Functional characterization of abiotic stress responsive With No Lysine kinase in *Oryza sativa*

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

RAKESH MANUKA

Under the Supervision of

Prof. Kundan Kumar

BITS Pilani

Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

(RAJASTHAN) INDIA

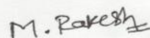
2019

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This thesis is submitted under Regulation 8.20 (a) of the Academic Regulations for Doctoral Programmes which allows a faculty member of the Institute/Professional to do Ph.D. research without the benefit of a supervisor.

This is to certify that the thesis entitled **Functional characterization of abiotic stress responsive With No Lysine kinase in *Oryza sativa*** and submitted by **Mr. Rakesh Manuka** ID No **2014PHXF0002G** for award of Ph.D. Degree of the Institute embodies my original work.

Signature: 

Name in Block Letters: RAKESH MANUKA

ID No.: 2014PHXF0002G

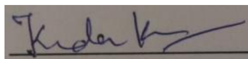
Designation: Ph.D. Scholar

Date: 13th May 2019

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled **Functional characterization of abiotic stress responsive With No Lysine kinase in *Oryza sativa*** and submitted by **Mr. Rakesh Manuka** ID No **2014PHXF0002G** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

Signature of the Supervisor: 

Name in capital letters: Prof. KUNDAN KUMAR

Designation: Associate Professor

Date: 13th May 2019

Acknowledgement

The journey of PhD life "Self-belief and hard work will always earn to your path of success". The time gone through journey it thought me a lot related to research explores and learning scientific studies. The establishment of lab leaded me stronger, for handling the experiments in a precise way. I would like to first thank my supervisor Dr. Kundan Kumar for his great support throughout the PhD journey, his ideas and advises edge me for achieving PhD.

I am grateful to thank Prof. Souvik Bhattacharyya (Vice Chancellor, BITS, Pilani), Prof. Raghurama G (Director, BITS Pilani K. K. Birla Goa Campus), Prof. Srinivas Krishnaswamy (Dean, Academic Research Division, BITS Pilani K. K. Birla Goa Campus), Prof. Sunil Bhand (Dean, Sponsored Research and Consulting Division, BITS Pilani K. K. Birla Goa Campus), Prof. Bharat M. Deshpande (Associate Dean, Academic Research Division, BITS Pilani K. K. Birla Goa Campus), and Prof. Meenal Kowshik (Associate Dean, Sponsored Research and Consulting Division, BITS Pilani K. K. Birla Goa Campus), for providing me the research facilities for performing my research work at BITS, Pilani K. K. Birla Goa Campus.

I would like to acknowledge the members of the Doctoral Advisory Committee, Dr. Sukanta Mondal and Dr. Malabika Biswas, Department of Biological Sciences for their support and guidance. I would like to express my sincere thanks for Prof. Srikanth Mutnuri Head of the Department, Biological Sciences, and Prof. Dibakar Chakrabarty, Convener, Departmental Research Committee, for their support and co-operation, all faculty members of Biological sciences Department Dr. Judith Braganca, Prof. Vijayashree Nayak, Dr. Anusuya Ganguly, Dr. Sumit Biswas, Dr. Angshuman Sarkar, Dr. Veeky Baths, Dr. Malabika Biswas, Dr. Sukanta Mondol, Dr. Indrani Talukdar, Dr. Raviprasad Aduri, Prof. Rajesh Mehrotra, Dr. Sandhya Mehrotra, and Dr. Arnab Banerjee for their timely help throughout the work.

I would like to acknowledge Dr. Pooja Bhatnagar Mathur and Dr. Srinivas Reddy, ICRISAT, patancheru, Hyderabad for allowing me to perform

qRT-PCR studies in their lab. I also thank Prof. Rajesh Mehrotra lab for helping me in the Arabidopsis transformation work. I would like to thank Dr. Sridev Mohapatra, BITS Pilani, Hyderabad campus for helping in confocal imaging studies.

I would like to acknowledge DST-SERB (Sponsored project) and BITS PILANI K.K. Birla goa campus for providing me fellowship throughout my work. I also thank DST-SERB for International travel grant to attend 16th International Symposium on Rice Functional Genomics (ISRFG 2018), Tokyo University of Agriculture, Tokyo, Japan.

I would like to thanks KK lab members, Ankush Ashok Saddhe, Nilesh Dahibhate, Manali Malvankar, Suhas Karle and Bhavya Nair for their help in my journey of PhD. I also thank for the support of all research scholars in the department of biological science. Moreover, I would like to specially thank Ansie and Divya for their great support in each and every time point of my work. Also, I would like to thank Ms. Kamna, Ms. Pallavi, Mr. Mahadev and Mr. Mahaling for their departmental help. I thank Mr. Sagar, Mr. Rahul and Mr. Deepak for helping in all technical works for carrying my work in a smooth way.

Finally, I would like to thank wholeheartedly for unconditional support of my parents Mr. Manuka Srinivas and Ms. Manuka Padma, giving me the support to pursue my dreams. I would like to thank my younger brother Mr. Rajesh Manuka for his support throughout my journey of PhD. I thank to the Almighty of god for all wonderful things given me in life.

The end note learned from my experience in life "Don't give up, the beginning is always hardest but life gives you fruitful thing at end for hard work we gone through".

RAKESH MANUKA

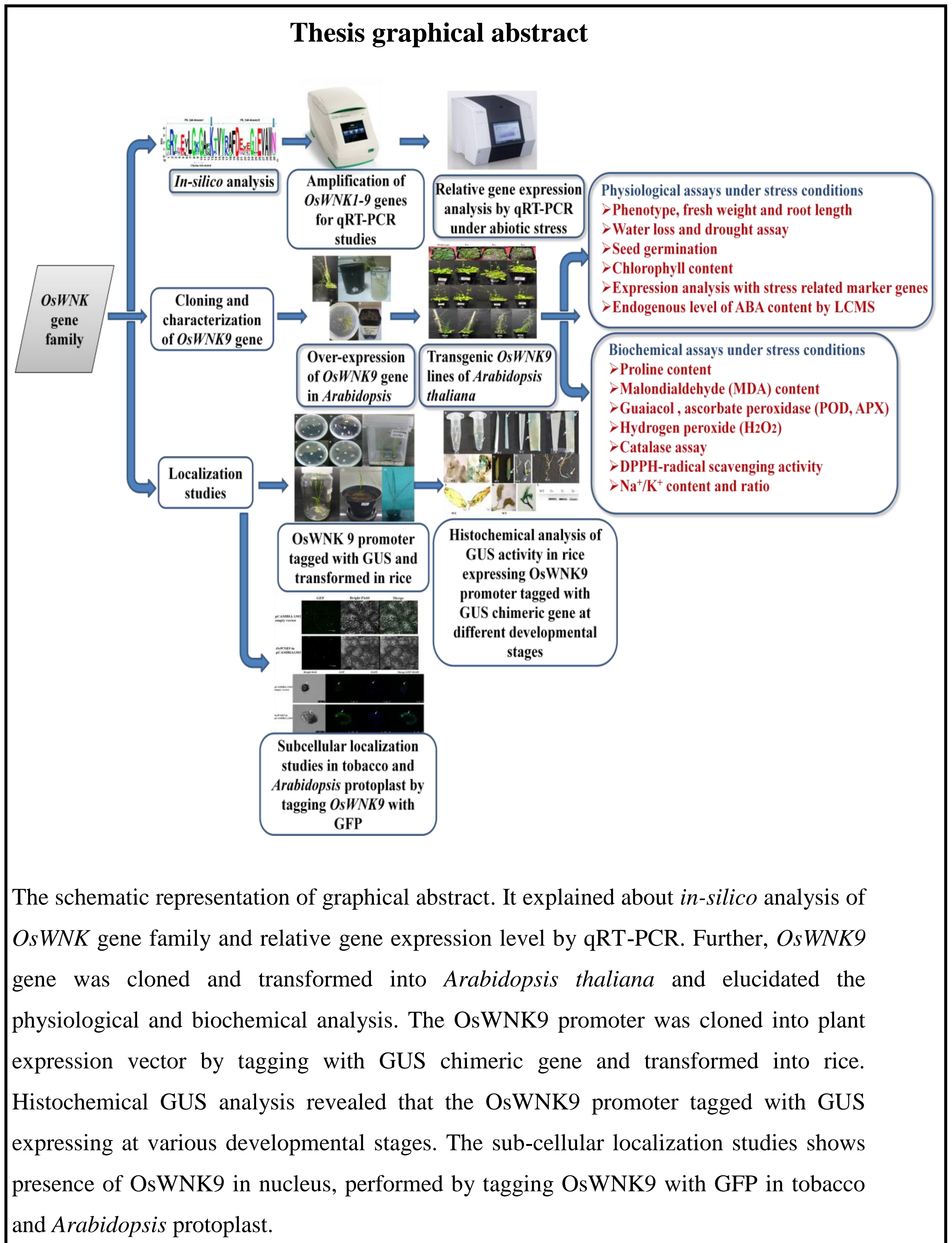
Abstract

The aim of this work was identification and characterization of stress responsive With No Lysine kinase (WNK) gene family in rice. WNKs are members of ser/thr protein kinase family, which lack conserved catalytic lysine (K) residue at protein kinase subdomain II and is replaced by either asparagine, serine or glycine residues and lysine residue is shifted to subdomain I. They are involved in regulation of flowering time, circadian rhythms and abiotic stresses in *Arabidopsis thaliana*. In this study, we have identified 9 members of WNK in rice, showed resemblance to *Arabidopsis* and human WNK and further clustered into five main clades phylogenetically. The predicted genes structure, bonafide conserved signature motif and domains strongly support their identity, as members of WNK kinase family. Further, transcript analysis of *OsWNK* by qRT-PCR revealed their differential regulation in tissue specific and abiotic stresses libraries.

Further, we cloned full-length coding region of *WNK9* from Oryza sativa (*OsWNK9*) and performed *in-silico* studies to confirm the presence of all kinase signature regulatory elements. The transcript analysis revealed that *OsWNK9* was strongly down regulated under salinity, drought and ABA (Abscisic acid) stress in shoots. Constitutive expression of *OsWNK9* in *Arabidopsis thaliana* imparted increased tolerance to salt, drought, and ABA stress. Promoter/gene expression studies revealed that *OsWNK9* were expressed throughout plant tissues with higher expression in roots. Subcellular localization studies of OsWNK9 showed their presence in the nucleus. The transcript analysis of abiotic stress marker genes and ABA dependent genes showed they were highly expressed in transgenic lines compared to WT in response to salt, drought and ABA stress. The endogenous ABA level under salt and drought stress in transgenic lines was higher than

wild type (WT).Further studies evaluated the biochemical properties of WT and *OsWNK9* transgenic *Arabidopsis* lines against the salt and drought stress conditions. Transgenic lines showed high levels of proline accumulation and much reduced membrane damage and hydrogen peroxide content compared to WT plants. Moreover, the transgenic lines exhibited improved activity of antioxidant enzymes such as catalase and ascorbate peroxidase along with the dynamism in peroxidase activity. Altogether, transgenic *Arabidopsis* lines evinced well tolerance to salt and drought stress environments in comparison with WT by signified involvement in biochemical activities of *OsWNK9* gene in the regulation of varied stress tolerance mechanisms.

Thesis graphical abstract



The schematic representation of graphical abstract. It explained about *in-silico* analysis of *OsWNK* gene family and relative gene expression level by qRT-PCR. Further, *OsWNK9* gene was cloned and transformed into *Arabidopsis thaliana* and elucidated the physiological and biochemical analysis. The OsWNK9 promoter was cloned into plant expression vector by tagging with GUS chimeric gene and transformed into rice. Histochemical GUS analysis revealed that the OsWNK9 promoter tagged with GUS expressing at various developmental stages. The sub-cellular localization studies shows presence of OsWNK9 in nucleus, performed by tagging OsWNK9 with GFP in tobacco and *Arabidopsis* protoplast.

TABLE OF CONTENTS

Page No

Thesis title page………………………………………………………………….. I

Certificate from Supervisor ……………………………………………………… II-III

Acknowledgements………………………………………………………………. IV-V

Abstract………………………………………………………………………….. VI-VIII

Table of contents ……….………………………….…………………………….. IX-XIV

List of figures…..………………………………………………………………… XV-XXII

List of tables...……………………………………………………………………. XXIII

List of abbreviations and symbols……...………………………………………… XXIV

CHAPTER 1………………………………………………………………………. 1-32

Introduction and review of literature

1.1 Abiotic stresses……………………..……………………………………………. 2

1.1.1 Plants response to abiotic stress……..………………………………………. 5

1.1.2 Salinity stress…………………………..…………………………………. 8

1.1.2.1 Responsive mechanism of salinity stress in rice…...…..……….. 9

1.1.3 Drought stress………………...……………………..……………….. 13

1.1.4 Cold stress…………………………………………………….……………….. 14

1.1.5 Heat stress…………………….……………………………..…………...…. 17

1.2 Protein kinases……………………………………...………………………. 19

1.3 Role of protein kinases in abiotic stress……………………………………….... 20

1.4 With No Lysine (WNK) kinases…………………………………………………. 24

1.4.1 WNK Kinase and Autoinhibitory Domains...…..…………………………. 25

1.4.2 Physiological Function of WNK…...………...…………………………… 27

1.5 Gaps in Existing Research….………………………...…………………….….. 32

1.6 To address the aforementioned gaps the following objectives were framed..……. 32

CHAPTER 2…………………………………………………………………………. 33-62

Genome-wide identification and expression analysis of WNK kinase gene family in rice

2.1 Introduction……………………….……………………………………………….. 34

2.2 Materials and methods……………………………....………………………….... 35

2.2.1 Database search and retrieval of WNK sequences….…...…………………. 35

2.2.2 Phylogenetic analysis…………………………………...…………………. 36

2.2.3 OsWNK sequence analysis and annotations……………..……………….. 36

2.2.4 *In-silico* analysis of *cis*-elements of OsWNK promoter region…...………. 37

2.2.5 Plant material and stress treatment…………………………………...……. 37

2.2.6 Quantitative real time PCR (qRT-PCR)…………………………………… 38

2.3 Results……….…………………………………...………………………. 39

2.3.1 Identification of WNK gene family across the plant kingdom……...…… 39

2.3.2 Phylogenetic analysis…………………………………………………… 41

2.3.3 Rice WNK annotation and analysis……………………………………... 43

2.3.4 *Cis*-acting regulatory elements present in the promoter region of OsWNK. 47

2.3.5 Tissue specific and abiotic stress responsive expression pattern of OsWNK gene family……………………………………………………………. 48

2.4 Discussion…………………………………………...………………………. 53

2.5 Conclusion…....……………………………………...……………………… 62

CHAPTER 3………………………………………………………………………. 63-121

Expression of *OsWNK9* in *Arabidopsis thaliana* and localization studies of OsWNK9 in rice

3.1 Introduction…………………….…………...…………………………….. 64

3.2 Materials and methods……………………….....……………………….... 65

3.2.1 Plant material and stress treatment………………….…...………………… 65

3.2.2 Quantitative real time PCR (qRT-PCR)……………………………………. 65

3.2.3 Cloning of OsWNK9 and *in silico* analysis...……...……………….....…….. 67

3.2.3.1 Full length *OsWNK9* cloning………………………………………. 67

3.2.3.2 Cloning of OsWNK9 for overexpression, promoter analysis and tissue specific expression………………………………………………... 68

3.2.3.3 Cloning of OsWNK9 promoter for tissue specific localization……. 68

3.2.3.4 Cloning of OsWNK9 for subcellular localization………………….. 68

3.2.4 Overexpression of *OsWNK9* in *Arabidopsis*……………………....………. 69

3.2.5 Evaluation of transgenic line exposed to salt, drought and ABA stress…… 72

3.2.6 Chlorophyll retention assay………………………………………………… 73

3.2.7 Stress treatment of *Arabidopsis* plant for qRT-PCR analysis……………… 73

3.2.8 ABA estimation by LC–MS/MS…………………………………………… 74

3.2.9 Tissue specific localization………………………………………………… 74

3.2.10 Subcellular localization of OsWNK9……………………………………… 78

3.2.11 Statistical analysis ………………………………………………………… 81

3.3 Results……….…………………………………………...……………………….. 81

3.3.1 *OsWNK9* is differentially regulated under salt, drought and ABA stress….. 81

3.3.2 *In-silico* analysis, phylogenetic relationship, and homology modeling predicted OsWNK9 belongs to protein kinase group………………………. 83

3.3.3 Transgenic *Arabidopsis* plants showed better phenotype under salt, drought and ABA stress………………………………………………….. 85

3.3.4 Overexpression of *OsWNK9* in *Arabidopsis* conferred increased tolerance to salt, drought, and ABA stress…………………………………………. 90

3.3.4.1 Seed germination………………………………………………… 90

3.3.4.2. Water loss assay……………………………………...…………. 94

3.3.4.3. Chlorophyll content………………………..……………………. 98

3.3.5 OsWNK9 regulates the transcription of salt, drought and ABA stress related genes…………………………………………………………... 101

3.3.6 Enhanced ABA level in transgenic lines under salt and drought stress……. 105

3.3.7 Tissue specific expression of OsWNK9 in rice……………………………. 106

3.3.8 OsWNK9 protein is localized in the cell nucleus………………………….. 111

3.4 Discussion…………………………………………...………………………. 113

3.5 Conclusion…...……………………………………...………………………. 120

CHAPTER 4……………………………………………………………......... 122-149

OsWNK9* mitigates salt and drought stress effects through the modulation of multiple antioxidant systems in *Arabidopsis

4.1 Introduction……………………..…………...……………………………….. 123

4.2 Materials and methods……………………………....……………………….. 124

4.2.1 Plant materials and growth conditions……………………………..……. 124

4.2.2 Proline content measurement……………………………………………. 125

4.2.3 Malondialdehyde (MDA) content measurement…………………………… 125

4.2.4 Guaiacol Peroxidase (POD) assay……………………………………….. 126

4.2.5 Ascorbate Peroxidase (APX) assay…………………………………………… 126
4.2.6 Catalase (CAT) assay………………………………………………………. 127
4.2.7 Hydrogen Peroxidase (H_2O_2) content measurement……………………….. 128
4.2.8 DPPH-radical scavenging activity…………………………………………. 128
4.2.9 Measurement of total Na+ and K+ ion content under salt stress…………... 129
4.3 Results……………..……………………………......………………………... 129

4.3.1 *OsWNK9* promotes osmotic balance by raising proline levels under salt and drought stress…………………………………………………………...129
4.3.2 Transgenic plants show reduced membrane damage under salinity and drought stress……………………………………………………………….. 131
4.3.3 WT and transgenic plants exhibit a dynamic pattern of peroxidase activity. 133
4.3.4 Transgenic plants show improved APX activity against salt and drought stress………………………………………………………………………... 135
4.3.5 Transgenic plants maintained increased catalase activity under salt and drought stress……………………………………………………………….. 137
4.3.6 Antioxidant defenses in transgenic lines alleviate over-accumulated H_2O_2... 139
4.3.7 Transgenic lines maintain total antioxidant system actively to the highest level………………………………………………………………………… 141
4.3.8 Transgenic lines exhibit an active ion homeostasis under salt stress……… 143
4.4 Discussion……..……..……………………………......………………………... 145
4.5 Conclusion………………………………………………………………………… 149
5.0 Summary……..………………………………….……..……......…………………... 150-153
6.0 Future scope of work………………………………………………………….. 154-155
References…………………………………………………………….......................... 156-177
Appendix I-List of chemical, reagents, and media compositions....………………….. 179-182

Appendix II-List of publications………………………………………………………..... 183

Appendix III-Poster presentation in conferences………..…………………………… 184

Appendix IV- Brief biography of candidate……………………………………...…. 185

Appendix IV-Brief biography of supervisor……………………………………...…. 186-187

Appendix V-Reprints of publications………………………………………………… 188

List of Figures

No.	Figure	Page No.
1.1	**Abiotic stresses.** (A) Representing different types of abiotic stresses such as salinity, drought, salinity, cold, heat, ozone, UV light, heavy metals, flood and anoxic stress. (B) Schematic representation of rice plant and the interaction with abiotic stresses such as drought, cold, heat, salinity and heavy metals	4
1.2	**Plant responses to abiotic stress.** The stress related genes are categorized into two groups based on their functions. The group I member includes membrane water transporters, osmoprotectants, detoxification enzymes and macromolecules protective enzymes. The group II member includes transcription factors, protein kinases and proteinases.	6
1.3	**Rice crop affected by salinity stress.** The crop subjecting to salinity stress at reproductive stage leads for the formation of sterile spikelets. The sterile spikelet lacks the proper seed formation in the grains and affects the productivity of crop (Source: IRRI https://www.slideshare.net/FOODCROPS/rk-singh-breeding-for-salt-tolerance-in-rice)	9
1.4	**Salt tolerance mechanism in rice**. An overview of some important genes involved at the level of leaf, shoot, and root. The influx and efflux mechanism of OsNHX1, OsSOS1, OsHKT2;1, OsNRT1;2, OsCAX1, OsCLC1 and OsKCO1 genes was represented in the figure under salinity conditions in rice plant. The effective role of Na^+/H^+ antiporters, Na^+/K^+ symporter, H^+/Ca^+ antiporter, Ca^{2+} permeable channel, K^+ inward-rectifying channel, Cl^- channel and nitrate transporter under salinity conditions in the cell was represented. The intake mechanism of salt from soil through roots to cell was represented at various levels such as ion exclusion and osmotic exclusion (Source: Reddy et al., 2017).	12
1.5	**Rice crop affected by drought stress.** The effect of diminish water level in rice field. Plants subjected to stress conditions due to lack of water levels leads to drying of plants and reduces the productivity of crop (Source: IRRI-Rice knowledge bank-http://www.knowledgebank.irri.org/).	14
1.6	**Rice grains affected by cold stress.** The decrease in temperature caused the sterile grains which leads to direct effect on the crop productivity. The low temperature levels at reproductive stage will affect the seed setting due to freezing stress and forms the empty grains without proper seeds. (Source: NAGOYA university-https;//satreps.agr.nagoya-u.ac.jp/eng/outline/background. html)	16
1.7	**Representing the shape of chalky and perfect seed formation.** The chalky grain formation occurs due to increasing in temperature at ripening stage of crop. The decrease in starch levels was found in chalky grain comparatively with the perfect grain (Source: Mitsui et al., 2013).	17
1.8	***Arabidopsis thaliana* and *Oryza sativa* WNK's.** Representing the size of	26

	full length protein sequences, kinase domain and autoinhibitory domains are represented with oval and square shape boxes in *Arabidopsis* and rice WNK members (# represents absence of auto inhibitory domain).	
1.9	**Overview of WNK's in plants.** ***Arabidopsis thaliana*** **and** ***Oryza sativa*** **WNK's.** The WNK kinases are oval shape, rectangular boxes representing the cellular processes. Black line represents inhibitory effects and blue line leads the activation. Question mark illustrates the functions of the various proteins and unknown outcomes. EDM2 (Enhanced Downy Mildew 2); AtVHA-C (Vacuolar H+ ATPase Subunit C) (Modified from Cao-pham et al., 2018).	28
2.1	**Distribution of WNK homologs in planta.** Total of 155 WNK homologs retrieved by performing blast from different plant species and represented according to the number of WNK homologs present in each individual plant.	39
2.2	**Graphical representation of conserved OsWNK amino acids subdomain I and II displayed using WebLogo tool.** The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. Given logo shows that highly conserved protein kinase subdomain I and II for plant WNK. N-terminal conserved signature glycine rich stretch shown by down horizontal arrow. Above horizontal arrow shows that protein kinase subdomain I and II. Catalytic lysine (K) residue of subdomain II replaced by asparagine (N)/serine (S) at position 30 and shifted to subdomain I at position 13.	40
2.3	**Phylogenetic relationships of WNKs from plant species.** The unrooted phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates. The bootstrap values are shown at the nodes. The tree was divided into five phylogenetic cluster designated as I to V.	41
2.4	**A physical map of OsWNK on the nine chromosomes of rice genome.** Chromosome numbers are indicated at the top of each bar. The chromosomal positions of putative OsWNKs were mapped according to the genome coordinates	45
2.5	**Phylogenetic relationships and gene structure of rice WNKs.** (A) The phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates. The bootstrap values are shown at the nodes. (B) Gene structure schematic diagram of OsWNK genes. Exons were demonstrated by filled gray boxes and introns were demonstrated by red lines. Untranslated regions 3′ and 5′ (UTR) were displayed by filled black boxes at both ends.	46
2.6	**Graphical representation of putative** ***cis*****-elements in the promoter sequences of 9** ***OsWNK's***. Eight putative *cis*-elements are represented by different colored symbols as indicated.	47
2.7	**Tissue specific expression analysis:** Tissue specific gene expression of OsWNKs analyzed by qRT-PCR in different organs of rice. qRT-PCR data was normalized using rice *eEF1α* gene and shown relative to culm tissue.	48

	A representative histogram with standard error from three replicates performed for each time point has been represented.	
2.8	**qRT-PCR of OsWNKs:** Relative gene expression analyzed by qRT-PCR of OsWNKs under abiotic stress. (A) Salt stress, (B) drought stress, (C) cold stress and (D) heat stress. qRT-PCR data was normalized using rice eEF1α gene and are shown relative to 0 h. A representative histogram with standard error from three replicates performed for each time point has been represented.	50
2.9	**Heat map showing comparison between differential expression profile of rice and Arabidopsis *WNK*.** (A) Expression profile of *OsWNK* in Tissue specific libraries determined by qRT-PCR analysis. (B) Expression profile of *AtWNK* in Tissue specific libraries. The values derived from microarray data available online. (C) Expression profile of *OsWNK* in abiotic stresse rice tissue determined by qRT-PCR analysis. (D) Expression profile of Arabidopsis *WNK* in abiotic stresse libraries. The values derived from microarray data available online (AtGenExpress visualization tool).	51
3.1	***Agrobacterium* mediated transformation in *Arabidopsis thaliana*.** (A) stage for floral dipping of *Arabidopsis thaliana* control plant, (B) Floral dipping in *Agrobacterium* cell suspension, (C) Selection of kanamycin-resistant *Arabidopsis thaliana* seedlings, (D) Selected positive T_2 line seedling transferred to pot, (E) Growth stages of transgenic lines along with WT.	69
3.2	***Agrobacterium* mediated transformation in rice callus.** (A) PB1 (Pusa Basmati) seeds on ½ MS+2, 4-D plate, (B)Untransformed callus, (C) *Agrobatcerium* mediated transformed callus on co-cultivation media, (D) Transformed callus on selection media, (E) Transformed callus on regeneration media, (F) Transformed T_1 lines onto rooting media, (G) T_1 line transferred to pots, & (H) T_1 line transferred to green house.	73
3.3	**Transcript analysis of *OsWNK9* under salt, drought, ABA AND UV stress.** Relative gene expression of *OsWNK9* was analyzed by qRT-PCR, (A) Shoots and (B) Roots (C) UV samples were analyzed by RT-PCR. Three weeks old rice plants were exposed to 200mM salt, 20%PEG and 100µM ABA and samples were collected at 0, 0.5, 3 and 12 h. qRT-PCR data were normalized using rice *eEF1α* gene and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.	76
3.4	**OsWNK9 sequence analysis, phylogeny and homology modeling.** (A) Multiple sequence alignment between OsWNK9, GmWNK1 and human WNK1 protein sequences, conserved domains and motif were highlighted, (B) Phylogenetic analysis based on NJ tree with 1000 bootstrap replicates between rice WNK and Arabidopsis WNK gene family members, (C) Predicted homology model of OsWNK9 using I-Tasser web server and shown highly conserved WNK kinase domain, (D) Predicted homology model of OsWNK9 with an autoinhibitory domain.	78

3.5	**Figure 3.5 Expression level of *OsWNK9* in transgenic lines.** (A) Schematic representation of the *OsWNK9* construct driven by constitutive CaMV 35S promoter and nopaline synthase terminator (NOS) in a pBI121 vector. (B). Expression of the *OsWNK9* mRNA transcripts in T_2 generation lines of *Arabidopsis* was confirmed by performing semi-quantitative RT-PCR with gene specific primers. (C) Relative gene expression analyzed by qRT-PCR of *OsWNK9* in T_2 homozygous lines. Error bars represent the mean ± standard error of relative expression level of three biological replicates.	80
3.6	**Abiotic stress tolerant phenotype of *Arabidopsis* expressing *OsWNK9*.** *Arabidopsis* transgenic lines 9, 11, 12 and 13 expressing *OsWNK9* as compared with WT. The 6 days old seedlings were grown for 2 weeks on MS medium supplemented with indicated concentrations (A) Control (MS), (B) Salt (125mM NaCl), (C) Mannitol (200mM Mannitol) and (D) ABA (12µM ABA). Each experiment was conducted for three times with biological replicates and displayed the representative phenotype results.	81
3.7	**Fresh shoot weight and primary root length of the transgenic lines compared with WT.** The 6 days old *Arabidopsis* seedlings were grown for 2 weeks on MS medium supplemented with indicated concentrations (A) Fresh shoot weight and (B) Primary root length under control condition (MS), (C) Fresh shoot weight and (D)Primary root length under salt stress (MS+125mM NaCl), (E) Fresh shoot weight and (F) Primary root length under mannitol stress (MS+200mM Mannitol), (G) Fresh shoot weight and, (H) Primary root length under ABA stress (MS+12µM ABA). The average and standard error values are represented for 15 biological replicates each for WT and all overexpressed lines. The asterisks represent the significant difference in biomass accumulation and root length compared with wild type (WT) plants, (*) $P<0.05$, (**) $P<0.01$.	82
3.8	**Assessment of seed germination under abiotic stresses**. The percentage of the radical emergence of WT and transgenic lines were calculated under different abiotic stress conditions. (A) 75mM NaCl, (B) 100mM NaCl, (C) 100mM Mannitol, (D) 200mM Mannitol (E) 0.5µM ABA and (F) 2µM ABA. Percentages were determined in relation to the total number of sown seeds. Error bars represent SEM (n=4 plates with ~50 seedlings each). The asterisks represent the significant difference in seed germination of transgenic lines compared with WT plants, (*) $P<0.05$.	85
3.9	**Assessment of seed germination under abiotic stress.** The green cotyledons percentage of WT and transgenic lines 11, 12 and 13 were calculated under different abiotic stress conditions. (A) 75mM NaCl, (B) 100mM NaCl, (C) 100mM mannitol, (D) 200mM mannitol (E) 0.5µM ABA and (F) 2µM ABA. Percentages were determined in relation to the total number of sown seeds. Error bars represent the mean ± standard error of four biological replicates (n=4, plates with ~50 seedlings each). The asterisks represent the significant difference in seed germination of transgenic lines compared with WT plants, (*) $P<0.05$.	86

3.10	**Phenotypic differentiation of the water loss from the detached leaves of the WT and transgenic lines at different time intervals.** The detached leaves from 4-week-old plants were placed on filter papers with the abaxial side of the leaf under temperature-controlled growth chamber.	88
3.11	**Water loss assay.** Time course of the water loss from the detached leaves of the transgenic lines and WT. The water loss was expressed as a percentage of the initial fresh weight (FW) at indicated time intervals. The values are presented as an average and standard error (SE) of four biological replicates. The asterisks represent the significant difference of transgenic lines compared with WT plants, (*) $P<0.05$	89
3.12	**Drought tolerance assay.** A phenotype of the WT and *OsWNK9* overexpressing plants before and after drought treatment. (A) The plants were grown under normal watering conditions for 3 weeks, (B) Further stressed by completely stopping of irrigation for 2 weeks (C) Phenotype of plants after rewatering for 3 weeks.	90
3.13	**Salt sensitivity assay.** Chlorophyll content of transgenic lines and WT *Arabidopsis* under salt stress. The leaves of 4 weeks old plants were detached and kept in different salt concentrations (100mM & 150mM) for 72 hrs and measured chlorophyll content. The values are presented as an average and standard error (SE) of biological replicates. The asterisks represent the significant difference in chlorophyll content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.	92
3.14	**Effect on chlorophyll content of transgenic lines and WT *Arabidopsis* plants exposed to different salt concentrations.** The leaves of four week old plants were detached and kept in a Petri dish with different salt concentrations (100mM & 150mM) for 72 hrs and measured chlorophyll content, (A) chlorophyll a, (B) chlorophyll b. The values are presented as an average and standard error (SE) of triplicates. The asterisks represent the significant difference in chlorophyll content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.	93
3.15	**Expression analysis of abiotic stress relevant marker genes and transcription factors related genes.** The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and transgenic line were placed in liquid MS medium with salt (200mM) for 0, 6, and 12 hr and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and *AtNACO19* were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.	95
3.16	**Expression analysis of abiotic stress related marker genes and transcription factors related genes.** The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and the transgenic line was placed in liquid MS medium with mannitol (300mM) for 0, 6, 12 hr and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and	96

	AtNACO19 were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.	
3.17	**Expression analysis of abiotic stress related marker genes and transcription factors related genes.** The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and the transgenic line was placed in liquid MS medium with ABA (100µM) for 0, 6, 12 h and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and *AtNACO19* were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.	97
3.18	**Endogenous ABA level analysis of transgenic line and WT at three time points (0hr, 24hr, and 48hr).** (A) ABA concentration under salt stress analyzed for WT and transgenic lines (WT, T_{11}, T_{12}, and T_{13}) (B) ABA concentration under drought stress analyzed for WT and transgenic lines (WT, T_{11}, T_{12}, and T_{13}). The values are presented as an average and standard error (SE) of three biological triplicates. The asterisks represent the significant difference in ABA content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.	98
3.19	**Histochemical GUS assay**. Histochemical analysis of GUS activity in rice expressing OsWNK9 promoter tagged with GUS chimeric gene at different developmental stages. (A) Schematic representation of the OsWNK9 promoter tagged with GUS chimeric gene and nopaline synthase terminator (NOS) in pCAMBIA1301 vector, (B) Young seedling, (C) Young leaf, (D) Flag leaf, (E) Mature leaf, (F) Callus, (G) Spikelet, (H) Young plant, (I) Roots, (J) Pollen, and (K) Female gametophyte, (L) Amplification of GUS gene in transgenic lines (White arrows represent GUS activity).	101
3.20	**Histochemical GUS activity in OsWNK9 rice transgenic line under different abiotic stress.** The 4 days old T_2 rice seedlings were transferred to stress conditions for 0, 6, 12 and 24 h and performed GUS assay, (A) Salt stress (200mM NaCl), (B) Mannitol (200mM Mannitol) and (C) ABA (100µM ABA). WT rice seedlings without stress condition were assayed for GUS activity.	102
3.21	**Subcellular localization of *OsWNK9*-GFP in tobacco and *Arabidopsis thaliana*.** (A) Schematic representation of the *OsWNK9* gene fused with mgfp5 and nopaline synthase terminator (NOS) in pCAMBIA 1302 vector (B) Subcellular localization of pCAMBIA1302-OsWNK9 GFP fusion protein along with empty vector as a control in tobacco leaves. The fluorescence was visualized using confocal microscopy. GFP fluorescence (green channel), bright field channel and merged were shown from left to right (C) Subcellular localization of pCAMBIA1302-OsWNK9 GFP fusion protein along with empty vector as a control in Arabidopsis protoplast through PEG mediated transformation by DAPI staining. The fluorescence was visualized using laser confocal microscopy. The bright field channel,	104

	GFP fluorescence (green channel), DAPI (blue staining) and merged were shown from left to right.	
4.1	**Proline content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	121
4.2	**Malonaldehyde (MDA) content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	122
4.3	**Peroxidase (POD) activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	124
4.4	**Ascorbate peroxidase (APX) activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	125
4.5	**Catalase activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	126
4.6	**Hydrogen peroxide (H_2O_2) content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	128
4.7	**Percent of radical scavenging activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress.** Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	129

4.8	**Na^+ and K^+ contents and ratio in WT and transgenic *Arabidopsis* under control and salt stress (200 mM NaCl) environment, (A) Na^+ content, (B) K^+ content, and (C) Na^+/K^+ ratio.** Plants were exposed to the stress conditions for 0 (control), and 48 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).	130
5.1	**Figure 5.1 Hypothetical schematic model for the regulatory function of OsWNK9 under the salt and drought stress.** Salt and drought stress triggered cumulatively osmotic and ionic stress which is sensed by unknown plasma membrane receptors and activate the downstream response. The probable unknown mechanisms (marked with '?') is represented here. OsWNK9 might be involved in the abiotic stress responsive cascade through ABA-dependant pathways. The activated OsWNK9 further phosphorylate other unknown proteins as well as transcription of abiotic stress-related genes. It may activate vacuolar and plasma membrane transporter by phosphorylating and maintaining ion homeostasis.	151

List of Tables

No.	Table	Page No.
1.1	Role of transcription factors under abiotic stress conditions.	6
1.2	Role of protein kinases reported to respond against abiotic stress conditions.	23
1.3	The functional roles of WNK genes reported in different plants.	30
2.1	List of oligonucleotide primers used for qRT-PCR experiments.	38
2.2	List of locus IDs of all WNK genes represented in Fig 2.3.	42
2.3	Physicochemical characteristics of OsWNK genes. Abbreviations used in the table. CDS-coding sequences, AA-amino acid, bp-base pair, MW-molecular weight, g/mol-gram/mole, pI-isoelectric point, C-cytoplasm, N-nucleus.	44
3.1	List of the *OsWNK9* qRT-PCR (OsWNK9), semi quantitative PCR full length (FlWNK9), promoter (PWNK9), subcellular localization (OsWNK9-1302) and GUS primers (GUS).	65
3.2	List of the q-RT PCR abiotic stress and transcription factors related gene primers of *Arabidopsis.*	65

List of Symbols and Abbreviations

Symbols	Description
%	Percentage
°C	Degree Celsius
<	Less than
>	Greater than
pH	Acidic/Basic measurement unit
mM	Milli molar
ml	Milli liter
mg	Milli gram
$mg.ml^{-1}$	Milligram per milliliter
g	Gram
µg	Micro gram
lt	Liter
mg/L	Milli gram/Liter
µl	Micro liter
µM	Micro molar
M	Molarity
3D	Three dimensional
nm	Nano meter
ng	Nano gram
ng/g	Nano gram/ gram
β	Beta

Abbreviations	Description
Na^+	Sodium ions
Cl^-	Chloride ions
K^+	Potassium ions
Ca^{2+}	Calcium ions
UV	Ultra violet
DERB	Dehydration responsive element-binding
MYB	Myeloblastosis oncogene
MYC	Myelocytomatosis oncogene
bZIP	basic leucine zipper domain
ABA3	ABA deficient 3
NCED3	Nine-cis-epoxy carotenoid dioxygenase 3
EREBP	Ethylene responsive element binding factor
WRKY	WRKY transcription factor
NACO19	NAC transcription factor O 19
RD22	Responsive to dehydration 22
h	Hours
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
WNK	With no lysine kinase
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
*eEF1*α	Elongation factor 1α
18S RNA	18S ribosomal RNA
Ct	Cycle thresholds
bp	Base pairs
Col-0	Columbia 0 phenotype
MS	Murashige and Skoog

WT	Wild type
ABA	Abscisic acid
NaCl	Sodium chloride
CaMV	Cauliflower mosaic virus
GFP	Green fluorescent protein
GUS	β-glucuronidase
DAPI	4′,6-diamidino-2-phenylindole
OE	Over-expression
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
EDTA	Ethylene diamine tetra acetic acid
H_2O_2	Hydrogen peroxide
PVP	Poly vinyl pyrrolidone

Chapter-1

Introduction & review of literature

1.1 Abiotic stresses

A recent report of the Food and Agricultural Organization of United Nation framed 17 Sustainable Development Goals (SDG). These mainly emphasized on the population growth in Africa and South Asian countries along with world hunger, food security and agricultural productivity, and climate change (FAO, 2017). The SDGs presented an urgent challenge to researchers for transforming agriculture and food sector towards the achievement of food and nutritional security with sustainability in natural resources for the coming decades. Global food requirement is predicted to increase by 70–85% as world population grows upto 9 billion in 2050. The African and South Asian countries would be the largest population contributors (FAO, 2017; Dhankher and Foyer, 2018). Climate change is a major concern to the global agriculture which negatively impacts on soil fertility and carbon sequestration, high temperature, water scarcity, soil salinity, soil micro-flora and fauna activity and their diversity (Dhankher and Foyer, 2018). It was speculated that drought and heavy precipitation, increased temperature, salinity and heavy metals contamination of soil has led to a sharp decline in agricultural productivity and increased risk of famine (Dhankher and Foyer, 2018).

