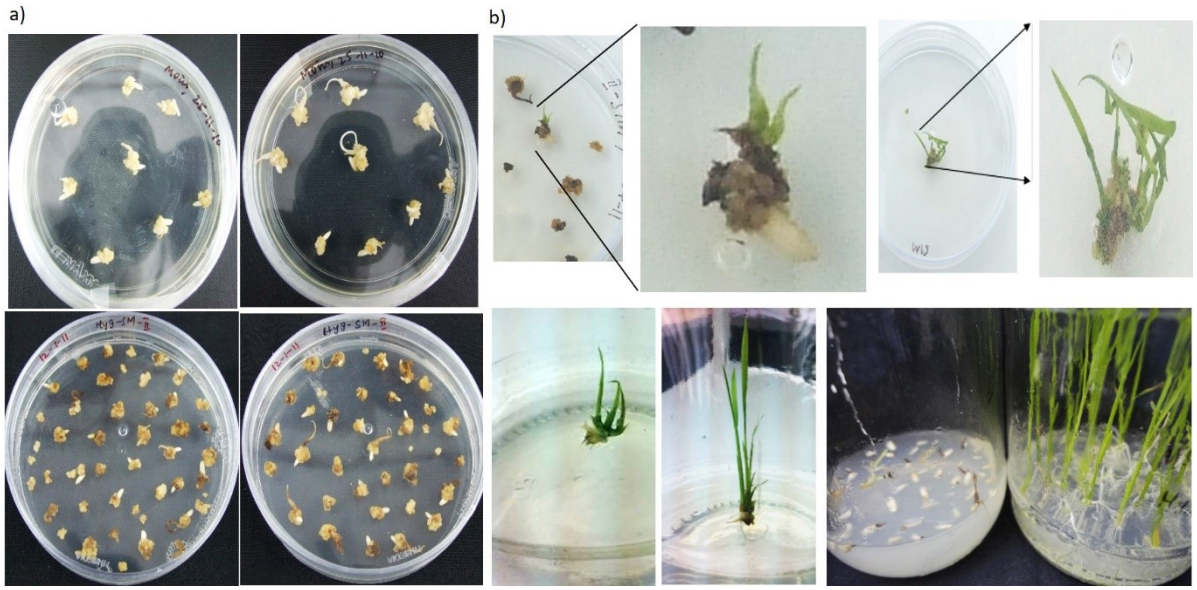


Fig. 2.4. Different stages involved in callus-mediated transformation and shoot regeneration

a) Dehusked rice seeds were cultured on callus-induction medium (containing MS salts along with 2, 4 D and kinetin) for two weeks. After *Agrobacterium* mediated transformation, calli were selected on antibiotic (Hygromycin 50 mg l^{-1}) medium. Actively dividing calli were

transferred to shoot-induction medium (MS salts containing BAP and NAA) until the emergence of shoots in light.

2.10.2. *In planta* transformation procedure of rice

Although the callus-mediated transformation was successful, a relatively rapid method with high transformation efficiency was required for generating a larger population of transgenic plants. This was achieved using a novel method using vacuum infiltration coupled with sudden release of vacuum. Mature seeds of Samba Mahsuri, a widely cultivated *indica* rice cultivar were used in this study. After surface sterilization, the dehulled seeds were incubated in water for 16 h at 28°C in a rotary shaker (100 rpm) to soften the embryos. This is in contrast to the use of 48 h old-husked rice seeds (Supartana et al., 2005, Lin et al., 2009); our observations showed the emergence of both radicle and hypocotyl during this stage and transformation of the explant with *Agrobacterium* after radicle emergence resulted in reduced transformation efficiency.

Dehulled seeds were surface sterilized with 70% Ethanol for 1 min, then twice for 10 min with 4% sodium hypochlorite containing a drop of Tween-20, followed by five washes with sterile double distilled water. The *Agrobacterium* culture carrying the binary vector pDEB was grown overnight at 28°C in 100 mL Luria Bertani (LB) medium, the culture was centrifuged at 5000 rpm for 5 min and the pellet was re-suspended in an equal volume of *Agrobacterium* suspension solution (MS basal salts supplemented with 60 g l^{-1} sucrose, 10 g l^{-1} glucose, 0.6 g l^{-1} proline, 200 µM Acetosyringone, pH 5.4). A fine sterile needle was dipped in the *Agrobacterium* suspension and used to gently pierce the basal part of the embryo which would later produce hypocotyl and subsequently cotyledons.

After infection, the seeds in the *Agrobacterium* suspension were kept in a diaphragm jar, using an air-out vacuum system; air was evacuated and a vacuum of about 15 mmHg was maintained for 20 min, after which the vacuum was released as quickly as possible by sudden and swift removal of the air outflow valve. This forces the *Agrobacterium* cells to enter into the explant by replacing intercellular air spaces (Fig. 2.5).

After three days of co-cultivation (MS salts containing 30 g l^{-1} sucrose, 1.0 g l^{-1} proline, 150 µM Acetosyringone and 7 g l^{-1} agar, pH 5.4) in dark at 28°C seeds were washed thrice with water and allowed to recover on blotting paper moistened with half strength MS solution

containing carbenicillin and cefotaxime (250 mg l^{-1} each) until the growth of seedlings was observed, which were then transferred to pots and grown under green-house conditions (Moin et al., 2016a).

2.10.3. Media composition used during the transformation process

The *Agrobacterium* suspension solution used MS basal salts supplemented with 60 g l^{-1} sucrose, 10 g l^{-1} glucose, 0.6 g l^{-1} proline, 200 µM Acetosyringone (always fresh stock), pH 5.4. The Co-cultivation medium used MS salts containing 30 g l^{-1} sucrose, 1.0 g l^{-1} proline, 150 µM Acetosyringone and7 g l^{-1} agar, pH 5.4.

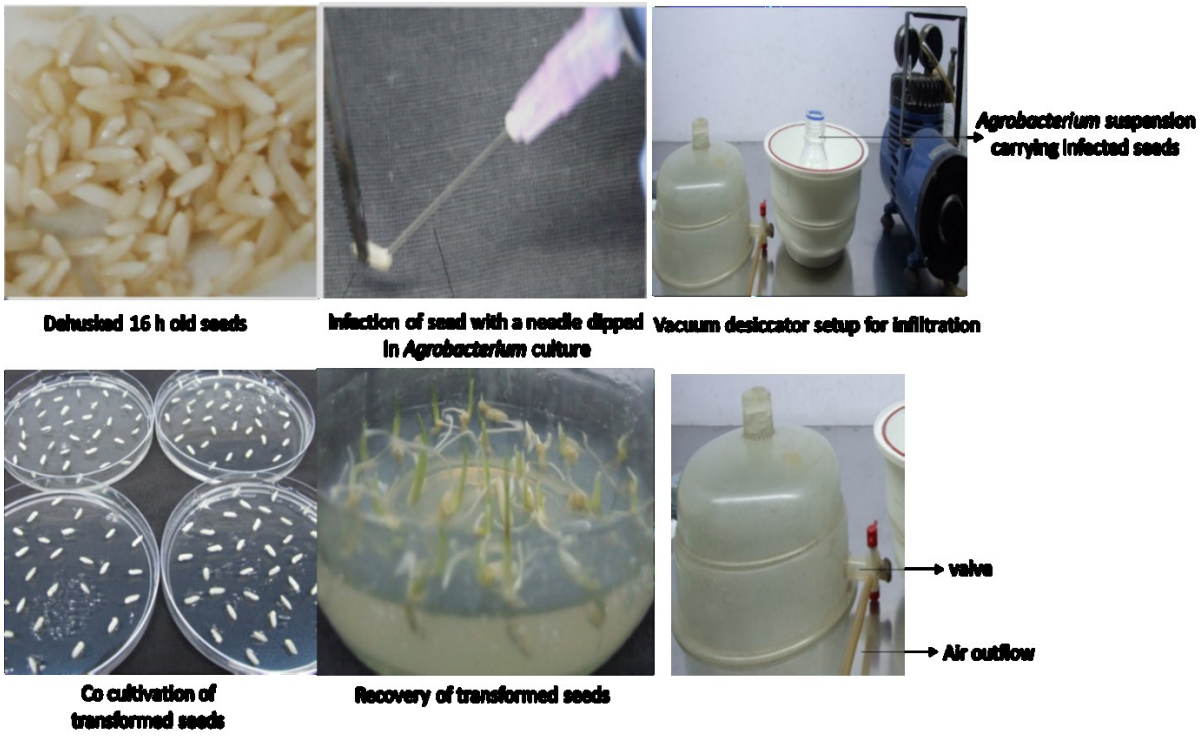


Fig. 2.5. Schematic representation of various steps involved in *in planta* transformation protocol of rice.

The 16 h old rice seeds were pierced with a sterile needle dipped in *Agrobacterium* suspension carrying activation tagging vectors (pDEB and pEB). After vacuum infiltration coupled with sudden release of vacuum infected seeds were cultured on co-cultivation medium for three days after which they were allowed to grow on MS medium until the growth of plantlets and transferred to green house for further characterization.

2.11. Selection of T_1 generation transgenic plants after *in planta* transformation

Seeds obtained from the *Agrobacterium*-treated plants were screened on 10 mg l^{-1} PPT selection medium and those seedlings that germinated on the selection medium were transferred to pots in the greenhouse and were further confirmed by PCR amplification of appropriate elements present in the T-DNA. The transgenic nature of the plants and copy number of T-DNA integration were finally confirmed through Southern-blot hybridization.

2.11.1 Polymerase Chain Reaction

PCR was used to amplify the target genes for cloning and also for the confirmation of the transgenic plants. Primers specific for all the DNA elements present in the T-DNA were designed using OligoCalc and Primer3 tools. The PCR components include 20-25 pmol/µl forward and reverse primes, 100-150 ng of genomic DNA or appropriate template, 200 µM of dNTP mix, 1.5 mM $MgCl_2$ and 2.5 U of recombinant Taq DNA polymerase (Invitrogen, USA) to the final volume of 25 µl reaction. PCR was performed in Eppendorf Master cycler Gradient (Germany) and/or Bio-Rad Laboratories Inc., (USA). The reaction conditions for PCR include an initial denaturation at 94°C for 3-5 min followed by 35 cycles of 94°C for 30-60 sec, an appropriate annealing temperature for 50 sec, extension at 72°C for 1 min (depending up on the size of the product). This was followed by a final extension of 72°C for 10 min. The PCR products were observed on 1% agarose gel under UV illuminator. The primers used in various analysis were provided in Table 2.1.

Table 2.1 Primers used in various analysis.

Primer name	Sequence	Purpose
bar gene FP	5’ GAA TTC ATG AGC CCA GAA CGA CGC 3’	Screening of plants
bar gene RP	5’ CCC GTC ACC GAG ATC TGA AAG CTT 3’	Screening of plants
bar cassette FP	5’ GAA TTC GAT AAG CTA GAG ATC CGT CA 3’	Cloning of vector
bar cassette RP	5’ AAG CTT AGA GAT CTA GTA ACA TAG ATG AC 3’	Cloning of vector
CaMV35S FP	5’AAG CTT GAT CCC CAA CAT GGT GGA GC 3’	Screening of plants

CaMV35S RP	5'TTC GGA TCT AGA TAT CAC ATC AAT C	Screening of plants
*hpt*II FP	5' TATTTCTTTGCCCTCGGACGAGTGCTG 3'	Screening
*hpt*II RP	5' ATGAAAAAGCCTGAACTCACCGCG 3'	Screening
Degenerate primer 1	5'-NGACGA(G/C)(A/T)GANA(A/T)GAA-3'	TAIL-PCR
Degenerate primer 2	5'-NGACGA(G/C)(A/T)GANA(A/T)GAC-3'	TAIL-PCR
DS.SP1	5'CTCACAGCACTTAGCAGTACAGCACGTCAG C 3'	Nested primer in Primary TAIL-PCR
DS.SP2	5' GTGCGCGTGGGCATGGATGTGGC 3'	Nested primer in Secondary TAIL-PCR
DS.SP3	5' ATA GTT TAG TTA AAG GTC AGT TGT GTC 3'	Nested primer in Tertiary TAIL-PCR

2.11.2. Southern-blot hybridization analysis of transgenic plants

Southern hybridization was performed to establish the integration pattern and copy number of the T-DNA inserted in the genome of T_1 plants using a DIG-DNA labelling and detection kit (Roche Life Science, Germany). Genomic DNA (15-20 µg) isolated from leaves was completely digested overnight using a restriction enzyme, *Kpn*I. The fully digested DNA fragments were electrophoresed on 0.8% agarose gel at 20 V for 14-16 h. The fragments were alkali denatured and transferred on to Hybond N^+ nylon membrane (GE Healthcare Life Sciences), followed by UV cross linking (120 kJ/ cm^2). The PCR amplified product of the *bar* gene was labelled with DIG-dUTP and used as a probe. After hybridization and stringency washes, binding of the probe was detected according to the manufacturer's protocol.

2.12. Growth of the transgenic plants under limited water conditions and physiological characterization

Transgenic plants carrying stable enhancers along with wild type (WT) were grown in pots under greenhouse conditions (temperature 32±2°C, relative humidity 55±5%) providing ample water (up to 3 cm overlay above the soil level that is required for normal growth of rice crop) for only first four weeks after transfer to soil from growth room conditions. After the fourth week, overlay water was withdrawn from the pots and watering was subsequently restricted to a level just to maintain barely moist conditions in the soil (field capacity). This condition was maintained till maturity. Two types of WT plants were maintained; one grown along with transgenic plants under limited water supply (field capacity) and the other grown with ample water as required under normal conditions. A total of 570 out of 1070 stable Ds^+ plants obtained from the *Ac*/*Ds* population and about 2500 from 3900 T_2 generation pDEB plants were screened for their ability to grow under limited water availability with sustained or improved productivity.

2.12.1. Chlorophyll Fluorescence

Chlorophyll fluorescence, which is a measure of activity of photosystem II (PSII) and also an indicator of the response of a plant to environmental stresses, was used to assess the overall photosynthetic performance of a plant (Murchie and Lawson 2013). Although chlorophyll (Chl) fluorescence accounts for only 1-2% of the total light absorbed by Chl, it gives an insight into the use of excitation energy by PSII. In our study, the fluorescence of chlorophyll *a* was monitored using a portable pulse-amplitude modulated photosynthesis yield analyser (MINI-PAM) essentially according to the manufacturer's protocol (Heinz Walz, Germany). It works on the principle that when leaves that have been adapted to a dark period of 30 min are exposed to a beam of low-intensity light (0.1 μmol m^{-2} s^{-1}), chlorophyll present in the reaction centres of PSII gets excited to a minimal level (F_o), but the electron transport system is not induced because of too low light intensity. Application of a saturating pulse (8000 μmol m^{-2} s^{-1}) results in the formation of a maximum possible yield of fluorescence (F_m). The difference between F_m and F_0 (F_m-F_o) gives the variability in fluorescence (F_v) and the effective quantum yield of photosystem II was calculated as F_v/F_m (Murchie and Lawson 2013). The quantum efficiency of unstressed plants grown under normal conditions had been in the range of 0.83-0.84 indicating that 16-17% of the radiation is not absorbed by the photosystem. While low F_v/F_m values indicates that plants are experiencing stress, higher values represents high quantum yield (Batra et al., 2014).

Chlorophyll fluorescence of the activation-tagged mutant populations was measured along with two types of wild types of the same age. Two readings were recorded in triplicates, each at four weeks interval after withdrawing water and the mean of F_v/F_m of two observations were plotted as a histogram.

2.12.2. Carbon isotope analysis ($\Delta^{13}C$ measurements) for water-use efficiency

Plants discriminate between the two isotopes of carbon (^{13}C and ^{12}C) during photosynthesis through stomatal diffusion and carboxylation by RuBisCo. During limited water supply, stomatal aperture tends to become reduced causing a decrease in the intercellular CO_2 concentration (Ci). The discrimination between the isotopes by RuBisCo is high when Ci is high, which decreases with a decrease in Ci. Thus $\Delta^{13}C$ value, which is the relative ratio of $^{12}C/^{13}C$, expressed relative to the PDB standard, of a plant tissue reflects the capacity of a plant for gaseous exchange through stomata, integral Ci and overall WUE of a plant (Martin et al., 1987, Bassette, 2013). To determine the carbon isotope composition and WUE, 500 mg of mature leaf samples were collected just before flowering from WT and five selected mutants grown under water deficit conditions and having high quantum efficiency. Samples were dried at 65°C for 3 d, finely powdered and carbon isotope ratios were analyzed with an Isotope Ratio Mass Spectrometer (IRMS).

2.13. Isolation of tag-end sequences of selected activation-tagged transgenic plants

Those mutants that had high quantum efficiency under limited water conditions with sustained productivity were selected for flanking sequence analysis. Thermal asymmetric interlaced PCR (Liu et al., 1995) was performed by using a degenerate primer and three nested primers specific for the *Ds* element for the identification of the location of activation-tag in the genome and to analyze the flanking sequences. Three nested primers (NP) were designed specific to the 5′ end of the *Ds* fragment, NP1 was 1 kb upstream, NP2 was 500 bp and NP3 was 100 bp upstream relative to the 5′ end of the *Ds* element. In the pDEB vector, the *Ds* element was flanked by T-DNA border sequences. The right border sequence was used as a third nested primer, while the first and second nested primers remained the same for flanking sequence analysis of pDEB plants. Genomic DNA isolated from the transgenic plants was used as a template in the first round TAIL-PCR, while the products of subsequent reactions were diluted 50-100-fold according to the observation on the gel and 1 µl each of the diluted samples was used for the second and third reactions. After the third round of PCR reaction, the specific and target amplicons were purified, cloned into the pTZ57R/T cloning vector and sequenced. The

sequences were submitted in a BLAST search against Rice Genome Annotation Project Database (RGAP-DB) to find the location of inserts in the genome. The detailed protocol of TAIL-PCR is represented as Table 2.2.

2.13.1. Important steps involved in TAIL-PCR

- Any of the two degenerate primers provided in the Table 2.1 can be used. Run about 10 µl of each reaction product on the gel to check the band pattern.
- First TAIL-PCR product normally gives a series of multiple bands. If the multiple bands persist in second/third reactions, the concentration of degenerate primers must be decreased.
- When the results of the second or third round is smear on Gel, the DNA template in the first round must be lowered or the reaction result of the first round PCR is diluted, or the annealing temperature in the second and third round is increased.
- If the intensity of bands is weak in the third PCR, increase the number of cycles accordingly or the template by 1-2-fold based on the results on gel.
- Third PCR product gives more specific and more intense band (Only one band in most of the cases). If multiple bands appear in the third reaction also (more than 3-4), dilute the third reaction product to 50-100 µl, and repeat one more reaction.
- Third PCR template can be used to perform another or fourth PCR to further increase the band specificity and intensity. Purify the desired product, it can either be cloned in cloning vector or sequenced directly using same primers and conditions.
- If the result is nothing, no obvious bands even no smear, and the situation is not improved through the adjustment of the template, the temperature and the cycles, it may be need to design the new nested primers.

Table 2.2. Various steps involved in TAIL-PCR

PCR type	Cycles	PCR reaction protocol	Important points
First PCR	1	95°C 3 min	In the first round PCR, nested/specific primer SP1: AD primer=1:4, every primer concentration is 10 µmol. In the 20 µl reaction, SP add 0.4 µl, AD add 2 µl.
	3	94°C 30s; 60°C 30s; 72°C 2 min (Annealing temp is as per nested primer)	
	2	94°C 30s; 25°C 2 min; 0.2 °C/sec;72°C 2 min	
	5-10	94°C 30s; 60°C 30s; 72°C 2 min 94°C 30s; 60°C 30s; 72°C 2 min	Ramping from 0.2°C from 25°C to 72°C is the most important. The 60°C is

		94°C 30s; **35°C** 30s; 72°C 2 min	according to the annealing temperature of nested primer. Make up to 100 µl with water to primary TAIL product and use 1 µl for the second round.
	1	72°C 10 min.	
Second PCR	1	95°C, 3 min 94°C 30s; 35°C 30s; 72°C 2 min 94°C 30s; 35°C 30s; 72°C 2 min	In the second round PCR, SP2 and AD add 1 µl. According to the results on Gel, Dilute PCR reaction with ddH_2O about 100-200 fold then use for the third round PCR.
	30	94°C 30s; 60°C 30s; 72°C 2 min	
	1	72°C 5 min.	
Third PCR	1	95°C, 3 min	The third round is the normal PCR, SP3 and AD add 1µl.
	15-20	94°C 30s; 60°C 30s; 72°C 2 min	
	1	72°C 10 min.	

2.14. Semiquantitative and Quantitative-PCR

To examine which of the tagged genes were activated by the enhancers and also the level of activation, semiquantitative and quantitative-PCR were performed on five selected transgenic plants; DEB.42, En.64, DEB.86, DEB.3 and En.16. The transcript levels of eighteen genes that were situated in a 20 kb stretch near the site of T-DNA integration in these five plants were analyzed with respect to the WT grown under the conditions of limited water availability.

RNA was isolated from the leaves of two month old mutant and WT plants to study the expression of tagged genes and also from shoot and root tissues of 7 d old WT seedlings subjected to abiotic treatments to determine the differential expression of RPL6 and RPL23 genes. The cDNA synthesized from 2 µg of RNA was diluted 7 times and 2 µl of it was used for Q-PCR experiments. Each Q-PCR reaction was repeated as three biological replicates along with *actin,* which served as an internal reference gene. The reaction conditions for

semiquantitative-PCR included an initial denaturation of 94°C for 3 min followed by 25-28 cycles of amplification (94°C, 30 sec; annealing temperature of 55-58°C, 30 sec; extension at 72°C for 30 sec) with a final extension at 72°C for 10 min. The relative transcript levels were calculated according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) using rice *Actin1* as a reference gene to normalize the gene expression.

2.15. Stress treatments

To check whether the patterns of expression of the two RP genes (RPL6 and RPL23A) tagged by CaMV35S enhancers under various abiotic conditions, different hormonal and stress treatments were applied. The 7 d old WT seedlings were grown at 4°C and 42°C for cold and heat treatments and submerged in polyethylene glycol (PEG) (10%) and sodium chloride (NaCl) (250 mM) for dehydration and salt treatments, respectively. For hormone treatments, solutions of Abscisic acid (ABA) (100 µM), salicylic acid (SA) (3 mM) and methyl jasmonate (MeJa) (100 µM) were used. After treatments shoot and root samples were collected separately at 5 min, 3 h, 6 h, 12 h, 24 h and 60 h intervals (Moin et al., 2016a). Treatment with water was served as a corresponding control. Quantitative analysis of gene expression in various treatments was performed using Quantitative-PCR as described earlier.

2.16. Transcript analysis of other members of 60S ribosomal subunit genes

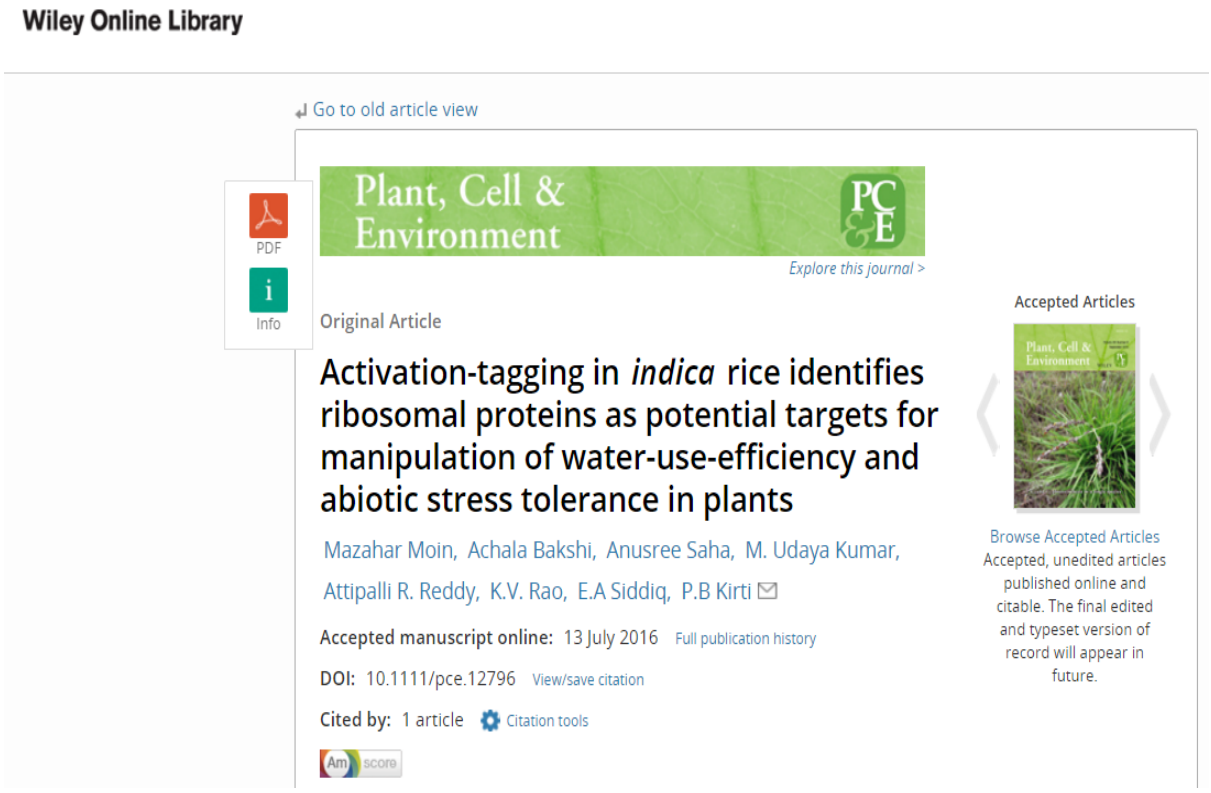
In addition to RPL6 and RPL23A, the induced expression pattern of 34 other RPL genes was also studied. In plants, RPs exist as multigene families with an average copy number of 2-3 for each gene (Barakat et al., 2001). According to recent release 7 of TIGR, the proteins of the large subunit of ribosomes in rice are encoded by at least 34 candidate genes. Several orthologues genes encode the same RP gene taking the total to 123 genes. Since the functions of the orthologues genes are conserved, we selected 34 candidate genes each representing one orthologues group and studied their expression under three different abiotic conditions. The stress treatments were given by dipping roots of 7 d old seedlings grown in MS medium in solutions of PEG-8000 (10%), ABA (100 µM) and NaCl (250 mM), after which shoot and root samples were collected separately in two biological replicates at 5 min, 3 h, 6 h, 12 h, 24 h and 60 h time intervals (Moin et al., 2016a). Seedlings dipped in double-distilled water were served as a corresponding control. The Q-PCR data of 34 RPL genes has been represented through heat maps which were generated by incorporating the mean values of fold change obtained from biological duplicates in GENE-E program.

2.17. Analysis of putative promoter sequences of RPL6 and RPL23A

To identify the presence of *cis*-regulatory elements that are stress specific/responsive in the promoter regions of RPL6 and RPL23A genes, which were activated by CaMV35S enhancers in high yielding activation-tagged mutants, we analyzed the upstream sequences of these two genes. A maximum of 1.1 kb sequence 5′ upstream to each gene was analyzed using Plant Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Chapter 3:

Identification of Candidate Genes Responsible for High Water-Use Efficiency



3. 1. Generation of *Ac/Ds* population and isolation of stable *Ds* plants

The transgenic plants carrying *Ac*/*Ds* elements developed through callus-mediated transformation were screened by antibiotic selection by germinating the seeds on Hygromycin medium (50 mg l^{-1}) and confirmed by PCR amplification of genes present in the T-DNA (Fig. 3.1). Since the gene for selection marker *hpt*II is present outside the *Ds* element, plants that are resistant to Hygromycin carry either the *Ac* element alone (Ac^+/Ds^-) or both *Ac* and *Ds* elements (Ac^+/Ds^+), which were then confirmed by PCR. Seeds that did not germinate on selection medium were either null (Ac^-/Ds^-) or stable *Ds* element carrying plants (Ac^-/Ds^+).

To isolate plants with Ds^+ element alone from the seed obtained from the transgenic plants carrying *Ac*/*Ds* elements, they were cultured on Hygromycin selection medium and allowed to germinate for four days. After the fourth day, those seeds that did not germinate or started to bleach were transferred to selection free medium (medium without Hygromycin) to recover those plants, which were either null, stable Ds^+ plants or Hygromycin low expression lines or escapes (Ac^+/Ds^+), which were later confirmed by duplex PCR for *hpt*II and RFP genes (Fig. 3.2).

About 150 primary transgenic plants were obtained from callus regeneration experiments, of which 50 were randomly selected to produce 8,000 T_1 seeds, which were germinated to isolate stable Ds^+ plants and plants carrying both elements, the latter were used to identify transpositions in subsequent generations. Transposition events, which were tracked through antibiotic selection and PCR amplification of DNA elements located within and outside the *Ds* element were not identified in T_1 and T_2 generations but were obtained from T_3 generation onwards (Fig. 3.3). The frequency of obtaining Ds^+ from *Ac/Ds* population was 6%, 15% and 21% from 2460, 3000 and 2480 seeds screened in T_3, T_4 and T_5 generations, respectively. A total of 1030 *Ds* stable plants were recovered from 7940 *Ac/Ds* plants in three generations. However, selection of a suitable starter line is a key factor as in one particular line the frequency of obtaining stable lines was as high as 45%. The frequency of obtaining Ds^+ plants was calculated as (Moin et al., 2016a):

RFP^+ and *hpt*II^- PCR plants obtained from *hpt*II^- seedlings /Number of seeds cultured on *hpt*II medium X 100

Because of the low frequency of obtaining stable *Ds* lines, plants carrying two component transposable elements (Ac^{+}/Ds^{+}) need to be thoroughly screened up to T_4 to T_5 generations by repeated selfing and plant maintenance, which is a difficult task and very time-consuming.

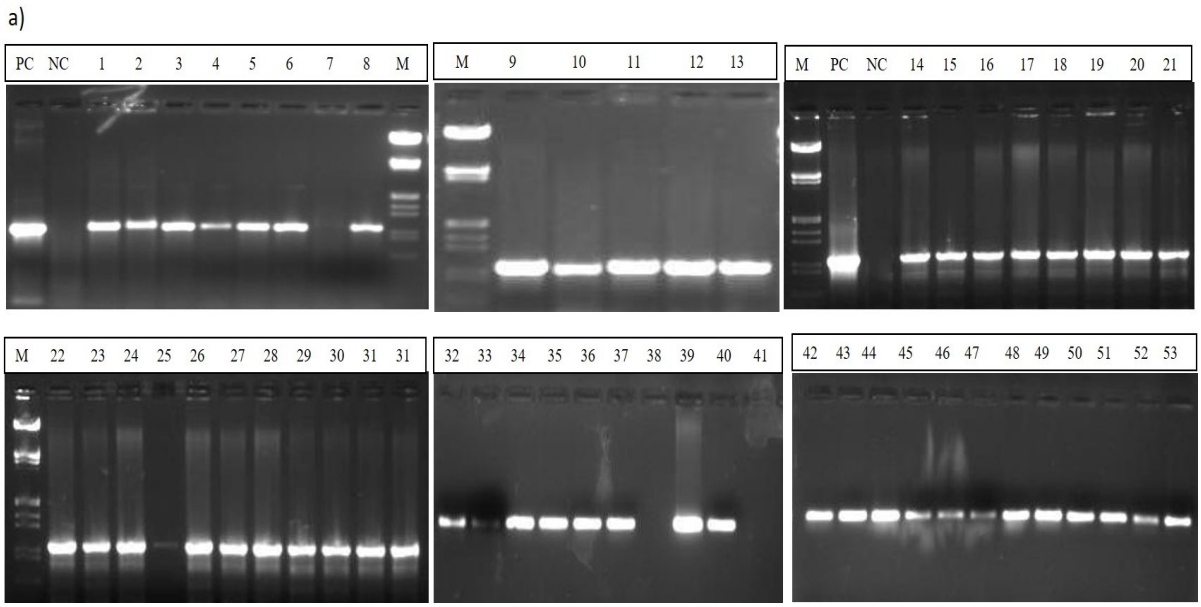


Fig. 3.1: PCR screening of transgenic plants generated through callus mediated transformation

PCR analysis of selected transgenic plants to check the presence of T-DNA using *hpt*II specific amplification. 1-53, genomic DNA of transgenic plants; M, λ-*Eco*RI-*Hin*dIII DNA marker; PC, positive control; NC, negative control.

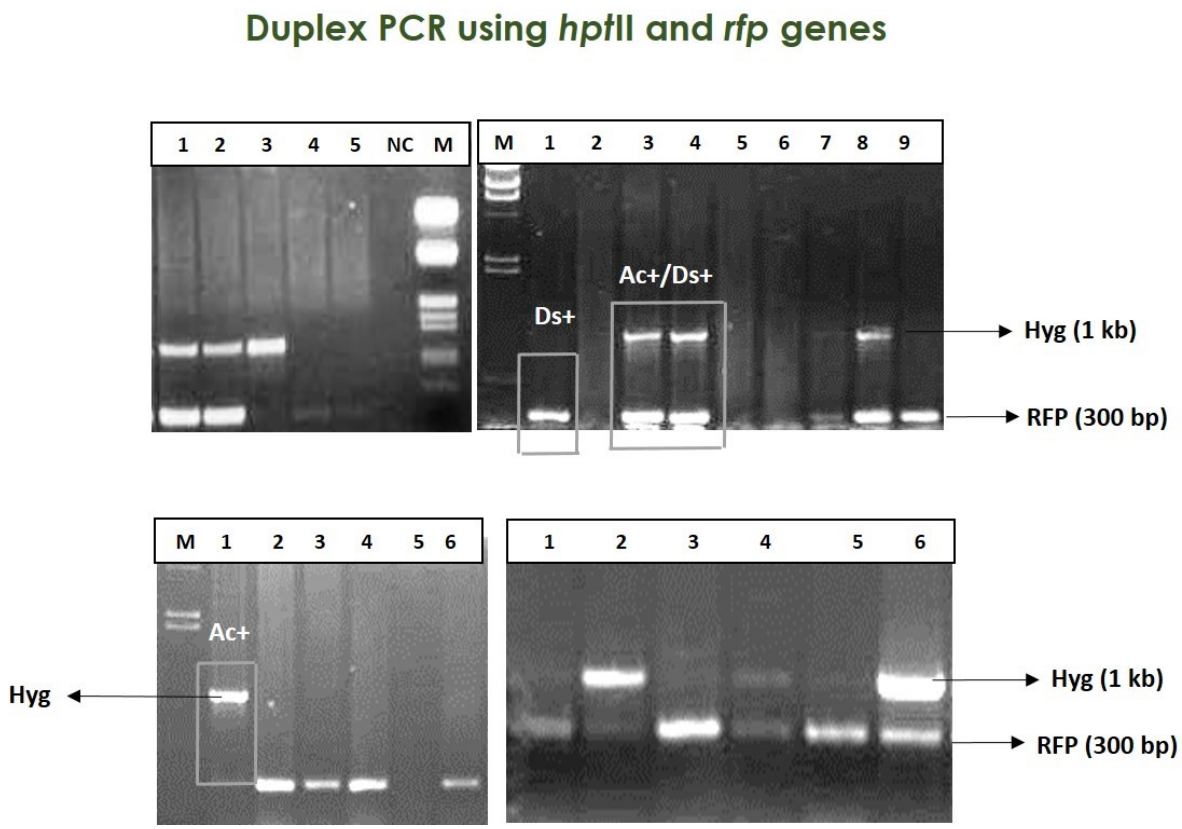


Fig. 3.2: Duplex PCR with *hpt*II (1000 bp) and RFP (300 bp) genes. Plants that show amplification with both *hpt*II and RFP genes (two bands) are *Ac+/Ds+*, those that amplify with *hpt*II alone (upper band) are *Ac+* plants and that exhibit amplification with RFP alone (lower band) are stable *Ds+* plants. M: λ *Eco*RI-*Hind*III marker.

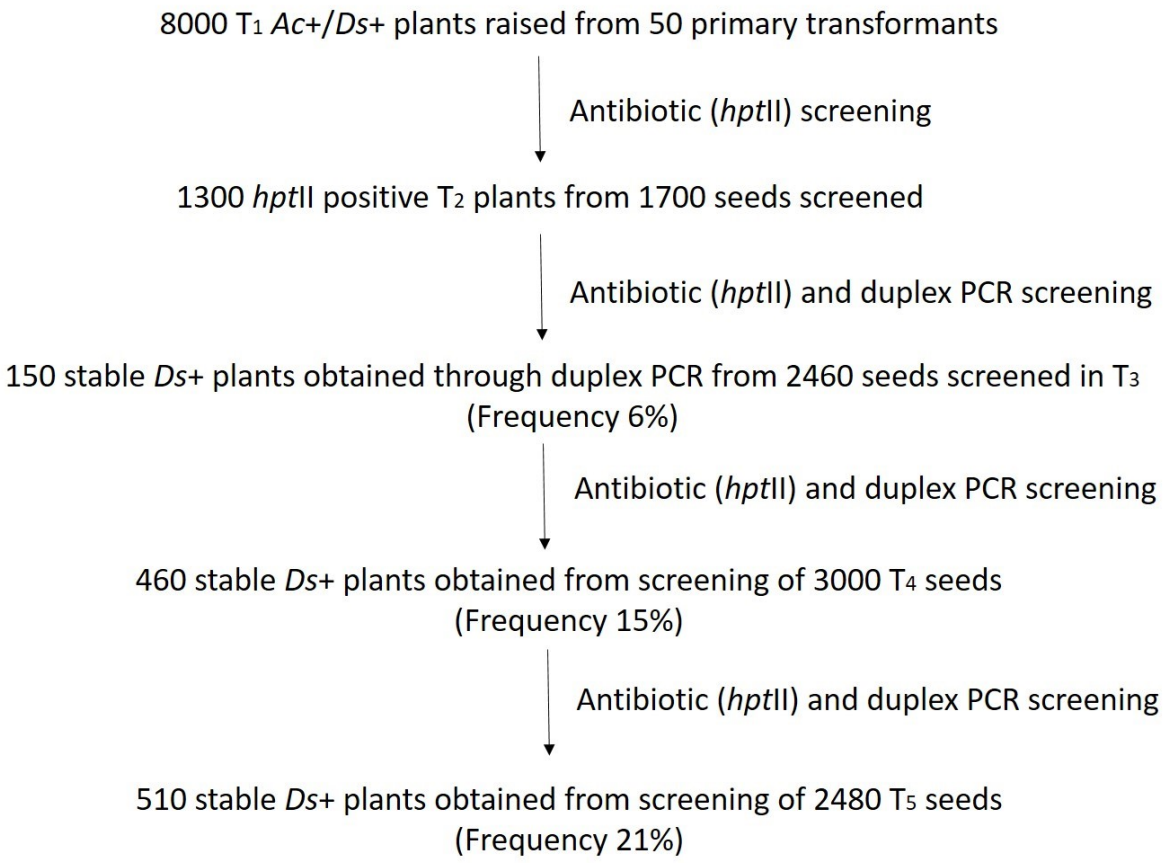


Fig. 3.3: Isolation of stable *Ds*+ plants at different generations from *Ac/Ds* population

Seeds obtained from the transgenic plants were screened on Hygromycin selection medium. Plants from the seeds that did not germinate on the antibiotic selection were recovered and analyzed through PCR to confirm whether they were null or *Ds* carrying plants. Using this approach stable *Ds* plants were obtained at every generation from *Ac*/*Ds* population.

3.2. Selection of transgenic plants obtained from *in planta* transformation

A 16 h time point after soaking in water was found to be the most suitable time for infection and the efficiency of stable transformation was increased from 0% (without vacuum), to 5% (with vacuum alone) and to more than 20% (a combinatorial treatment of vacuum and swift removal of vacuum) (Fig. 3.4). Seeds obtained from the *Agrobacterium*-treated plants were screened on 10 mg l^{-1} PPT selection medium. The progeny seeds (T_1 generation) that carried the T-DNA started germinating on the selection medium within 3-4 days after inoculation, while the seeds that did not carry the T-DNA because of segregation and seeds of the non-transformed control plants did not germinate and became bleached. About 18,000 seeds obtained from the plants infected with *Agrobacterium* carrying activation tagging vector,

pDEB were screened for resistance to PPT, of which 3,900 T_1 plants were selected to be positive. These were further confirmed by PCR amplification of appropriate DNA fragments present within the T-DNA (Fig. 3.5). Some of these were progressed to T_2 generation and PPT resistant progeny exhibited Mendelian segregation ratio of 3:1 for the integrated T-DNA (Table 3.1).

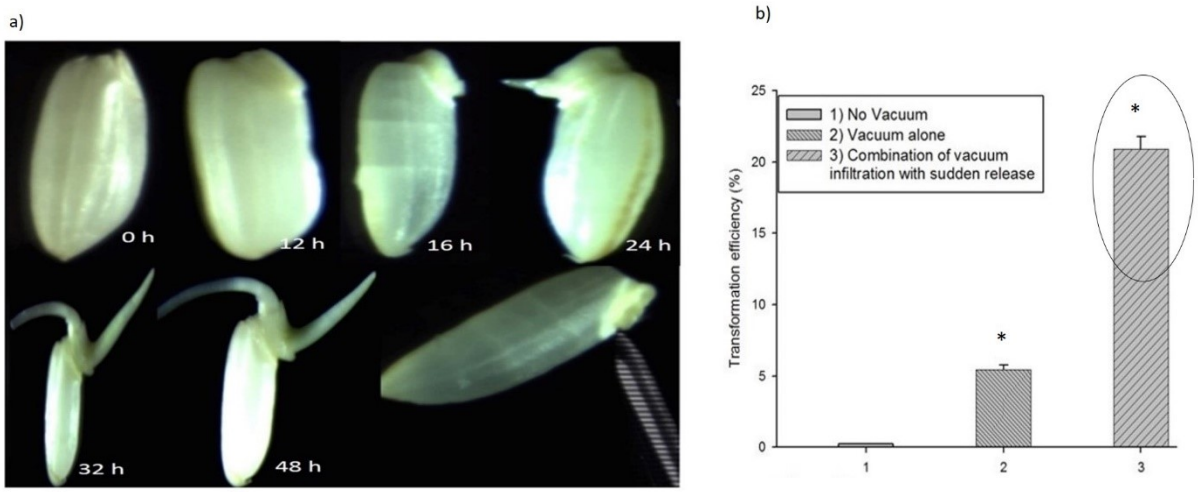


Fig. 3.4: Germination process of rice seed and *in planta* transformation efficiency

(a) After soaking in water, during the first 10-12 h seeds imbibe water and embryonic elongation occurs while hypocotyl and radicle emergence occurred from 36 h. The 16 h old embryos were considered as a suitable stage for transformation with a sterile needle dipped in the *Agrobacterium* culture. (b) Among the factors that affect *in planta* transformation, a combination of vacuum infiltration and sudden release of vacuum was found to increase the transformation efficiency up to 20%.

Of the 23 PPT resistant and PCR positive transformants analyzed for T-DNA integration through Southern hybridization (Fig. 3.6), 18 plants were found to be positive. Among these, 16 plants exhibited a single copy T-DNA insertion (frequency of 70%) and two plants showed the integration of two copies (8.6%). The independent nature of integration of T-DNA into the genome of transgenic plants was indicated by the different restriction fragments binding to the probe. Five PPT resistant plants did not give a positive signal in the Southern analysis, probably because of the inferior quality of isolated genomic DNA (Moin et al., 2016a).

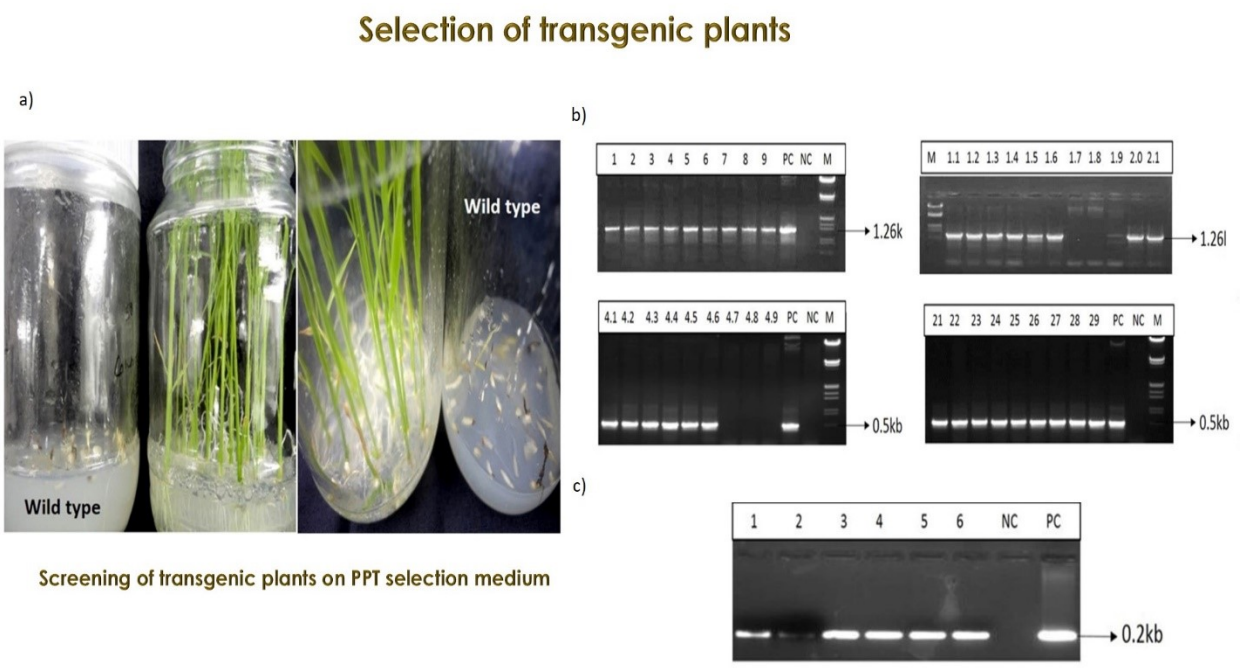


Fig. 3.5: Screening and molecular confirmation of transgenic plants.

(a) Selection of seeds derived from the *Agrobacterium*-treated rice plants on PPT (10 mg l^{-1}) medium. (b) PCR screening of transformants selected on PPT medium with *bar* gene (560 bp) and expression cassette of *bar* gene (1260 bp) in T_1 T_2 generations. (c) Semiquantitative-PCR analysis of selected transgenic plants to check the expression of *bar* gene. M, λ-*Eco*RI-*Hin*dIII DNA marker; PC, positive control; NC, negative control.

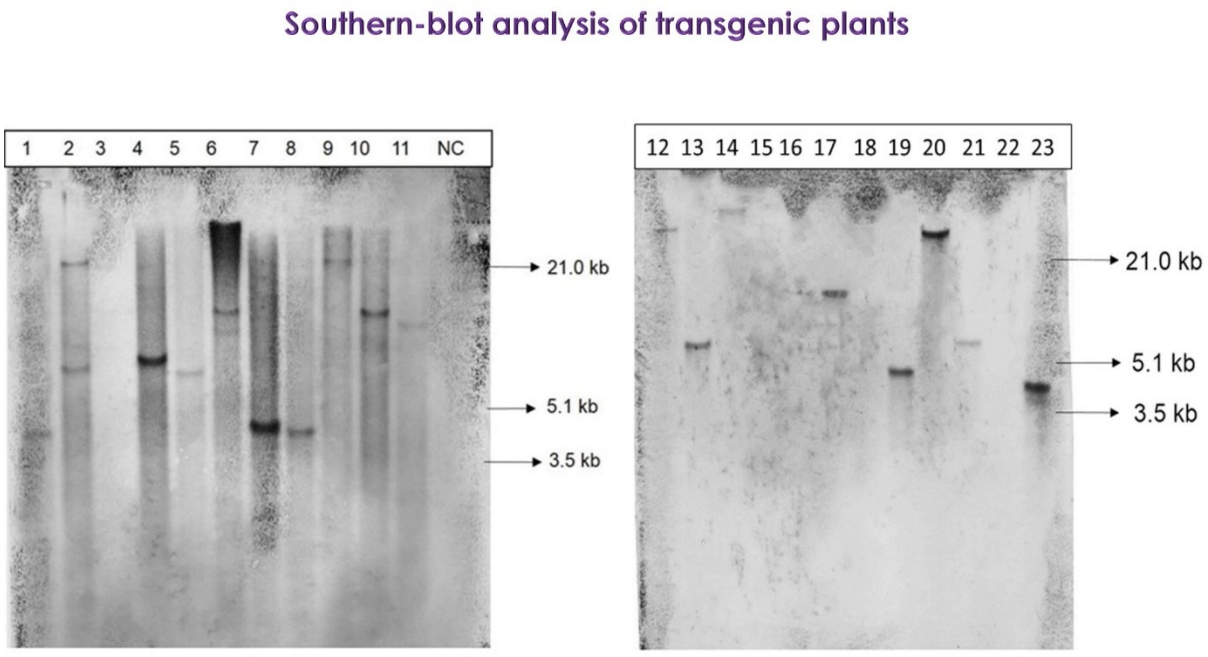


Fig. 3.6: Southern-blot hybridization of the selected transgenic plants.

Genomic DNA from T_1 generation transgenic plants (lanes 1–23) along with NC was digested with the restriction enzyme, *Kpn*I and hybridized with the DIG-dUTP labelled bar gene probe.

Table 3.1: Transmission of T-DNA to T_2 plants (Segregation Analysis).

No. of seeds screened	PPT positive (Germinated)	PPT negative (non - Germinated)	∑ Chi Square value $\chi 2$
175	134	41	0.23047619
157	117	40	0.01910828
101	76	25	0.00330033
151	103	48	3.710816777
127	87	40	2.858267717
120	83	37	2.177777778
135	97	38	0.713580247
97	65	32	3.302405498
98	69	29	1.102040816
152	123	29	2.842105263
106	85	21	1.522012579
141	105	36	0.021276596
113	76	37	3.613569322
23	15	8	1.173913043
85	56	29	3.768627451
114	83	31	0.292397661
158	114	44	0.683544304
124	86	38	2.107526882
160	113	47	1.633333333
170	128	42	0.007843137
38	27	11	0.315789474
21	12	9	3.571428571
154	110	44	1.047619048
126	86	40	3.058201058
75	52	23	1.284444444
23	16	7	0.362318841
60	46	14	0.088888889
33	24	9	0.090909091
34	26	8	0.039215686
83	62	21	0.004016064

66	46	20	0.98989899
80	53	27	3.266666667
114	88	26	0.292397661
173	132	41	0.156069364
181	130	51	0.974217311
120	83	37	2.177777778
93	76	17	2.240143369
91	65	26	0.619047619
46	31	15	1.420289855
186	129	57	3.161290323
121	82	39	3.374655647
89	65	24	0.183520599
128	103	25	2.041666667
210	146	64	3.358730159
73	46	27	5.593607306
25	16	10	1.613333333
80	56	24	1.066666667
24	16	8	0.888888889
60	39	21	3.2
166	123	43	0.072289157

Most of the plants in T_2 generation exhibited Mendelian 3:1 ratio.

3.3. Phenotypic analysis of mutants

Transgenic plants that showed better yield related parameters with distinct phenotypes such as increased tillering (Fig. 3.7a), plant height (Fig. 3.7b), panicle number, productive panicles (Fig. 3.7c) and seed yield (Fig. 3.7d) with respect to both WT samples were selected for further analysis. WT grown with adequate water have 12-15 tillers per plant, reached a height of 90 cm and the total seed yield was ~15-20 g in contrast to the WT grown under limited water availability which had 4-5 tillers, 3-5 panicles per plant and started to undergo wilting six weeks after continuous water stress. These plants also flowered early during 70-80 days and produced a limited number (80-100 number) of partially filled grains. The transgenic plants exhibited a wide variation in growth; some of the mutants had high tiller number ranging from 5 to as high as 18 per plant, up to 10 panicles, and seed yield of 15-25 g with respect to both controls. About 120 Ds^+ and 370 pDEB plants were found to have sustained productivity of which 42 and 158 plants were, respectively selected for flanking sequence analysis.

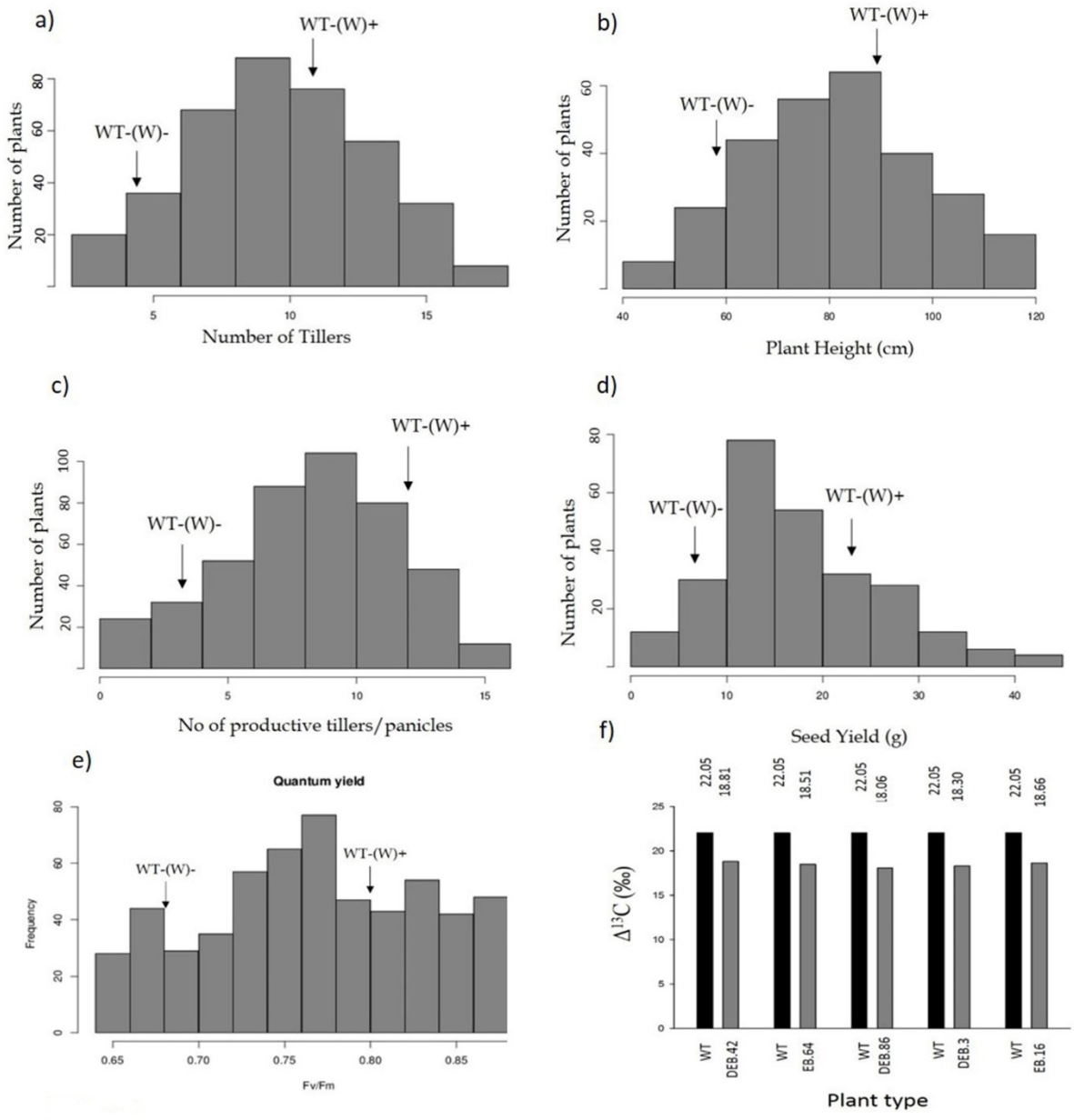


Fig. 3.7: Various growth and physiological parameters observed under limited water conditions in activation-tagged mutant population with respect to WT.