Abiotic stress is one of the major ongoing challenges and limiting factors for agricultural productivity throughout the world which includes drought, heat, cold, chilling, freezing, salinity, ozone (O_3), heavy metals, and anoxia; being most extensively studied (Fig.1.1) (Munns and Tester, 2008; Chinnusamy and Zhu, 2009; Mittler and Blumwald, 2010; Gupta and Huang, 2014; Dhankher and Foyer, 2018). The current climatic models have predicted that the average surface temperature will increase by 3–5°C in the next 50–100 years. This will significantly affect world's agriculture by

increasing the frequency of flood, heat waves and drought (IPCC, 2007, 2008; Mittler and Blumwald, 2010). The global warming nowadays is a burning issue which gives rise to many abiotic stress problems such as heat, drought, increased sea level, flooding, salinity and hypoxic stress (Wani et al., 2014). Most of agricultural crops are glycophytes (salt sensitive) and cereals among them are more susceptible to abiotic stresses. Wani et al. (2014) reported that rice production in 1996 was 257 million tons and in 2011 it increased up to 718 million tons but still 800 million people are lacking access to adequate food every day. Similarly Ashikari and Ma reported that the world population by 2050 will reach to nine billion and there will be a huge demand for food. Almost 50% of food sources mainly depend on the three major crops, rice with 23% stands first and wheat 17% followed by maize 10% (Ashikari and Ma, 2015).

Rice is a most staple food crop in the world and grown in 160 million hectares worldwide, which supply calories to half of the world's population (Latha et al., 2017). Among the abiotic stresses, drought and salt stress are most prevalent and affect the crops by triggering osmotic imbalance, oxidative damage, accumulation of Na^+ and Cl^-, nutrient depletion and increased generation of nitrogen and reactive oxygen species leading to alteration in metabolism, enhanced lipid peroxidation thereby membrane damage as well as reduced cell growth and development (Kumar et al., 2013). To overcome such adverse effects of abiotic stress, there is an urgent need to device strategies for increasing the crop production which will help in feeding the increasing population across the world (Pandey et al., 2016). The developing abiotic stress tolerant genotype along with high yield is a major objective. Genetic engineering method is one of the approach by transferring the abiotic stress resistant gene into rice genome and

developing the transgenic rice crops. The transgenic crops are now entity assessed in different countries under various field conditions (Latha et al., 2017).

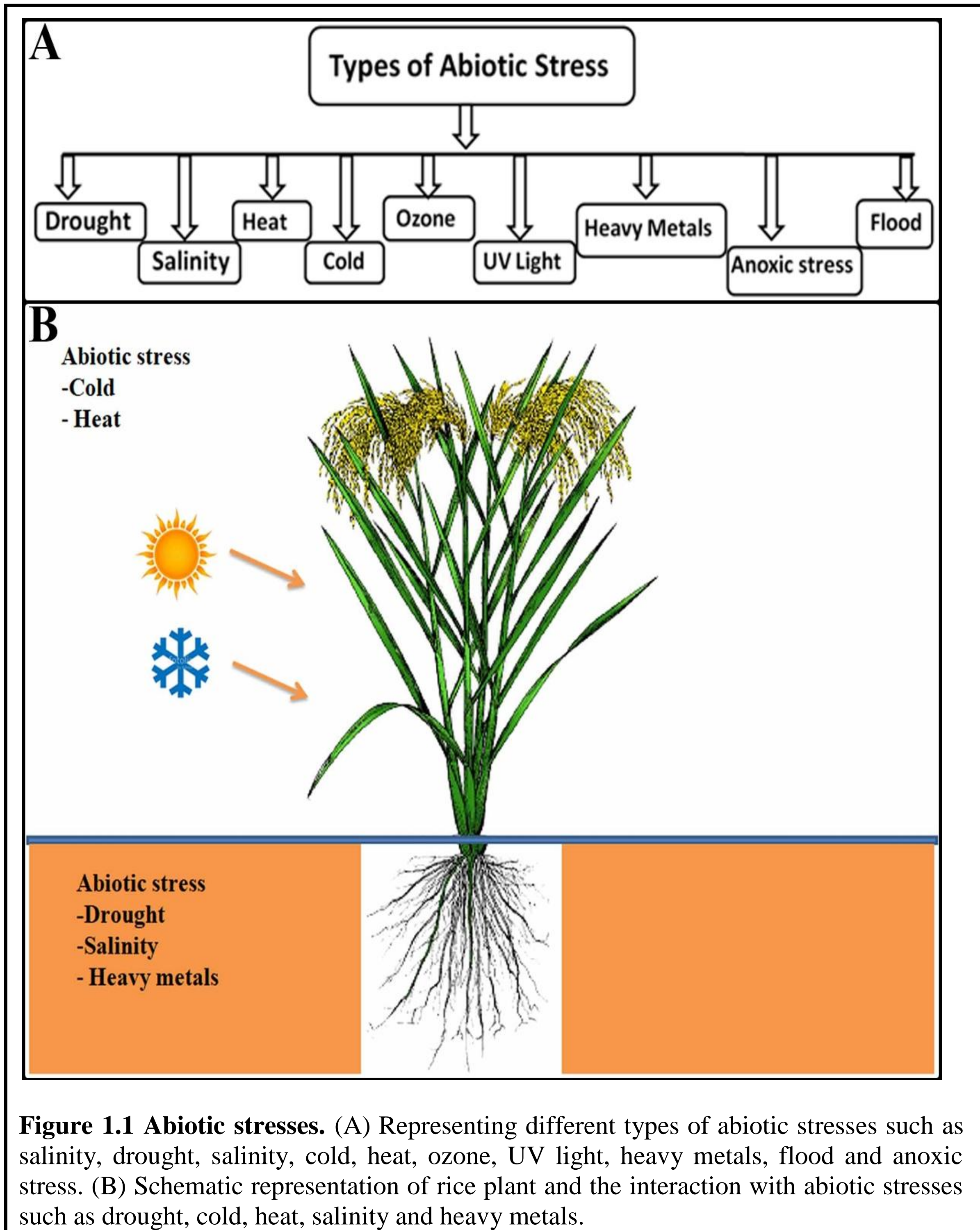


Figure 1.1 Abiotic stresses. (A) Representing different types of abiotic stresses such as salinity, drought, salinity, cold, heat, ozone, UV light, heavy metals, flood and anoxic stress. (B) Schematic representation of rice plant and the interaction with abiotic stresses such as drought, cold, heat, salinity and heavy metals.

1.1.1 Plants response to abiotic stress

Plants sense and respond to abiotic stresses which involve interactions and crosstalk's between several plant hormones, membrane and intra-cellular receptors, signaling pathways and metabolic networks. Recent advanced genomics and proteomics approaches would be helpful to decipher various stress related metabolic and signaling genes and proteins. All these stress-related genes and expressed protein products can be categorized into two groups based on their functions (Liang et al., 2018). The first group consists of membrane transporters that regulates water transport through membranes (major intrinsic proteins and transporters); osmoprotectants (proline, betaine and sugars); enzymes of the antioxidant system (catalase, glutathione *S*-transferase, superoxide dismutase, ascorbate peroxidase and hydrolase, etc.) and macromolecules protecting proteins such as LEA proteins, osmotin, antifreeze proteins, chaperons and mRNA binding protein. The second group comprises regulatory proteins, such as transcription factors (DERB, MYB, bZIP, WRKY, and MYC) (Table 1.1), protein kinases (MAP kinase, ribosomal-protein kinase, CDP kinase, transcription-regulation protein kinase and receptor protein kinases) and proteinases (phospholipase C and phosphoesterases) involved in the regulation of signal transduction and gene expression as shown in Fig. 1.2. The transcription regulators bind and interact with the *cis*-elements present at upstream region of several abiotic stress related genes and regulates expression of many genes conferring tolerance against abiotic stresses (Agarwal et al., 2018).

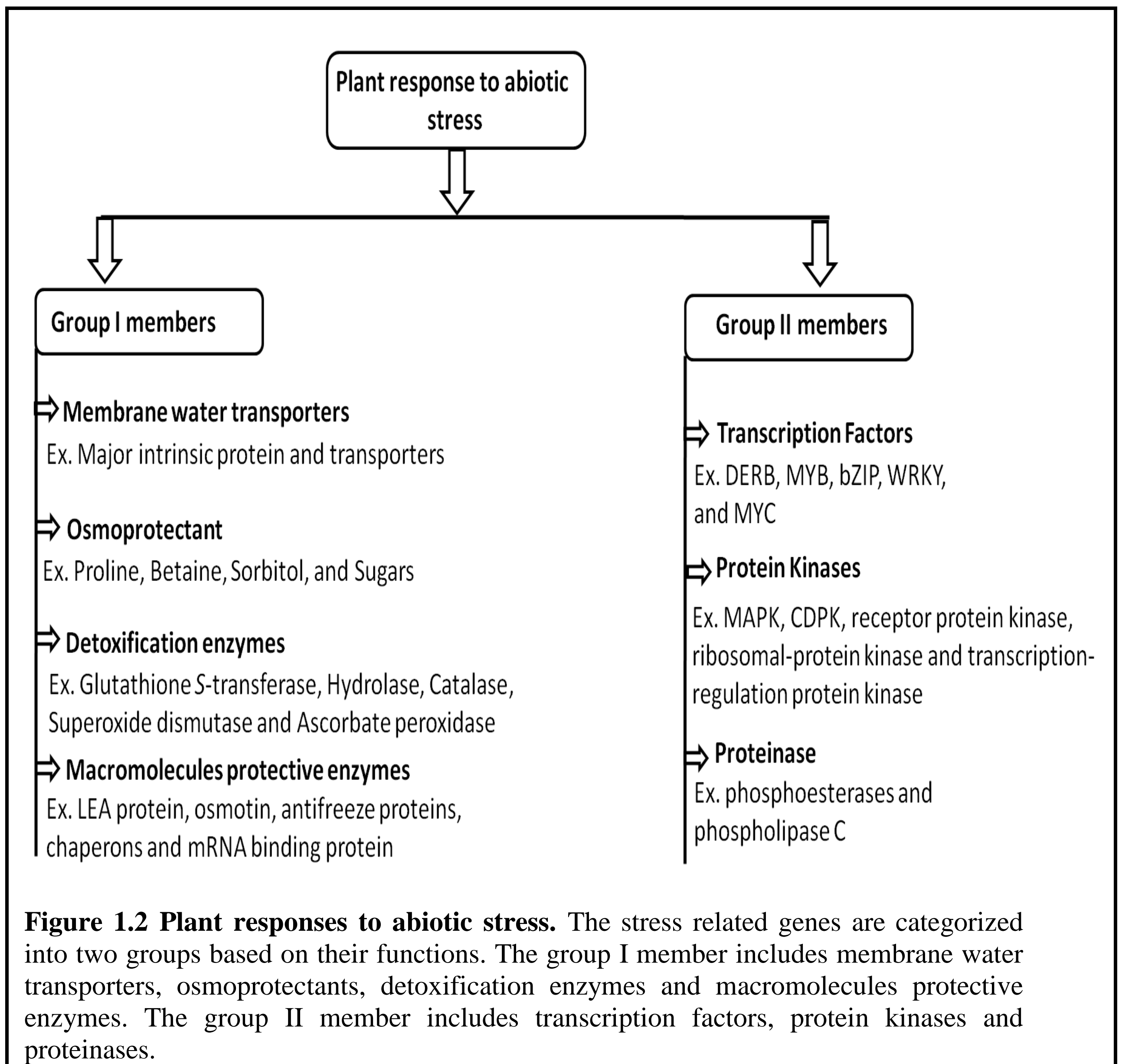


Figure 1.2 Plant responses to abiotic stress. The stress related genes are categorized into two groups based on their functions. The group I member includes membrane water transporters, osmoprotectants, detoxification enzymes and macromolecules protective enzymes. The group II member includes transcription factors, protein kinases and proteinases.

Table 1.1 Role of transcription factors under abiotic stress conditions.

Sr. no	Gene name	Plant species	Characteristics of transgenic plant	References
1	AtWRKY11 and AtWRKY17	*Arabidopsis thaliana*	Role under salinity, drought and ABA stress	Ali et al., 2018
2	AtWRKY28	*Arabidopsis thaliana*	Tolerance to salinity stress	Babitha et al.,2013

3	AtMYB15	*Arabidopsis thaliana*	Role under salinity, drought and cold stress	Ding et al., 2009
4	ONAC022	*Oryza sativa*	Role under salinity, drought	Huang et al.,2016
5	OsNAP	*Oryza sativa*	Role under salinity, drought and cold stress	Chen et al., 2014
6	OsbZIP71	*Oryza sativa*	Role under salinity and drought stress	Liu et al., 2014
7	OsERF4a	*Oryza sativa*	Tolerance to drought stress	Joo et al., 2013
8	OsMYB2	*Oryza sativa*	Role under salinity, drought and cold stress	Yang et al., 2012
9	OsWRKY45	*Oryza sativa*	Role under salinity and drought stress	Qiu and Yu, 2009
10	OsNAC063	*Oryza sativa*	Role under salinity and osmotic tolerance	Yokotani et al., 2009
11	OsNAC6	*Oryza sativa*	Role under salinity, drought and Cold stress	Nakahsima et al., 2007
12	GmNAC20	*Glycine max*	Role under salinity and freezing tolerance	Hao et al., 2011
13	MtWRKY76	*Medicago truncatula*	Role under salinity and drought stress	Liu et al., 2016
14	MdSIMYB1	*Malus*	Role under salinity, drought and cold stress	Wang et al., 2014
15	MsDREB2C	*Malus sieversii Roem*	Role under salt, drought, heat and ABA stress	Zhao et al., 2013
16	SsDREB	*Suaeda salsa*	Role under salt and drought stress	Zhang et al., 2015
17	TaWRKY93	*Triticum*	Role under salinity, drought and cold stress	Qin et al., 2015
18	TabZIP60	*Triticum*	Role under salinity, drought, and freezing tolerance	Zhang et al., 2015
19	VrDREB2A	*Vigna radiata*	Role under salinity and drought stress	Chen et al., 2016
20	ZmWRKY58	*Zea maize*	Role under salinity and drought stress	Cai et al., 2014
21	ZmSNAC1	*Zea maize*	Role under salinity, drought and cold stress	Lu et al., 2012
22	ZmbZIP72	*Zea maize*	Role under salinity and drought stress	Ying et al., 2012

1.1.2 Salinity stress

Salinity is one of the major abiotic stress factors which leads to osmotic stress, ionic imbalance and disruption of metabolic activities (Munns and Tester, 2008). The higher concentration of salts in soil hampers water uptake by roots, which induce osmotic stress and also imbalance in ionic homeostasis in the intracellular regions of the plant (Singh and Jwa, 2013). The excess ion accumulation in leaf blades, shoots causes the cell death and photo inhibition (Zhu, 2002). The increased salt concentration in soil is a global issue which has detrimentally affected approximately around 45 million hectares of agriculture land and around 1.5 million hectares have become uncultivable as a relating consequence (Munns and Tester, 2008). Previous studies have identified numerous adaptive responses against salt stress at physiological, biochemical and molecular levels. Plants respond to different stresses similar to an individual cell as well as interdependently as a whole organism. The effect of growth retardation by salinity can be determined by measuring effects as a function of addition of salt in a time dependent manner (Roy et al., 2014). Salinity affects the stomatal closure which leads to increase in inhibition of shoot elongation and leaf temperature (Rajendran et al., 2009; Sirault et al., 2009). The response to extended salinity phase involves growth inhibition over a particular period of time and premature senescence of older leaves, which was determined as ionic phase (Munns and Tester 2008).

Rice is salt sensitive crop plants having great importance worldwide. Globally, rice is the second largest cultivated crops and covers one tenth of world's arable land making it the single largest source of food (FAO, 2015). Salinity retards rice growth and development, stress responses and adaptation. Salinity in rice during pollination and

fertilization leads to sterility (Reddy et al., 2017). Akbar and Yabuno (1977) reported that salinity led to panicle sterility in some rice varieties, indicating some level of genetic control. Salt stress delays seed development process in rice which has a negative impact on several yield components (Grattan et al., 2002). Salinity stress affects shoot and root length, leaf size, shoot growth, seed germination, flowering stage, seedling growth, shoot fresh and dry weight, spikelet number, number of tillers per plant, percent of sterile florets and productivity (Fig1.3) (Reddy et al., 2017). Higher salt will reduce pollen viability at flowering stage, which in turn affects grain yield. The differences of growth among different genotypes in response to salinity are dependent on concentration of the salt and degree of tolerance to salt (Eynard et al., 2005).

Figure 1.3 Rice crop affected by salinity stress. The crop subjecting to salinity stress at reproductive stage leads for the formation of sterile spikelets. The sterile spikelet lacks the proper seed formation in the grains and affects the productivity of crop (Source:IRRI- https://www.slideshare.net/FOODCROPS/rk-singh-breeding-for-salt-tolerance-in-rice).

1.1.2.1 Responsive mechanism of salinity stress in rice

To increase productivity of rice under salinity, it is crucial to understand basic molecular mechanism of multiple genes regulated under salt tolerance (Chinnusamy et al., 2005). Rice has been categorized as the salt susceptible cereal at its younger growth stage (Lutts et al., 1995) and salinity depletes efficiency of production at its mature stage (Todaka et al., 2012). Rice being transplantable crop allows for control in salinity by managing the seedling transplantation at the mature stage as compared to the flowering stage (Singh et al., 2004). In general, rice can tolerate salinity mainly by two mechanisms, ion exclusion and osmoregulation. Further ion exclusion majorly involves transportation of Na^+ and $Cl^−$ in roots by Na^+ uptake from xylem followed by efflux into the soil (Rajendran et al., 2009; Roy et al., 2014; Reddy et al., 2017). The possible mechanisms of following genes involved in salinity tolerance are shown in Fig 1.4. The OsNHX1, OsSOS1 (Na^+/H^+ antiporters), OsHKT2;1 (Na^+/K^+ symporter) (Kumar et al., 2013; Amin et al., 2016; Mishra et al., 2016), OsNRT1;2 (nitrate transporter), OsCAX1 (H^+/Ca^+ antiporter), OsTPC1 (Ca^{2+} permeable channel), OsAKT1 (K^+ inward- rectifying channel), OsCLC1 ($Cl^−$ channel), and OsKCO1 (K^+ outward-rectifying channel) (Diedhiou and Golldack, 2006; Wang et al., 2012; Kurusu et al., 2012; Kumar et al., 2013; Yang et al., 2014).

Recent studies have identified various adaptive responses to salinity stress at cellular, molecular, physiological and biochemical levels. Plants restrict the transport of ions by selective uptake through root cells and further, ions entering into the root along with water through symplastic and apoplastic regions (Das et al, 2015). In apoplastic pathway, initially water enters into the root hairs and it travels cell to cell through intracellular spaces. In symplastic pathway, the Na^+ is taken up through an ion channels

and/or membrane transporter proteins on the plasma membranes (Das et al, 2015). The Na^+ enters into the cell through various membrane transporters families such as high or low affinity K^+ transporter (HKT) family and plasma-membrane non-selective cation channels (NSCCs) (Gupta and Huang, 2014). Sodium ions could enter cells through nonselective cation channels (NSCC) which are influenced by Ca^{2+}. However, plants are evolved with several counter mechanisms to cope with salinity stress including minimum uptake of Na^+, compartmentalization into the vacuoles and effluxing surplus Na^+ out of the cell (Ji et al., 2013). Discovery of a novel salt overly sensitive (SOS) pathway in plants opened the new horizon of salt tolerance mechanism (Ji et al., 2013). Currently, there are three key components of SOS pathways which regulates the SOS activity in salt stress such as SOS1 (NHX), SOS2 (protein kinase) and SOS3 (SCaBP8, calcium sensor) as shown in Fig. 1.4 (Ji et al., 2013; Sandhu et al., 2017). The prevalent function of SOS1 (NHX) under salt stress is extrusion of Na^+ out of cell. However, the sequestration of excess Na^+ into vacuole is another crucial process to minimize cellular $Na^{+,}$ toxicity and also helped to build osmotic potential inside the cell to facilitate water uptake into the cell (Gupta and Huang, 2014).

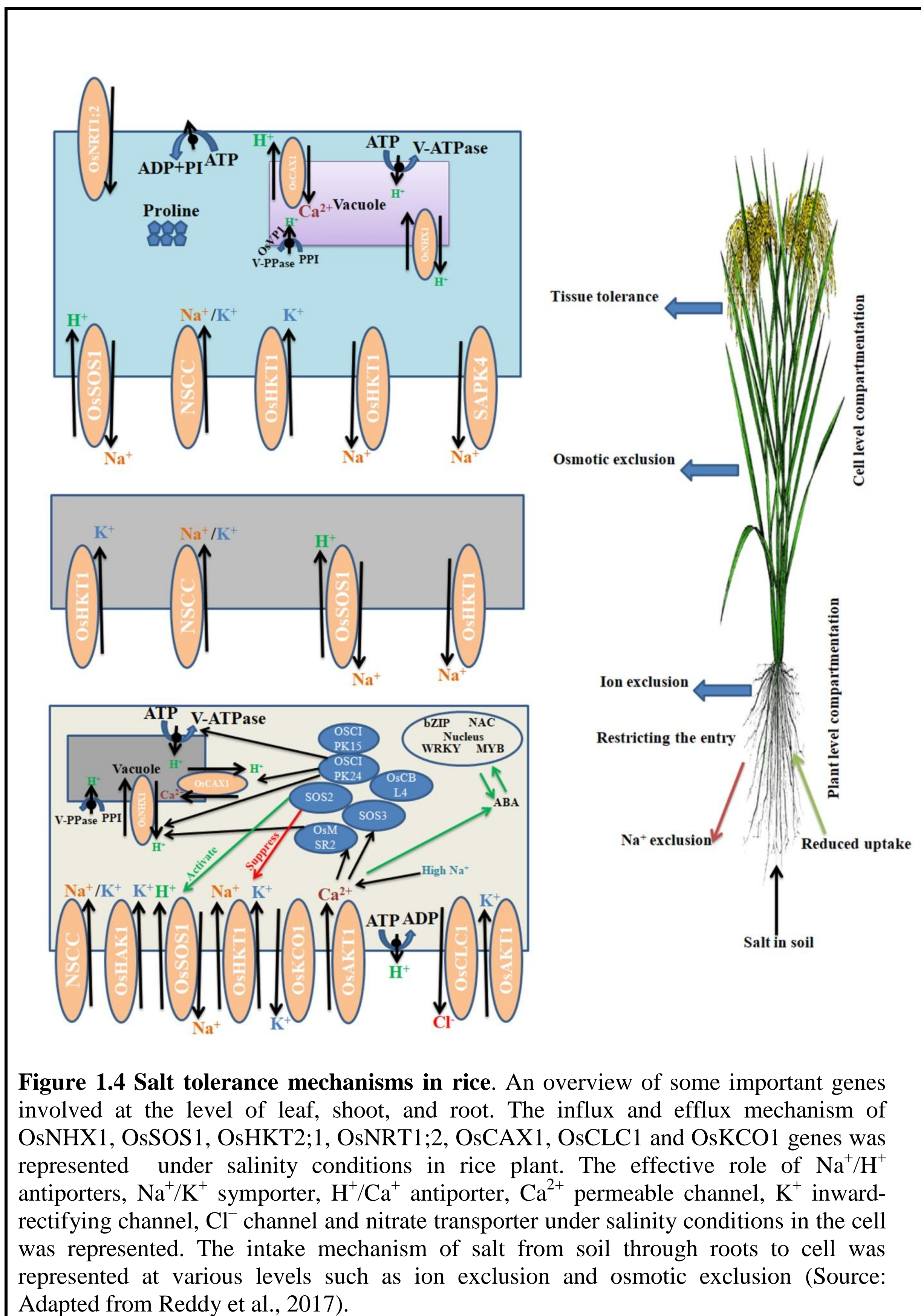


Figure 1.4 Salt tolerance mechanisms in rice. An overview of some important genes involved at the level of leaf, shoot, and root. The influx and efflux mechanism of OsNHX1, OsSOS1, OsHKT2;1, OsNRT1;2, OsCAX1, OsCLC1 and OsKCO1 genes was represented under salinity conditions in rice plant. The effective role of Na^+/H^+ antiporters, Na^+/K^+ symporter, H^+/Ca^+ antiporter, Ca^{2+} permeable channel, K^+ inward-rectifying channel, Cl^- channel and nitrate transporter under salinity conditions in the cell was represented. The intake mechanism of salt from soil through roots to cell was represented at various levels such as ion exclusion and osmotic exclusion (Source: Adapted from Reddy et al., 2017).

1.1.3 Drought stress

Drought stress is related to low water content and cellular dehydration. Drought-resistant crop plants can maintain water balance within cells, withstand low water content, and are capable of quick recovery after re-watering (Singh et al., 2012). Cereal plants mainly rice and maize is very sensitive to reduced water content in soil. Since these crops are cultivated under flood irrigational environmental conditions, consequently large amount of water is required for production of rice crop. To produce one kg of rice seeds, 3000 to 5000 liters of water is required (Singh et al., 2002). Improving the efficiency of water-use for rice cultivation will contribute to conservation of water used for irrigation. Yield of rice cultivation majorly depend on water acquisition and use (Todaka et al., 2015).

In rice, drought stress affects crop in many ways. It is mainly related to loss of water, which causes stomatal closure as well as it leads to limited gaseous exchange. Drought stress refers to reduction in water content, turgor pressure, decreases of cell enlargement and growth, stomatal activity and shrinkage of leaf water potential. Critical water loss may lead to impairment of metabolic processes, photosynthesis and may ultimately lead to death of the plant (Fig 1.5) (Jaleel et al., 2008). Drought conditions hamper growth of plants by overwhelming different physiological and biochemical processes such as respiration, photosynthesis, carbohydrates, translocation, and ion uptake, nutrient metabolism and growth promoter activity (Singh et al., 2012). Effect of drought stress on plant will differ according to various organizational levels which depend on the duration of stress, intensity, plant species and as well as its varied growth stages (Chaves et al., 2002).

Figure 1.5 Rice crop affected by drought stress. The effect of diminish water level in rice field. Plants subjected to stress conditions due to lack of water levels leads to drying of plants and reduces the productivity of crop (Source: IRRI-Rice knowledge bank-http://www.knowledgebank.irri.org/).

The key genes of transcriptional networks can be studied at molecular level and can be employed for development of transgenic plants exhibiting tolerance against stress conditions. The two important pathways are ABA (abscissic acid) dependent and ABA-independent signaling pathways mediated by DREB (dehydration responsive element-binding) type transcription factors. The key transcriptional activators are AREB/ABFs that function in regulation of ABRE-dependent gene expression under ABA signaling during the drought conditions (Todaka et al., 2015).

1.1.4 Cold

Cold is one of the major environmental stress factors that significantly reduce productivity and quality of crop plants. In the life cycle of crop, plants are exposed to

different environmental conditions. Cold stress is mainly defined as chilling conditions (0–15°C) and freezing conditions (<0°C) which are major environmental stress factors leading to limitation in growth and productivity of crops (Fig 1.6) (Zhu et al., 2007). Rice originates from tropical and subtropical regions. It is more sensitive to cold conditions compared to rest of cereal crops such as barley and wheat. In temperate regions, rice production was critically restricted by cold stress (Xie et al., 2012). The low temperature condition at crucial reproductive stages have huge negative impact on grain quality as well as yield in the high-latitude regions of Japan, Korea, China and some other parts of the world (Jena et al., 2012). Cold stress leads to cellular dehydration by limiting nutrient and water uptake and has considerable impact on plant proteome (Xin and Browse, 2000). In rice, differential expression of genes related to cold stress at the level of protein abundance has broadly investigated under cold stress conditions (Kosova et al., 2011). The rice seedlings while exposed to low temperature for deferential proteomic analysis for 48 h at 5°C have shown increased abundance of the proteins related to energy metabolism (adenylate kinase, putative glyoxysomal malate dehydrogenase, UDP-glucose pyrophosphorylase and putative fructose-bisphosphate aldolase) and decrease in the relative abundance of defense response related proteins (phenylalanine ammonia lyase and β-1-3-glucanase). This reveals that energy production pathways are induced under the cold conditions and lead to decreased abundance of defense related proteins (Hashimoto and Komatsu, 2007).

Figure 1.6 Rice grains affected by cold stress. The decrease in temperature caused the sterile grains which leads to direct effect on the crop productivity. The low temperature levels at reproductive stage will affect the seed setting due to freezing stress and forms the empty grains without proper seeds. (Source:https://satreps.agr.nagoyau.ac.jp/eng/outline/background.html).

The photosynthesis in rice is interrupted through effects on fluorescence and chlorophyll content under cold stress conditions (Kanneganti and Gupta, 2008; Kim et al., 2009). Furthermore, malondialdehyde (MDA) and reactive oxygen species (ROS) over-accumulate in rice owing to cold stress. This can impair metabolism through the cellular oxidative damage (Nakashima et al., 2007; Xie et al., 2009). Rice plants also exhibit certain adaptive mechanisms to cope up with the cold stress. For example, rice plants treated with cold can accumulate osmoprotectant proline; an amino acid which maintains optimal functions of cells and stabilizes protein synthesis (Kandpal and Rao, 1985). The contents of antioxidant species also increase ROS scavenging and protect rice plants against oxidative damage (Sato et al., 2011). Some of the physiological changes in plants which occur upon the cold exposure are indicators of cold tolerance in rice (Zhang et al., 2014).

1.1.5 Heat

High temperature is one of major environmental stresses that affect agricultural productivity in plants around the world. Chalky grain formation occurs when plant is subjected to high temperature stress during the ripening stage of grains (Fig 1.7) (Ishimaru et al., 2009; Mitsui et al., 2013). Some of the studies have shown that the white, immature kernels are produced when an average air temperature of first 20 days was more than the 27 °C (Wakamatsu et al., 2007). In rice, ripening period is one of the crucial phases for seed setting; when plant is exposed to high temperature stress during ripening period, it disrupts sink-source balance of the carbohydrates leading to formation of white immature kernels (Morita, 2008). Under high temperatures, carbohydrate supply from source organs depletes essentially resulting in panicle clipping or shading during ripening stages. It also affects growth of the grains (Tsukaguchi and Iida, 2008).

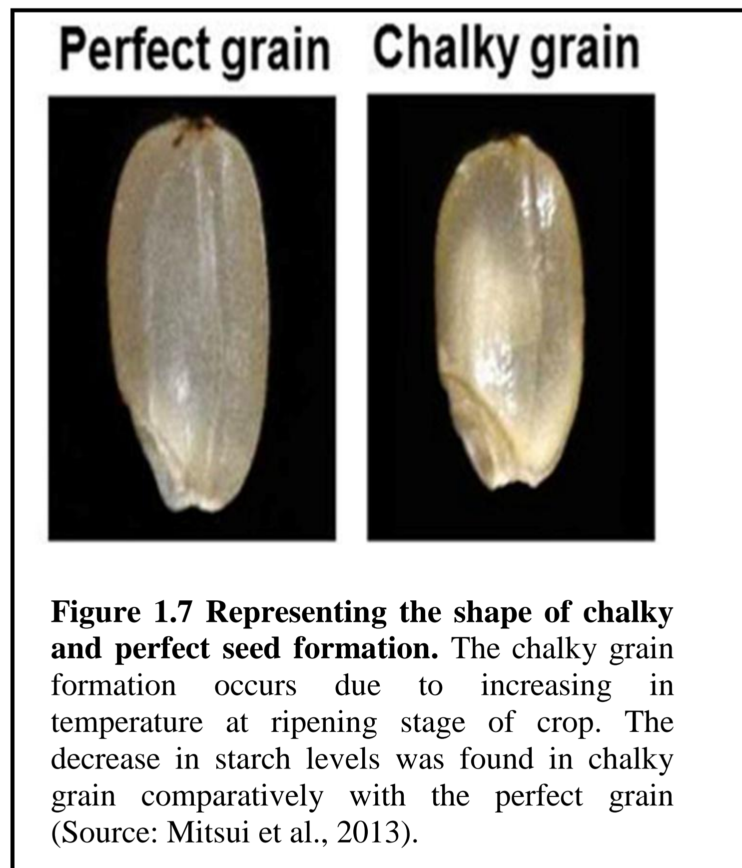


Figure 1.7 Representing the shape of chalky and perfect seed formation. The chalky grain formation occurs due to increasing in temperature at ripening stage of crop. The decrease in starch levels was found in chalky grain comparatively with the perfect grain (Source: Mitsui et al., 2013).

One of the correlation studies have shown decline in yield by higher temperatures during night times due to global warming from 1979 to 2003. Moreover, reports confirm that heat affect the yield of the dry season rice crop by 15% with increase in 1°C temperature every growing year (Peng et al., 2004). To overcome this, all developmental stages must be observed (Triboi et al., 2002). The major challenge for improving rice production was to increase in the stability of yield along with simultaneous reduction in cultivation cost. Some of the strategies could be anther culture, embryo rescuing, inducing *in vitro* fertilization, somaclonal variations, and protoplast fusion between distantly related varieties of rice and developing the genetic markers for identifying specific gene blocks to encourage breeding process. Also, constant variations of forecasted climatic changes affect the yield (Barnabas et al., 2008). Higher temperatures are characterized by reduction in water transport, so the developing cultivars should be capable for overcoming both the stresses (Tester and Bacic, 2005). However, advanced genetic engineering and molecular breeding methods can provide additional tools for development of crop cultivars adapted towards heat tolerance (Kondamudi et al., 2012).

Overall, abiotic stresses such as salinity and drought along with temperature stress cause immense problems for crop plants because they are unfavorable environmental factors that deter plants from acting against stressful conditions. The signaling of salt and drought stress can be differentiated into the three functional categories: osmotic and ionic stress signaling which helps in re-establishment of the cellular homeostasis under stress conditions, detoxification signaling for the controlling and repair of stress induced damage and signaling pathways involved in the coordination of cell division. Cellular

homeostasis along with detoxification signaling would impart stress tolerance and negatively regulate the responses of growth inhibition (Zhu, 2002).

1.2 Protein kinases

Protein kinases are one of the largest enzyme superfamilies in eukaryotes. They are broadly classified into nine groups, 134 families and 196 subfamilies (Manning et al., 2002). Protein kinases transfer gamma phosphate group of adenosine triphosphate (ATP) to serine, threonine or tyrosine residues of the proteins or polypeptides. The catalytic domain of protein kinases usually consist of 250–300 amino acid residues and distinguished into 12 conserved regions called subdomains (Hanks and Quinn, 1991). They are crucial regulators of several cellular processes such as growth, development, cell cycle and signal transduction. Protein phosphorylation triggers conformational changes in the protein structure which regulates and conveys the signal. In plants different types of protein kinases exhibit responses to external stimuli. The expression of protein kinases generate various signals which are recognized and transduced into cells through interactions between complex networks. For plants, it is essential to adapt accordingly with the changing environmental conditions. Some of the protein kinases found in plants include MAPK (Mitogen activated protein kinases), WNK (With no lysine kinases), CDK (Cyclin-dependent kinases), SNF1 (Sucrose non-fermenting 1), CTR1 (Constitutive triple response 1), CK2 (Casein kinase 2) and NPK15 (A tobacco protein-serine/threonine kinase). Some unique protein kinases in plants are considerably different from eukaryotes of similar type including receptor like kinases (RLKs), calmodulin-like domain protein kinases (CDPKs) or calcium-dependent protein kinases

and PVPK1. These protein kinases may possess some of the unique characteristics involved in plant development and growth (Ho, 2015).