(a) Activation-tagged mutants showed variation in tiller number that ranges from 2 to as high as 18. (b) Plant height (40 cm to 120 cm). (c) Number of panicles/productive tillers per plant. (d) Total seed yield (grams) of a plant compared with WT. (e) Quantum efficiency of activation-tagged population monitored through MINI-PAM ranging from 0.65 to 0.85, while WT with and without water had 0.80 and 0.68, respectively. (f) Δ13C, which is a surrogate of WUE, measured in WT and five selected mutants, viz. DEB.42, EB.64, DEB.86, DEB.3 and En.16, are in the range of 22.05‰ and 18.81‰, 18.51‰, 18.60‰, 18.31‰ and 18.66‰,

respectively. $\Delta^{13}C$ is inversely related to WUE; the lesser the Δ13C, the higher is the WUE. The phenotypic readings were taken as a mean of five plants, whereas the physiological data are a mean of three biological replicates.

3.4. Physiological characterization and flanking sequence analysis

The WT grown with ample water exhibited a mean quantum efficiency of 0.80 and WT under water stress conditions showed a quantum yield of 0.65 to 0.68. While the quantum efficiency of activation-tagged population grown under limiting water conditions ranged from 0.60 to as high as 0.85 (Fig. 3.7e). Further, the carbon isotope ratio, $\Delta^{13}C$, which is inversely related to WUE (lesser the $\Delta^{13}C$, higher will be WUE) was measured in five mutants viz., DEB.42, EB.64, DEB.86, DEB.3, En.16 had 18.81‰, 18.51‰, 18.06‰, 18.30‰ and 18.66‰, respectively whereas WT showed a $\Delta^{13}C$ value of 22.05‰, indicating that the selected mutants have high WUE in addition to their productivity related traits (Fig. 3.7f).

The transgenic plants with quantum efficiency ≥0.80 were selected for flanking sequence analysis. The 200 sequences obtained from selected plants after tertiary TAIL-PCR were submitted in a BLAST search against Rice Genome Annotation Project Data Base (TIGR) and their locations were mapped on corresponding rice chromosomes. Of these, the highest number of insertions were found on chromosome-3 (35), followed by chromosome-6 (26); chromosome-11 had very few insertions (4), other insertions were almost equally distributed on remaining chromosomes (Fig. 3.8).

Further, it had been found that 57% of the *Ds*-Red insertions were intergenic and 43% were intragenic, while 44% of pDEB T-DNA insertions were intergenic and 56% were intragenic (Table 3.2). This analysis not only identified the genes flanking the *Ds* element at the site of insertion but also demonstrated the wide distribution of activation-tags (*Ds*-4xEnhancer) on various chromosomes throughout the rice genome (Moin et al., 2016a).

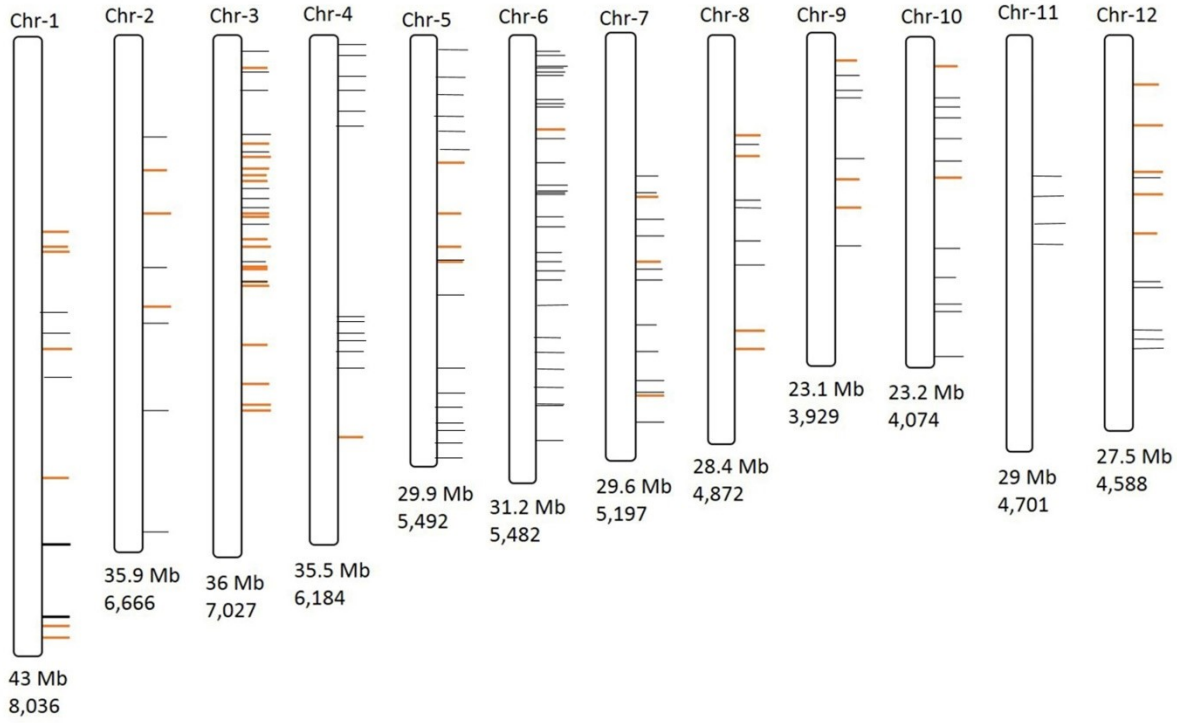


Fig. 3.8: Distribution of genetically mapped insertions of selected plants on corresponding chromosomes.

About 200 sequences obtained from the BLAST search were mapped on corresponding chromosomes. Orange bars mark the Ds insertions (derived from *Ac*/*Ds* population), while black bars represent the position of T-DNA insertions. The highest number of insertions was found on chromosome-3 (35), while chromosome-11 has only four insertions.

Table 3.2: Study of inter and intragenic flanking sequences of selected activation-tagged transgenic plants

T-DNA insertions					Ds insertions				
Chromosome No.	No. of insertions	Intragenic		Intergenic	Chromosome No.	No. of insertions	Intragenic		Intergenic
		Exonic	Intronic				Exonic	Intronic	
1	12	3	3	6	1	7	2	1	4
2	7	2	2	3	2	3	0	0	3
3	24	8	4	12	3	14	7	0	7
4	12	0	4	8	4	1	0	0	1
5	15	5	5	5	5	4	0	0	4
6	30	7	8	15	6	3	0	0	3
7	12	4	1	7	7	3	0	1	2

8	8	3	1	4	8	2	0	2	0
9	10	4	2	4	9	1	1	0	0
10	13	1	6	6	10	1	1	0	0
11	4	0	4	0	11	0	0	0	0
12	11	4	5	2	12	3	1	0	0
Total	158	41	45	72		42	12	6	24

Sequences obtained from BLAST search against RGAP-DB were mapped to identify the intergenic and intragenic insertions. Nearly 57% of the *Ds*-Red insertions (derived from *Ac/Ds* population) were intergenic and 43% were intragenic, while 44% and 56% of pDEB T-DNA insertions were inter and intragenic, respectively.

3.5. Transcript analysis of the tagged genes

Since CaMV35S enhancers elicit the expression of genes placed over a stretch of 10 kb on either side of the insertion, a minimum of a 20 kb region was mapped. Five mutants viz., DEB.42, En.64, DEB.86, DEB.3 and En.16, with a quantum efficiency of 0.85, 0.80, 0.82, 0.85 and 0.84, respectively along with low $\Delta^{13}C$, increased tillering and seed yield under limited water conditions (Fig. 3.9) with respect to both the controls were selected for the analysis of transcript levels of all the candidate genes residing within a 20 kb region from the site of insertion of the activation-tag. The details of the physiological parameters and genes tagged in these five selected mutants with their location in the genome have been provided in Table 3.3.

Fig. 3.9: Phenotypic characterization of selected mutants under limited water conditions. (a) After 4 weeks of transfer to the pots, water overlay was withdrawn to maintain barely moist conditions. (b) Mutant EB.64 had 10 tillers grown under limited water conditions. (c) Mutants DEB.103, 150 and DEB.280 exhibited increased plant height than WT. (d) Six weeks after continuous growth under water deficiency, a maximum of three to four tillers was observed in WT with limited water that subsequently started to wilt, whereas DEB.42 and DEB.3 had 15 and 18 tillers, respectively, with each tiller further giving productive panicles. (e) En.16 displayed profuse tillering and panicles compared with another dwarf mutant En.15. Phenotype observations were recorded as a mean of five transgenic plants grown along with WT.

In DEB.42 three genes; LOC_Os12g32240 (translation initiation factor 5A), LOC_Os12g32250 (WRKY96 transcription factor), and LOC_Os12g32260 (putative expressed protein) were located within a 20 kb region of the *Ds* insertion (Fig. 3.10a). The transcript level of LOC_Os12g32250, which is situated ~2.1 kb upstream from the enhancers was elevated about 15-fold in DEB.42 with respect to WT (Fig. 3.10b), while there was no significant activation of other two genes.

In En.64, four genes flanked the enhancers (Fig. 3.11a). These were LOC_Os03g57260 (GRF zinc finger family protein), LOC_Os03g57270 (ubiquitin carboxyl-terminal hydrolase),

LOC_Os03g57280 (N-dimethyl guanosine tRNA methyltransferase protein) and LOC_Os03g57290 (cullin-4 protein). LOC_Os03g57290, situated 9 kb downstream from the insert became activated 20-fold (Fig. 3.11b).

In DEB.86, the insert was flanked by four genes; LOC_Os03g40070 (transposon protein, putative expressed), LOC_Os03g40080 (GRAS family transcription factor containing protein), LOC_Os03g40084 (expressed protein) and LOC_Os03g40090 (expressed protein) (Fig. 3.12a). Of these, the transcripts of two adjacent genes; LOC_Os03g40080 and LOC_Os03g40084 showed a 6-fold elicitation (Fig. 3.12b).

Another phenotypic variant DEB.3 has three genes LOC_Os04g39700 (RPL6), LOC_Os04g39710 (retrotransposon protein), LOC_Os04g39720 (retrotransposon protein) flanking the enhancers (Fig. 3.13a). LOC_Os04g39700 (RPL6), located 1 kb downstream of the insert became 26-fold activated compared with WT (Fig. 3.13b).

In En.16, four genes, LOC_Os01g24680 (3-hydroxyacyl-CoA dehydrogenase), LOC_Os01g24690 (RPL23A), LOC_Os01g24700 (putative protein) and LOC_Os01g24710 (Jacalin-like lectin domain containing protein) were situated (Fig. 3.14a). LOC_Os01g24690 which is located 1 kb downstream of enhancers became activated 18-fold (Fig. 3.14b) (Moin et al., 2016a).

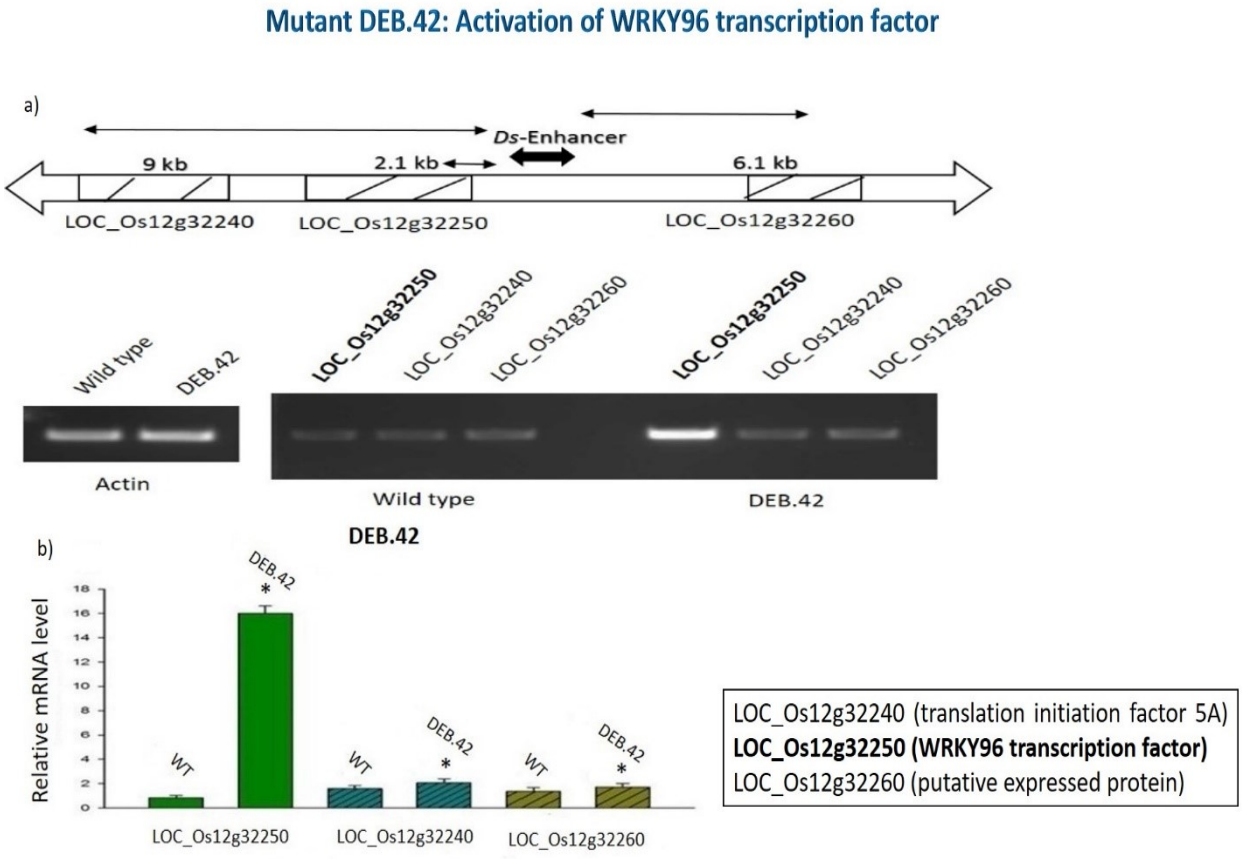


Fig. 3.10: Semi-quantitative-PCR analysis of enhancer-tagged genes in selected mutants. (a) In DEB.42, three genes (LOC_Os12g32240, LOC_Os12g32250 and LOC_Os12g32260) were located on chromosome-12, of which the transcript level of LOC_Os12g32250 present at 10 kb upstream was up-regulated with respect to WT. In DEB.42, the locus, LOC_Os12g32250, was activated 16-fold compared with other loci in transgenic and WT. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

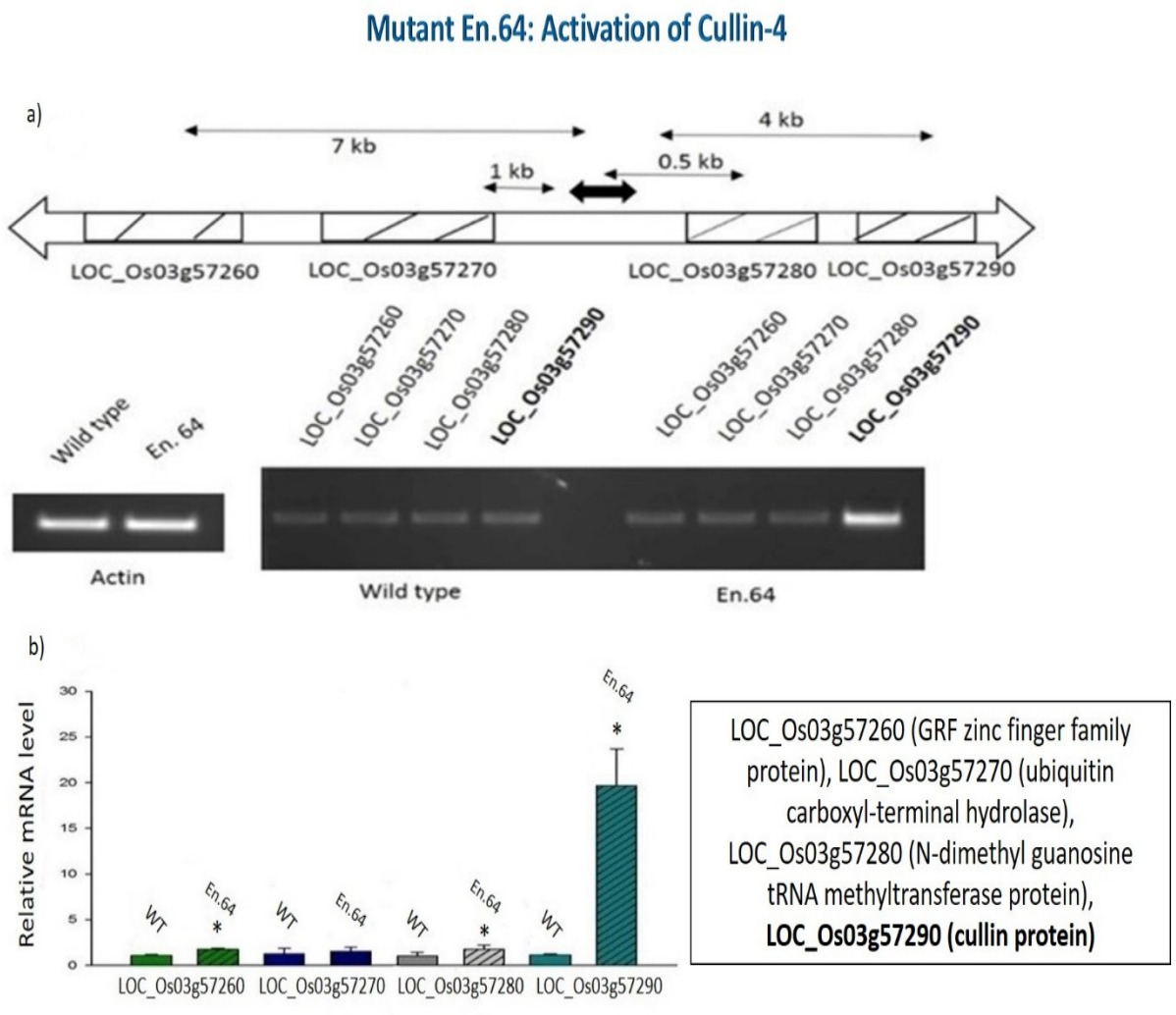


Fig. 3.11: Activation of Cullin-4 gene involved in protein ubiquitination by semi-Q and Q-PCR

(a) In En.64, four genes flanked the enhancers in a 20 kb region on chromosome-3. LOC_Os03g57290, which is situated 9 kb downstream from the insert, became activated. (b) In EB.64, LOC_Os03g57290 became activated 15-fold. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

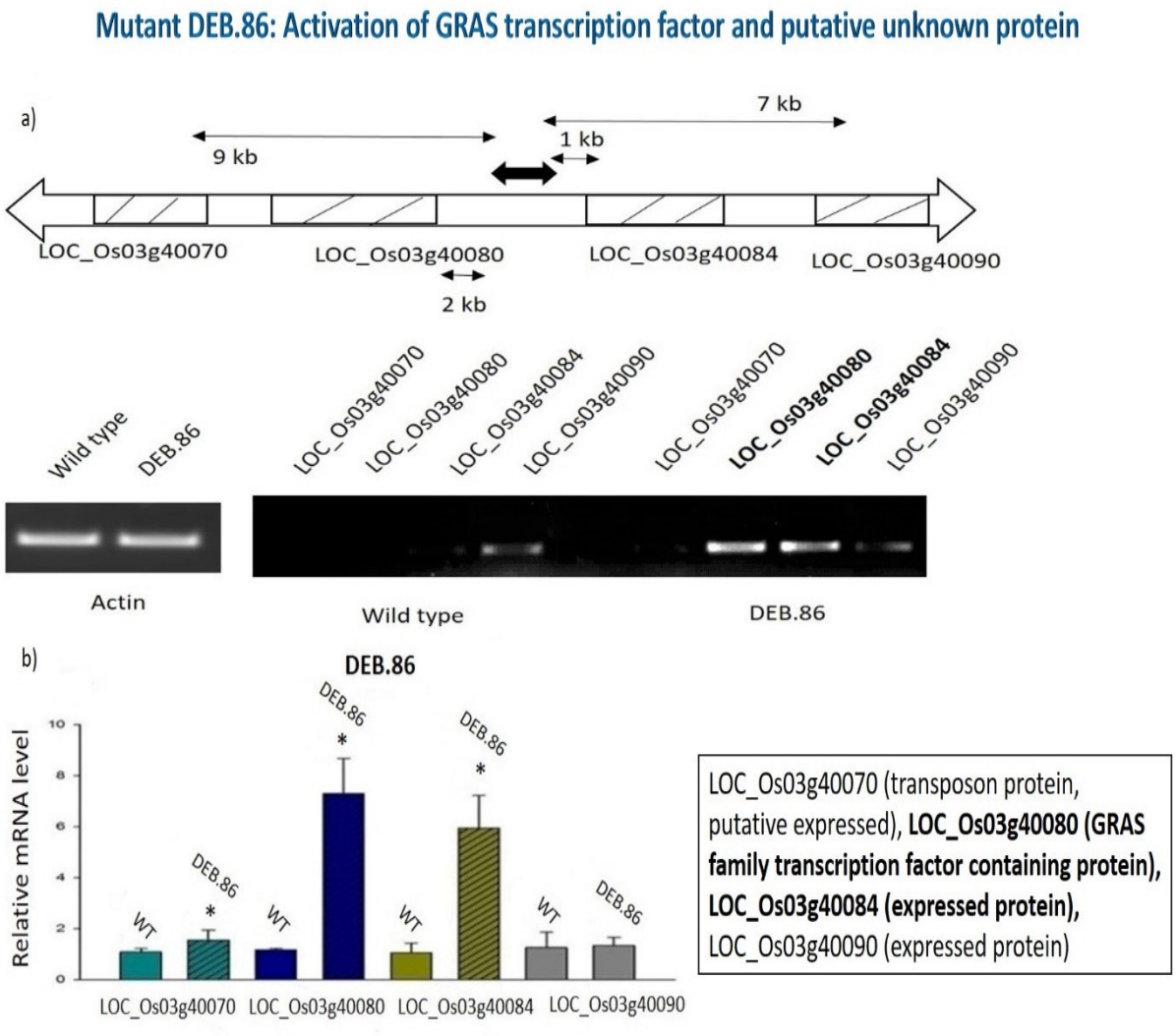


Fig. 3.12: Activation of two genes by tetrameric enhancers

(a) In DEB.86, two genes (LOC_Os03g40080 and LOC_Os03g40084) were up-regulated concurrently. In mutant DEB.86, of the four flanking genes, two loci LOC_Os03g40080 and LOC_Os03g40084 located 2 kb downstream and at 1 kb upstream, became elicited six-fold and eightfold, respectively observed through Q-PCR. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

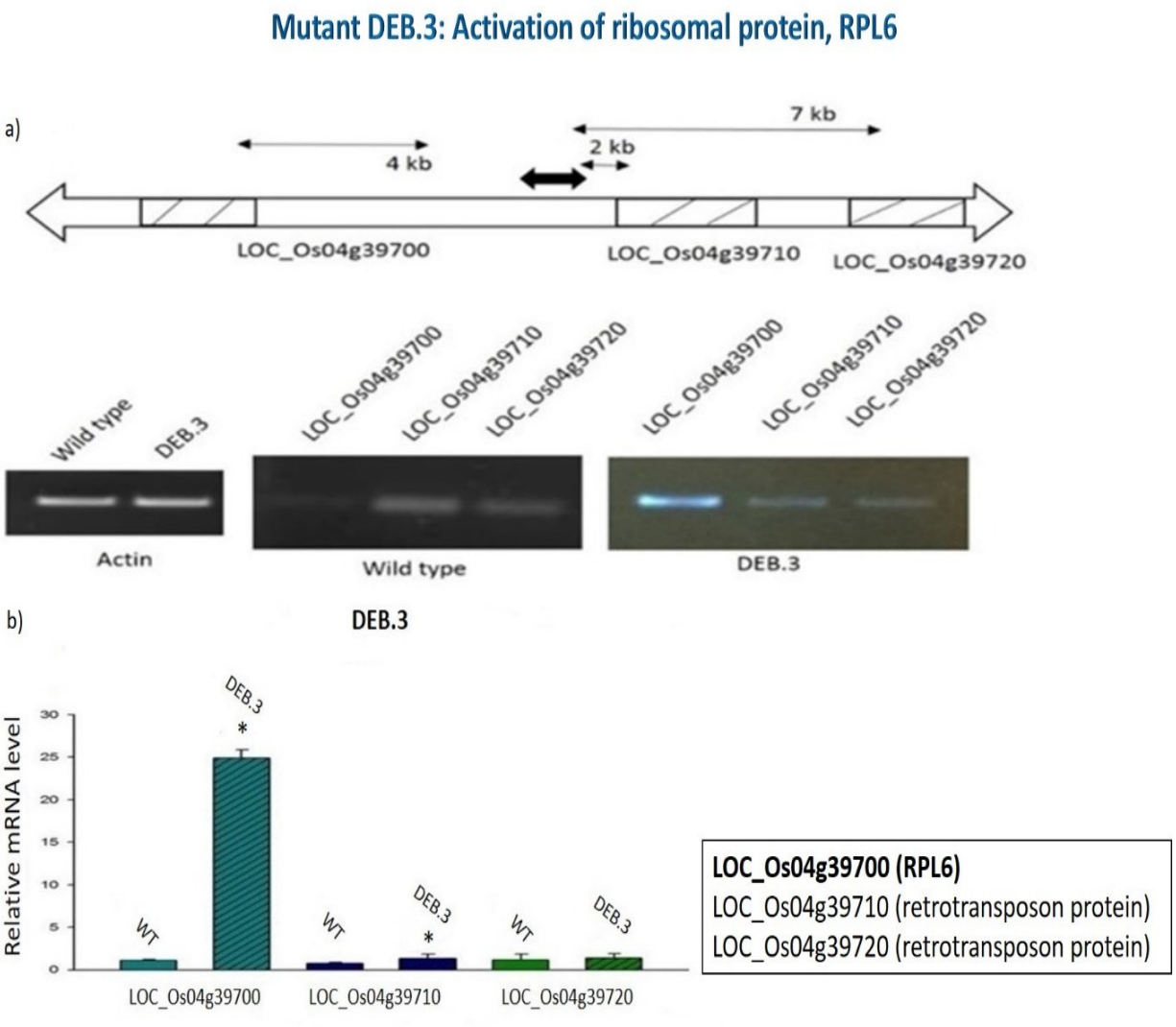


Fig. 3.13: Activation of Ribosomal Protein Large Subunit gene (RPL6) by enhancers

(a) In DEB.3, the transcript of LOC_Os04g39700 became up-regulated, (b) (d) In DEB.3, LOC_Os04g39700, located 1 kb downstream from the site of insert location was activated 24-fold with respect to control. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

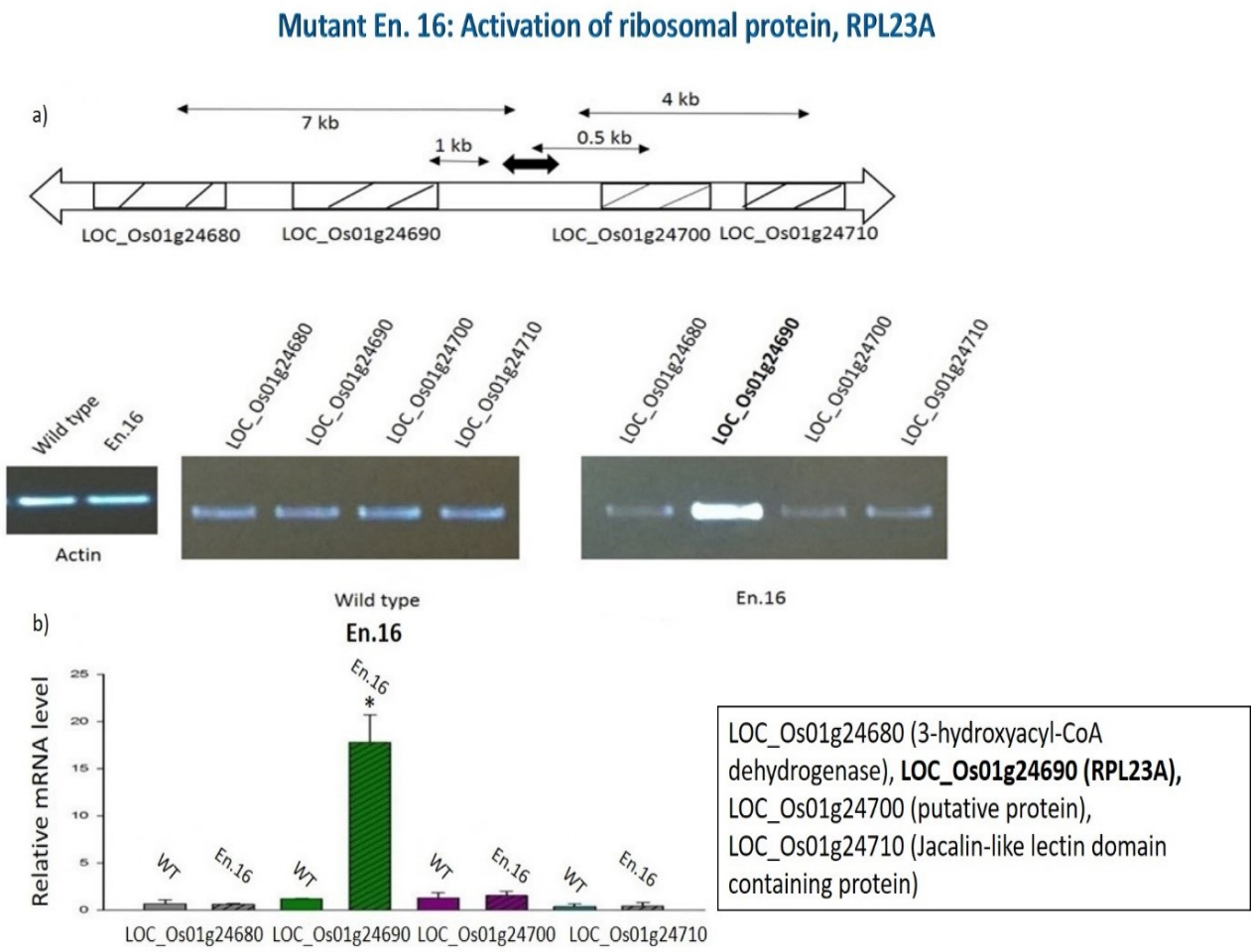


Fig. 3.14: Activation of Ribosomal Protein Large Subunit gene (RPL23A) by enhancers

(a) In En.64, LOC_Os01g24690 was up-regulated, (b) In En.16, the transcript level of LOC_Os01g24690 located adjacent to enhancer insertion became 15-fold activated. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

Table 3.3: Physiological and transcript details of five selected mutants

Mutant Name	Quantum Yield	Δ13C (‰)	Insert location in the genome	Genes located within a 20 kb region	Gene(s) tagged by enhancer	Protein
DEB.42	0.85	18.81	Chr12:19462000-19482000	LOC_Os12g32240, LOC_Os12g32250, LOC_Os12g32260	LOC_Os12g32250	WRKY96
En.64	0.80	18.51	Chr3:32665000-32675000	LOC_Os03g57260, LOC_Os03g57270, LOC_Os03g57280, LOC_Os03g57290	LOC_Os03g57290	Cullin protein
DEB.86	0.82	18.06	Chr3:22267000-22274000	LOC_Os03g40070, LOC_Os03g40080, LOC_Os03g40090	LOC_Os03g40080, LOC_Os03g40084	GRAS transcription factor,

						Expressed Unknown protein
DEB.3	0.85	18.30	Chr4:23654000-23674000	LOC_Os04g39700, LOC_Os04g39710, LOC_Os04g39720	LOC_Os04g39700	RPL6
En.16	0.84	18.66	Chr1:13889000-13909000	LOC_Os01g24680, LOC_Os01g24690, LOC_Os01g24700, LOC_Os01g24710	LOC_Os01g24690	RPL23A

3.6. Inducible expression pattern of RPL6 and RPL23A genes

To check the inducible expression pattern of RPL6 and RPL23A genes that were activated by enhancers to abiotic conditions, we subjected 7 d old WT seedlings of same rice variety, BPT-5204 (Samba Mahsuri), which was used to generate the activation-tagged mutant population to four different abiotic stress factors namely cold (4°C), heat (42°C), dehydration (PEG), salt (NaCl) and three plant hormones (ABA, SA and MeJa). The expression of these genes was studied in shoot and root tissues separately at different time intervals. The transcripts of RPL6 and RPL23A had responded immediately to almost every stress tested, following which their expression was gradually ascended with the progression of the stress duration and finally reached a maximum level at a certain time point. In shoots, the level of RPL6 mRNA had reached up to 150-fold after 12 h of dehydration, 250-fold and 120-fold in response to ABA and NaCl, respectively after 60 h of treatment. SA and MeJa also caused a significant up-regulation by more than 30-fold. Similarly, the activation of RPL6 was also observed in roots, but the level was less compared with shoots (Fig. 3.15a & 3.15b). The RPL23A transcripts also responded as early as 5 min after the onset of stress. Unlike RPL6, although the level of RPL23A activation was not several hundred fold, it was more in root tissues than in shoots (Fig. 3.15c & 3.15c). Dehydration, ABA and NaCl treatments lead to the activation by more 40-fold in roots, whereas SA and MeJa caused the up-regulation by 17-fold in roots and 26-fold in shoots, respectively (Moin et al., 2016a).

3.7. *In silico* analysis of upstream sequences of genes for two ribosomal proteins

Since RPL6 and RPL23A had responded to various abiotic treatments with a very high level of activation, we checked for the presence of any stress-specific/responsive elements in their promoters. The upstream 1.1 kb putative promoter regions of RPL6 and RPL23A were found to have various *cis*-regulatory elements that possibly respond to stress/hormone specific signals. The promoter region of RPL6 has three TGA or auxin-responsive elements and a

GARE-motif that responds to plant hormones, auxins and gibberellic acid, respectively. It also has three HSEs (Heat-stress elements), two MeJa responsive elements and an ABRE (ABA-responsive elements)-motif (Fig. 3.15e). High level of up-regulation of RPL6 in response to ABA and MeJa treatment is likely in congruence with the presence of the corresponding stress responsive elements. RPL6 was also activated by heat stress (42°C), although the level of activation was comparatively low. Also, it has TC-rich repeats, which have been found to be involved in plant defence and stress responsiveness (Diaz-De-Leon et al., 1993).

The promoter region of RPL23A also exhibited several *cis*-regulatory elements, which include two MeJa responsive elements, an auxin-responsive element, an ABRE-motif, and a LTF (low temperature-responsive element) motif. The presence of these elements supports the transcript abundance of RPL23A in response to ABA, MeJa and cold treatments (Fig. 3.15f). RPL23A promoter also possesses Box-W1 motif, which is a fungal elicitor-responsive element, MBS-motif, a MYB transcription factor binding site and a W-box motif, which is a preferential binding site for WRKY transcription factors. MBS-motifs have been reported to be essential for binding of MYB transcription factors for drought-inducible gene expressions (Baldoni et al., 2015). Although WRKY transcription factors have diverse roles in plants, their major functions appear to be inducing tolerance against biotic and abiotic stresses (Ülker et al., 2004).

3.8. Differential expression of RPL genes in abiotic stress response

Two out of 8 genes that were activated by the enhancers among the activation-tagged mutants in search of candidate genes responsible for high WUE belonged to 60S ribosomal subunits. We have investigated whether other members of 60S ribosomal gene family respond differentially to abiotic stress treatments. We, therefore, subjected 7 d old rice seedlings to three abiotic treatments viz., ABA, PEG and NaCl and analyzed the expression pattern of 34 rice ribosomal protein genes in root and shoot tissues. Among the 34 genes, the expression of almost all genes responded to at least one of the three treatments in the form of either down or up-regulation over a 60 h period of stress (Moin et al., 2016a). The genes that became up-regulated were more in number than those that were down-regulated. Among the genes that responded to the abiotic treatments (either up-regulation or down-regulation), some were specific to a particular stress in a particular tissue, while others exhibited an overlap in the pattern of expression in response to one or more treatments.

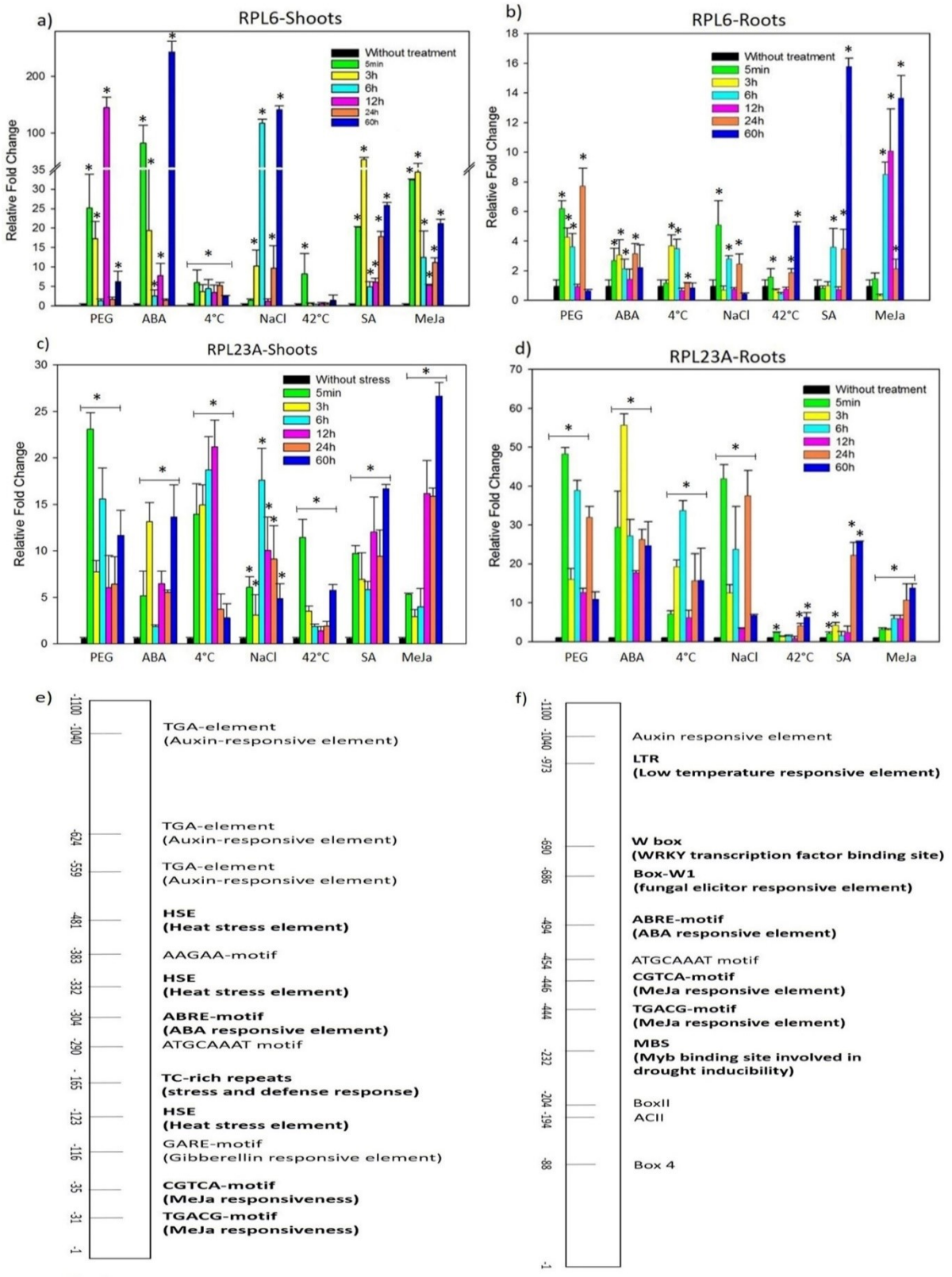


Fig. 3.15: Tissue-specific expression of RPL6 and RPL23A under seven abiotic treatments and identification of cis-regulatory elements.

Tissue-specific differential expression of (a, b) RPL6 and (c, d) RPL23A were carried out using 7-day-old WT rice seedlings treated with PEG, ABA, 4 °C, NaCl, 42 °C, SA and MeJa. Expression of these genes in water (without treatment) at corresponding time point was used as a calibrator to determine the relative expression under treated conditions. Rice actin (*Act*1) was used as an internal reference gene. The Q-PCR data is a mean of three biological replicates. The relative expression was considered statistically significant at P value < 0.05 (represented with asterisks) based on one-way ANOVA in all the analysed genes. The 1.1 kb 5′ upstream regions of (e) RPL6 and (f) RPL23A genes have a wide range of cis-regulatory elements that respond to various environmental stresses and act as binding sites for stress-specific transcription factors. Stress-specific elements are highlighted in bold.

We investigated the expression pattern of 34 genes at seven different time intervals. At every time point, we short-listed the genes that were specifically expressive under a given treatment or common to more than one treatment. Among the transcripts that became up-regulated, majority of them responded instantaneously and these were categorized as immediate-early (IE) genes. About 44% of ABA, 70% of PEG and 29% of salt induced RPL genes in shoots and 61% (ABA), 58% (PEG) and 58% (NaCl-responsive) in roots became activated within 5 min to 3 h of exposure to stress. Since this immediate transcriptional activation of genes might not be stress-specific and probably has been induced because of the activity of overall plant environmental stress responsive genes (Killian et al., 2007), we looked for a split in the expression pattern of IE-RPL genes. The transcriptional activity of some of the IE-RPL genes that were common to any two treatments such as L6, L7, L11, L12, L13b, L18P, L23A; L24a; L30, L32, L37, L44 was initially declined, which was followed by an elevation in their expression as the treatments progressed in both shoot and root tissues. However, some of the IE-RPL genes like L23A, L19.3, L38, maintained a high level of expression throughout the duration of any two or three treatments. The level of transcriptional up-regulation of these two classes of genes varied as some exhibited a very high level of up-regulation up to several hundred fold (RPL6, L8, L12, L19.3 and L35), some had moderate expression and others have comparatively low transcript levels (Moin et al., 2016a).

These two classes of IE-RPL genes, which were activated instantaneously after stress and exhibited a split in the expression or maintained a continuous high level of expression might function as an immediate defence to stress (Kawasaki et al., 2001). The expression of another set of IE-RPL genes declined and became stationary after continuous exposure to ABA, PEG and NaCl treatments (Table 3.4 presents a list of the expression pattern of different IE-RPL

genes under three treatments). The differential transcriptional regulation of 34 RPL genes in response to PEG, (Fig. 3.16), ABA (Fig. 3.17), and salt treatments (Fig. 3.18) were represented as heat maps. Each row, which depicts the differential expression of a gene through variation in coloration at different time points was followed by an expression profile spark line that shows the respective pattern of expression (Moin et al., 2016a). The transcripts, which became activated between 3 h to 12 h and after 12 h of exposure to stress were categorized as early (E) and late (L) responding genes, respectively. Fig. 3.19 describes an overlap in the expression pattern of 34 genes in three abiotic treatments in roots and shoots separately in the form of Venn diagrams. Table 3.5 detailed the list of IE, E and L-RPL genes with fold changes under three treatments in shoots and roots. Among those that were down-regulated, NaCl-induced the down-regulation of more number of genes compared to PEG and ABA treatments. RPL30, L44, L22, L14, L29, L36, L13a and L15 were commonly down-regulated in all the three treatments. Salt-induced down-regulation of RPL32 has been consistent as shown earlier (Mukhopadhyay et al., 2011).

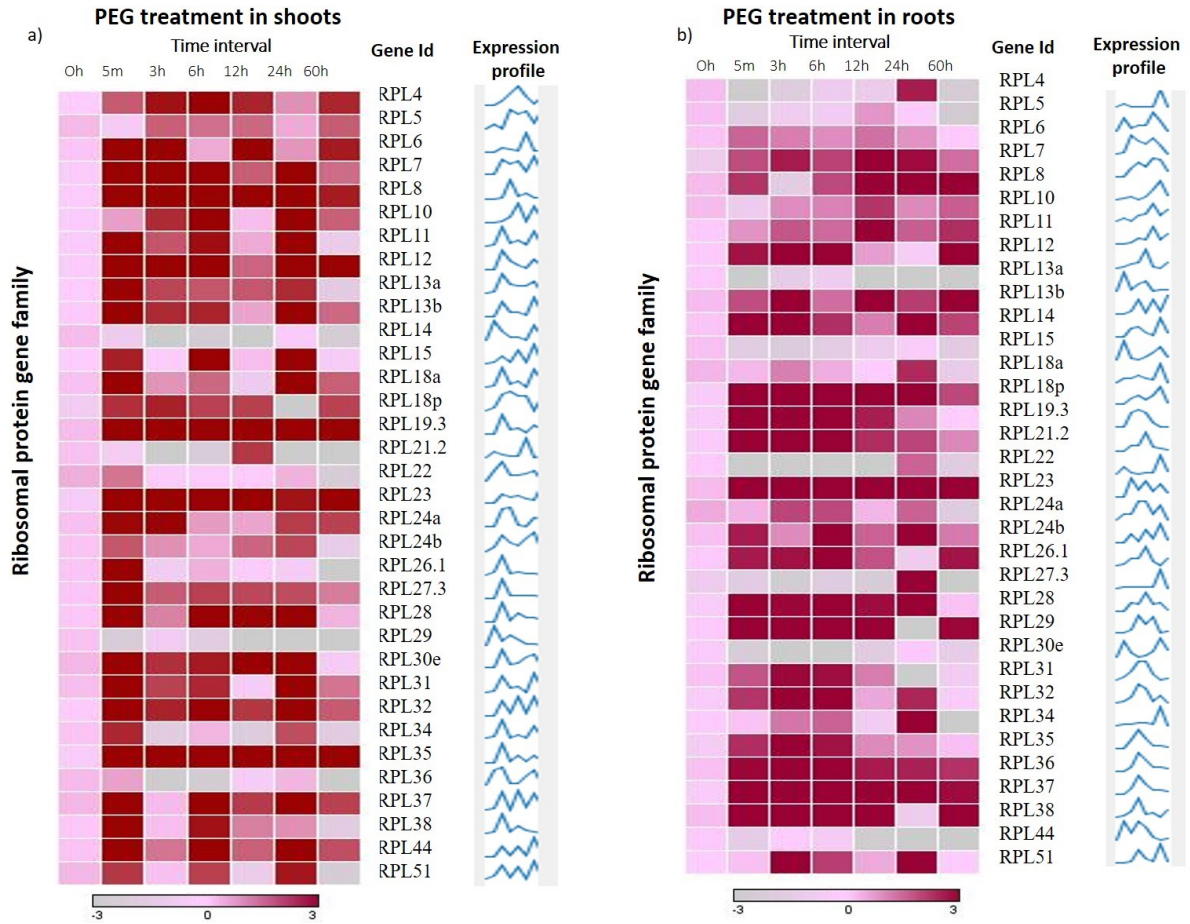


Fig. 3.16: Heat map depiction of differential expression of 34 rice 60S ribosomal protein candidate genes in response to PEG treatment

The heat map generated for expression profiling showed differential transcript abundancy/inadequacy of each ribosomal gene in response to PEG in (a) shoot and (b) root tissues. About 70% of PEG induced RPL genes in shoots and 58% in roots are activated instantaneously (IE). Dark- and light-colored grids indicate transcript abundancy and inadequacy, respectively. Heat maps were generated using the GENE-E program.

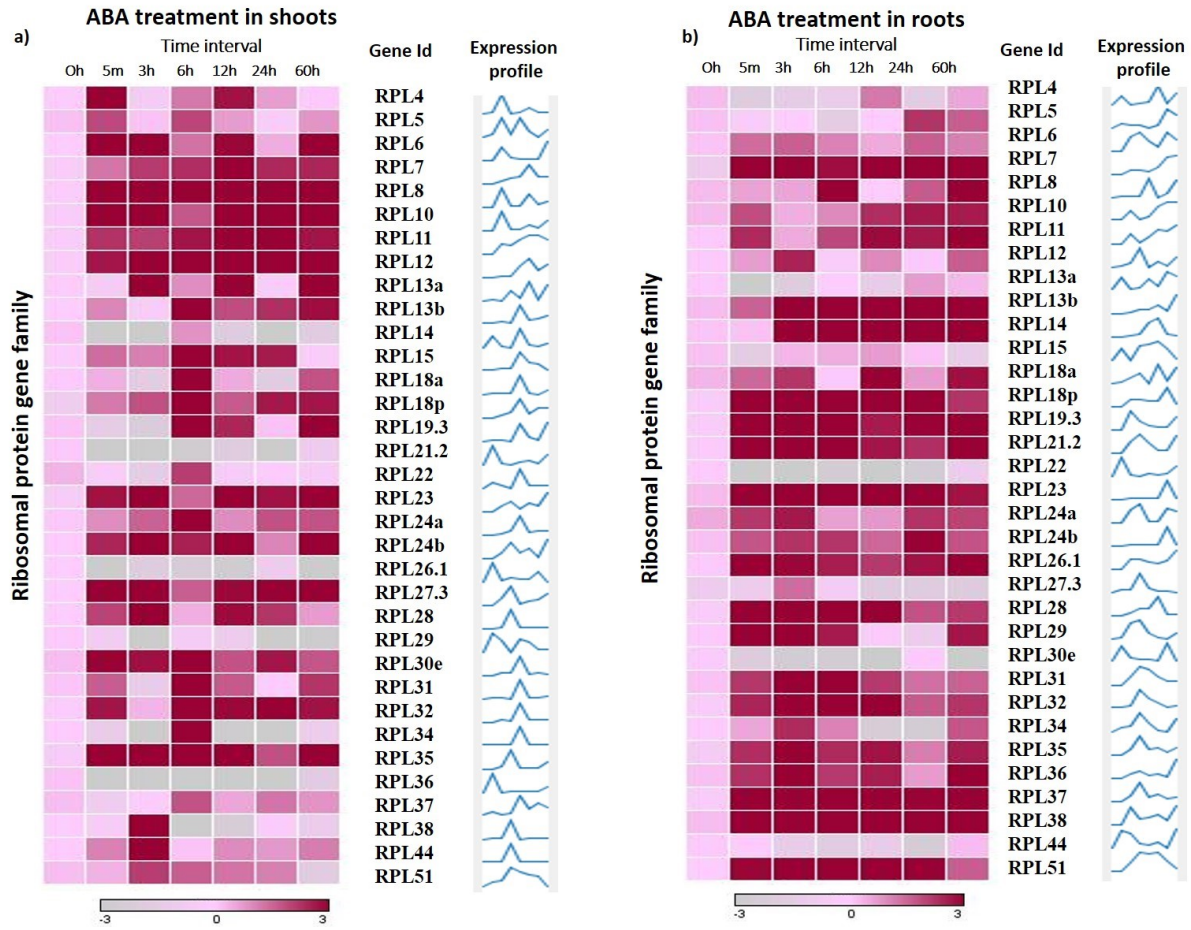


Fig. 3.17: Heat map depiction to show the differential expression of 34 RPL genes under ABA treatment

The heat map generated for expression profiling showed differential transcript abundancy/inadequacy of each ribosomal gene in response to ABA in (a) shoot and (b) root tissues. Dark- and light-colored grids indicate transcript abundance and inadequacy, respectively.

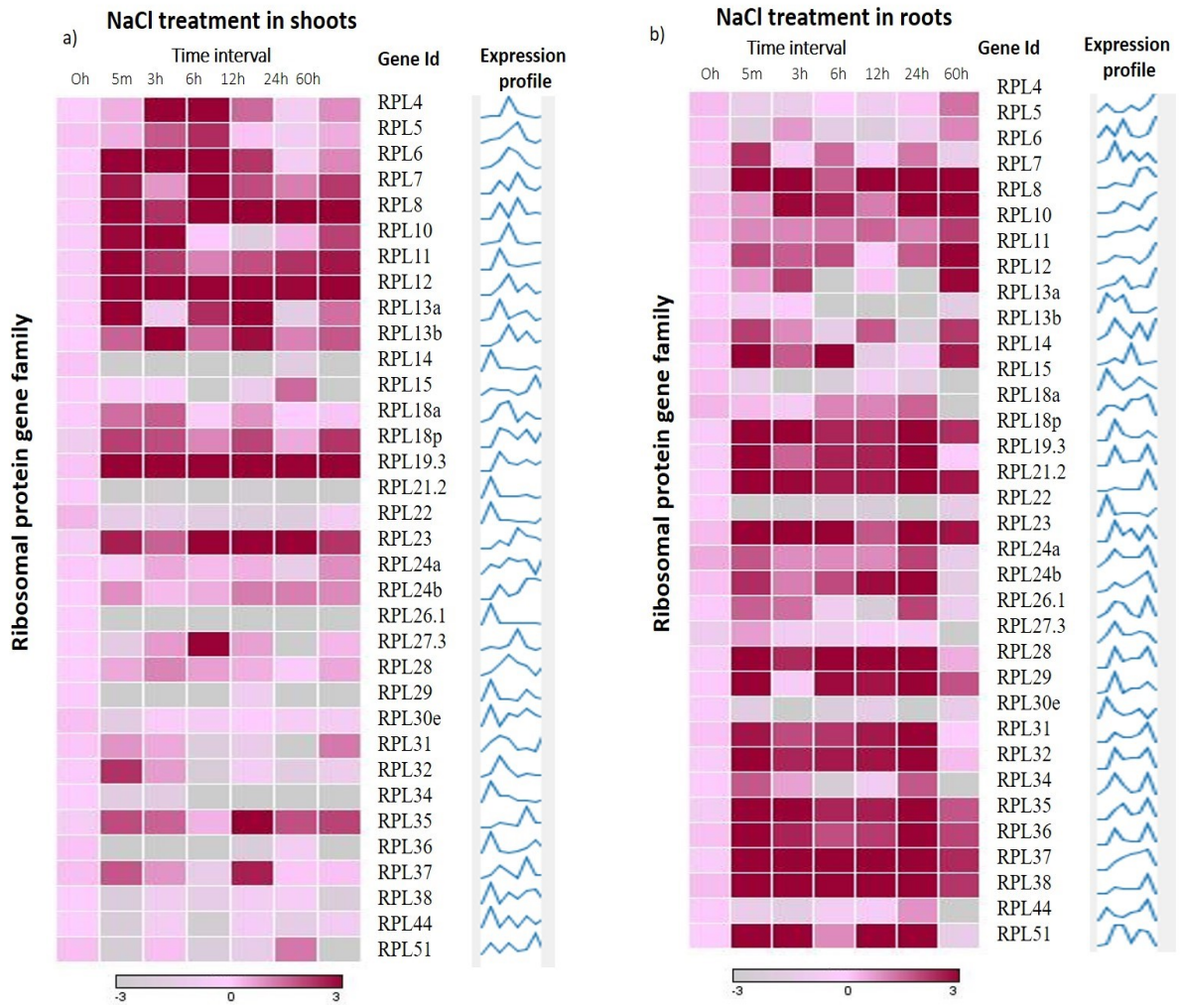


Fig. 3.18: Heat map depiction of 34 RPL genes in response to NaCl treatment

The heat map generated for expression profiling showed differential transcript abundancy/inadequacy of each ribosomal gene in response to NaCl treatment in (a) shoot and (b) root tissues. About 29% of salt induced RPL genes in shoots and 58% in roots became activated within 5 min to 3 h of exposure to stress.

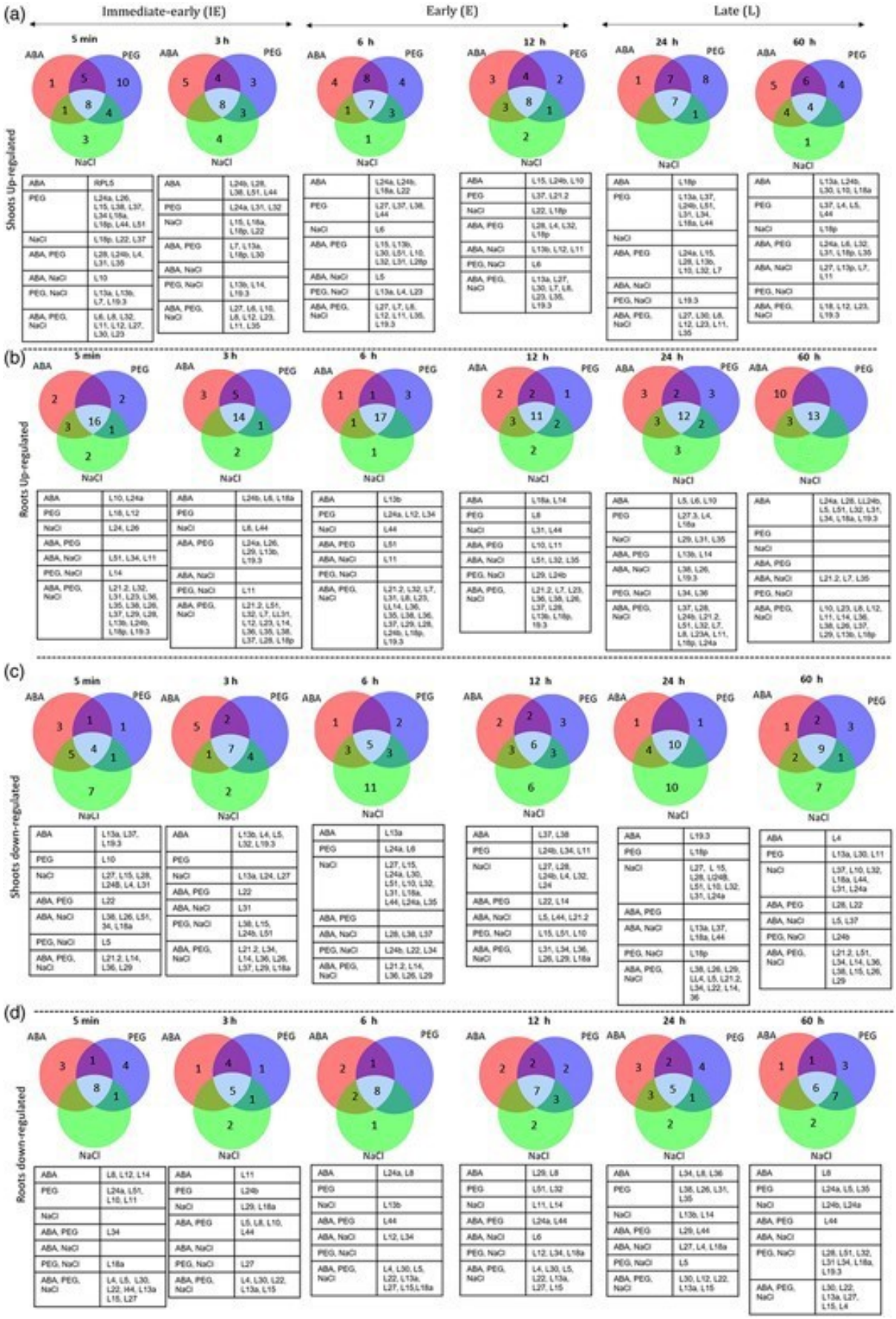

(a)
Immediate-early (IE)
Early (E)
Late (L)
5 min
3 h
6 h
12 h
24 h
60 h
ABA
PEG
NaCl
Shoots Up-regulated
(b)
Roots Up-regulated
(c)
Shoots down-regulated
(d)
Roots down-regulated

Fig. 3.19: Venn diagrams representing the stress and spatiotemporal specific up/down-regulation of ribosomal genes.

The Venn diagrams depict the total number and list of genes (presented in a Table at the bottom of every chart) that were (a, b) up-regulated and (c, d) down-regulated in shoots and roots at each time point (5 min, 3 h, 6 h, 12 h, 24 h and 60 h) after subjected to each stress treatment.

Table 3.4: Comparative regulation of large subunit of ribosomal protein gene family under various stress treatments.

		Shoot				Root			
TIGR Locus	Protein	Regulation	Response	Maximum fold change	*P*-value	Regulation	Response	Maximum fold change	*P*-value
ABA									
LOC_Os03g58204	RPL4	Up	IE (24.34)	24.3442	0.005	down	-		
LOC_Os05g11710	RPL5	Up	IE (3.85)	3.8548	0.199	Up	L (4.76)	3.1732	0.009
LOC_Os04g39700	RPL6	Up	IE (82.38)	250.307	0.017	Up	IE (3.08)	3.085	0.589
LOC_Os04g51630	RPL7	Up	IE (4.48)	13.7817	0.001	Up	IE (12.2)	62.584	0.001
LOC_Os12g38000	RPL8	Up	IE (239)	239.0753	0.004	Up	L (13.6)	3.2611	0.001
LOC_Os11g01420	RPL10	Up	IE (143.4)	143.4743	0.01	Up	IE (3.66)	6.4347	0.001
LOC_Os04g50990	RPL11	Up	IE (4.89)	8.8797	0.004	Up	IE (5.23)	9.5285	0.001
LOC_Os02g10540	RPL12	Up	IE (6.5)	280.5248	0.004	Up	E (5.73)	5.7364	0.007
LOC_Os07g01870	RPL13a	Up	E (8.75)	16.1879	0.006	Down	-	-	0.007
LOC_Os03g37970	RPL13b	Up	E (14.7)	14.7666	0.004	Up	IE (3.007)	58.1498	0.005
LOC_Os02g40880	RPL14	down	-	-		Up	IE (9.4)	99.0597	0.007
LOC_Os03g40180	RPL15	Up	E (16.2)	16.2582	0.009	down	-	-	0.038
LOC_Os05g49030	RPL18a	Up	E (18.92)	18.9234	0.006	Up	IE (4.72)	8.0658	0.001
LOC_Os01g67134	RPL18p	Up	IE (3.65)	13.1343	0.014	Up	IE (10.90)	32.9422	0.001
LOC_Os03g21940	RPL19.3	Up	E (30.06)	33.485	0.005	Up	IE (52.83)	52.832	0.009
LOC_Os10g32820	RPL21.2	Down	-	-	0.011	Up	IE (24.72)	41.5009	0.018
LOC_Os09g08430	RPL22	Up	E (4.35)	4.3518	0.009	Down	-	-	0.041
LOC_Os01g24690	RPL23A	Up	IE (6.87)	14.6887	0.166	Up	IE (29.42)	56.2693	0.014
LOC_Os01g24690	RPL24	Up	E (3.02)	12.5439	0.008	Up	IE (4.65)	6.3795	0.026
LOC_Os01g04730	RPL24b	Up	IE (5.64)	17.5887	0.004	Up	IE (3.42)	170.1135	0.028
LOC_Os11g05370	RPL26.1	Down	-	-	0.005	Up	IE (8.11)	14.5404	0.005
LOC_Os02g18380	RPL27.3	Up	IE (12.36)	36.0092	0.004	Down	-	-	0.043
LOC_Os07g36090	RPL28	Up	IE ()4.16	111.9472	0.004	Up	IE (19.3)	164.6033	0.004
LOC_Os06g51530	RPL29	Down	-	-	0.026	Up	IE (29.12)	25.3614	0.007
LOC_Os07g44230	RPL30e	Up	IE 7.89()	63.9518	0.153	Down	-	-	0.014
LOC_Os06g21480	RPL31	Up	IE (3.17)	48.2484	0.005	Up	IE (4.66)	10.0181	0.03

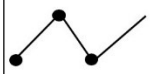
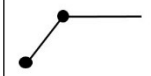
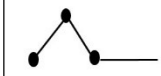
LOC_Os09g32532	RPL32	Up	IE (6.62)	68.5485	0.01	Up	IE (5.65)	39.8725	0.016
LOC_Os09g24690	RPL34	Up	L (21.1)	21.1681	0.005	Up	IE (5.27)	5.2783	0.011
LOC_Os05g48310	RPL35	Up	IE (30.18)	248.9023	0.006	Up	IE (5.13)	16.4339	0.064
LOC_Os01g62350	RPL36	Down	-	-	0.004	Up	IE (4.95)	24.7725	0.009
LOC_Os01g62350	RPL37	down	-	-	0.005	Up	IE (17.42)	58.5086	0.006
LOC_Os11g24610	RPL38	Up	IE (13.9)	12	0.007	Up	IE (64.93)	75.4605	0.0104
LOC_Os07g33898	RPL44	Up	IE (25.8)	2.3058	0.001	Down	-	-	0.022
LOC_Os03g10930	RPL51	Up	IE (4.31)	4.3108	0.001	Up	IE (7.86)	16.2279	0.001
PEG									
LOC_Os03g58204	RPL4	Up	IE (3.01)	11.7354	0.0159	Up	L (6.11)	7.51108	0.023
LOC_Os05g11710	RPL5	Up	L (3.08)	3.0871	0.05	Up	down	-	-
LOC_Os04g39700	RPL6	Up	IE (25.02)	145.159	0.007	Up			0.024
LOC_Os04g51630	RPL7	Up	IE (11.03)	13.9149	0.095	Up	IE (3.7)	8.426	0.015
LOC_Os12g38000	RPL8	Up	IE (28.2)	876.214	0.004	Up	E (4.8)	36.9725	0.004
LOC_Os11g01420	RPL10	Up	E (5.2)	19.0453	0.005	Up	E (4.71)	4.7194	0.006
LOC_Os04g50990	RPL11	Up	IE (26.7)	26.7152	0.003	Up	IE (3.4)	8.0905	0.001
LOC_Os02g10540	RPL12	Up	IE (163.7)	163.7058	0.004	Up	IE (6.7)	36.0333	0.007
LOC_Os07g01870	RPL13a	Up	IE (9.3)	9.3299	0.007	Down	-	-	0.004
LOC_Os03g37970	RPL13b	Up	IE (15.4)	21.2421	0.005	Up	IE (3.7)	22.8814	0.001
LOC_Os02g40880	RPL14	Down	-	-	0.001	Up	IE (8.28)	8.2814	0.001
LOC_Os03g40180	RPL15	Up	IE (5.95)	14.4504	0.011	D	-	-	0.017
LOC_Os05g49030	RPL18a	Up	IE (9.5)	9.5136	0.004	Up	L (5.28)	5.2851	0.001
LOC_Os01g67134	RPL18p	Up	IE (4.91)	5.8181	0.001	Up	IE (9.9)	29.562	0.006
LOC_Os03g21940	RPL19.3	Up	IE (213.1)	213.122	0.001	Up	IE (15.6)	19.6832	0.006
LOC_Os10g32820	RPL21.2	Up	E (4.5)	4.5621	0.008	Up	IE (8.96)	8.9631	0.005
LOC_Os09g08430	RPL22	down	-	-	-	down	-	-	-
LOC_Os01g24690	RPL23A	Up	IE (23.5)	15.7592	0.0109	Up	IE (48.3)	48.3154	0.004
LOC_Os01g24690	RPL24a	Up	IE (7.69)	7.874	0.01	Up	IE (4.025)	4.0253	0.006
LOC_Os01g04730	RPL24b	Up	L (3.82)	3.2808	0.0159	Up	IE (6.2)	13.3898	0.006
LOC_Os11g05370	RPL26.1	Up	IE (10.6)	10.6831	0.004	Up	IE (6.2)	18.2521	0.027
LOC_Os02g18380	RPL27.3	Up	IE (24.05)	24.0152	0.001	Up	L (13.05)	13.0502	0.007
LOC_Os07g36090	RPL28	Up	E (39.2)	39.2292	0.004	Up	IE (9.27)	19.9663	0.012
LOC_Os06g51530	RPL29	Down	-	-	0.006	Up	IE (13.2)	57.5807	0.004
LOC_Os07g44230	RPL30e	Up	IE (22.09)	16.3594	0.008	Down	-	-	0.001
LOC_Os06g21480	RPL31	Up	IE (17.4)	20.6065	0.004	Up	IE (3.45)	7.5798	0.005
LOC_Os09g32532	RPL32	Up	IE (24.98)	34.2632	0.005	Up	IE (4.07)	15.2326	0.005
LOC_Os09g24690	RPL34	Up	IE (5.44)	5.4433	0.005	Up	IE (3.04)	30.2738	0.006
LOC_Os05g48310	RPL35	Up	IE (496.9)	496.906	0.005	Up	IE (5.08)	12.1035	0.012
LOC_Os01g62350	RPL36	Down	-	-	0.006	Up	IE (7.6)	30.7245	0.001
LOC_Os01g62350	RPL37	Up	IE (27.05)	30.054	0.004	Up	IE (17.6)	57.8211	0.016
LOC_Os11g24610	RPL38	Up	IE (21.45)	21.4577	0.003	Up	IE (14.06)	57.0962	0.001
LOC_Os07g33898	RPL44	Up	IE (18.51)	36.3906	0.006	Down	-	-	0.004

LOC_Os03g10930	RPL51	Up	IE (4.51)	6.3857	0.006	Up	IE (14.2)	22.4642	0.009
NaCl									
LOC_Os03g58204	RPL4	Up	E (40.2)	40.2494	0.007	Up	down	-	-
LOC_Os05g11710	RPL5	Up	E (3.3)	5.2903	0.048	Up	down	-	-
LOC_Os04g39700	RPL6	Up	IE (10.2)	127.13	0.005	Up	IE (45.9)	45.979	0.009
LOC_Os04g51630	RPL7	Up	E (6.7)	10.6238	0.028	Up	IE (26.76)	139.0102	0.004
LOC_Os12g38000	RPL8	Up	IE (48.4)	64.8624	0.001	Up	IE (7.06)	9.9032	0.009
LOC_Os11g01420	RPL10	Up	IE (7.8)	60.9375	0.01	Up	L (4.4)	4.4393	0.017
LOC_Os04g50990	RPL11	Up	IE (18.1)	18.1352	0.001	Up	IE (11)	10.2093	0.001
LOC_Os02g10540	RPL12	Up	IE (59.6)	170.3959	0.001	Up	IE (4.3)	7.7927	0.009
LOC_Os07g01870	RPL13a	Up	E (20.5)	20.5578	0.003	Down	-	-	0.005
LOC_Os03g37970	RPL13b	Up	IE (3.02)	10.0838	0.001	Up	IE (4.3)	4.6203	0.006
LOC_Os02g40880	RPL14	Down	-	-	0.011	Up	IE (14.9)	51.4105	0.008
LOC_Os03g40180	RPL15	down	-	-	-	Down	-	-	-
LOC_Os05g49030	RPL18a	down	-	-	-	down	-	-	-
LOC_Os01g67134	RPL18p	Up	IE (18.4)	4.8979	0.001	Up	IE (32.9)	32.9	0.005
LOC_Os03g21940	RPL19.3	Up	IE (48.04)	48.4087	0.001	Up	IE (21.2)	25.5497	0.005
LOC_Os10g32820	RPL21.2	Down	-	-	0.001	Up	IE (11.4)	106.6211	0.018
LOC_Os09g08430	RPL22	Down	-	-	0.009	Down	-	-	0.045
LOC_Os01g24690	RPL23A	Up	IE (6.06)	17.6154	0.0124	Up	IE (41.90	41.9289	0.036
LOC_Os01g24690	RPL24	down	-	-	-	Up	IE (3.45)	4.1919	0.001
LOC_Os01g04730	RPL24b	down	-	-	-	Up	IE (5.1)	11.0093	0.009
LOC_Os11g05370	RPL26.1	Down	-	-	0.045	Up	IE (3.6)	4.1897	0.006
LOC_Os02g18380	RPL27.3	down	-	-	-	down	-	-	-
LOC_Os07g36090	RPL28	Down	-	-	0.009	Up	IE (18.9)	18.9877	0.005
LOC_Os06g51530	RPL29	Down	-	-	0.032	Up	IE (30.5)	30.5064	0.001
LOC_Os07g44230	RPL30e	Down	-	-	0.011	Down	-	-	0.008
LOC_Os06g21480	RPL31	down	-	-	-	Up	IE (6.6)	13.1491	0.001
LOC_Os09g32532	RPL32	Down	-	-	0.006	Down	-	-	0.01
LOC_Os09g24690	RPL34	Down	-	-	0.004	Up	IE (3.3)	3.317	0.005
LOC_Os05g48310	RPL35	Up	IE (3.83)	17.0443	0.243	Up	IE (9.9)	22.742	0.001
LOC_Os01g62350	RPL36	Down	-	-	0.005	Up	IE (18.3)	18.3195	0.001
LOC_Os01g62350	RPL37	Up	IE (3.47)	6.0471	0.001	Up	IE (9.5)	26.1374	0.005
LOC_Os11g24610	RPL38	Down	-	-	0.001	Up	IE (26.05)	69.2948	0.005
LOC_Os07g33898	RPL44	Down	-	-	0.024	Down	-	-	0.001
LOC_Os03g10930	RPL51	down	-	-	-	Up	IE (16.3)	16.4535	0.004

The expression of 34 RPL genes was studied in response to three abiotic treatments (ABA, PEG and NaCl) through Q-PCR. 'Down' indicates a lower abundance and 'Up' indicates a comparatively greater abundance of each RPL gene transcript under every treatment. The relative expression was considered statistically significant at *P* value <0.05 based on one-way

ANOVA in all the analyzed genes. With respect to time-based expression of genes, they were classified as IE: Immediate-early that expressed within 5 min-3 h after treatment; E: early which expressed between 3 h to 12 h and L: late responsive genes that expressed after 12 h of treatment. Under the column 'response', the values in brackets represents the fold change at that time point.

Table 3.5: Overlap in the expression pattern of the IE-RPL genes

Expression Pattern	ABA		PEG		NaCl	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
	**L7**, L24b**, L11, L30,** L31, **L32,** L27, **L23A**	**L6**, L14, L36, L24a, L35, L24b, L26, **L37,** L31, **L32,** L51	**L6**, **L7, L37**, L51, L8, **L12,** L13a, **L13b,** L15, **L18P, L30**, **L24a,** L44	**L6**, **L32,** L36, **L37**, L38, **L11, L12,** L18a, L21.2, **L23A,** L24a, L26	**L6**, L8, **L12,** L13b, L18P, L23, **L37**	**L6**, **L7,** L19.3, L26, L35, L8, **L11, L12**, L36, **L13b**, **L18P,** L14, L28, L34, L29, **L32, L24a,**
	L6, L8, L10, L12, L35	L7, L21.2, L11, **L23A,** L28, L18p, **L19.3**, **L38**	**L19.3**, **L23A,** L35, L32	L13b, L14, L29	**L19.3**	L24b, **L38**
	L4, L5	L29, **L34**	L11, L30, L18a, L38, L26, L2, **L34**	L19.3, L24a, L28, L31, L35	L41, L32	L51

List of IE-RPL genes under different abiotic treatments. Marked in bold are those that are common to any two treatment.

Chapter 4:

Identification of Rice Ribosomal Protein Large Subunit Genes

4.1 Introduction

Ribosomes are tiny (200-300Å) ribonucleoprotein complexes typically existing as two unequal sized subunits in all organisms and constituting 25-30% of total cell mass (Alberts et al., 2002). The ribosome complex, as a whole, performs mRNA-directed protein synthesis. Specific interaction of ribosomal proteins and rRNA with mRNA, tRNA and other non-ribosomal protein cofactors ensure that the process of initiation of protein synthesis, amino acid assembly and termination occurs appropriately in the cells (Maguire et al., 2001). About 65-80% of the total mass of ribosomes accounts for rRNA while ribosomal proteins constitute 10-35% (Ramakrishnan et al., 2001, Yoshihama et al., 2002). The 70S prokaryotic ribosomes consist of large 50S subunit, having 23S rRNA, 5S rRNA, and 30-40 r-proteins, and small 30S subunit contains 16S rRNA with four domains and single copies of 20 r-proteins (Wimberley et al., 2000, Ramakrishnan et al., 2001). Eukaryotic ribosomes have a sedimentation coefficient of 80S with the large 60S subunit having 25S, 5.8S, 5S rRNA, and the small 40S subunit consisting of 18S rRNA (Ben-Shem et al., 2010). The number of RPs in ribosomes varies between organisms, with eukaryotes having up to 80 RPs and prokaryotes possess a total of only 54 RPs in both the subunits (Doudna and Rath, 2002). The presence of additional r-proteins, r-protein extensions, large sized rRNAs and an additional rRNA in the form of expansion segments contributed to the high degree of complexity and increased molecular mass of the eukaryotic ribosomes (Wilson et al., 2012). The r-protein extensions form complex networks with rRNA expansion segments and also with other proteins. Interaction of these specific segments with other proteins through shared beta sheets results in the formation of functional dimeric r-proteins (Grebenyuk et al., 2009, Klinge et al., 2011, Shem et al., 2011).

The ribosomal gene family has more than 200 genes, but less than 100 corresponding RPs are incorporated into the ribosomes in all organisms including yeast, animals and plants (Ban et al., 2000; Barakat et al., 2001; Hanson et al., 2004). This supports the fact that each RP-gene exists as 2-5 identical members with 95-100% nucleotide and predicted protein similarity. An RP synthesized from only one gene copy of a group incorporates into a ribosome under a given condition/tissue (Guarinos et al., 2003; Schuwirth et al., 2005). For example, the *Arabidopsis* genome has 249 genes for 80 RPs (48-large subunit proteins, 32-small subunit proteins) with each gene having 3-4 expressed copies and none exists as a single gene copy (Wool et al., 1996). RPs, in addition to their universal roles of stabilizing the ribosomal complex and mediating polypeptide synthesis, also have extra-ribosomal functions such as their involvement in response to the environmental stresses (Warner et al., 2009; Sormani et al., 2011).). These

are also involved in replication, RNA splicing, chaperone activity, DNA repair, cell growth, cell proliferation, interaction with extra-r-proteins in response to stresses, and so forth (Wool 1996, Semrad et al., 2004, Kovacs et al., 2009, Singh et al., 2009, Warner and McIntosh 2009). In humans, mutations in RP genes have been associated with multiple genetic disorders; reduced synthesis of small subunit proteins, RPS4, and RPS6 was observed in cases of Turner syndrome. RPL6 gene is situated in a locus responsible for Noonan syndrome and has been considered an important candidate for both Noonan and Turner syndrome (Kanimochi et al., 2002).

Mutations in plant RP genes have been implicated in perturbed phenotypes as has been seen in animal systems including humans. Earlier studies with *Arabidopsis* showed that mutations in many RP genes (RPS18A, RPL24B, RPS5B, RPS13B and RPL27A) resulted in a 'pointed first leaf' phenotype characterized by reduced cell division and growth, and genotoxic sensitive plants (Lijsebettens et al., 1994; Revenkova et al., 1999; Ito et al., 2000; Szakonyi and Byrne, 2011). A T-DNA insertion mutation in the *Arabidopsis* AtRPL10 gene caused lethal female gametophytes, while overexpression complemented the same with the recovery of the severe dwarf phenotype that resulted from the disruption of the ACL5 gene (Imai et al., 2008). A transposon insertion mutation in one of the three copies of the AtRPS13A gene resulted in reduced cell division, late flowering, retarded root and leaf growth (Ito et al., 2000). Similar effects of plant growth retardation and reduced fertility were observed after knockdown of AtRPL23aA resulting in reduced synthesis of the RPL23aA protein, while knockout of its paralog, RPL23aB, had no effect on growth (Degenhardt et al., 2008a). RPL23aB is the only RP paralog that did not produce any visible phenotypic defects upon knockout (Degenhardt et al., 2008b).

These RP-gene knockout studies clearly show that although RP genes exist as multiple gene copies, the maximum possible expression of all the gene copies is required for them to be incorporated into the ribosomes during specific stages of growth and development and under certain stress conditions (Schmid et al., 2005; Byrne, 2009). The variation in the composition of ribosomes by the incorporation of RPs derived from identical members could be a major factor in the translational regulation of transcripts in different cell types and under various specific conditions (Giavalisco et al., 2005; Carroll et al., 2008). The change in the composition of RPs upon feeding of *Arabidopsis* leaves with sucrose, further supports the heterogeneity of ribosomes in response to external stimuli (Hummel et al., 2012).

The expression of RP genes has also been shown to be differentially regulated by signaling molecules and environmental stresses. The transcript levels of *Arabidopsis* RPS15a (RPS15aA, C, D and F) were up-regulated in response to phytohormone and heat treatments (Hulm et al., 2005). Similar transcript abundance under BAP treatment was detected for *Arabidopsis* RPS14, RPL13 and RPL30 genes (Cherepneva et al., 2003). Low temperature induced the expression of three RP genes; RPS6, RPS13 and RPL37 in soybean (Kim et al., 2004) and a homologue of RPL13, BnC24 in Brassica and *E. coli* (Sáez-Vásquez et al., 2000; Tanaka et al., 2001). The overexpression of RPL13 also resulted in tolerance against a fungal pathogen, *Verticillium dahliae* in transgenic potato with coordinated up-regulation of genes coding for defense and antioxidant enzymes (Yang et al., 2013) implying that RPs function in stress-response/ tolerance through a network of multiple stress-related genes. In maize and *Arabidopsis*, RPL10A and RPL10C were shown to be significantly up-regulated under UV-B stress (Ferreyra et al., 2010a; Ferreyra et al., 2010b). RPL44 was found to be up-regulated under osmotic stresses, and the overexpression of *Aspergillus glaucus* RPL44 in yeast and tobacco ensured increased tolerance to salt and drought stresses (Liu et al., 2014). The majority of studies on RPs were undertaken in *Arabidopsis* largely because of the availability of insertion mutant lines and smaller genome size.

Despite producing similar/identical proteins, the paralogs of RP genes are differentially expressed under certain conditions. The paralogs of *Arabidopsis RPL23a*; *RPL23aA,* and *RPL23aB* were differentially regulated by cold and heavy metal treatments (McIntosh & Bonham-Smith, 2005). This differential response of two isoforms to a given treatment implies that their site of location in the genome and promoter sequences which might contain regulatory elements determine the amount of transcripts synthesized. Out of 249 RP genes in *Arabidopsis*, about 244 up-regulated in response to phosphate and iron deficiency in roots (Wang et al., 2013). This differential expression is not only specific to cytosolic r-proteins, but also observed in the case of chloroplast and mitochondrial RP genes.

Until now, not much emphasis has been placed on the differential expression patterns of RP genes of rice in response to external stimuli. We had generated a large-scale enhancer based activation-tagged gain-of-function mutant population in *indica* rice, which was screened for water-use efficiency. Among the potential mutants with sustained productivity under prolonged water-limiting conditions, two of them were found to have enhanced expression of large subunit ribosomal genes because of their being tagged by the enhancers (Moin et al.,

2016). This has prompted us to investigate the other rice RPL genes in the context of stress-responsiveness.

In the present study, we describe the genome-wide organization of predicted 123 RPL genes in rice including the individual gene copies. We investigated their overall expression pattern in selected tissues covering the major growth stages of rice. Also, we have provided an overview of their differential expression pattern under biotic and abiotic stress conditions that limit rice productivity. We identified specific RP genes, whose expression is unique or overlapping under native and treated conditions. In summary, the information presented in this study provides a resource for subsequent exploitation of RPL genes to ameliorate abiotic and biotic stress conditions in rice and also other crop plants in future.

4.2. MATERIALS AND METHODS

4.2.1. Nucleotide sequence retrieval of RPL genes

To identify the total members of the large subunit ribosomal gene family, a keyword search using "ribosomal" was performed under the putative function search of Rice Genome Annotation Project Data Base (RGAP-DB v7)[1] and Phytozome v11[2]. The large subunit members were shortlisted by selecting the genes starting with prefix 'L', for large subunit as opposed to 'S' that specifies small subunit genes. A total of 123 RPL genes were identified, and since the number of RPL genes in both the databases was same, the gene sequences were downloaded from RGAP-DB. When further looked for the presence of identical members or copies of each gene in RGAP-DB, we observed that each RPL gene has an average of 2-3 gene copies in the genome. From these 123 genes, we selected 34 candidate genes each representing one orthologous group excluding the identical copies for expression studies. All the identified 123 sequences were also confirmed through nucleotide and protein BLAST search in the NCBI[3] and Hidden Markov Model (HMM) of Pfam[4] databases, respectively. The predicted protein sequences of all the 123 RPL genes were also verified in NCBI conserved domain database[5]. To minimize the missing of potential RPL genes and to ensure that all the identified sequences belong to the ribosomal large subunit gene family, multiple databases were employed (Moin et al., 2016b).

4.2.2. Chromosomal distribution of RPL genes

To determine the chromosomal distribution, the locus number of each of the 123 RPL genes obtained from RGAP-DB was submitted to the OryGenesDB[6]. Based on the output generated

in OryGenesDB, the position of each gene at its corresponding locus on the chromosome was located manually.

4.2.3. RPL gene structures

The structure of each of the 34 RPL genes was determined to study the number and position of introns and exons, GC-content, gene orientation in the genome and alternative splice forms. The full-length sequences of each gene and cDNA were submitted to the Gene Structure Display Server (GSDSv2)[7] to predict the structure.

4.2.4. Protein properties, secondary structure, homology modeling and phylogenetic analysis

The predicted sequences of 34 RPL proteins were obtained from the RGAP-DB and analyzed using an online tool, PSORT[8] to predict the protein properties such as size, molecular weight and isoelectric point (*p*I). The amino acid sequences of these proteins were aligned in ClustalW[9] and submitted to the Molecular Evolutionary Genetic Analysis (MEGAv6)[10] program for constructing an unrooted phylogenetic tree to identify the protein similarities in the RPL family in rice. The domains and motifs in proteins were identified using SMART[11] (Simple Modular Architecture Research Tool). The GRAVY (Grand average of hydropathicity) indices of RPL proteins, which are the determinants of the hydrophobicity of whole protein was calculated using ExPASy ProtParam[12]. The GRAVY values of most of the proteins are usually in the range of +2 to -2, and values in negative range or less than zero indicate that the proteins are hydrophilic in nature (Song et al., 2015).

Although the detailed crystal structure of ribosomal complex has been well-characterized (Ben-Shem et al., 2010), we tried to study the properties of individual ribosomal proteins. To gain an insight into the secondary structure of RPL proteins and to characterize the presence of metal-ligand/ protein/RNA interacting sites, the three-dimensional secondary structures of 34 RPL proteins were predicted using Phyre2[13] program (Protein Homology/AnalogY Recognition Engine v2) (Kelley et al., 2015). Individual protein sequences were submitted in Phyre2 in FASTA format and after studying the properties such as α-helices and β-strands, they were directed to 3DLigandSite[14] to predict the metal/non-metal ligands and their binding sites in each protein.

4.2.5. *In silico* putative promoter analysis of 34 RPL genes

To determine the presence of stress-responsive *cis*-regulatory elements, the nucleotide sequence ≤1kb upstream of each RPL gene was retrieved from RGAP-DB and submitted to the Plant *Cis*-Acting Regulatory Elements[15] database. The location and number of repeats of each *cis*-regulatory sequence in the putative promoter regions of each RPL gene were identified.

4.2.6. Plant material and growth conditions

The seeds of *Oryza sativa* L. sp. *indica* var. Samba Mahsuri (BPT-5204) maintained in greenhouse conditions were surface sterilized with 70% ethanol for 50-60 sec followed by 4% sodium hypochlorite for 20 min. Seeds were then washed thrice with sterile double-distilled water, blot dried and cultured on solid MS medium at 28 ± 2°C and 16 h light/8 h dark photoperiods.

To analyze the native tissue-specific expression pattern of 34 RPL genes at different stages of rice development, samples were collected from 13 different tissues covering major stages of rice growth. After sterilization, seeds were soaked in water in a rotary shaker and after 16 h of incubation, the embryonic portion was manually cut under a stereo-microscope to collect the embryos and endosperm. Some of these seeds were allowed to continue to germinate on MS medium. After 3 d and 6 d of germination, the plumules, radicles, shoot and leaf tissues were collected separately. After two weeks of growth on MS medium, some of the seedlings were transferred to pots containing alluvial soil and grown under greenhouse conditions (30 ± 2°C, 16 h light/8 h dark photoperiods). Plants were amply watered with RO (Reverse Osmosis) purified water up to 3 cm overlay in the pots as required for normal growth of rice. About 45 days after transfer (DAT) to the greenhouse, rice plants were uprooted to collect shoot, root, flag leaf and root-shoot transition tissues. After 60 DAT, flowers, partially filled grains and spikes were collected (Moin et al., 2016b).

4.2.7. Abiotic stress treatments to seedlings

To analyze the differential expression pattern of 34 RPL genes and to distinguish RPL genes that are up/down-regulated under abiotic conditions, 7 d old seedlings were exposed to five different abiotic treatments such as Methyl Jasmonate (MeJa), Salicylic acid (SA), cold stress (4°C), heat stress (42°C) and oxidative stress (H_2O_2). The 7 d old seedlings were dipped in the solutions of 100 µM MeJa (Wang et al., 2007), 3 mM SA (Mitsuhara et al., 2008) and 10 µM H_2O_2. The root and shoot tissues were collected separately at 5 min, 3 h, 6 h, 24 h and 60 h

after treatments. For cold and heat induced stresses, seedlings in water were exposed to 4°C and 42°C (Jami et al., 2012), respectively and root and shoot samples were collected at time intervals as described. Since WT rice seedlings started to wilt after 24 h of exposure to 42°C, heat stress samples were collected up to 24 h only. Seedlings in water at corresponding time intervals served as controls to normalize the expression patterns. Tissue samples were collected as three biological replicates after each time interval (Moin et al., 2016b). The primer details of 34 RPL genes used in the expression analysis were provided in Table 4.1.

Table 4.1: Primers used in gene expression studies

Primer Name	Sequence (5'-3')
RPL3 RT FP	TGGACTTGTGGCCTATGTGA
RPL3 RT RP	CCGGCATCGCTATCATACTT
RPL4 RT FP	AAGAAGCTCGACGAGGTGTA
RPL4 RT RP	CCACATTCTTCAGAGGGTTC
RPL5 RT FP	GATCTTGGCATCAAGTACGAC
RPL5 RT RP	GACACCCTCATACTTGACCTG
RPL6 RT FP	GTTCCTCAAGCAGCTCAAAT
RPL6 RT RP	CTTCTGCTTCTTGTCCCTAGA
RPL7 RT FP	TACCCAAACCTGAAGAGTGTC
RPL7 RT RP	GACAGTCATGATCTCGTGGA
RPL8 RT FP	ACTACGCCATCGTCATCAG
RPL8 RT RP	GGTACTTGTGGTAGGCGTTT
RPL10 RT FP	AGAAGAAGCCTGGATTAGAGC
RPL10 RT RP	ATATCCTGCTGGAGGACTTG
RPL11 RT FP	AAGAAGATCGGTGAGGACATC
RPL11 RT RP	TCTTGACCTTCTTCCTGTCC
RPL12 RT FP	GCTCATTTGTACAGCACAGAG
RPL12 RT RP	TTGGTTCAGTCTGAGAAGGAG
RPL13a RT FP	GAACTACCACGACACCATCAG
RPL13a RT RP	GGGGCCAAAATATCTATCTG
RPL13b RT FP	AAGCACTGGCAGAACTATGTC
RPL13b RT RP	CCCTCGACTTCATGTTGTACT
RPL14 RT FP	GTGAACTACGGCAAGGACTAC

RPL14 RT RP	TAACATCAGCCTCCTCCATAG
RPL15 RT FP	ACAAGTACGTGTCGGAGCTAT
RPL15 RT RP	GACACGGTAAACCACATAACC
RPL18a RT FP	TCCAAGTTCTGGTACTTCCTG
RPL18a RT RP	GTTGTGGTAACCTGTTCTGCT
RPL18p RT FP	TGGGGAGGACTACTATGTTGA
RPL18p RT RP	AAACCTCTTGTCACTGTGAGG
RPL19.3 RT FP	AGTATCGTGAGGCCAAGAAG
RPL19.3 RT RP	CTTAGCCTCAAACTGGTCAGA
RPL21.2 RT FP	CTGAGGAAGATCAAGAACGAC
RPL21.2 RT RP	AACCACCCTTGAGATCATTG
RPL22 RT FP	GAGGTGAAAGGTCTGGATGTT
RPL22 RT RP	TCACTGGTTCTTCCTTCTCTG
RPL23A RT FP	GACCAAAGACCCTGAAGAAGG
RPL23A RT RP	ACGATGAAGACAAGGGTGTTG
RPL24b RT FP	GTTGGTGCTACACTGGAAGTT
RPL24b RT RP	CCTTCGACTGTGTCTTCTGAG
RPL26.1 RT FP	ACAAGTACAACGTGGTGAGG
RPL26.1 RT RP	GTCCTTGTCGAGCTTGAGTT
RPL27.3 RT FP	CTTCCTCAAGCTCGTCAACT
RPL27.3 RT RP	CTTGGTGAAGAACCACCTGT
RPL28 RT FP	TAGACGAATACCTCCTGAAGA
RPL28 RT RP	AAACCCTGTTCGATCTTAGTC
RPL29 RT FP	CCCAACAAGCTCTCCAATATA
RPL29 RT RP	AGAAACAGAAGCATTCCCTG
RPL30e RT FP	GAGCAAGAAGAAGAACAAGTC
RPL30e RT RP	GCTTCATCCATATCTTTTCCG
RPL31 RT FP	TCAAGGAGATCAGGAAGTTTG
RPL31 RT RP	AACAGTGACCAGAGAGTAGAG
RPL32 RT FP	GCCTAATATTGGCTATGGTTC
RPL32 RT RP	CTTCTTCGTTGAGACATTGTG
RPL34 RT FP	GAAGAAGATCCAGGGAATTCC

RPL34 RT RP	CACAATCTTCTGCTCTTCAAC
RPL35a.3 RT FP	CTACGTCTACAAGGCCAAG
RPL35a.3 RT RP	TGCTGGGGTACATGAAGA
RPL36.2 RT FP	GGAAAAGTACCAAGAGAGTGA
RPL36.2 RT RP	CTTCTTCTTTGCTCTCTTGTG
RPL37 RT FP	CTTCCACCTGCAGAAGAG
RPL37 RT RP	CCCCTCTCTGAAGTTACTCT
RPL38 RT FP	CACGAGATCAAGGACTTCC
RPL38 RT RP	AAAGGTGGATGAAATGTAGGC
RPL44 RT FP	AAGAAGACCTACTGCAAGAAC
RPL44 RT RP	CCTTACCCTTCTTGTACTGAG
RPL51 RT FP	GTGACAGAGTTAGTCCGTGGA
RPL51 RT RP	TCTCAGCTTCACCACTTTCCT

4.2.8. Biotic stress treatment

To check the expression pattern of rice ribosomal genes in response to biotic stress, we used the bacterial pathogen *Xanthomonas oryzae* pv. o*ryzae* (*Xoo*) that causes Bacterial Leaf Blight (BLB) of rice, which is one of the most severe yield constraints of rice worldwide (Sundaram et al., 2015). At the seedling stage, the infected leaves start to roll-up, and as the disease progresses, the leaves turn yellow and wilt, leading to drying up and death. This drastically reduces the total seed yield of the plant. The yield loss may be as high as 70% when plants are grown in conditions favorable to the disease (Ryan et al., 2011). The bacterial suspension of *Xoo* was applied on the leaves of two-month-old plants grown under greenhouse conditions, and leaf samples were collected after 11 d of infection. Leaf samples of untreated plants grown under similar conditions were used as a control to normalize the expression (Moin et al., 2016b).

Because the transcript level of RPL10 was significantly up-regulated 11 d after treatment, we selected this gene in particular to analyze its expression at successive time points such as 3 h, 6 h, 1 d, 2 d, 3 d, up to 11 d postinfection of rice leaves with *Xoo* pathogen. The qRT-PCR was performed with *Xoo* treated and untreated samples collected as three biological and three technical replicates. Rice specific *act1* and β-*tub* genes were used as controls for normalization,

and the mean of the fold change was represented as bar diagrams constructed using SigmaPlot v11.

4.2.9. RNA isolation, cDNA synthesis and Quantitative-PCR (qRT-PCR)

Total RNA was isolated from stress-treated and untreated tissues using TriReagent (Takara Bio, UK) following the manufacturer's protocol. The quality of extracted RNA was checked on 1.2% agarose gel prepared in TBE (Tris-borate-EDTA) buffer and quantified using Nanodrop. Total RNA (2 µg) was used to synthesize the first strand cDNA using reverse transcriptase (Takara Bio, UK). The cDNA was diluted in 1:7 proportions and 2 µl of it was used in qRT-PCR. Primers specific for each RPL gene sequence retrieved from RGAP-DB was designed using the primer-3[16] online tool, and 10 µM of each was used per reaction. The reaction conditions for qRT-PCR included an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, an appropriate annealing temperature for 25 sec and an extension of 72°C for 30 sec. At the end of the reaction, a melting curve step was inserted to analyze the specificity of amplification of each gene. Rice specific actin (*act1*) and tubulin (β-*tub*) were used as internal reference genes to normalize the expression patterns. The mean values of relative fold change, which was calculated as per $\Delta\Delta C_T$ method (Livak et al., 2001) obtained from each reference gene was considered as the final fold change in the transcript levels. Each qRT-PCR reaction was performed as three biological and three technical replicates.

The relative fold change of the 34 genes in 13 tissues and under five abiotic treatments was illustrated in the form of heat maps. A dendrogram was constructed to represent the Hierarchical clustering of relative fold change of 34 genes under each treatment using the GENE-E[17] program.

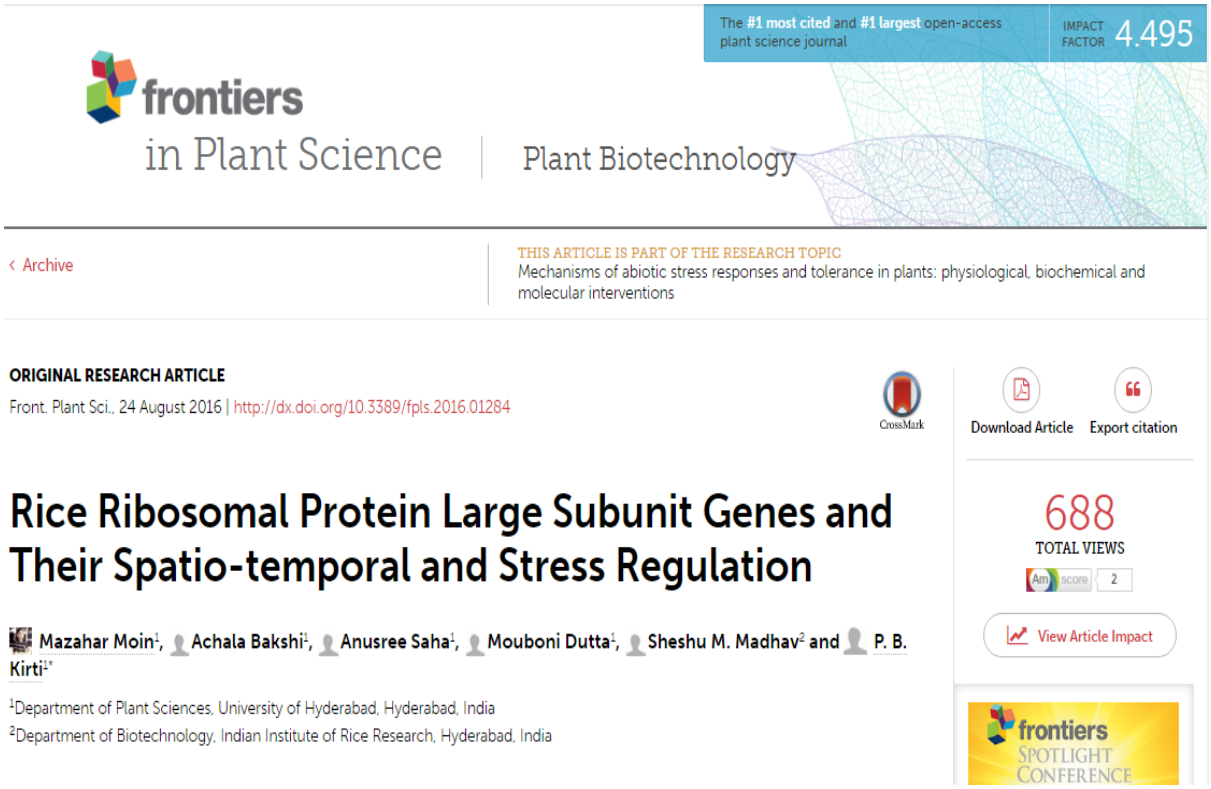
4.2.10. Various web-links employed in the study

1. http://rice.plantbiology.msu.edu/index.shtml
2. https://phytozome.jgi.doe.gov/pz/portal.html
3. http://blast.ncbi.nlm.nih.gov/Blast.cgi
4. http://pfam.xfam.org/
5. http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
6. http://orygenesdb.cirad.fr/tools.html
7. http://gsds.cbi.pku.edu.cn/
8. http://psort.hgc.jp/form.html

9. http://www.genome.jp/tools/clustalw/
10. http://www.megasoftware.net/
11. http://smart.embl-heidelberg.de/
12. http://web.expasy.org/cgi-bin/protparam/protparam
13. http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
14. http://www.sbg.bio.ic.ac.uk/3dligandsite/
15. http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
16. http://bioinfo.ut.ee/primer3-0.4.0/
17. http://www.broadinstitute.org/cancer/software/GENE-E/
18. http://rapdb.dna.affrc.go.jp/

Chapter 5:

Rice Ribosomal Protein Large Subunit Genes are Stress and Developmentally Regulated



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

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5.1. Genome-wide identification and chromosomal distribution of RPL genes

To explore the cytoplasmic large subunit (60S) ribosomal gene family members in rice, we used a keyword search "ribosomal" in the putative function search of RGAP-DB and Phytozome databases, which resulted in the identification of 428 and 754 genes, respectively and these included genes belonging to cytoplasmic 60S and 40S subunits and 50S and 30S subunits of chloroplast and mitochondrial ribosomes. Keyword search and homology-based identification through HMM are widely used practices in identifying genome-wide copies of the annotated genes (Kapoor et al., 2008; Liang et al., 2016). We then searched for genes starting with the prefix 'L' to select large subunit genes. This process excluded small subunit genes and identified 215 genes that included large subunit members of cytoplasmic (60S) and chloroplast and mitochondrial (50S) ribosomal subunits. We then shortlisted the cytoplasmic 60S subunit genes by their putative cellular localization using the information available in RGAP-DB. A similar process was applied in shortlisting the 60S subunit genes from Phytozome. Both these approaches identified 123 genes belonging to the cytoplasmic 60S subunit. Each of these genes was then confirmed by a BLAST search of their nucleotide and predicted amino acid sequences in other rice databases like RAP-DB[18] and OryGenesDB. BLASTn and BLASTp results in NCBI and HMM of Pfam and NCBI conserved domain databases, respectively, further confirmed that these genes belong to the 60S ribosomal family by the presence of ribosomal domains.

The locus numbers of 123 genes were submitted in OryGenesDB and, based on the output generated, the location of each gene on the corresponding chromosome was mapped manually using OryGenesDB. The location of these 123 genes was found on all chromosomes, indicating their wide distribution throughout the rice genome. Chromosome-7 has 19; chromosome-1, being the largest of rice chromosomes has 18; chromosome-2 has 16; chromosome-5 has 14 and chromosome-3 showed 13 genes. Chromosomes-9, 10 and 11 exhibited four genes each, while chromosomes-4, 8 and 9 evidenced 5, 10 and 9 RPL genes, respectively (Fig. 5.1). The nucleotide sequence alignment of genes within an orthologous group exhibited 100% similarity, but their chromosomal locations are different. We selected the 34 candidate genes, each representing one orthologous group for a detailed characterization to understand their gene and protein structures, and comprehensive expression analysis in response to a wide range of stress treatments.

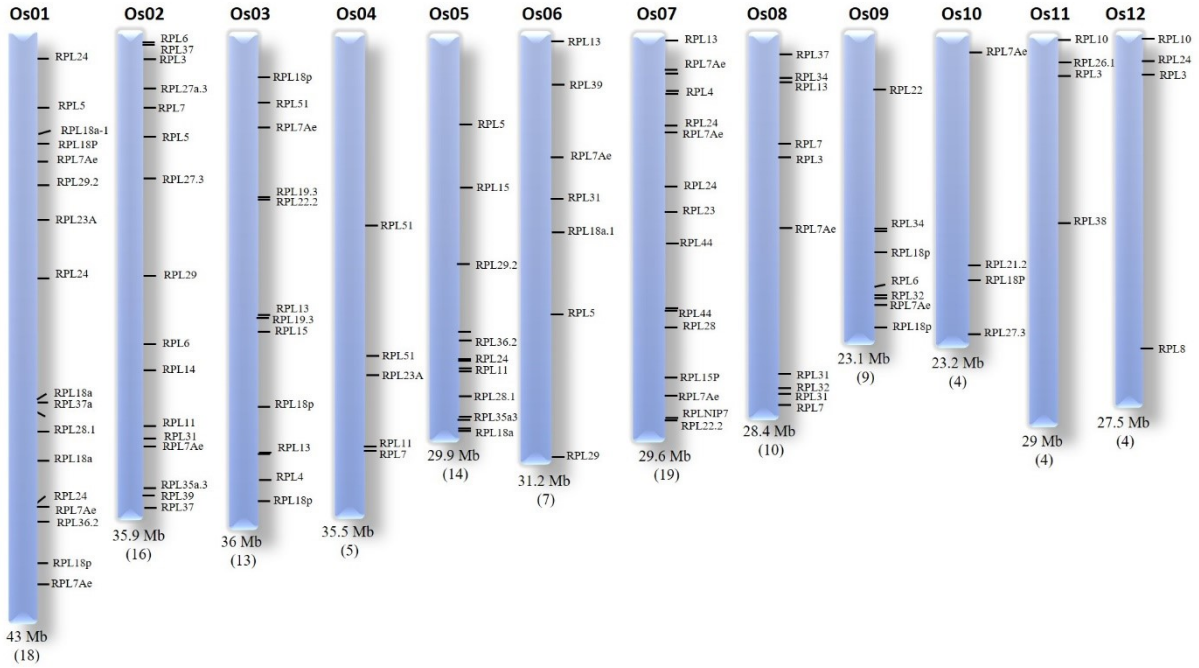


Fig. 5.1: Chromosomal organization of RPL genes

The chromosomal number and size is represented at the top and bottom of each chromosome, respectively. The number of RPL genes is given within brackets at the bottom of each corresponding chromosome.