The changes in kinase activity by phosphorylation have correlation with extracellular signals. Some of the examples for regulation of cellular responses involving kinases include initiation of mitosis, self-incompatibility, cytoplasmic streaming, isoprenoid biosynthesis, MSERK1 activity, sucrose phosphate synthase activity and phosphoenol pyruvate carboxylase activity (Xing et al., 2007). Apart from that, many signaling molecules are common in different pathways, such as Ca^{2+}, G-proteins and inositol phospholipids (Ho, 2015).

1.3 Role of protein kinases in abiotic stress

Mitogen-activated protein kinase (MAPK) is activated by protein kinases through three-tiered signaling congregation in a sequence of MAPKKK, MAPKK and MAPK. The activation of cascade will occurs at upstream of MAPKKK via receptor like kinase, heterotrimeric GTPases, transmembrane receptors and other protein kinases (Komis et al., 2018). MAPK cascade plays a vital role to respond against different abiotic stresses including salt, cold, heat and drought. The three major MAPKKK such as AtMEKK (MAP Kinase kinase), ANP1-3, and CTR1 have been identified and shown to trigger responses against abiotic stresses such as drought, mechanical stimulation and cold (Teige et al., 2004). MAPKs are involved in various cellular processes such as hormonal signaling, development, abiotic and biotic stress signaling (Moustafa and Abuqamar, 2014). Moreover, several MAPKs belonging to yeasts and animals are involved in cell division, differentiation as well as responses to stress conditions. Some MAPKs at sub-

domain VIII are highly conserved with threonine and tyrosine residues capable of tyrosine and threonine phosphorylations. This particular dual specific MAPKK has been activated by serine/threonine MAPKKK (Xie et al., 2012). Overall, kinase cascade mediate distinct signal transduction pathways. The transcript level of *AtMPK3* which belongs to *Arabidopsis* MAPK was found to be increased after exposing the plant to salt, drought, cold and touch stress (Mizoguchi et al., 1996). Plant hormones such as ABA and MAPKs respond to the cold and drought stress conditions, even though they operate in their own independent pathways. ABA functions as a negative regulator for seed germination similarly responding to abiotic stress such as drought condition. The major role of ABA is controlling water balance as well as osmotic stress apart from tolerating cellular dehydration in plants. It also regulates closing and opening of stomata by influencing ion transportation to stomatal guard cells through changes in water availability from environment. Some of the reports state that closing of stomata occurs by virtue of the ability of guard cells to lose K^+ leading to turgidity of cells, the same being stimulated by ABA (Ho, 2015). ABA is a key regulatory hormone which balances the osmotic pressure within plant cells through opening and closing of stomata simultaneously utilizing combined action of stress response genes. Similar results were proposed by Hugouvieux et al. (Hugouvieux et al., 2001) in *Arabidopsis* about the role of ABA in stomatal closing through loss of turgor pressure. Based on their results, they deduced the responses of ABA for controlling water loss at much lower rate than the wild type. The boosting up of ABA biosynthesis under osmotic stress conditions proves role of ABA in thermal and drought stress tolerance. Similarly, ABA biosynthesis is also enhanced under salinity conditions comparatively being less in case of cold stress (Knight

and Knight, 2001). Apart from MAPKs, ABA is also an important hormone mediating a response to cold and water logging conditions. The MEKK-MMKK2 is a protein kinase cascade consisting of MPK4/MPK6 which responds to the cold and salt stresses in *Arabidopsis* (Teige et al., 2004; Ichimura et al., 2006). Calcium dependent protein kinase and their upstream kinases are activated under different stress conditions. CDPKs are involved in enabling the plant to cope with various stress conditions such as oxidative, mechanical stimulation and cold stress. For example, the characterization of CDPK have suggested that few stress-induced Ca^{2+} signals are transmitted by CDPKs. Overall, CDPKs are activated by abiotic stress (Hwang et al 2000). In *Arabidopsis*, *AtCDPK1* and *AtCDPK2* genes are involved in signaling pathways working against salt and drought stresses (Ho, 2015). Calcium is a secondary signaling molecule perceived by different CDPKs while operating in biotic and abiotic stress signaling pathways. CDPKs belong to one of the serine/ threonine protein kinase families which exhibits C-terminal calmodulin-like domain including presence of 4EF hand motifs, which bind to Ca^{2+} directly. Overexpression of *OsCDPK7* kinase gene in the rice has shown tolerance against drought and salt stress (Saijo et al 2000). Few kinase members, which are involved in different stress responses are reported in table 1.2. With no lysine kinase (WNK) belongs to ser/thr subfamily that differs from other MAP kinases with respect to the replacement of lysine residue in their kinase subdomain. Many reports claim that WNK kinases are involved in various abiotic stress conditions, though the mechanism has not been fully investigated. Moreover, most of the plant WNK kinase members are still uncharacterized. It is thus essential to explore the WNK regulatory mechanism and their signaling cascade under various stress conditions.

Table 1.2 Role of protein kinases reported to respond against abiotic stress conditions.

S.no	Gene name	Plant	Characteristics of transgenic plant	References
1	*AtCPK6*	*Arabidopsis thaliana*	Tolerant to salt and drought stresses	Xu J et al., 2010
2	*AtMEK1*	*Arabidopsis thaliana*	Tolerant to drought stresses	Xing et al., 2007
3	*AtMKK2*	*Arabidopsis thaliana*	MKK2 Pathway Mediates Cold and Salt Stress Signaling	Teige et al., 2004
4	*OsSIK2*	*Oryza sativa*	Tolerant to salt and drought stresses and delays dark-induced leaf senescence	Chen et al., 2013
5	*OsMKK6*	*Oryza sativa*	Enhanced tolerance to chilling stress	Xie et al., 2012
6	*OsMKKs*	*Oryza sativa*	OsMKKs differentially regulated by Salinity, drought, cold, and heat stresses	Kumar et al., 2008
7	*OsMAPK5*	*Oryza sativa*	MAPKs plays role in modulating the interaction of defense pathways activated by biotic and abiotic factors	Xiong and Yang, 2003
8	*OsCDPK7*	*Oryza sativa*	Tolerant to salt and drought stresses	Saijo et al., 2000
9	*GsRLCK*	*Glycine soja*	Tolerant to salt and drought stresses	Sun et al., 2013
10	*TaCIPK2*	*Tobacco*	Enhanced tolerance under drought condition by regulating stomatal moment	Wang et al., 2016
11	*ZmSIMK1*	*Tobacco*	Conferred tolerance under drought conditions and increased antioxidants and ROS-scavenging enzyme	Wang et al., 2014
12	*ZmMPK3*	*Zea mays*	Plays role under Salinity, drought, cold, ultraviolet light, mechanical wounding and heavy metal	Wang et al., 2010
13	*NPK1*	*Zea mays*	Enhanced tolerance under drought condition	Shou et al., 2004

1.4 With No Lysine (WNK) kinases

Eukaryotic protein kinases represent the largest gene families which are mostly involved in diverse regulatory functions (Hanks and Hunter, 1995). With No Lysine (WNK) kinases are unique subfamily of serine/threonine (S/T) protein kinases related to the STE20/PAK-like family, first discovered in mammals while screening for novel members of mitogen activated protein kinase family (Xu et al., 2000). WNK family members are not present in yeast and distribution is restricted to higher organisms (Xu et al., 2002). A peculiar feature of WNK is the transfer of a catalytic lysine (K) residue in kinase subdomain II to subdomain I, which is conserved among all other kinases (Xu et al., 2000). It has been reported that the function of shifted K in kinase subdomain I is involves in phosphorylation process (McCormick and Ellison, 2011). The WNK homologs are present in higher organisms except for yeast (Verissimo and Jordon, 2001). Four WNK kinases are reported in human namely WNK1, WNK2, WNK3, and WNK4. However, single WNK homolog was reported in *Drosophila melanogaster* and *Caenorhabditis elegans* respectively (Verssimo and Jordan, 2001; Wilson et al., 2001; Gagnon et al., 2006). WNKs are engaged in several physiological functions such as maintenance of ions and pH homeostasis, cell communication, proliferation and organ development. Few WNK members are known to respond for osmotic stress and regulate epithelial ion transport like Solute Carrier 12 (SLC12) family of cation chloride co-transporters (CCCs) in mammals through a diverse range of catalytic dependent mechanisms and cell volume homeostasis by modulating ion transporter and channels (Wilson et al., 2001; Reilly et al., 2003; Kahle et al., 2005; Gagnon et al., 2006; Kahle et al., 2006). In humans, a mutation in WNK1 and WNK4 kinases cause several disorders

such as loss of renal ion transport, hypertension, and cancer (Wilson et al., 2001; Reilly et al., 2003; Kahle et al., 2005). WNK's also plays critical role in angiogenesis of zebrafish (Lai et al., 2014) and regulates the Wnt signaling in Drosophila (Serysheva et al., 2013).

In *Arabidopsis*, 11 members of WNK family have been predicted and few of the members have so far been well characterized under various abiotic stresses (Huang et al., 2007; Wang et al., 2008). All *Arabidopsis* WNK members except *AtWNK6* are expressed in all the tissues from the seedling to the flowering plants. In plants, WNK members are reported to be involved in several physiological and cellular roles, such as regulation of flowering time, maintaining the circadian cycle, root architect and regulation of various abiotic stresses (Uchida et al., 2014).

1.4.1 WNK and autoinhibitory domains

Mammalian WNKs are well characterized at structural and functional level (Yamada et al., 2016). On the other hand, plant WNKs are not explored much except few recent studies. Currently, WNK's structural information has been elucidated from rat and human WNK (Yamada et al., 2016). Plant WNK sequence analysis has predicted and confirmed the presence of two predominant active domains and conserved motifs. It has an N-terminal kinase domain and C-terminal autoinhibitory domain of which, kinase domain is highly conserved and the point of attention/discovery is the shifted conserved lysine (K) residue from subdomain II to subdomain I. In human, WNK1 have five Arg-Phe-Xaa-Val (RFXV) motifs, out of which one is present in the kinase domain and four in the C terminal region (Min et al., 2004). Autoinhibitory domain interacts with RFxV motif and inhibits the kinase activity. However, RFxV motifs are absent in *Arabidopsis* and rice

WNKs. AtWNK and OsWNK sequences are aligned and their domains are depicted in the form of boxes. AtWNK11 and OsWNK5 have kinase domain but lack the autoinhibitory domain in the sequence (Fig. 1.8).

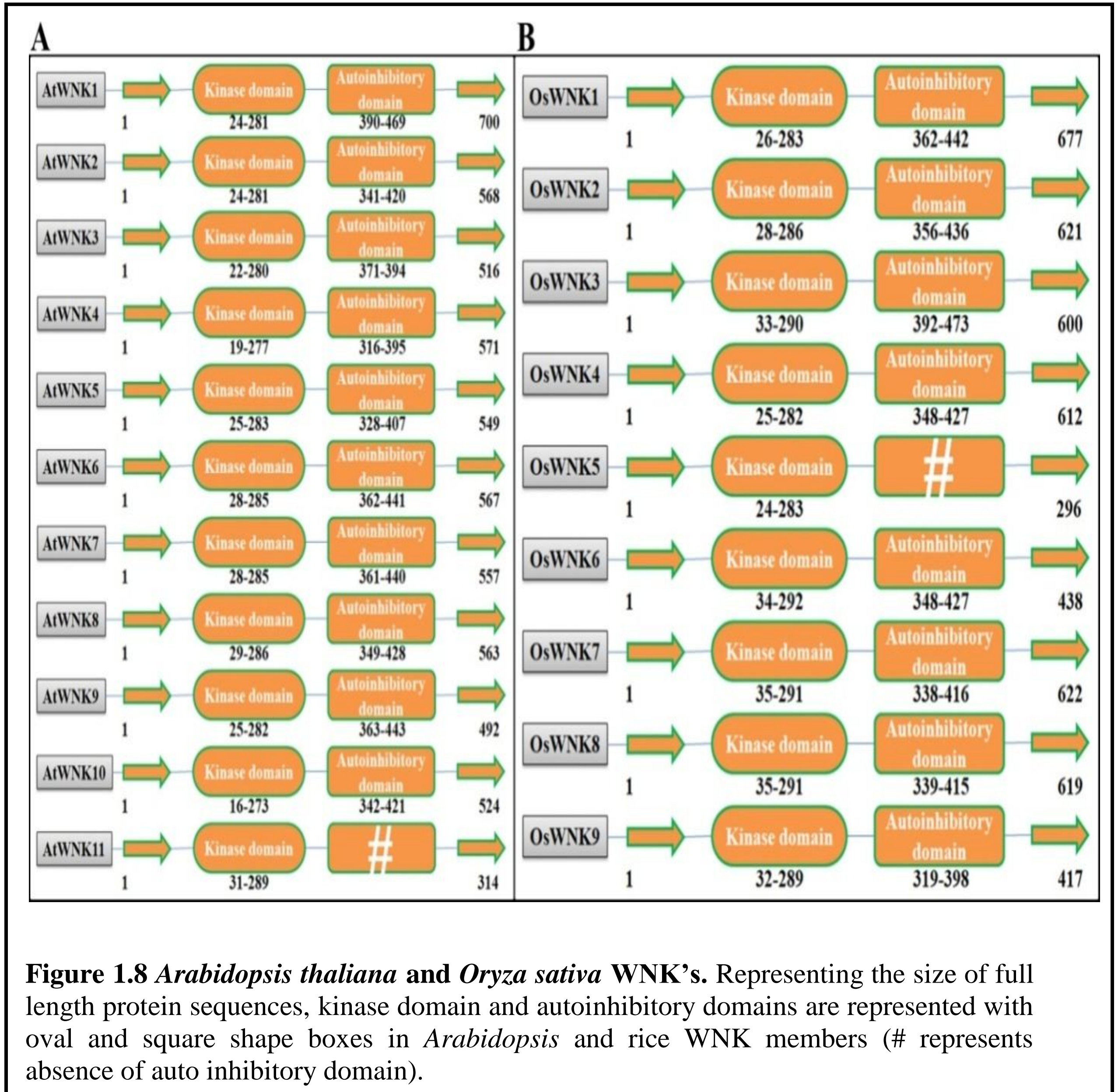


Figure 1.8 *Arabidopsis thaliana* and *Oryza sativa* WNK's. Representing the size of full length protein sequences, kinase domain and autoinhibitory domains are represented with oval and square shape boxes in *Arabidopsis* and rice WNK members (# represents absence of auto inhibitory domain).

Human WNK protein kinases are too long compared to plant WNK protein size. Human WNK ranges from 1243 to 2382 amino acids long while *Arabidopsis* WNK

ranged from 492 to 701 amino acids (Xu et al., 2000; Verissimo and Jordan, 2001; Wilson et al., 2001). However, rice WNKs ranged from 296-677 amino acids long as shown in the Fig. 1.8. Comparatively, C-terminal region of the human WNK kinases are most variable (Cao-Pham et al., 2018). WNK kinase activity is suppressed until appropriate signals induce the release of the inhibitory domain from its binding site.

1.4.2 Physiological function of WNKs

WNKs are involved in various physiological and patho-physiological conditions. Human *WNK1* or *WNK4* gene mutation causes an autosomal dominant syndrome of hypertension and hyperkalemia. Human WNKs are involved in the regulation of ion transport in kidneys and the maintenance of blood pressure and electrolyte homeostasis. Moreover, recent studies have shown that WNKs are involved in the regulation of vascular and neuronal development. Additionally, WNKs are also involved in cancer and the immune response.

Arabidopsis WNK1 regulates circadian rhythms through the phosphorylation of circadian clock component APRR3 (Nakamichi et al., 2002; Kojima et al., 2002). The circadian rhythms are controlled at the transcriptional level of *AtWNK1 AtWNK2, AtWNK4* and *AtWNK6* (Nakamichi et al., 2002). Besides circadian cycle, *AtWNK1* is involved in regulation of flowering time in *Arabidopsis,* which was demonstrated by *wnk1* knockout showing delayed flowering during long-day hours. Moreover, *AtWNK2, AtWNK5* and *AtWNK8* mediated regulation of flowering time, which was confirmed by T-DNA mutant analysis (Wang et al., 2008). AtWNK8 was shown to be involved in the regulation of ion transport through the interaction between C-terminal domain of

AtWNK8 and subunit C of H^+-ATPase (V-ATPase) (Hermesdorf et al., 2006). Further, MALDI-TOF MS analysis revealed that AtWNK8 was regulated by auto-phosphorylation and which in turn phosphorylated multiple sites of vacuolar H^+-ATPase subunit C. *AtWNK8* knockout significantly improved tolerance to salt and osmotic stresses by modulating proline content, catalase and peroxidase enzymes activity (Zhang et al., 2013). *AtWNK8* is the well characterized member of AtWNK family which functions in different processes such as floral transition, regulation of the intracellular signaling by G protein and accumulation of proline during osmotic stresses. *AtWNK2* and *AtWNK5* act as crucial negative regulator of the pathway for flowering and circadian clock through their inhibitory effects on ELF4 (Early flowering 4), TOC1 (Timing of CAB expression 1), FT expression (Flowering locus T) and CO (Constans) (Fig 1.9). AtWNK1 and AtWNK8 are involved in the coordination with AtRGS1 and leads to inter cellular signaling pathway. AtWNK1 promotes FT and CO and regulate circadian rhythms via APRR3 (*Arabidopsis* Pseudo-Response Regulator 3) (Nakamichi et al., 2002) (Fig. 1.9). *AtWNK9* showed role in the accumulation of proline under drought stress conditions (Xie et al., 2014).

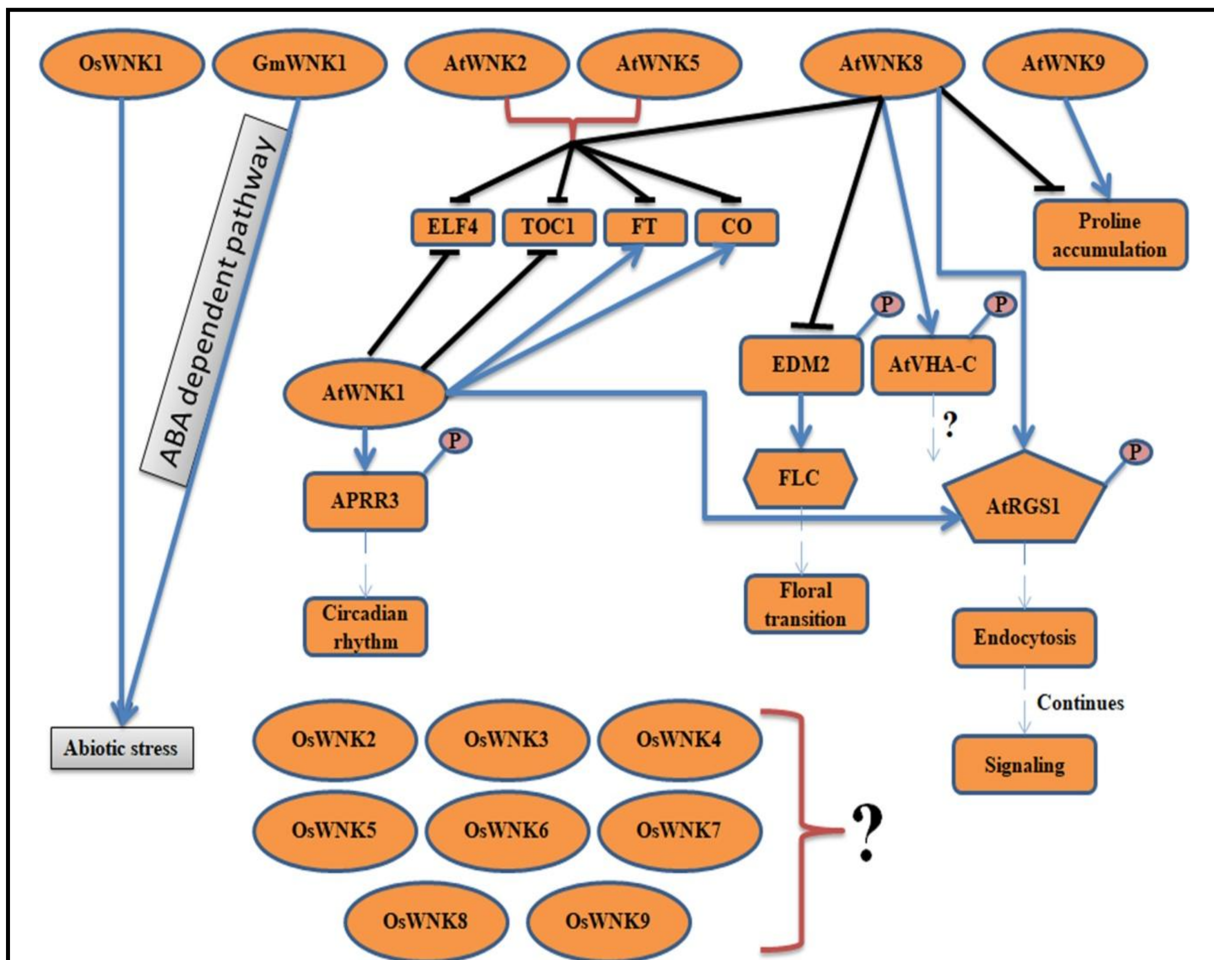


Figure 1.9 Overview of WNKs in plants. Role of *Arabidopsis thaliana* and *Oryza sativa* WNKs. The WNK kinases are oval shape, rectangular boxes representing the cellular processes. Black line represents inhibitory effects and blue line leads the activation. Question mark illustrates the functions of the various proteins and unknown outcomes. EDM2 (Enhanced Downy Mildew 2); AtVHA-C (Vacuolar H+ ATPase Subunit C) (Source: Adapted from Cao-pham et al., 2018).

AtWNK8 physically interact with the defence and developmental components of EDM2 (enhanced downy mildew 2) in nucleus and regulates floral transition by modulating expression of floral repressor gene FLC (Tsuchiya and Eulgem, 2010). In another study, the AtWNK8 protein was shown to interact with the receptor of activated C kinase1 (RACK1) scaffold protein and phosphorylated at Ser122 and Thr162 (Urano et al., 2015). *AtWNK9* overexpression in *Arabidopsis* showed enhanced drought tolerance

by positive regulation in ABA signaling cascade (Xie et al., 2014). Soybean root specific protein kinase, *GmWNK1* was functionally characterized and demonstrated that it interacted with ABA 8'-hydroxylase protein. Further, it was demonstrated that *GmWNK1* is involved in the regulation of stress response through ABA-dependent pathway in the root system (Wang et al., 2010). Over-expression study of *GmWNK1* in *A. thaliana* confirmed that it confers tolerance to salt and osmotic stresses (Wang et al., 2011). The rice *OsWNK1* transcript analysis suggested that it may be involved in the regulation of circadian cycle and probably in abiotic stresses (Table 1.3) (Kumar et al., 2011). A significant transcript regulation of rice WNK members was observed when the rice seedlings were subjected to potassium (K^+) deficiency (Ma et al., 2012).

Studying pathways associated with WNK will lead to novel findings on mechanism under various abiotic stress conditions, as this kinase family is thus far uncharacterized. The *GmWNK1* is having a role in salt and osmotic stress but the signaling pathway is yet unknown. The *OsWNK1* transcript analysis suggests that the rice WNK family might have roles in abiotic stresses. Further research into rice WNKs may reveal their probable roles and mechanism under abiotic stress conditions.

Table 1.3 The functional roles of WNK genes reported in different plants.

S.no	Gene name	Plant species	Role of the gene	References
1	*AtWNK9*	*Arabidopsis thaliana*	Role under drought conditions by positive response in ABA signaling cascade	Xie et al., 2014
2	*AtWNK8*	*Arabidopsis thaliana*	Role under salt and osmotic stress by knockout the gene	Zhang et al., 2013
3	*AtWNK2, AtWNK5* and *AtWNK8*	*Arabidopsis thaliana*	Role in the regulation of flowering time	Wang et al., 2008
4	*AtWNK2, AtWNK4* and *AtWNK6*	*Arabidopsis thaliana*	Role in circadian rhythms	Nakamichi et al., 2002
5	*OsWNK1*	*Oryza sativa*	Regulation of circadian cycle and probably in abiotic stresses	Kumar et al., 2011
6	*GmWNK1*	*Glycine max*	Altered sensitivity to salt and osmotic stress	Wang et al., 2011
7	*GmWNK1*	*Glycine max*	Regulates stress-responsive ABA signaling on root system architecture	Wang et al., 2010

1.5 Gaps in existing research

1) The role of WNKs is poorly understood in plants.

2) Functional characterization of *OsWNK* genes in abiotic stress is yet to be explored.

3) No published reports on functional and molecular characterization of *OsWNK* gene family.

1.6 To address the aforementioned gaps the following objectives were framed

1) Genome-wide identification and expression analysis of WNK kinase gene family in rice.

2) Functional characterization of the identified rice *OsWNK* in planta.

3) Localization studies of the identified *OsWNK* in planta.

Chapter 2

Genome-wide identification and expression analysis of WNK kinase gene family in rice

2.1 Introduction

Protein kinases are one of the largest superfamilies, broadly classified into nine groups, 134 families and 196 subfamilies in eukaryotes (Manning et al., 2002). They are capable of transferring gamma phosphate group of adenosine triphosphate (ATP) to phosphorylate serine, threonine or tyrosine residues of protein (Hanks and Hunter, 1995). They are essential regulator of various cellular processes such as development, cell cycle and signal transduction. The catalytic domain of protein kinases are 250–300 amino acid residues in length and demarcate as 12 conserved regions, referred to as subdomains (Hanks and Quinn, 1991). With No lysine (WNK) kinase, a subfamily of serine/threonine protein kinases related to the STE20/PAK-like family is found in higher organisms (Xu et al., 2002). A unique feature of WNK subfamily is marked by the absence of catalytic lysine (K) residue in kinase subdomain II, which is essential for the coordination of ATP in the active center and conserved among all other kinases (Xu et al., 2000; Wilson et al., 2001; Xu et al., 2002). In contrast, the catalytic lysine of WNK kinases is shifted towards sub-domain I and replaced by cysteine (C) residue in human. The WNK homologues have been recorded in various eukaryotes except yeast and it is proposed that they may be restricted to multicellular organisms (Verissimo and Jordon, 2001). They are involved in diverse physiological function such as regulation of ion balance, cell signaling, proliferation and organ development in animals. In plants, WNK members are involved in various physiological processes, such as regulation of flowering time by modulating the photoperiod and maintaining circadian cycle (Kahle et al., 2006; Uchida et al., 2014).

In the present study, putative WNK homologues were identified and confirmed by absence of catalytic lysine (K) residue in the kinase subdomain II which is replaced by

asparagine/serine/glycine residue. The current study was mainly focused on rice WNK family, consisting of nine putative members distributed on 5 different chromosomes. Furthermore, we analyzed protein domain, phylogenetic relationship and gene structure of *OsWNK*. Moreover, we performed tissue specific gene expression patterns of rice *OsWNK* genes under the normal growth condition and against various abiotic stresses (drought, salt, heat and cold). It underscored functional importance of OsWNK in the different tissues and might be involved in regulation of various abiotic stresses.

2.2 Materials and methods

2.2.1 Database search and retrieval of WNK sequences

With No lysine (WNK) kinase protein sequences were obtained from the NCBI (www.ncbi.nlm.nih.gov/) protein database with WNK protein as the query search. The search for plant WNK gene was carried out using BLASTP and 50% identity was taken as the threshold (Altschul et al., 1990). The obtained sequences were searched in various plant genome databases such as TAIR (http://www.*arabidopsis*.org/), NCBI (www.ncbi.nlm.nih.gov/), Gramene (http://www.gramene.org/), rap-db (http://rapdb.dna.affrc.go.jp/), TIGR (http://rice.plantbiology.msu.edu/), Phytozome (http://www.phytozome.net/) and *Medicago* genome project (http://www.jevi.org/medicago). 155 WNK sequences from various plant species *viz*. *Cyanidioschyzon merolae, Chlamydomonas reinhardtii, Physcomitrella patens, Amborella trichopoda, Selaginella moellendorffi, Brachypodium distachyon, Sorghum bicolor, Zea mays, Oryza sativa, Hordeum vulgare, A. thaliana, Medicago truncatula, Solanum lycopersicum, Populus trichocarpa, Brassica rapa* and *Glycine max* were

retrieved. All WNK sequences were aligned and HMM profile was constructed (Siever et al., 2011). Non redundant protein database was searched using HMMER 3.0 (hmmer.janelia.org) (Finn, et al., 2011). HMMER and BLAST hits were compared and manually edited to avoid redundant sequences. Plant WNKs were aligned and submitted to weblogo, which is a web based tool to make of sequence logos (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004).

2.2.2 Phylogenetic analysis

All WNK amino acid sequences were aligned using Clustal W (multiple sequence alignment) tool and a phylogenetic tree was constructed by neighbor joining (NJ) method. The NJ tree with following parameters: p-distance, pairwise deletion, and bootstrap analysis with 1000 replicates. Further tree were constructed using MEGA 6.06 tool (Tamura et al., 2013).

2.2.3 OsWNK sequence analysis and annotation

Rice OsWNK amino acid sequences were analyzed for the presence of protein kinase domain using Interproscan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan/), Prosite (http://prosite.expasy.org/) and SMART databases (Schultz et al., 2000; Zdobnov and Apweiler, 2001; Sigrist et al., 2012). Physiochemical data were generated from the ExPASy ProtParam server (http://web.expasy.org/protparam/) including sequence length, molecular weight and theoretical isoelectric point (pI) values (Gasteiger et al., 2005). Protein subcellular localization was predicted by LOCTREE3 and plant mPLoc predictive system (https://www.rostlab.org/services/loctree3) (Chou and Shen, 2010;

Goldberg et al., 2014). Gene structure, exons and introns were obtained by comparing open reading frame (ORF) and genomic sequences. Gene structures were displayed using a GSDS 2.0 (http://gsds.cbi.pku.edu.cn) (Guo et al., 2007).

2.2.4 *In-silico* analysis of *cis*-elements of OsWNK promoter region

The promoter regions of OsWNK (−1500 to −1 bp) were retrieved from NCBI *O. sativa* Genome map viewer database. Sequence analysis was performed using different databases including PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot et al., 2002). The *cis*-acting elements were searched in both forward and reverse strands. Eight *cis*-elements were predicted in this study: dehydration responsive element (MBS:TAACTG), abscisic acid responsive element (ABRE:TACGTG), circadian control responsive element (circadian: CAANNNNATC), light responsive element (G-box: CACGTG), heat shock responsive element (HSE: AAAAAATTTC), auxin responsive element (TGA: AACGAC), ethylene responsive element (ERE: ATTTCAAA), anaerobic induction regulatory element (ARE: TGGTTT).

2.2.5 Plant material and stress treatment

Rice seeds (*O. sativa* ssp. Indica cultivar IR64) were surface sterilized with 5% sodium hypochlorite and imbibed in water in the dark at 28 °C for 2 days. Germinated seeds were planted in plastic trays and grown hydroponically in Hoagland solution with a 16 h light/8 h dark cycle. After three weeks, the seedlings were subjected to the abiotic stress treatments. For salt and drought treatments, seedlings were treated with Hoagland

solution containing 200 mM NaCl and 20% PEG-6000 respectively. For cold and heat shock treatments, seedlings were exposed to 4 °C and 45 °C respectively. After stress treatment, samples were collected separately at 0, 0.5, 3, 6 and 12 h intervals. For tissue specific expression young leaf, flag leaf, culm, panicle and roots were collected from pot grown rice plants under controlled stress free condition. Three replicates for each experiment were performed in this study. After harvest samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2.6 Quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the RNeasy plant mini-kit (Qiagen) and quantified by nanodrop (Thermo scientific). Specific primer pairs were designed for all the nine *OsWNK* genes (Table 2.1) and their specificities were confirmed by running the RT-PCR product in agarose gel, before they were used for qRT-PCR analysis. The amplicon size of each of them was around 200 bp. qRT-PCR was performed in an eppendorf realplex 2.2 PCR system (Eppendrof) using SYBR Green master mix (DyNAmo ColorFlash SYBR Green qPCR kit F-416L, Thermo scientific). The dissociation curve was used to detect the presence of primer dimer or other nonspecific amplified products that would produce a detectable CT value and negatively skew the results. Eukaryotic elongation factor (*eEF1*α) was used as housekeeping genes for normalization. The relative expression change of OsWNK in response to abiotic stresses and tissue specific expression was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2.1: List of oligonucleotide primers used for qRT-PCR experiments.

S.no	Primer label	Primer sequence 5'-3'
1	OsWNK1-F OsWNK1-R	AGATGATTGATGGCGAGGTTAG GATCCGCAGTTATGGCAGTAT
2	OsWNK2-F OsWNK2-R	TCTGAGTTGCGTAATGAGCC AAAAGACTGAGGACTGGTGC
3	OsWNK3-F OsWNK3-R	GAGTCATCTCCGGGATCAAG TAGTAGCCAACGCCAAAACC
4	OsWNK4-F OsWNK4-R	GATAAGGTGGACGATGCTGAG GACGATACTGCCTGAGACTTC
5	OsWNK5-F OsWNK5-R	GCAGCAACGTCTTCATCAAC CTGTGGTAGATCTGCACGAC
6	OsWNK6-F OsWNK6-R	ATGGGACAGTCAATGGGAAG CAAGCTGCTCAACCATTTCA
7	OsWNK7-F OsWNK7-R	CATCAAAGTGAACATGCCCTTAAT GTCTCATGGCGAAACTCTCAT
8	OsWNK8-F OsWNK8-R	CGATCAGATGCAGAGTACGTAAG ACACTCATATATGCACTTCTCTCC
9	OsWNK9-F OsWNK9-R	AACGGTCTACAAAGCCTTCG TTCTGCGGTACTGTGTCAAG
10	eEF1 alpha-F eEF1 alpha-R	AGCGTGTGATTGAGAGGTTC AGATACCAGCCTCAAAACCAC

Rice qRT-PCR tissue specific and abiotic stress data were used for heatmaps construction using CIMminer online tool (http://discover.nci.nih.gov/cimminer) (Weinstein et al., 1994). We retrieved *A. thaliana* microarray expression values from AtGenExpress visualization tool database using respective gene IDs (http://jsp.weigelworld.org/expviz/expviz.jsp). The heatmaps were generated for tissue specific and abiotic stress values using CIMminer tool.

2.3 Results

2.3.1 Identification of WNK gene family across the plant kingdom

In the present study, sixteen plant genome databases belonging to algae, bryophyte, lycophytes and angiosperms were searched and analyzed for distribution of WNK

homologues. 155 non redundant plant WNK sequences were retrieved by performing BLASTP (Fig. 2.1). The WNK genes were identified and confirmed on the basis of the replacement of catalytic lysine residue with either asparagine (N)/ serine (S) or glycine (G) residues in plant (Fig. 2.2). Sequence logo was developed for WNK kinase subdomain I and II showing highly conserved protein kinase subdomain. The catalytic lysine (K) residue of subdomain II replaced by either asparagine (N) or serine (S) at position 30 and shifted to subdomain I at position 13 (Fig. 2.2).

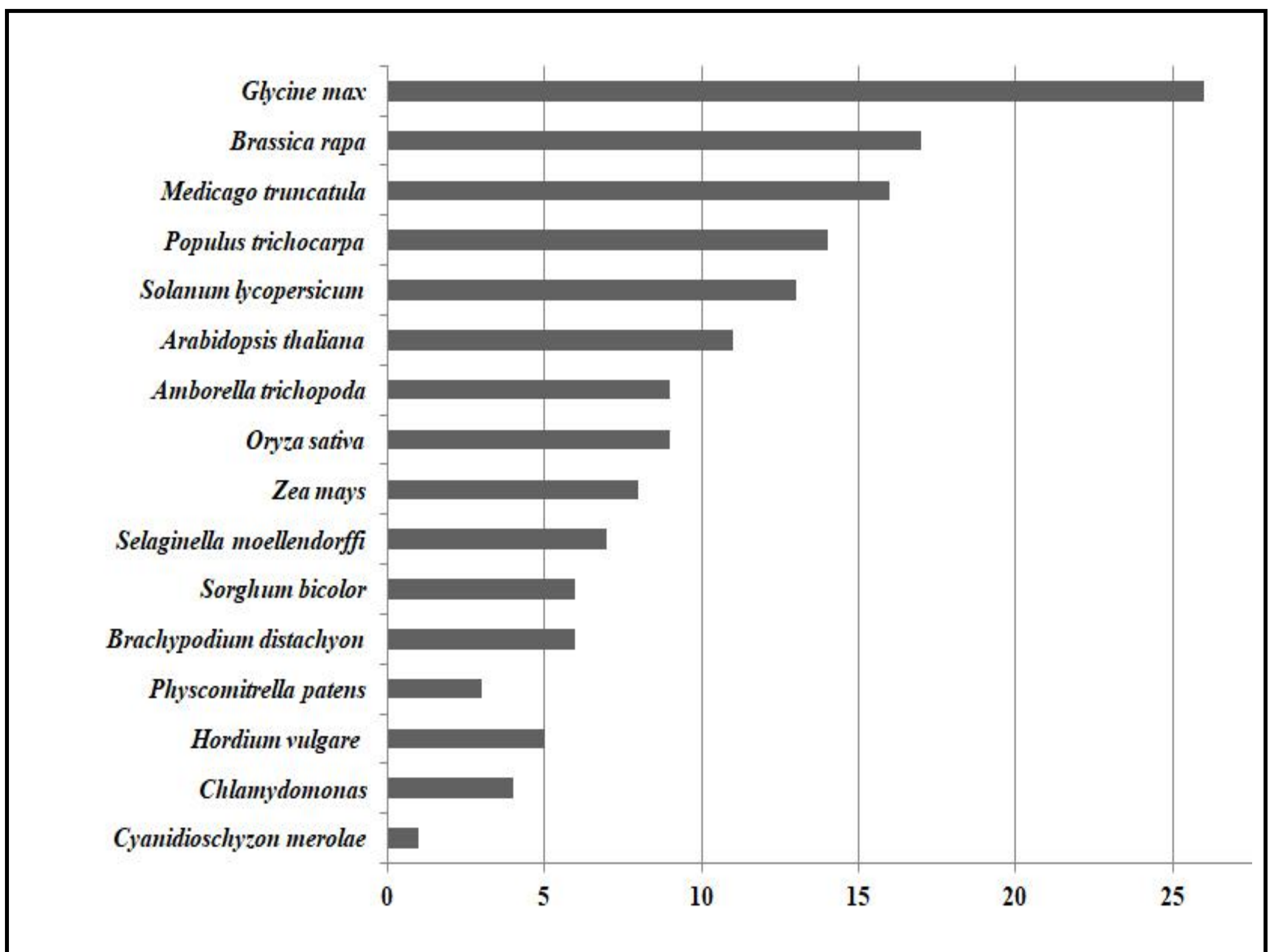


Figure 2.1 Distribution of WNK homologs in planta. Total of 155 WNK homologs retrieved by performing blast from different plant species and represented according to the number of WNK homologs present in each individual plant.