5.2. Analysis of RPL gene structures

A comparative study between 34 RPL genes was performed to determine the number and position of introns and exons, GC-content and 5' and 3' untranslated regions. The number of introns varied from none to seven. The RPL10 and RPL18p have the highest number of introns (7), whereas RPL4, 8, 21.2, 23A, 26.1, 28, and 31 have only one intron. RPL7, 11, 24b, 27.3 and 38.2 do not contain any introns in their coding regions; these genes also have high GC-content. For example, RPL24b, 27.3 and 38 have 54%, 60% and 51% GC-content, respectively. Genes with introns are reported to be crucial for gene expression, and a high number of introns is linked with increased expression of a gene (Callis and Virginia, 1987; Karve et al., 2011). We observed that RPL18p and RPL10 with seven introns exhibited high expression in various tissues studied compared with other RPL members. Introns when particularly present at the start site or 5' end of a gene have an enhanced ability for expression (Callis and Virginia, 1987; Donath et al., 1995). RPL35a.3, 51, 32, 30e, 22, 19.3, 18a, 10 and 5 have their first introns within 500 bp regions from the transcription start site, which might be the reason for their constitutive expression in almost all the tissues studied. Similarly, the number of exons also

varied among 34 RPL genes. Genes like RPL10 and RPL18p have the highest number of exons (8), whereas RPL7, 11, 24b, 27.3 and 38.2 have only one exon. Some of these RPL genes (RPL4, 13a, 14, 15, 23A, 28, 30e, 32, 35a and 51) undergo alternative splicing to produce 2-5 splice-variants (Table 5.1) with similar nucleotide sequence. For expression analysis, we used the sequence of only one variant of a gene as it is difficult to design the primers for alternative splice forms exhibiting high nucleotide similarity. In addition, the 5' and 3' UTRs of these genes varied in size and positions. RPL28 has an unusually long UTR of 2.5 kb at the 3' end (Fig. 5.2). Table 5.1 provides detailed information about their gene structure, site of location in the genome and copy number of individual genes.

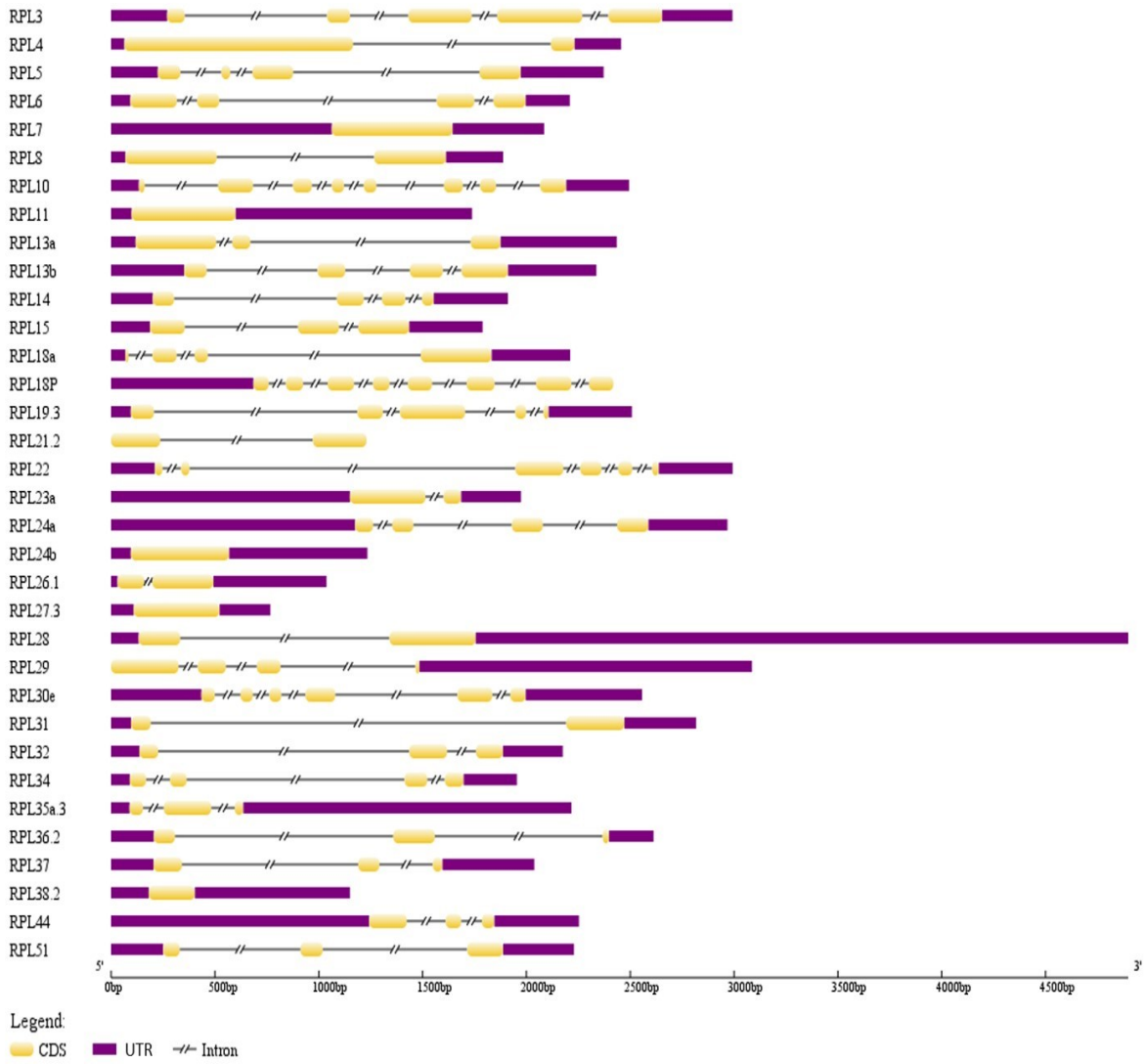


Fig. 5.2: Representation of structures of 34 RPL genes

The yellow and blue boxes represent exon and UTR regions, respectively whereas introns are indicated as the split line.

Table 5.1: Details of rice RPL genes

Gene Id	Chromosome number	Location (bp)	Protein name	Gene Structure						Gene copies
				Gene Size (bp)	GC (%)	Introns	Exons	Alternative splice forms	Orientation	
LOC_Os11g06750	Os11	3294148-3297139	RPL3	2991	46%	5	5		5'-3'	2
LOC_Os03g58204.1	Os03	33143597-33146052	RPL4	2456	41%	2	2	2	3'-5'	3
LOC_Os05g11710.1	Os05	6649548-6651919	RPL5	2372	43%	4	4		5'-3'	5
LOC_Os04g39700.1	Os04	23660833-23663041	RPL6	2209	44%	3	4		3'-5'	2
LOC_Os02g10540.1	Os02	5550032-5552116	RPL7/L12	2085	46%		1		3'-5'	5
LOC_Os12g38000.1	Os12	23349992-23351879	RPL8	1888	49%	1	2		5'-3'	1
LOC_Os11g01420.1	Os11	252426-254919	RPL10	2494	41%	7	8		3'-5'	2
LOC_Os04g50990.1	Os04	30180315-30182052	RPL11	1738	44%		1		5'-3'	2
LOC_Os07g01870.1	Os07	523361-525795	RPL13a	2435	47%	2	3	2	3'-5'	4
LOC_Os03g37970.1	Os03	21086229-21088565	RPL13b	2337	46%	4	4		3'-5'	3
LOC_Os02g40880.1	Os02	24777727-24779637	RPL14	1911	42%	4	4	2	3'-5'	2
LOC_Os03g40180.1	Os03	22336536-22338324	RPL15	1789	48%	3	3	2	3'-5'	4
LOC_Os05g49030.1	Os05	28118285-28120495	RPL18a	2211	43%	3	3		5'-3'	3
LOC_Os01g67134.1	Os01	38973740-38976158	RPL18p/L5e	2419	43%	7	8		3'-5'	2
LOC_Os03g21940.1	Os03	12542185-12544692	RPL19.3	2508	43%	4	5		3'-5'	3
LOC_Os10g32820.1	Os10	17184530-17185759	RPL21.2	1230	46%	1	2		3'-5'	2
LOC_Os09g08430.1	Os09	4390880-4393872	RPL22	2993	41%	6	6		3'-5'	2
LOC_Os01g24690.1	Os01	13896291-13898264	L23A	1974	41%	2	2	3	5'-3'	2
LOC_Os01g59990.1	Os01	34682518-34685485	RPL24a	2968	42%	5	5		5'-3'	4
LOC_Os01g04730.1	Os01	2137763-2138996	RPL24b	1234	54%		1		5'-3'	3
LOC_Os11g05370.1	Os11	2382184-2383220	RPL26.1	1037	52%	1	2		3'-5'	3
LOC_Os02g18380.1	Os02	10700750-10701516	RPL27.3	767	60%	-	1		3'-5'	2
LOC_Os07g36090.3	Os07	21574435-21579332	RPL28	4898	42%	3	2	3	3'-5'	1
LOC_Os06g51530.1	Os06	31223501-31226586	RPL29	3086	46%	7	4		3'-5'	7
LOC_Os07g44230.1	Os07	26432701-26435257	RPL30e	2557	42%	6	6	2	3'-5'	1
LOC_Os06g21480.1	Os06	12412525-12415341	RPL31	2817	43%	1	2		5'-3'	4

LOC_Os09g32532.1	Os09	19418910-19421084	RPL32	2175	42%	3	3	2	3'-5'	4
LOC_Os09g24690.1	Os09	14691168-14693121	RPL34	1954	41%	3	4		3'-5'	3
LOC_Os05g48310.1	Os05	27695307-27697523	RPL35a.3	2217	46%	3	3	2	5'-3'	3
LOC_Os01g62350.1	Os01	36083051-36085662	RPL36.2	2612	38%	3	3		3'-5'	2
LOC_Os02g56990.1	Os02	34918111-34920148	RPL37	2038	44%	2	3		5'-3'	3
LOC_Os11g24610.1	Os11	14044207-14045356	RPL38	1150	516 %		1		5'-3'	4
LOC_Os07g33898.1	Os07	20273588-20275840	RPL44	2253	42%	3	3		5'-3'	11
LOC_Os03g10930.2	Os03	5613250-5615444	RPL51	2229	43%	3	3	2	3'-5'	1

Table 5.1: RPL gene sequences were retrieved from RGAP-DB. Gene properties such as size, GC content, orientation in the genome, chromosomal distribution, copy number of each gene, number of introns and exons were studied in the OryGenesDB.

5.3. Domain recognition, ligand binding sites and phylogeny of rice RPL proteins

Among the 34 proteins, RPL4 is the largest with a predicted molecular mass of 44.5 kDa. All the RPL proteins have similar isoelectric points ranging from 9.5-12. The maintenance of similar *p*I values in RPL proteins might be to reduce coulombic repulsions as these proteins are interactive in nature. They have a varied percentage of α, β and distorted regions with RPL proteins interacting with other r– and non-r proteins exhibiting high content of α-helices. For example, RPL7, which interacts with elongation factors-Tu and -G, has 55% α-helical structure. RPL13a, existing at the interface of RPL3 has 57% of α-helix content, whereas RPL29 that interacts with RPL23A and initiation factors has 48% α-helix content. Furthermore, all the RPL proteins have a GRAVY value <0, which indicate their high hydrophilicity. The proteins with high hydrophilic nature tend to undergo conformational changes and form flexible structures with other molecules and also contribute towards inducing tolerance during stress conditions (Fuxreiter et al., 2004; Liang et al., 2016).

All the RPs are characterized by the presence of ribosomal domain(s). They also have several other domains that participate in interaction with other proteins. RPL 14, 19.3, 21.2, 24b, 26 and 27 have KOW-SH3 motifs at their N-terminal regions, which are involved in protein-protein interactions. KOW-motifs link RPs with transcription factors (Kyrpides et al., 1996). RPL18p (135-226 amino acids) has an FCD domain (FadR C-terminal Domain) that is involved in the regulation of transcription of genes. RPL18p also has a XPGN domain (208-281 amino

acids) that is associated with cancer and Xeroderma pigmentosum in humans. RPL21.2 has cheY motif at the C-terminus activated by phosphorylation through histidine kinases. RPL22 has a DUF1087 domain (amino acids 1-67) that is involved in chromatin remodelling and a WWE domain that is associated with poly-ADP-ribosylation and ubiquitin-mediated proteolysis. RPL29 has a carboxyl-terminal domain (CTD) and proteins with such domains are related to pre-mRNA processing by binding with mRNA capping enzymes (Schwer et al., 2009). It also has RQC, a DNA binding domain found in RecQ helicases.

Ligand-mediated signal transmission is essential for proper functioning of a majority of proteins including certain RPs. However, recognition of the core structures or amino acids involved in protein-ligand interactions is of paramount importance for understanding the dynamic and kinetic properties of the proteins. Analysis of RPL proteins for the presence of sites for ligand binding reveals that out of 34, 20 RPL proteins have sites for binding with ligands (that include metals ions and cofactors) whereas no such ligand binding sites were observed in 14 proteins (RPL3, 4, 6, 10, 11, 13b, 14, 15, 18a, 24a, 29, 34, 36 and 37). RPL8 (Lys198), 13a (Pro114, 115), 19.3 (Glu179, Arg180), 21.2 (Arg70), 22 (Ile52), 24b (Leu118, Lys121, Ala122), 26 (Leu105), 28 (Tyr49), 38 (Lys35) and 44 (Gly51) have Mg^{+2} ion binding sites. RPL7, 31 and 51 bind with Cu^{+2} and RPL5 (Asp30, Thr33) and 23A (Lys104) have sites for binding with Ca^{2+} ions. RPL19, 35, 38 and 51 also have Zn^{2+} binding sites. RPL29 present at the interface of RPL23A binds with the cofactor FAD. The RPL35 binds with cofactors FUC and FAD. Metal ions in RPs serve important biological functions by interacting with nucleic acids, particularly RNA. Non-metal ligands are cofactors involved in catalytic activities. Cofactors in RPs ensure that the processes of protein initiation, amino acid assembly and termination are correctly undertaken (Maguire et al., 2001). Because of this, RPs might have binding sites for both metal and non-metal ligands. The details of the protein properties such as size, *p*I, the percentage of α-helices and β-sheets, GRAVY indices, the presence of metal/non-metal ligands and their binding sites are detailed in Table 5.2. The secondary structures of selected RPL proteins with ligand binding properties are represented in Fig. 5.3.

Table 5.2: RPL protein properties

Protein type	Length (aa)	MW (kDa)	p*I*	LCR	Disordered protein (%)	Alpha helix (%)	Beta-Strand (%)	Ligands	Ligand Binding residues	GRAVY	Specific interactions
RPL3	390	44.4	10.73	one (76-87)	32	12	15			-0.616	

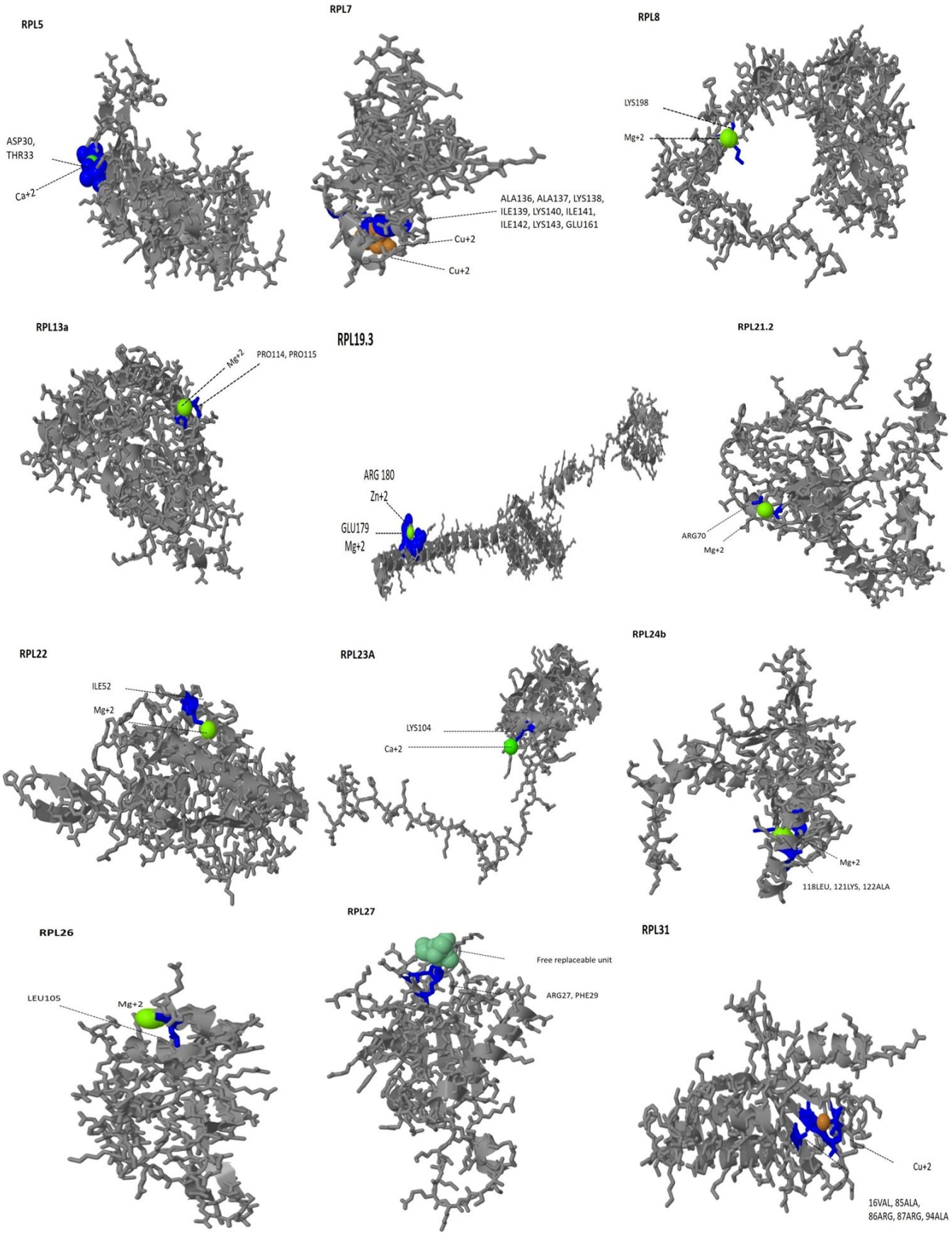
RPL4	405	44.48	11.21	two (126-142) (339-35)	36	45	8			-0.342	
RPL5	183	20.8	10.48	one (135-149)	16	30	25	Ca+2	ASP30, THR33	-0.499	
RPL6	223	24.6	10.8	35-56	41	39	16			-0.532	IF-2
RPL7/L12	194	20.7	9.5	three (106-123, 135-143, 173-186)	45	55	6	two Cu+2	ALA136, ALA137, LYS138, ILE139, LYS140, ILE141, ILE142, LYS143, GLU161	-0.230	EF-Tu, EF-G
RPL8	262	28.2	11.5	239-260	39	5	39	Mg+2	LYS198	-0.498	
RPL10	234	26.5	9.3		15	35	26			-0.573	L12 interface
RPL11	167	17.7	10		29	41	22			-0.228	L7/L12 interface, L26 interface
RPL13a	207	23.7	11.12	159-177	17	57	11	Mg+2	PRO114, PRO115	-0.472	RPL3 interface
RPL13b	204	24.05	11.55	two (27-42, 194-208)	34	54	1			-0.911	
RPL14	135	15.3	11.01		15	43	31			-0.313	
RPL15	205	24.33	12.05	58-78	32	40	19			-1.042	
RPL18a	179	21.45	11.12		21	26	38			-0.749	
RPL18p/L5e	302	34.3	9.48	15-28, 158-169	32	42	12			-0.812	RPL5 interface
RPL19.3	204	24.14	11.95	two (58-67, 94-107)	32	67	3	Zn+2, Mg+2	GLU179, ARG180	-1.058	
RPL21.2	165	18.7	11.21		33	20	35	Mg+2	ARG70	-0.687	
RPL22	172	19.48	10.9	153-163	29	34	16	Mg+2	ILE52	-0.818	
L23A	153	17.05	11.01	two (24-41, 95-111)	36	34	20	Ca+2	LYS104	-0.671	
RPL24a	163	18.45	11.48	two (116-138, 149-161)	25	68	9			-1.058	RPL3, RPL14 interface
RPL24b	158	17.47	11.71	three (102-115, 119-133, 135-151)	45	29	25	Mg+2	LEU118, LYS121, ALA122	-0.625	
RPL26.1	141	15.5	11.7	two (89-102, 127-134)	44	36	26	Mg+2	LEU105	-0.606	

RPL27.3	138	15.6	11.19	one (107-117)	29	30	31		ARG27, PHE29	-0.377	
RPL28	205	23.06	10.58	one (160-178)	33	52	8	Mg+2	TYR49	-0.377	
RPL29	198	21.08	12.93	three (26-89, 90-110, 155-192)	40	48	16	Mo, FAD,		-0.422	RPL23A interface, Signal recognition particle interaction site, trigger factor interaction site
RPL30e	190	20.42	10.25	two (80-94, 103-115)	49	64	7			-0.124	
RPL31	125	14.23	10.65	one (74-88)	30	23	35	Cu+2	16VAL, 85ALA, 86ARG, 87ARG, 94ALA	-0.824	
RPL32	134	15.67	11.2	one (8-20)	31	24	19			-0.745	
RPL34	130	13.67	11.94	one (96-111)	32	32	15			-0.913	
RPL35a.3	112	12.66	11.25		23	10	45	Zn+2, FUC, FAD	52ARG, 72TRP, 74LYS	-0.699	
RPL36.2	111	12.42	12.016		35	65				-0.904	
RPL37	96	10.76	12.2		53	54	3			-1.028	
RPL38	75	8.7	10.6		16	30	42	Zn+2, Mg+2, AMP, CTP	24ARG, 35LYS, 71LEU	-0.430	
RPL44	106	12.13	10.94	one (60-67)	32	32	15	Mg+2	GLY51	-1.272	
RPL51	130	13.72	11.22		17	25	33	Zn+2, Cu+2	PRO35	-0.461	

To evaluate the evolutionary relationships within the RPL protein family of rice, three phylogenetic trees were constructed using full-length amino acid sequences and amino acid sequences derived from ribosomal domains and Low Complexity Regions (LCR) (Fig. 5.4). The unrooted phylogenetic tree was constructed by using a 'neighbour joining algorithm' with a bootstrap value of 1000. The homologous proteins having significant bootstrap value (>95%) were considered as having the highest similarity with respect to others.

The phylogenetic tree of full-length RPL proteins was divided into four clades or groups (A, B, C and D). The RPL proteins, RPL24b and 26.1 have the highest similarity indicating that these two genes might have become duplicated recently in the rice genome. The phylogenetic relationship has also been used for gene function identification and the proteins with the highest similarity perhaps exhibit similar functions and expression patterns (Lijavetzky et al., 2013). The expression of RPL24b and RPL26.1 was similar in shoots indicating their similar roles in shoot growth and development; their expression was also similar in oxidative stress indicating functional similarity.

The phylogenetic trees of two separate domains, the ribosomal domain and LCR were also divided into groups to check domain-wise similarity. The ribosomal-domain analysis showed maximum sequence similarity between RPL24b and 26, which exhibited similarity with RPL18a and 19.3 proteins. RPL18a and 19.3 showed similar expression patterns in different tissues like spikes, endosperm, plumules, radicles, 45 d shoot and root tissues and root-shoot transition indicating their functional similarity during growth and development. All the RPL proteins except RPL10, 11, 14, 21.2, 35, 36, 37, 38 and 51 have predicted LCR. The phylogenetic analysis of LCR showed that RPL6 and RPL26 exhibited the maximum sequence similarity followed by RPL5 and 31 and RPL27 and RPL8, whereas RPL3 formed a separate clade, which showed its possible divergence from the other RPL proteins.


RPL5
ASP30,
THR33
Ca+2
RPL7
ALA136, ALA137, LYS138,
ILE139, LYS140, ILE141,
ILE142, LYS143, GLU161
Cu+2
Cu+2
RPL8
LYS198
Mg+2
RPL13a
Mg+2
PRO114, PRO115
RPL19.3
ARG 180
Zn+2
GLU179
Mg+2
RPL21.2
ARG70
Mg+2
RPL22
ILE52
Mg+2
RPL23A
LYS104
Ca+2
RPL24b
Mg+2
118LEU, 121LYS, 122ALA
RPL26
LEU105
Mg+2
RPL27
Free replaceable unit
ARG27, PHE29
RPL31
Cu+2
16VAL, 85ALA,
86ARG, 87ARG, 94ALA

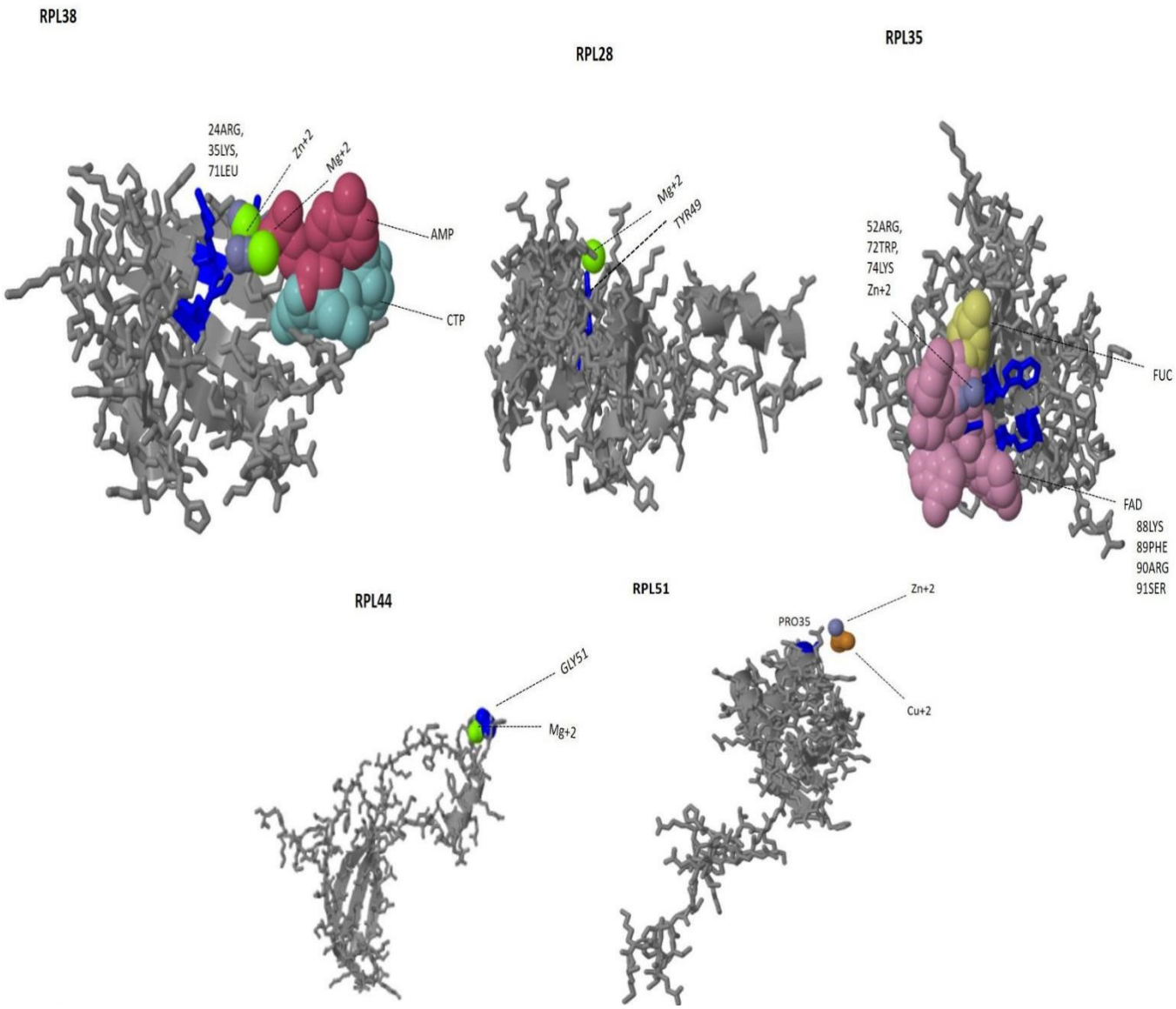


Fig. 5.3: RPL protein predicted secondary structures and ligand binding sites

The secondary structures of selected 17 RPL proteins with predicted ligand binding sites. The metal ligands or cofactors are represented with different colored balls followed by amino acids involved in ligand binding indicated as split lines.

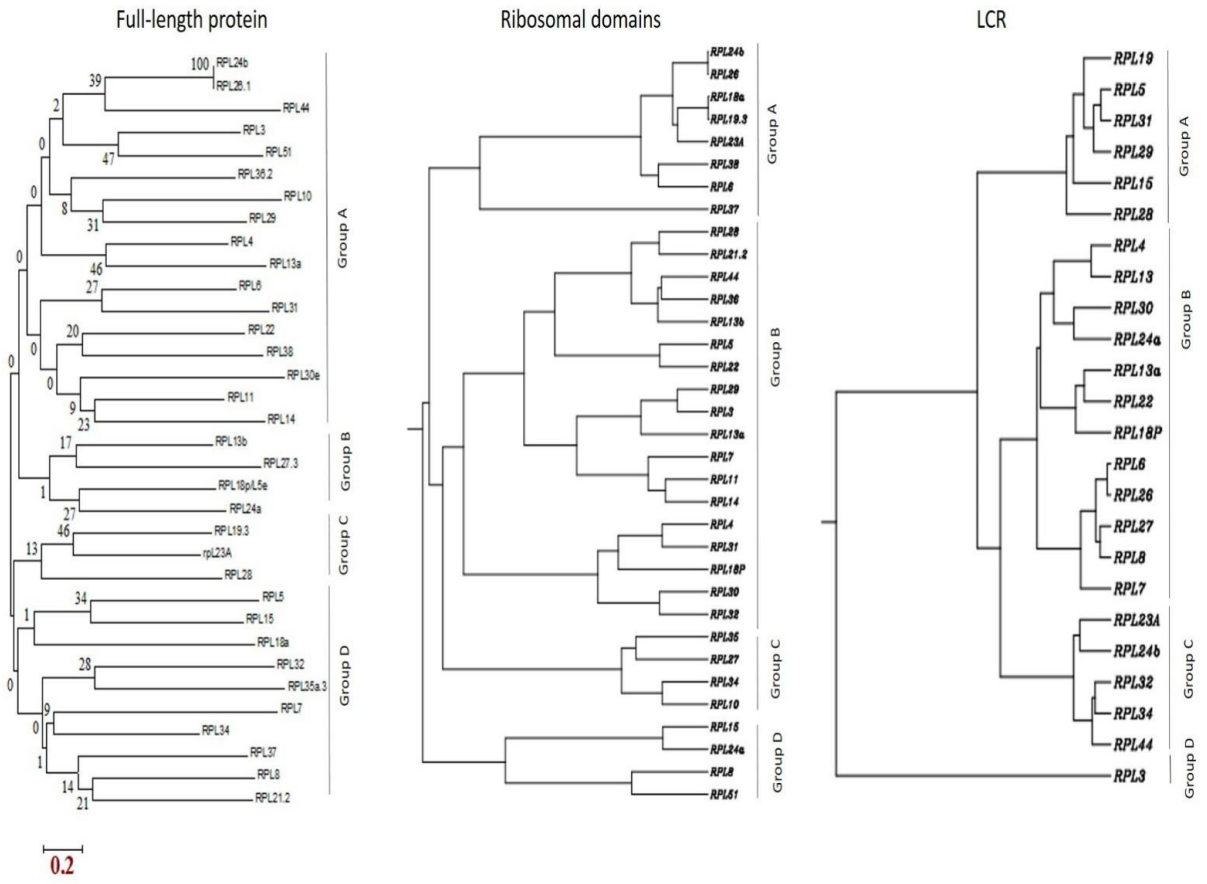


Fig. 5.4: Phylogenetic relations within RPL proteins

Three phylogenetic trees were constructed using full-length amino acid sequences and amino acid sequences derived from ribosomal domains and Low Complexity Regions (LCR). The unrooted phylogenetic tree was constructed by using a 'neighbour joining algorithm' with a bootstrap value of 1000.

5.4. *In silico* analysis of putative promoter regions of rice RPL genes

The expression studies showed that many RPL genes are differentially regulated in various tissues and under various abiotic treatments. To assess whether this differential regulation is due to the presence of stress or signal-responsive elements in their regulatory regions, nucleotide sequences ≤1kb upstream to each of the 34 genes were retrieved and searched using the PlantCARE database. This analysis resulted in the identification of multiple stress-responsive elements in the putative promoter regions of all the genes. Abiotic stress-responsive elements that are associated with heat and cold temperatures such as HSE (Heat Stress Elements) and LTR (Low-Temperature Response) and dehydration stress such as MBS (Myb Binding Site) are widely distributed within the putative promoter regions of RPL genes. MBS

is a binding site for MYB-related transcription factors that are involved in the regulation of genes responsive to water-deficit conditions (Urao et al., 1993). The presence of these elements in the promoter regions suggests that the corresponding genes become activated under water stress or drought conditions. In addition to abiotic stresses, elements that respond to phytohormones such as ABA (ABRE-Abscisic acid responsive element and Motif IIb), MeJa (TGACG-motif and CGTCA-motif), SA (TCA-motif), Gibberellic acid (GARE-Gibberellic acid responsive element) and Auxin (TGA-motif and AuxR-Auxin responsiveness) are also present in multiple copies.

Except RPL37, which did not exhibit any abiotic-responsive element in its upstream region, the putative promoters of all other genes had one or the other stress-responsive elements. MBS, ABRE, TGACG and CGTCA motifs are commonly found in multiple copies. RPL8 has five repeats of ABRE and two repeats of each MBS, TGACG, CGTCA and TGA elements. RPL10 exhibited 5 repeats of each TGACG and CGTCA motifs that respond to MeJa treatment and three copies of MBS elements. RPL14 has four repeats of dehydration responsive elements. RPL18a showed four and three copies of Motif IIb and ABRE, respectively that respond to ABA and two copies of TGACG and CGTCA motifs. RPL28 showed five repeats of ABRE and four repeats of MeJa responsive elements. RPL29 has six copies of ABRE and three copies of TGA element. RPL31 showed three copies of TCA element and two repeats of TGACG and CGTCA motifs. RPL35 has four copies of ABRE and two copies of TCA element. RPL36 and 38 had three copies of ABRE and MBS elements, respectively. RPL44 has two copies of ABRE, CGTCA and TGACG motifs (Fig. 5.5). In addition, TC-rich repeats that are involved in defense and stress-responsiveness (Diaz-De-Leon et al., 1993), W-box motifs which are the binding sites for stress-responsive WRKY transcription factors (Eulgem and Somssich, 2007), a WUN-motif, a wound-responsive element that is associated with biotic stress (Jiang et al., 2014), a Box-W1 motif, a fungal elicitor element that binds with WRKY33 transcription factor in response to phytopathogens (Rushton et al., 1996; Lippok et al., 2007) are present in single copies in the putative *cis*-elements. Table 5.3 presents a detailed analysis of both abiotic and biotic responsive elements and their repeats in the putative promoter regions (Moin et al., 2016b).

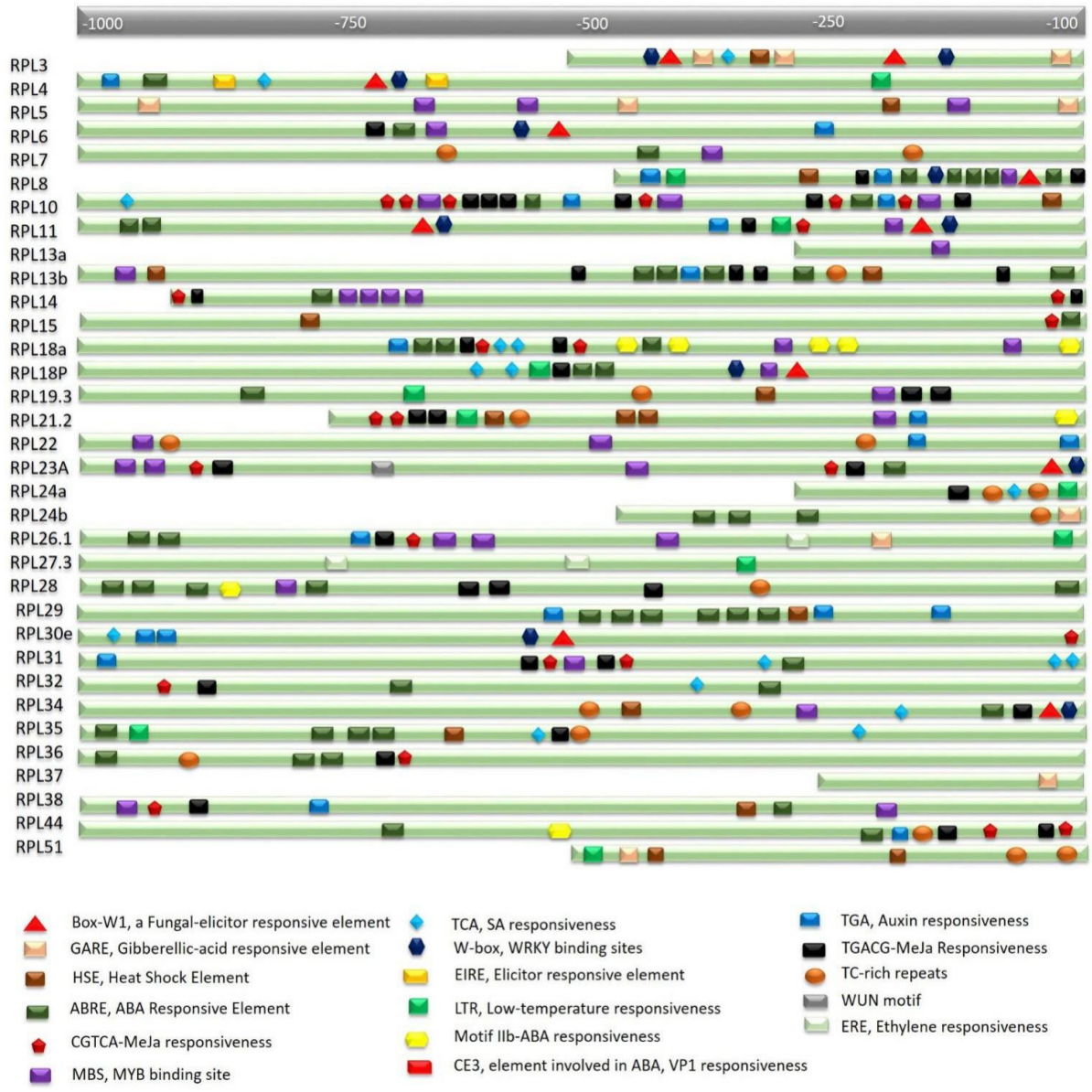


Fig. 5.5: *In silico* analysis of rice RPL promoters for the identification of *cis*-regulatory elements

Nucleotide sequence ≤1kb upstream of the transcription start site of each gene carries multiple stress and signal-responsive elements. Each element is represented with a different shape and color which is described at the bottom of the figure. A scale at the top indicates the putative localization of corresponding elements.

Table 5.3: Putative promoter analysis of RPL genes

Gene	MBS	HSE	LTR	TCA-element	TC-rich repeats	TGACG motif	CGTCA motif	BOXW1	DRE	GARE	W box	ABRE	Motif IIb	TGA element	AuxR	AT rich	WUN motif	ERE
RPL3		*		*				**		**	*							
RPL4			*	*				*			*	*		*				
RPL5	*	*								***								
RPL6	*					*	*	*			*	*		**				
RPL7/L12	*				**					*		*						
RPL8	**	*	*			**	**	*			*	****		**				
RPL10	***	*		*		*****	****					*	*	**				
RPL11	*		*			*	*	**		*	**	**			*			
RPL13a	*																	
RPL13b	**	**			*							****		*				
RPL14	****						**			*		*						
RPL15		*				*						*				**		
RPL18a				**		**	**		*			***	****	*				
RPL18P	*		*	**			*	*			*	**						
RPL19.3		*	***		*	**	**					*						
RPL21.2	*	***	*		*	**	**						*		*			
RPL22	**				**									**				
RPL23A	***				*		**	*			*	*					*	
RPL24a			*		**	*	*							*				
RPL24b	*				*					*		***						
RPL26.1	***		*			*						**		*				*
RPL27.3			*															**

RPL28	*				*	***	***					*****				*		
RPL29		*										******		***				
RPL30e	*			*		*		*		**	*			*	*			
RPL31	*			***		**	**					*		*				
RPL32				*		*	*					**						
RPL34	*	*		*	**			*			*	*						
RPL35a.3		*	*	**	*	*	*					****						
RPL36.2					*	*	*					***						
RPL37										*								
RPL38	***					*						*		*				
RPL44					*	**	**					**	*	*				
RPL51		*	*		**					*								

The presence or *cis*-regulatory elements were analyzed using PlantCARE database. MBS (MYB binding site), DRE (Drought Responsive Element), W-box (WRKY transcription factor binding elements), HSE (Heat Stress Elements), LTR (Low Temperature Responsiveness), TCA-element (Salicylic Acid responsiveness), TC-rich repeats (Defence and stress responsiveness), TGACG and CGTCA -motif (MeJa responsiveness), BOXW1 (Fungal elicitor-responsive element), ERE (Ethylene Responsive Element), GARE (Gibberellin-Responsive Element), WUN-motif (Wound responsiveness), ABRE (ABA Responsive Element), TGA-element, Motif IIb (Auxin-responsive element), AuxR and TGA-element (Auxin Responsiveness).

5.5. Spatial expression of rice RPL genes

To obtain insights into the tissue-specific and native expression patterns of RPL genes, we studied the expression of 34 RPL genes in 13 different tissues including 16 h embryo and endosperm, plumule and radicle of 3 d old seedlings, root and shoot tissues of 7 d old seedlings,

two month old tissues of mature flag leaf, shoot, root, root-shoot transition, partially filled grains, flowers and spikes.

Two-month-old shoot and root tissues induced an up-regulation of 30 and 22 RPL genes, respectively, which is larger than any other tissue. Out of 34, root-shoot transition and grains induced the expression of 17 RPL genes. Embryo, 6 d root and floral organs induced the expression of 12 RPL genes, whereas endosperm, plumule, radicle, 6 d shoot, flag leaf and spikes induced the expression of a total of 16, 13, 14, 10, 21 and 19 RPL genes, respectively. RPL5 and RPL24a were highly up-regulated in all the tissues. The expression of RPL27 and RPL37 was detected only in three tissues; endosperm, 45 d shoot, flowers and 6 d shoot, 45 d root and flowers, respectively. RPL8 and RPL15 were expressive only in endosperm and 6 d roots, respectively, but non-expressive in the remaining tissues studied. Ten RPL genes *viz*., RPL5, 7, 8, 18P, 19.3, 21.2, 22, 24a, 31 and 34 were commonly up-regulated in embryo and endosperm indicating that they can be implicated in early embryonic development. RPL4-6, 13a, 19.3, 23A and 24a were up-regulated in plumules and radicle suggesting their role in root and shoot initiation. RPL5, 6, 24a, 31, 34 and 51 were highly expressive in shoot and root tissues of 7 d old seedlings. RPL4-6, 11, 13a, 14, 18, 19, 21.2, 22, 23A, 24a, 31, 34, 35, 38 and 44 were commonly up-regulated in root, shoot and flag leaf indicating that these genes are involved in vegetative growth and plant maturity.

RPL13a, 14, 19.3, 22, 24a, 26 and 34 were highly expressive in spikes, flowers and partially filled grains indicating that these are likely associated with the development of reproductive organs and grain filling. RPL13a, 14 and 24a were expressive from the 6 d old seedling stage to the grain filling stage in all the tissues studied indicating that they play a major role in the growth and development of both vegetative and reproductive organs such as root, shoot, flowers and grains. RPL5, 19.3, 23A and 24a were expressive in mitotically active tissues like embryo, endosperm, plumule and radicles suggesting that these genes are involved in the early maturity and emergence of shoot and root (Moin et al., 2016b). RPL10 and RPL29 were specifically expressed only in endosperm and flowers, respectively (Table 5.4). The spatial expression of 34 RPL genes in 13 different tissues has been represented in the form of heat maps generated by incorporating the qRT-PCR data obtained from tissue samples collected as three biological replicates (Fig. 5.6).

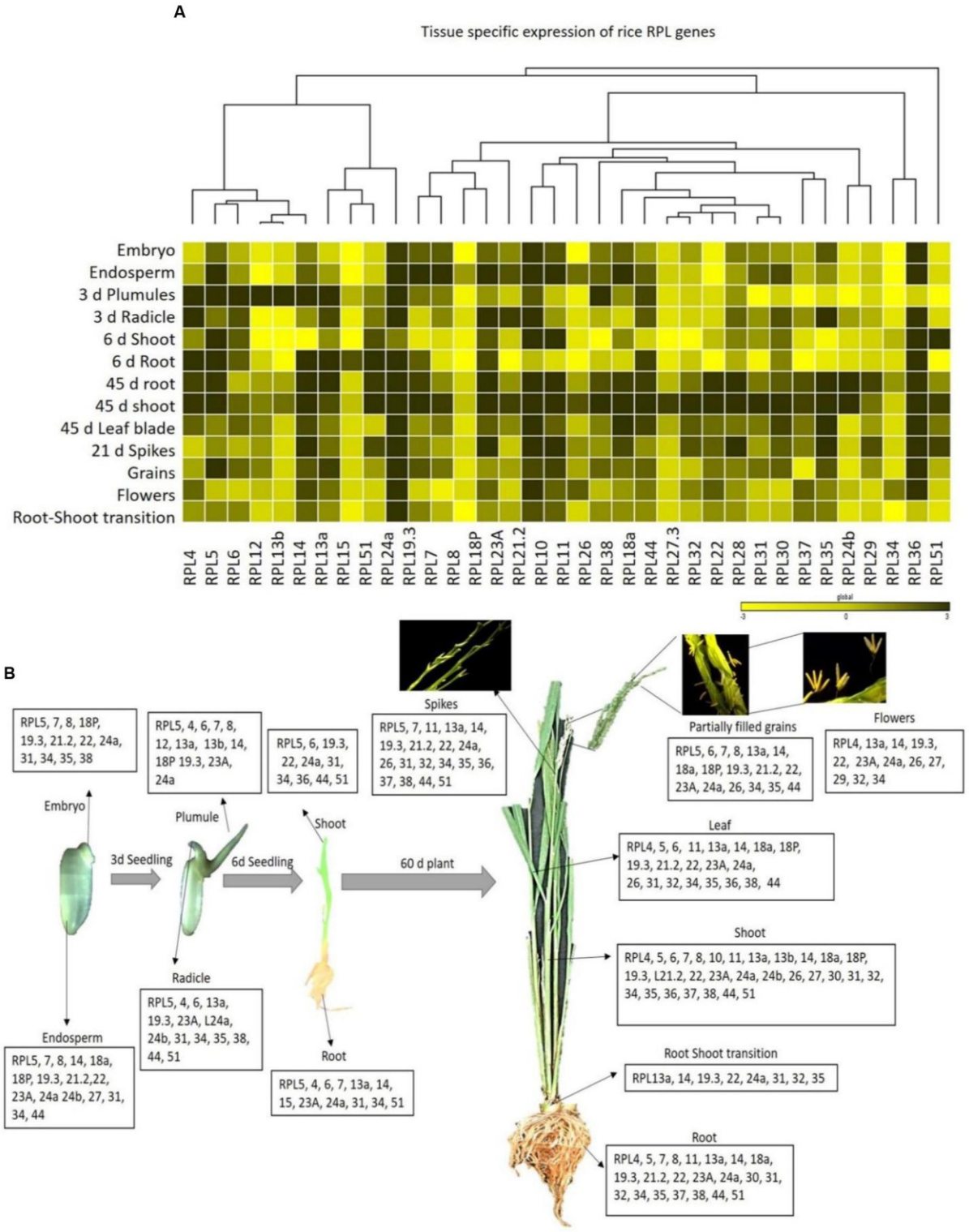


Fig. 5.6: Tissue-specific expression of rice RPL genes

The qRT-PCR of 34 RPL genes was performed in 13 different tissues and the level of expression was normalized with rice actin. The mean values of fold change of biological and

technical triplicates were represented in the form of heat maps. A dendrogram was constructed to represent the Hierarchical clustering. The RPL transcripts that are significantly up-regulated in each tissue are represented pictorially at the bottom.

Table 5.4: RPL genes with fold levels ≥2 on the log_2 scale were considered as up-regulated.

Embryo	Endosperm	Plumules	Radicles	6 d S	6 d R	Roots	Shoots	Leaves	Panicles	Grains	Flowers	Root-shoot transition
L5	L5	L5	L5	L5	L5	L5	L5	L5	L5	L5		
		L4	L4		L4	L4	L4	L4			L4	
		L6	L6	L6	L6		L6	L6		L6		
L7	L7	L7			L7	L7	L7		L7	L7		
L8	L8	L8				L8	L8			L8		
							L10					
						L11	L11	L11	L11			
		L12										
		L13a	L13a		L13a	L13a	L13a	L13a	L13a	L13a	L13a	L13a
		L13b					L13b					
	L14	L14			L14	L14	L14	L14	L14	L14	L14	L14
					L15							
	L18a					L18a	L18a	L18a		L18a		
L18P	L18P	L18P					L18P	L18P		L18P		
L19.3	L19.3	L19.3	L19.3	L19.3		L19.3	L19.3	L19.3	L19.3	L19.3	L19.3	L19.3
L21.2	L21.2					L21.2	L21.2	L21.2	L21.2	L21.2		
L22	L22			L22		L22	L22	L22	L22	L22	L22	L22
	L23	L23	L23		L23	L23	L23	L23		L23	L23	
L24	L24	L24	L24	L24	L24	L24	L24	L24	L24	L24	L24	L24
	L24b		L24b				L24b					
							L26	L26	L26	L26	L26	
	L27						L27				L27	
											L29	
						L30	L30					
L31	L31		L31	L31	L31	L31	L31	L31	L31			L31
						L32	L32	L32	L32		L32	L32
L34	L34		L34	L34	L34	L34	L34	L34	L34	L34	L34	
L35			L35			L35	L35	L35	L35	L35		L35
				L36			L36	L36	L36			
						L37	L37		L37			
L38			L38			L38	L38	L38	L38			
	L44		L44	L44		L44	L44	L44	L44	L44		
			L51	L51	L51	L51	L51		L51			

The Table details the RPL genes expressing in each tissue specifically.

5.6. Differential transcriptional regulation of rice RPL genes under various abiotic stress treatments

The motivation for studying rice large subunit ribosomal genes in stress-response stems from our results on activation-tagged mutant population generated for an important agronomical trait called water-use efficiency (Moin et al., 2016a). The mutants with sustained/improved seed productivity under the conditions of limited water availability were selected for flanking sequence analysis and subsequently for studying the expression pattern of the enhancer tagged genes. The five short-listed mutant plants that appeared to have high productivity were then characterized with physiological parameters related to WUE such as measuring their photosynthetic efficiency and carbon isotope analysis. Among these, two mutants were found to have ribosomal large subunit genes, RPL6 and RPL23A activated by the integrated enhancers. The presence of multiple stress-responsive elements in their putative promoter regions and their significant up-regulation in response to ABA, NaCl and dehydration stresses further corroborated our findings (Moin et al., 2016a). This study not only suggested that RPL6 and RPL23A are potential candidates for abiotic-stress amelioration, but more importantly it provides a basis for the exploration of other members of large subunit ribosomal genes for stress-responsiveness.

Taking a cue from these observations, we assessed the abiotic and biotic stress responsive roles of other rice 60S ribosomal genes (Moin et al., 2016b). For this, we selected 34 genes, one from each orthologous group as described earlier and comprehensively studied the differential transcriptional regulation of 34 genes under phytohormone (MeJa and SA), temperature (heat; 42°C and cold; 4°C) and oxidative stress treatments in shoot and root tissues at 6 different time intervals (5 min, 3 h, 6 h, 12 h, 24 h and 60 h). After applying the abiotic treatments, tissue samples were collected as early as 5 min to check the immediate responsiveness of the RPL genes and continued up to 60 h. All the RPL genes responded to the treatments in the form of either up or down-regulation.

The genes that exhibited ≥3-fold transcript level on the $\log_2$ scale were considered as significantly up-regulated. MeJa, SA and cold treatments induced the up-regulation of more genes (>60%) than heat and H_2O_2 treatments, which caused the down-regulation of 75% of the genes. In shoots, MeJa and SA-induced the up-regulation of 27 (79%) RPL genes each, cold treatment up-regulated 19 genes (55%), while heat and H_2O_2 treatments up-regulated 6 (17%) RPL genes each. In roots, MeJa, SA, cold, heat and H_2O_2 treatments up-regulated 19 (55%),

22 (64%), 16 (47%), 6 (17%) and 14 (41%) RPL genes, respectively. Genes that were up-regulated in both the shoot and root tissues include; RPL7, 8, 12, 13b, 18P, 19.3, 24a, 32, 35 and 51 under MeJa treatment, whereas SA-induced the expression of RPL7, 8, 12, 13b, 19.3, 24a, 26, 32, 35 and 51. Genes such as RPL6, 7, 23A, 28, 32, 35 and 37 were up-regulated in cold treatment, while RPL6, 12, 23A and RPL18a and 13a were up-regulated under heat and H_2O_2 treatments, respectively.

To study the detailed regulation of RPL genes at various time points, we categorized the genes that responded within 5 min to 3 h after treatment as immediate-early (IE), those that responded between 3 h to 12 h as early (E) and those that were regulated after 12 h of treatment up to 60 h were considered as late (L) responding genes. The majority of the genes that were up-regulated had responded immediately within 5 min to 3 h after the onset of the stress. In shoots, among the genes that were expressive, a total of 17, 21, 19, 6 and one genes belonged to the IE-responsive class with instantaneous up-regulation under MeJa, SA, cold, heat and H_2O_2 treatments, respectively. Among this IE-responsive class of genes, some continued to maintain a high level of expression at all the time points observed, while others exhibited a split in the expression followed by again an increase in the level of their expression. These probably form an important set of genes that respond to environmental stresses and might function as an immediate defense after the onset of the stress (Kawasaki et al., 2001).

The other class of IE genes was down-regulated after an immediate-early response. Under MeJa and SA treatments, RPL7, 8, 12, 13b, 19.3, 24a, 28 and 35 maintained a high level of expression throughout the duration of stress in both shoot and root tissues. However, the level of transcriptional up-regulation varied with some exhibiting a very high level of up-regulation up to 100-fold (RPL8, 12, 19.3, 24a and 35), while some had moderate expression up to 30-fold (RPL7) and others showed low transcript levels with <10-fold (RPL28). RPL6, 7, 12 and 24a exhibited a consistent up-regulation under cold and heat treatments, of which RPL6 and 12 became up-regulated more than 50-fold. In H_2O_2 treatment, RPL18a was up-regulated in both roots and shoots up to 65-fold whereas 24a, 24b, 30 and 34 showed significant up-regulation in roots. Since stress signals are transmitted through the roots to other parts of the plant body, genes that were significantly up-regulated particularly in roots might have an important role in combating the stress and providing early defense.

The differential expression patterns in response to MeJa and SA (Fig. 5.7) and cold, heat (Fig. 5.8) and oxidative treatments (Fig. 5.9) have been represented in the form of heat maps. These

were generated by incorporating the mean values of fold change normalized using $\Delta\Delta C_T$ method obtained from three biological and three technical replicates. The overlap in the up-regulation (Fig. 5.10) and down-regulation (Fig. 5.11) of 34 RPL genes in both shoot and root tissues were represented as Venn diagrams (Moin et al., 2016b).

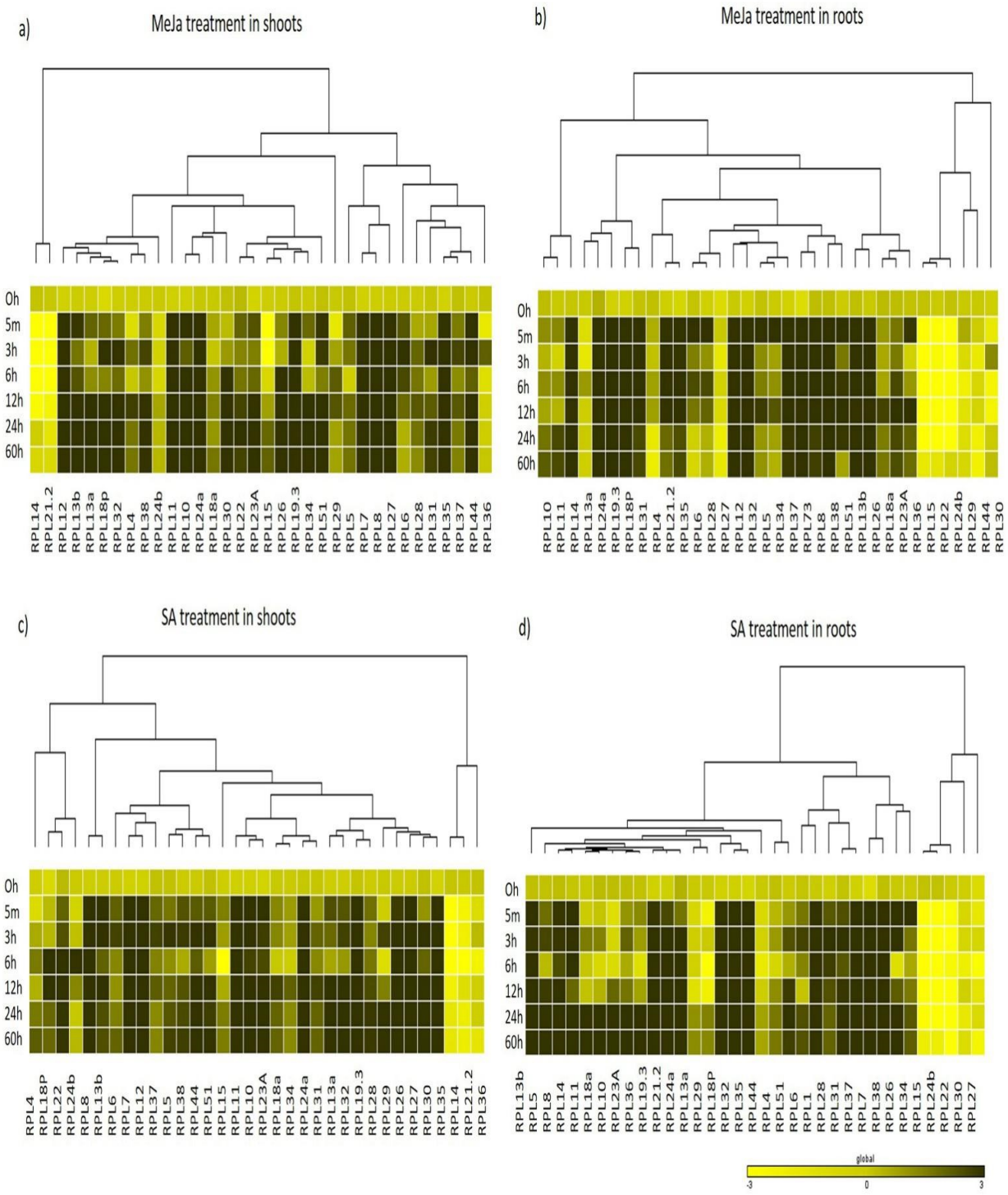


Fig. 5.7: Heat map representation of RPL genes in rice treated with MeJa and SA

Seven-day-old rice seedlings were exposed to different abiotic stresses such as MeJa; 100 µM (a, b) and SA; 3 mM at six different time points as indicated on the left. The qRT-PCR is used to determine the expression levels of RPL genes and the fold change was normalized using $\Delta\Delta C_T$ method relative to that in unstressed seedlings dipped in water at corresponding time points. Rice actin (*act1*) and β-*tub* genes were used as internal controls. Three biological replicates and two technical replicates were included in the study. A dendrogram was constructed to represent the Hierarchical clustering of genes.

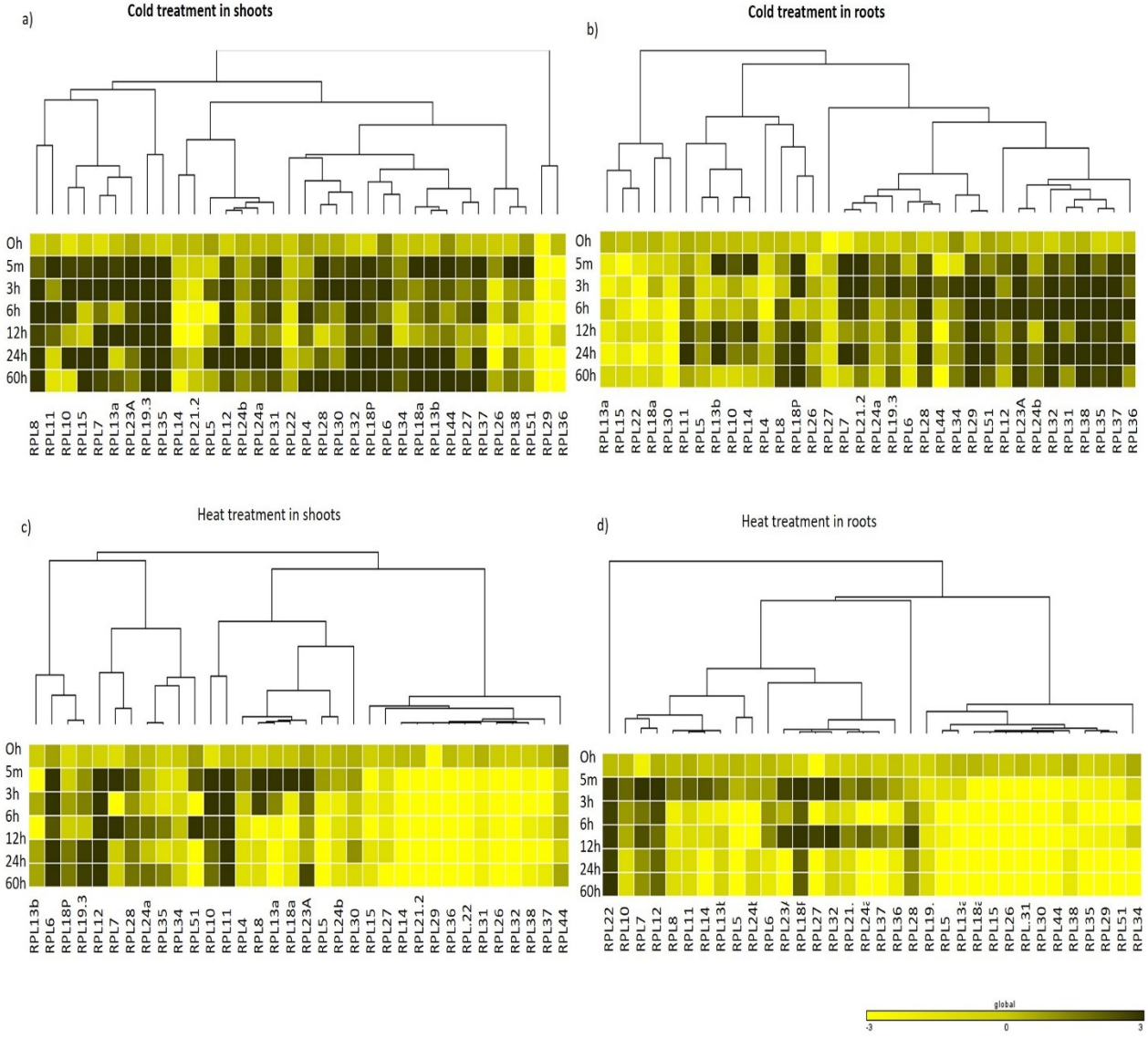


Fig. 5.8: Heat map representation of RPL genes in rice in response to cold and heat treatments

Seven-day-old rice seedlings were exposed to different abiotic stresses such as cold stress at 4°C (a, b) and heat stress at 42°C (c, d) at six different time points as indicated on the left. The qRT-PCR is used to determine the expression levels of RPL genes and the fold change was normalized relative to that in unstressed seedlings dipped in water at corresponding time points. Rice actin *(act1*) and β-tubulin were used as internal reference genes. Three biological replicates and two technical replicates were included in the study. A dendrogram was constructed to represent the Hierarchical clustering of genes.

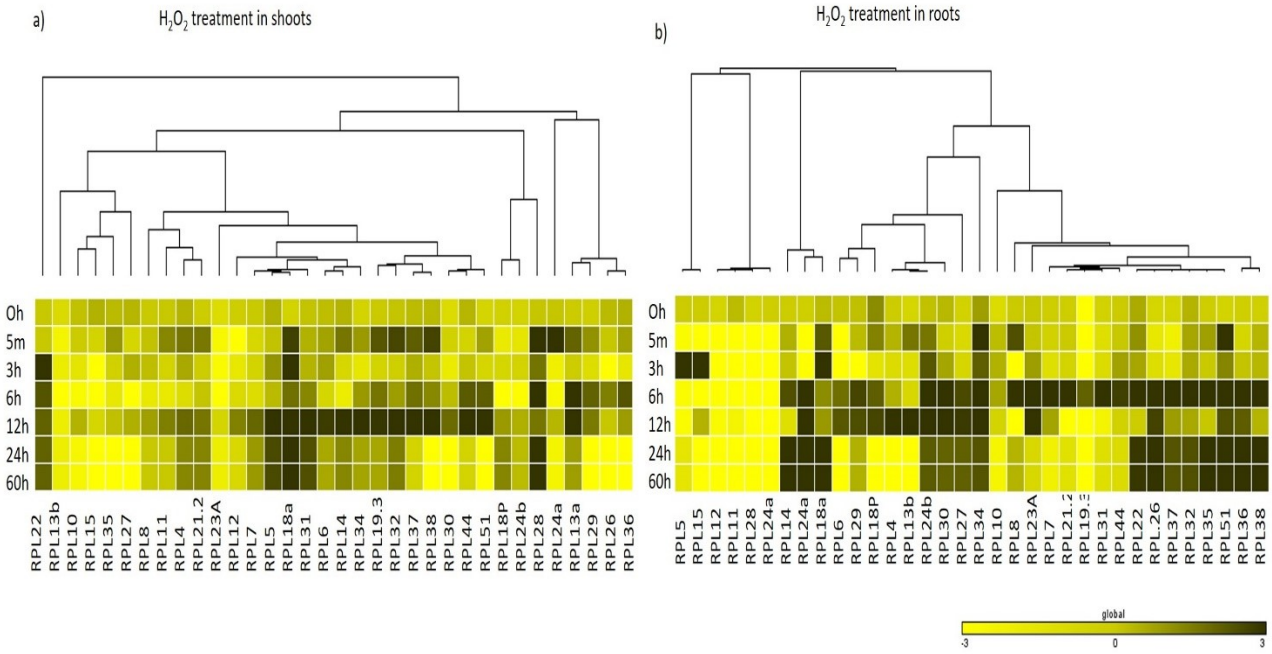


Fig. 5.9: Heat map representation of RPL genes in rice in response to H_2O_2 treatments

Seven-day-old rice seedlings were exposed to oxidative stress with H_2O_2; 10 µM (a, b) at six different time points as indicated on the left. The qRT-PCR is used to determine the expression levels of RPL genes and the fold change was normalized relative to that in unstressed seedlings dipped in water at corresponding time points. Rice actin *(act1*) and β-tubulin were used as internal reference genes. Three biological replicates and two technical replicates were included in the study. A dendrogram was constructed to represent the Hierarchical clustering of genes.

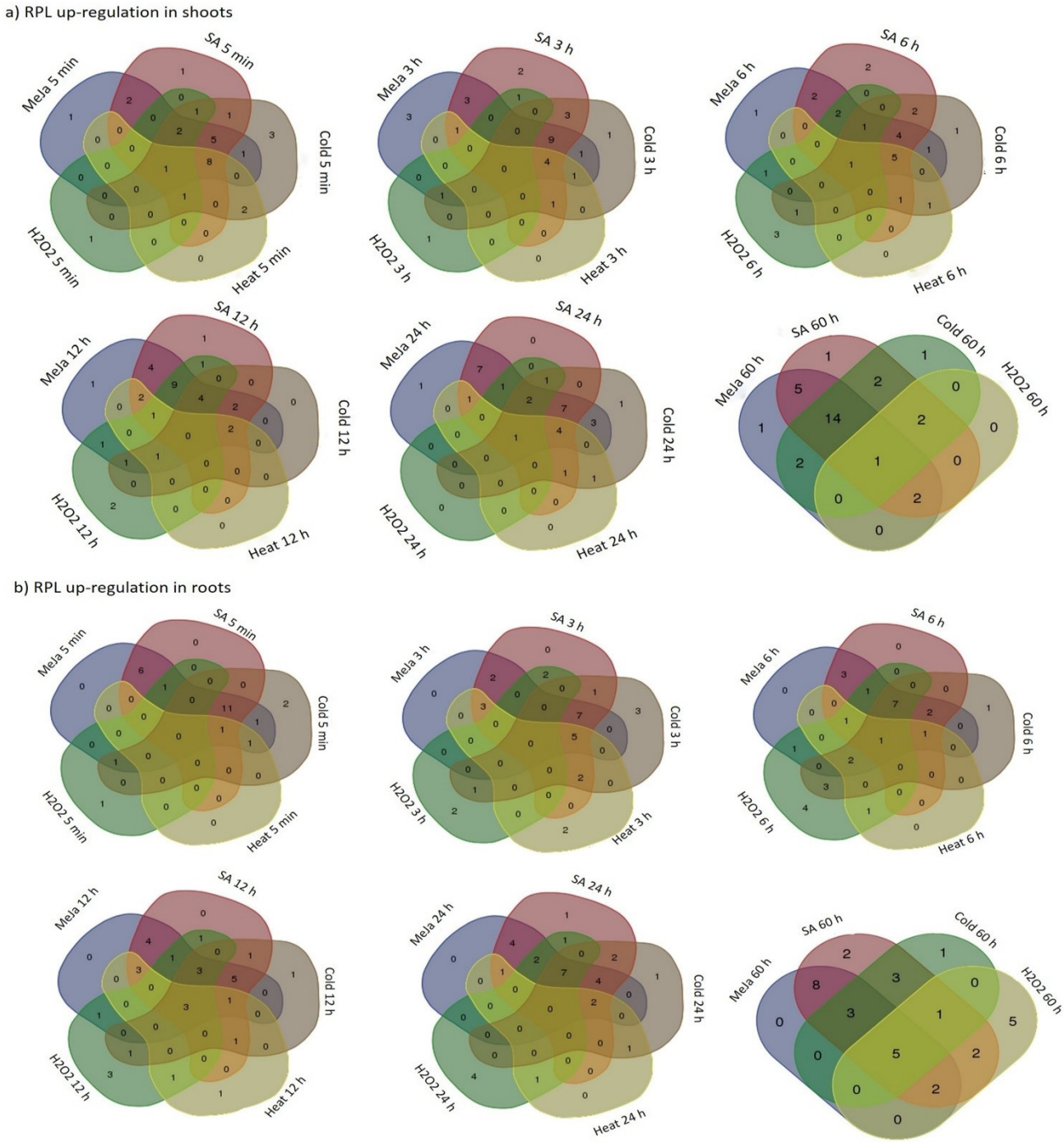


Fig. 5.10: Overlap in the up-regulation of rice RPL genes under five abiotic conditions

The RPL genes that exhibited ≥3-fold transcript level on the $\log_2$ scale were considered as significantly up-regulated while others were considered as down-regulated or without any change in expression. Venn diagrams are used to show the overlap in the up-regulation in shoot (a) and root (b) tissues.

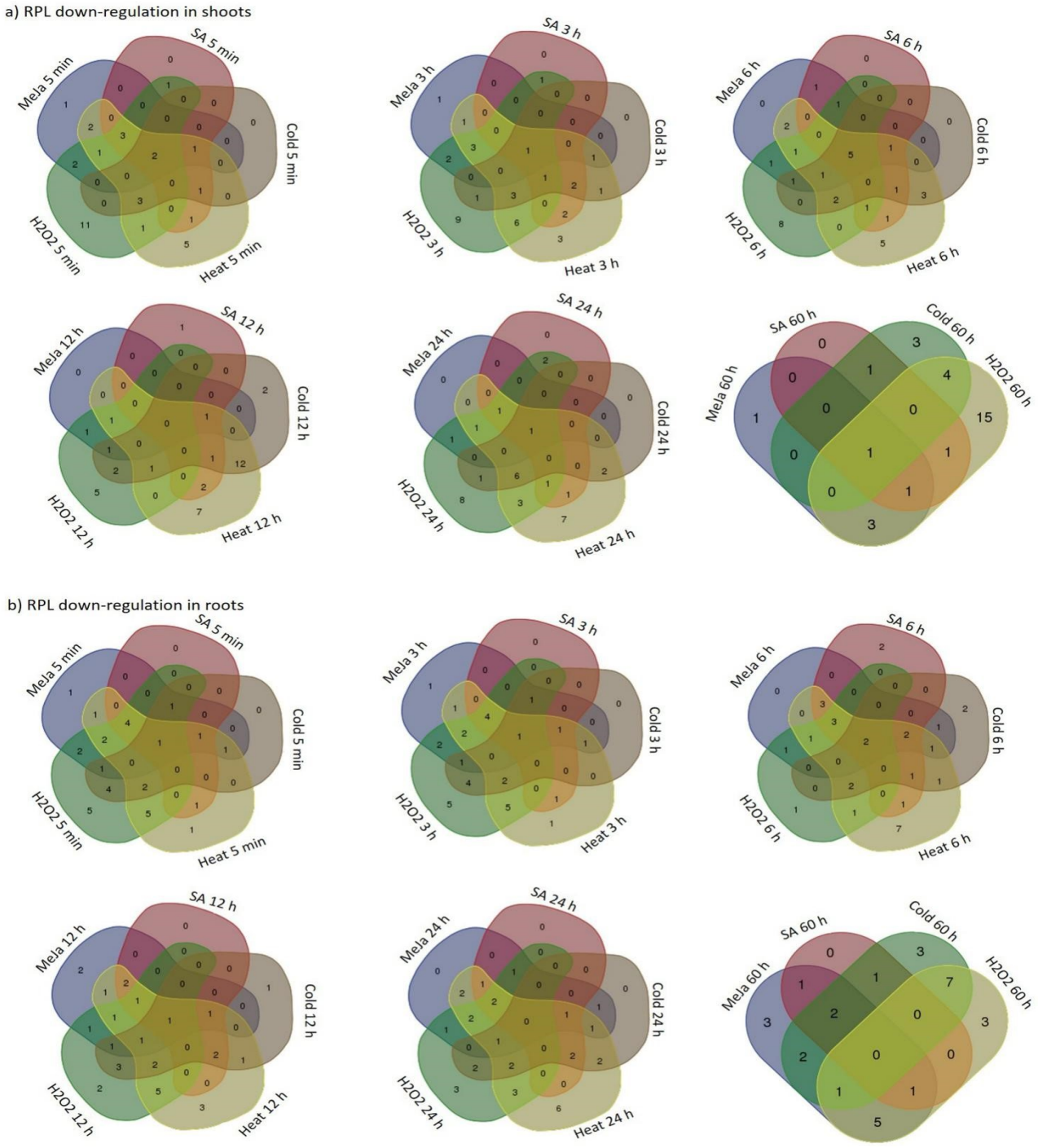


Fig. 5.11: Overlap in the down-regulation of rice RPL genes under five abiotic conditions

The RPL genes that exhibited <3-fold transcript level on the $\log_2$ scale were considered as down-regulated with respect to others. Venn diagrams are used to show the overlap in the down-regulation in shoot (a) and (b) root tissues separately.

5.7. Differential transcriptional regulation of RPL genes in response to treatment with the *Xoo* pathogen

The qRT-PCR analysis of 34 genes showed that they are differentially regulated under abiotic treatments, with many of them becoming significantly and immediately up-regulated. Hence, we simultaneously examined the expression levels of 34 RPL genes in response to the *Xoo* pathogen that causes BLB of rice. Out of 34 genes, 6 (17%) were down-regulated, RPL38 was non-responsive, and the remaining genes became activated (80%). RPL12, 28, 30, 36, 44 and 51 were among those that were down-regulated, and the transcript level of RPL38 did not change significantly, while all other genes studied were up-regulated. Among those that were expressive, RPL10, 11, 15, 24a, 26, 27 and 37 up-regulated more than 10-fold. The transcript level of RPL10 was the highest with more than 75-fold up-regulation (Fig. 5.12a). In addition to significant expression at 11 d, the transcript level of RPL10 also exhibited a gradual increase at 3 h, 6 h, 1 d and 2 d up to 7 d post-infection with the *Xoo* pathogen (Fig. 5.12b) (Moin et al., 2016b).

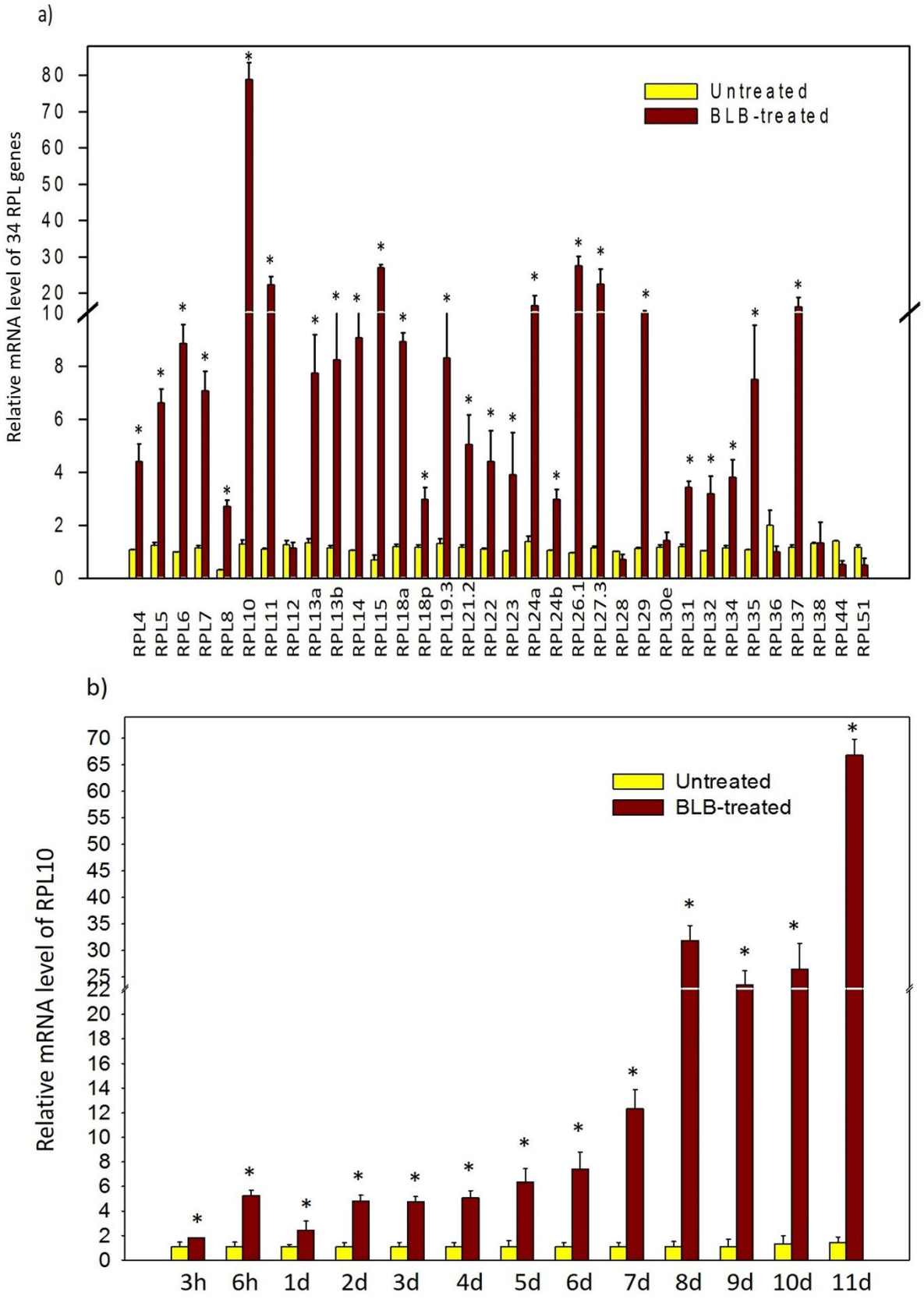


Fig. 5.12: Expression of RPL genes upon infection of rice with the *Xoo* pathogen

The expression of RPL genes was determined in response to the bacterium, *Xanthomonas oryzae,* which causes leaf blight. (a) The bacterial suspension was applied on 60 d old rice plants, and after 11 d of treatment, leaf samples were analyzed for differential transcript levels of 34 RPL genes. (b) Since the up-regulation of RPL10 was significant, we analyzed its expression at progressive time courses such as 3 h, 6 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, 8 d, 9 d, 10 d and 11 d post-infection with *Xoo* pathogen. The expression was normalized with untreated samples of same age grown under identical conditions. The statistical significance was calculated using one-way ANOVA at $P<0.05$ and represented with asterisks.

Chapter 6:

Discussion and Conclusion

6.1. Yield limiting factors in rice

Stress factors such as heat, cold, dehydration and pathogen attack can exacerbate the global agriculture system with an estimated >50% crop yield loss per annum (Wang et al., 2003). Each of these stresses elicits a cascade of signaling pathways to ensure plant survival (Pareek et al., 2010, Pandey, 2015). It is important to identify the genes that contribute to sustainable plant productivity under conditions of stress to further improve their productivity potential. Although more than 500 genes have been overexpressed and characterized in rice for stress-tolerance, many remain to be identified and examined.

Plant tolerance to water stress can be improved by drought avoidance or drought tolerance among which drought avoidance tends to conserve water by water-use efficiency (WUE). While drought avoidance is the maintenance of high water status under water deficit conditions, drought tolerance refers to the ability of one genotype to yield better than the other in a severely dehydrated state (Blum et al., 2005). In drought tolerant genotypes, yield parameters are not adversely affected as grain yield is supported by mobilization of stem reserves, which occurs predominantly during drought stress (Blum et al., 1994, Yang et al., 2001). The genotypes with high WUE experience reduced biomass and yield due to stomatal closure to avoid transpirational water loss, which is also accompanied by reduced carbon assimilation under the conditions of limited water availability (Blum et al., 2005). However, overexpression of WUE genes in rice and other crops resulted in transgenic plants with higher biomass and seed yield compared to the WT plants under limited water conditions (Sivamani et al., 2000, Karaba et al., 2007). Hence, identification of genes having potential in improving plant yield under water deficit conditions assumes importance in the generation of transgenic plants with enhanced WUE without suffering a reduction in yield and biomass. Proteins that most probably function in abiotic stress tolerance include heat shock proteins, LEA proteins, transcription factors like bHLH, bZIP, NAC, AP2/ERF, MYB, Zinc finger, WRKY, kinases (calcium-dependent kinases, MAP kinases), osmoprotectants, water channels, detoxifying and metabolic enzymes. Overexpression of these endogenous genes in transgenic rice resulted in increased yield under limited water conditions (Dubouzet et al., 2003, Zhang et al., 2004, Nakashima et al., 2007, Karaba et al., 2007, Hu et al., 2008, Jeong et al., 2010).

6.2. Activation tagging, a suitable gain-of-function mutagenesis strategy in crops

In *Arabidopsis,* with a genome size of 134 Mb and 27,416 protein coding genes (TAIR, 2011) averaging 2940 bp size and a gene density of 4.35 kb/gene, 60% of the genome constitutes the transcriptionally active region and insertion of the *Ds* in the genic region is possible and expected. In rice, with about 165 Mb transcriptionally active area (including 54 Mb TE-related genic region and 111 Mb non-TE related genic region) from 373 Mb of non-overlapping sequence (comprising 44% of total coding area), high frequency of insertion of the *Ds* between the coding regions (57%) indicated that the *Ds* element preferentially transposed to genic sites. Preferential intergenic transposition makes the activation tagging technique more feasible and can be extended to other crop systems as well.

6.3. *In planta* transformation for functional genomics

The transformation protocol developed in this study completely eliminated the need for tissue culture and the transgenic plants obtained can be comparable to WT with respect to the genome constitution except for the integrated T-DNA (Moin et al., 2016a). Hence, this method can be suitable for developing transgenic rice plants of agronomic value and producing large populations that are required for functional genomic studies without interference from the endogenous transposable elements (TEs). These TEs become activated during stressful conditions that result from *in vitro* tissue culture (Mieulet et al., 2013).

The excision of the *Ds* element was very low in *indica* rice and the highest frequency of obtaining stable Ds^+ plants even in the T_5 generation was 21%, which is in contrast to the highest transposition frequency of up to 80% observed in *japonica* cultivars (Qu et al., 2008). Because of this, the transposon carrying plants needed to be screened through selfing in several generations to obtain a larger population of stable *Ds* plants. As an alternative, we developed another activation tagging vector (pDEB) that carries tetrameric enhancers.

6.4. Identification of potential candidate genes for high water-use efficiency

The transgenic plants carrying stable *Ds* element with tetrameric enhancers or pDEB vector based enhancer elements were grown under limited water conditions (at field capacity without normal water overlay that is required for rice cultivation) till plant maturity to select for plants with enhanced WUE. Among the 200 mutants, we selected five plants having high quantum efficiency, low $\Delta^{13}C$ and exhibiting suitable growth and productivity related phenotypes to study the expression of all the candidate genes located within a 20 kb stretch on either side of the enhancer insertions on the chromosomes. It was found that only one of the 3 to 4 genes

residing in the 20 kb region were activated in four out of five plants analyzed, while two genes became activated in one plant indicating that an enhancer can activate more than one gene simultaneously near the site of insertion.

Transcript analysis of the tagged genes studied in the five mutants revealed the activation of transcription factors (WRKY96, GRAS), proteins involved in protein ubiquitination (cullin4) and ribosome biogenesis (RPL6 and RPL23A). Approximately 100 WRKY transcription regulator family genes have been identified in rice having diverse roles including tolerance to biotic and abiotic stress (Bakshi et al., 2014). With respect to this, activation of WRKY96 by enhancers in DEB.42 and sustenance of this mutant to limited water conditions could be easily correlated.

In mutant En.64, the locus LOC_Os03g57290 encodes cullin-4, a hydrophobic protein that acts as a scaffold to carry E2-ubiquitin binding RING protein RING box1 (RBX 1) and the substrate-recruiting protein. Cullin-RING ligases (CRLs) have been shown to exhibit diverse roles in *Arabidopsis* that includes light perception, hormone signaling and response to biotic and abiotic stress (Stone, 2004, Lyzenga et al., 2011). In DEB.86, two genes encoding a GRAS (Gibberellic-acid insensitive Repressor of GA1 and Scarecrow) transcription factor (LOC_Os03g40080) and an unclassified expressed protein (LOC_Os03g40084) became activated simultaneously. Recently, a member of GRAS transcription factor OsGRAS23 located on chromosome 4 was shown to improve tolerance of transgenic rice to drought stress (Xu et al., 2015).

6.5 Ribosomal Protein Large Subunit (RPL) genes are stress and developmentally regulated

Ribosomal genes encode proteins that are the components of the two-subunit ribosomal complex, which together with the members of the same group and other proteins participate in protein synthesis. There have been limited reports on the role of ribosomal genes in the stress-responses. The availability of full-length and high-quality rice genome sequence and databases, further helped us to exploit the information on these genes. Based on the information available in rice databases, we identified 123 genes that are the components of rice ribosomal large subunit, of which 2-3 genes exist as identical gene copies in the genome. These genes are distributed throughout the 12 chromosomes of rice genome with chromosome 7 and 1 having the highest number of genes (Moin et al., 2016b). The present investigation that reports on the analysis of native and differential expression of the RPL gene family also corroborated the

earlier reports that RPL genes are regulated spatio-temporally (Sormani et al., 2011; Carroll, 2013; Zheng et al., 2016). In rice, RPL genes appear to be developmentally regulated as they are widely expressed in all the 13 tissues studied starting from as early as embryonic initiation to plant maturity. Among the 19 RPL genes that were expressive in grain filling stage, the expression of RPL5, 7, 13a, 14, 19.3, 22, 24a, 34 and 35 were conspicuously detected. Further characterization of these genes would throw useful insights into their role in grain production, which is a significant yield-related trait in rice.

In addition to their housekeeping functions such as ribosome biogenesis, protein translation and post translational modifications, RPs participate in developmental and growth processes and also respond to various stresses. Out of 241 RP genes in *Arabidopsis* (143 large subunit and 98 small subunit genes), almost all were up-regulated under macro elements deficiency (Wang et al., 2013). In Maize, the expression of a large number of ribosomal proteins was up-regulated along with RPL6 in response to UV-B exposure (Casati and Walbot, 2003). Knockout of *Arabidopsis At*RPL23A gene through RNAi resulted in retarded plant growth, irregular leaf and root morphology and loss of apical dominance (Degenhardt et al., 2008). Therefore, activation of RPL23A by enhancers in En.16 mutant with high yielding phenotype suggests the fact that RPL23A plays an important role in fitness traits (Moin et al., 2016a). Corroborating this, the up-regulation was instantaneous and high in various stress treatments indicating its significant involvement in abiotic stress amelioration. In *Arabidopsis*, the location of RPL23A has been mapped adjacent to the polypeptide exit tunnel suggesting its important role in protein translocation and secretion (Maier et al., 2005).

An orthologue of RPL6 (LOC_Os02g37862) has also been shown to be up-regulated in response to biotic stress attack by the brown planthopper (BPH) in BPH susceptible rice lines (Wei et al., 2009). This is in correlation with the up-regulation of RPL6 by SA and MeJa treatments, which are the key plant hormones involved in plant defense against insects and necrotrophic pathogens in the present study. RPL6, whose role has been demonstrated earlier in biotic stress tolerance, has also been shown to be involved in enhancing WUE in the present study along with its immediate and several hundred fold up-regulation in ABA, PEG and NaCl treatments (Moin et al., 2016a).

The presence of significant stress-responsive *cis*-regulatory elements in addition to many other motifs in the promoter regions of RPL6 and RPL23A, their activation by enhancers in WUE mutants and significant up-regulation in response to various stresses support our findings that

these two genes play an important role in alleviating plant abiotic stress. This alleviation might be through an interplay with stress-responsive transcription factors. However, the level and the underlying mechanism of stress tolerance needs to be investigated further by raising independent transgenic plants of these two genes. Currently efforts are underway to overexpress RPL6 and RPL23A genes for a detailed functional characterization to further validate their role in enhancing WUE and also drought and other stress tolerance in rice. Other RP genes of rice such as RPL35 and RPL32 became up and down-regulated in heat and salt stress treatments, respectively (Mukhopadhyaya et al., 2011). Also, RPL10 was up-regulated under radiation exposure in *Arabidopsis* (Ferreyra et al., 2010), indicating that both large and small subunit of RP genes respond differentially to various biotic and abiotic stress conditions, likely playing a significant role in abiotic stress tolerance including WUE.

The instantaneous and high up-regulation of RPL genes in response to stress might function as an immediate defence. Since, these are ribosomal genes, the defence might also occur by maintaining the mRNA integrity/stability during protein translation under the conditions of stress. The coordinated transcriptional up-regulation of translational related genes might also be a necessity for the cells to maintain the crucial cellular function under the conditions of stress.

Flanking sequence analysis of selected mutants having sustained growth and productivity under the condition of limited water availability revealed the activation of the two ribosomal genes (RPL6 and RPL23A) by the integrated enhancers (Moin et al., 2016a). This has persuaded us further to analyze the importance of several of RPL genes in stress-responses. We therefore, performed a comprehensive native tissue-specific and differential expression of 34 RPL genes under various abiotic and biotic stress conditions at different time intervals.

In the present work, we report on the comprehensive expression profiling of rice ribosomal large subunit genes under multiple abiotic and biotic stress treatments at progressive time points and also identified their putative promoter sequences. The information provided here can be exploited further in the functional characterization of these stress-responsive genes, which might help in augmenting rice yields by generating independent transgenic plants. We also identified the genes that exhibited an overlap in the expression patterns in response to two or more stresses (Moin et al., 2016a & b). We propose that such genes are particularly promising in bringing about the tolerance to multiple stresses as the presence of a second stress factor can enhance the detrimental effects of the first one (Atkinson and Urwin, 2012).

Although many of the RPL genes that were expressive in all the tissues, they cannot be considered as house-keeping as their level of expression changed in response to environmental signals. Similar expression profiling of small and large subunit genes was reported in response to macro-elements deficiency in *Arabidopsis* in which about 244 among 249 RP genes became up-regulated (Wang et al., 2013). The up-regulation of the RPL genes is likely to maintain or improve protein synthesis and hence, proper functioning of ribosomes, the basic cellular moieties under the conditions of stress (Kim et al., 2004). Plants being sessile acclimate to environmental cues by undergoing many metabolic changes, one of them being increased protein turnover that includes both protein biosynthesis and ubiquitination (Kosová et al., 2014). Proteomic studies revealed variations in the levels of translation-related proteins such as initiation factors, elongation factors and proteins of both small and large subunits during the process of acclimation particularly, to dehydration, salt and temperature stresses in cereals (Fatehi et al., 2012; Budak et al., 2013; Ghabooli et al., 2013; Gharechahi et al., 2013).

In addition to their significant up-regulation, the presence of multiple *cis*-regulatory elements in the putative promoter regions of RPL genes further corroborates our findings that these genes might play also play a role in alleviating plant biotic and abiotic stress. RPL6 and RPL23A, in addition to their role in WUE, became up-regulated in almost all the stresses studied illustrating their possible involvement in inducing tolerance to abiotic stresses (Moin et al., 2016a). Cold, MeJa and SA treatments induced the up-regulation of a majority of RPL genes, while H_2O_2 and heat treatments down-regulated 75% of the genes (Moin et al., 2016b).

The up-regulation of RPL genes by cold treatment is to enhance the process of polypeptide synthesis at low temperatures (Kim et al., 2004). RPL7, 8, 12, 13b, 19.3, 24a, 28 and 35 were up-regulated and constantly maintained a high level of expression throughout the duration of stress in response to SA and MeJa, the two phytohormones involved in plant defense against pathogen attack. These genes also contain TC-rich repeats, which are known for their involvement in plant defense and stress response (Diaz-De-Leon et al., 1993). RPL18a, 24a, 24b, 30 and 34 were expressed at higher levels when exposed to H_2O_2 treatment. This may reflect that these genes might have potential in combating oxidative stress. High temperature appeared to cause detrimental effects on the expression of RPL genes as heat stress had down-regulated >75% of the genes. RPL6, 12 and 23A were among those whose expression was detected. Down-regulation of RPL genes under high temperature might be because of decreased stability of RNA molecules with increasing temperatures.

Infection with *Xoo*, which causes BLB also up-regulated a large number of RPL genes. Among those that were expressive (RPL10, 11, 15, 24a, 26, 27 and 37), the expression of RPL10 was more evident as its transcript levels gradually increased from 3 h post-infection and reached a peak at 11 d after treatment. Also, RPL10 was activated in shoots under MeJa and SA up to 60 h after treatment, further suggesting its involvement in biotic stress response.

By studying the transcript levels of 34 genes, we made an attempt to provide an overview of their differential expression under abiotic treatments. Considering the stability of mRNA and proteins and their downstream targets and mechanisms, there is no direct link between the levels of transcripts of a gene produced with the degree of tolerance under a given treatment. Because of this, these genes might not be considered as stress-tolerant with their high up-regulation at this stage. Rather, they can be classified as stress-responsive. However, since two ribosomal protein genes became activated by the 35S enhancers in two mutants with high WUE (RPL6 and RPL23A), this study suggests that these two ribosomal proteins are potential targets for genetic manipulation. The other RPL genes also could be valuable resources for manipulating abiotic stress tolerance in rice and other crops for future as shown by their instantaneous and significant up-regulation (Moin et al., 2016a & b).

6.6 Conclusion

The present investigation reports on the development of the activation-tagged population in *indica* rice and screening the mutants for an agronomically important trait, water-use efficiency. It also reports on the tagging of ribosomal protein genes and their detailed expression profiling in response to various abiotic treatments, which revealed their possible role in abiotic stress amelioration along with WUE.

Our exploration on the detailed expression analysis underpins that RPL genes regulate tissue-specific development and respond rapidly to the environmental cues and might function as facilitators of immediate defense against stresses (Moin et al., 2016a & b). The coordinated transcriptional up-regulation of translation-related genes is a necessity for the cells to maintain the crucial functions of ribosomes under the conditions of stress. The increase in the expression of RPL genes under a wide range of stress-treatments including both biotic and abiotic conditions demonstrate that these are potential targets for the manipulation of stress-tolerance in rice and other related cereal crops as well. However, the level of tolerance induced by each of these genes needs to be analyzed by their independent overexpression in the transgenic rice plants.

One key purpose of this work is to identify novel genes for WUE, which could further be exploited in manipulating tolerance to other abiotic stresses in rice. One group of such genes identified in this study are those encoding proteins involved in protein synthesis. Interestingly, two out of the five mutants analyzed for WUE turned out to be genes for RPs. This gives an indication that analysis of a larger number of mutants for WUE might throw more plants with activation of genes for other RPs as well and genes for RPs could be very important targets for WUE and abiotic stress tolerance in rice and other crops. These ribosomal genes have not been previously shown to be regulated by dehydration stress in rice, further illustrating the fact that activation tagging is a powerful tool for the discovery of novel gene functions.

Chapter 7:

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Chapter 8:

Publications from the Thesis

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Original Article

Activation tagging in *indica* rice identifies ribosomal proteins as potential targets for manipulation of water-use efficiency and abiotic stress tolerance in plants

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ABSTRACT

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

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

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

INTRODUCTION

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

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

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

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efficiency of this process. There are no reports on the development of an activation-tagged population in *indica* rice because of the fact that *indica* cultivars are more recalcitrant to tissue culture regeneration, which can only be used to produce a limited number of transgenic plants. However, functional genomic approaches necessitate the development of large populations of transgenic plants.

While *Arabidopsis* genome has provided a basis for comparative genomics in dicotyledons, this is not directly applicable to monocotyledons. Rice is an appropriate monocot model crop because of the availability of the high-quality genome sequence (Goff *et al.* 2002; Yu *et al.* 2002) and its much smaller genome size (370 Mb) compared with related cereals such as sorghum (700 Mb), maize (2800 Mb), barley (4900 Mb) and wheat (16000 Mb). Because rice has syntenic relationships with these cereals, functional characterization of genes in rice will provide key genetic insights that would lead to comparative genomics among closely related grass family members.

Post-genome sequencing, large-scale insertional mutagenesis has been undertaken to investigate the role of unknown genes in the rice genome. Generation of insertion mutant populations in rice greatly relied on elements such as T-DNA, *Tos17*, *Ac/Ds* and En/Spm transposons. These generate unbiased, random loss-of-function mutations and provide unique signatures in the genome, and the flanking sequences at the integration sites can be identified through appropriate genome walking methods. However, these loss-of-function screens cannot uncover genes that function redundantly, which is attributed to the fact that plants have natural tendency to undergo large-scale random genome duplications, a phenomenon called 'polyploidization' (Bowers *et al.* 2003). Knockout of any one of the homologous duplicated genes with complete redundancy might result in an indistinguishable phenotype (Briggs *et al.* 2006).

The alternative approach is to use gain-of-function mutagenesis through activation tagging based on the insertion of transcriptional CaMV35S enhancers into the genome. These enhancers cause endogenous overexpression of genes near the sites of integration, which could give rise to novel phenotypes (Weigel *et al.* 2000). Insertional mutagenesis approaches including activation tagging can only be successful when sufficiently large populations of tagged lines covering as many genes as possible in a genome are developed through genetic transformation. Rice has a genome of about 373 Mb with 56 081 genes. Of these, 16 979 are transposable element (TE) related genes, and 39 102 are non-TE-related genes (Kawahara *et al.* 2013). It has 44% of the genome that is transcriptionally active, with a minimum gene density of one gene per 9.9 kb (International Rice Genome Sequencing Project 2005). A substantial number of independent transgenic plants are needed to tag each of these 56 081 genes with activation tags (4×-Enhancers) to enhance their endogenous expression patterns. Once such a population is developed, it can be screened for all the possible phenotypes as desired, and the population can be mined for novel genes controlling agronomically important characters.

Activation-tagged mutant populations have so far been developed only in *japonica* varieties (Kolesnik *et al.* 2004; Qu *et al.* 2008; Droc *et al.* 2013; Yang *et al.* 2013; Lo *et al.* 2015). Similar efforts could not be made in any of the *indica* cultivars because of their recalcitrance to genetic manipulation.

The current communication is the first one to report on the development of an activation-tagged population using an improved and efficient *in planta* transformation method. The transgenic plants generated were screened for WUE by growing them under limited water conditions in an *indica* rice, Samba Mahsuri (BPT-5204), which is a very widely cultivated, high-yielding variety with superior cooking quality. We also report for the first time on the tagging of ribosomal proteins (RPs) in rice plants that performed well under water-limiting conditions (with 100% field capacity) indicating their involvement in ameliorating abiotic stress including water-limiting conditions. This was corroborated by the confirmation of their significant and immediate up-regulation in various stress treatments, and these observations are reported here.

MATERIALS AND METHODS

Activation tagging vector, pSQ5

The T-DNA of the *Ac*/*Ds* activation tagging vector, pSQ5 (Qu *et al.* 2008) contains an *Activator* (*Ac*) transposase and a *Dissociator* (*Ds*) element carrying tetrameric enhancer sequences derived from the CaMV35S promoter. Because the *Ds* element tends to jump in the presence of an *Ac* transposase, a large number of events can be generated from a small number of primary transposon-carrying transgenic plants by the repeated and controlled selfing. Hence, this vector was transformed into rice through a callus-transformation protocol to develop transgenic plants. However, the excision frequency of the *Ds* element in BPT-5204 was very low, and it took three to four generations to obtain plants carrying stable *Ds* element by controlled selfing and plant maintenance. Therefore, the *Ds*-Red from pSQ5 was replaced with *Ds-bar* and cloned in a binary vector pCAMBIA 2300 (Fig. S1) to select stable *Ds* plants directly and efficiently. The T-DNA of the pDEB vector carries tetrameric repeats of CaMV35S enhancers cloned within the *Ds* element along with the *bar* gene, a resistant marker for the herbicide phosphinothricin (PPT).

Construction of the vector pDEB for activation tagging

To construct pDEB, a restriction map of the *Ds* element of pSQ5 (Fig. S1) was developed by digesting it with various restriction enzymes for subsequent cloning. Because the *Ds* element of pSQ5 contains multiple sites for most of the commonly used enzymes, a multi-step strategy was followed in vector development. The cloning of pDEB vector is detailed in Fig. S2.

Because pDEB does not contain an *Ac* element and the *Ds* insertions in the transgenic plants are stable and cannot jump in the absence of an active *Ac* transposase, a large population of independent transgenic plants needs to be developed to saturate the rice genome with tetrameric enhancers. Hence, a modified *in planta* transformation protocol was established to transform this vector into rice.

In planta transformation procedure

Although the callus-mediated transformation was successful, a relatively rapid method with high transformation efficiency was required for generating a larger population of transgenic plants. This was achieved using a novel method using vacuum infiltration coupled with sudden release of vacuum. Mature seeds of Samba Mahsuri, a widely cultivated *indica* rice cultivar, were used in this study. After surface sterilization, the dehulled seeds were incubated in water for 16 h at 28 °C in a rotary shaker (100 rpm) to soften the embryos. This is in contrast to the use of 48-hour-old husked rice seeds (Supartana *et al.* 2005; Lin *et al.* 2009); our observations showed the emergence of both radicle and hypocotyl during this stage (Fig. S3), and transformation of the explant with *Agrobacterium* after radicle emergence resulted in reduced transformation efficiency. The detailed *in planta* transformation protocol is represented in Fig. S4.

Selection of T_1 generation transgenic plants after *in planta* transformation

Seeds obtained from the *Agrobacterium*-treated plants were screened on 10 mg L^{-1} PPT selection medium, and those seedlings that germinated on the selection medium were transferred to pots in the greenhouse and were further confirmed by PCR amplification of appropriate elements present in the T-DNA. The transgenic nature of the plants and copy number of T-DNA integration were finally confirmed through Southern blot hybridization.

Growth of the transgenic plants under limited water conditions

Transgenic plants carrying stable enhancers along with wild type (WT) were grown in pots under greenhouse conditions (temperature 32 ± 2 °C, relative humidity 55 ± 5%) providing ample water (up to 3 cm overlay above the soil level that is required for normal growth of rice crop) for only first 4 weeks after transfer to soil from growth room conditions. After the fourth week, overlay water was withdrawn from the pots, and watering was subsequently restricted to a level just to maintain barely moist conditions in the soil (field capacity). This condition was maintained till maturity. Two types of WT plants were maintained: one grown along with transgenic plants under limited water supply (field capacity) and the other grown with ample water as required under normal conditions. A total of 570 out of 1070 stable Ds^+ plants obtained from the *Ac*/*Ds* population and about 2500 from 3900 T_2 generation pDEB plants were screened for their ability to grow under limited water availability with sustained or improved productivity.

Genomic DNA isolation and Southern blot hybridization analysis of transgenic plants

Genomic DNA was isolated from transgenic plants using the CTAB method with certain modifications to the standard protocols to obtain high-quality and high-yield DNA (Fig. S5). Genomic DNA (10 μg), isolated from the leaves of T_1 plants was digested overnight, and the fragments were electrophoresed on 0.8% agarose gel at 20 V for 14–16 h. Digested fragments were alkali denatured and transferred onto Hybond N^+-nylon membrane (GE Healthcare Life Sciences, Massachusetts, USA), followed by UV cross-linking (120 kJ/cm^2). After hybridization with DIG-dUTP labelled *bar* gene probe and stringency washes, binding of the probe was detected according to the manufacturer's protocol (Roche Life Science, Germany).

Chlorophyll fluorescence

Chlorophyll fluorescence, which is a measure of activity of photosystem II (PSII) and also an indicator of plant response to environmental stresses, was used to assess the overall photosynthetic performance of a plant (Murchie & Lawson 2013). Although chlorophyll (Chl) fluorescence accounts for only 1–2% of the total light absorbed by Chl, it gives an insight into the use of excitation energy by PSII and its efficiency.

In our study, the fluorescence of chlorophyll *a* was monitored using a portable pulse-amplitude modulated photosynthesis yield analyser (MINI-PAM) essentially according to the manufacturer's protocol (Walz, Effeltrich, Germany; Murchie & Lawson 2013). The quantum efficiency (F_v/F_m) of unstressed rice plants grown under normal conditions were in the range of 0.83–0.84, indicating that 16–17% of the radiation is not absorbed by the photosystem. A low F_v/F_m (where F_m is the maximum possible yield of fluorescence resulting from the application of a saturating pulse of 8000 μmol m^{-2} s^{-1} and F_v is the variability in fluorescence) indicates that plants are experiencing stress, and higher values represent high quantum efficiency (Batra *et al.* 2014).

Chlorophyll fluorescence of the activation-tagged mutant populations was measured along with two types of WT of the same age. Two readings were recorded in biological triplicates, each at 4 week interval after withdrawing water, and the mean of F_v/F_m of two observations were plotted as a histogram.

Carbon isotope analysis ($\Delta^{13}C$ measurements) for water-use efficiency

Plants discriminate between the two isotopes of carbon (^{13}C and ^{12}C) during photosynthesis through stomatal diffusion and carboxylation by Rubisco. During limited water supply, stomatal aperture tends to become reduced causing a decrease in the intercellular CO_2 concentration (Ci). The discrimination between the isotopes by Rubisco is high when Ci is high, which decreases with a decrease in Ci. Thus, $\Delta^{13}C$ value, which is the relative ratio of $^{12}C/^{13}C$, expressed relative to the PDB standard, of a plant tissue reflects the capacity of a plant for gaseous exchange through stomata, integral Ci and overall WUE of a plant (Martin & Thorstenson 1988; Bassett 2013). To determine the carbon isotope composition and WUE, 500 mg of mature leaf samples were collected just before flowering from WT

and five selected mutants grown under water deficit conditions and having high quantum efficiency. Samples were dried at 65 °C for 3 d and finely powdered, and carbon isotope ratios were analysed with an isotope ratio mass spectrometer.