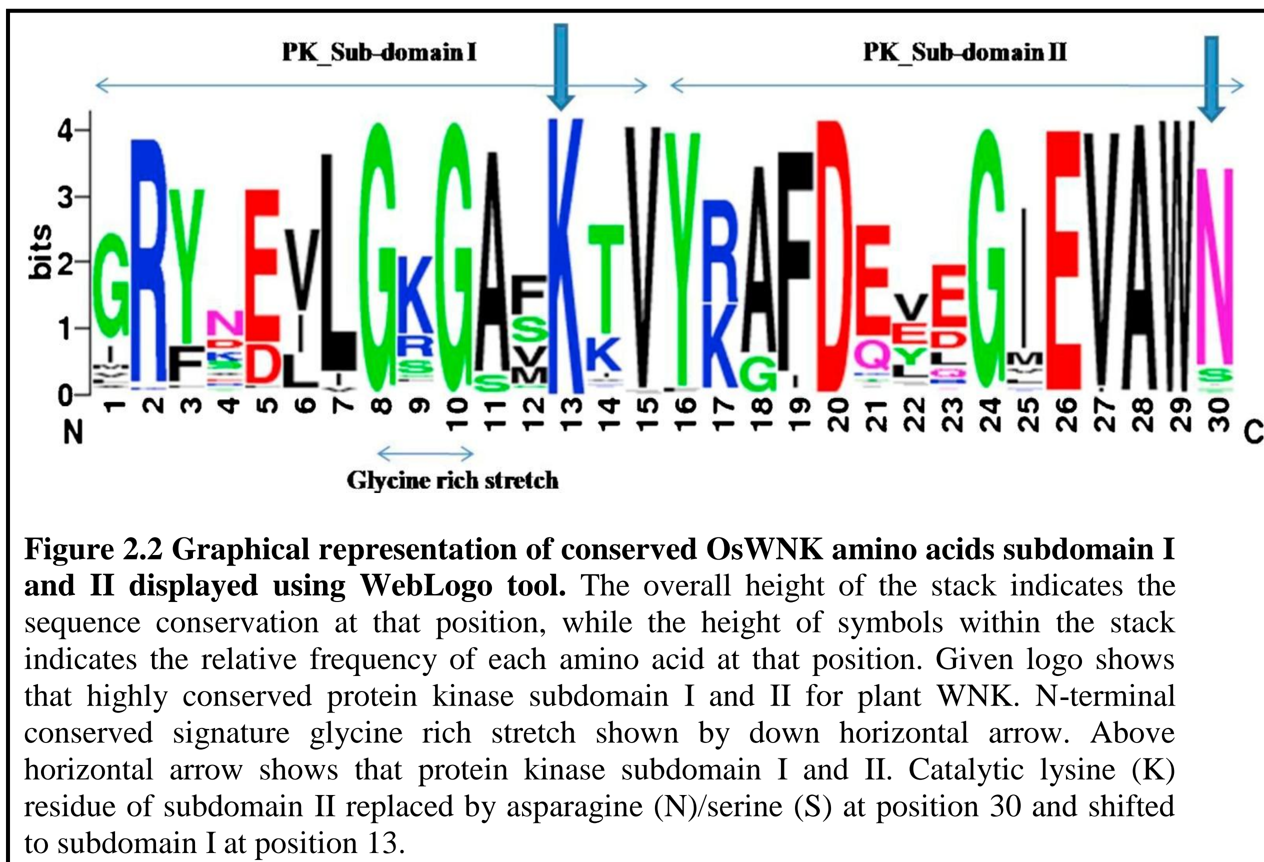


Figure 2.2 Graphical representation of conserved OsWNK amino acids subdomain I and II displayed using WebLogo tool. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. Given logo shows that highly conserved protein kinase subdomain I and II for plant WNK. N-terminal conserved signature glycine rich stretch shown by down horizontal arrow. Above horizontal arrow shows that protein kinase subdomain I and II. Catalytic lysine (K) residue of subdomain II replaced by asparagine (N)/serine (S) at position 30 and shifted to subdomain I at position 13.

2.3.2 Phylogenetic analysis

Phylogenetic relationship among all plant WNKs were demonstrated using neighbor joining method. According to NJ all plant WNKs were clustered into 5 different clades (Class I–V) (Fig. 2.3). Clade I and clade II comprised of three members each belonging to *Physcomitrella*, *Cyanidioschyzon* and *Chlamydomonas*. Similarly clade III has 54 members with further subdivision into 2 subclades. Subclade IIIA has 27 members with OsWNK7 and OsWNK8, while subclade IIIB has 27 members with OsWNK5. Clade IV is subdivided into two subclades, subclade IVA and subclade IVB with 22 and 16 members respectively. OsWNK4 is a member of subclade IVA, however OsWNK6 and OsWNK9 are members of subclade IVB. Clade V is divided into two subclades, subclade

VA and subclade VB. OsWNK2 is the member of subclade VA, while OsWNK1 and OsWNK3 are members of subclade VB. All rice OsWNKs belongs to clades III, IV and V (Fig. 2.3).

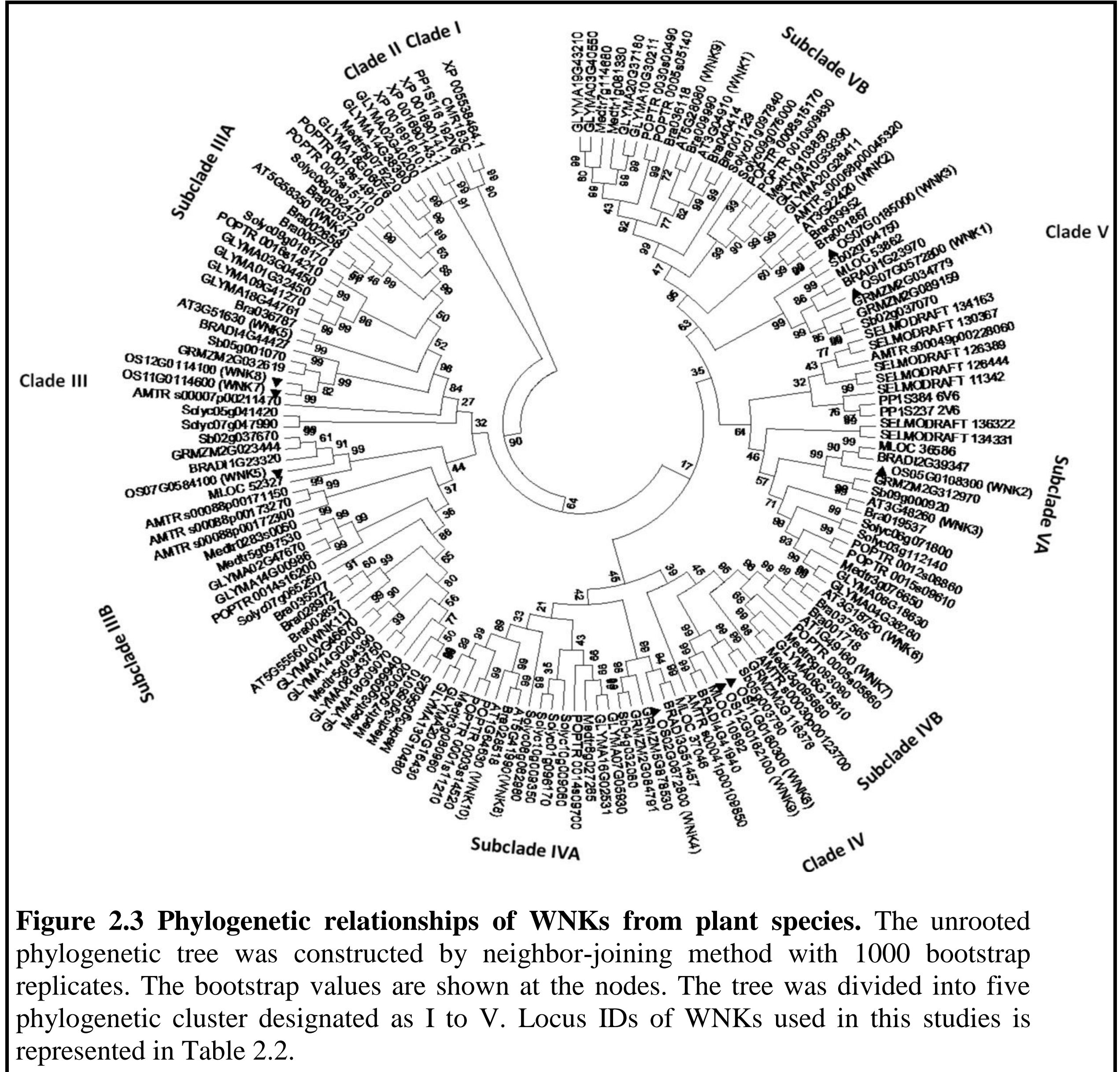


Figure 2.3 Phylogenetic relationships of WNKs from plant species. The unrooted phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates. The bootstrap values are shown at the nodes. The tree was divided into five phylogenetic cluster designated as I to V. Locus IDs of WNKs used in this studies is represented in Table 2.2.

Table 2.2 List of locus IDs of all WNK genes represented in Fig. 2.3.

S.No	Plant species	Locus IDs
1	*Oryza sativa*	Os02G0672800, Os11G0114600, Os12G0114100, Os11G0160300, Os07G0584100,Os12G0162100, Os07G0572800, Os05G0108300, Os07G0185000
2	*Solanum lycopersicum*	Solyc01g096170, Solyc10g009350, Solyc10g009060, Solyc08g082980, Solyc05g041420, Solyc09g018170, Solyc06g082470, Solyc06g071800, Solyc01g097840, Solyc09g076000, Solyc03g112140, Solyc07g047990, Solyc07g065250
3	*Zea mays*	GRMZM2G084791, GRMZM2G023444, GRMZM2G032619, GRMZM2G116376,

		GRMZM2G312970, GRMZM2G034779, GRMZM2G089159, GRMZM5G878530
4	*Glycine max*	GLYMA07G05930, GLYMA16G02531, GLYMA13G10480, GLYMA01G32450, GLYMA03G04450, GLYMA18G44761, GLYMA09G41270, GLYMA06G15610, GLYMA18G06076, GLYMA14G38390, GLYMA02G40200, GLYMA06G18630, GLYMA19G43210, GLYMA10G39390, GLYMA20G28411, GLYMA04G36260, GLYMA03G40550, GLYMA20G37180, GLYMA10G30211, GLYMA20G16430, GLYMA18G09070, GLYMA08G43750, GLYMA14G00986, GLYMA14G02000, GLYMA02G46670, GLYMA02G47670
5	*Brassica rapa*	Bra028518, Bra020372, Bra002658, Bra006771, Bra036787, Bra037565, Bra039952,Bra040414, Bra009990, Bra036118, Bra001129, Bra019537, Bra001867, Bra002658, Bra002897, Bra028972, Bra035577
6	*Sorghum bicolor*	Sb04g032080, Sb02g037670, Sb05g003790, Sb05g001070, Sb09g000920, Sb02g037070, Sb02g004750) *Hordeum vulgare* (MLOC_10892, MLOC_37046, MLOC_52327, MLOC_53862, MLOC_36586
7	*Populus trichocarpa*	POPTR_0030s00490, POPTR_0005s05140, POPTR_0008s15170, POPTR_0010s09830, POPTR_0016s14210, POPTR_0013s15110, POPTR_0019s14910, POPTR_0012s08860, POPTR_0015s09610, POPTR_0014s09700, POPTR_0003s14520, POPTR_0001s11210, POPTR_0005s05860, POPTR_0014s16200
8	*Medicago truncatula*	Medtr3g076650.1, Medtr7g114680.1, Medtr1g103850, Medtr1g081330.1, Medtr8g093090.1, Medtr8g027265.1, Medtr3g080960.1, Medtr3g095680.1, Medtr5g075220.1, Medtr5g094390.1, Medtr3g099940.1, Medtr5g097530.2, Medtr7g029020.1, Medtr3g056025.1, Medtr0283s0050.1, Medtr3g056010.1
9	*Arabidopsis thaliana*	At1G64630, At5G41990, At1G49160, At3G18750, At3G51630, At5G58350, At3G48260, At3G04910, At3G22420, At5G28080, At5G5556
10	*Cyanidioschyzon merolae*	XP_005538464.1, XP_001690143.1, XP_001690141.1, XP_001691610.1
11	*Physcomitrella patens*	PP1S116_192V6, PP1S384_6V6, PP1S237_2V6
12	*Selaginella moellendorffi*	SELMODRAFT_134163, SELMODRAFT_130367, SELMODRAFT_136322, SELMODRAFT_134331, SELMODRAFT_11342, SELMODRAFT_126389, SELMODRAFT_126444
13	*Amborella trichopoda*	AMTR_s00041p00109850, AMTR_s00007p00211470, AMTR_s00030p00123700, AMTR_s00049p00228060, AMTR_s00088p00173270, AMTR_s00088p00171150, AMTR_s00088p00172300, AMTR_s00068p00045320
14	*Brachypodium distachyon*	BRADI4G41940, BRADI3G51457, BRADI4G44427, BRADI2G39347, BRADI1G23320, BRADI1G23970
15	*Hordeum vulgare*	MLOC_10892, MLOC_37046, MLOC_52327, MLOC_53862, MLOC_36586

2.3.3 Rice WNK annotation and analysis

Genome wide analysis of rice revealed 9 members of WNK gene family (designated as OsWNK1–OsWNK9). The summary of OsWNK genes, such as: names, locus IDs, ORF lengths, AA length, nucleotide length, locations on chromosome and basic

physiochemical parameters of putative protein, were recorded. Nucleotide length range between (984–2115 bp), protein length (328–705 AA), molecular weight (36,623.5–79,820.5 g/mol) and isoelectric point (pI) (4.64–6.81) were observed (Table 2.3).

Table 2.3 Physicochemical characteristics of OsWNK genes. Abbreviations used in the table. CDS-coding sequences, AA-amino acid, bp-base pair, MW-molecular weight, g/mol-gram/mole, pI-isoelectric point, C-cytoplasm, N-nucleus.

OsWNK	**CDS (5'-3') Coordinates**	**Nt bp**	**AA length**	**MW (g/mol)**	**pI**	**Subcellular Localization**	**Description**
OsWNK 1	07/23145979 -23149609	2115	705	79,820.5	5.47	C, N	Uncharacterized, Protein kinase-like
OsWNK 2	05/471400-476283	1866	622	70,101.4	4.78	C, N	Uncharacterized, Protein kinase-like
OsWNK 3	07/4501918-4498924	1806	602	65,437.6	4.64	C, N	Uncharacterized, Protein kinase-like
OsWNK 4	02/27376763 -27372028	1839	613	68,709.7	4.69	C, N	Uncharacterized, Protein kinase-like
OsWNK 5	07/23677863 -23679948	984	328	36,623.5	6.39	C, N	Uncharacterized, Protein kinase-like
OsWNK 6	11/2929173-2925595	1320	440	49,676.1	6.13	C, N	Uncharacterized, Protein kinase-like
OsWNK 7	11/661236-654974	1869	623	68,663.6	6.81	C, N	Uncharacterized, Protein kinase-like
OsWNK 8	12/705258-698936	1860	620	68,517.4	6.43	C, N	Uncharacterized, Protein kinase-like
OsWNK 9	12/3131907-3128498	1254	418	47,055.1	5.05	C, N	Uncharacterized, Protein kinase-like

Chromosomal distribution and locations were determined for OsWNK, based on their mapped coordinates from the rice genomic database (http://rice.plantbiology.msu.edu/). Rice WNKs were distributed on the chromosome no.

2, 5, 7, 11 and 12. *OsWNK1* (Os07g0572800), *OsWNK3* (Os07g0185000) and *OsWNK5* (Os07g0584100) genes were located on chromosome number 7; *OsWNK6* (Os11G0160300) and *OsWNK7* (Os11g0114600) were present on chromosome number 11; *OsWNK8* (Os12g0114100) and *OsWNK9* (Os12g0162100) were found to be present on chromosome number 12 whereas *OsWNK2* (Os05g0108300) and *OsWNK4* (Os02g0672800) genes were located on chromosome number 5 and 2, respectively as shown (Fig. 2.4).

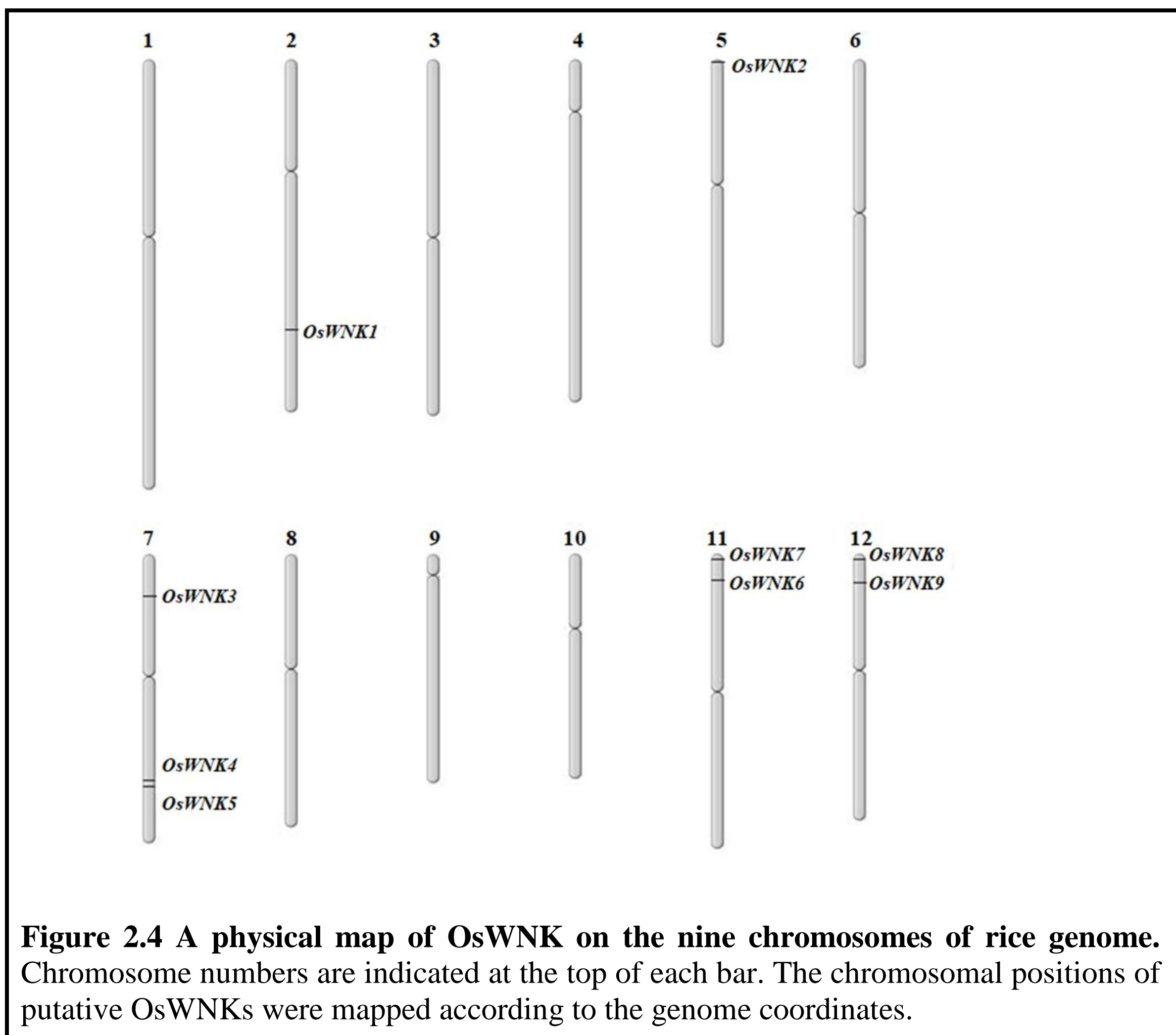


Figure 2.4 A physical map of OsWNK on the nine chromosomes of rice genome. Chromosome numbers are indicated at the top of each bar. The chromosomal positions of putative OsWNKs were mapped according to the genome coordinates.

Similarly, we have observed 3 pairs of OsWNK proteins with high levels of protein sequence similarity. The entire protein sequences of OsWNK7 and OsWNK8

shared 92.6%, OsWNK6 and OsWNK9 shared 72% while OsWNK1 and OsWNK3 shared 52% sequence resemblance (Fig. 2.5A). All OsWNK have introns in their genomic structure and the number of exons varied from 2 to 9, while highest number of introns and exons were observed in OsWNK2 (Fig. 2.5B). The super family database of structural and functional protein annotations showed that protein kinase domain is found in all rice OsWNK sequences. The Prosite analysis confirmed the presence of protein kinase domain in the identified OsWNK.

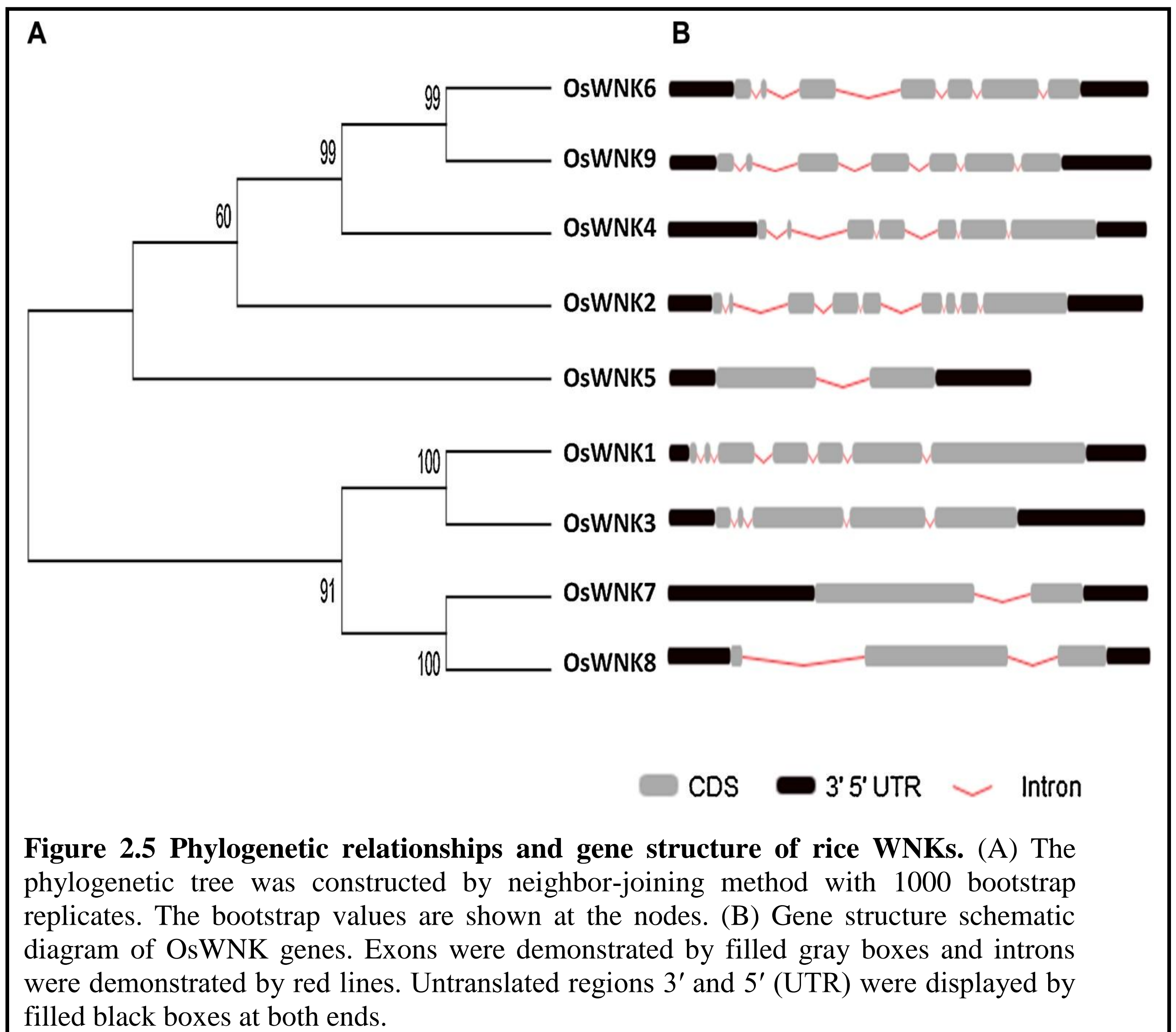


Figure 2.5 Phylogenetic relationships and gene structure of rice WNKs. (A) The phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replicates. The bootstrap values are shown at the nodes. (B) Gene structure schematic diagram of OsWNK genes. Exons were demonstrated by filled gray boxes and introns were demonstrated by red lines. Untranslated regions 3′ and 5′ (UTR) were displayed by filled black boxes at both ends.

2.3.4 *Cis*-acting regulatory elements present in the promoter region of OsWNK

The putative *cis*-acting elements were predicted from 1500 bp upstream promoter region of OsWNKs. Sequence analysis from PlantCARE revealed that the promoter of each gene contained important putative *cis*-acting elements. The predicted *cis*-acting elements are as follows: ABA responsive element (ABRE), environmental signal response (G-box), dehydration responsive elements (MBS), heat shock element (HSEs), ethylene responsive elements (ERE), anaerobic induction regulatory element (ARE), auxin-responsive element (TGA) and circadian regulatory elements (CAANNNNATC). The dehydration responsive element (MBS) was predicted in 6 out of 9 *OsWNKs*. The ABREs element was observed in upstream of all *OsWNKs* except *OsWNK3* and *OsWNK7*. The heat shock element (HSEs) was observed in the promoters of *OsWNK1*, *OsWNK7* and *OsWNK9*. The ERE element is distributed in *OsWNK2*, *OsWNK4* and *OsWNK7*, similarly ARE was recorded in the *OsWNK1, OsWNK2, OsWNK3, OsWNK6, OsWNK8* and *OsWNK9* promoter region (Fig. 2.6). The distribution of TGA, were predicted in the *OSWNK1, OsWNK2, OsWNK4* and *OsWNK5*. The circadian regulatory element was predicted in the *OsWNK2, OsWNK4, OsWNK5, OsWNK7* and *OsWNK9* which may regulate biological circadian mechanism. An environmental signal response element (G-box) was observed in the almost all OsWNK except *OsWNK3* (Fig. 2.6).

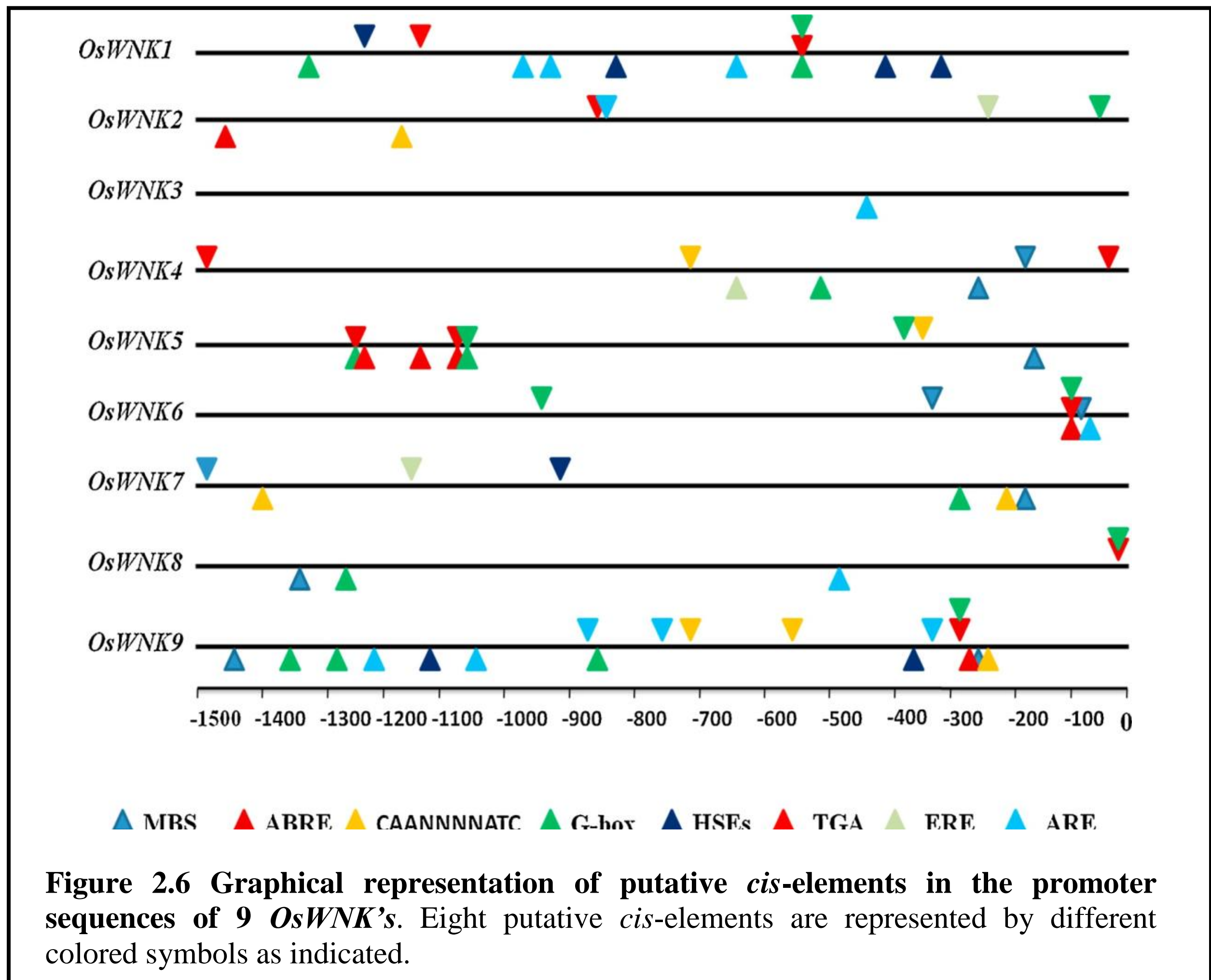


Figure 2.6 Graphical representation of putative *cis*-elements in the promoter sequences of 9 *OsWNK's*. Eight putative *cis*-elements are represented by different colored symbols as indicated.

2.3.5 Tissue specific and abiotic stress responsive expression pattern of *OsWNK* gene family

Analysis of *OsWNK* gene expression patterns in different tissues provided us new insights into their role in several organs in rice. The *OsWNK* transcript level was monitored using qRT-PCR in different organs *viz.* young leaf, root, flag leaf, culm and panicle. *OsWNK5* and *OsWNK6* showed higher (more than 10 fold) expression in root tissues in contrast to other organs of rice plants. Moreover, *OsWNK8* also showed higher transcript expression in young leaf, while *OsWNK6* expression was low. The quantitative analysis of tissue specific expression revealed that most of the *OsWNK* exhibit higher

transcript expression level in roots as compared with young leaf, flag leaf, panicle and culm (Fig. 2.7). Our results indicate that *OsWNK* might have a major role in the root formation and architecture.

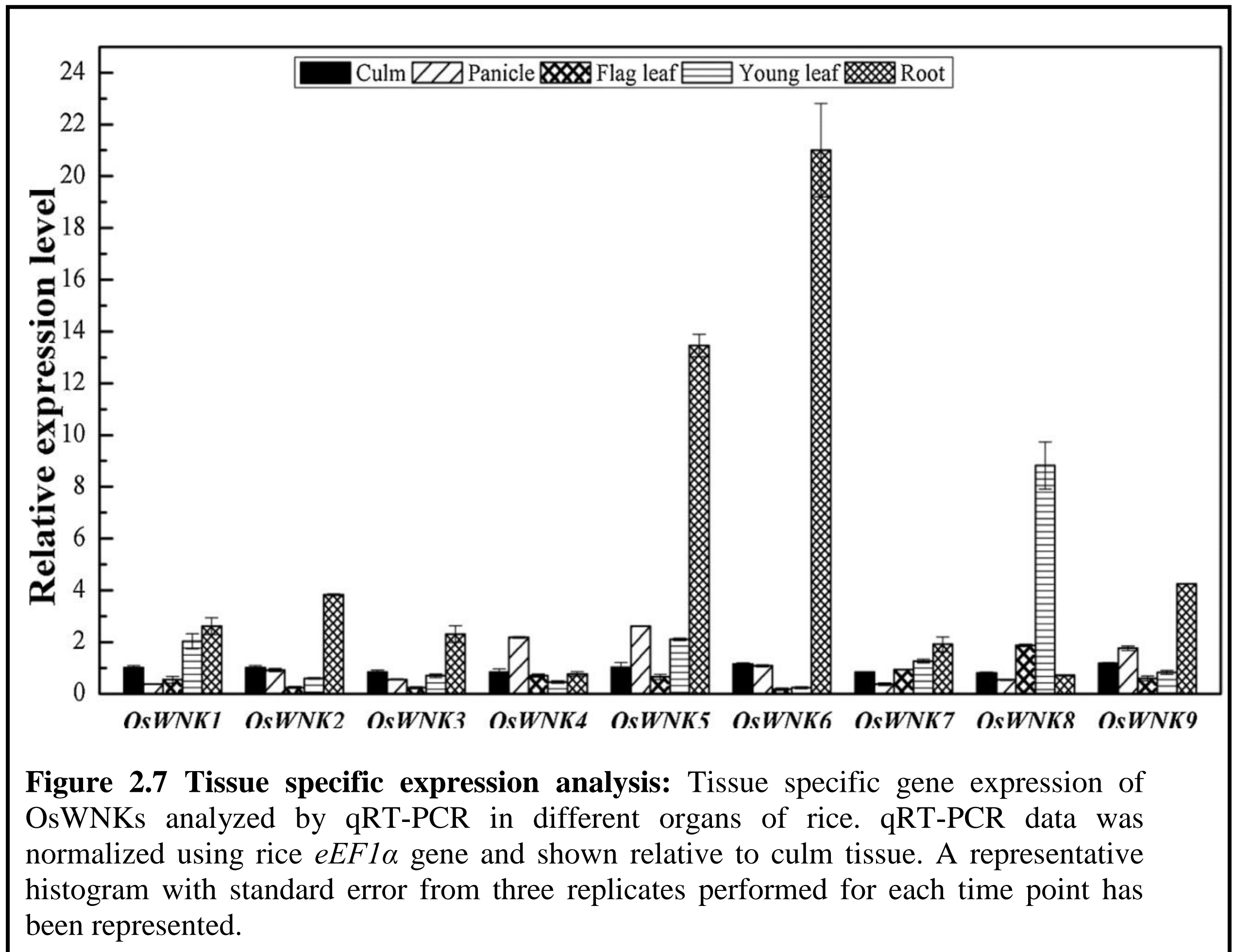


Figure 2.7 Tissue specific expression analysis: Tissue specific gene expression of OsWNKs analyzed by qRT-PCR in different organs of rice. qRT-PCR data was normalized using rice *eEF1α* gene and shown relative to culm tissue. A representative histogram with standard error from three replicates performed for each time point has been represented.

To understand the *OsWNK* response and regulation under the abiotic stresses, qRT-PCR was performed. The *OsWNK* transcript accumulations were confirmed in different abiotic stresses such as salt, drought, heat and cold. Under salt stress, transcript level of *OsWNK3, OsWNK5, OsWNK6* and *OsWNK7* exhibited significant differential expression patterns. *OsWNK9* displayed lower expression pattern during salt stress. The transcript regulation pattern indicated that *OsWNK3, OsWNK5, OsWNK6* and *OsWNK7*

might be involved in the salt stress regulation (Fig. 2.8A). Under drought stress, *OsWNK1, OsWNK2, OsWNK6* and *OsWNK8* showed higher transcript accumulation at 12 h (Fig. 2.8B). During cold stress *OsWNK6* showed higher transcript accumulation at 3 h, while *OsWNK1*, *OsWNK2*, *OsWNK6*, *OsWNK8*, *OsWNK9* exhibited down regulation at 12 h intervals. *OsWNK9* showed the down regulation pattern (Fig. 2.8C). In heat stress *OsWNK2* was up regulated to 5.1 fold at 12 hr. *OsWNK6* showed 10.5 fold up regulation at 6 h and highly up regulated to 40 fold at 12 h of heat stress. *OsWNK7* and *OsWNK8* showed very high (more than 15 times) up regulation pattern at 0.5 and 12 h of heat stress. (Fig. 2.8D). The higher transcript accumulation of *OsWNK6*, *OsWNK7*, and *OsWNK8* revealed their probable role during heat stress.

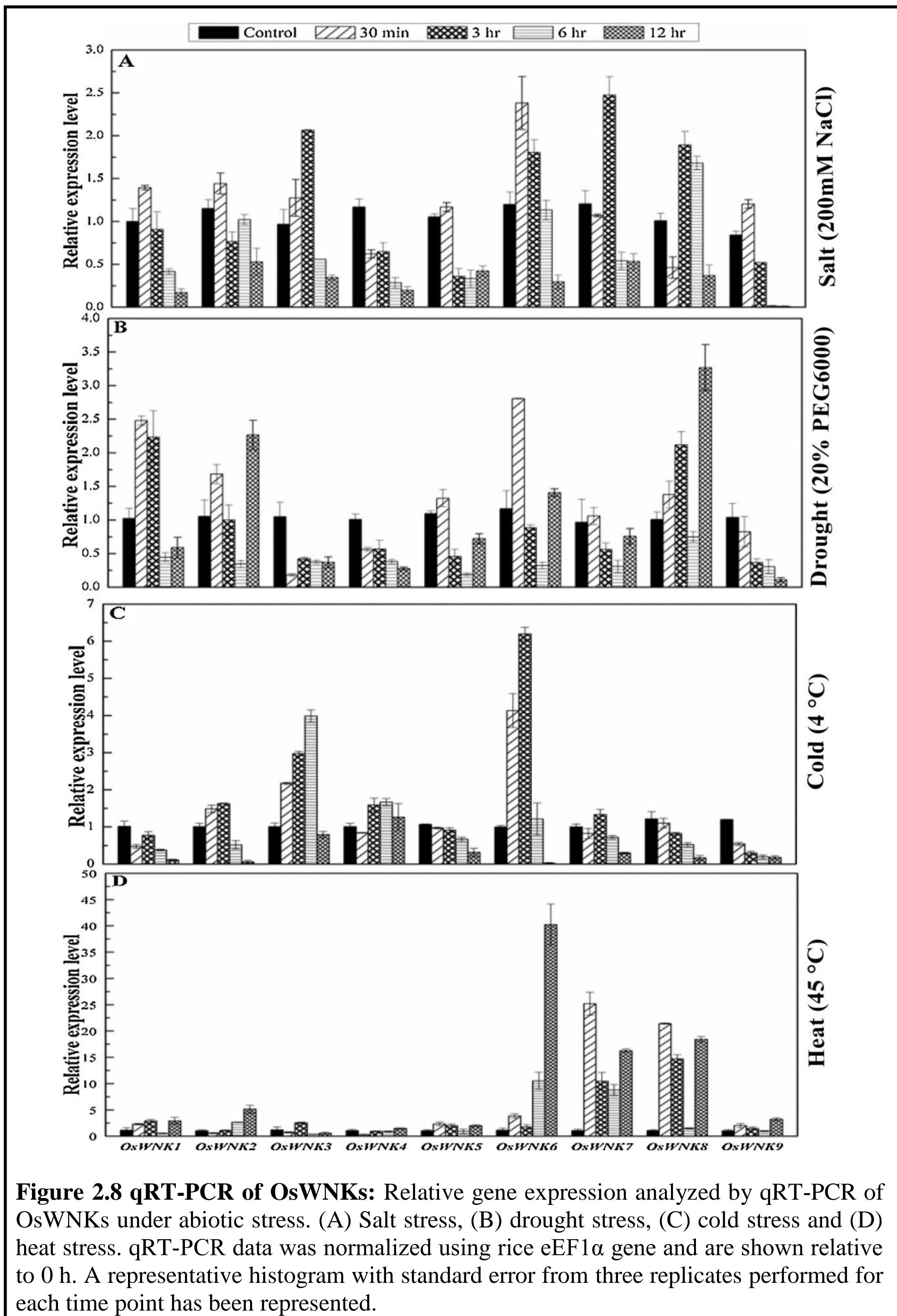


Figure 2.8 qRT-PCR of OsWNKs: Relative gene expression analyzed by qRT-PCR of OsWNKs under abiotic stress. (A) Salt stress, (B) drought stress, (C) cold stress and (D) heat stress. qRT-PCR data was normalized using rice eEF1α gene and are shown relative to 0 h. A representative histogram with standard error from three replicates performed for each time point has been represented.

Rice WNK transcript expression profile compared with available *Arabidopsis* microarray data which provided more insight into WNK function (Fig. 2.9). In both plants, root is a major organ, where most of WNKs are highly expressed. *OsWNK8* is highly expressed in leaf, in contrast *OsWNK1, OsWNK5* and *OsWNK7* showed moderate level expression (Fig. 2.9A). *Arabidopsis AtWNK1* showed higher transcript accumulation in leaf tissue compared with other *AtWNKs* (Fig. 2.9B). The rice panicle shows higher transcript accumulation of *OsWNK4* and *OsWNK5* (Fig. 2.9C). Similarly, *Arabidopsis* exhibit higher transcript accumulation of *AtWNK2* and *AtWNK4* in flower (Fig. 2.9D). Abiotic stress libraries were compared between rice and *Arabidopsis*. Rice *OsWNK* expression data showed higher differential regulation pattern under various abiotic stresses in comparison to *Arabidopsis*.

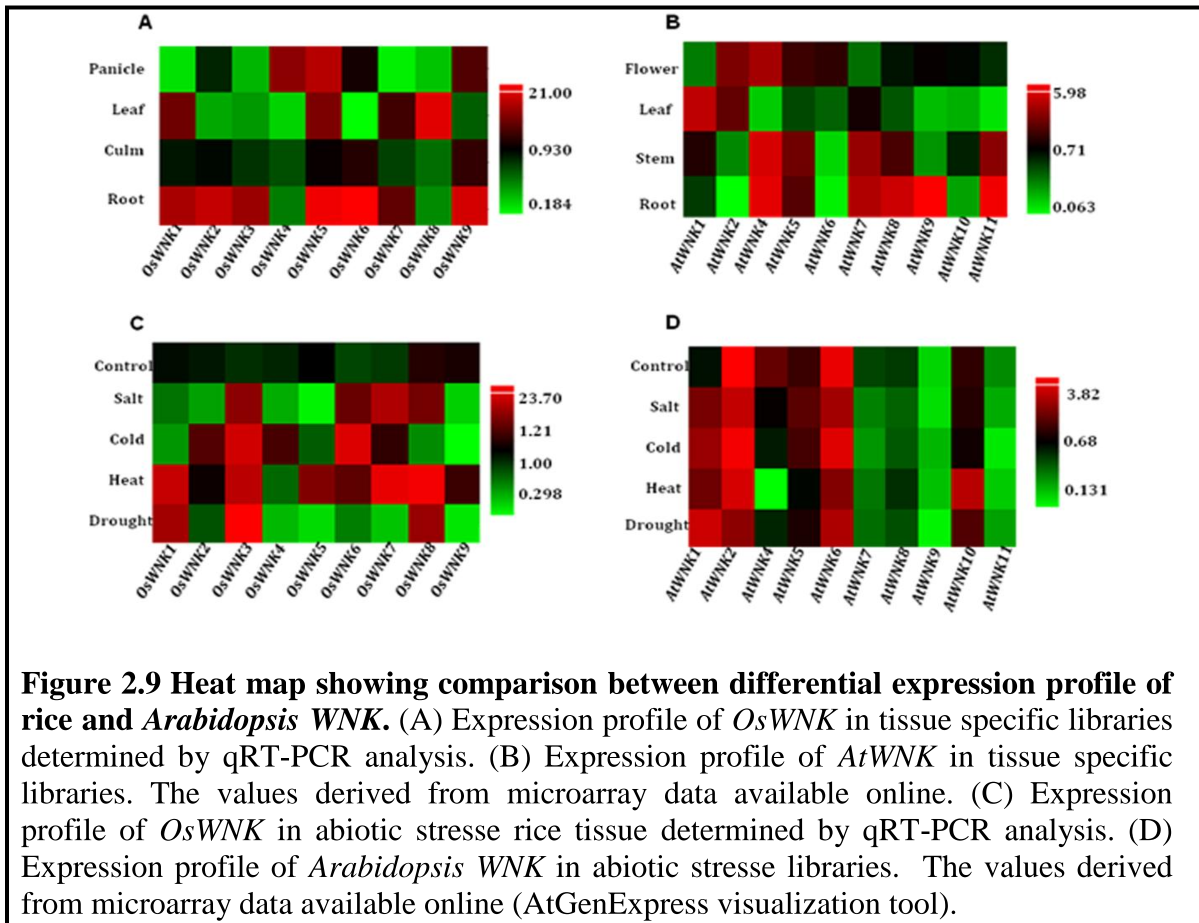


Figure 2.9 Heat map showing comparison between differential expression profile of rice and *Arabidopsis WNK.* (A) Expression profile of *OsWNK* in tissue specific libraries determined by qRT-PCR analysis. (B) Expression profile of *AtWNK* in tissue specific libraries. The values derived from microarray data available online. (C) Expression profile of *OsWNK* in abiotic stresse rice tissue determined by qRT-PCR analysis. (D) Expression profile of *Arabidopsis WNK* in abiotic stresse libraries. The values derived from microarray data available online (AtGenExpress visualization tool).

2.4 Discussion

Arabidopsis kinome was categorized into two groups, receptor protein kinase and soluble protein kinase. Receptor protein kinase clades represented by large superfamily of receptor kinases (RLK), transmembrane leucine-rich-repeat (LRR) receptor kinases and receptor-like cytoplasmic kinases (RLCK). Soluble protein kinase includes cyclin dependent kinase (CDK), mitogen activated protein kinases (MAPK, MAPKK, MAPKKK), AGC kinases, and calcium dependant protein kinases (CDPK) (Zulawski, et al., 2014). With no lysine (WNK) are soluble serine/threonine protein kinases, belong to a unique branch of the kinome. They are called so due to unusual placement of an essential catalytic lysine. With No Lysine (WNK) kinases are responsible for the human hereditary hypertensive disease called pseudo hypoaldosteronism type II (PHAII). It is involved in the WNK-OSR1/SPAK-NCC signaling cascade with oxidative stress-responsive gene 1 (OSR1), Ste20-related proline-alanine rich kinase (SPAK), and thiazide-sensitive NaCl cotransporter (NCC). The distribution of WNK is restricted to higher multicellular organism. However very few and scattered reports are available on plant WNK kinases distribution and their function in the plant kingdom.

Rice (*O. sativa* L.) is the most important food crop and source of food for more than half of the world population (Kumar et al., 2013). We focused on rice crop and performed genome wide analysis to identify WNK gene family in rice. In the current study, we searched various plant genome databases and predicted 155 WNK kinase genes in the plant kingdom and formed HMM profile for the identification of rice WNKs. All the WNK gene sequences were confirmed based on their kinase domain knowledge and unusual position of lysine (K) residue. Subdomain I, at the NH2 terminus of the kinase

domain, contains the consensus motif Gly-X-GIy-X-X-Gly- X-Val, but in WNK kinase third glycine replaced by lysine (K) residue which alter consensus motif to Gly-X-Gly-X-X-Lys-X-Val. A glycine-rich loop in the sub-domain I of protein kinase is highly conserved motifs and proved that by replacing third glycine had minimal effects on steady-state kinetic parameters. In contrast Gly-1 and Gly-2 had major effects on both *Km* and *k*cat values which showed importance of the N-terminus glycine rich loop for catalysis. The first and second glycines of GXGXXG motif are essentially invariant, whereas the third is somewhat more variable (Hemmer et al., 1997). However subdomain II contains invariant lysine has been recognized as enzymatically active residue which helps in anchoring and orientation of ATP (Hanks and Hunter, 1995). Therefore, we can hypothesize that, during evolutionary process variable glycine might be replaced by lysine residue and formed independent WNK protein kinase group. In WNK group functional residues from two different sub-domains merge and formed single active center G-X-G-X-X-K-X-V. Phylogeny is important for addressing and analysis of various molecular differences, mainly in DNA/amino acid sequences, to gain insight into an organism's evolutionary relationships (Yang and Rannala, 2012). Earlier phylogenetic analysis of AtWNK was attempted with other protein family members such as RLK, MAPKKK and MAPK. It showed that WNK family is a novel protein family and the kinase domain is similar to MAPKKKs (Nakamichi et al., 2002). First time, we attempted to construct a complete phylogenetic tree of WNK gene family members and established the evolutionary relationship among WNKs in plants. It demonstrated that all plant WNKs were clustered into 5 major different clades (Class I-V). Clade I includes unicellular red algae and green algae WNK members. Similarly clade II has members of

C. reinhardtii and one member of *P. patens.* Almost all the WNK genes from higher plants were clustered into clade III, IV and V When we moved from simple unicellular algae to more complex dicots plant, multiple genome duplication events were recorded which might leads to higher no of WNK gene. Gene families are group of genes formed by duplication and their size reflects the number of duplicated events, called paralogues. As we know the gene duplications event might be one of the crucial evolutionary force for either neo-functionalization or sub-functionalization (Grassi et al., 2008). However, distribution of WNK genes in the rice (9 WNK genes) is almost similar to *Arabidopsis* (11 WNK genes). Four different major genome duplication events were predicted for *Arabidopsis* over 100–200 million years ago (Simillion et al., 2002; Saddhe and Kumar, 2015).

The physical locations of the *OsWNK* genes were assigned and it is distributed on chromosomes 2, 5, 7, 11 and 12. Based on phylogenetic clustering and sequence similarity, we have observed a pair of OsWNK gene showing high level of sequence similarity. The protein sequences of OsWNK6 and OsWNK9 shared 72% sequence similarity and clustered into subclade IVB with 99% internal node bootstrap value. However OsWNK7 and OsWNK8 shared 92.6% sequence resemblance and clustered into subclade IIIA with 99% internal node bootstrap value. Based on this observation, OsWNK6 and OsWNK9 genes may be considered as paralogues of each other. On similar note, OsWNK7 and OsWNK8 are paralogues of each other, which might have evolved by duplication events. So it could be possible that OsWNK6 shows functional redundancy with OsWNK9, similarly OsWNK7 might exhibit functional redundancy with OsWNK8, which needs further confirmation by functional genomics approach.

We identified 9 members of putative WNK gene family from rice and further performed their domain analysis, gene structure and subcellular localization *in silico*. Putative amino acid length of OsWNK ranges from 328 to 705 AA. However, human WNK1amino acid length was reported around 2382 AA, which is three times longer than rice WNK (Verissimo and Jordon, 2001). Similarly soybean GmWNK1 encoded putatively 610 amino acid long chain (Wang et al., 2010). Here we predicted gene structure of OsWNK, which showed that varied numbers of exons and introns ranges from 2 to 9. Interestingly, we observed OsWNK6 and OsWNK9 have equal numbers of exons and introns in their genomic structure. The human WNK1 gene spans 160 kb, contains 28 exons and WNK2 gene spans 136 kb and contains 30 exons. WNK3 and WNK4 gene spans 165 kb with 24 exons and 16 kb with containing 19 exons respectively (McCormick and Ellison, 2011). An *in silico* subcellular localization was predicted using two different web based servers, according to LOCTREE3, all OsWNK localized to cytoplasm but mPLoc was predicted their localization in nucleus. The subcellular localization of GFP-tagged WNK2 in HeLa cells was predominantly localized in cytoplasm (Jordan, 2011). Human WNK1 was localized to cytoplasm but WNK4 showed localization to intercellular junctions and cytoplasm of kidney (Wilson et al., 2001).

To investigate *cis*-acting elements in the promoters that regulate gene expression at the transcriptional level are crucial for improving our basic understanding of gene regulation (Garcia and Finer, 2014). The *cis*-acting regulatory elements provide binding site for transcription factor, which synergistically regulates gene transcription. Earlier reports underscored the OsWNK regulatory function during circadian cycle and abiotic stresses. For same we predicted distribution of circadian, abiotic stress and phytohormone

specific regulatory elements in promoter. *WNK1* gene in *Arabidopsis* has been reported to be under the control of circadian rhythms, indicating a circadian associated function to the member of WNK family (Nakamichi et al., 2002). The circadian elements were recorded in the OsWNK which gives more insight into regulation of circadian cycle. In *Arabidopsis* there are three major classes of *cis*-regulatory modules within the plant circadian network: the morning (ME, GBOX), evening (EE, GATA), and midnight (PBX/TBX/SBX) modules (Filichkin et al., 2011). G-box is conserved circadian rhythm regulatory motif and mutant analysis showed that reduction the amplitude of oscillation (Spensley et al., 2009). When we scanned OsWNK for conserved circadian motif, we found that G-box dominantly present in the all OsWNK (except OsWNK3) promoter regions. However, we also predicted circadian regulatory elements (CAANNNNATC) in the 5 OsWNK (OsWNK2, 4, 5, 7 and 9) which is indicate involvement of WNK in circadian rhythm controlled expression. The circadian regulatory elements (CAANNNNATC) were identified from promoter region of various genes (example- S-adenosylmethionine decarboxylase) and their expression is circadian clock controlled (Dresselhaus et al., 1996). Based on this data, we can hypothesize that all OsWNK (except *OsWNK3*) might have some roles in circadian regulation or might be their expression is under circadian control. This will enrich our understanding about circadian rhythm and will shed lights on new players of circadian rhythm. Similarly we also searched for abiotic stress responsive *cis*-elements. The promoters containing different combinations and copy number of elements (ABRE, MYB, MYC, as1, rps1 site 1-like) from stress-inducible promoters in *Arabidopsis* (Hou et al., 2012). Similarly, we searched for plants stress responsive *cis*-elements including MBS, HSE and our data supports

OsWNK involvement in the abiotic stresses. Kumar et al. (2011) reported the involvement of *OsWNK1* in the abiotic stresses such drought, cold and heat. The stress-responsive elements comprise the dehydration-responsive element DRE (A/GCCGAC) implicated in the regulation of cold and dehydration responses in *Arabidopsis* (Yamaguchi and Shinozaki, 1994). The Study of class I HSP promoters showed that heat shock elements (HSEs), the *cis*-acting elements necessary for the heat shock response (Carranco et al., 1999). We also searched for phytohormonal regulatory elements as follows ABRE, ERE, TGA. The distribution of ABRE in all OsWNK (except *OsWNK3* and *OsWNK7*) may suggest that, OsWNKs are involved in the abscisic acid signaling cascade. Two well known ABA-responsive *cis*-regulatory elements are ABRE and CE3, which are involved in stomatal closure, seed and bud dormancy, and physiological responses to cold, drought and salinity stress. However previous study proved that a single copy of ABRE is not enough to induce transcription, but multiple ABREs or the combination of an ABRE with coupling element (CE) can form a minimal ABA-responsive complex (ABRC) (Porras et al., 2007). Consistent with this data, we also predicted that, only OsWNK5, OsWNK6 and OsWNK9 have multiple ABRE regions. Similarly ethylene regulatory elements also recorded in OsWNK2, OsWNK4 and OsWNK7. This predictive study of regulatory elements in the OsWNK might provide insight into regulation of abiotic stresses, hormone and developmental patterns of different tissue in rice plant.

Analysis of the tissues specific expression of *OsWNKs* provided us new insights into their role in different organs of rice. To determine the spatio-expression analysis in different tissues and organs, qRT-PCR was performed to monitor the transcript

accumulation of OsWNKs in the roots, young leaves, flag leaf, culm and panicles. The *OsWNK* genes transcripts were recorded in the various organs and tissue of rice. The quantitative analysis of tissue specific expression revealed that most of the *OsWNK* exhibit higher transcript expression level in roots as compared with other organs (Fig. 2.7). The result demonstrated that *OsWNK* might have major role in the root formation and architecture. In the *Arabidopsis* all *AtWNK* (except *AtWNK6*) was expressed in the various organs from seedling to flowering plants (Wang et al., 2008). *AtWNK8* is mainly expressed in primary root, hypocotyl, stamen and pistil (Zhang et al., 2013). Higher expression of *OsWNK8* has been observed in root and young leaf predicting their possible role in the early development and cell proliferation. *GmWNK1* is involved in the repression of lateral root formation in an ABA-dependent mechanism distinct from known ABA signaling pathways (Wang et al., 2010).

Rice (*O. sativa* L.) an important monocot crop worldwide and classified as a salt sensitive crop in their early seedling stages. Abiotic stresses including salinity, drought, cold, heat and metal toxicity, cause lower agriculture crop productivity. In plant, protein kinases play important roles in regulating the stress signal transduction pathways, plant growth and development (Kumar et al., 2013). *OsWNK1* responded differentially under several abiotic stresses like cold, heat, salt, drought and showed rhythmic expression pattern under diurnal and circadian conditions at the transcription level (Kumar et al., 2011). Our result showed that, under the salt stress condition transcript level of *OsWNK3, OsWNK5, OsWNK6* and *OsWNK7* exhibited significant differential expression patterns. *OsWNK9* displayed lower expression pattern during salt stress. Similarly under drought stress, *OsWNK1, OsWNK2, OsWNK6* and *OsWNK8* showed significant transcript

accumulation. *OsWNK3*, *OsWNK4* and *OsWNK9* exhibited down-regulation patterns under drought stress. Under cold stress *OsWNK6* showed higher transcript accumulation at 3 h, while *OsWNK1*, *OsWNK2*, *OsWNK6*, *OsWNK8*, *OsWNK9* exhibited down regulation at 12 h intervals. *OsWNK3* was up regulated and showed 4 fold up regulation at 6 hr of cold stress, while *OsWNK9* showed the down regulation pattern. In heat stress *OsWNK2, OsWNK6*, *OsWNK7*, and *OsWNK8* showed higher transcript accumulation, revealed their significant role in heat stress. An overexpression study of *GmWNK1* in *Arabidopsis* showed that, it modulates cellular response to osmotic stresses in plants (Wang et al., 2010). *AtWNK8* and *AtWNK9* are involved in the abiotic stress modulation (Zhang et al., 2013; Xie et al., 2014). The gene expression pattern and protein product in plant varies when exposed to the low temperature (Sanghera et al., 2011). The relative gene expression level of *OsWNK*1 in our studies was correlated with our earlier studies under cold stress (Kumar et al., 2011).

The comparative transcriptomic studies will help us to understand functional aspect of gene family between two organisms. Therefore, we compared *Arabidopsis* WNK gene family expression pattern with rice qRT-PCR data by constructing heat maps (Fig. 2.9). Interestingly, rice showed 3.5 times higher tissue specific expression and 7 times higher expression under various abiotic stresses than *Arabidopsis*. Most of WNKs are highly expressed in the root system followed by panicle/flower, leaf and culm/stem tissues. This observation is consistent with previous *WNK1* reports in rice and soybean (Wang et al., 2010; Kumar et al., 2011). According to Kumar et al. (2011) rice *WNK1* was highly expressed in the panicle followed by stem, root and leaf. However, they also reported regulation of the *OsWNK1* under different abiotic stress such as cold, heat,

salinity and drought stress. Interestingly rice *OsWNK3* showed highest upregulation pattern in all stresses, this indicate *OsWNK3* might have some regulatory function during abiotic stresses. In *Arabidopsis AtWNK1* is highly unregulated similarly *OsWNK1* showed significant expression level under heat and drought stress. One more significant observation is the down regulation pattern of *OsWNK9* under cold stress followed by salt and drought stresses. Similar observation recorded for *AtWNK9*, which indicates importance of *WNK9* member under various abiotic stresses. Under heat stress *AtWNK4* showed significant down regulation pattern, similarly in rice *OsWNK4* showed down regulation pattern. Overall this comparative expression analysis will help us somehow to understand the involvement of WNK in various abiotic stresses.

2.5 Conclusion

In the present study, we identified 9 members of WNK in rice, that showed resemblance to *Arabidopsis* and human WNK. Phylogenetic analysis of all WNK was performed and categorized into five main clades and subclades. Conserved motif and domains in the deduced amino acid sequences of rice WNK strongly supported their identity as members of WNK kinase protein. Their gene expression pattern was further investigated using qRT-PCR in tissue specific and various abiotic stresses. Significant expression level of most *OsWNK* was observed in root tissues but in contrast *OsWNK8* was observed in young leaf. However, *OsWNKs* showed differential transcript accumulation pattern under various abiotic stresses *viz.* salt, drought, cold and heat. The presence of various abiotic stress responsive *cis*-elements in the promoter region of OsWNK also predict their importance in providing basal tolerance to rice during critical conditions. Our observations may further elucidate the significance of functional analysis of rice WNK genes and unravel their biological roles using functional genomics approach.

Chapter 3

Functional characterization of *OsWNK9* in *Arabidopsis* and localization studies in rice

3.1 Introduction

In plants, WNK members were reported to be involved in several physiological and cellular roles, such as regulation of flowering time, maintaining the circadian cycle, root architect and regulation of various abiotic stresses. In *Arabidopsis thaliana* 11 members of WNK family have been predicted and few of them members have so far been well characterized (Huang et al., 2007; Wang et al., 2008). *Arabidopsis WNK1* was involved in phosphorylation of circadian clock component APRR3 and hence regulates the circadian rhythm. The circadian rhythm was controlled at the transcription level of *AtWNK1 AtWNK2, AtWNK4*, and *AtWNK6* indicating an involvement of *AtWNKs* in the regulation of the circadian clock (Nakamichi et al., 2002). Besides circadian cycle, *AtWNK1* was involved in regulation of flowering time in *Arabidopsis*, which was demonstrated by wnk1 knockout showing delayed flowering during long-day hours (Wang et al., 2008). Another *AtWNK* member, *AtWNK9* overexpression in *Arabidopsis* showed enhanced drought tolerance by positive regulation in ABA signaling cascade (Xie et al., 2014). Soybean root specific protein kinase, *GmWNK1* was characterized functionally and signifies that it interacted with ABA 8′-hydroxylase protein. Further, it was indicated that *GmWNK1* is involved in the regulation of stress response through an ABA-dependent pathway in root system (Wang et al., 2010). Overexpression of *GmWNK1* in *Arabidopsis* showed tolerance to salt and osmotic stresses (Wang et al., 2011). The rice *OsWNK1* transcript analysis suggested that it may be involved in the regulation of circadian cycle and their probable roles in abiotic stresses (Kumar et al., 2011).

Rice is a major staple food crop worldwide. WNK kinase is one of the most important gene family, which is involved in the cellular function. But, there are no significant reports available on rice WNK family members and their physiological roles are not reported so far. In the present study, we functionally characterized a novel rice protein kinase *OsWNK9* under salt and drought stress by overexpression studies in *Arabidopsis*.

3.2 Materials and methods

3.2.1 Plant material and stress treatment

Rice seeds (*Oryza sativa* L. ssp. indica cultivar IR64) were surface sterilized by 5% sodium hypochlorite and germinated at 28°C. The germinated seeds were transferred to a hydroponic culture containing Hoagland's nutrient and allowed to grow for 3 weeks. After three weeks, the seedlings were subjected to abiotic stress treatments. For salt and drought treatments, seedlings were treated with Hoagland's solution containing 200mM NaCl and 20% PEG-6000 respectively. ABA stress treatment was administered by adding 100µM ABA in Hoagland's nutrient solution. After stress treatments, root and shoot tissues were harvested separately at 0, 0.5, 3, and 12 h intervals. Harvested tissues were frozen in liquid nitrogen and stored at -80°C until further use.

3.2.2 Quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the Ribozol (aMResco) method following the manufacturer's instructions. The concentration of RNA was quantified using the Nanodrop and subjected to first-strand cDNA synthesis. All gene-specific primers used

for the quantitative real time PCR analysis were designed using PrimerQuest (Integrated DNA Technologies) and the specificities were confirmed by the RT-PCR product in agarose gel before used for qRT-PCR. The primer sequences are listed in Table 3.1 and 3.2. Quantitative RT-PCR was performed on Real-Time PCR system (AriaMx Agilent Technologies) using SYBR green master mix (2X Brilliant III SYBR® Green QPCR, Agilent Technologies). The melting curve was used to detect the presence of primer dimer or other nonspecific amplified products that would produce a detectable C_T value. The relative expression changes of *OsWNK9* and stress marker genes were quantified using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Three biological replicates with two technical replicates of each sample were used for qPCR analysis and the transcript levels relative to *eEF1α* or At*Actin2* were reported as mentioned in figure legends.

Table 3.1. List of the *OsWNK9* qRT-PCR (OsWNK9), PCR full length (FlWNK9), promoter (PWNK9), subcellular localization (OsWNK9-1302) and GUS primers (GUS).

S.No	Primer label	Primer sequence 5'-3'
1	eEF1 alpha-F eEF1 alpha-R	AGCGTGTGATTGAGAGGTTC AGATACCAGCCTCAAAACCAC
2	18s rRNA-F 18s rRNA-R	CCGTCCTAGTCTCAACCATAAAC GCTCTCAGTCTGTCAATCCTTG
3	OsWNK9-F OsWNK9-R	AACGGTCTACAAAGCCTTCG TTCTGCGGTACTGTGTCAAG
4	FlWNK9-F FlWNK9-R	TACGTCTCTAGAATGGATCTGGTGGAGGCGGA TAGCTGCCCGGGTCAGCGCTTCTCAATTGTCAAG
5	PWNK9-F PWNK9-R	TACGTCCCATGGATCAAAAACCGACTCAAACG TAGCTGAGATCTAGATTGTCAACTGCTTCCCA
6	OsWNK9-1302-F OsWNK9-1302-R	TACGTCCCATGGATGGATCTGGTGGAGGCGGA TAGCTGACTAGTGCGCTTCTCAATTGTCAAGC
7	GUS-F GUS-R	CGAAGTCACAGCCAAAAGCC GCGAAATATTCCCGTGCACC

Table 3.2. List of the q-RT PCR abiotic stress and transcription factors related gene primers of *Arabidopsis.*

S.no	Primer label	Primer sequence 5'-3'
1	AtActin2-F AtActin2-R	TGGATTCTGGTGATGGTGTG GTTTCCATCTCCTGCTCGTAG
2	AtRD22-F AtRD22-R	CGTCTTCCTCTGATCTGTCTTC TGGGAATGGGAGTGTTTGG
3	AtABA3-F AtABA3-R	TCAAGTCGCTTACACCTTCTG TCCAAGTAGCCAAACCAAGAG
4	AtNCED3-F AtNCED3-R	CCAGCAAGTCGTTTTCAAGC TCATCTGTTTCTGGCTCTTCC
5	AtEREBP-F AtEREBP-R	AAGAACGGAGCTAGGGTTTG GTTAACTGATCGGAGACTGACG
6	AtWRKY28-F AtWRKY28-R	GAAACCTCCCATCACAAAACG CCCTAGTTTCAGTCTCGTTGC
7	ANACO19-F ANACO19-R	TCTAACCCAAACCGCATCTC ATTAAACCCGTGACTGCTCTC
8	AtDREB2C-F AtDREB2C-R	GCTCGGTTACTGCATTTTCTG CTTCCCTCACTTCCTCTTTTCC

3.2.3 Cloning of OsWNK9 and *in silico* analysis

3.2.3.1 Full length *OsWNK9* cloning

The full-length CDS of *OsWNK9* was amplified from *O. sativa* L. indica cultivar IR64 using OsWNK9-F and OsWNK9-R primers (Table 3.1). Full-length CDS was cloned into pGEM T-easy vector system (Promega, UK) as per manufacturer's instruction. Clones were sequenced and submitted to GenBank. A phylogenetic tree was constructed by neighbor-joining (NJ) tree based on the bootstrap value of 1000 replicates using MEGA 6.0.6 tool (Tamura et al., 2013). Threading of OsWNK9 3D model was performed on the I-TASSER web server with default parameters (Roy et al., 2010) (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The secondary structure of the target protein OsWNK9 was predicted using position-specific iterated prediction (PSI-

PRED) and position specific iterated-BLAST (PSI-BLAST). An assembly and refinement of protein structure were evaluated on the Support Vector Machine SVMSEQ and SPICKER programs. The quality of the model was estimated based on C-score, cluster density, TM-score, and root mean squared deviation (RMSD). The PyMOL (Schrodinger Inc) was used to visualize the model and generate publishable images.

3.2.3.2 Cloning of OsWNK9 for overexpression, promoter analysis and tissue specific expression

Full-length fragment of *OsWNK9* was cloned into the CaMV 35S promoter driven expression cassette of pBI121 using XbaI/SmaI restriction sites. Full-length fragment of *OsWNK9* with a stop codon was amplified by RT-PCR (Fig. 3.3 A).

3.2.3.3 Cloning of OsWNK9 promoter for tissue specific localization

For tissue specific localization, the promoter region of OsWNK9 (1kb upstream) was amplified from rice genomic DNAusing PWNK9-F and PWNK9-R pair of primers by RT-PCR (Table 3.1). Promoter sequence was cloned into pGEM T-easy vector system (Promega) and confirmed by sequencing. Further, the OsWNK9 promoter sequence was cloned into the pCAMBIA1301 binary vector containing fused GUS reporter gene to create a recombinant cassette, rice PWNK9:GUS with NOS terminator (Fig. 3.19A).

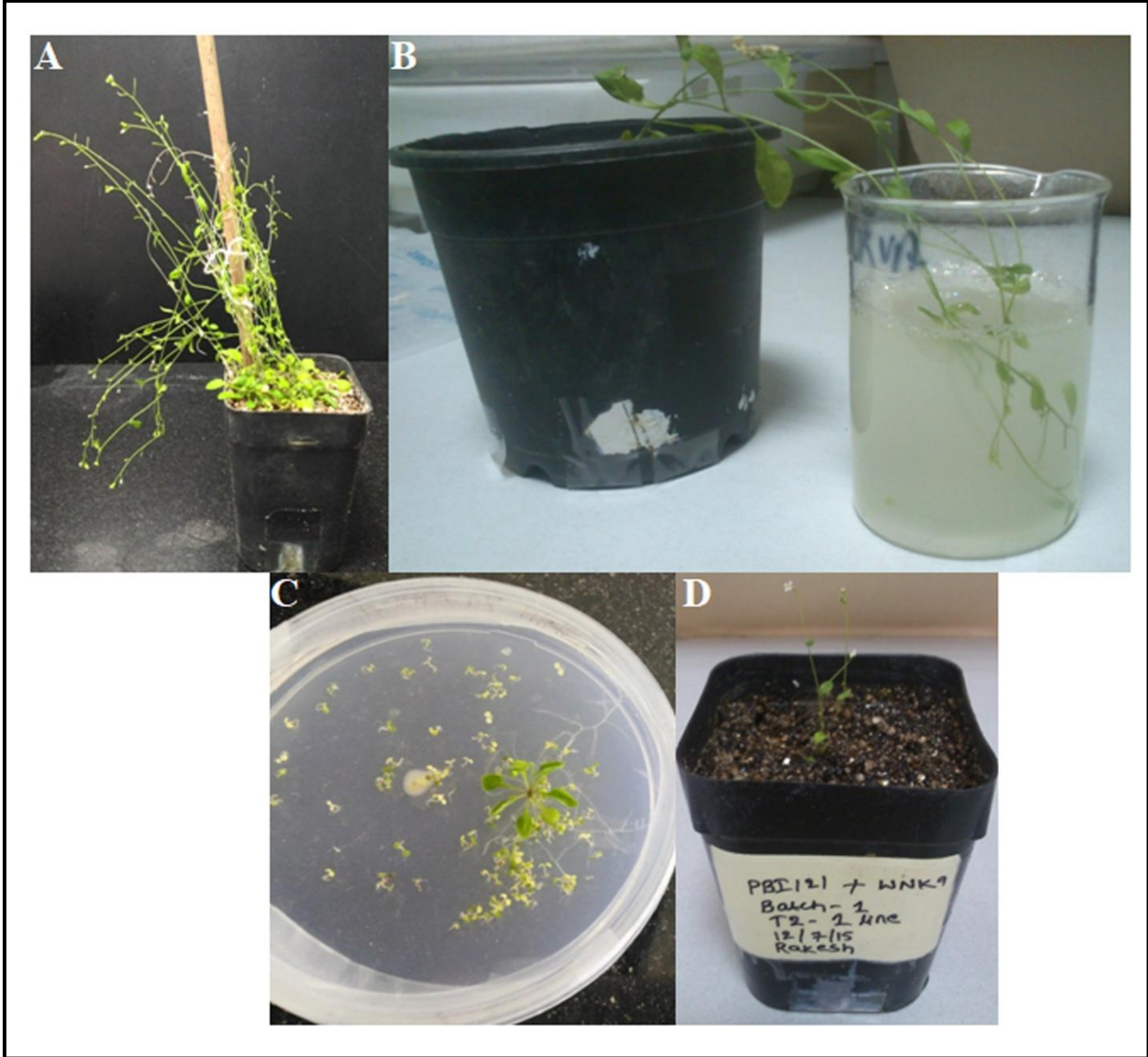
3.2.3.4 Cloning of OsWNK9 for subcellular localization

For subcellular localization, full-length OsWNK9 without stop codon sequence was cloned into pGEM T-easy vector system (Promega) and sequenced for confirmation.

Further, the *OsWNK9* sequence was cloned into the pCAMBIA1302 binary vector with green fluorescent protein Further, protein (GFP) reporter gene to create a recombinant cassette, rice OsWNK9:GFP (Fig. 3.21A).

3.2.4 Overexpression of *OsWNK9* in *Arabidopsis*

Arabidopsis thaliana (Col-0) were grown with the following conditions: temperature 22°C, relative humidity 55-65%, and photoperiod 16h/8h (light/dark). Three week old *Arabidopsis* seedlings were transferred to pot containing soilrite, perlite and vermiculite (1:1:1 ratio). The plasmid construct CaMV35S:*OsWNK9* was transformed in *Agrobacterium tumefaciens* strain GV3101 containing *FlWNK9*::pBI121. The primary culture was grown in LB broth+Rifampicin (25mg/L)+Kanamycin (50mg/L) at 28°C for 24 h. Secondary culture was grown upto OD_{600} to 0.6-0.8 by inoculating 100µl of primary culture. The secondary culture was used for floral dip (Bechtold, 1993). The floral buds of wild type plant were dipped in *Agrobacterium* culture for 20 seconds and kept in dark covered with plastic bag to maintain humidity for overnight then transferred back to growth chamber. Kanamycin resistant T_0 seedlings of *Arabidopsis* expressing the *OsWNK9* were selected. The independent T_1 transgenic lines were screened for Mendelian segregation ratios 3:1 (resistant: sensitive) and obtained single insert homozygous T_2 lines. Transcript analysis was performed for the T_2 transgenic *Arabidopsis* lines by qRT-PCR. Four independent transgenic lines (T_9, T_{11}, T_{12}, and T_{13}) out of 13 transgenic lines based on transcript analysis were selected for further studies (Fig. 3.1).