Isolation of tag-end sequences of selected activation-tagged transgenic plants

Those mutants that had high quantum efficiency under limited water conditions with sustained productivity were selected for flanking sequence analysis. Thermal asymmetric interlaced PCR (Liu *et al.* 1995) was performed by using a degenerate primer and three nested primers specific for the *Ds* element for the identification of the location of activation tag in the genome and to analyse the flanking sequences. Three nested primers (NP) were designed specific to the 5′ end of the *Ds* fragment, NP1 was 1 kb upstream, NP2 was 500 bp and NP3 was 100 bp upstream relative to the 5′ end of the *Ds* element. In the pDEB vector, the *Ds* element was flanked by T-DNA border sequences. The right border sequence was used as a third nested primer, while the first and second nested primers remained the same for flanking sequence analysis of pDEB plants. Genomic DNA isolated from the transgenic plants was used as a template in the first round TAIL-PCR, while the products of subsequent reactions were diluted 50- to 100-fold according to the observation on the gel and 1 μL each of the diluted samples was used for the second and third reactions. After the third round of PCR reaction, the specific and target amplicons were purified, cloned into the pTZ57R/T cloning vector and sequenced. The sequences were submitted in a BLAST search against Rice Genome Annotation Project Database (RGAP-DB) to find the location of inserts in the genome.

Semiquantitative and Quantitative-PCR

To examine which of the tagged genes were activated by the enhancers and also the level of activation, semiquantitative and quantitative-PCR were performed on five selected transgenic plants; DEB.42, En.64, DEB.86, DEB.3 and En.16. The transcript levels of 18 genes that were situated in a 20 kb stretch near the site of T-DNA integration in these five plants were analysed with respect to the WT grown under the conditions of limited water availability.

RNA was isolated from the leaves of 2-month-old mutant and WT plants to study the expression of tagged genes and also from shoot and root tissues of 7-day-old WT seedlings subjected to abiotic treatments to determine the differential expression of RPL6 and RPL23A genes. The cDNA synthesized from 2 μg of RNA was diluted seven times, and 2 μL of it was used for Q-PCR experiments (primer details are given in Table S1). Each Q-PCR reaction was repeated as three biological replicates along with *actin*, which served as an internal reference gene. The reaction conditions for semi-quantitative-PCR included an initial denaturation of 94 °C for 3 min followed by 25–28 cycles of amplification (94 °C, 30 s; annealing temperature of 55–58 °C, 30 s; 72 °C, 30 s) with a final extension step at 72 °C for 10 min.

Stress treatments

To check whether the patterns of expression of the two RP genes (RPL6 and RPL23A) tagged by CaMV35S enhancers under various abiotic conditions, different hormonal and stress treatments were applied. The 7-day-old WT seedlings were grown at 4 and 42 °C for cold and heat treatments and submerged in polyethylene glycol (PEG; 10%) and sodium chloride (NaCl; 250 mM) for dehydration and salt treatments, respectively. For hormone treatments, solutions of abscisic acid (ABA; 100 μM), salicylic acid (SA; 3 mM) and methyl jasmonate (MeJa; 100 μM) were used. After treatments, shoot and root samples were collected separately at 5 min, 3 h, 6 h, 12 h, 24 h and 60 h intervals. Treatment with water served as a corresponding control. Quantitative analysis of gene expression in various treatments was performed using Quantitative-PCR as described earlier.

Transcript analysis of other members of 60S ribosomal subunit genes

In addition to RPL6 and RPL23A, the induced expression pattern of 34 other RPL genes was also studied. In plants, RPs exist as multigene families with an average copy number of 2–3 for each gene (Barakat *et al.* 2001). According to recent release 7 of TIGR, the proteins of the large subunit of ribosomes in rice are encoded by at least 34 candidate genes. Several orthologous genes encode the same RP gene taking the total to 123 genes. Because the functions of the orthologous genes are conserved, we selected 34 candidate genes each representing one orthologous group and studied their expression under three different abiotic conditions. The stress treatments were given by dipping roots of 7-day-old seedlings grown in MS medium in solutions of PEG-8000 (10%), ABA (100 μM) and NaCl (250 mM), after which shoot and root samples were collected separately in two biological replicates at 5 min, 3 h, 6 h, 12 h, 24 h and 60 h time intervals. Seedlings dipped in double-distilled water were served as a corresponding control. The Q-PCR data of 34 RPL genes have been represented through heat maps, which were generated by incorporating the mean values of fold change obtained from biological duplicates in the GENE-E program.

Analysis of putative promoter sequences of RPL6 and RPL23A

To identify the presence of *cis*-regulatory elements that are stress specific/responsive in the promoter regions of RPL6 and RPL23A genes, which were activated by CaMV35S enhancers in high-yielding activation-tagged mutants, we analysed the upstream sequences of these two genes. A maximum of 1.1 kb sequence 5′ upstream to each gene was analysed using the Plant Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RESULTS

Generation of *Ac/Ds* population and isolation of stable *Ds* plants

The transgenic plants carrying *Ac*/*Ds* elements developed through callus-mediated transformation were screened by antibiotic selection by germinating the seeds on Hygromycin medium (50 mg L^{-1}). Because the gene for selection marker *hpt*II is present outside the *Ds* element, plants that are resistant to Hygromycin carry either the *Ac* element alone (Ac^+/Ds^-) or both *Ac* and *Ds* elements (Ac^+/Ds^+), which were then confirmed by PCR. Seeds that did not germinate on selection medium were either null (Ac^-/Ds^-) or stable *Ds* element carrying plants (Ac^-/Ds^+).

To isolate plants with Ds^+ element alone from the seed obtained from the transgenic plants carrying *Ac*/*Ds* elements, they were cultured on Hygromycin selection medium and allowed to germinate for 4 d. After the fourth day, those seeds that did not germinate or started to bleach were transferred to selection free medium (medium without Hygromycin) to recover those plants, which were either null, stable Ds^+ plants or Hygromycin low expression lines or escapes (Ac^+/Ds^+), which were later confirmed by duplex PCR for *hpt*II and RFP genes (Fig. S6).

About 150 primary transgenic plants were obtained from callus regeneration experiments, of which 50 were randomly selected to produce 8000 T_1 seeds, which were germinated to isolate stable Ds^+ plants and plants carrying both elements, the latter were used to identify transpositions in subsequent generations. Transposition events, which were tracked through antibiotic selection and PCR amplification of DNA elements located within and outside the *Ds* element, were not identified in T_1 and T_2 generations but were obtained from T_3 generation onwards (Fig. S7). The frequency of obtaining Ds^+ from *Ac/Ds* population was 6%, 15% and 21% from 2460, 3000 and 2480 seeds screened in T_3, T_4 and T_5 generations, respectively. A total of 1030 *Ds* stable plants were recovered from 7940 *Ac/Ds* plants in three generations. However, selection of a suitable starter line is a key factor as in one particular line the frequency of obtaining stable lines was as high as 45%. The frequency of obtaining Ds^+ plants was calculated as follows:

$$\text{RFP}^+ \text{and } hpt\text{II}^- \text{ PCR plants obtained from } hpt\text{II}^- \text{ seedlings} \\ / \text{Number of seeds cultured on } hpt\text{II medium} \times 100$$

Because of the low frequency of obtaining stable *Ds* lines, plants carrying two-component TEs (Ac^+/Ds^+) need to be thoroughly screened up to T_4 to T_5 generations by repeated selfing and plant maintenance, which is a difficult task and very time-consuming.

Selection of transgenic plants after *in planta* transformation

A 16 h time point after soaking in water was found to be the most suitable time for infection, and the efficiency of stable transformation was increased from 0% (without vacuum), to 5% (with vacuum alone) and to more than 20% (a combinatorial treatment of vacuum and swift removal of vacuum; Fig. S3). Seeds obtained from the *Agrobacterium*-treated plants were screened on 10 mg L^{-1} PPT selection medium (Fig. 1a). The progeny seeds (T_1 generation) that carried the T-DNA started germinating on the selection medium within 3–4 d after inoculation, while the seeds that did not carry the T-DNA because of segregation and seeds of the non-transformed control plants did not germinate and became bleached. About 18 000 seeds obtained from the plants infected with *Agrobacterium* carrying activation tagging vector pDEB were screened for resistance to PPT, of which 3900 T_1 plants were selected to be positive. These were further confirmed by PCR amplification of appropriate DNA fragments present within the T-DNA (Fig. 1b,c,d,e,f). Some of these were progressed to T_2 generation, and PPT resistant progeny exhibited Mendelian segregation ratio of 3:1 for the integrated T-DNA (Table S2).

Of the 23 PPT-resistant and PCR-positive transformants analysed for T-DNA integration through Southern hybridization (Figs 1g & S8), 18 plants were found to be positive. Among these, 16 plants exhibited a single copy T-DNA insertion (frequency of 70%), and two plants showed the integration of two copies (8.6%). The independent nature of integration of T-DNA into the genome of transgenic plants was indicated by the different restriction fragments binding to the probe. Five PPT-resistant plants did not give a positive signal in the Southern analysis, probably because of the inferior quality of isolated genomic DNA.

Phenotypic analysis of mutants

Transgenic plants that showed better yield-related parameters with distinct phenotypes such as increased tillering (Fig. 2a), plant height (Fig. 2b), panicle number, productive panicles (Fig. 2c) and seed yield (Fig. 2d) with respect to both WT samples were selected for further analysis. WT grown with adequate water have 12–15 tillers per plant and reached a height of 90 cm, and the total seed yield was ~15–20 g in contrast to the WT grown under limited water availability, which had four to five tillers, three to five panicles per plant and started to undergo wilting 6 weeks after continuous water stress. These plants also flowered early during 70–80 d and produced a limited number (80–100 number) of partially filled grains. The transgenic plants exhibited a wide variation in growth; some of the mutants had high tiller number ranging from 5 to as high as 18 per plant, up to 10 panicles, and seed yield of 15–25 g with respect to both controls. About 120 Ds^+ and 370 pDEB plants were found to have sustained productivity of which 42 and 158 plants were, respectively selected for flanking sequence analysis.

Physiological characterization and flanking sequence analysis

The WT grown with ample water exhibited a mean quantum efficiency of 0.80, and WT under water stress conditions showed a quantum yield of 0.65 to 0.68, while the quantum efficiency of activation-tagged population grown under limiting

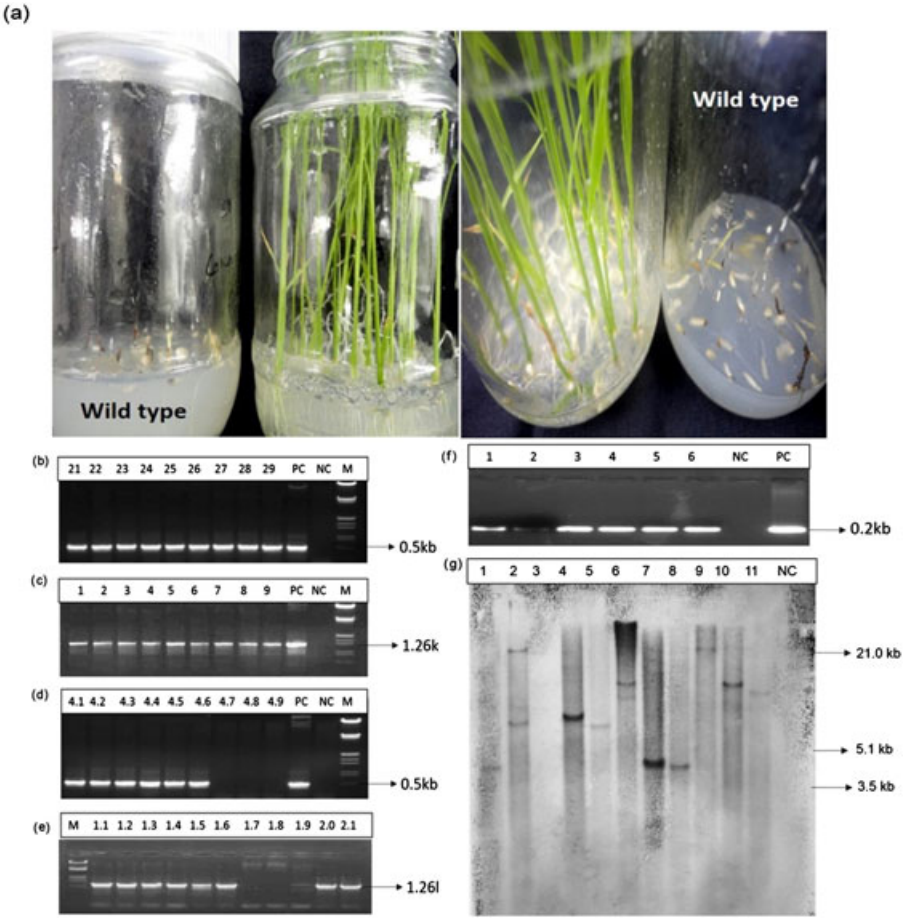

Figure 1. Screening and molecular confirmation of transgenic plants. (a) Selection of seeds derived from the *Agrobacterium*-treated rice plants on PPT (10 mg L^{-1}) medium. PCR screening of transformants selected on PPT medium with (b) *bar* gene (560 bp), (c) expression cassette of *bar* gene (1260 bp) in T_1 and (d, e) T_2 generations. (f) Semiquantitative-PCR analysis of selected transgenic plants to check the expression of *bar* gene. M, λ-*Eco*RI-*Hin*dIII DNA marker; PC, positive control; NC, negative control. (g) Southern blot hybridization. Genomic DNA from T_1 generation transgenic plants (lanes 1–11) along with NC was digested with the restriction enzyme, *Kpn*I and hybridized with the DIG-dUTP labelled *bar* gene probe.

water conditions ranged from 0.60 to as high as 0.85 (Fig. 2e). Further, the carbon isotope ratio, $\Delta^{13}C$, which is inversely related to WUE (the lesser the $\Delta^{13}C$, the higher will be WUE), was measured in five mutants; *viz.* DEB.42, EB.64, DEB.86, DEB.3 and En.16 had 18.81‰, 18.51‰, 18.06‰, 18.30‰ and 18.66‰, respectively, whereas WT showed a $\Delta^{13}C$ value of 22.05‰, indicating that the selected mutants have high WUE in addition to their productivity-related traits (Fig. 2f).

Transgenic plants with quantum efficiency ≥0.80 were selected for flanking sequence analysis. The 200 sequences obtained from selected plants after tertiary TAIL-PCR (Fig. S9) were submitted in a BLAST search against Rice Genome Annotation Project Data Base (TIGR), and their locations were mapped on corresponding rice chromosomes. Of these, the highest number of insertions were found on chromosome-3 (35), followed by chromosome-6 (26); chromosome-11 had very few insertions (4), other insertions were almost equally distributed on remaining chromosomes (Fig. 3). Further, it had been found that 57% of the *Ds*-Red insertions were intergenic and 43% were intragenic, while 44% of pDEB T-DNA insertions were intergenic and 56% were intragenic (Table 1). This analysis not only identified the genes flanking the *Ds* element at the site of insertion but also demonstrated the wide distribution of activation tags (*Ds*-4×Enhancer) on various chromosomes throughout the rice genome.

Transcript analysis of the tagged genes

Because CaMV35S enhancers elicit the expression of genes placed over a stretch of 10 kb on either side of the insertion, a minimum of a 20 kb region was mapped. Five mutants, *viz.* DEB.42, En.64, DEB.86, DEB.3 and En.16, with a quantum efficiency of 0.85, 0.80, 0.82, 0.85 and 0.84, respectively, along with low $\Delta^{13}C$, increased tillering and seed yield under limited water conditions (Fig. 4) with respect to both the controls were selected for the analysis of transcript levels of all the candidate genes residing within a 20 kb region on either side of the site of insertion of the activation tag. The details of the physiological parameters and genes tagged in these five selected mutants with their location in the genome have been provided in Table S3.

In DEB.42, three genes, LOC_Os12g32240 (translation initiation factor 5A), LOC_Os12g32250 (WRKY96 transcription

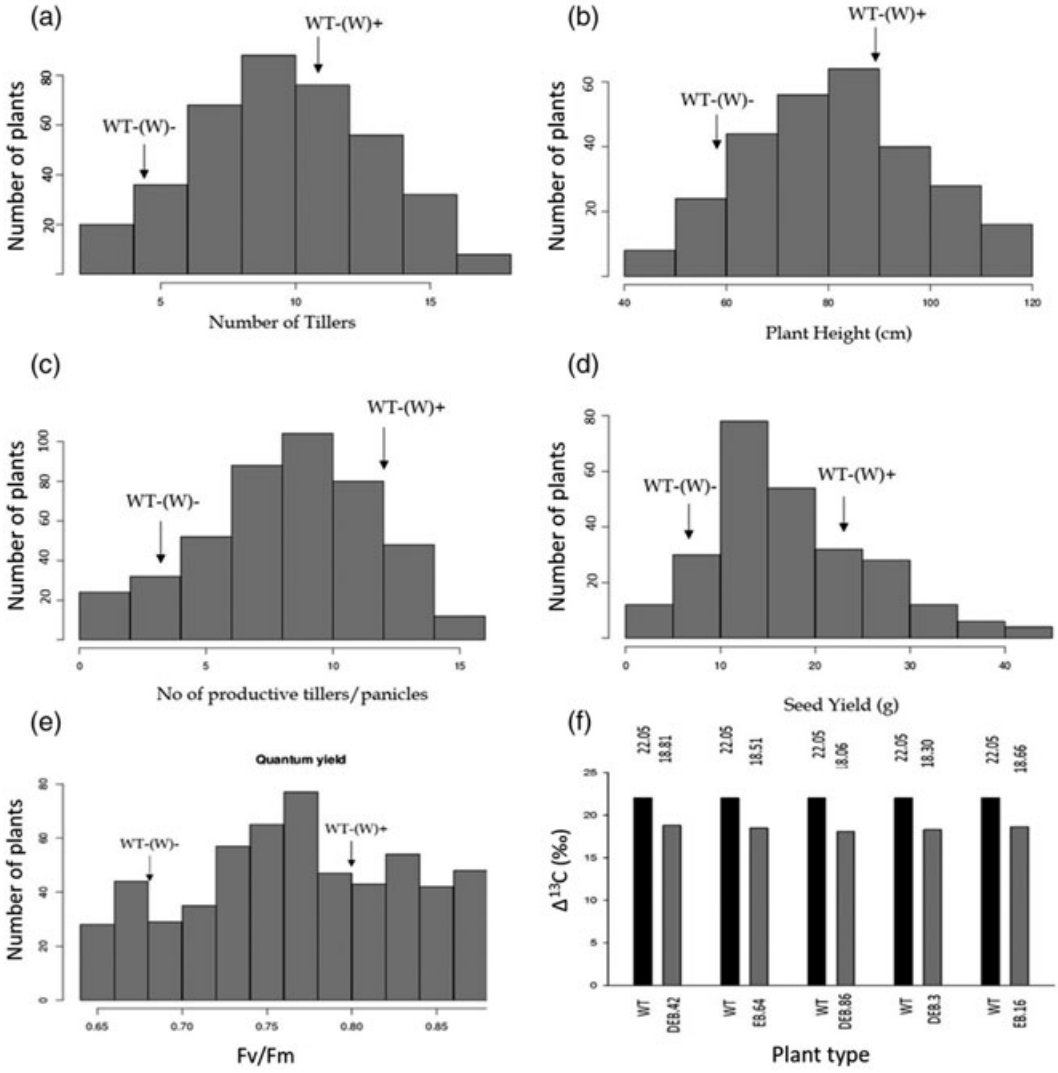

Figure 2. Various growth and physiological parameters observed under limited water conditions in activation-tagged mutant population with respect to WT. (a) Activation-tagged mutants showed variation in tiller number that ranges from 2 to as high as 18. (b) Plant height (40 cm to 120 cm). (c) Number of panicles/productive tillers per plant. (d) Total seed yield (grams) of a plant compared with WT. (e) Quantum efficiency of activation-tagged population monitored through MINI-PAM ranging from 0.65 to 0.85, while WT with and without water had 0.80 and 0.68, respectively. (f) $\Delta^{13}C$, which is a surrogate of WUE, measured in WT and five selected mutants, *viz.* DEB.42, EB.64, DEB.86, DEB.3 and En.16, are in the range of 22.05‰ and 18.81‰, 18.51‰, 18.60‰, 18.31‰ and 18.66‰, respectively. $\Delta^{13}C$ is inversely related to WUE; the lesser the $\Delta^{13}C$, the higher is the WUE. The phenotypic readings were taken as a mean of five plants, whereas the physiological data are a mean of three biological replicates.

factor) and LOC_Os12g32260 (putative expressed protein), were located within a 20 kb region of the *Ds* insertion (Fig. 5a). The transcript level of LOC_Os12g32250, which is situated ~2.1 kb upstream from the enhancers, was elevated about 15-fold in DEB.42 with respect to WT (Fig. 6a), while there was no significant activation of other two genes.

In En.64, four genes flanked the enhancers (Fig. 5b). These were LOC_Os03g57260 (GRF zinc finger family protein), LOC_Os03g57270 (ubiquitin carboxyl-terminal hydrolase), LOC_Os03g57280 (*N*-dimethyl guanosine tRNA methyltransferase protein) and LOC_Os03g57290 (cullin protein). LOC_Os03g57290, situated 9 kb downstream from the insert, became activated 20-fold (Fig. 6b). In DEB.86, the insert was flanked by four genes, LOC_Os03g40070 (transposon protein, putative expressed), LOC_Os03g40080 (GRAS family transcription factor containing protein), LOC_Os03g40084 (expressed protein) and LOC_Os03g40090 (expressed protein; Fig. 5c). Of these, the transcripts of two adjacent genes, LOC_Os03g40080 and LOC_Os03g40084, showed a six-fold elicitation (Fig. 6c).

Another phenotypic variant DEB.3 has three genes LOC_Os04g39700 (RPL6), LOC_Os04g39710 (retrotransposon protein) and LOC_Os04g39720 (retrotransposon protein) flanking the enhancers (Fig. 5d). LOC_Os04g39700 (RPL6), located 1 kb downstream of the insert, became 26-fold activated compared with WT (Fig. 6d). In En.16, four genes, LOC_Os01g24680 (3-hydroxyacyl-CoA dehydrogenase), LOC_Os01g24690 (RPL23A),

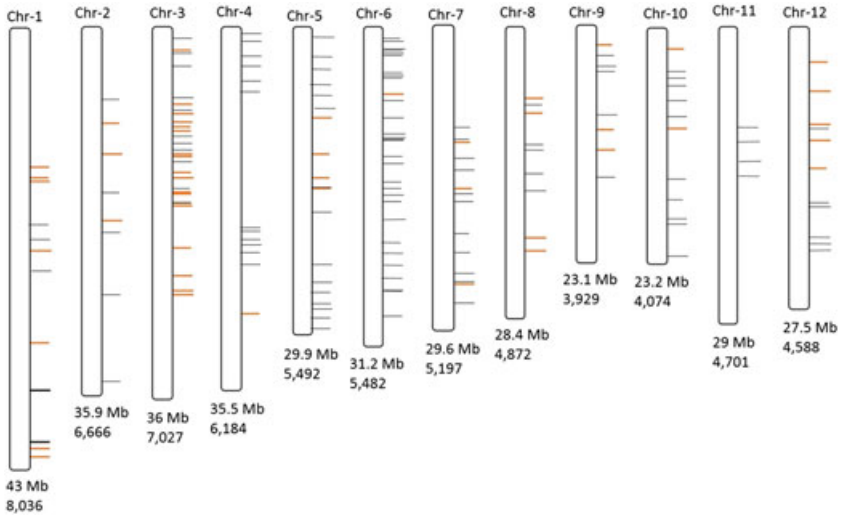

Figure 3. Distribution of genetically mapped insertions of selected plants on corresponding chromosomes. About 200 sequences obtained from the BLAST search were mapped on corresponding chromosomes. Orange bars mark the *Ds* insertions (derived from *Ac/Ds* population), while black bars represent the position of T-DNA insertions. The highest number of insertions was found on chromosome-3 (35), while chromosome-11 has only four insertions.

Table 1. Study of intergenic and intragenic flanking sequences of selected activation-tagged transgenic plants

T-DNA insertions					*Ds* insertions				
		Intragenic					Intragenic		
Chromosome no.	No. of insertions	Exonic	Intronic	Intergenic	Chromosome no.	No. of insertions	Exonic	Intronic	Intergenic
1	12	3	3	6	1	7	2	1	4
2	7	2	2	3	2	3	0	0	3
3	24	8	4	12	3	14	7	0	7
4	12	0	4	8	4	1	0	0	1
5	15	5	5	5	5	4	0	0	4
6	30	7	8	15	6	3	0	0	3
7	12	4	1	7	7	3	0	1	2
8	8	3	1	4	8	2	0	2	0
9	10	4	2	4	9	1	1	0	0
10	13	1	6	6	10	1	1	0	0
11	4	0	4	0	11	0	0	0	0
12	11	4	5	2	12	3	1	0	0
Total	158	41	45	72		42	12	6	24

Sequences obtained from BLAST search against RGAP-DB were mapped to identify the intergenic and intragenic insertions. Nearly 57% of the *Ds*-Red insertions (derived from *Ac/Ds* population) were intergenic and 43% were intragenic, while 44% and 56% of pDEB T-DNA insertions were intergenic and intragenic, respectively.

LOC_Os01g24700 (putative protein) and LOC_Os01g24710 (Jacalin-like lectin domain containing protein), were situated (Fig. 5e). LOC_Os01g24690, which is located 1 kb downstream of enhancers, became activated 18-fold (Fig. 6e).

Inducible expression pattern of RPL6 and RPL23A genes

To check the inducible expression pattern of RPL6 and RPL23A genes that were activated by enhancers to abiotic conditions, we subjected 7-day-old WT seedlings of the same rice variety, BPT-5204 (Samba Mahsuri), which was used to generate the activation-tagged mutant population to four different abiotic stress factors, namely cold (4 °C), heat (42 °C), dehydration (PEG), salt (NaCl) and three plant hormones (ABA, SA and MeJa). The expression of these genes was studied in shoot and root tissues separately at different time intervals. The transcripts of RPL6 and RPL23A had responded immediately to almost every stress tested, following which their expression was gradually ascended with the progression of the stress duration and finally reached a maximum level at a certain time point. In shoots, the level of RPL6 mRNA had reached up to 150-fold after 12 h of dehydration, 250-fold and 120-fold in

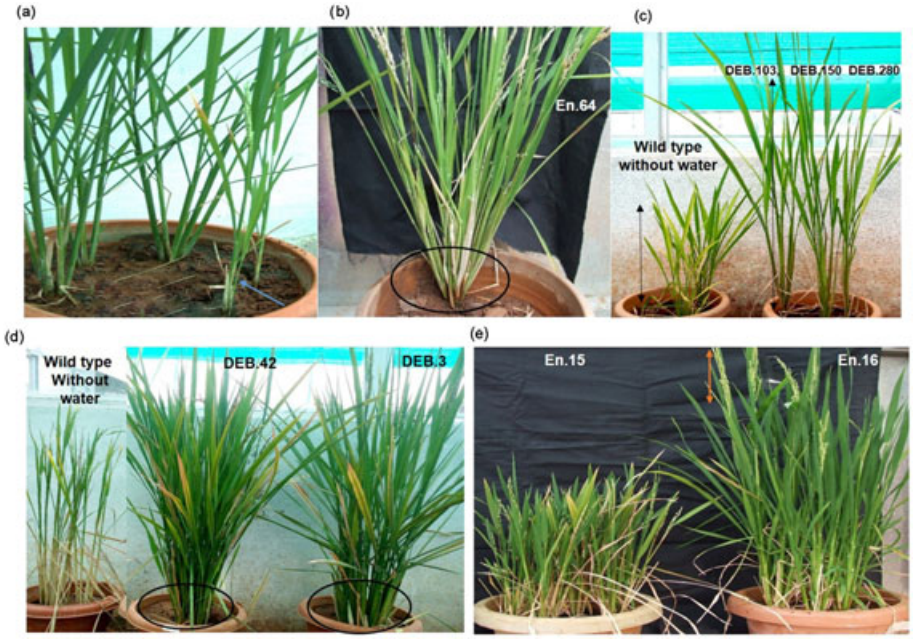

Figure 4. Phenotypic characterization of selected mutants under limited water conditions. (a) After 4 weeks of transfer to the pots, water overlay was withdrawn to maintain barely moist conditions. (b) Mutant EB.64 had 10 tillers grown under limited water conditions. (c) Mutants DEB.103, 150 and DEB.280 exhibited increased plant height than WT. (d) Six weeks after continuous growth under water deficiency, a maximum of three to four tillers was observed in WT with limited water that subsequently started to wilt, whereas DEB.42 and DEB.3 had 15 and 18 tillers, respectively, with each tiller further giving productive panicles. (e) En.16 displayed profuse tillering and panicles compared with another dwarf mutant En.15. Phenotype observations were recorded as a mean of five transgenic plants grown along with WT.

response to ABA and NaCl, respectively after 60 h of treatment. SA and MeJa also caused a significant up-regulation by more than 30-fold. Similarly, the activation of RPL6 was also observed in roots, but the level was less compared with shoots (Fig. 7a,b). The RPL23A transcripts also responded as early as 5 min after the onset of stress. Unlike RPL6, although the level of RPL23A activation was not several hundred folds, it was more in root tissues than in shoots (Fig. 7c,d). Dehydration, ABA and NaCl treatments lead to the activation by more than 40-fold in roots, whereas SA and MeJa caused the up-regulation by 17-fold in roots and 26-fold in shoots, respectively.

In silico analysis of upstream sequences of genes for two RPs

Because RPL6 and RPL23A had responded to various abiotic treatments with a very high level of activation, we checked for the presence of any stress-specific/responsive elements in their promoters. The upstream 1.1 kb putative promoter regions of RPL6 and RPL23A were found to have various *cis*-regulatory elements that possibly respond to stress/hormone-specific signals. The promoter region of RPL6 has three TGA or auxin-responsive elements and a GARE-motif that responds to plant hormones, auxins and gibberellic acid, respectively. It also has three heat-stress elements, two MeJa responsive elements and an ABA-responsive elements (ABRE) motif (Fig. 7e). High level of up-regulation of RPL6 in response to ABA and MeJa treatment is likely in congruence with the presence of the corresponding stress-responsive elements. RPL6 was also activated by heat stress (42 °C), although the level of activation was comparatively low. Also, it has TC-rich repeats, which have been found to be involved in plant defence and stress responsiveness (Diaz-De-Leon *et al.* 1993).

The promoter region of RPL23A also exhibited several *cis*-regulatory elements, which include two MeJa responsive elements, an auxin-responsive element, an ABRE-motif, and a low temperature-responsive element motif. The presence of these elements supports the transcript abundance of RPL23A in response to ABA, MeJa and cold treatments (Fig. 7f). RPL23A promoter also possesses Box-W1 motif, which is a fungal elicitor-responsive element, MBS-motif, a MYB transcription factor binding site and a W-box motif, which is a preferential binding site for WRKY transcription factors. MBS-motifs have been reported to be essential for binding of MYB transcription factors for drought-inducible gene expressions (Baldoni *et al.* 2015). Although WRKY transcription factors have diverse roles in plants, their major functions appear to be inducing tolerance against biotic and abiotic stresses (Ülker & Somssich 2004).

Differential expression of RPL genes in abiotic stress response

Two out of eight genes that were activated by the enhancers among the activation-tagged mutants in search of candidate genes responsible for high WUE belonged to 60S ribosomal subunits. We have investigated whether other members of 60S ribosomal gene family respond differentially to abiotic

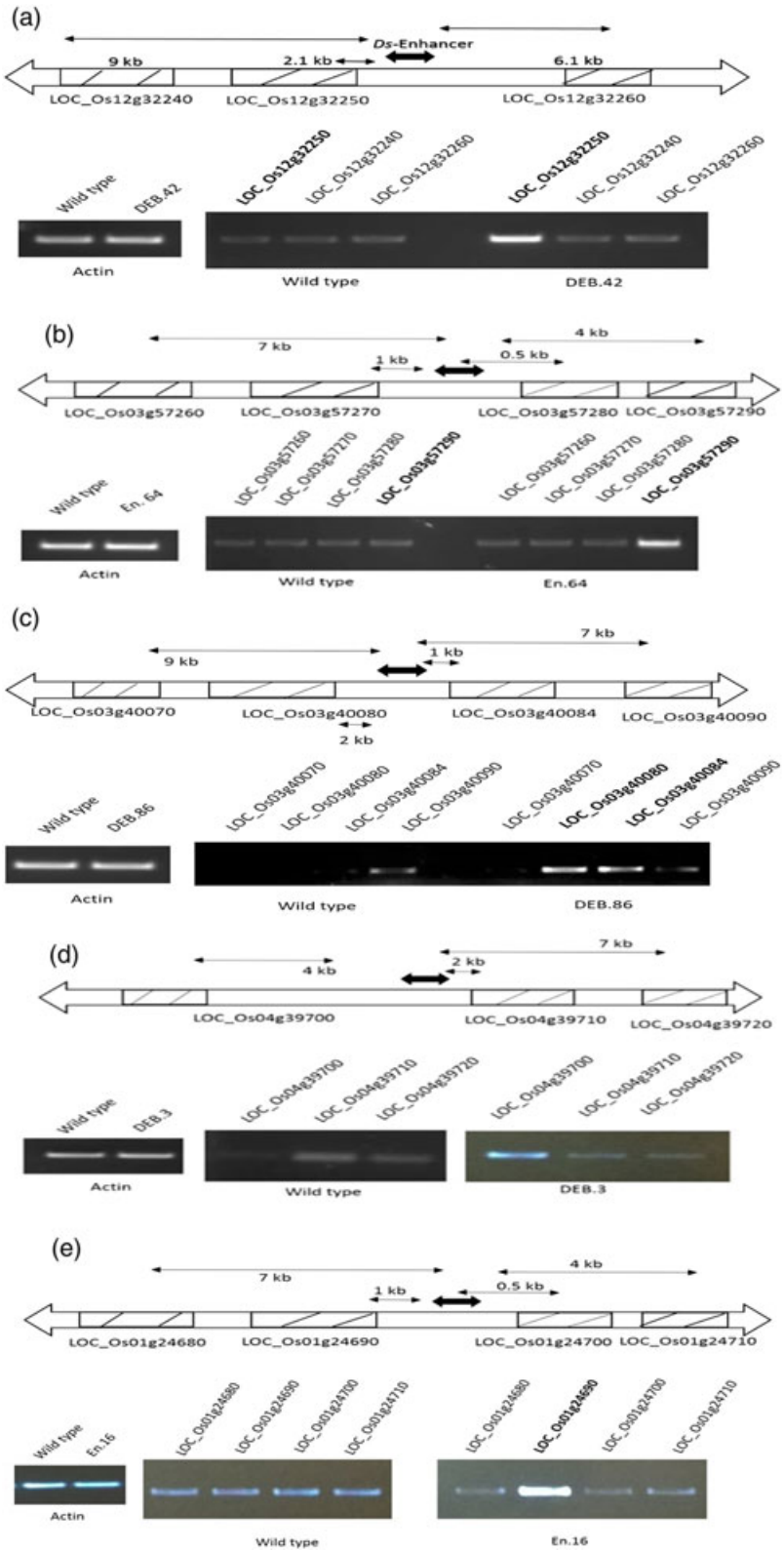

Figure 5. Semi-quantitative-PCR analysis of enhancer-tagged genes in selected mutants. (a) In DEB.42, three genes (LOC_Os12g32240, LOC_Os12g32250 and LOC_Os12g32260) were located on chromosome-12, of which the transcript level of LOC_Os12g32250 present at 10 kb upstream was up-regulated with respect to WT. (b) In En.64, four genes flanked the enhancers in a 20 kb region on chromosome-3. LOC_Os03g57290, which is situated 9 kb downstream from the insert, became activated. (c) In DEB.86, two genes (LOC_Os03g40080 and LOC_Os03g40084) were up-regulated concurrently. (d) In DEB.3, the transcript of LOC_Os04g39700 became up-regulated, whereas (e) in En.64, LOC_Os01g24690 was up-regulated. The map of insert location on each chromosome was based on RGAP-DB.

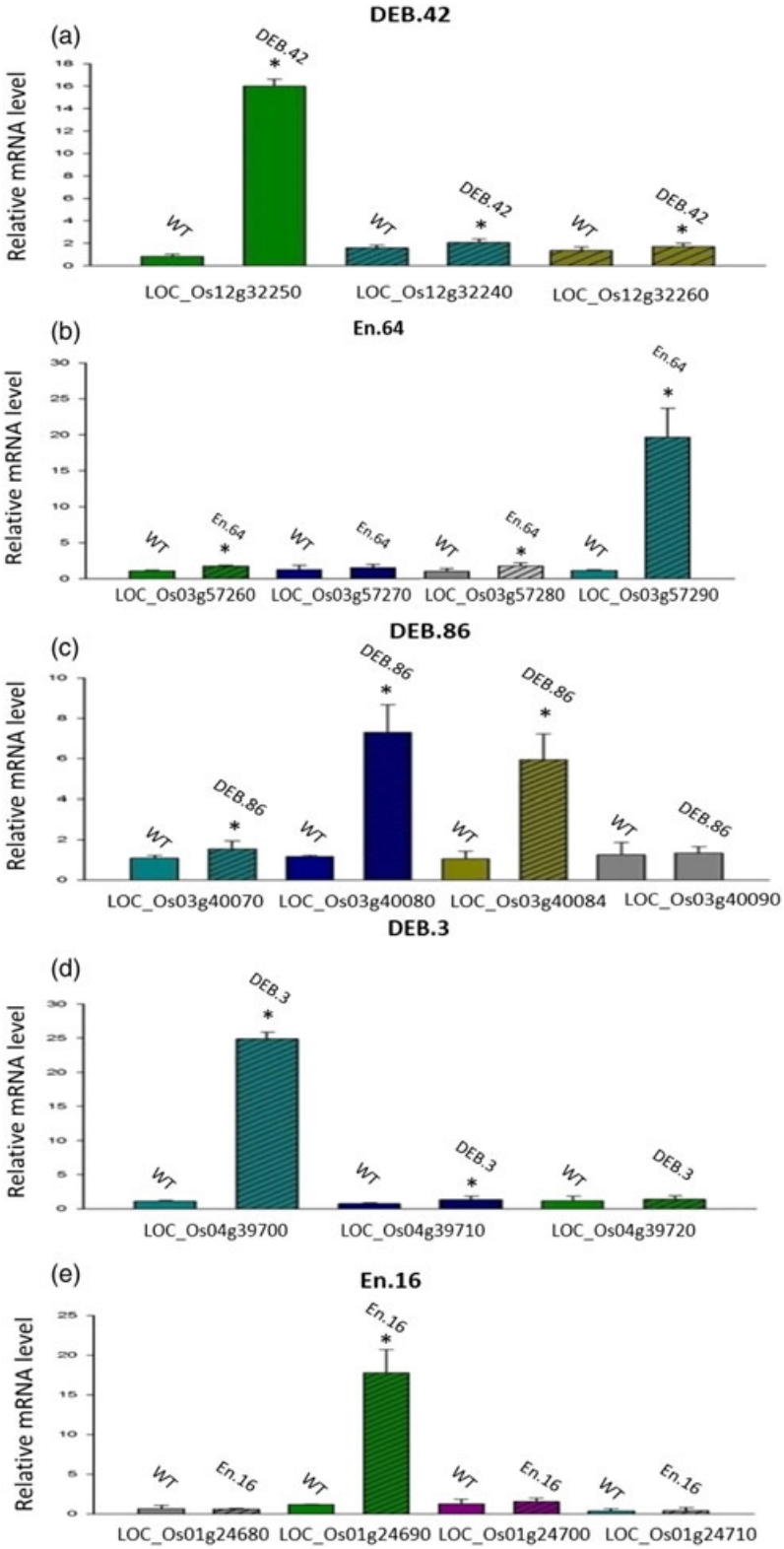

Figure 6. Quantitative-PCR analysis of genes tagged by enhancers in five selected mutants with respect to WT. Q-PCR was performed to calculate the level of activation of the enhancer-tagged genes in five selected mutants. (a) In DEB.42, the locus, LOC_Os12g32250, was activated 16-fold compared with other loci in transgenic and WT. (b) In EB.64, LOC_Os03g57290 became activated 15-fold. (c) In mutant DEB.86, of the four flanking genes, two loci LOC_Os03g40080 and LOC_Os03g40084 located 2 kb downstream and at 1 kb upstream, became elicited sixfold and eightfold, respectively. (d) In DEB.3, LOC_Os04g39700, located 1 kb downstream from the site of insert location was activated 24-fold with respect to control. (e) In En.16, the transcript level of LOC_Os01g24690 located adjacent to enhancer insertion became 15-fold activated. The relative expression was considered statistically significant at $P < 0.05$ using one-way ANOVA and represented with asterisks.

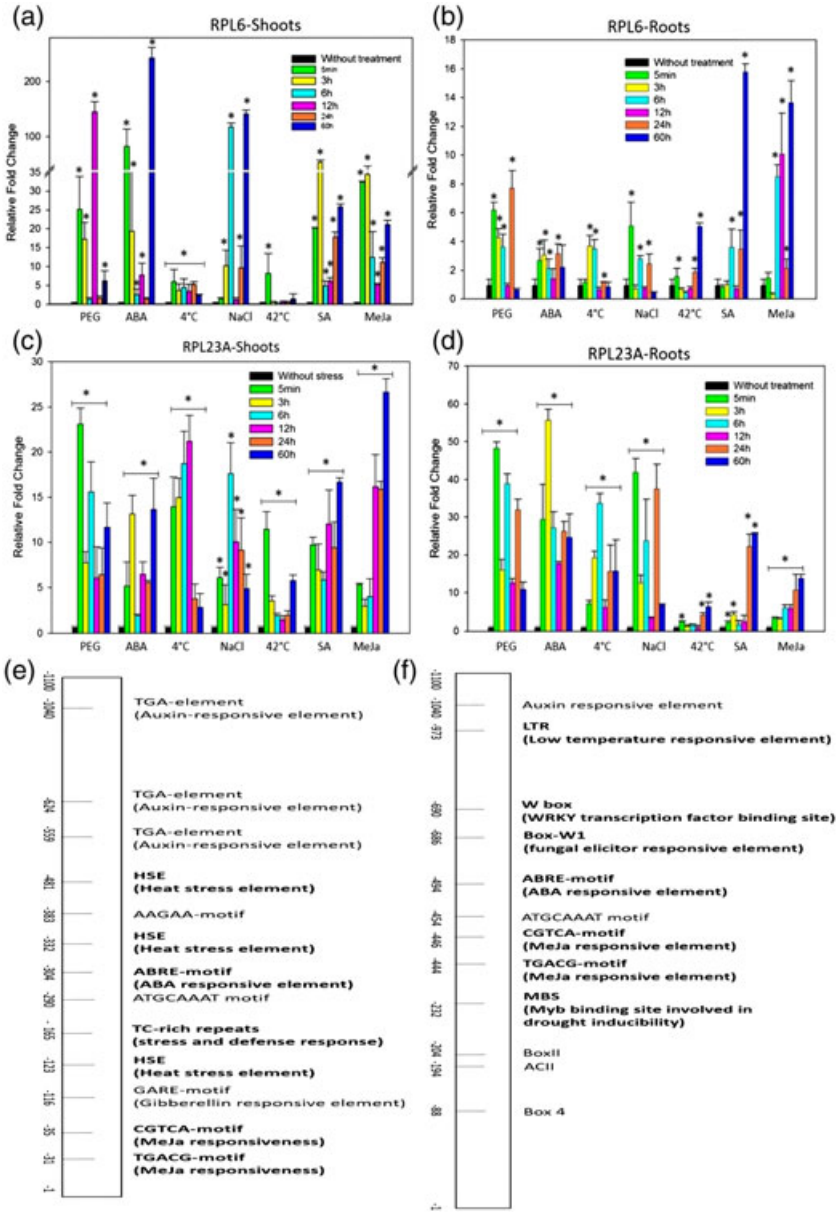

Figure 7. Tissue-specific expression of RPL6 and RPL23A under seven abiotic treatments and identification of *cis*-regulatory elements. Tissue-specific differential expression of (a, b) RPL6 and (c, d) RPL23A were carried out using 7-day-old WT rice seedlings treated with PEG, ABA, 4 °C, NaCl, 42 °C, SA and MeJa. Expression of these genes in water (without treatment) at corresponding time point was used as a calibrator to determine the relative expression under treated conditions. Rice actin (*Act1*) was used as an internal reference gene. The Q-PCR data is a mean of three biological replicates. The relative expression was considered statistically significant at P-value < 0.05 (represented with asterisks) based on one-way ANOVA in all the analysed genes. The 1.1 kb 5′ upstream regions of (e) RPL6 and (f) RPL23A genes have a wide range of *cis*-regulatory elements that respond to various environmental stresses and act as binding sites for stress-specific transcription factors. Stress-specific elements are highlighted in bold.

stress treatments. We, therefore, subjected 7-day-old rice seedlings to three abiotic treatments, *viz.* ABA, PEG and NaCl, and analysed the expression pattern of 34 rice RP genes in root and shoot tissues. Among the 34 genes, the expression of almost all genes responded to at least one of the three treatments in the form of either down- or up-regulation over a 60 h period of stress.

The genes that became up-regulated were more in number than those that were down-regulated. Among the genes that responded to the abiotic treatments (either up-regulation or down-regulation), some were specific to a particular stress in a particular tissue, while others exhibited an overlap in the pattern of expression in response to one or more treatments. We investigated the expression pattern of 34 genes at seven different time intervals. At every time point, we short-listed the genes that were specifically expressive under a given treatment or common to more than one treatment. Figure 8 describes an overlap in the expression pattern of 34 genes in three abiotic

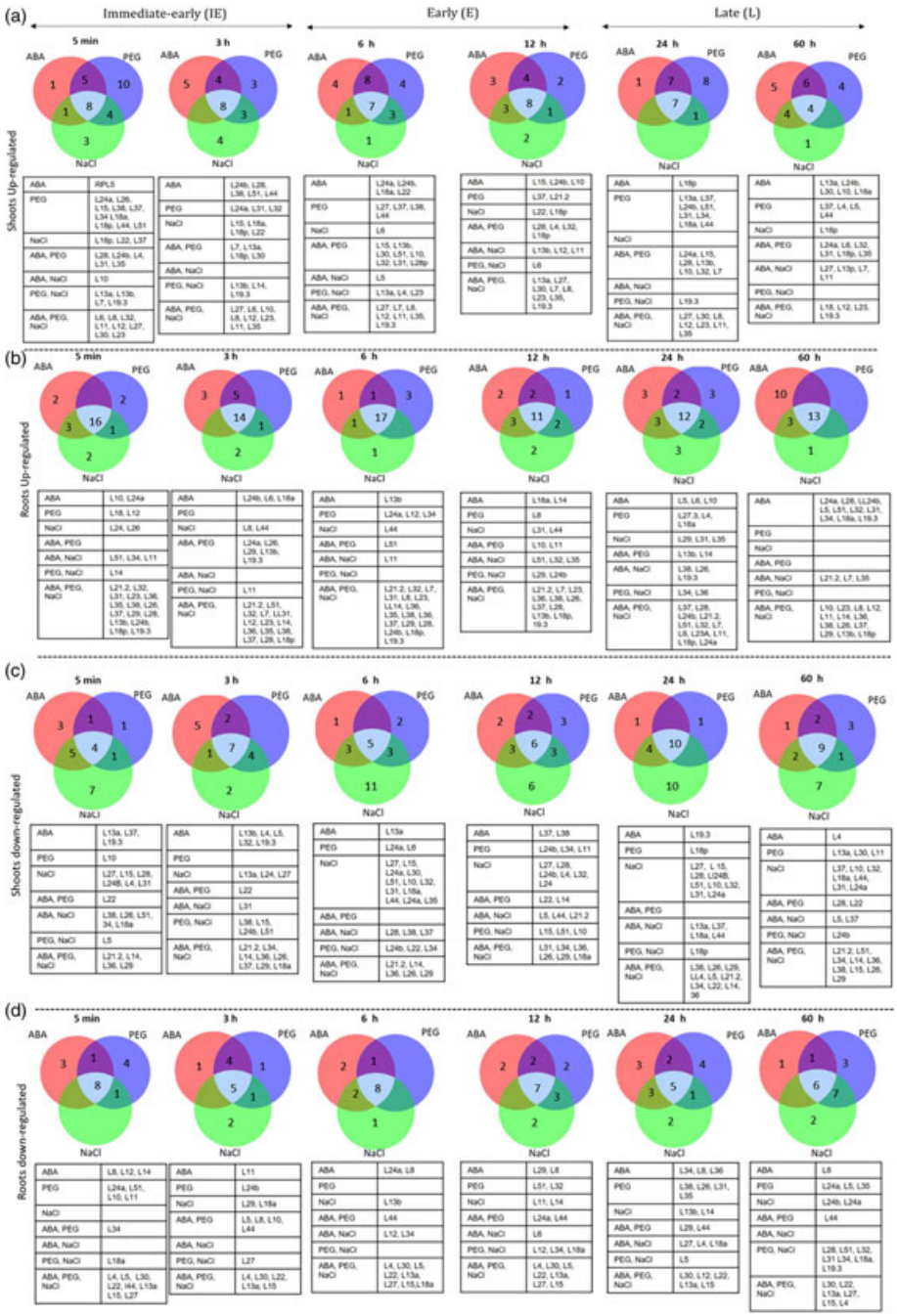

Figure 8. Venn diagrams representing the stress and spatiotemporal specific up/down-regulation of ribosomal genes. The Venn diagrams depict the total number and list of genes (presented in a table at the bottom of every chart) that were (a, b) up-regulated and (c, d) down-regulated in shoots and roots at each time point (5 min, 3 h, 6 h, 12 h, 24 h and 60 h) after subjected to each stress treatment.

treatments in roots and shoots separately in the form of Venn diagrams.

Among the transcripts that became up-regulated, majority of them responded instantaneously, and these were categorized as immediate-early (IE) genes. About 44% of ABA, 70% of PEG and 29% of salt-induced RPL genes in shoots and 61% (ABA), 58% (PEG) and 58% (NaCl-responsive) in roots became activated within 5 min to 3 h of exposure to stress. Because this immediate transcriptional activation of genes might not be stress-specific and probably has been induced because of the activity of overall plant environmental stress responsive genes (Kilian *et al.* 2007), we looked for a split in the expression pattern of IE-RPL genes. The transcriptional activity of some of the IE-RPL genes that were common to any two treatments such as RPL6, L7, L11, L12, L13b, L18P, L23A, L24a, L30, L32, L37 and L44 was initially declined, which was followed by an elevation in their expression as the treatments progressed in both shoot and root tissues. However, some of the IE-RPL genes such as RPL23A, L19.3 and L38 maintained a high level of expression throughout the duration of any two or three

treatments. The level of transcriptional up-regulation of these two classes of genes varied as some exhibited a very high level of up-regulation up to several hundred folds (RPL6, L8, L12, L19.3 and L35), some had moderate expression and others have comparatively low transcript levels.

These two classes of IE-RPL genes, which were activated instantaneously after stress and exhibited a split in the expression or maintained a continuous high level of expression, might function as an immediate defence to stress (Kawasaki *et al.* 2001). The expression of another set of IE-RPL genes declined and became stationary after continuous exposure to ABA, PEG and NaCl treatments (Table S4 presents a list of the expression pattern of different IE-RPL genes under the three treatments). Figure 9 represents a heat map of differential transcriptional regulation of genes in response to ABA, PEG and salt treatments. Each row, which depicts the differential expression of a gene through variation in coloration at different time points, was followed by an expression profile spark line that shows the respective pattern of expression. The transcripts, which became activated between 3 to 12 h and after 12 h of exposure to stress were categorized as early (E) and late (L) responding genes, respectively. Table 2 detailed the list of IE, E and L-RPL genes with fold changes under the three treatments in shoots and roots. Among those that were down-regulated, NaCl-induced the down-regulation of more number of genes compared with PEG and ABA treatments. RPL30, L44, L22, L14, L29, L36, L13a and L15 were commonly down-regulated in all the three treatments. Salt-induced down-regulation of RPL32 has been consistent as shown earlier (Mukhopadhyay *et al.* 2011).

DISCUSSION

Plant tolerance to water stress can be improved by drought avoidance or drought tolerance among which drought avoidance tends to conserve water by WUE. While drought avoidance is the maintenance of high water status under water deficit conditions, drought tolerance refers to the ability of one genotype to yield better than the other in a severely dehydrated state (Blum 2005). In drought tolerant genotypes, yield parameters are not adversely affected as grain yield is supported by mobilization of stem reserves, which occurs predominantly during drought stress (Blum *et al.* 1994; Yang *et al.* 2001). The genotypes with high WUE experience reduced biomass and yield because of stomatal closure to avoid transpirational water loss, which is also accompanied by reduced carbon assimilation under the conditions of limited water availability (Blum *et al.* 2005). However, overexpression of WUE genes in rice and other crops resulted in transgenic plants with higher biomass and seed yield compared with the WT plants under limited water conditions (Sivamani *et al.* 2000; Karaba *et al.* 2007). Hence, identification of genes having potential in improving plant yield under water deficit conditions assumes importance in the generation of transgenic plants with enhanced WUE without suffering a reduction in yield and biomass. Proteins that most probably function in abiotic stress tolerance include heat shock proteins, LEA proteins, transcription factors such as bHLH, bZIP, NAC, AP2/ERF, MYB, Zinc finger, WRKY, kinases (calcium-dependent kinases, MAP kinases), osmoprotectants, water channels, detoxifying and metabolic enzymes. Overexpression of these endogenous genes in transgenic rice resulted in increased yield under limited water conditions (Dubouzet *et al.* 2003; Zhang *et al.* 2004; Karaba *et al.* 2007; Nakashima *et al.* 2007; Hu *et al.* 2008; Jeong *et al.* 2010).