A
B
C
D
PBI121 + WNK9
Batch- 1
T2- 1 line
12/7/15
Rakesh

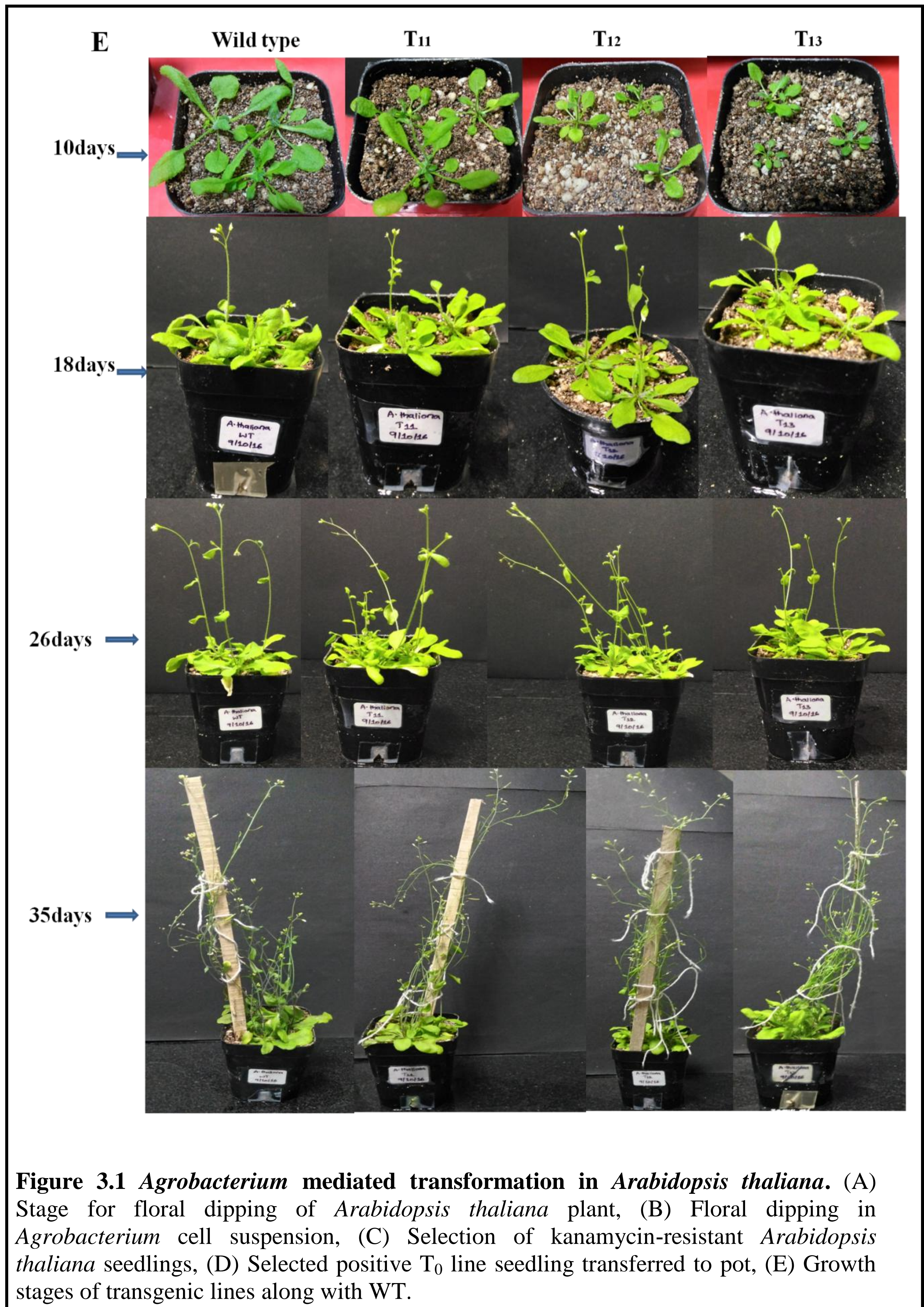


Figure 3.1 ***Agrobacterium*** **mediated transformation in** ***Arabidopsis thaliana*****.** (A) Stage for floral dipping of *Arabidopsis thaliana* plant, (B) Floral dipping in *Agrobacterium* cell suspension, (C) Selection of kanamycin-resistant *Arabidopsis thaliana* seedlings, (D) Selected positive T_0 line seedling transferred to pot, (E) Growth stages of transgenic lines along with WT.

3.2.5 Evaluation of transgenic line exposed to salt, drought and ABA stress

To analyze salt, drought and ABA stress tolerance of *OsWNK9* overexpressing transgenic *Arabidopsis*, T_2 seeds were germinated in ½ MS medium for 1 week and transferred to 125mM NaCl, 200mM mannitol and 12μM ABA containing MS medium and placed vertically in the growth chamber under controlled conditions. To reduce variations, WT and transgenic plants were grown side by side on the same plate and their growth was compared. Plants were photographed, shoots and roots were harvested separately, fresh weight of shoots and their root lengths were measured. Fresh weight and root length of 15 independent plants were taken from 3 different plates. For calculation of a percentage of seed germination, WT *Arabidopsis* seeds and transgenic line seeds were surface sterilized and placed on MS plate supplemented with NaCl (75mM and 100mM), mannitol (100mM and 200mM) and ABA (0.5μM and 2μM). The results were obtained from 4 biological replicate with 50 seeds each collected from independent seeds stocks. For the drought stress treatment, *Arabidopsis* seeds (40 each) of WT and transgenic lines were grown in pots for 3 weeks and then subjected to water deprivation stress by withholding water for 2 weeks. Plants were rewatered again after 2 weeks and surviving plants were observed after 3 weeks. The experiment was repeated for three times. To measure the water loss, detached leaves from 4 weeks old plants were placed on filter paper and weighed at regular intervals of time points (1h, 2h, 4h, 6h, 8h, 10h and 12h). The water loss was calculated as the percentage of initial fresh weight at each time point (Xie et al., 2014). The water loss experiments were performed four times using leaves grown from independent plants.

3.2.6 Chlorophyll retention assay

Four weeks old leaves from WT and four transgenic lines (T_9, T_{11}, T_{12}, and T_{13}) were detached and exposed to NaCl (100mM and 150mM) stress for 72 hrs. Total protein concentration of leaves was estimated by Bradford method (Ni et al., 2009). Leaves (200mg) were homogenized in 80% acetone and centrifuged at 3,000 ×g for 15 min. The absorbance of supernatant was recorded at 663 and 645 nm, and the chlorophyll content was calculated per mg of protein (Jones et al., 1989).

Chlorophyll a = (12.7xA663-2.69xA645) x v/1000 x W

Chlorophyll b = (22.9x A645-4.86 x A663) x v/1000 x W

Chlorophyll a+b = (8.02 x A663 + 20.20 x A645) x v/1000 x W

(V= Volume of the extract (ml), W= Weight of fresh leaves (g)).

3.2.7 Stress treatment of *Arabidopsis* plant for qRT-PCR analysis

Four weeks old *Arabidopsis* seedlings (WT and T_{13} line) were grown in hydroponic solution with MS medium. After 4 weeks, the *Arabidopsis* seedlings were subjected to salt, drought and ABA stress with concentrations of 200mM NaCl and 300mM mannitol in MS media respectively. Shoot tissues were harvested at 0, 6 and 12 hrs and immediately flash frozen in liquid nitrogen followed by storage at -80°C until further use. *AtActin2* was used as an internal control and gene expression level were shown relative to 0 hr in both WT and T_{13} line. For each treatment, values for three biological replicates with two technical replicates were obtained.

3.2.8 ABA estimation by LC–MS/MS

ABA was estimated in plant samples by multiple reactions monitoring method (MRM). Samples were prepared according to previously reported method with some modifications (Lopez and Jauregui, 2005; Almeida et al., 2014). All samples were crushed in liquid nitrogen and weighed 250mg in each 1.5 ml micro-centrifuge tube for 1g of plant material for each sample and 1ml of ethyl acetate was added in each tube. Samples were incubated at -20°C for 1 hour by vortexing followed by 30 min shaking incubation at 10°C. After incubation samples were centrifuge at 16,000g for 5 min at 4°C and collected supernatant into fresh tube followed by dry in speed vac. Samples were reconstituted in 500µL of methanol, and centrifuged at 16,000g at 4°C for 10 min. The 5µl of supernatant were injected into LC-MS/MS system. The LC-MS/MS experiments were performed on an Agilent technology 6460 triple quad LC-MS/ using C18 column with length 2.1x150mm, 3.5uM diameter. The analyses were performed using the electrospray ion source in negative ion mode.

3.2.9 Tissue specific localization

The PWNK9:GUS construct was transformed into *Agrobacterium* strain EHA105 by $CaCl_2$ method (Holsters et al., 1978).

- The LB plate was streaked with EHA 105 & GV3101 of *Agrobacterium strain* (used kanamycin and rifampicin for selection antibiotics) and grown for 2 days at 28°C.
- A single colony was inoculated into 5ml LB broth (primary culture) and grown overnight at 28°C on rotating shaker.

- Primary culture (500µl) was inoculated into a secondary culture of volume 50ml LB broth and grown overnight at 28°C on a rotating shaker.
- The overnight grown culture flask was kept on ice for 30 min.
- Chilled culture (45ml) was transferred into a pre-chilled falcon centrifuge tube and then centrifuged at 4,000 rpm for 10 min at 4°C.
- The supernatant was removed and gently resuspended pellet in 5.0 ml ice cold 20 mM calcium chloride by holding the tube on ice.
- The tubes were centrifuged at 4,000 rpm for 5 min at 4°C and gently resuspended pellet in 1.0 ml of ice cold 20 mM calcium chloride by holding the tube on ice. Aliquoted the culture into 50 ul each in pre-chilled 1.5 ml micro centrifuge tubes.
- Further snap freezed the tubes in liquid nitrogen and stored at -80°C.
- **Freeze-Thaw Transformation**
- The plasmid DNA (500 ng) was added to a micro centrifuge tube of competent cells, tube was then placed on ice for 15-30 min and freezed in liquid nitrogen for 5 min.
- The heat shock was given at 37°C water bath for 5 min, then returned to ice for ~5 minutes. Further 1.0 ml of LB was added to the tube and then incubated on a 28°C rotating shaker for 3-4 hrs.
- The incubated culture (50-200ul) was plated on a LB plate containing an appropriate selection agent.
- The plates were grown for 2 days at 28°C and carried forward to colony PCR for confirming transformation.

The rice seeds (Pusa Basmati-1) were surface sterilized by 5% sodium hypochlorite and placed on 1/2x MS media with 2,4-Dichlorophenoxyacetic acid (2mg/l) for 3 weeks at 28°C under 14h light/10h dark photoperiod. The *Agrobacterium* EHA105 strain with pCAMBIA1301::*PWNK9* were transformed to rice callus and maintained on MS media supplemented with acetosyringone (400µM) for 4 days. After 4th days, callus were washed with sterile water containing cefotaxime (500 mg/l) and further transferred to co-cultivation media supplemented with hygromycin (25mg/l) and gelrite (0.4%) for 6 weeks under dark. Callus was subcultured regularly after every 2 weeks. After 6 weeks callus was transferred to regeneration media containing MS with cefotaxime (250mg/l), hygromycin (25mg/l), BAP (3mg/l), NAA (0.5mg/l), Tryptone (50mg/l) and 0.4% gelrite. The regenerated plants were transferred to rooting medium containing MS with cefotaxime (250mg/l), hygromycin (25mg/l) and gelrite (0.2%). Further plantlets were transferred to green house for hardening, plants were grown till maturity and seeds were harvested (Fig. 3.2).

Figure 3.2 *Agrobacterium* mediated transformation in rice callus. (A) PB1 (Pusa Basmati-1) seeds on ½ MS+2, 4-D plate, (B) Untransformed callus, (C) *Agrobatcerium* mediated transformed callus on co-cultivation media, (D) Transformed callus on selection media, (E) Transformed callus on regeneration media, (F) Transformed T_0 lines onto rooting media, (G) T_0 line transferred to pots, & (H) T_0 line transferred to green house.

GUS histochemical staining of WT and transgenic rice plants containing PWNK9:GUS fusion were performed by the previously described method with few modifications (Jefferson 1987). The samples were incubated in GUS solution containing 50mM phosphate buffer (pH 7.0), 0.1% (v/v) Triton-X100, 100mM potassium ferricyanide, 100mM potassium ferrocyanide, 0.5M EDTA, 20% (v/v) methanol and 100mM 5-Bromo-4-chloro-3-indolyl-β-D glucuronide (X-gluc) at 37°C for 16h-24h. The samples were examined with an inverted microscope (Leica Q500MC, Cambridge, England) at a low magnification and photographed with a digital camera.

The GUS activities of T_2 line under abiotic stresses were performed with different time points. The 4 days old T_2 rice seedlings were transferred to stress conditions (200mM NaCl, 200mM Mannitol and 100µM ABA) for 6, 12 and 24 hr and performed GUS assay.

3.2.10 Subcellular localization of OsWNK9

The pCAMBIA1302 vector with Os*WNK9*:GUS construct was transformed into tobacco (*Nicotiana benthamiana*) leaves using *Agrobacterium* strain EHA105 by agro-infiltration method. The tobacco leaves were agro in-filtered on the bottom side of leaf by the agro culture and incubation for 48h. The transformed leaf incubated up to 48 hrs and images were captured by an Olympus IX81/FV500 confocal microscopy using argon laser (488nm) and a green helium/neon laser (543nm) (Zhao et al., 2017).

Further the same construct were transformed into *Arabidopsis* protoplast by PEG mediated transformation (Yoo et al., 2007). The transformed protoplast images were

captured by a Leica DMi 8 laser confocal microscopy in bright field, GFP (509nm) and DAPI (440nm). The protoplast isolation and transformation as follows

- The well-expanded leaves from 3–4 week-old plants (5-7 leaves) before flowering was selected and cut it to 0.5–1-mm leaf strips from the middle part of leaf using a fresh sharp razor blade without tissue crushing at the cutting site (10–20 leaves can be digested in 5–10 ml enzyme solution).
- Leaf strips transferred quickly and gently onto prepared enzyme solution (10–20 leaves in 5–10 ml) by dipping both sides of the strips using a pair of flat-tip forceps.
- Vacuum infiltratation was carried out for 30 min in dark using desiccator.
- The digestion was continued without shaking in dark for at least 3h at room temperature. The enzyme solution should turn to green after a gentle swirling motion, which indicates release of the protoplasts.
- The release of protoplasts in solution was checked under the microscope and the size of *Arabidopsis* mesophyll protoplasts is approximately 30–50 mm.
- The enzyme/protoplast solution was diluted with an equal volume of W5 solution before filtration to remove undigested leaf tissues.
- A clean 75-mm nylon mesh was washed with water to remove ethanol (the clean mesh should be kept in 95% ethanol), then removed excess water before protoplast filtration. Filtered the enzyme solution containing protoplasts after wetting with 75-mm nylon mesh with W5 solution.

- The flow-through was centrifuged at 1000g to pellet the protoplasts in a 30-ml round-bottomed tube for 1–2 min. Removed supernatant as much as possible and re-suspended the protoplast pellet by gentle swirling.
- The protoplast was re-suspended with W5 solution after counting cells under microscope using a hemocytometer. Protoplasts were incubated on ice for 30 min.
- Protoplasts should begin to settle at the bottom of tube by gravity after 15 min. Removed W5 solution as much as possible without touching the protoplast pellet. Re-suspended protoplasts in MMG solution and kept at room temperature.
- **DNA PEG calcium Transfection**
- DNA (1 ml) was added to a 2-ml micro centrifuge tube. Further 100 ml of protoplasts was added and mixed gently.
- PEG solution (110 ml) was added and then mixed completely by gently tapping on the tube.
- The transfection mixture was incubated at room temperature up to 15 min and diluted mixture with 400–440 ml of W5 solution at room temperature, mixed well by gently inverting the tube to stop the transfection process.
- The mixture was centrifuged at 1000g for 4 min at room temperature using a bench-top centrifuge and removed supernatant.
- The protoplast was re-suspended gently with 1 ml of WI in each well of 6-well tissue culture plate. Incubated the protoplasts at room temperature for 15 minutes and harvested protoplasts by centrifugation at 1000g for 4 min.

- The supernatant was removed and cellular image of GFP fusion proteins was photographed using a confocal microscope.

3.2.11. Statistical analysis

The statistical significance of each parameter across treatments was determined using a one-way analysis of variance (One-way ANOVA) followed by post-hoc t-test. $p < 0.01$ is considered as highly significant labeled as **, and $p < 0.05$ is considered significant and labeled as *.

3.3 Results

3.3.1 *OsWNK9* is differentially regulated under salt, drought and ABA stress

Transcript accumulation was performed to understand *OsWNK9* response and regulation under abiotic stresses. *OsWNK9* mRNA levels were quantified under salt, drought and ABA stresses in shoots and roots tissues by qRT-PCR. In shoots under salt, drought and ABA stresses transcript accumulation showed consistent down regulation pattern at different time intervals. Under salt stress, *OsWNK9* showed 10-fold down regulation at 12 h. Similarly, under drought stress transcript of *OsWNK9* was down regulated by 4-fold at 1 h and 8-fold at 12 h. Under ABA stress *OsWNK9* transcript accumulation was 6-fold down regulated at 30 min and maintained at low level till 12 h (Fig. 3.3A). Interestingly, in the root tissue under salt stress, *OsWNK9* showed an increased level of transcript abundance up to 3.5-folds at 30 min and 2.9-fold increased transcript accumulation at 3 h. Under drought stress, *OsWNK9* showed increased transcript accumulation of 2.5-folds at

6 and 12 h. However, there was increase of 4-fold transcript accumulation under ABA stress at 30 min and 1 hour in root tissues (Fig. 3.3B).

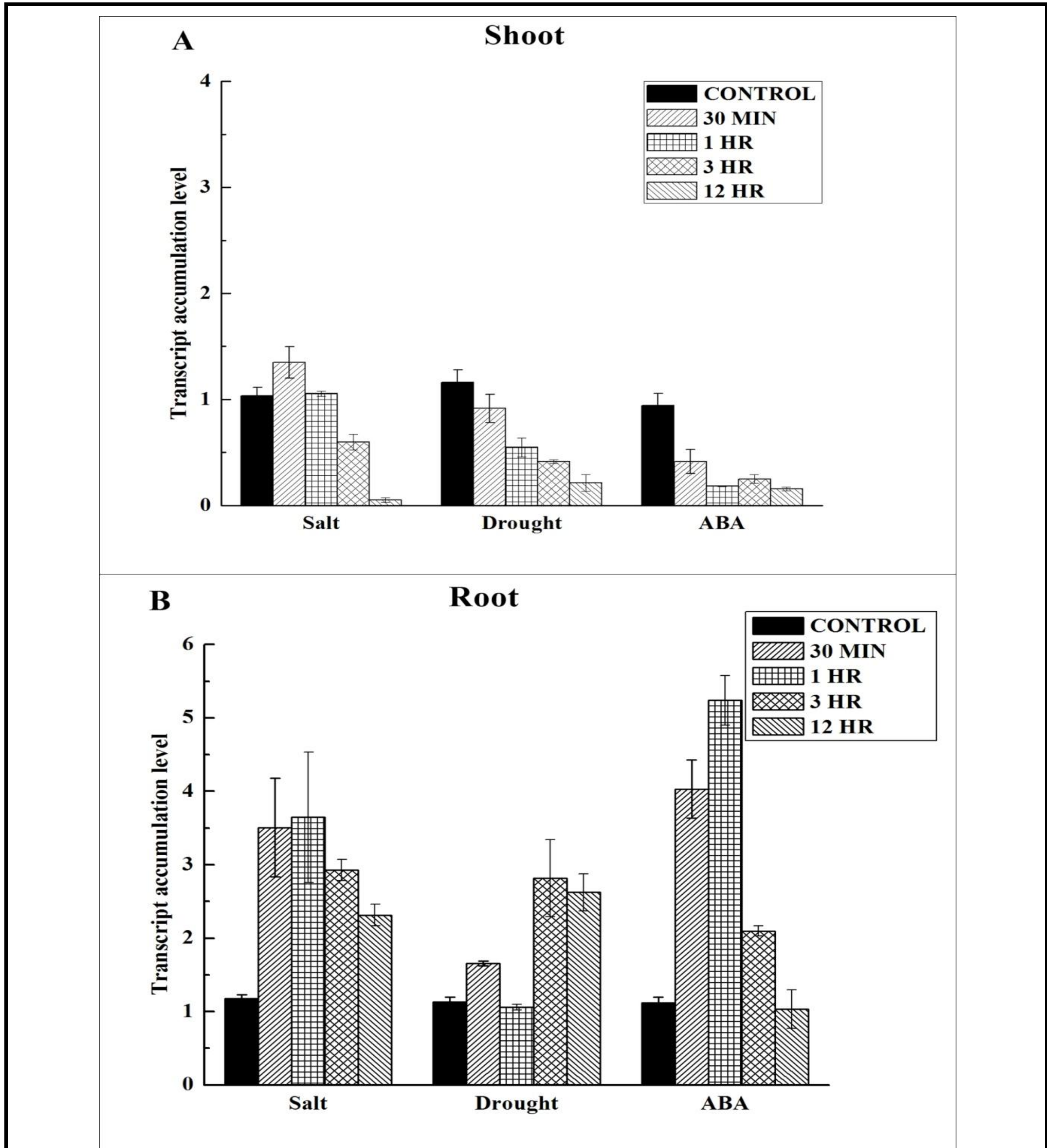


Figure 3.3 Transcript analysis of *OsWNK9* under salt, drought and ABA stress. Relative gene expression of *OsWNK9* was analyzed by qRT-PCR, (A) Shoots and (B) Roots. Three weeks old rice plants were exposed to 200mM salt, 20%PEG and 100µM ABA and samples were collected at 0, 0.5, 3 and 12 h. qRT-PCR data were normalized using rice *eEF1α* gene and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.

3.3.2 *In-silico* analysis predicted OsWNK9 belongs to protein kinase group

The full-length sequence of OsWNK was deposited to GenBank and received accession no. KY774844. The OsWNK9 predicted protein sequence contains 417 residues and 47.11kDa molecular weight. The domains and motifs analysis confirm presence of N-terminal kinase domain in OsWNK9, which was similar to plant and animal WNKs. The protein kinase domain was fragmented into 12 classical subdomains. The motifs 'IIHRDLKCDNIFI' in subdomain VIb and 'GTPEFMAPE' in subdomain VIII were observed in OsWNK9 underscored that this protein belongs to ser/thr kinase group (Fig. 3.4A). A conserved activation-loop (A-loop), Asp-Phe-Gly (DFG) motif was involved in ATP binding and located in the N-terminal region. Besides kinase domain, C-terminal has an autoinhibitory conserved domain with FXF motif (Fig. 3.4A). Phylogenetic relationship clustered all WNKs into 4 different clades (group I–IV) (Fig. 3.4B). The OsWNK1, OsWNK3, OsWNK7, and OsWNK8 along with AtWNK9 were grouped into group 1. In group II, cloned OsWNK9 showed perfectly clustering with OsWNK9 of subspecies Japonica. However, OsWNK4, OsWNK6, and OsWNK9 were clustered with AtWNK6, AtWNK7, AtWNK8, and AtWNK10. The top ranked threading fold of human WNK1 kinase domain (3fpqA) shared significant identity with the OsWNK9. I-TASSER server produced five top-ranked 3D homology models using humanWNK1 as a template. The model 1 with the highest C-score (−2.70) considered a model of best quality with a structural similarity between the predicted model and the native structure (TM-score 0.40 ± 0.14 and RMSD 13.5 ± 4.0Å) (Fig. 3.4C, D). The OsWNK9 model showed the presence of two lobed structure, N-lobe (amino lobe) and C- lobe (carboxy lobe). The N-lobe contained a 5-stranded beta sheet, an alpha helix (αC), which contributes to ATP

binding (Fig. 3.4C). The C-lobe made up of multiple alpha helices and the peptide substrate binding site (Fig. 3.4D). The N-terminal kinase domain made up of beta strands and the catalytic lysine residue (Lys-44) was present within the glycine motif (GXGXXK) in the beta strand (Fig. 3.4C). A novel C-terminal domain referred to auto-inhibitory domain (~60 AA) contain FXF motif is important for the auto-inhibition activity (Fig. 3.4D).

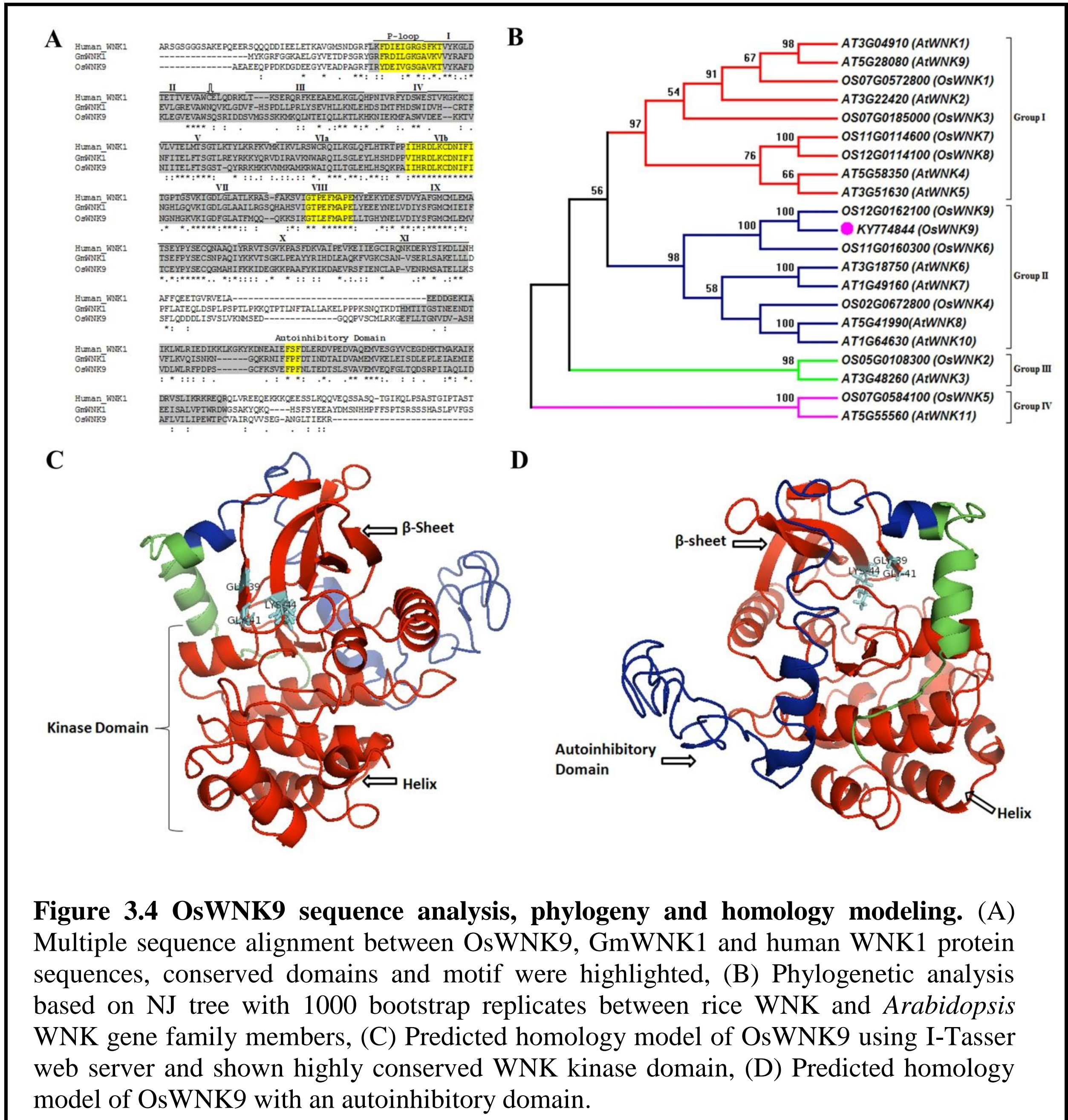


Figure 3.4 OsWNK9 sequence analysis, phylogeny and homology modeling. (A) Multiple sequence alignment between OsWNK9, GmWNK1 and human WNK1 protein sequences, conserved domains and motif were highlighted, (B) Phylogenetic analysis based on NJ tree with 1000 bootstrap replicates between rice WNK and *Arabidopsis* WNK gene family members, (C) Predicted homology model of OsWNK9 using I-Tasser web server and shown highly conserved WNK kinase domain, (D) Predicted homology model of OsWNK9 with an autoinhibitory domain.

3.3.3 Transgenic *Arabidopsis* plants showed better phenotype under salt, drought and ABA stress

To functionally characterize *OsWNK9*, we overexpressed full-length *OsWNK9* under a strong constitutive CaMV 35S promoter in *Arabidopsis* (Fig. 3.5A). Expression of the *OsWNK9* mRNA transcripts in 13 (T_4, T_5, T_6, T_7, T_8, T_9, T_{10}, T_{11}, T_{12}, T_{13}, T_{14}, T_{17}, T_{21}) T_2 generation lines of *Arabidopsis* was confirmed by performing semi-quantitative RT-PCR with gene specific primers. In wild type, *Arabidopsis* Col-0 showed no expression, whereas all the other all transgenic T_2 generation lines showed the expression of *OsWNK9* gene (Fig 3.5B). The four best independent T_2 generation homozygous lines (T_9, T_{11}, T_{12}, and T_{13}) were subsequently selected for further analysis. These four overexpressed (OE) lines along with control wild-type (WT) were evaluated for relative *OsWNK9* mRNA transcript levels by qRT-PCR (Fig. 3.5C). T_{13} line showed the highest transcript accumulation up to 1120 folds. The lines T_9, T_{11} and T_{12} showed 321, 401 and 517 folds levels of *OsWNK9* transcript accumulation respectively (Fig. 3.5C).

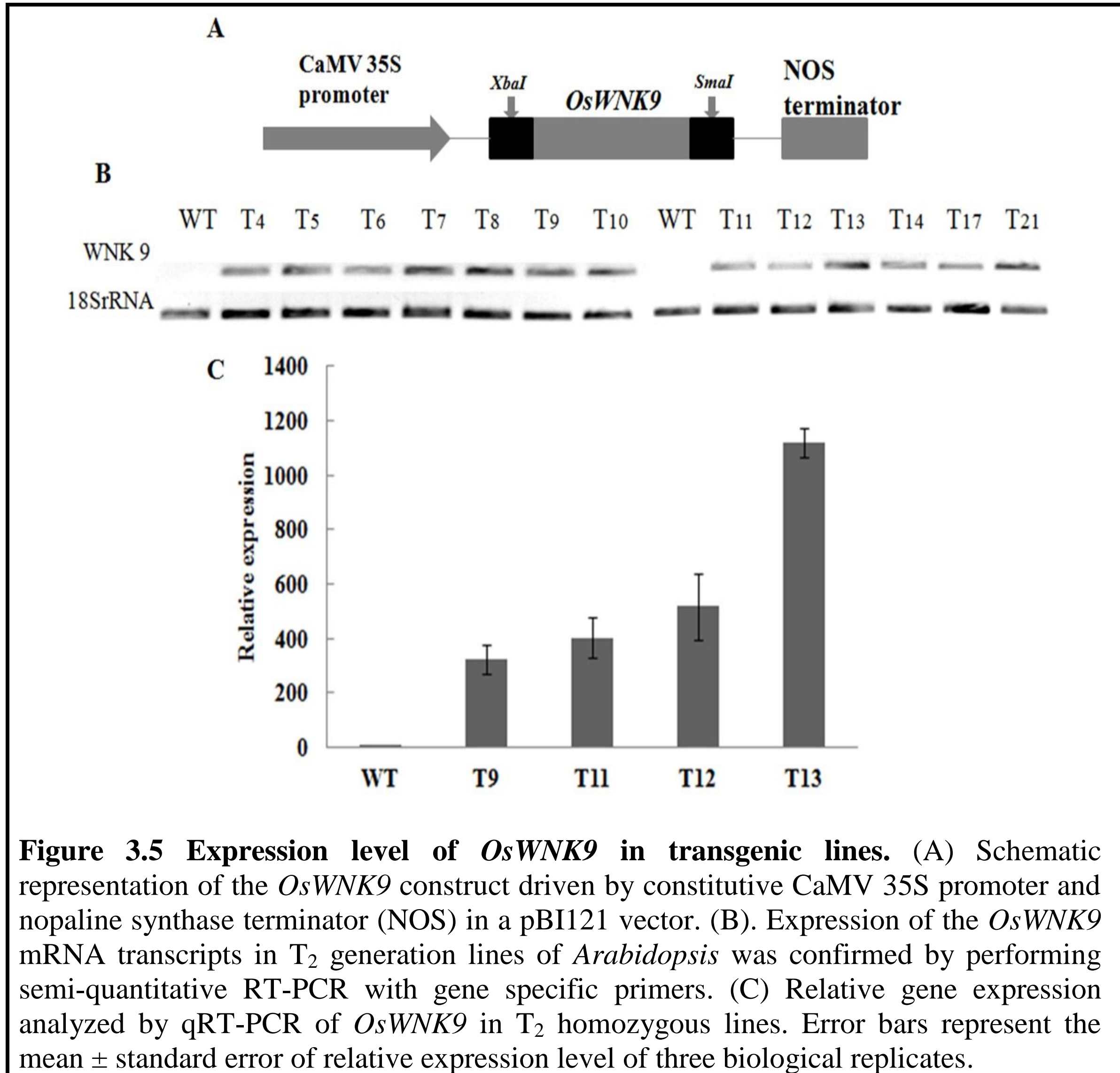


Figure 3.5 Expression level of *OsWNK9* in transgenic lines. (A) Schematic representation of the *OsWNK9* construct driven by constitutive CaMV 35S promoter and nopaline synthase terminator (NOS) in a pBI121 vector. (B). Expression of the *OsWNK9* mRNA transcripts in T_2 generation lines of *Arabidopsis* was confirmed by performing semi-quantitative RT-PCR with gene specific primers. (C) Relative gene expression analyzed by qRT-PCR of *OsWNK9* in T_2 homozygous lines. Error bars represent the mean ± standard error of relative expression level of three biological replicates.

The transgenic lines showed better phenotypes with green healthy shoots and better primary roots under various abiotic stress conditions compared to WT (Fig. 3.6). There were no phenotypic differences observed between WT and transgenic lines in controlled condition as shown (Fig. 3.6A). Further, phenotypic evaluation was performed on MS plates supplemented with 125mM salt stress and drastic phenotypic variation were observed between transgenic lines and WT. Transgenic lines showed significantly higher growth of the roots and shoots compared to WT (Fig. 3.6B). Interestingly, under salt

stress, WT were affected severely and showed stunted roots and shoot growth, chlorosis and wilting of leaves as compared to transgenic lines. These result demonstrated that *OsWNK9* played important roles during salinity stress and significantly improved plant phenotypes. Similarly, drought is simulated by adding mannitol, sorbitol, or polyethylene glycol, which lowers the water potential of the medium (Lopez and Jauregui, 2005). The transgenic lines showed the vigorous growth with green rosette, while in a WT rosette was stressed and damaged in drought stress (Fig. 3.6C). ABA is a well-known for multiple functions as it maintain seed dormancy, stress and inhibits the root growth of plants. The growth medium supplemented with ABA has a significant effect on the phenotype of *Arabidopsis* roots (Verslues et al., 2006). Further, we investigated the response of overexpression of *OsWNK9* under ABA and observed healthy and vigorous leaves and enhanced root length compared to WT (Fig. 3.6D). It reflects that OsWNK9 respond to exogenous ABA and improved transgenic lines phenotype compared to WT.

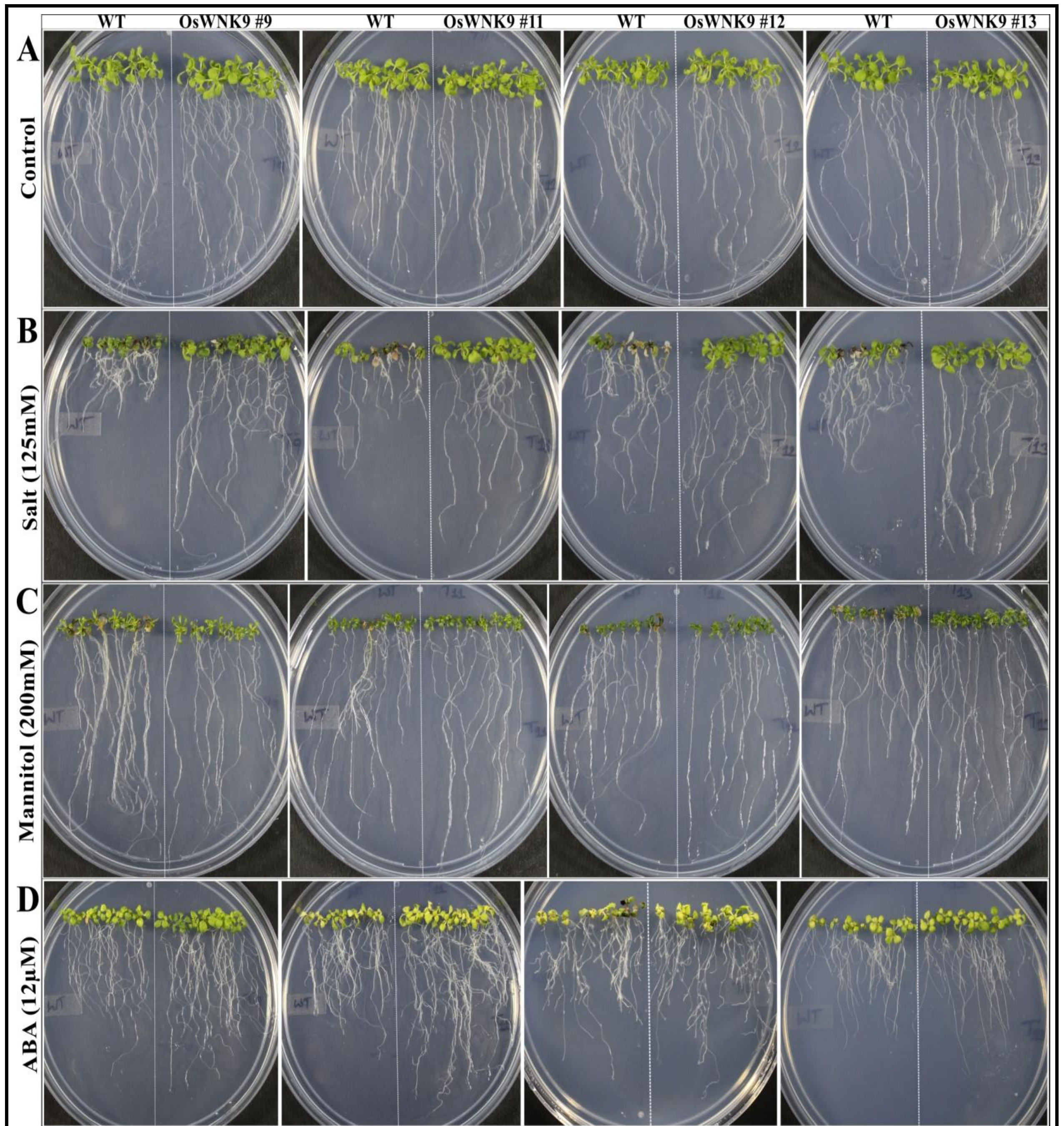


Figure 3.6 Abiotic stress tolerant phenotype of *Arabidopsis* expressing *OsWNK9*. *Arabidopsis* transgenic lines 9, 11, 12 and 13 expressing *OsWNK9* as compared with WT. The 6 days old seedlings were grown for 2 weeks on MS medium supplemented with indicated concentrations (A) Control (MS), (B) Salt (125mM NaCl), (C) Mannitol (200mM Mannitol) and (D) ABA (12μM ABA). Each experiment was conducted for three times with biological replicates and displayed the representative phenotype results.

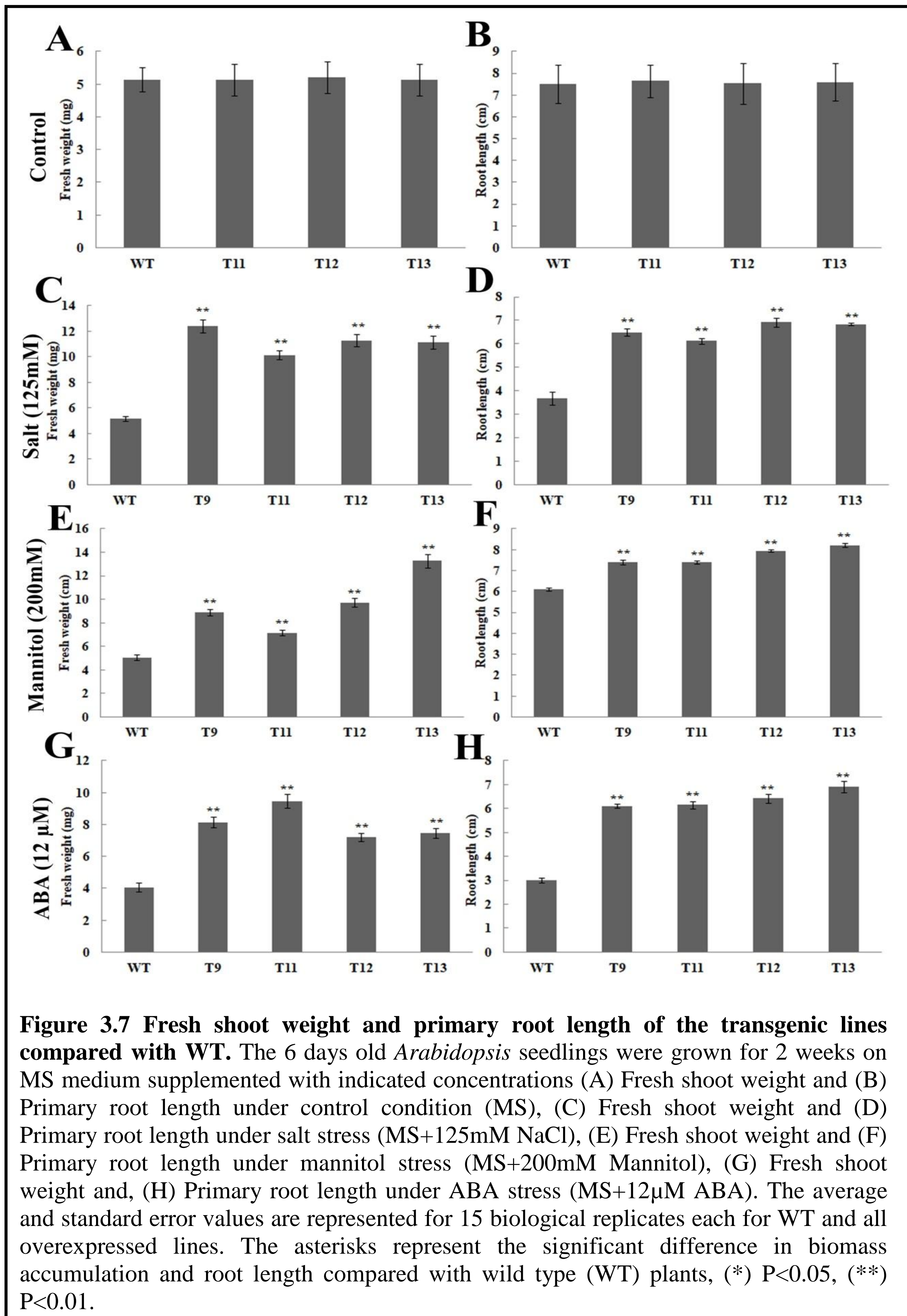


Figure 3.7 Fresh shoot weight and primary root length of the transgenic lines compared with WT. The 6 days old *Arabidopsis* seedlings were grown for 2 weeks on MS medium supplemented with indicated concentrations (A) Fresh shoot weight and (B) Primary root length under control condition (MS), (C) Fresh shoot weight and (D) Primary root length under salt stress (MS+125mM NaCl), (E) Fresh shoot weight and (F) Primary root length under mannitol stress (MS+200mM Mannitol), (G) Fresh shoot weight and, (H) Primary root length under ABA stress (MS+12µM ABA). The average and standard error values are represented for 15 biological replicates each for WT and all overexpressed lines. The asterisks represent the significant difference in biomass accumulation and root length compared with wild type (WT) plants, (*) $P<0.05$, (**) $P<0.01$.

The WT and transgenic lines did not show any significant difference in fresh weight and root length under stress free conditions (Fig. 3.7A-B). The transgenic lines showed most significant results approximately 2-fold higher fresh biomass and root length as compared to WT in salt stress (Fig.3.7C-D). Similarly, under drought stress, transgenic lines showed significantly higher biomass and primary root length compared to WT (Fig. 3.7E-F). Under ABA stress, transgenic lines exhibited 2-fold increased fresh weight in all four lines (Fig. 3.7G) and primary root length higher than WT plants (Fig. 3.7G-H). Overall overexpressed lines showed significant improvement of fresh biomass and primary root length under abiotic stresses.

3.3.4 Overexpression of *OsWNK9* in *Arabidopsis* conferred increased tolerance to salt, drought, and ABA stress

Here, we tested the tolerance of transgenic seeds to salt, mannitol and ABA stress based on the percentage of radicle emergence, green cotyledon, water loss, drought tolerance and chlorophyll content.

3.3.4.1 Seed germination

The radicle emergence and green cotyledons both are equally informative to evaluate tolerance assay. The germination rate of transgenic line and WT seeds have not shown any difference under controlled condition on MS medium plate. Percentage of radicle emergence under 75mM salt stress showed 12-20% emergence in all three lines (T_{11}, T_{12}, and T_{13}) but WT showed only 6% emergence on the 3rd day (Fig.3.8A). On the 9th day, radicle emergence reached up to 91.97% in transgenic lines, while WT showed 79%.

However, under 100mM salt stress, on the 9th day, 69-88% radicle emergences were observed in transgenic seeds and only 57% was observed in WT (Fig. 3.8B). Under drought stress with mannitol (100mM and 200mM) stress, at 9th day there was not much significant radicle emergence difference observed between transgenic and WT (Fig. 3.8C-D). A similar result was observed under ABA (0.5μM), where radicle emergence on 4th day was around 34-46% in transgenic seeds and 28% in WT. At 2μM ABA, on 9th day ~90% radicle was emerged in transgenic seeds and 88% for WT (Fig. 3.8E-F). Simultaneously, we also recorded the percentage of green cotyledon germination under salt, mannitol and ABA stress. Under 75mM NaCl treatment, on 10th days in WT, 71% of seeds showed green cotyledon, while 75%, 81% and 89% of the T_{11}, T_{12}, and T_{13} transgenic seeds showed green cotyledons respectively (Fig. 3.9A). Similarly, under 100mM salt stress, first green cotyledons were observed in transgenic lines on the 6th day with 9-12% emergence and WT showed 5% cotyledon emergence. On 10th day, WT showed only 40% green cotyledons while, transgenic seeds of T_{11}, T_{12} and T_{13} showed 46%, 52% and 59% green cotyledons respectively (Fig. 3.9B). Under 100mM mannitol stress, on 10th day, WT showed 89% cotyledon, while transgenic seeds showed 95-97% green cotyledons (Fig. 3.9C). In contrast, under 200mM mannitol stress, green cotyledons emergence reduced up to 21% for transgenic seeds and 9% for WT on the 5th day (Fig. 3.9D). Surprisingly, on 10th day, all transgenic seeds showed 79-84% green cotyledons, while WT showed 70% green cotyledon (Fig. 3.9D).

The ABA hormone inhibits and delays the germination process as shown (Fig. 3.9E-F). Green cotyledons emergence was recorded on the 7th and 11th day under 0.5μM and 2μM ABA stress respectively (Fig. 3.9E-F). Under ABA of 0.5μM concentration,

8% green cotyledons were observed in WT and 11-15% was recorded in transgenic seeds (Fig. 3.9E). On 12th day green cotyledon was observed up to 47% in WT and 67-75% in transgenic seeds. Similarly, under 2μM ABA concentration, less than 10% green cotyledons were observed in all seeds on 11th day of seed germination (Fig. 3.9F). It indicated that exogenous ABA treatment delayed transgenic and WT seeds germination process compared to salt and mannitol stress.

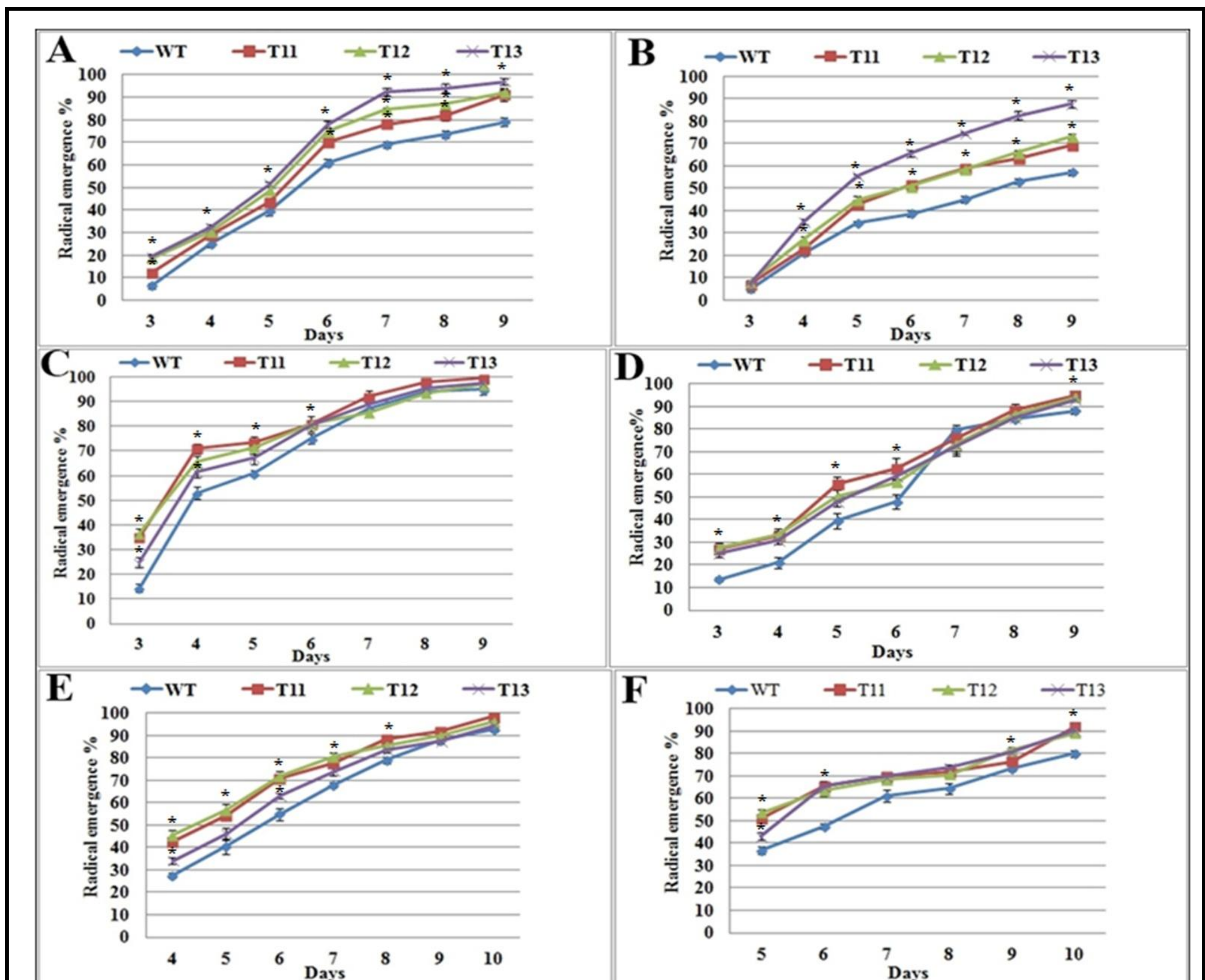


Figure 3.8 Assessment of seed germination under abiotic stresses. The percentage of the radical emergence of WT and transgenic lines were calculated under different abiotic stress conditions. (A) 75mM NaCl, (B) 100mM NaCl, (C) 100mM Mannitol, (D) 200mM Mannitol (E) 0.5μM ABA and (F) 2μM ABA. Percentages were determined in relation to the total number of sown seeds. Error bars represent SEM (n=4 plates with ~50 seedlings each). The asterisks represent the significant difference in seed germination of transgenic lines compared with WT plants, (*) $P<0.05$.

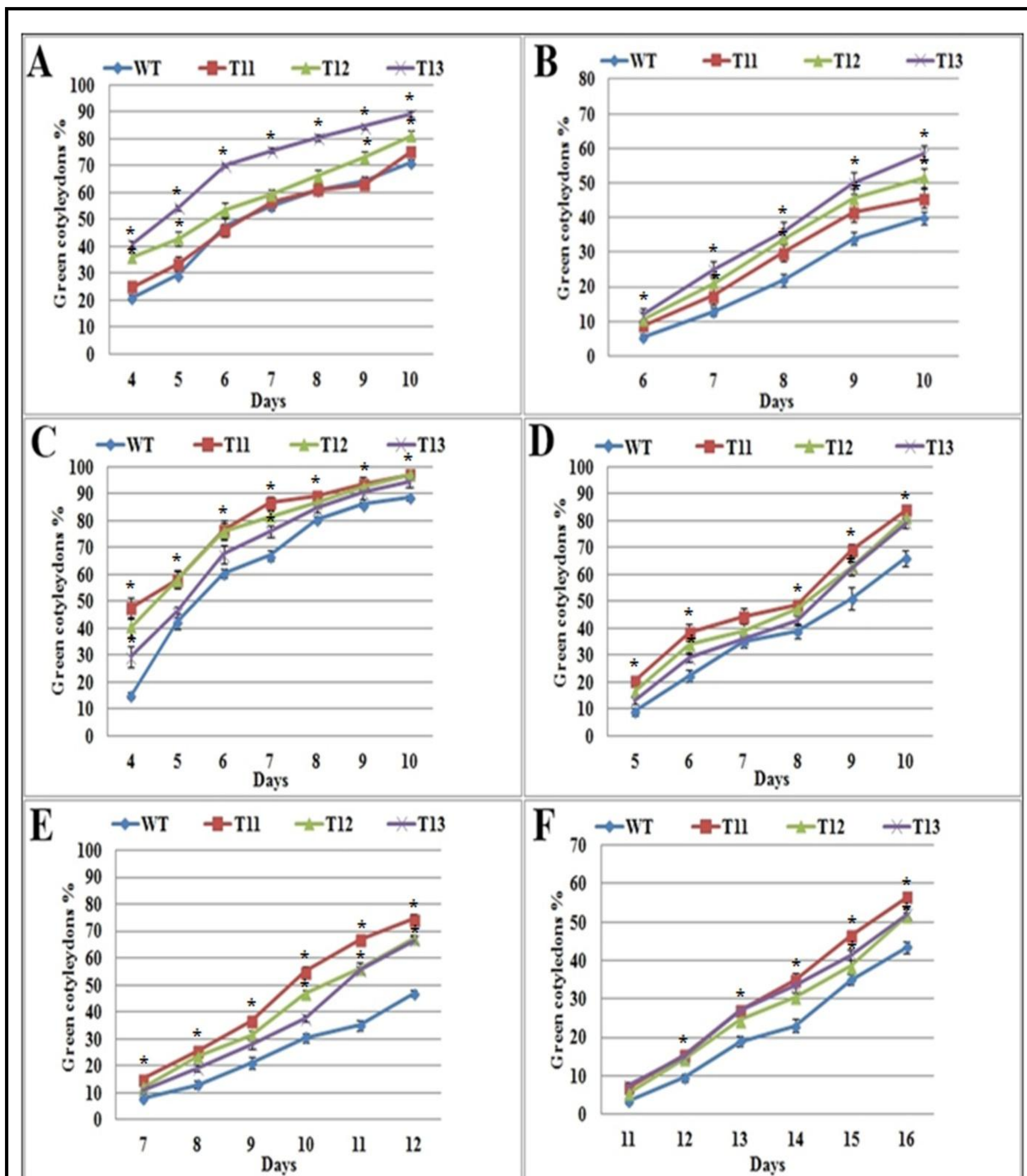


Figure 3.9 Assessment of seed germination under abiotic stress. The green cotyledons percentage of WT and transgenic lines 11, 12 and 13 were calculated under different abiotic stress conditions. (A) 75mM NaCl, (B) 100mM NaCl, (C) 100mM mannitol, (D) 200mM mannitol (E) 0.5µM ABA and (F) 2µM ABA. Percentages were determined in relation to the total number of sown seeds. Error bars represent the mean ± standard error of four biological replicates (n=4, plates with ~50 seedlings each). The asterisks represent the significant difference in seed germination of transgenic lines compared with WT plants, (*) $P<0.05$.

3.3.4.2 Water loss assay

During water stress condition plant keeps their leaf water potentials above the soil water potentials through shut off stomata and consequently reduced photosynthesis as well as transpiration activity (Leung and Giraudat, 1998). The variation in drought tolerance of transgenic plants could be attributed to changes in transpiration rate. To test this hypothesis, we performed leaf water loss assay between WT and transgenic plants. After detaching leaves, WT plant lost more than 50% water, while transgenic plants (T_9, T_{11}, and T_{12}) lost ~22% water and T_{13} line lost 15% water in 1hr (Fig. 3.11). After 12 hrs, WT lost almost 96% water but T_9, T_{11}, T_{12} and T_{13} lines showed water loss up to 78%, 71%, 78% and 59% respectively (Fig. 3.10 & 3.11). The result indicated that transgenic plants retained more water in drought conditions and plays an important role during drought tolerance.

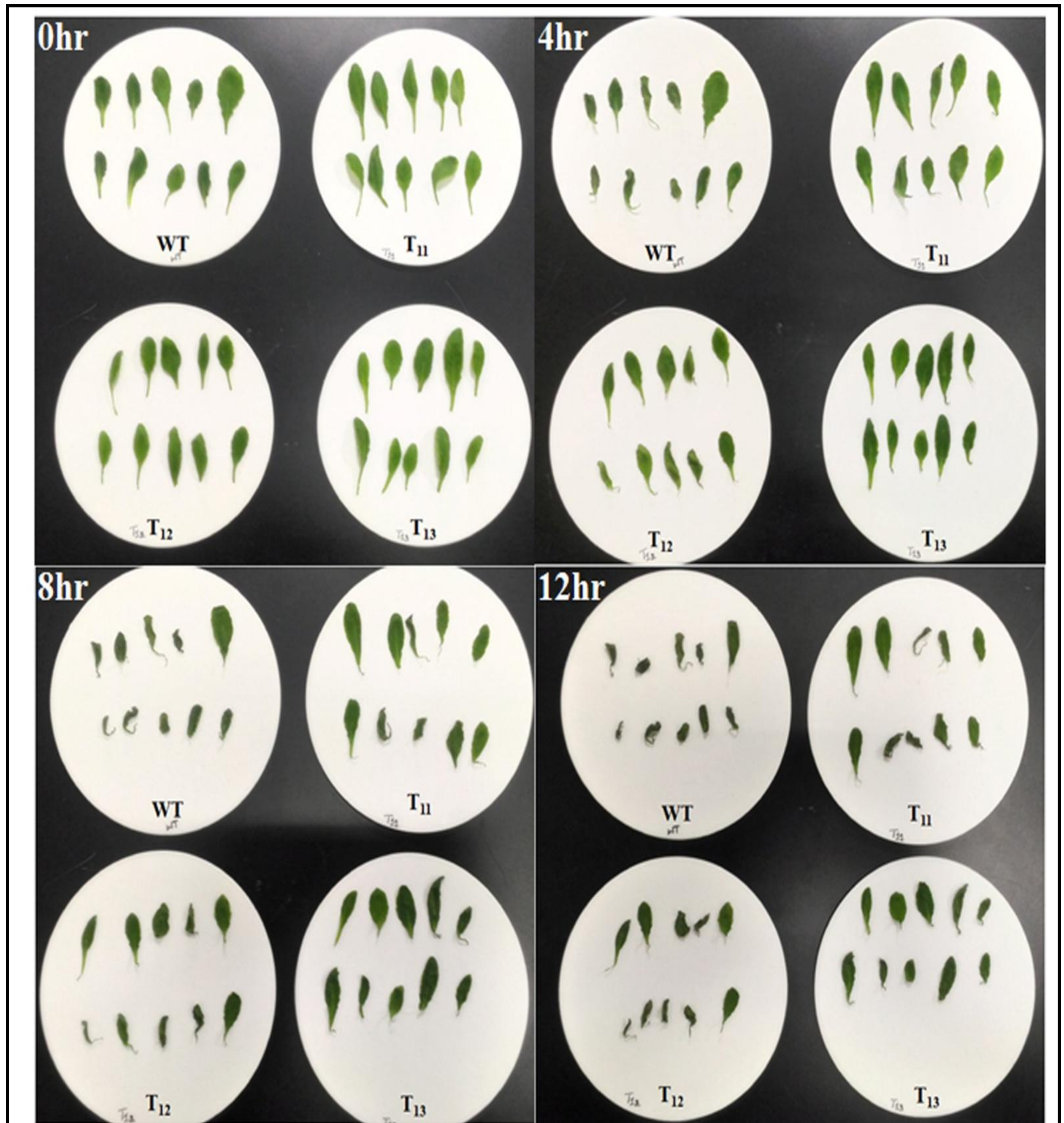


Figure 3.10 Phenotypic differentiation of the water loss from the detached leaves of the WT and transgenic lines at different time intervals. The detached leaves from 4-week-old plants were placed on filter papers with the abaxial side of the leaf under temperature-controlled growth chamber.

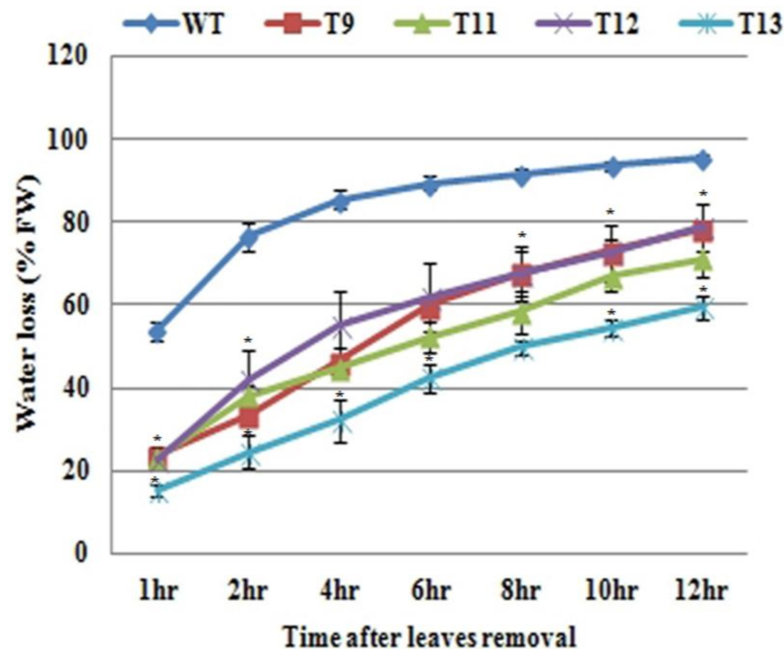


Figure 3.11 Water loss assay. Time course of the water loss from the detached leaves of the transgenic lines and WT. The water loss was expressed as a percentage of the initial fresh weight (FW) at indicated time intervals. The values are presented as an average and standard error (SE) of four biological replicates. The asterisks represent the significant difference of transgenic lines compared with WT plants, (*) $P<0.05$.

It was expected that *OsWNK9* transgenic lines may have improved the tolerance against drought stress. To test this, WT and transgenic lines (T_{11}, T_{12}, and T_{13}) were grown under normal growth conditions for three weeks. No plants showed obvious morphological or developmental abnormalities before drought treatment (Fig. 3.12A). Thereafter, plants were challenged with drought by depriving water for 2 weeks and were photographed. When subjected to drought stress, the wild-type plants were wilted and showed an impaired morphology compared to transgenic lines (Fig. 3.12B). After stopping watering for 2 weeks, plants were again watered for 3 weeks and checked for revival. Moreover, transgenic lines showed healthy and green leaves with inflorescence compared to WT leaves which showed wilting symptoms (Fig. 3.12C). This strongly

suggests that the *OsWNK9* transgenic plants, as opposed to the WT plants, were tolerant to drought stress.

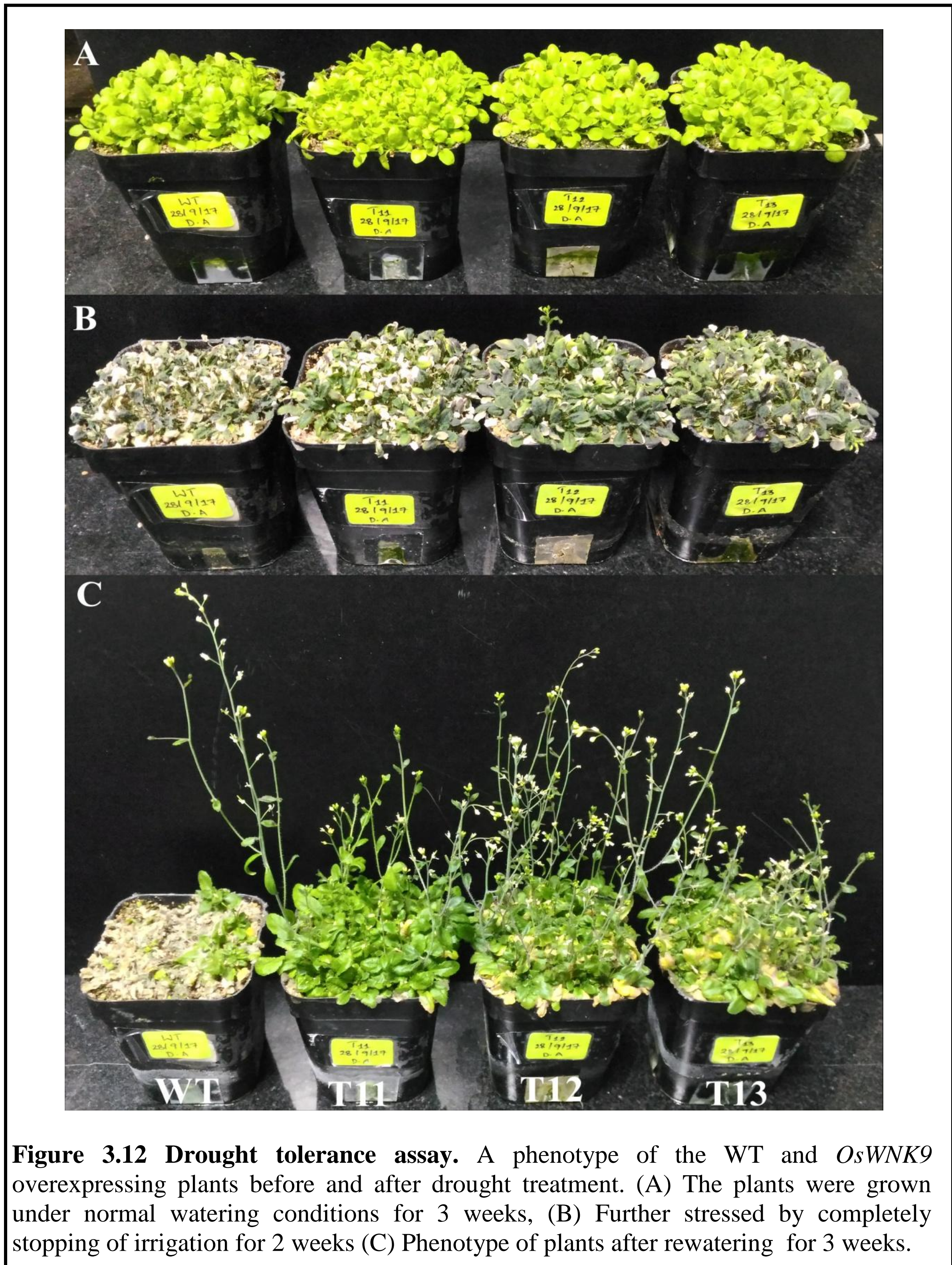


Figure 3.12 Drought tolerance assay. A phenotype of the WT and *OsWNK9* overexpressing plants before and after drought treatment. (A) The plants were grown under normal watering conditions for 3 weeks, (B) Further stressed by completely stopping of irrigation for 2 weeks (C) Phenotype of plants after rewatering for 3 weeks.

3.3.4.3 Chlorophyll content

Salinity stress could affect the chlorophyll content by modulating chlorophyll synthesis pathways or promoting its degradation, which leads to reducing photosynthetic capacity (Kiani and Rasouli, 2014). Here we used four transgenic lines for chlorophyll retention assay. The chlorophyll retention of transgenic lines and WT were measured from leaves of 4-week old plants. In the control experiments, the chlorophyll content between transgenic and WT plants were almost equal. The result demonstrated that chlorophyll content of 4-week-old transgenic plants was higher than those of WT (Fig. 3.13). Further, the measurement of chlorophyll content after 72 h of treatment with 100mM and 150mM salt concentrations were shown significant variation in chlorophyll content. Under 100mM salt stress, WT showed 6.4 μg/mg of protein of chlorophyll content while T_9 and T_{13} lines showed 2-fold and 2.8-fold higher chlorophyll content compared to WT respectively. At 150mM salt stress, WT showed 6μg/mg of chlorophyll content while T_9 and T_{13} showed 2.6-fold and 2.1-fold higher chlorophyll content (Fig. 3.13). Interestingly the loss of chlorophyll content was lower in the transgenic lines compared to WT. Further, measurement of chlorophyll a, chlorophyll b separately suggested that the transgenic lines were playing a better role under salt stress conditions (Fig. 3.14A-B).

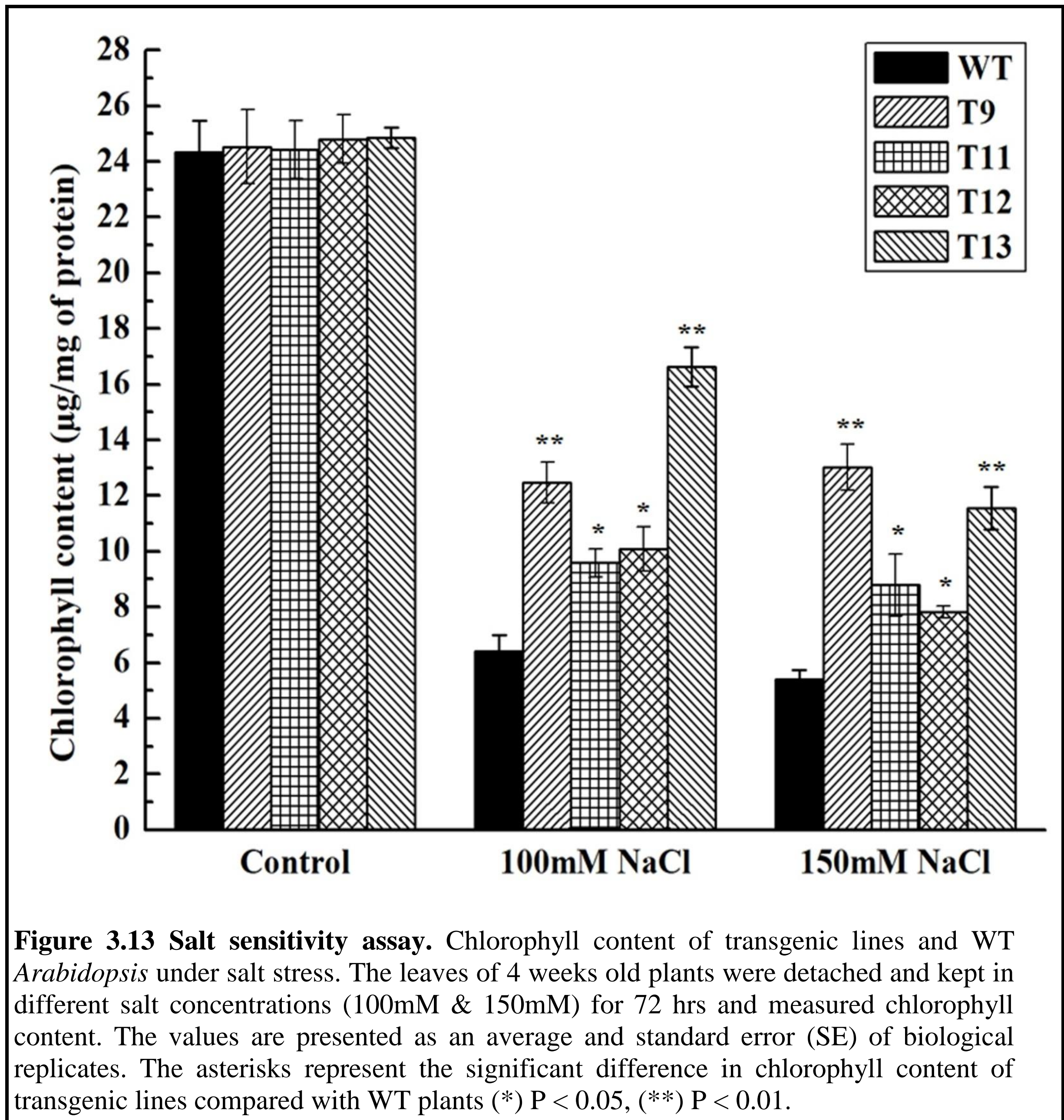
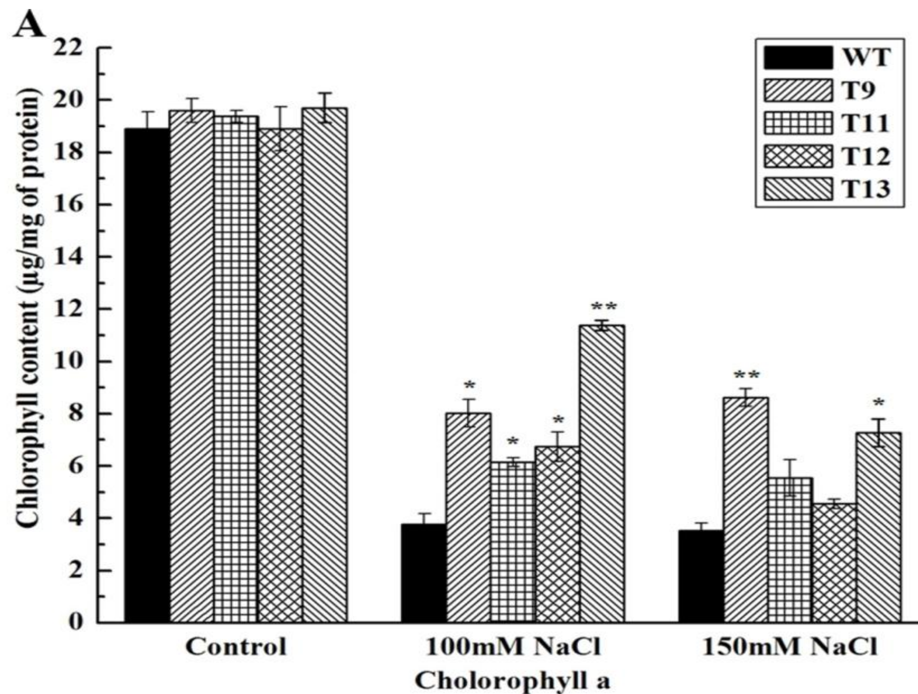


Figure 3.13 Salt sensitivity assay. Chlorophyll content of transgenic lines and WT *Arabidopsis* under salt stress. The leaves of 4 weeks old plants were detached and kept in different salt concentrations (100mM & 150mM) for 72 hrs and measured chlorophyll content. The values are presented as an average and standard error (SE) of biological replicates. The asterisks represent the significant difference in chlorophyll content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.