In *Arabidopsis,* with a genome size of 134 Mb and 27 416 protein coding genes (Lamesch *et al*. 2012) averaging 2940 bp size and a gene density of 4.35 kb/gene, 60% of the genome constitutes the transcriptionally active region, and insertion of the *Ds* in the genic region is possible and expected. In rice, with about 165 Mb transcriptionally active area (including 54 Mb TE-related genic region and 111 Mb non-TE related genic region) from 373 Mb of non-overlapping sequence (comprising 44% of total coding area), high frequency of insertion of the *Ds* between the coding regions (57%) indicated that the *Ds* element preferentially transposed to genic sites. Preferential intergenic transposition makes the activation tagging technique more feasible and can be extended to other crop systems as well.

The transformation protocol developed in this study completely eliminated the need for tissue culture, and the transgenic plants obtained can be comparable to WT with respect to the genome constitution except for the integrated T-DNA. Hence, this method can be suitable for developing transgenic rice plants of agronomic value and producing large populations that are required for functional genomic studies without interference from the endogenous TEs. These TEs become activated during stressful conditions that result from *in vitro* tissue culture (Mieulet *et al.* 2013).

The excision of the *Ds* element was very low in *indica* rice, and the highest frequency of obtaining stable Ds^+ plants even in the T_5 generation was 21%, which is in contrast to the highest transposition frequency of up to 80% observed in *japonica* cultivars (Qu *et al.* 2008). Because of this, the transposon-carrying plants needed to be screened through selfing in several generations to obtain a larger population of stable *Ds* plants. As an alternative, we developed another activation tagging vector (pDEB) that carries tetrameric enhancers.

The transgenic plants carrying stable *Ds* element with tetrameric enhancers or pDEB vector based enhancer elements were grown under limited water conditions (at field capacity without normal water overlay that is required for rice cultivation) until plant maturity to select for plants with enhanced WUE. Among the 200 mutants, we selected five plants having high quantum efficiency, low $\Delta^{13}C$ and exhibiting suitable growth and productivity-related phenotypes to study the expression of all the candidate genes located within a 20 kb stretch on either side of the enhancer insertions on the chromosomes. It was found that only one of the three to four genes residing in the 20 kb region were activated in four out of five plants analysed, while two genes became activated in one plant indicating that an enhancer can activate more than one gene simultaneously near the site of insertion.

Transcript analysis of the tagged genes studied in the five mutants revealed the activation of transcription factors (WRKY96, GRAS), proteins involved in protein ubiquitination (cullin4) and ribosome biogenesis (RPL6 and

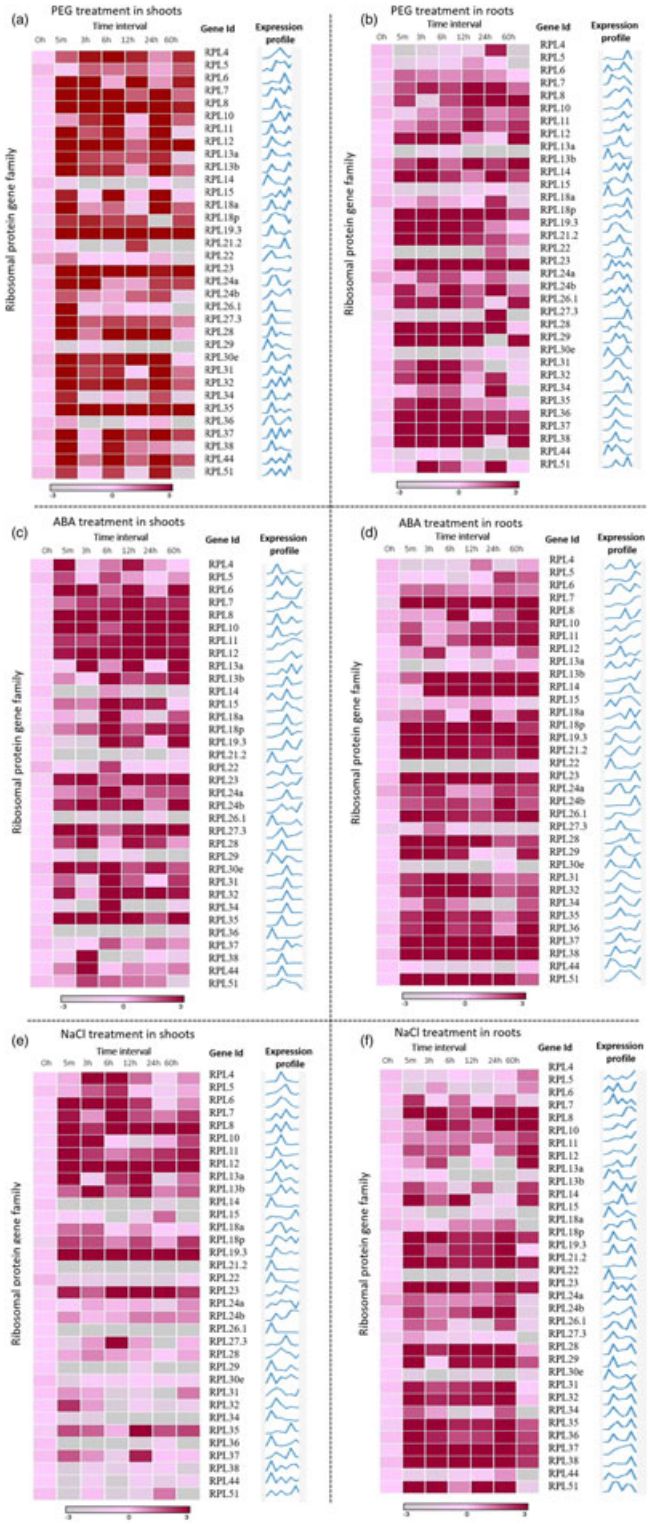

Figure 9. Heat map depiction of differential expression of 34 rice 60S ribosomal protein candidate genes under abiotic stress conditions. The heat map generated for expression profiling showed differential transcript abundancy/inadequacy of each ribosomal gene in response to (a, b) PEG, (c, d) ABA and (e, f) NaCl treatment in shoot and root tissues. Dark- and light-coloured grids indicate transcript abundancy and inadequacy, respectively. Heat maps were generated using the GENE-E program.

RPL23A). Approximately 100 WRKY transcription regulator family genes have been identified in rice having diverse roles including tolerance to biotic and abiotic stress (Bakshi & Oelmüller 2014). With respect to this, activation of WRKY96 by enhancers in DEB.42 and sustenance of this mutant to limited water conditions could be easily correlated.

Table 2. Comparative regulation of large subunit of ribosomal protein gene family under various stress treatments

		Shoot				Root			
TIGR locus	Protein	Regulation	Response	Maximum fold change	*P*-value	Regulation	Response	Maximum fold change	*P*-value
ABA									
LOC_Os03g58204	RPL4	Up	IE (24.34)	24.3442	0.005	Down	—		
LOC_Os05g11710	RPL5	Up	IE (3.85)	3.8548	0.199	Up	L (4.76)	3.1732	0.009
LOC_Os04g39700	RPL6	Up	IE (82.38)	250.307	0.017	Up	IE (3.08)	3.085	0.589
LOC_Os04g51630	RPL7	Up	IE (4.48)	13.7817	0.001	Up	IE (12.2)	62.584	0.001
LOC_Os12g38000	RPL8	Up	IE (239)	239.0753	0.004	Up	L (13.6)	3.2611	0.001
LOC_Os11g01420	RPL10	Up	IE (143.4)	143.4743	0.01	Up	IE (3.66)	6.4347	0.001
LOC_Os04g50990	RPL11	Up	IE (4.89)	8.8797	0.004	Up	IE (5.23)	9.5285	0.001
LOC_Os02g10540	RPL12	Up	IE (6.5)	280.5248	0.004	Up	E (5.73)	5.7364	0.007
LOC_Os07g01870	RPL13a	Up	E (8.75)	16.1879	0.006	Down	—	—	0.007
LOC_Os03g37970	RPL13b	Up	E (14.7)	14.7666	0.004	Up	IE (3.007)	58.1498	0.005
LOC_Os02g40880	RPL14	Down	—	—		Up	IE (9.4)	99.0597	0.007
LOC_Os03g40180	RPL15	Up	E (16.2)	16.2582	0.009	Down	—	—	0.038
LOC_Os05g49030	RPL18a	Up	E (18.92)	18.9234	0.006	Up	IE (4.72)	8.0658	0.001
LOC_Os01g67134	RPL18p	Up	IE (3.65)	13.1343	0.014	Up	IE (10.90)	32.9422	0.001
LOC_Os03g21940	RPL19.3	Up	E (30.06)	33.485	0.005	Up	IE (52.83)	52.832	0.009
LOC_Os10g32820	RPL21.2	Down	—	—	0.011	Up	IE (24.72)	41.5009	0.018
LOC_Os09g08430	RPL22	Up	E (4.35)	4.3518	0.009	Down	—	—	0.041
LOC_Os01g24690	RPL23A	Up	IE (6.87)	14.6887	0.166	Up	IE (29.42)	56.2693	0.014
LOC_Os01g24690	RPL24	Up	E (3.02)	12.5439	0.008	Up	IE (4.65)	6.3795	0.026
LOC_Os01g04730	RPL24b	Up	IE (5.64)	17.5887	0.004	Up	IE (3.42)	170.1135	0.028
LOC_Os11g05370	RPL26.1	Down	—	—	0.005	Up	IE (8.11)	14.5404	0.005
LOC_Os02g18380	RPL27.3	Up	IE (12.36)	36.0092	0.004	Down	—	—	0.043
LOC_Os07g36090	RPL28	Up	IE (4.16)	111.9472	0.004	Up	IE (19.3)	164.6033	0.004
LOC_Os06g51530	RPL29	Down	—	—	0.026	Up	IE (29.12)	25.3614	0.007
LOC_Os07g44230	RPL30e	Up	IE 7.89 ()	63.9518	0.153	Down	—	—	0.014
LOC_Os06g21480	RPL31	Up	IE (3.17)	48.2484	0.005	Up	IE (4.66)	10.0181	0.03
LOC_Os09g32532	RPL32	Up	IE (6.62)	68.5485	0.01	Up	IE (5.65)	39.8725	0.016
LOC_Os09g24690	RPL34	Up	L (21.1)	21.1681	0.005	Up	IE (5.27)	5.2783	0.011
LOC_Os05g48310	RPL35	Up	IE (30.18)	248.9023	0.006	Up	IE (5.13)	16.4339	0.064
LOC_Os01g62350	RPL36	Down	—	—	0.004	Up	IE (4.95)	24.7725	0.009
LOC_Os01g62350	RPL37	Down	—	—	0.005	Up	IE (17.42)	58.5086	0.006
LOC_Os11g24610	RPL38	Up	IE (13.9)	12	0.007	Up	IE (64.93)	75.4605	0.0104
LOC_Os07g33898	RPL44	Up	IE (25.8)	2.3058	0.001	Down	—	—	0.022
LOC_Os03g10930	RPL51	Up	IE (4.31)	4.3108	0.001	Up	IE (7.86)	16.2279	0.001
PEG									
LOC_Os03g58204	RPL4	Up	IE (3.01)	11.7354	0.0159	Up	L (6.11)	7.51108	0.023
LOC_Os05g11710	RPL5	Up	L (3.08)	3.0871	0.05	Up	Down	—	—
LOC_Os04g39700	RPL6	Up	IE (25.02)	145.159	0.007	Up			0.024
LOC_Os04g51630	RPL7	Up	IE (11.03)	13.9149	0.095	Up	IE (3.7)	8.426	0.015
LOC_Os12g38000	RPL8	Up	IE (28.2)	876.214	0.004	Up	E (4.8)	36.9725	0.004
LOC_Os11g01420	RPL10	Up	E (5.2)	19.0453	0.005	Up	E (4.71)	4.7194	0.006
LOC_Os04g50990	RPL11	Up	IE (26.7)	26.7152	0.003	Up	IE (3.4)	8.0905	0.001
LOC_Os02g10540	RPL12	Up	IE (163.7)	163.7058	0.004	Up	IE (6.7)	36.0333	0.007
LOC_Os07g01870	RPL13a	Up	IE (9.3)	9.3299	0.007	Down	—	—	0.004
LOC_Os03g37970	RPL13b	Up	IE (15.4)	21.2421	0.005	Up	IE (3.7)	22.8814	0.001
LOC_Os02g40880	RPL14	Down	—	—	0.001	Up	IE (8.28)	8.2814	0.001
LOC_Os03g40180	RPL15	Up	IE (5.95)	14.4504	0.011	D	—	—	0.017
LOC_Os05g49030	RPL18a	Up	IE (9.5)	9.5136	0.004	Up	L (5.28)	5.2851	0.001
LOC_Os01g67134	RPL18p	Up	IE (4.91)	5.8181	0.001	Up	IE (9.9)	29.562	0.006
LOC_Os03g21940	RPL19.3	Up	IE (213.1)	213.122	0.001	Up	IE (15.6)	19.6832	0.006
LOC_Os10g32820	RPL21.2	Up	E (4.5)	4.5621	0.008	Up	IE (8.96)	8.9631	0.005
LOC_Os09g08430	RPL22	Down	—	—	—	Down	—	—	—
LOC_Os01g24690	RPL23A	Up	IE (23.5)	15.7592	0.0109	Up	IE (48.3)	48.3154	0.004
LOC_Os01g24690	RPL24a	Up	IE (7.69)	7.874	0.01	Up	IE (4.025)	4.0253	0.006
LOC_Os01g04730	RPL24b	Up	L (3.82)	3.2808	0.0159	Up	IE (6.2)	13.3898	0.006
LOC_Os11g05370	RPL26.1	Up	IE (10.6)	10.6831	0.004	Up	IE (6.2)	18.2521	0.027
LOC_Os02g18380	RPL27.3	Up	IE (24.05)	24.0152	0.001	Up	L (13.05)	13.0502	0.007

(*Continues*)

Table 2. (Continued)

		Shoot				Root			
TIGR locus	Protein	Regulation	Response	Maximum fold change	*P*-value	Regulation	Response	Maximum fold change	*P*-value
LOC_Os07g36090	RPL28	Up	E (39.2)	39.2292	0.004	Up	IE (9.27)	19.9663	0.012
LOC_Os06g51530	RPL29	Down	—	—	0.006	Up	IE (13.2)	57.5807	0.004
LOC_Os07g44230	RPL30e	Up	IE (22.09)	16.3594	0.008	Down	—	—	0.001
LOC_Os06g21480	RPL31	Up	IE (17.4)	20.6065	0.004	Up	IE (3.45)	7.5798	0.005
LOC_Os09g32532	RPL32	Up	IE (24.98)	34.2632	0.005	Up	IE (4.07)	15.2326	0.005
LOC_Os09g24690	RPL34	Up	IE (5.44)	5.4433	0.005	Up	IE (3.04)	30.2738	0.006
LOC_Os05g48310	RPL35	Up	IE (496.9)	496.906	0.005	Up	IE (5.08)	12.1035	0.012
LOC_Os01g62350	RPL36	Down	—	—	0.006	Up	IE (7.6)	30.7245	0.001
LOC_Os01g62350	RPL37	Up	IE (27.05)	30.054	0.004	Up	IE (17.6)	57.8211	0.016
LOC_Os11g24610	RPL38	Up	IE (21.45)	21.4577	0.003	Up	IE (14.06)	57.0962	0.001
LOC_Os07g33898	RPL44	Up	IE (18.51)	36.3906	0.006	Down	—	—	0.004
LOC_Os03g10930	RPL51	Up	IE (4.51)	6.3857	0.006	Up	IE (14.2)	22.4642	0.009
NaCl									
LOC_Os03g58204	RPL4	Up	E (40.2)	40.2494	0.007	Up	Down	—	—
LOC_Os05g11710	RPL5	Up	E (3.3)	5.2903	0.048	Up	Down	—	—
LOC_Os04g39700	RPL6	Up	IE (10.2)	127.13	0.005	Up	IE (45.9)	45.979	0.009
LOC_Os04g51630	RPL7	Up	E (6.7)	10.6238	0.028	Up	IE (26.76)	139.0102	0.004
LOC_Os12g38000	RPL8	Up	IE (48.4)	64.8624	0.001	Up	IE (7.06)	9.9032	0.009
LOC_Os11g01420	RPL10	Up	IE (7.8)	60.9375	0.01	Up	L (4.4)	4.4393	0.017
LOC_Os04g50990	RPL11	Up	IE (18.1)	18.1352	0.001	Up	IE (11)	10.2093	0.001
LOC_Os02g10540	RPL12	Up	IE (59.6)	170.3959	0.001	Up	IE (4.3)	7.7927	0.009
LOC_Os07g01870	RPL13a	Up	E (20.5)	20.5578	0.003	Down	—	—	0.005
LOC_Os03g37970	RPL13b	Up	IE (3.02)	10.0838	0.001	Up	IE (4.3)	4.6203	0.006
LOC_Os02g40880	RPL14	Down	—	—	0.011	Up	IE (14.9)	51.4105	0.008
LOC_Os03g40180	RPL15	Down	—	—	—	Down	—	—	—
LOC_Os05g49030	RPL18a	Down	—	—	—	Down	—	—	—
LOC_Os01g67134	RPL18p	Up	IE (18.4)	4.8979	0.001	Up	IE (32.9)	32.9	0.005
LOC_Os03g21940	RPL19.3	Up	IE (48.04)	48.4087	0.001	Up	IE (21.2)	25.5497	0.005
LOC_Os10g32820	RPL21.2	Down	—	—	0.001	Up	IE (11.4)	106.6211	0.018
LOC_Os09g08430	RPL22	Down	—	—	0.009	Down	—	—	0.045
LOC_Os01g24690	RPL23A	Up	IE (6.06)	17.6154	0.0124	Up	IE (41.90	41.9289	0.036
LOC_Os01g24690	RPL24	Down	—	—	—	Up	IE (3.45)	4.1919	0.001
LOC_Os01g04730	RPL24b	Down	—	—	—	Up	IE (5.1)	11.0093	0.009
LOC_Os11g05370	RPL26.1	Down	—	—	0.045	Up	IE (3.6)	4.1897	0.006
LOC_Os02g18380	RPL27.3	Down	—	—	—	Down	—	—	—
LOC_Os07g36090	RPL28	Down	—	—	0.009	Up	IE (18.9)	18.9877	0.005
LOC_Os06g51530	RPL29	Down	—	—	0.032	Up	IE (30.5)	30.5064	0.001
LOC_Os07g44230	RPL30e	Down	—	—	0.011	Down	—	—	0.008
LOC_Os06g21480	RPL31	Down	—	—	—	Up	IE (6.6)	13.1491	0.001
LOC_Os09g32532	RPL32	Down	—	—	0.006	Down	—	—	0.01
LOC_Os09g24690	RPL34	Down	—	—	0.004	Up	IE (3.3)	3.317	0.005
LOC_Os05g48310	RPL35	Up	IE (3.83)	17.0443	0.243	Up	IE (9.9)	22.742	0.001
LOC_Os01g62350	RPL36	Down	—	—	0.005	Up	IE (18.3)	18.3195	0.001
LOC_Os01g62350	RPL37	Up	IE (3.47)	6.0471	0.001	Up	IE (9.5)	26.1374	0.005
LOC_Os11g24610	RPL38	Down	—	—	0.001	Up	IE (26.05)	69.2948	0.005
LOC_Os07g33898	RPL44	Down	—	—	0.024	Down	—	—	0.001
LOC_Os03g10930	RPL51	Down	—	—	—	Up	IE (16.3)	16.4535	0.004

The expression of 34 RPL genes was studied in response to three abiotic treatments (ABA, PEG and NaCl) through Q-PCR. 'Down' indicates a lower abundance, and 'Up' indicates a comparatively greater abundance of each RPL gene transcript under every treatment. The relative expression was considered statistically significant at P-value < 0.05 based on one-way ANOVA in all the analysed genes. With respect to time-based expression of genes, they were classified as follows: IE, immediate-early that expressed within 5 min to 3 h after treatment; E, early that expressed between 3 h to 12 h; L, late responsive genes that expressed after 12 h of treatment. Under the column 'response', the values in brackets represent the fold change at that time point.

In mutant En.64, the locus LOC_Os03g57290 encodes cullin-4, a hydrophobic protein that acts as a scaffold to carry E2-ubiquitin binding RING protein RING box1 and the substrate-recruiting protein (Zhihua & Vierstra 2011). Cullin-RING ligases have been shown to exhibit diverse roles in *Arabidopsis* that include light perception, hormone signalling

and response to biotic and abiotic stress (Lyzenga & Sophia 2011; Stone 2014). In DEB.86, two genes encoding a GRAS (gibberellic acid insensitive repressor of GA1 and Scarecrow) transcription factor (LOC_Os03g40080) and an unclassified expressed protein (LOC_Os03g40084) became activated simultaneously. Recently, a member of GRAS transcription factor OsGRAS23 located on chromosome 4 was shown to improve tolerance of transgenic rice to drought stress (Xu *et al.* 2015).

In addition to their housekeeping functions such as ribosome biogenesis, protein translation and post translational modifications, RPs participate in developmental and growth processes and also respond to various stresses. Out of the 241 RP genes in *Arabidopsis* (143 large subunit and 98 small subunit genes), almost all were up-regulated under macro elements deficiency (Wang *et al.* 2013). In Maize, the expression of a large number of RPs was up-regulated along with RPL6 in response to UV-B exposure (Casati & Walbot 2003). Knockout of *Arabidopsis At*RPL23A gene through RNAi resulted in retarded plant growth, irregular leaf and root morphology and loss of apical dominance (Degenhardt & Bonham-Smith 2008). Therefore, activation of RPL23A by enhancers in En.16 mutant with high-yielding phenotype suggests the fact that RPL23A plays an important role in fitness traits. Corroborating this, the up-regulation was instantaneous and high in various stress treatments indicating its significant involvement in abiotic stress amelioration. In *Arabidopsis*, the location of RPL23A has been mapped adjacent to the polypeptide exit tunnel suggesting its important role in protein translocation and secretion (Maier *et al.* 2005).

An orthologue of RPL6 (LOC_Os02g37862) has also been shown to be up-regulated in response to biotic stress attack by the brown planthopper (BPH) in BPH susceptible rice lines (Wei *et al.* 2009). This is in correlation with the up-regulation of RPL6 by SA and MeJa treatments, which are the key plant hormones involved in plant defence against insects and necrotrophic pathogens in the present study. RPL6, whose role has been demonstrated earlier in biotic stress tolerance, has also been shown to be involved in enhancing WUE in the present study along with its immediate and several hundred fold up-regulation in ABA, PEG and NaCl treatments.

The presence of significant stress-responsive *cis*-regulatory elements in addition to many other motifs in the promoter regions of RPL6 and RPL23A, their activation by enhancers in WUE mutants and significant up-regulation in response to various stresses support our findings that these two genes play an important role in alleviating plant abiotic stress. This alleviation might be through an interplay with stress-responsive transcription factors. However, the level and the underlying mechanism of stress tolerance needs to be investigated further by raising independent transgenic plants of these two genes. Currently, efforts are underway to overexpress RPL6 and RPL23A genes for a detailed functional characterization to further validate their role in enhancing WUE and also drought and other stress tolerance in rice. Other RP genes of rice such as RPL35 and RPL32 became up- and down-regulated in heat and salt stress treatments, respectively (Mukhopadhyay *et al.* 2011). Also, RPL10 was up-regulated under radiation exposure in *Arabidopsis* (Ferreyra *et al.* 2010), indicating that both large and small subunit of RP genes respond differentially to various biotic and abiotic stress conditions, likely playing a significant role in abiotic stress tolerance including WUE.

The instantaneous and high up-regulation of RPL genes in response to stress might function as an immediate defence. Because these are ribosomal genes, the defence might also occur by maintaining the mRNA integrity/stability during protein translation under the conditions of stress. The coordinated transcriptional up-regulation of translational related genes might also be a necessity for the cells to maintain the crucial cellular function under the conditions of stress.

By studying the transcript levels of 34 genes, we made an attempt to provide an overview of their differential expression under abiotic treatments. Considering the stability of mRNA and proteins and their downstream targets and mechanisms, there is no direct link between the levels of transcripts of a gene produced with the degree of tolerance under a given treatment. Because of this, these genes might not be considered as stress-tolerant with their high up-regulation at this stage. Rather, they can be classified as stress-responsive. However, because two RP genes became activated by the 35S enhancers in two mutants with high WUE (RPL6 and RPL23A), this study suggests that these two RPs are potential targets for genetic manipulation. The other RPL genes also could be valuable resources for manipulating abiotic stress tolerance in rice and other crops for future as shown by their instantaneous and significant up-regulation.

One key purpose of this work is to identify novel genes for WUE, which could further be exploited in manipulating tolerance to other abiotic stresses in rice. One group of such genes identified in this study are those encoding proteins involved in protein synthesis. Interestingly, two out of the five mutants analysed for WUE turned out to be genes for RPs. This gives an indication that analysis of a larger number of mutants for WUE might throw more plants with activation of genes for other RPs as well and genes for RPs could be very important targets for WUE and abiotic stress tolerance in rice and other crops. These ribosomal genes have not been previously shown to be regulated by dehydration stress in rice, further illustrating the fact that activation tagging is a powerful tool for the discovery of novel gene functions.

Author contribution statement

P. B. K., K. V. R., E. A. S. and M.M. designed the experiments. M. M. and A. B. performed all the experiments such as cloning, transformation and molecular investigations. A. R. R. analysed the quantum yield (PAM) data. M. U. K. helped in the $\Delta^{13}C$ analysis. A. S. helped in the analysis of Q-PCR data. P. B. K. supervised the work; M. M. and P. B. K. prepared the manuscript.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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

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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

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

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INTRODUCTION

Ribosomes are tiny (200–300Å) ribonucleoprotein complexes typically existing as two unequal sized subunits in all organisms and constituting 25–30% of total cell mass (Alberts et al., 2002). The ribosome complex, as a whole, performs mRNA-directed protein synthesis. Specific interaction of RPs and rRNA with mRNA, tRNA, and other non-ribosomal protein cofactors ensure that the process of initiation of protein synthesis, amino acid assembly and termination occurs appropriately in the cells (Maguire and Zimmermann, 2001). Eukaryotic ribosomes have a sedimentation coefficient of 80S with the large 60S subunit having 25S, 5.8S, 5S rRNA, and the small 40S subunit consisting of 18S rRNA (Ben-Shem et al., 2010). The number of RPs in ribosomes varies between organisms, with eukaryotes having up to 80 RPs and prokaryotes possess a total of only 54 RPs in both the subunits (Doudna and Rath, 2002).

The ribosomal gene family has more than 200 genes, but less than 100 corresponding RPs are incorporated into the ribosomes in all organisms including yeast, animals, and plants (Ban et al., 2000; Barakat et al., 2001; Hanson et al., 2004). This supports the fact that each RP-gene exists as 2–5 identical members with 95–100% nucleotide and predicted protein similarity. An RP synthesized from only one gene copy of a group incorporates into a ribosome under a given condition/tissue (Guarinos et al., 2003; Schuwirth et al., 2005). For example, the *Arabidopsis* genome has 249 genes for 80 RPs (48-large subunit proteins, 32-small subunit proteins) with each gene having 3–4 expressed copies and none exists as a single gene copy (Wool et al., 1996). RPs, in addition to their universal roles of stabilizing the ribosomal complex and mediating polypeptide synthesis also have extra-ribosomal functions such as their involvement in response to the environmental stresses (Warner and McIntosh, 2009; Sormani et al., 2011).

Mutations in plant RP genes have been implicated in perturbed phenotypes as has been seen in animal systems including humans. Earlier studies with *Arabidopsis* showed that mutations in many RP genes (*RPS18A, RPL24B, RPS5B, RPS13B,* and *RPL27A*) resulted in a 'pointed first leaf' phenotype characterized by reduced cell division and growth, and genotoxic sensitive plants (Lijsebettens et al., 1994; Revenkova et al., 1999; Ito et al., 2000; Szakonyi and Byrne, 2011). A T-DNA insertion mutation in the *Arabidopsis AtRPL10* gene caused lethal female gametophytes, while overexpression complemented the same with the recovery of the severe dwarf phenotype that resulted from the disruption of the *ACL5* gene (Imai et al., 2008). A transposon insertion mutation in one of the three copies of the *AtRPS13A* gene resulted in reduced cell division, late flowering, retarded root and leaf growth (Ito et al., 2000). Similar effects of plant growth retardation and reduced fertility were observed after knockdown of *AtRPL23aA* resulting in reduced synthesis of the RPL23aA protein, while knockout of its paralog, *RPL23aB*, had no effect on growth (Degenhardt and Bonham-Smith, 2008a). *RPL23aB* is the only RP paralog that did not produce any visible phenotypic defects upon knockout (Degenhardt and Bonham-Smith, 2008b).

These RP-gene knockout studies clearly show that although RP genes exist as multiple gene copies, the maximum possible expression of all the gene copies is required for them to be incorporated into the ribosomes during specific stages of growth and development and under certain stress conditions (Schmid et al., 2005; Byrne, 2009). The variation in the composition of ribosomes by the incorporation of RPs derived from identical members could be a major factor in the translational regulation of transcripts in different cell types and under various specific conditions (Giavalisco et al., 2005; Carroll et al., 2008). The change in the composition of RPs upon feeding of *Arabidopsis* leaves with sucrose further supports the heterogeneity of ribosomes in response to external stimuli (Hummel et al., 2012).

The expression of RP genes has also been shown to be differentially regulated by signaling molecules and environmental stresses. The transcript levels of *Arabidopsis RPS15a* (*RPS15aA*, *C*, *D* and *F*) were up-regulated in response to phytohormone and heat treatments (Hulm et al., 2005). Similar transcript abundance under BAP treatment was detected for *Arabidopsis RPS14*, *RPL13,* and *RPL30* genes (Cherepneva et al., 2003). Low temperature induced the expression of three RP genes; *RPS6*, *RPS13,* and *RPL37* in soybean (Kim et al., 2004) and a homolog of RPL13, *BnC24* in Brassica and *E. coli* (Sáez-Vásquez et al., 2000; Tanaka et al., 2001). The overexpression of RPL13 also resulted in tolerance against a fungal pathogen, *Verticillium dahliae* in transgenic potato with coordinated up-regulation of genes coding for defense and antioxidant enzymes (Yang et al., 2013) implying that RPs function in stress-response/tolerance through a network of multiple stress-related genes. In maize and *Arabidopsis*, RPL10A and RPL10C were shown to be significantly up-regulated under UV-B stress (Casati and Virginia, 2003; Ferreyra et al., 2010, 2013). *RPL44* was found to be up-regulated under osmotic stresses, and the overexpression of *Aspergillus glaucus RPL44* in yeast and tobacco ensured increased tolerance to salt and drought stresses (Liu et al., 2014). The majority of studies on RPs were undertaken in *Arabidopsis* largely because of the availability of insertion mutant lines and smaller genome size.

Until now, not much emphasis has been placed on the differential expression patterns of RP genes of rice in response to external stimuli. We had generated a large-scale enhancer based activation-tagged gain-of-function mutant population in *indica* rice, which was screened for water-use efficiency. Among the potential mutants with sustained productivity under prolonged water-limiting conditions, two of them were found to have enhanced expression of large subunit ribosomal genes because of their being tagged by the enhancers (Moin et al., 2016). This has prompted us to investigate the other rice RPL genes in the context of stress-responsiveness.

In the present study, we describe the genome-wide organization of predicted 123 RPL genes in rice including the individual gene copies. We investigated their overall expression pattern in selected tissues covering the major growth stages of rice. Also, we have provided an overview of their differential expression pattern under biotic and abiotic stress conditions that limit rice productivity. We identified specific RP

genes, whose expression is unique or overlapping under native and treated conditions. In summary, the information presented in this study provides a resource for subsequent exploitation of RPL genes to ameliorate abiotic and biotic stress conditions in rice and also other crop plants in future.

MATERIALS AND METHODS

Nucleotide Sequence Retrieval of RPL Genes

To identify the total members of the large subunit ribosomal gene family, a keyword search using "ribosomal" was performed under the putative function search of Rice Genome Annotation Project Data Base (RGAP-DB v7)[1] and Phytozome v11[2]. The large subunit members were shortlisted by selecting the genes starting with prefix 'L,' for large subunit as opposed to 'S' that specifies small subunit genes. A total of 123 RPL genes were identified, and since the number of RPL genes in both the databases was same, the gene sequences were downloaded from RGAP-DB. When further looked for the presence of identical members or copies of each gene in RGAP-DB, we observed that each RPL gene has an average of 2–3 gene copies in the genome. From these 123 genes, we selected 34 candidate genes each representing one orthologous group excluding the identical copies for expression studies. All the identified 123 sequences were also confirmed through nucleotide and protein BLAST search in the NCBI[3] and Hidden Markov Model (HMM) of Pfam[4] databases, respectively. The predicted protein sequences of all the 123 RPL genes were also verified in NCBI conserved domain database[5]. To minimize the missing of potential RPL genes and to ensure that all the identified sequences belong to the ribosomal large subunit gene family, multiple databases were employed.

Chromosomal Distribution of RPL Genes

To determine the chromosomal distribution, the locus number of each of the 123 RPL genes obtained from RGAP-DB was submitted to the OryGenesDB[6]. Based on the output generated in OryGenesDB, the position of each gene at its corresponding locus on the chromosome was located manually.

RPL Gene Structures

The structure of each of the 34 RPL genes was determined to study the number and position of introns and exons, GC-content, gene orientation in the genome and alternative splice forms. The full-length sequences of each gene and cDNA were submitted to the Gene Structure Display Server (GSDSv2)[7] to predict the structure.

[1]http://rice.plantbiology.msu.edu/index.shtml

[2]https://phytozome.jgi.doe.gov/pz/portal.html

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

[4]http://pfam.xfam.org/

[5]http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

[6]http://orygenesdb.cirad.fr/tools.html

[7]http://gsds.cbi.pku.edu.cn/

Protein Properties, Secondary Structure, Homology Modeling, and Phylogenetic Analysis

The predicted sequences of 34 RPL proteins were obtained from the RGAP-DB and analyzed using an online tool, PSORT[8] to predict the protein properties such as size, molecular weight and isoelectric point (*p*I). The amino acid sequences of these proteins were aligned in ClustalW[9] and submitted to the Molecular Evolutionary Genetic Analysis (MEGAv6)[10] program for constructing an unrooted phylogenetic tree to identify the protein similarities in the RPL family in rice. The domains and motifs in proteins were identified using SMART[11] (Simple Modular Architecture Research Tool). The GRAVY (Grand average of hydropathicity) indices of RPL proteins, which are the determinants of the hydrophobicity of whole protein was calculated using ExPASy ProtParam[12]. The GRAVY values of most of the proteins are usually in the range of $+2$ to -2, and values in negative range or less than zero indicate that the proteins are hydrophilic in nature (Song et al., 2015).

Although the detailed crystal structure of ribosomal complex has been well-characterized (Ben-Shem et al., 2010), we tried to study the properties of individual RPs. To gain an insight into the secondary structure of RPL proteins and to characterize the presence of metal–ligand/protein/RNA interacting sites, the three-dimensional secondary structures of 34 RPL proteins were predicted using Phyre2[13] program (Protein Homology/AnalogY Recognition Engine v2; Kelley et al., 2015). Individual protein sequences were submitted in Phyre2 in FASTA format and after studying the properties such as α-helices and β-strands, they were directed to 3DLigandSite[14] (Wass et al., 2010) to predict the metal/non-metal ligands and their binding sites in each protein.

In silico Putative Promoter Analysis of 34 RPL Genes

To determine the presence of stress-responsive *cis*-regulatory elements, the nucleotide sequence $\leq$1 kb upstream of each RPL gene was retrieved from RGAP-DB and submitted to the Plant *Cis*-Acting Regulatory Elements[15] database. The location and number of repeats of each *cis*-regulatory sequence in the putative promoter regions of each RPL gene were identified.

Plant Material and Growth Conditions

The seeds of *Oryza sativa* L. sp. *indica* var. Samba Mahsuri (BPT-5204) maintained in greenhouse conditions were surface sterilized with 70% ethanol for 50–60 s followed by 4% sodium hypochlorite for 20 min. Seeds were then washed thrice with sterile double-distilled water, blot dried and cultured on solid MS medium at 28 $\pm$ 2°C and 16 h light/8 h dark photoperiods.

[8]http://psort.hgc.jp/form.html

[9]http://www.genome.jp/tools/clustalw/

[10]http://www.megasoftware.net/

[11]http://smart.embl-heidelberg.de/

[12]http://web.expasy.org/cgi-bin/protparam/protparam

[13]http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

[14]http://www.sbg.bio.ic.ac.uk/3dligandsite/

[15]http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

To analyze the native tissue-specific expression pattern of 34 RPL genes at different stages of rice development, samples were collected from 13 different tissues covering major stages of rice growth. After sterilization, seeds were soaked in water in a rotary shaker and after 16 h of incubation, the embryonic portion was manually cut under a stereo-microscope to collect the embryos and endosperm. Some of these seeds were allowed to continue to germinate on MS medium. After 3 and 6 days of germination, the plumules, radicles, shoot, and leaf tissues were collected separately. After 2 weeks of growth on MS medium, some of the seedlings were transferred to pots containing alluvial soil and grown under greenhouse conditions (30 ± 2°C, 16 h light/8 h dark photoperiods). Plants were amply watered with RO (Reverse Osmosis) purified water up to 3 cm overlay in the pots as required for normal growth of rice. About 45 days after transfer (DAT) to the greenhouse, rice plants were uprooted to collect shoot, root, flag leaf, and root–shoot transition tissues. After 60 DAT, flowers, partially filled grains and spikes were collected.

Abiotic Stress Treatments to Seedlings

To analyze the differential expression pattern of 34 RPL genes and to distinguish RPL genes that are up/down-regulated under abiotic conditions, 7-day-old seedlings were exposed to five different abiotic treatments such as MeJa, SA, cold stress (4°C), heat stress (42°C), and oxidative stress (H_2O_2). The 7-d-old seedlings were dipped in the solutions of 100 μM MeJa (Wang et al., 2007), 3 mM SA (Mitsuhara et al., 2008), and 10 μM H_2O_2 (Fuller, 2007). The root and shoot tissues were collected separately at 5 min, 3 h, 6 h, 24 h, and 60 h after treatments. For cold and heat induced stresses, seedlings in water were exposed to 4°C and 42°C (Jami et al., 2012), respectively, and root and shoot samples were collected at time intervals as described. Since WT rice seedlings started to wilt after 24 h of exposure to 42°C, heat stress samples were collected up to 24 h only. Seedlings in water at corresponding time intervals served as controls to normalize the expression patterns. Tissue samples were collected as three biological replicates after each time interval.

Biotic Stress Treatment

To check the expression pattern of rice ribosomal genes in response to biotic stress, we used the bacterial pathogen *Xanthomonas oryzae* pv. o*ryzae* that causes Bacterial Leaf Blight (BLB) of rice, which is one of the most severe yield constraints of rice worldwide (Sundaram et al., 2014). At the seedling stage, the infected leaves start to roll-up, and as the disease progresses, the leaves turn yellow and wilt, leading to drying up and death. This drastically reduces the total seed yield of the plant. The yield loss may be as high as 70% when plants are grown in conditions favorable to the disease (Ryan et al., 2011). The bacterial suspension of *Xanthomonas oryzae* pv. o*ryzae* was applied on the leaves of 2-month-old plants grown in greenhouse conditions, and leaf samples were collected after 11 days of infection. Leaf samples of untreated plants grown under similar conditions were used as a control to normalize the expression.

Because the transcript level of RPL10 was significantly up-regulated 11 days after treatment, we selected this gene in particular to analyze its expression at progressive time-points such as 3 h, 6 h, 1 day, 2 days, 3 days, up to 11 days post-infection of rice leaves with *Xanthomonas oryzae* pv. o*ryzae* pathogen. The qRT-PCR was performed with *Xanthomonas oryzae* pv. o*ryzae* treated and untreated samples collected as three biological and three technical replicates. Rice specific *act1* and β-*tub* genes were used as controls for normalization and the mean of the fold change was represented as bar diagrams constructed using SigmaPlot v11.

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

Total RNA was isolated from stress-treated and untreated tissues using TriReagent (Takara Bio, UK) following the manufacturer's protocol. The quality of extracted RNA was checked on 1.2% agarose gel prepared in TBE (Tris-borate-EDTA) buffer and quantified using Nanodrop. Total RNA (2 μg) was used to synthesize the first strand cDNA using reverse transcriptase (Takara Bio, UK). The cDNA was diluted in 1:7 proportions and 2 μl of it was used in qRT-PCR. Primers specific for each RPL gene sequence retrieved from RGAP-DB was designed using the primer-3[16] online tool and 10 μM of each was used per reaction. The reaction conditions for qRT-PCR included an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, an appropriate annealing temperature for 25 s and an extension of 72°C for 30 s. At the end of the reaction, a melting curve step was inserted to analyze the specificity of amplification of each gene. Rice specific actin (*act1*) and tubulin (β-*tub*) were used as internal reference genes to normalize the expression patterns. The mean values of relative fold change, which was calculated as per $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) obtained from each reference gene was considered as the final fold change in the transcript levels. Each qRT-PCR reaction was performed as three biological and three technical replicates.

The relative fold change of the 34 genes in 13 tissues and under five abiotic treatments was illustrated in the form of heat maps. A dendrogram was constructed to represent the Hierarchical clustering of relative fold change of 34 genes under each treatment using the GENE-E[17] program.

RESULTS

Genome-Wide Identification and Chromosomal Distribution of RPL Genes

To explore the cytoplasmic large subunit (60S) ribosomal gene family members in rice, we used a keyword search "ribosomal" in the putative function search of RGAP-DB and Phytozome databases, which resulted in the identification of 428 and 754 genes, respectively, and these included genes belonging to cytoplasmic 60S and 40S subunits and 50S and 30S subunits of chloroplast and mitochondrial ribosomes. Keyword search and homology-based identification through HMM are widely used

[16] http://bioinfo.ut.ee/primer3-0.4.0/

[17] http://www.broadinstitute.org/cancer/software/GENE-E/

practices in identifying genome-wide copies of the annotated genes (Kapoor et al., 2008; Liang et al., 2016). We then searched for genes starting with the prefix 'L' to select large subunit genes. This process excluded small subunit genes and identified 215 genes that included large subunit members of cytoplasmic (60S) and chloroplast and mitochondrial (50S) ribosomal subunits. We then shortlisted the cytoplasmic 60S subunit genes by their putative cellular localization using the information available in RGAP-DB. A similar process was applied in shortlisting the 60S subunit genes from Phytozome. Both these approaches identified 123 genes belonging to the cytoplasmic 60S subunit. Each of these genes was then confirmed by a BLAST search of their nucleotide and predicted amino acid sequences in other rice databases like RAP-DB[18] and OryGenesDB. BLASTn and BLASTp results in NCBI and HMM of Pfam and NCBI conserved domain databases, respectively, further confirmed that these genes belong to the 60S ribosomal family by the presence of ribosomal domains.

The locus numbers of 123 genes were submitted in OryGenesDB and, based on the output generated, the location of each gene on the corresponding chromosome was mapped manually using OryGenesDB. The location of these 123 genes was found on all chromosomes, indicating their wide distribution throughout the rice genome. Chromosome-7 has 19; chromosome-1, being the largest of rice chromosomes has 18; chromosome-2 has 16; chromosome-5 has 14; and chromosome-3 showed 13 genes. Chromosomes-9, 10, and 11 exhibited four genes each, while chromosomes-4, 8, and 9 evidenced 5, 10, and 9 RPL genes, respectively (**Figure 1**). The nucleotide sequence alignment of genes within an orthologous group exhibited 100% similarity, but their chromosomal locations are different. We selected the 34 candidate genes, each representing one orthologous group for a detailed characterization to understand their gene and protein structures, and comprehensive

[18]http://rapdb.dna.affrc.go.jp/

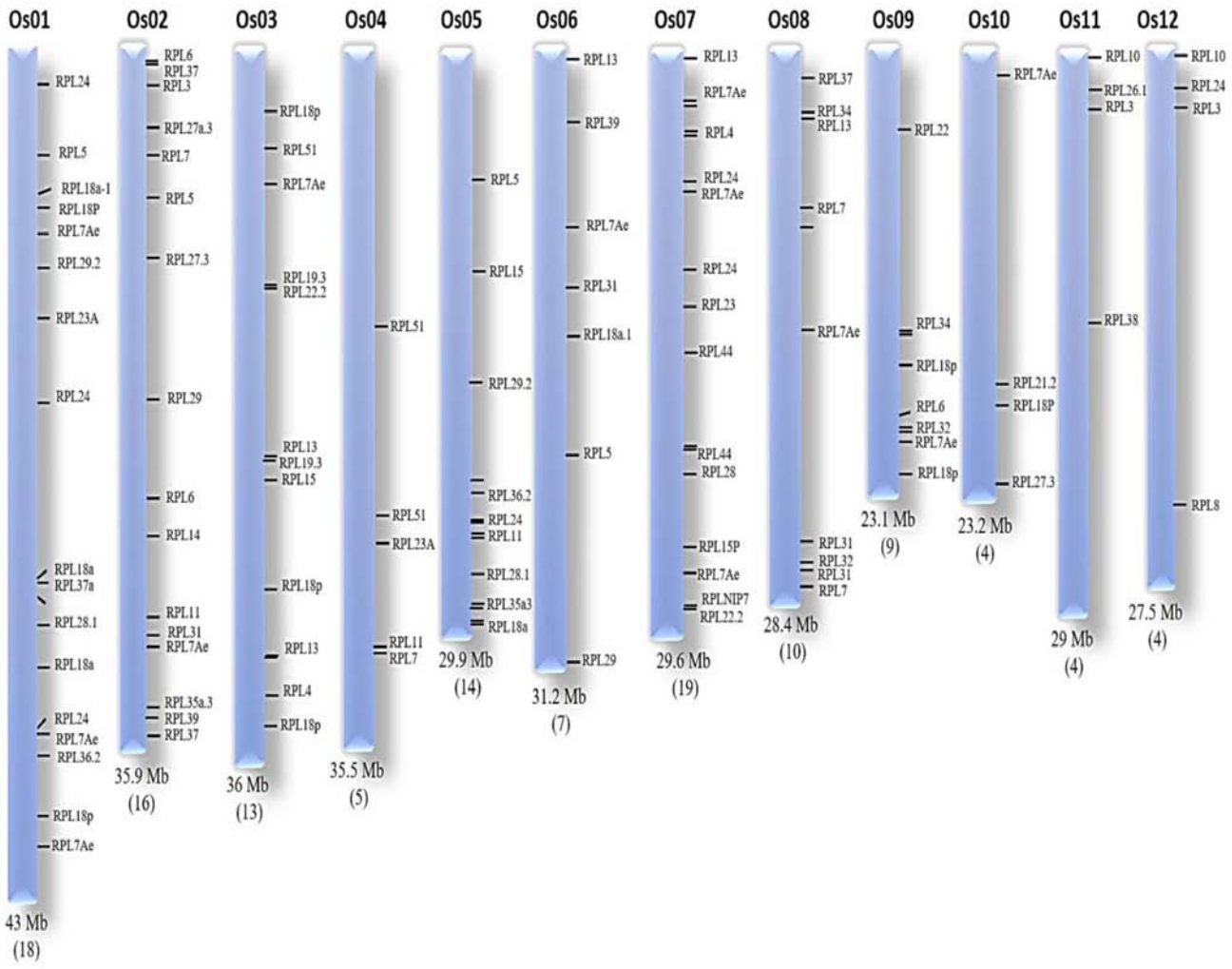

FIGURE 1 | Chromosomal organization of RPL genes. The chromosomal number and size is represented at the top and bottom of each chromosome, respectively. The number of RPL genes is given within brackets at the bottom of each corresponding chromosome.

expression analysis in response to a wide range of stress treatments.

Analyses of RPL Gene Structures

A comparative study between 34 RPL genes was performed to determine the number and position of introns and exons, GC-content and 5′ and 3′ untranslated regions. The number of introns varied from none to seven. The RPL10 and RPL18p have the highest number of introns (7), whereas RPL4, 8, 21.2, 23A, 26.1, 28, and 31 have only one intron. RPL7, 11, 24b, 27.3, and 38.2 do not contain any introns in their coding regions; these genes also have high GC-content. For example, RPL24b, 27.3 and 38 have 54, 60, and 51% GC-content, respectively. Genes with introns are reported to be crucial for gene expression, and a high number of introns is linked with increased expression of a gene (Callis et al., 1987; Karve et al., 2011). We observed that RPL18p and RPL10 with seven introns exhibited high expression in various tissues studied compared with other RPL members. Introns when particularly present at the start site or 5′ end of a gene have an enhanced ability for expression (Callis et al., 1987; Donath et al., 1995). RPL35a.3, 51, 32, 30e, 22, 19.3, 18a, 10, and 5 have their first introns within 500 bp regions from the transcription start site, which might be the reason for their constitutive expression in almost all the tissues studied. Similarly, the number of exons also varied among 34 RPL genes. Genes like RPL10 and RPL18p have the highest number of exons (8), whereas RPL7, 11, 24b, 27.3, and 38.2 have only one exon. Some of these RPL genes (RPL4, 13a, 14, 15, 23A, 28, 30e, 32, 35a, and 51) undergo alternative splicing to produce 2–5 splice-variants (**Table 1**) with similar nucleotide sequence. For expression analysis, we used the sequence of only one variant of a gene as it is difficult to design the primers for alternative splice forms exhibiting high nucleotide similarity. In addition, the 5′ and 3′ UTRs of these genes varied in size and positions. RPL28 has an unusually long UTR of 2.5 kb at the 3′ end (Supplementary Figure S1). **Table 1** provides detailed information about their gene structure, site of location in the genome and copy number of individual genes.

Domain Recognition, Ligand Binding Sites, and Phylogeny of Rice RPL Proteins

Among the 34 proteins, RPL4 is the largest with a predicted molecular mass of 44.5 kDa. All the RPL proteins have similar isoelectric points ranging from 9.5 to 12. The maintenance of similar *p*I values in RPL proteins might be to reduce coulombic repulsions as these proteins are interactive in nature. They have a varied percentage of α, β, and distorted regions with RPL proteins interacting with other r– and non-r proteins exhibiting high content of α-helices. For example, RPL7, which interacts with elongation factors-Tu and -G, has 55% α-helical structure. RPL13a, existing at the interface of RPL3 has 57% of α-helix content, whereas RPL29 that interacts with RPL23A and initiation factors has 48% α-helix content. Furthermore, all the RPL proteins have a GRAVY value < 0, which indicate their high hydrophilicity. The proteins with high hydrophilic nature tend to undergo conformational changes and form flexible structures with other molecules and also contribute toward inducing tolerance during stress conditions (Fuxreiter et al., 2004; Liang et al., 2016).

All the RPs are characterized by the presence of ribosomal domain(s). They also have several other domains that participate in interaction with other proteins. RPL 14, 19.3, 21.2, 24b, 26, and 27 have KOW-SH3 motifs at their N-terminal regions, which are involved in protein–protein interactions. KOW-motifs link RPs with transcription factors (Kyrpides et al., 1996). RPL18p (135–226 amino acids) has an FCD domain (FadR C-terminal Domain) that is involved in the regulation of transcription of genes. RPL18p also has a XPGN domain (208–281 amino acids) that is associated with cancer and Xeroderma pigmentosum in humans. RPL21.2 has cheY motif at the C-terminus activated by phosphorylation through histidine kinases. RPL22 has a DUF1087 domain (amino acids 1–67) that is involved in chromatin remodeling and a WWE domain that is associated with poly-ADP-ribosylation and ubiquitin-mediated proteolysis. RPL29 has a carboxyl-terminal domain (CTD) and proteins with such domains are related to pre-mRNA processing by binding with mRNA capping enzymes (Schwer et al., 2009). It also has RQC, a DNA binding domain found in RecQ helicases.

Ligand-mediated signal transmission is essential for proper functioning of a majority of proteins including certain RPs. However, recognition of the core structures or amino acids involved in protein–ligand interactions is of paramount importance for understanding the dynamic and kinetic properties of the proteins. Analysis of RPL proteins for the presence of sites for ligand binding reveals that out of 34, 20 RPL proteins have sites for binding with ligands (that include metals ions and cofactors) whereas no such ligand binding sites were observed in 14 proteins (RPL3, 4, 6, 10, 11, 13b, 14, 15, 18a, 24a, 29, 34, 36, and 37). RPL8 (Lys198), 13a (Pro114, 115), 19.3 (Glu179, Arg180), 21.2 (Arg70), 22 (Ile52), 24b (Leu118, Lys121, Ala122), 26 (Leu105), 28 (Tyr49), 38 (Lys35), and 44 (Gly51) have Mg^{+2} ion binding sites. RPL7, 31, and 51 bind with Cu^{+2} and RPL5 (Asp30, Thr33) and 23A (Lys104) have sites for binding with Ca^{2+} ions. RPL19, 35, 38, and 51 also have Zn^{2+} binding sites. RPL29 present at the interface of RPL23A binds with the cofactor FAD. The RPL35 binds with cofactors FUC and FAD. Metal ions in RPs serve important biological functions by interacting with nucleic acids, particularly RNA. Non-metal ligands are cofactors involved in catalytic activities. Cofactors in RPs ensure that the processes of protein initiation, amino acid assembly and termination are correctly undertaken (Maguire and Zimmermann, 2001). Because of this, RPs might have binding sites for both metal and non-metal ligands. The details of the protein properties such as size, *p*I, the percentage of α-helices and β-sheets, GRAVY indices, the presence of metal/non-metal ligands and their binding sites are detailed in Supplementary Table S1. The secondary structures of selected RPL proteins with ligand binding properties are represented in Supplementary Figure S2.

To evaluate the evolutionary relationships within the RPL protein family of rice, three phylogenetic trees were constructed

TABLE 1 | Details of rice RPL genes.

Gene Id	Chromosome number	Location (bp)	Protein name	Gene Structure						Gene copies
				Gene Size (bp)	GC (%)	Introns	Exons	Alternative splice forms	Orientation	
LOC_Os11g06750	Os11	3294148–3297139	RPL3	2991	46%	5	5		5′–3′	2
LOC_Os03g58204.1	Os03	33143597–33146052	RPL4	2456	41%	2	2	2	3′–5′	3
LOC_Os05g11710.1	Os05	6649548–6651919	RPL5	2372	43%	4	4		5′–3′	5
LOC_Os04g39700.1	Os04	23660833–23663041	RPL6	2209	44%	3	4		3′–5′	2
LOC_Os02g10540.1	Os02	5550032–5552116	RPL7/L12	2085	46%		1		3′–5′	5
LOC_Os12g38000.1	Os12	23349992–23351879	RPL8	1888	49%	1	2		5′–3′	1
LOC_Os11g01420.1	Os11	252426–254919	RPL10	2494	41%	7	8		3′–5′	2
LOC_Os04g50990.1	Os04	30180315–30182052	RPL11	1738	44%		1		5′–3′	2
LOC_Os07g01870.1	Os07	523361–525795	RPL13a	2435	47%	2	3	2	3′–5′	4
LOC_Os03g37970.1	Os03	21086229–21088565	RPL13b	2337	46%	4	4		3′–5′	3
LOC_Os02g40880.1	Os02	24777727–24779637	RPL14	1911	42%	4	4	2	3′–5′	2
LOC_Os03g40180.1	Os03	22336536–22338324	RPL15	1789	48%	3	3	2	3′–5′	4
LOC_Os05g49030.1	Os05	28118285–28120495	RPL18a	2211	43%	3	3		5′–3′	3
LOC_Os01g67134.1	Os01	38973740–38976158	RPL18p/L5e	2419	43%	7	8		3′–5′	2
LOC_Os03g21940.1	Os03	12542185–12544692	RPL19.3	2508	43%	4	5		3′–5′	3
LOC_Os10g32820.1	Os10	17184530–17185759	RPL21.2	1230	46%	1	2		3′–5′	2
LOC_Os09g08430.1	Os09	4390880–4393872	RPL22	2993	41%	6	6		3′–5′	2
LOC_Os01g24690.1	Os01	13896291–13898264	L23A	1974	41%	2	2	3	5′–3′	2
LOC_Os01g59990.1	Os01	34682518–34685485	RPL24a	2968	42%	5	5		5′–3′	4
LOC_Os01g04730.1	Os01	2137763–2138996	RPL24b	1234	54%		1		5′–3′	3
LOC_Os11g05370.1	Os11	2382184–2383220	RPL26.1	1037	52%	1	2		3′–5′	3
LOC_Os02g18380.1	Os02	10700750–10701516	RPL27.3	767	60%	–	1		3′–5′	2
LOC_Os07g36090.3	Os07	21574435–21579332	RPL28	4898	42%	3	2	3	3′–5′	1
LOC_Os06g51530.1	Os06	31223501–31226586	RPL29	3086	46%	7	4		3′–5′	7
LOC_Os07g44230.1	Os07	26432701–26435257	RPL30e	2557	42%	6	6	2	3′–5′	1
LOC_Os06g21480.1	Os06	12412525–12415341	RPL31	2817	43%	1	2		5′–3′	4
LOC_Os09g32532.1	Os09	19418910–19421084	RPL32	2175	42%	3	3	2	3′–5′	4

(Continued)

TABLE 1 | Continued

Gene Id	Chromosome number	Location (bp)	Protein name	Gene Structure						Gene copies
				Gene Size (bp)	GC (%)	Introns	Exons	Alternative splice forms	Orientation	
LOC_Os09g24690.1	Os09	14691168–14693121	RPL34	1954	41%	3	4		3′–5′	3
LOC_Os05g48310.1	Os05	27695307–27697523	RPL35a.3	2217	46%	3	3	2	5′–3′	3
LOC_Os01g62350.1	Os01	36083051–36085662	RPL36.2	2612	38%	3	3		3′–5′	2
LOC_Os02g56990.1	Os02	34918111–34920148	RPL37	2038	44%	2	3		5′–3′	3
LOC_Os11g24610.1	Os11	14044207–14045356	RPL38	1150	516%		1		5′–3′	4
LOC_Os07g33898.1	Os07	20273588–20275840	RPL44	2253	42%	3	3		5′–3′	11
LOC_Os03g10930.2	Os03	5613250–5615444	RPL51	2229	43%	3	3	2	3′–5′	1

RPL gene sequences were retrieved from RGAP-DB. Gene properties such as size, GC content, orientation in the genome, chromosomal distribution, copy number of each gene, number of introns and exons were studied in the OryGenesDB.

using full-length amino acid sequences and amino acid sequences derived from ribosomal domains and Low Complexity Regions (LCR; Supplementary Figure S3). The unrooted phylogenetic tree was constructed by using a 'neighbor joining algorithm' with a bootstrap value of 1000. The homologous proteins having significant bootstrap value (>95%) were considered as having the highest similarity with respect to others.

The phylogenetic tree of full-length RPL proteins was divided into four clades or groups (A, B, C, and D). The RPL proteins, RPL24b and 26.1 have the highest similarity indicating that these two genes might have become duplicated recently in the rice genome. The phylogenetic relationship has also been used for gene function identification and the proteins with the highest similarity perhaps exhibit similar functions and expression patterns (Lijavetzky et al., 2013). The expression of RPL24b and RPL26.1 was similar in shoots indicating their similar roles in shoot growth and development; their expression was also similar in oxidative stress indicating functional similarity.

The phylogenetic trees of two separate domains, the ribosomal domain and LCR were also divided into groups to check domain-wise similarity. The ribosomal-domain analysis showed maximum sequence similarity between RPL24b and 26, which exhibited similarity with RPL18a and 19.3 proteins. RPL18a and 19.3 showed similar expression patterns in different tissues like spikes, endosperm, plumules, radicles, 45 days shoot and root tissues and root–shoot transition indicating their functional similarity during growth and development. All the RPL proteins except RPL10, 11, 14, 21.2, 35, 36, 37, 38, and 51 have predicted LCR. The phylogenetic analysis of LCR showed that RPL6 and RPL26 exhibited the maximum sequence similarity followed by RPL5 and 31 and RPL27 and RPL8, whereas RPL3 formed a separate clade, which showed its possible divergence from the other RPL proteins.

In silico Analysis of Putative Promoter Regions of Rice RPL Genes

The expression studies showed that many RPL genes are differentially regulated in various tissues and under various abiotic treatments. To assess whether this differential regulation is due to the presence of stress or signal-responsive elements in their regulatory regions, nucleotide sequences ≤1 kb upstream to each of the 34 genes were retrieved and searched using the PlantCARE database. This analysis resulted in the identification of multiple stress-responsive elements in the putative promoter regions of all the genes. Abiotic stress-responsive elements that are associated with heat and cold temperatures such as HSE (Heat Stress Elements) and LTR (Low-Temperature Response) and dehydration stress such as MBS (Myb Binding Site) are widely distributed within the putative promoter regions of RPL genes. MBS is a binding site for MYB-related transcription factors that are involved in the regulation of genes responsive to water-deficit conditions (Urao et al., 1993). The presence of these elements in the promoter regions suggests that the corresponding genes become activated under water stress or drought conditions. In addition to abiotic stresses, elements that respond to phytohormones such as ABA (ABRE-Abscisic acid responsive element and Motif IIb), MeJa (TGACG-motif and CGTCA-motif), SA (TCA-motif), Gibberellic acid (GARE-Gibberellic acid responsive element), and Auxin (TGA-motif and AuxR-Auxin responsiveness) are also present in multiple copies.

Except RPL37, which did not exhibit any abiotic-responsive element in its upstream region, the putative promoters of all other genes had one or the other stress-responsive elements. MBS, ABRE, TGACG, and CGTCA motifs are commonly found in multiple copies. RPL8 has five repeats of ABRE and two repeats of each MBS, TGACG, CGTCA and TGA

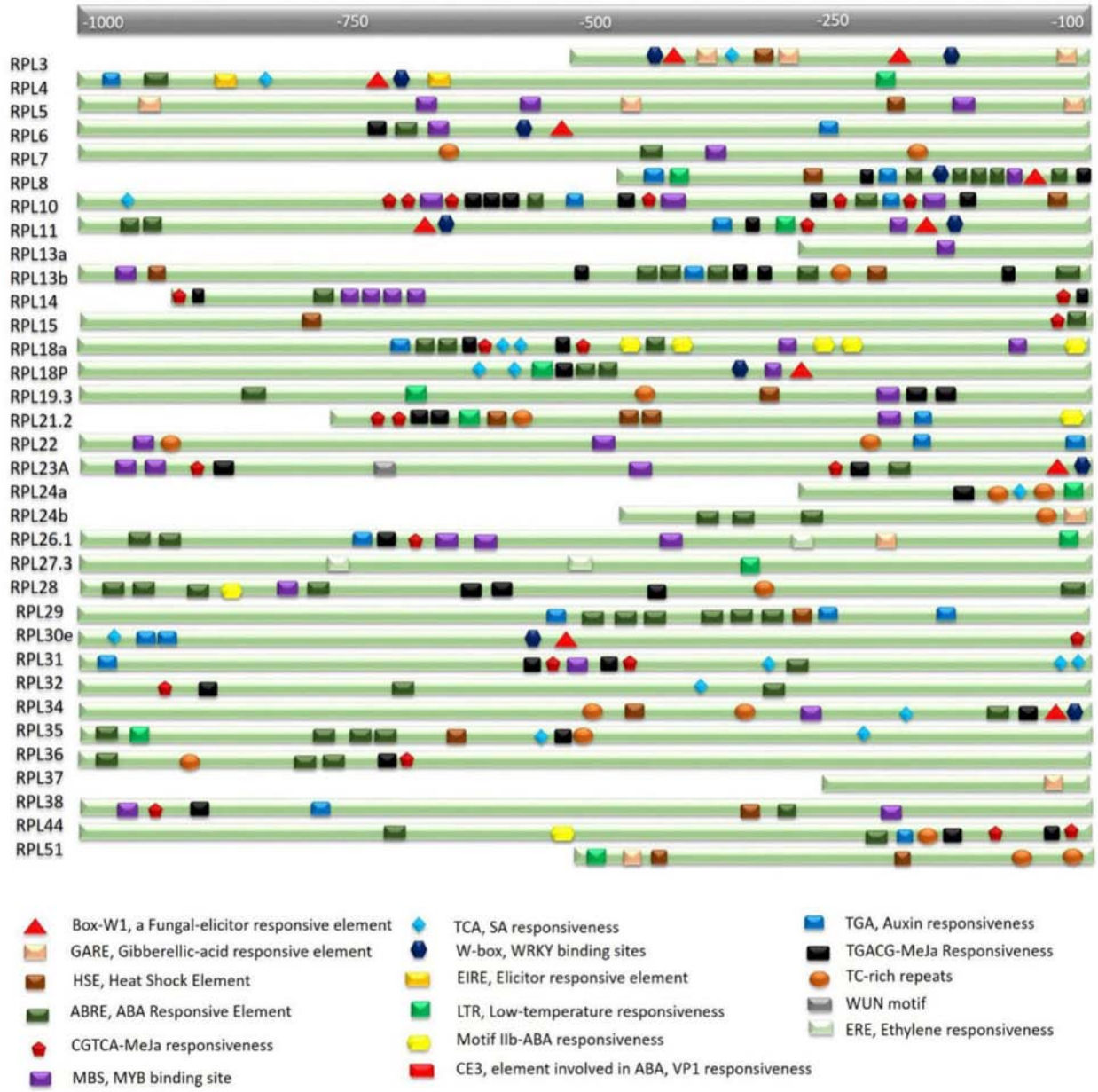

FIGURE 2 | *In silico* analysis of rice RPL promoters for the identification of *cis*-regulatory elements. Nucleotide sequence ≤1 kb upstream of the transcription start site of each gene carries multiple stress and signal-responsive elements. Each element is represented with a different shape and color which is described at the bottom of the figure. A scale at the top indicates the putative localization of corresponding elements.

elements. RPL10 exhibited five repeats of each TGACG and CGTCA motifs that respond to MeJa treatment and three copies of MBS elements. RPL14 has four repeats of dehydration responsive elements. RPL18a showed four and three copies of Motif IIb and ABRE, respectively, that respond to ABA and two copies of TGACG and CGTCA motifs. RPL28 showed five repeats of ABRE and four repeats of MeJa responsive elements. RPL29 has six copies of ABRE and three copies of TGA element. RPL31 showed three copies of TCA element and two repeats of TGACG and CGTCA motifs. RPL35 has four copies of ABRE and two copies of TCA element. RPL36 and 38 had three copies of ABRE and MBS elements, respectively. RPL44 has two copies of ABRE, CGTCA and TGACG motifs (**Figure 2**). In addition, TC-rich repeats that are involved in defense and stress-responsiveness (Diaz-De-Leon et al., 1993), W-box motifs which are the binding sites for stress-responsive WRKY transcription factors (Eulgem and Somssich, 2007), a WUN-motif, a wound-responsive element that is associated with biotic stress (Jiang et al., 2014), a Box-W1 motif, a fungal elicitor element that binds with WRKY33 transcription factor in

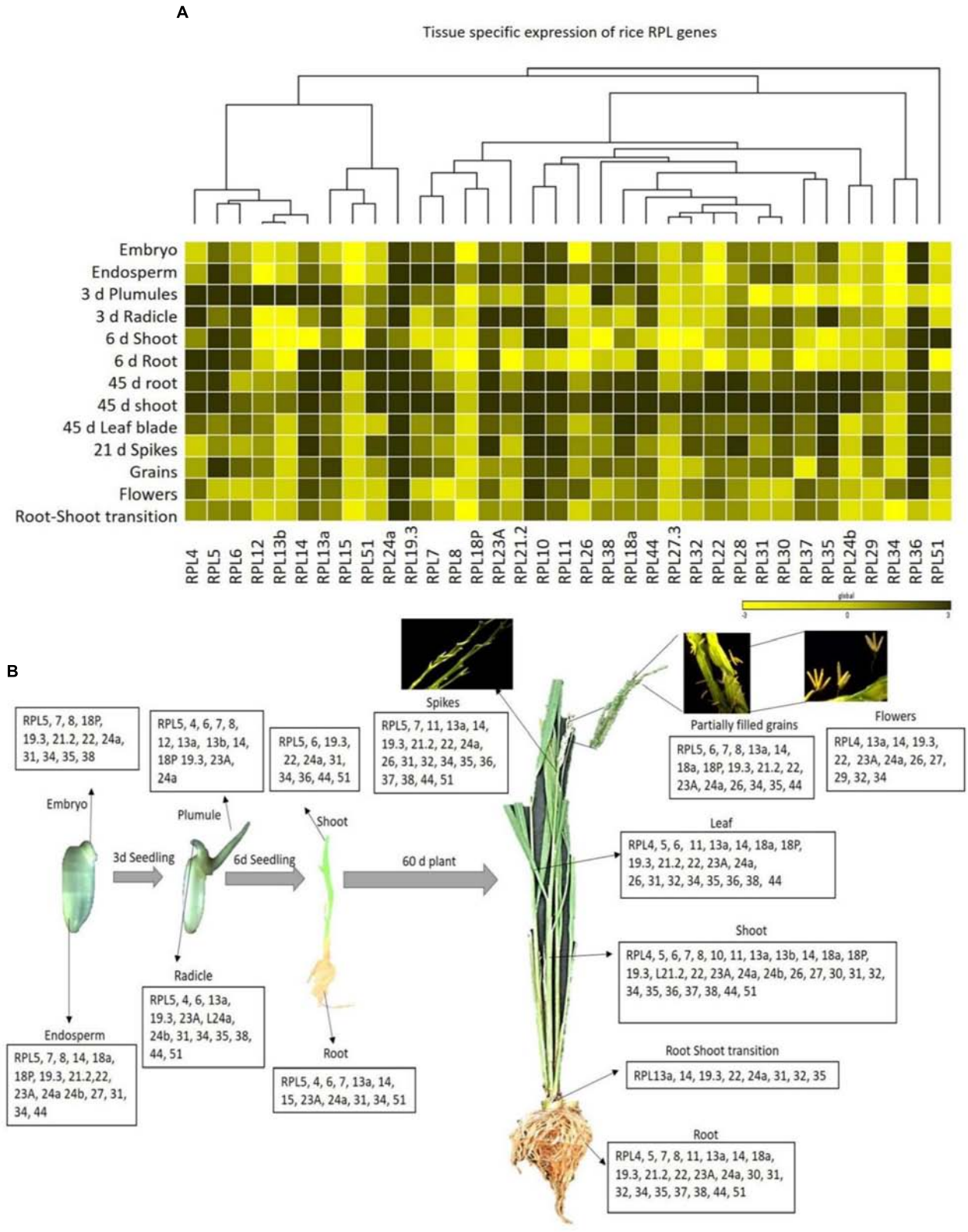

FIGURE 3 | Tissue-specific expression of rice RPL genes. (A) The qRT-PCR of 34 RPL genes was performed in 13 different tissues and the level of expression was normalized with rice actin. The mean values of fold change of biological and technical triplicates were represented in the form of heat maps. A dendrogram was constructed to represent the Hierarchical clustering. **(B)** The RPL transcripts that are significantly up-regulated in each tissue are represented pictorially at the bottom.

response to phytopathogens (Rushton et al., 1996; Lippok et al., 2007) are present in single copies in the putative *cis*-elements. Supplementary Table S2 presents a detailed analysis of both abiotic and biotic responsive elements and their repeats in the putative promoter regions.

Spatial Expression of Rice RPL Genes

To obtain insights into the tissue-specific and native expression patterns of RPL genes, we studied the expression of 34 RPL genes in 13 different tissues including 16 h embryo and endosperm, plumule and radicle of 3-day-old seedlings, root and shoot tissues of 7-day-old seedlings, 2-month-old tissues of mature flag leaf, shoot, root, root–shoot transition, partially filled grains, flowers and spikes. The primer details of 34 RPL genes used in the expression analysis were provided in Supplementary Table S3.

Two-month-old shoot and root tissues induced an up-regulation of 30 and 22 RPL genes, respectively, which is larger than any other tissue. Out of 34, root–shoot transition and grains induced the expression of 17 RPL genes. Embryo, 6 days root and floral organs induced the expression of 12 RPL genes, whereas endosperm, plumule, radicle, 6 days shoot, flag leaf, and spikes induced the expression of a total of 16, 13, 14, 10, 21, and 19 RPL genes, respectively. RPL5 and RPL24a were highly up-regulated in all the tissues. The expression of RPL27 and RPL37 was detected only in three tissues; endosperm, 45 days shoot, flowers and 6 days shoot, 45 days root and flowers, respectively. RPL8 and RPL15 were expressive only in endosperm and 6 days roots, respectively, but non-expressive in the remaining tissues studied. Ten RPL genes *viz*., RPL5, 7, 8, 18P, 19.3, 21.2, 22, 24a, 31, and 34 were commonly up-regulated in embryo and endosperm indicating that they can be implicated in early embryonic development. RPL4-6, 13a, 19.3, 23A, and 24a were up-regulated in plumules and radicle suggesting their role in root and shoot initiation. RPL5, 6, 24a, 31, 34, and 51 were highly expressive in shoot and root tissues of 7-day-old seedlings. RPL4-6, 11, 13a, 14, 18, 19, 21.2, 22, 23A, 24a, 31, 34, 35, 38, and 44 were commonly up-regulated in root, shoot and flag leaf indicating that these genes are involved in vegetative growth and plant maturity. RPL13a, 14, 19.3, 22, 24a, 26, and 34 were highly expressive in spikes, flowers and partially filled grains indicating that these are likely associated with the development of reproductive organs and grain filling. RPL13a, 14 and 24a were expressive from the 6-day-old seedling stage to the grain filling stage in all the tissues studied indicating that they play a major role in the growth and development of both vegetative and reproductive organs such as root, shoot, flowers and grains. RPL5, 19.3, 23A, and 24a were expressive in mitotically active tissues like embryo, endosperm, plumule, and radicles suggesting that these genes are involved in the early maturity and emergence of shoot and root. RPL10 and RPL29 were specifically expressed only in endosperm and flowers, respectively (Supplementary Table S4). The spatial expression of 34 RPL genes in 13 different tissues has been represented in the form of heat maps generated by incorporating the qRT-PCR data obtained from tissue samples collected as three biological replicates (**Figure 3**).

Differential Transcriptional Regulation of RPL Genes under Various Abiotic Stress Treatments

The motivation for studying rice large subunit ribosomal genes in stress-response stems from our results on activation-tagged mutant population generated for an important agronomical trait called water-use efficiency (Moin et al., 2016). The mutants with sustained/improved seed productivity under the conditions of limited water availability were selected for flanking sequence analysis and subsequently for studying the expression pattern of the enhancer tagged genes. The seven short-listed mutant plants that appeared to have high productivity were then characterized with physiological parameters related to WUE such as measuring their photosynthetic efficiency and carbon isotope analysis. Among these, two mutants were found to have ribosomal large subunit genes, RPL6 and RPL23A activated by the integrated enhancers. The presence of multiple stress-responsive elements in their putative promoter regions and their significant up-regulation in response to ABA, NaCl, and dehydration stresses further corroborated our findings (Moin et al., 2016). This study not only suggested that RPL6 and RPL23A are potential candidates for abiotic-stress amelioration, but more importantly it provides a basis for the exploration of other members of large subunit ribosomal genes for stress-responsiveness.

Taking a cue from these observations, we assessed the abiotic stress responsive roles of other rice 60S ribosomal genes. For this, we selected 34 genes, one from each orthologous group as described earlier and comprehensively studied the differential transcriptional regulation of 34 genes under phytohormone (MeJa and SA), temperature (heat; 42°C and cold; 4°C) and oxidative stress treatments in shoot and root tissues at six different time intervals (5 min, 3 h, 6 h, 12 h, 24 h, and 60 h). After applying the abiotic treatments, tissue samples were collected as early as 5 min to check the immediate responsiveness of the RPL genes and continued up to 60 h. All the RPL genes responded to the treatments in the form of either up or down-regulation.

The genes that exhibited $\geq$3-fold transcript level on the $\log_2$ scale were considered as significantly up-regulated. MeJa, SA, and cold treatments induced the up-regulation of more genes (>60%) than heat and H_2O_2 treatments, which caused the down-regulation of 75% of the genes. In shoots, MeJa and SA-induced the up-regulation of 27 (79%) RPL genes each, cold treatment up-regulated 19 genes (55%), while heat and H_2O_2 treatments up-regulated 6 (17%) RPL genes each. In roots, MeJa, SA, cold, heat, and H_2O_2 treatments up-regulated 19 (55%), 22 (64%), 16 (47%), 6 (17%), and 14 (41%) RPL genes, respectively. Genes that were up-regulated in both the shoot and root tissues include; RPL7, 8, 12, 13b, 18P, 19.3, 24a, 32, 35, and 51 under MeJa treatment, whereas SA-induced the expression of RPL7, 8, 12, 13b, 19.3, 24a, 26, 32, 35, and 51. Genes such as RPL6, 7, 23A, 28, 32, 35, and 37 were up-regulated in cold treatment, while RPL6, 12, 23A, and RPL18a and 13a were up-regulated under heat and H_2O_2 treatments, respectively.

To study the detailed regulation of RPL genes at various time points, we categorized the genes that responded within 5 min to 3 h after treatment as immediate-early (IE), those that responded

between 3 h to 12 h as early (E) and those that were regulated after 12 h of treatment up to 60 h were considered as late (L) responding genes. The majority of the genes that were up-regulated had responded immediately within 5 min to 3 h after the onset of the stress. In shoots, among the genes that were expressive, a total of 17, 21, 19, 6, and 1 genes belonged to the IE-responsive class with instantaneous up-regulation under MeJa, SA, cold, heat, and H_2O_2 treatments, respectively. Among this IE-responsive class of genes, some continued to maintain a high level of expression at all the time points observed, while others exhibited a split in the expression followed by again an increase in the level of their expression. These probably form an important set of genes that respond to environmental stresses and might function as an immediate defense after the onset of the stress (Kawasaki et al., 2001).

The other class of IE genes was down-regulated after an IE response. Under MeJa and SA treatments, RPL7, 8, 12, 13b, 19.3, 24a, 28, and 35 maintained a high level of expression throughout the duration of stress in both shoot and root tissues. However, the level of transcriptional up-regulation varied with some exhibiting a very high level of up-regulation up to 100-fold (RPL8, 12, 19.3, 24a, and 35), while some had moderate expression up to 30-fold (RPL7) and others showed low transcript levels with <10-fold (RPL28). RPL6, 7, 12, and 24a exhibited a consistent up-regulation under cold and heat treatments, of which RPL6 and 12 became up-regulated more than 50-fold. In H_2O_2 treatment, RPL18a was up-regulated in both roots and shoots up to 65-fold whereas 24a, 24b, 30, and 34 showed significant up-regulation in roots. Since stress signals are transmitted through the roots to other parts of the plant body, genes that were significantly up-regulated particularly in roots might have an important role in combating the stress and providing early defense.

The differential expression patterns in response to MeJa and SA (**Figure 4**) and cold, heat and oxidative treatments (**Figure 5**) have been represented in the form of heat maps. These were generated by incorporating the mean values of fold change normalized using $\Delta\Delta C_T$ method obtained from three biological and three technical replicates. The overlap in the up-regulation (**Figure 6**) and down-regulation (**Figure 7**) of 34 RPL genes in both shoot and root tissues were represented as Venn diagrams. Supplementary Tables S5 and S6 provide a detailed list of genes that exhibited overlap in the up and down-regulation, respectively, in shoot and root tissues at each time point.

Differential Transcriptional Regulation of RPL Genes in Response to Treatment with the *Xanthomonas oryzae* pv. *oryzae* Pathogen

The qRT-PCR analysis of 34 genes showed that they are differentially regulated under abiotic treatments, with many of them becoming significantly and immediately up-regulated. Hence, we simultaneously examined the expression levels of 34 RPL genes in response to the *Xanthomonas oryzae* pv. o*ryzae* pathogen that causes BLB of rice. Out of 34 genes, 6 (17%) were down-regulated, RPL38 was non-responsive, and the remaining genes became activated (80%). RPL12, 28, 30, 36, 44, and 51 were among those that were down-regulated, and the transcript level of RPL38 did not change significantly, while all other genes studied were up-regulated. Among those that were expressive, RPL10, 11, 15, 24a, 26, 27, and 37 up-regulated more than 10-fold. The transcript level of RPL10 was the highest with more than 75-fold up-regulation (**Figure 8A**). In addition to significant expression at 11 days, the transcript level of RPL10 also exhibited a gradual increase at 3 h, 6 h, 1 day, and 2 days up to 7 days post-infection with the *Xanthomonas oryzae* pv. o*ryzae* pathogen (**Figure 8B**).

DISCUSSION

Stress factors such as heat, cold, dehydration, and pathogen attack can exacerbate the global agriculture system with an estimated >50% crop yield loss per annum (Wang et al., 2003). Each of these stresses elicits a cascade of signaling pathways to ensure plant survival. It is important to identify the genes that contribute to sustainable plant productivity under conditions of stress to further improve their productivity potential. Although more than 500 genes have been overexpressed and characterized in rice for stress-tolerance, many remain to be identified and examined.

In the present work, we report on the comprehensive expression profiling of rice ribosomal large subunit genes under multiple abiotic and biotic stress treatments at progressive time points and also identified their putative promoter sequences. The information provided here can be exploited further in the functional characterization of these stress-responsive genes, which might help in augmenting rice yields by generating independent transgenic plants. We also identified the genes that exhibited an overlap in the expression patterns in response to two or more stresses. We propose that such genes are particularly promising in bringing about the tolerance to multiple stresses as the presence of a second stress factor can enhance the detrimental effects of the first one (Atkinson and Urwin, 2012).

Ribosomal genes encode proteins that are the components of the two-subunit ribosomal complex, which together with the members of the same group and other proteins participate in protein synthesis. There have been limited reports on the role of ribosomal genes in the stress-responses. We had generated an activation-tagged transgenic rice plant population carrying CaMV35S tetrameric enhancers. These rice mutants were screened for water-use efficiency by growing them under the provision of limited water supply compared to the level that required for normal growth of rice. Flanking sequence analysis of selected mutants having sustained growth and productivity under the condition of limited water availability revealed the activation of the two ribosomal genes (RPL6 and RPL23A) by the integrated enhancers (Moin et al., 2016). This has persuaded us further to analyze the importance of several of RPL genes in stress-responses. We therefore, performed a comprehensive native tissue-specific and differential expression of 34 RPL genes under various abiotic and biotic stress conditions at different time intervals.

The availability of full-length and high-quality rice genome sequence and databases, further helped us to exploit the information on these genes. Based on the information available

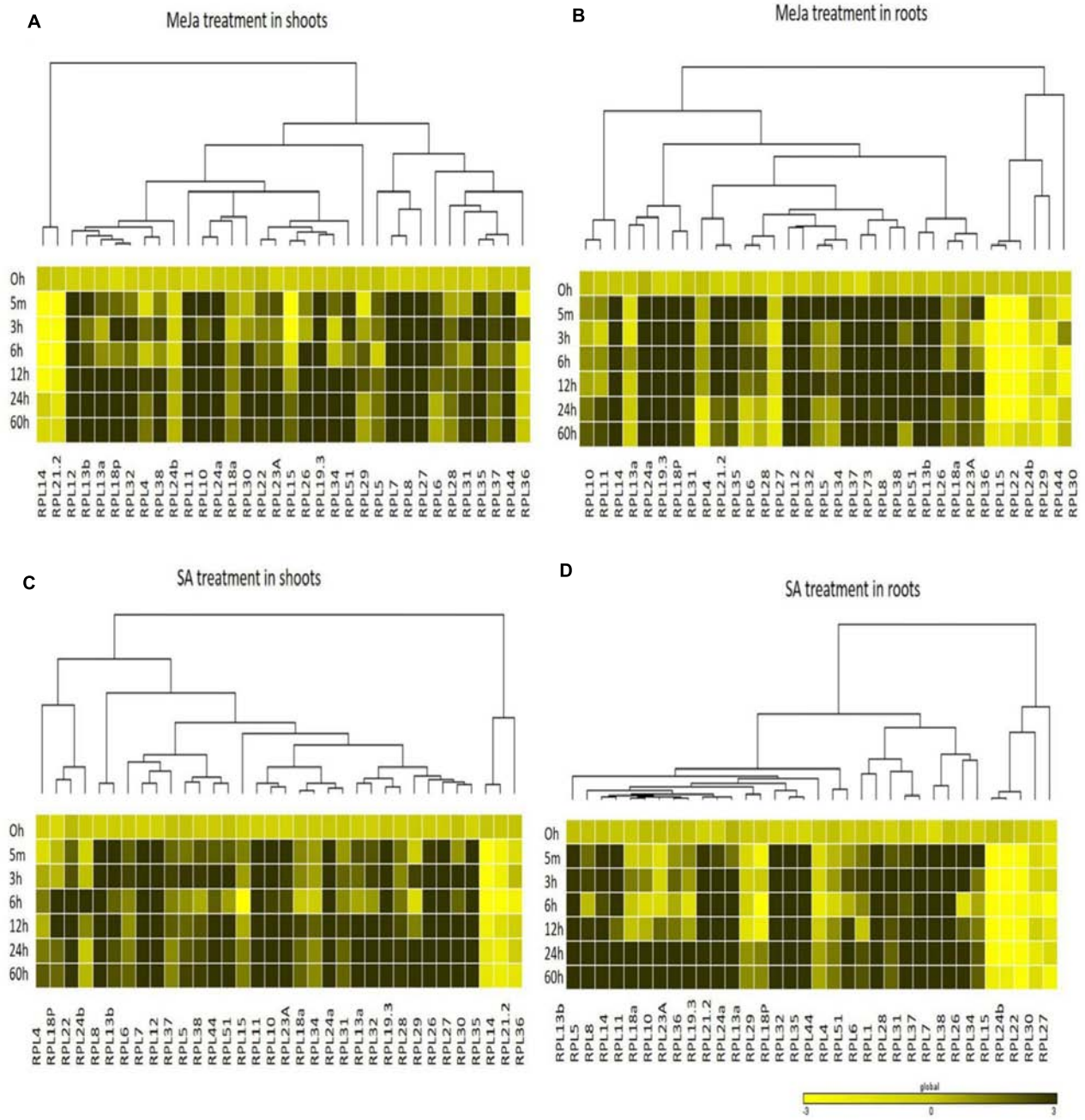

FIGURE 4 | Heat map representation of RPL genes in rice treated with MeJa and SA. Seven-day-old rice seedlings were exposed to different abiotic stresses such as MeJa; 100 μM **(A,B)** and SA; 3 mM **(C,D)** at six different time points as indicated on the left. The qRT-PCR is used to determine the expression levels of RPL genes and the fold change was normalized using $\Delta\Delta C_T$ method relative to that in unstressed seedlings dipped in water at corresponding time points. Rice actin (*act1*) and β-*tub* genes were used as internal controls. Three biological replicates and two technical replicates were included in the study. A dendrogram was constructed to represent the Hierarchical clustering of genes.

in rice databases, we identified 123 genes that are the components of rice ribosomal large subunit, of which 2–3 genes exist as identical gene copies in the genome. These genes are distributed throughout the 12 chromosomes of rice genome with chromosome 7 and 1 having the highest number of genes. The present investigation that reports on the analysis of native and differential expression of the RPL gene family also corroborated the earlier reports that RPL genes are regulated spatio-temporally (Sormani et al., 2011; Carroll, 2013; Zheng et al., 2016). In rice, RPL genes appear to be developmentally regulated as they are widely expressed in all the 13 tissues studied starting from as early as embryonic initiation to plant maturity. Among the 19 RPL

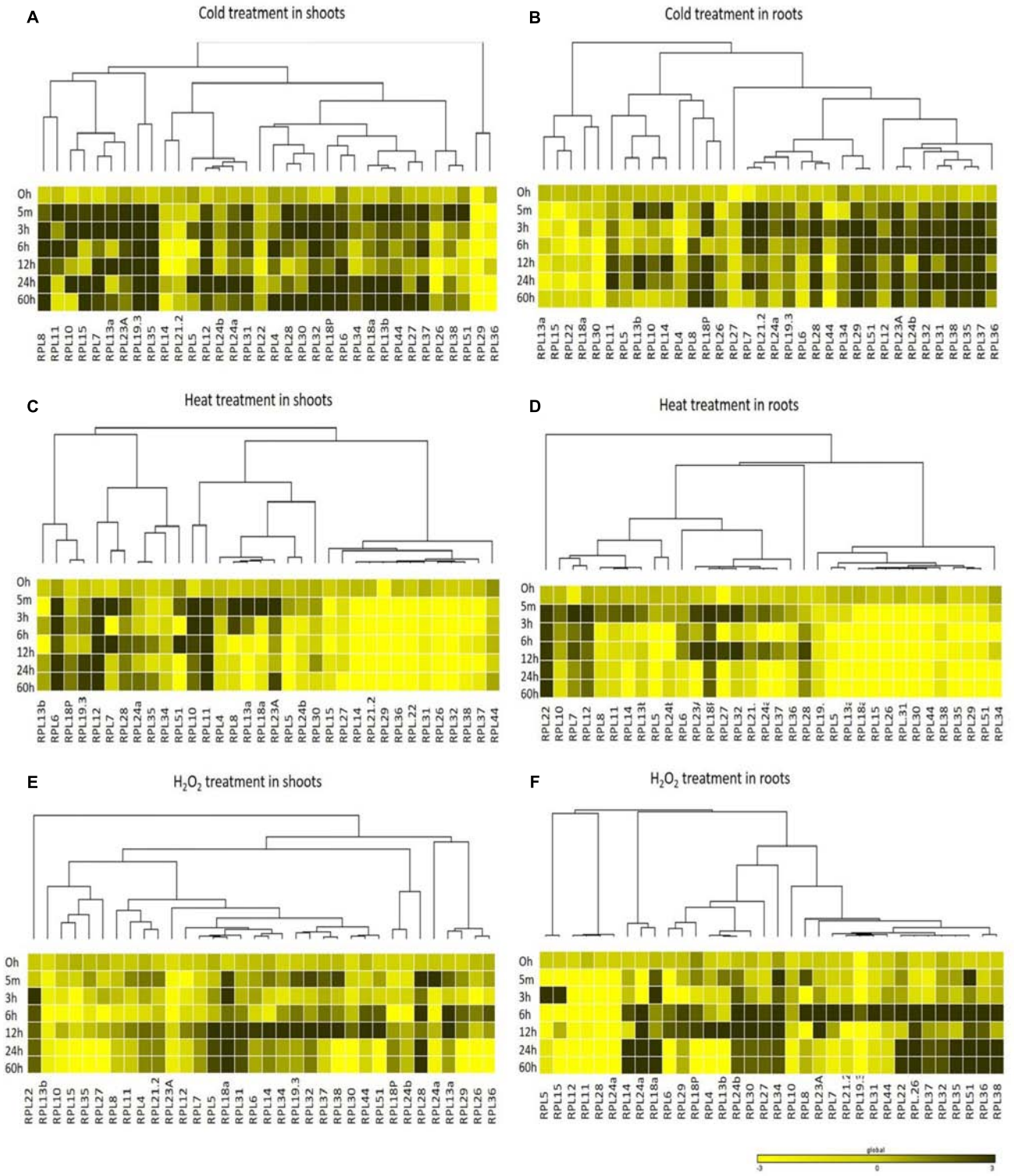

FIGURE 5 | Heat map representation of RPL genes in rice in response to cold, heat and H_2O_2 treatments. Seven-day-old rice seedlings were exposed to different abiotic stresses such as cold stress at 4°C **(A,B)**, heat stress at 42°C **(C,D)** and oxidative stress with H_2O_2; 10 μM **(E,F)** at six different time points as indicated on the left. The qRT-PCR is used to determine the expression levels of RPL genes and the fold change was normalized relative to that in unstressed seedlings dipped in water at corresponding time points. Rice actin *(act1)* and β-tubulin were used as internal reference genes. Three biological replicates and two technical replicates were included in the study. A dendrogram was constructed to represent the Hierarchical clustering of genes.

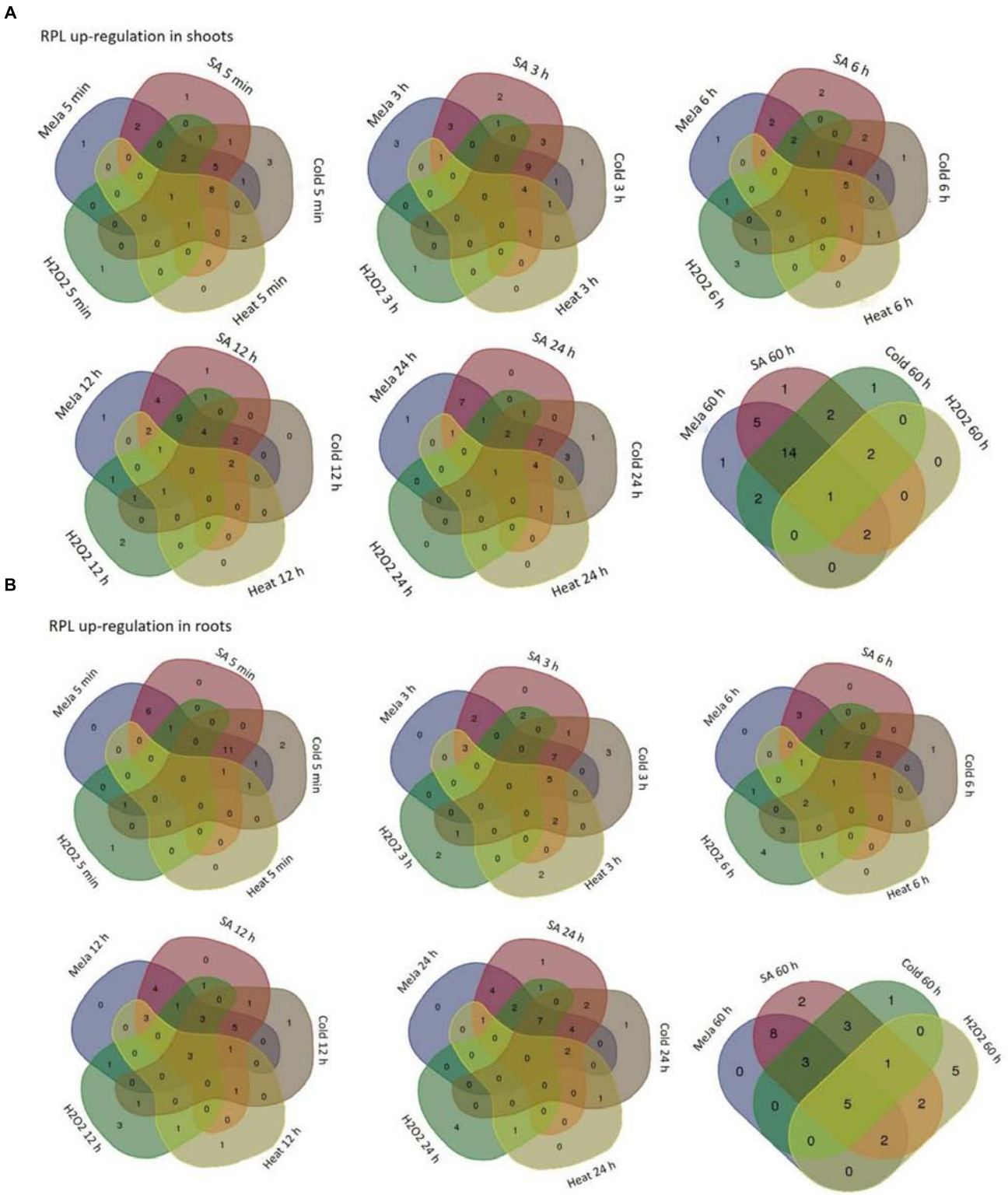

FIGURE 6 | Overlap in the up-regulation of rice RPL genes under five abiotic conditions. The RPL genes that exhibited ≥3-fold transcript level on the $\log_2$ scale were considered as significantly up-regulated while others were considered as down-regulated or without any change in expression. Venn diagrams are used to show the overlap in the up-regulation in shoot **(A)** and root **(B)** tissues.

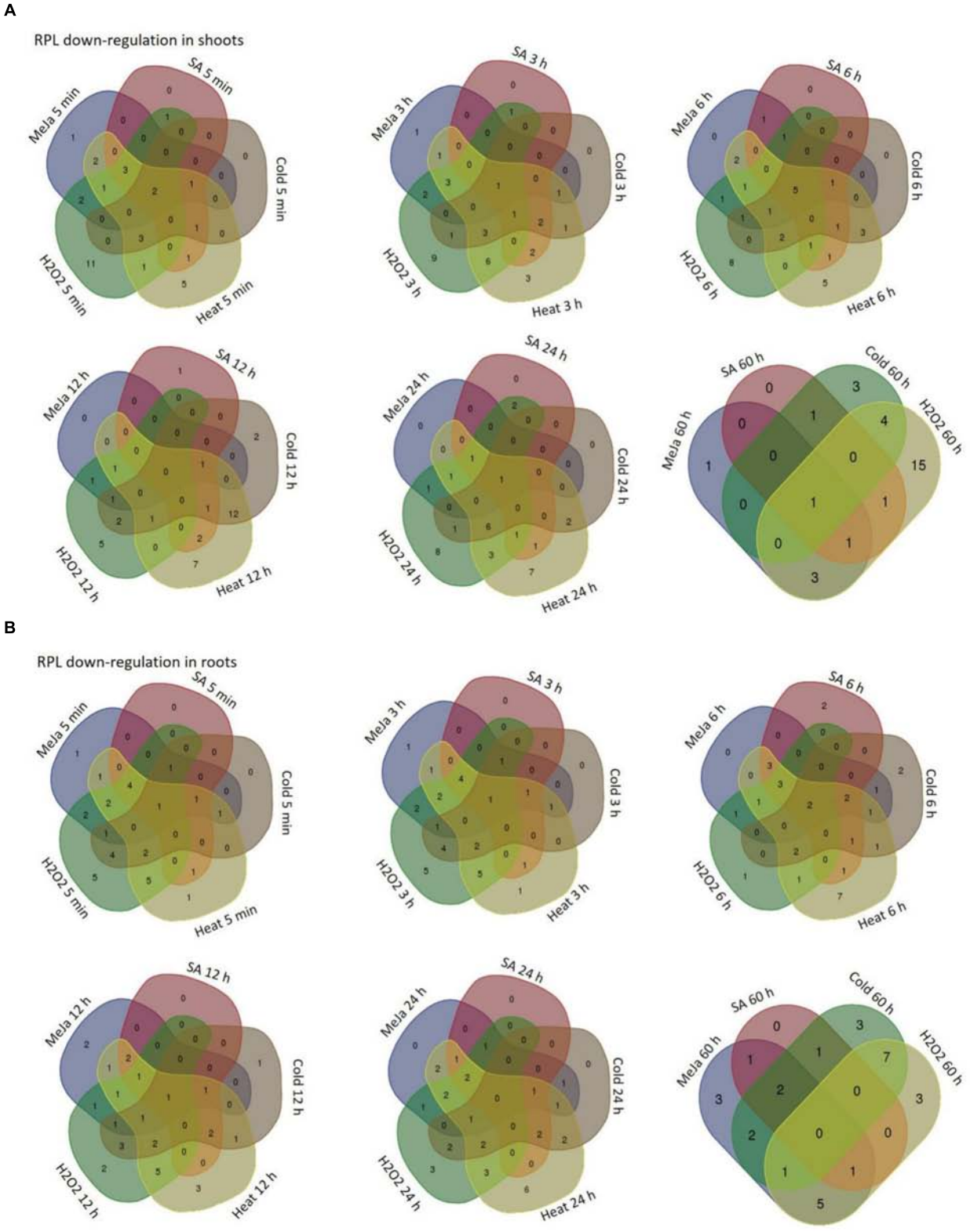

FIGURE 7 | Overlap in the down-regulation of rice RPL genes under five abiotic conditions. The RPL genes that exhibited <3-fold transcript level on the $\log_2$ scale were considered as down-regulated with respect to others. Venn diagrams are used to show the overlap in the down-regulation in shoot **(A)** and root **(B)** tissues separately.

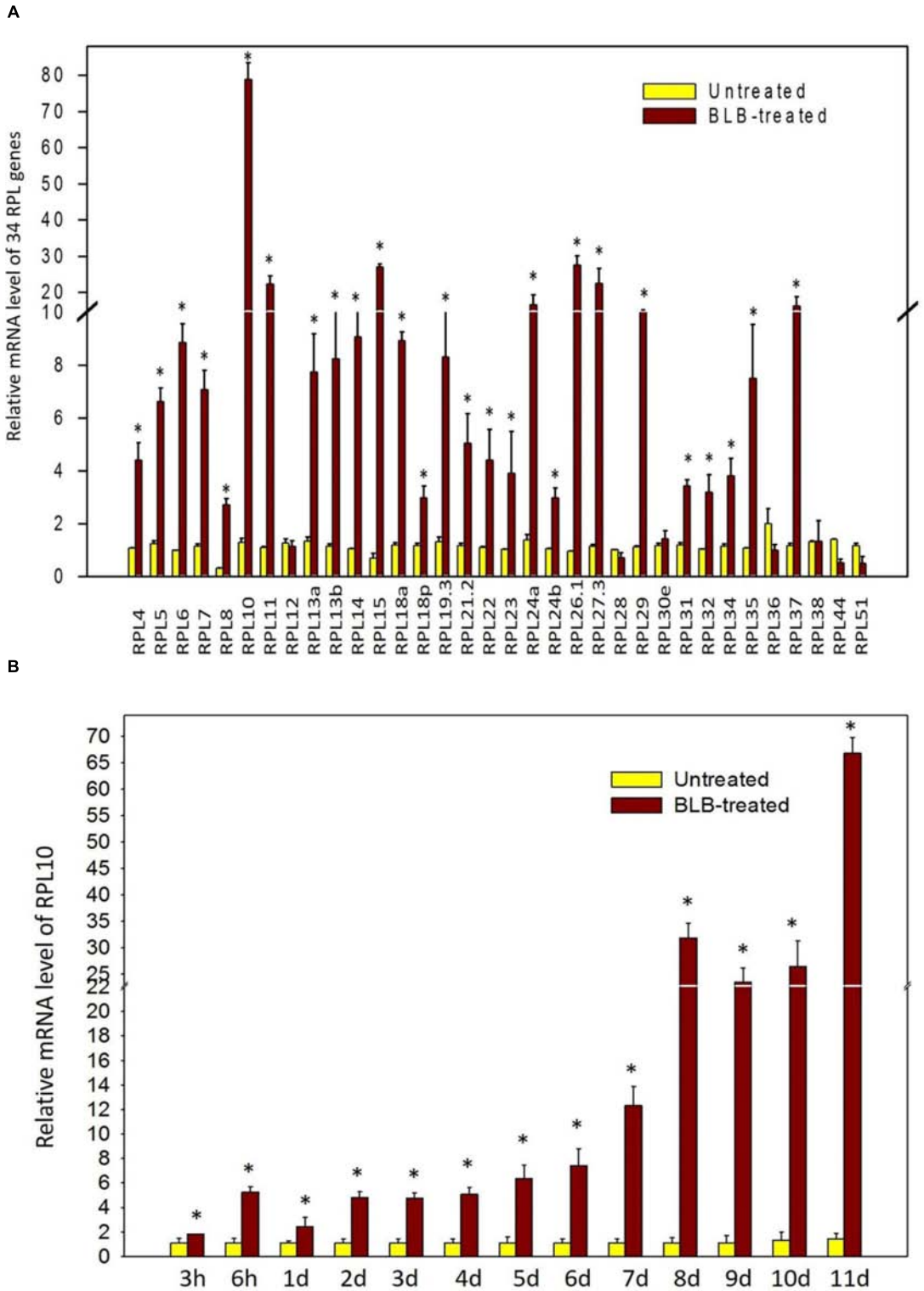

FIGURE 8 | Expression of RPL genes upon infection of rice with the *Xanthomonas oryzae* pv. *oryzae* pathogen. The expression of RPL genes was determined in response to the bacterium, *Xanthomonas oryzae,* which causes leaf blight. **(A)** The bacterial suspension was applied on 60-day-old rice plants, and after 11 days of treatment, leaf samples were analyzed for differential transcript levels of 34 RPL genes. **(B)** Since the up-regulation of RPL10 was significant, we analyzed its expression at progressive time courses such as 3 h, 6 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, and 11 days post-infection with *Xanthomonas oryzae* pv. *oryzae* pathogen. The expression was normalized with untreated samples of same age grown under identical conditions. The statistical significance was calculated using one-way ANOVA at $P < 0.05$ and represented with asterisks.

genes that were expressive in grain filling stage, the expression of RPL5, 7, 13a, 14, 19.3, 22, 24a, 34, and 35 were conspicuously detected. Further characterization of these genes would throw useful insights into their role in grain production, which is a significant yield-related trait in rice.

Although many of the RPL genes were expressive in all the tissues, they cannot be considered as house-keeping as their level of expression changed in response to environmental signals. Similar expression profiling of small and large subunit genes was reported in response to macro-elements deficiency in *Arabidopsis* in which about 244 among 249 RP genes became up-regulated (Wang et al., 2013). The up-regulation of the RPL genes is likely to maintain or improve protein synthesis and hence, proper functioning of ribosomes, the basic cellular moieties under the conditions of stress (Kim et al., 2004). Plants being sessile acclimate to environmental cues by undergoing many metabolic changes, one of them being increased protein turnover that includes both protein biosynthesis and ubiquitination (Kosová et al., 2014). Proteomic studies revealed variations in the levels of translation-related proteins such as initiation factors, elongation factors, and proteins of both small and large subunits during the process of acclimation particularly, to dehydration, salt and temperature stresses in cereals (Fatehi et al., 2012; Budak et al., 2013; Ghabooli et al., 2013; Gharechahi et al., 2014).

In addition to their significant up-regulation, the presence of multiple *cis*-regulatory elements in the putative promoter regions of RPL genes further corroborates our findings that these genes might also play a role in alleviating plant biotic and abiotic stress. RPL6 and RPL23A, in addition to their role in WUE, became up-regulated in almost all the stresses studied illustrating their possible involvement in inducing tolerance to abiotic stresses (Moin et al., 2016). Cold, MeJa, and SA treatments induced the up-regulation of a majority of RPL genes, while H_2O_2 and heat treatments down-regulated 75% of the genes.

The up-regulation of RPL genes by cold treatment is to enhance the process of polypeptide synthesis at low temperatures (Kim et al., 2004). RPL7, 8, 12, 13b, 19.3, 24a, 28, and 35 were up-regulated and constantly maintained a high level of expression throughout the duration of stress in response to SA and MeJa, the two phytohormones involved in plant defense against pathogen attack. These genes also contain TC-rich repeats, which are known for their involvement in plant defense and stress response (Diaz-De-Leon et al., 1993). RPL18a, 24a, 24b, 30, and 34 were expressed at higher levels when exposed to H_2O_2 treatment. This may reflect that these genes might have potential in combating oxidative stress. High temperature appeared to cause detrimental effects on the expression of RPL genes as heat stress had down-regulated >75% of the genes. RPL6, 12, and 23A were among those whose expression was detected. Down-regulation of RPL genes under high temperature might be because of decreased stability of RNA molecules with increasing temperatures.

Infection with *Xanthomonas oryzae* pv. o*ryzae*, which causes BLB also up-regulated a large number of RPL genes. Among those that were expressive (RPL10, 11, 15, 24a, 26, 27, and 37), the expression of RPL10 was more evident as its transcript levels gradually increased from 3 h post-infection and reached a peak at 11 days after treatment. Also, RPL10 was activated in shoots under MeJa and SA up to 60 h after treatment, further suggesting its involvement in biotic stress response.

Our exploration on the detailed expression analysis underpins that these genes regulate tissue-specific development and respond rapidly to the environmental cues and might function as facilitators of immediate defense against stresses. The coordinated transcriptional up-regulation of translation-related genes is a necessity for the cells to maintain the crucial functions of ribosomes under the conditions of stress. The increase in the expression of RPL genes under a wide range of stress-treatments including both biotic and abiotic conditions demonstrate that these are potential targets for the manipulation of stress-tolerance in rice and other related cereal crops as well. However, the level of tolerance induced by each of these genes needs to be analyzed by their independent overexpression in the transgenic rice plants.

AUTHOR CONTRIBUTIONS

PK and MM designed the experiments. MM performed all the experiments. AB, MD, and AS helped in the analysis of qRT-PCR data. SM performed the *Xanthomonas oryzae* pv. o*ryzae* infection on rice leaves. MM and PK prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01284

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

ACTIVATION TAGGING FOR THE IDENTIFICATION OF CANDIDATE GENES RESPONSIBLE

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