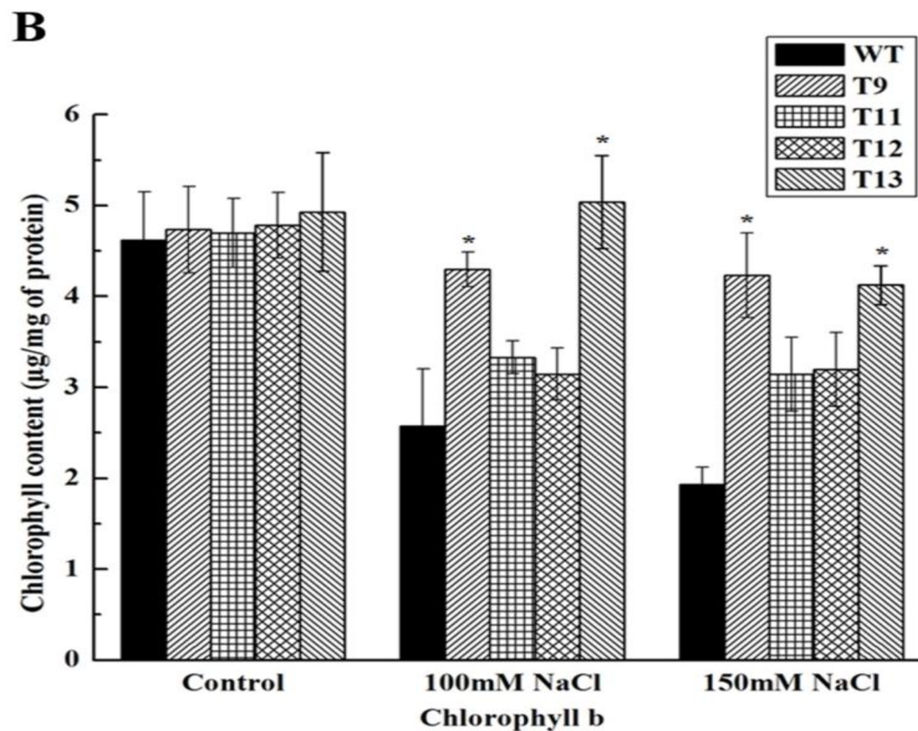


Figure 3.14 Effect on chlorophyll content of transgenic lines and WT *Arabidopsis* plants exposed to different salt concentrations. The leaves of four week old plants were detached and kept in a Petri dish with different salt concentrations (100mM & 150mM) for 72 hrs and measured chlorophyll content, (A) chlorophyll a, (B) chlorophyll b. The values are presented as an average and standard error (SE) of triplicates. The asterisks represent the significant difference in chlorophyll content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.

3.3.5 OsWNK9 regulates the transcription of salt, drought and ABA stress related genes

The mechanism and regulation pathway of *OsWNK9* is unknown. In order to understand their roles, we performed expressing profiling of transcription factors related to salinity, drought, and genes related to ABA signaling and biosynthesis. The T_{13} transgenic line showed a significant response to various abiotic stresses and exhibited 1120 fold higher expression of *OsWNK9* henceforth further experiments performed using T_{13} transgenic line along with WT. The expression level of drought inducible gene *AtRD22* increased significantly up to 13-folds at 12 h in transgenic line under salt, drought and ABA stress (Fig. 3.15, 3.16 & 3.17). The dehydration responsive element-binding protein (*DREB2C*) transcription factor showed increase expression pattern under salt, drought and ABA stress in transgenic lines compared to WT. The transcript accumulation of *AtWRKY28* showed significant up regulation pattern under salt, drought and ABA stress. The most significant expression pattern of *AtNAC019* was observed under salt stress up to 9.3-folds (Fig 3.15) and under ABA stress upto 22 folds (Fig 3.17). Under drought stress, *AtEREBP* were significantly up regulated in transgenic line compared to WT (Fig 3.16). Moreover, ABA3 plays important regulatory role during ABA biosynthesis and considered as a stress-responsive gene. The expression level of *AtABA3* was recorded higher in transgenic line under salt, drought and ABA stresses compared to WT. The expression level of *AtNCED3* under salt, drought and ABA stress was up regulated in transgenic lines (Fig. 3.15, 3.16 & 3.17). Compilation of this expression result helped us to understand the roles of *OsWNK9* OE lines under salt, mannitol and ABA stress. It

might be possible that *OsWNK9* regulate abiotic stress tolerance through ABA dependant signaling pathway and improved stress tolerance.

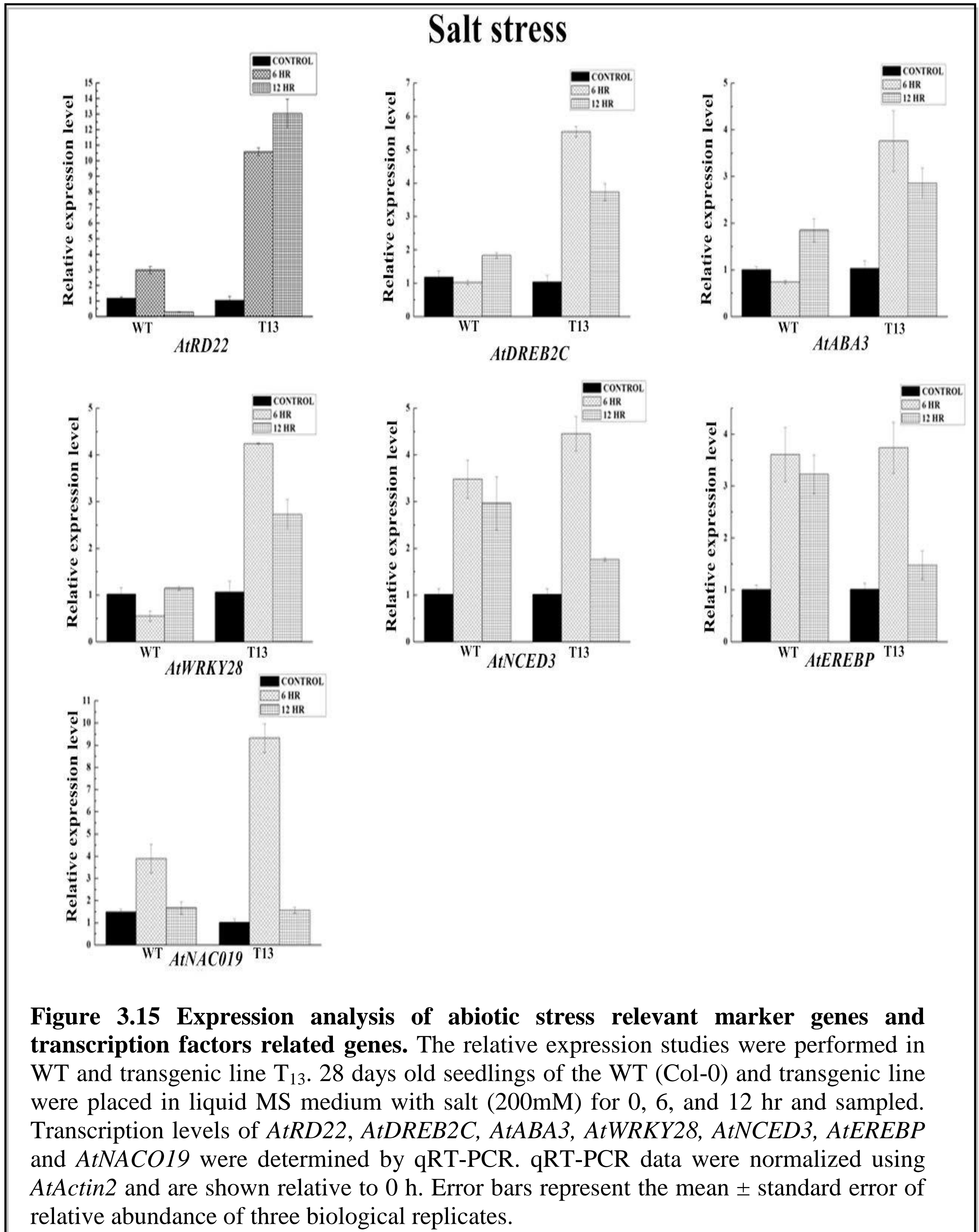


Figure 3.15 Expression analysis of abiotic stress relevant marker genes and transcription factors related genes. The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and transgenic line were placed in liquid MS medium with salt (200mM) for 0, 6, and 12 hr and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and *AtNACO19* were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.

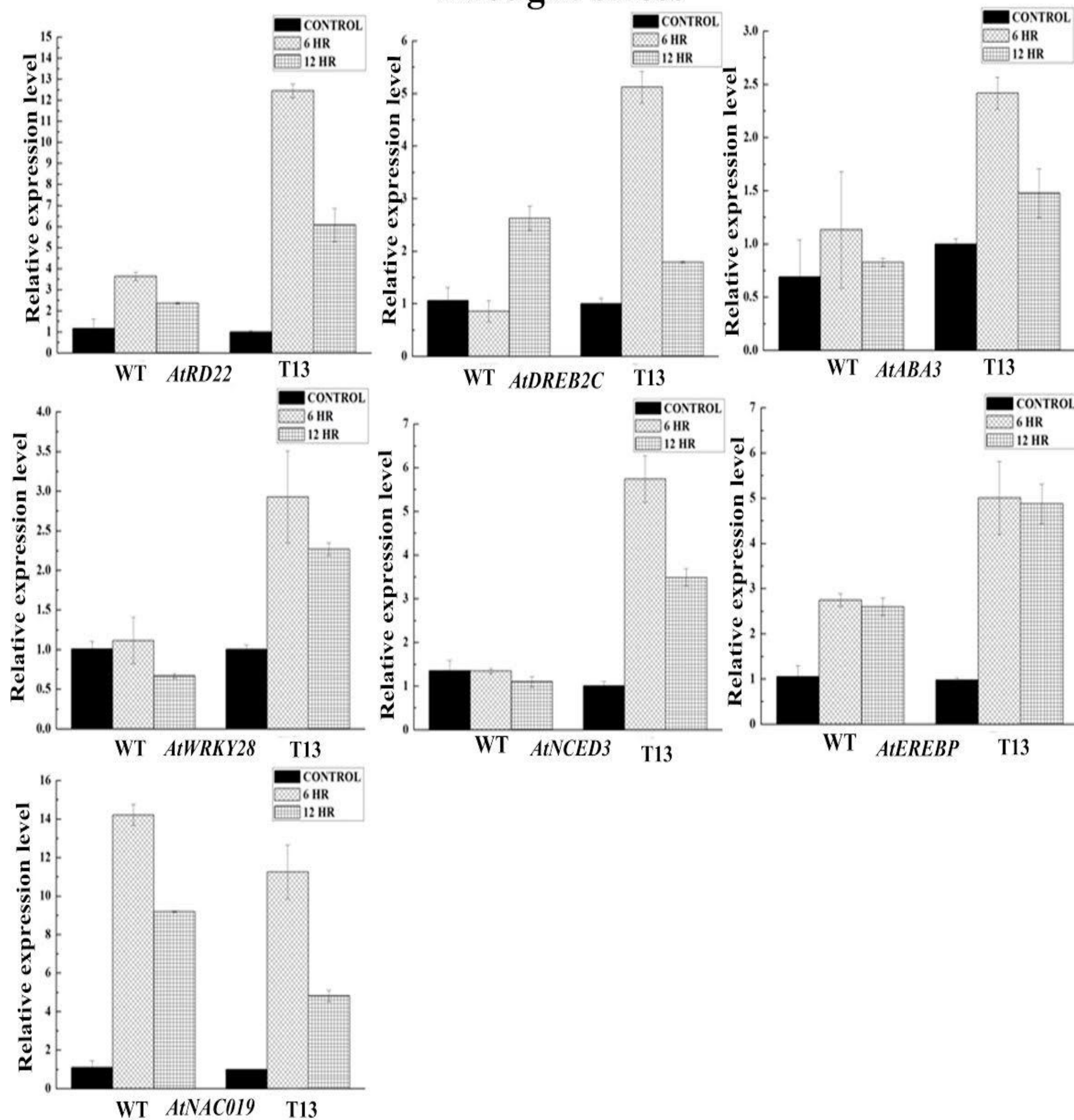


Figure 3.16 Expression analysis of abiotic stress related marker genes and transcription factors related genes. The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and the transgenic line was placed in liquid MS medium with mannitol (300mM) for 0, 6, 12 hr and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and *AtNACO19* were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.

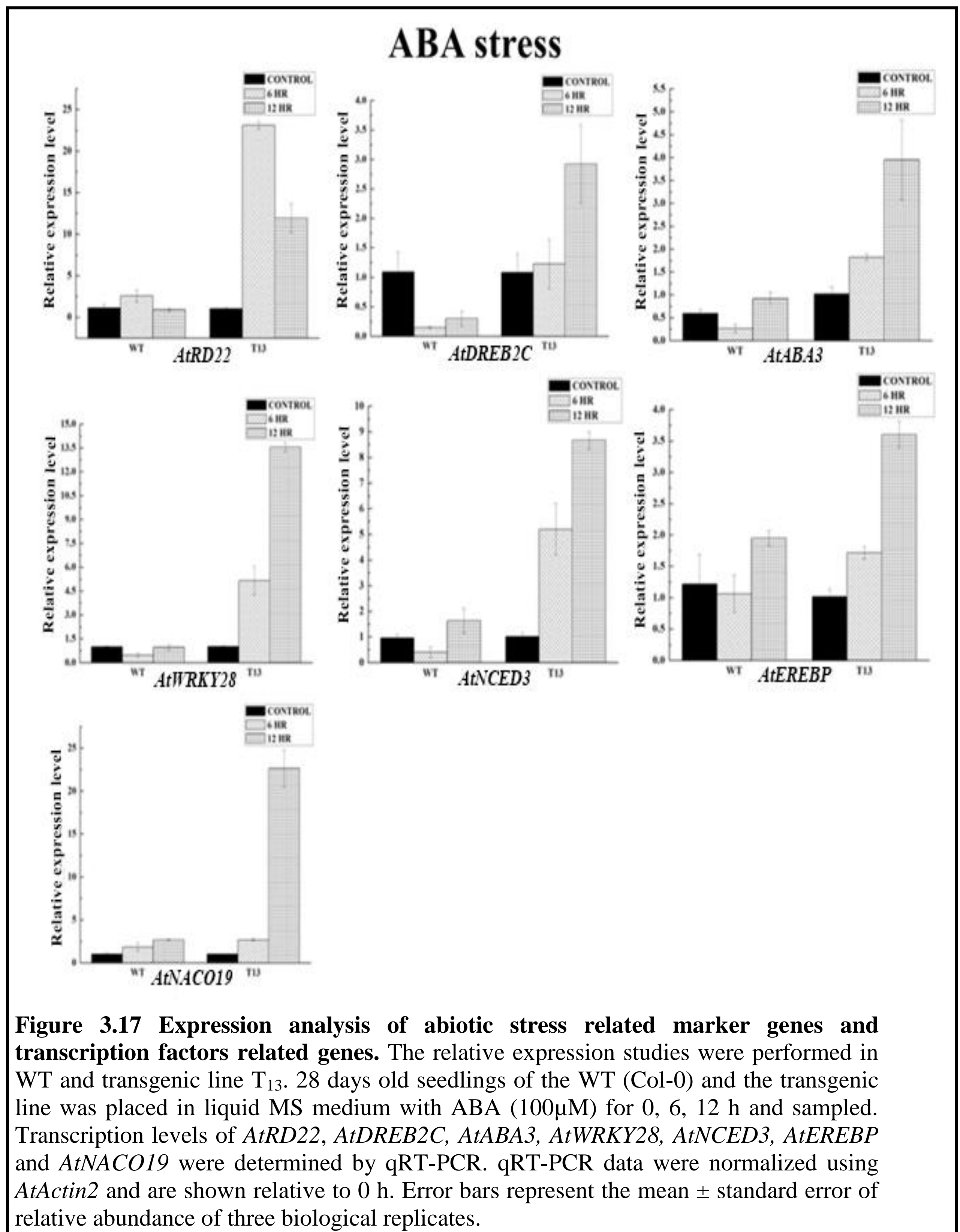
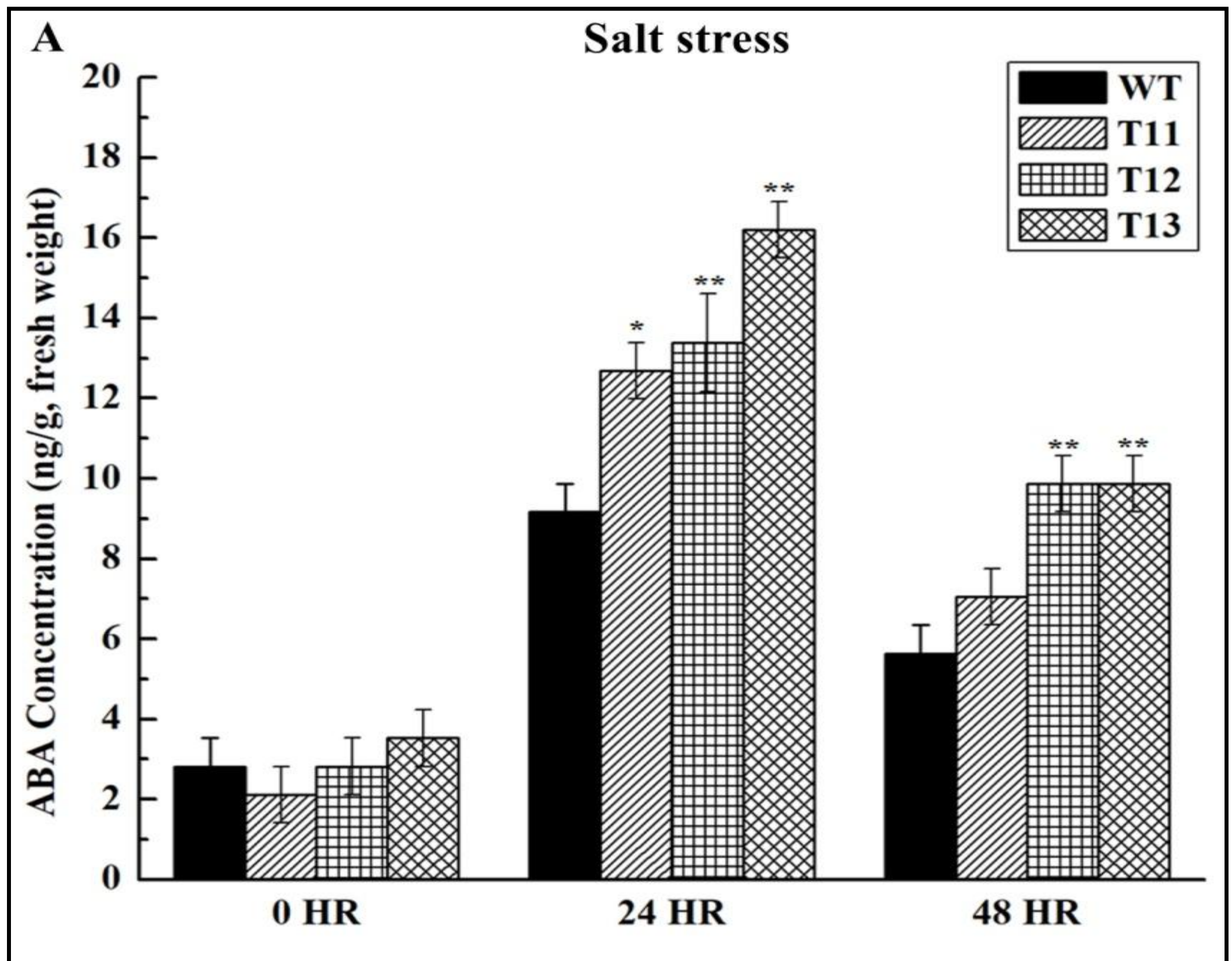


Figure 3.17 Expression analysis of abiotic stress related marker genes and transcription factors related genes. The relative expression studies were performed in WT and transgenic line T_{13}. 28 days old seedlings of the WT (Col-0) and the transgenic line was placed in liquid MS medium with ABA (100μM) for 0, 6, 12 h and sampled. Transcription levels of *AtRD22*, *AtDREB2C, AtABA3, AtWRKY28, AtNCED3, AtEREBP* and *AtNACO19* were determined by qRT-PCR. qRT-PCR data were normalized using *AtActin2* and are shown relative to 0 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates.

3.3.6 Enhanced ABA level in transgenic lines under salt and drought stress

The endogenous ABA concentration was determined in salt and drought stresses. The ABA level under salt and drought stress in transgenic lines was significantly higher than WT. The highest concentration of ABA in salt stress was 16 ng/g of fresh weight in T_{13} line at 24 hr and at the same time point in WT it was only 9 ng/g of fresh weight. Interestingly under drought stress in transgenic line T_{13} ABA level was 30 ng/g of fresh weight (Fig. 3.18). The results showed an average of three biological replicates.



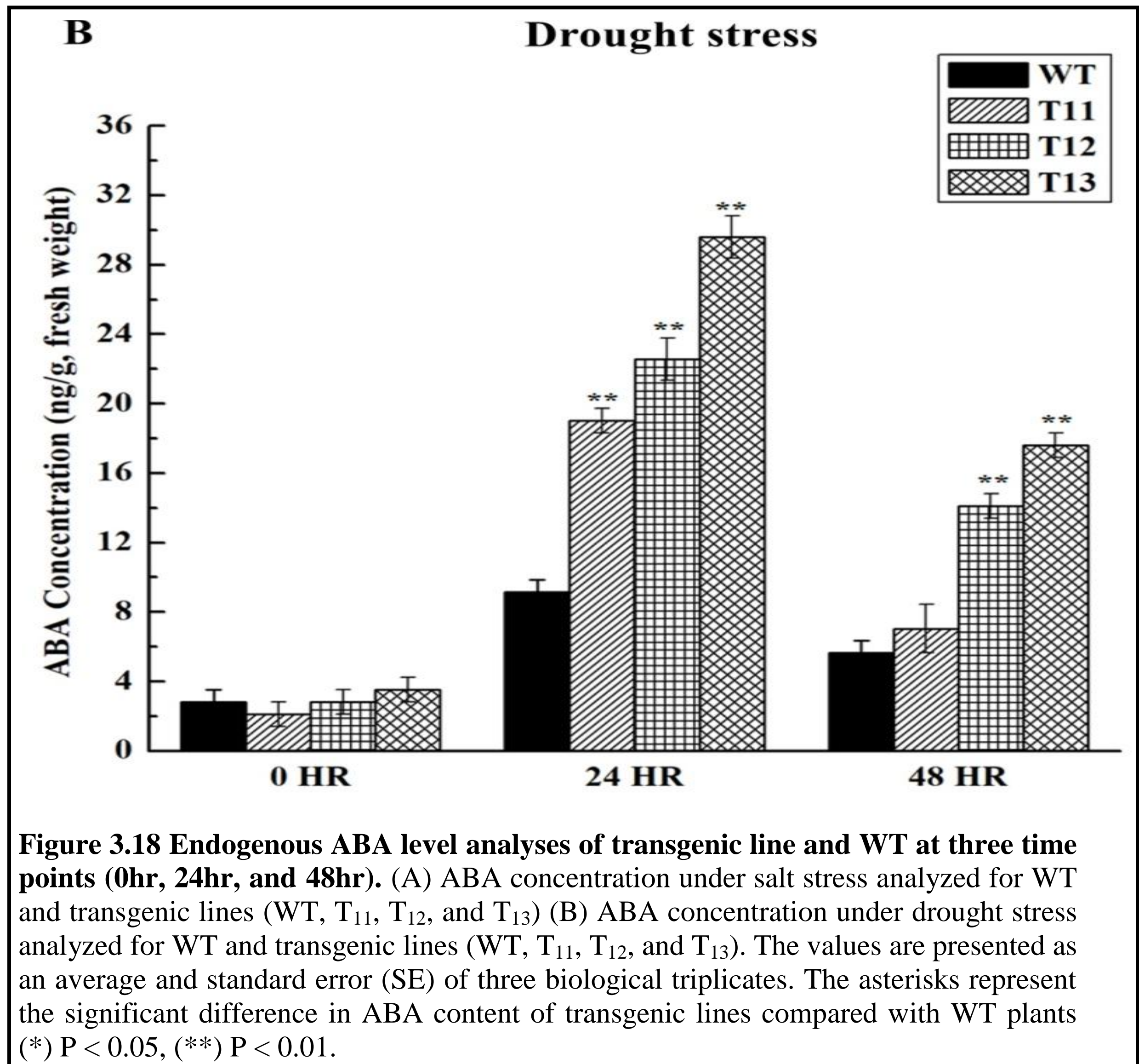


Figure 3.18 Endogenous ABA level analyses of transgenic line and WT at three time points (0hr, 24hr, and 48hr). (A) ABA concentration under salt stress analyzed for WT and transgenic lines (WT, T_{11}, T_{12}, and T_{13}) (B) ABA concentration under drought stress analyzed for WT and transgenic lines (WT, T_{11}, T_{12}, and T_{13}). The values are presented as an average and standard error (SE) of three biological triplicates. The asterisks represent the significant difference in ABA content of transgenic lines compared with WT plants (*) $P < 0.05$, (**) $P < 0.01$.

3.3.7 Tissue specific expression of *OsWNK9* in rice

In rice plant, to determine the tissue-specific expression of *OsWNK9* gene, we generated transgenic lines of rice (Pusa Basmati-1) carrying the GUS gene fused to the 1 kb promoter region of OsWNK9 (Fig 3.2 & 3.19A). The transformed lines were confirmed by semi-quantitative PCR using the GUS specific gene primer (Fig 3.19L). The histochemical analysis detected GUS staining in the various organs of transformed lines such as young seedlings (Fig. 3.19B), young leaf (Fig. 3.19C), flag leaf (Fig. 3.19D),

matured leaf (Fig. 3.19E), transformed callus (Fig. 3.19F), spikelet (Fig. 3.19G), shoots and roots (Fig. 3.19H & I). However, the GUS staining was also observed in reproductive organs such as pollen (Fig. 3.19J) and female gametophyte (Fig. 3.19K). Interestingly the GUS activity was found more in roots (Fig. 3.19I) which correlates with the transcript abundance of our data shown in Figure 3.3B, the previous reports showed that *GmWNK1* was mostly expressed in roots (Wang et al., 2010). The female gametophyte region was shown more expression than pollen it concludes that OsWNK9 was more expressive in female gametophyte. Overall from the callus to seed stage almost in all organs of rice the OsWNK9 was expressive in all different developmental stages of leafs such as young, mature and flag leafs. It could be possible that, OsWNK9 plays important role during signalling pathways and it may regulate various functions such as tolerance to abiotic stress.

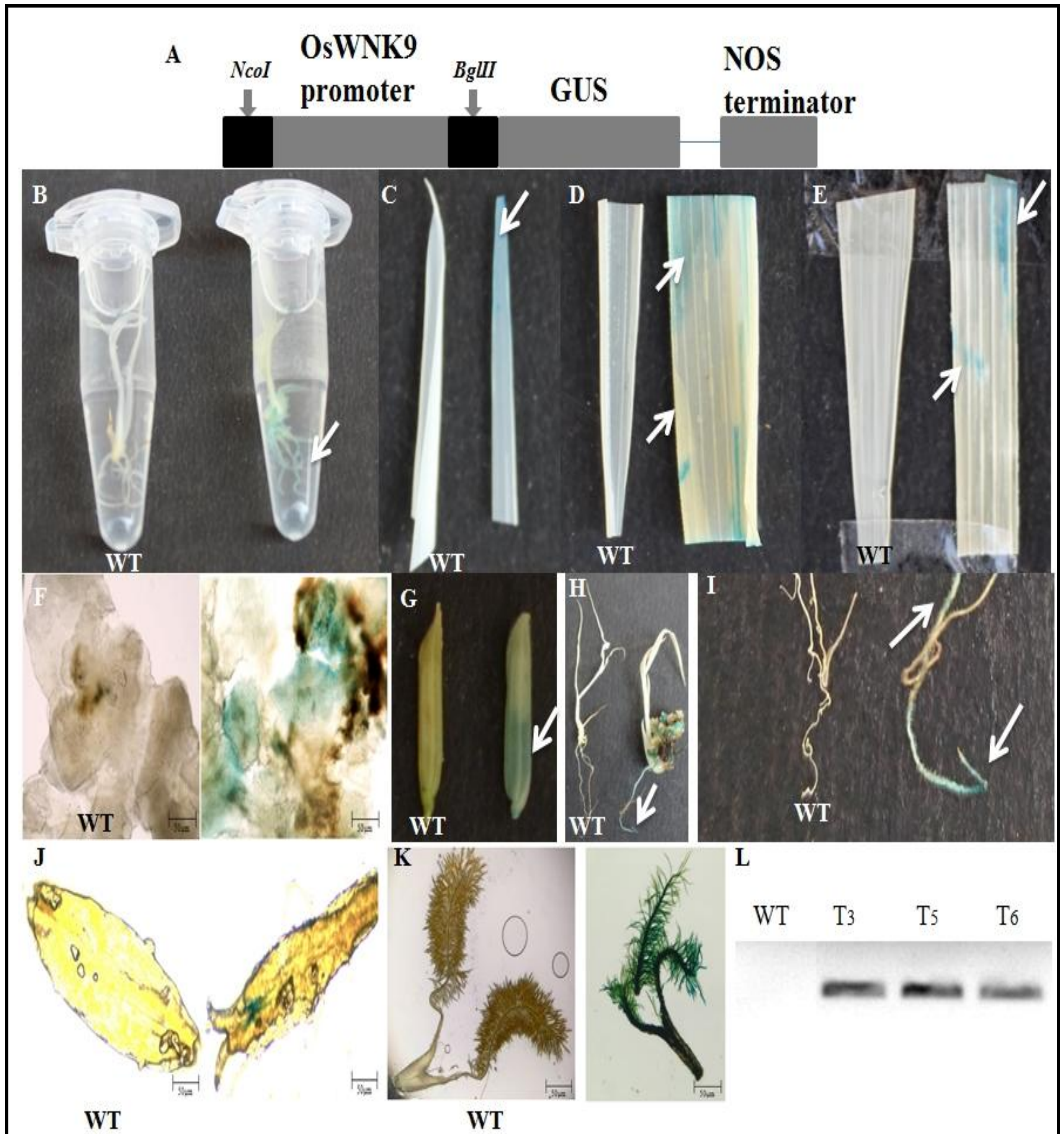


Figure 3.19 Histochemical GUS assay. Histochemical analysis of GUS activity in rice expressing OsWNK9 promoter tagged with GUS chimeric gene at different developmental stages. (A) Schematic representation of the OsWNK9 promoter tagged with GUS chimeric gene and nopaline synthase terminator (NOS) in pCAMBIA1301 vector, (B) Young seedling, (C) Young leaf, (D) Flag leaf, (E) Mature leaf, (F) Callus, (G) Spikelet, (H) Young plant, (I) Roots, (J) Pollen, and (K) Female gametophyte, (L) Amplification of GUS gene in transgenic lines (White arrows represent GUS activity).

The abiotic stress responsive cis-acting regulatory elements were present in the OsWNK9 promoter region. Further, we investigated the seedlings of PWNK9:GUS transgenic lines (T_2 generation) which were exposed to various abiotic stresses such as salt, mannitol and ABA at different time intervals. In salt stress, under 200mM concentration, the T_2 generation seedlings were subjected to stress at different time intervals, without stress transgenic line was used as control (0 h). At 0 h the GUS was expressive at coleoptile region, 6 h showed highest GUS activity in coleoptile and crown region of seedlings followed by 12 h and 24 h. Surprisingly at 6 h time point it showed highest GUS expression almost in entire seedling along with the roots (Fig. 3.20A). Similarly, 200mM mannitol stress showed higher GUS activity at all different time points, the expression of GUS was simultaneously increased in coleoptiles and crown region as the increasing in stress time points from 0 h to 12 h. At 12 h under mannitol stress it is highly expressive in complete seedling along with the root region (Fig. 3.20B). Finally under 100µM ABA stress condition it showed highest GUS activity at 24 h in coleoptile and crown region compared to 6 h and 12 h (Fig. 3.20C). Overall it shows that the OsWNK9 will be more expressive at developmental stages especially in coleoptile and crown regions, and also it revealed interesting results by showing GUS expression in roots which can be correlated with the data accumulated at transcript level regulation expression pattern (Fig 3.3B). These GUS expression findings leads to an impression that, during salt and drought stress OsWNK9 might be regulating tolerance at early stage while ABA induced late expression of OsWNK9.

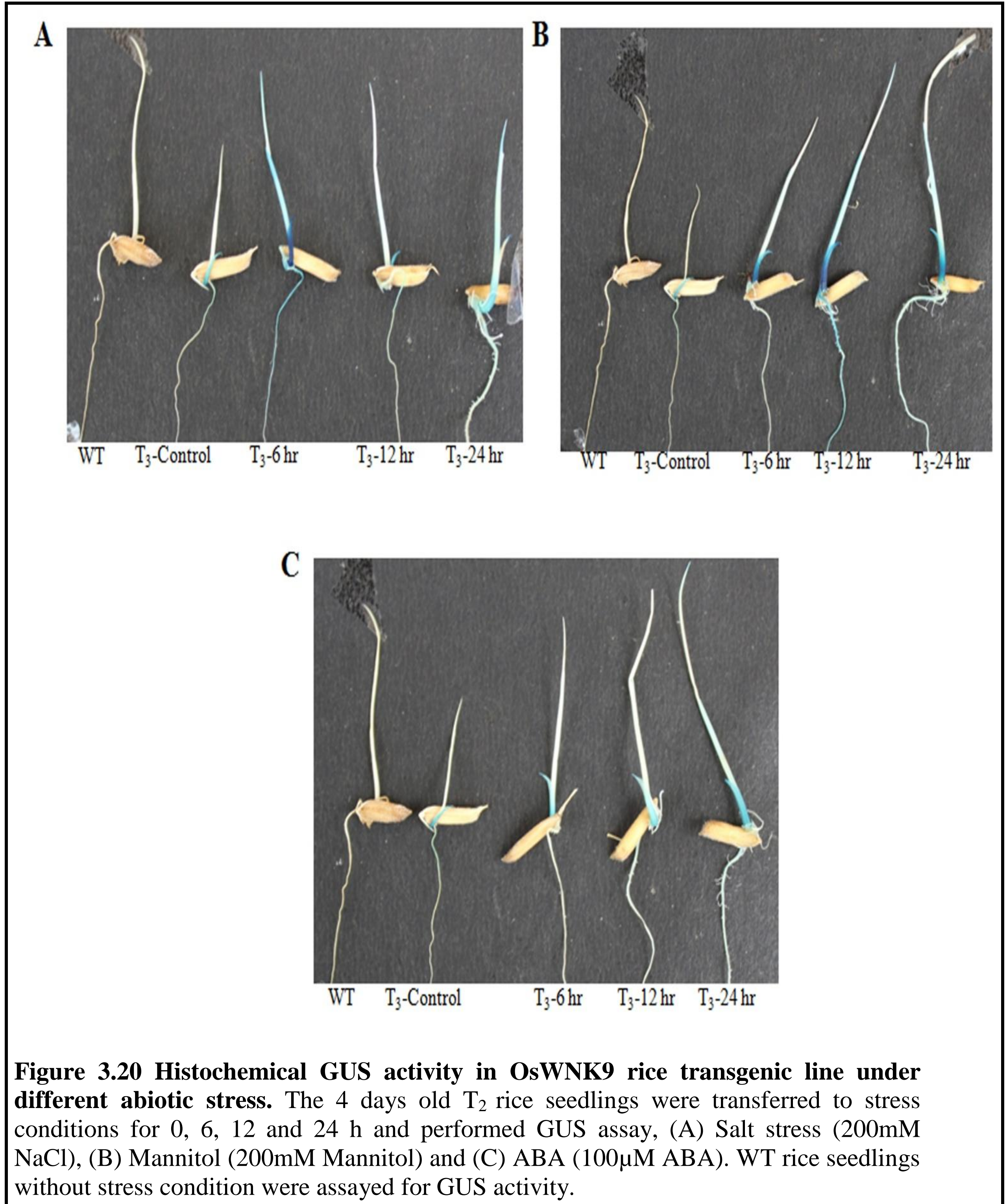


Figure 3.20 Histochemical GUS activity in OsWNK9 rice transgenic line under different abiotic stress. The 4 days old T_2 rice seedlings were transferred to stress conditions for 0, 6, 12 and 24 h and performed GUS assay, (A) Salt stress (200mM NaCl), (B) Mannitol (200mM Mannitol) and (C) ABA (100µM ABA). WT rice seedlings without stress condition were assayed for GUS activity.

3.3.8 OsWNK9 protein is localized in the cell nucleus

To determine the subcellular localization, *OsWNK9* was fused in frame to the coding region at the N-terminal side of green fluorescent protein (GFP) under the control of a 35S promoter of CaMV in pCAMBIA1302 (Fig 3.21A). The resulting binary construct was transformed in tobacco leaves. The transient expression of a fusion protein of GFP was observed in tobacco epidermal cells using confocal microscopy. Strong GFP signals were found in the nucleus of tobacco epidermal cell suggesting a functional role in nucleus (Fig. 3.21B). Similarly, localization studies in *Arabidopsis* protoplast through PEG mediated transformation shown the OsWNK9 localized in nucleus. The strong DAPI stain was observed in the nucleus and there is no signal of DAPI stain in empty pCAMBIA1302 vector (Fig. 3.21C). Further, *in silico* analysis of OsWNK9 using plant mPLoc server predicted OsWNK9 localized to the nucleus.

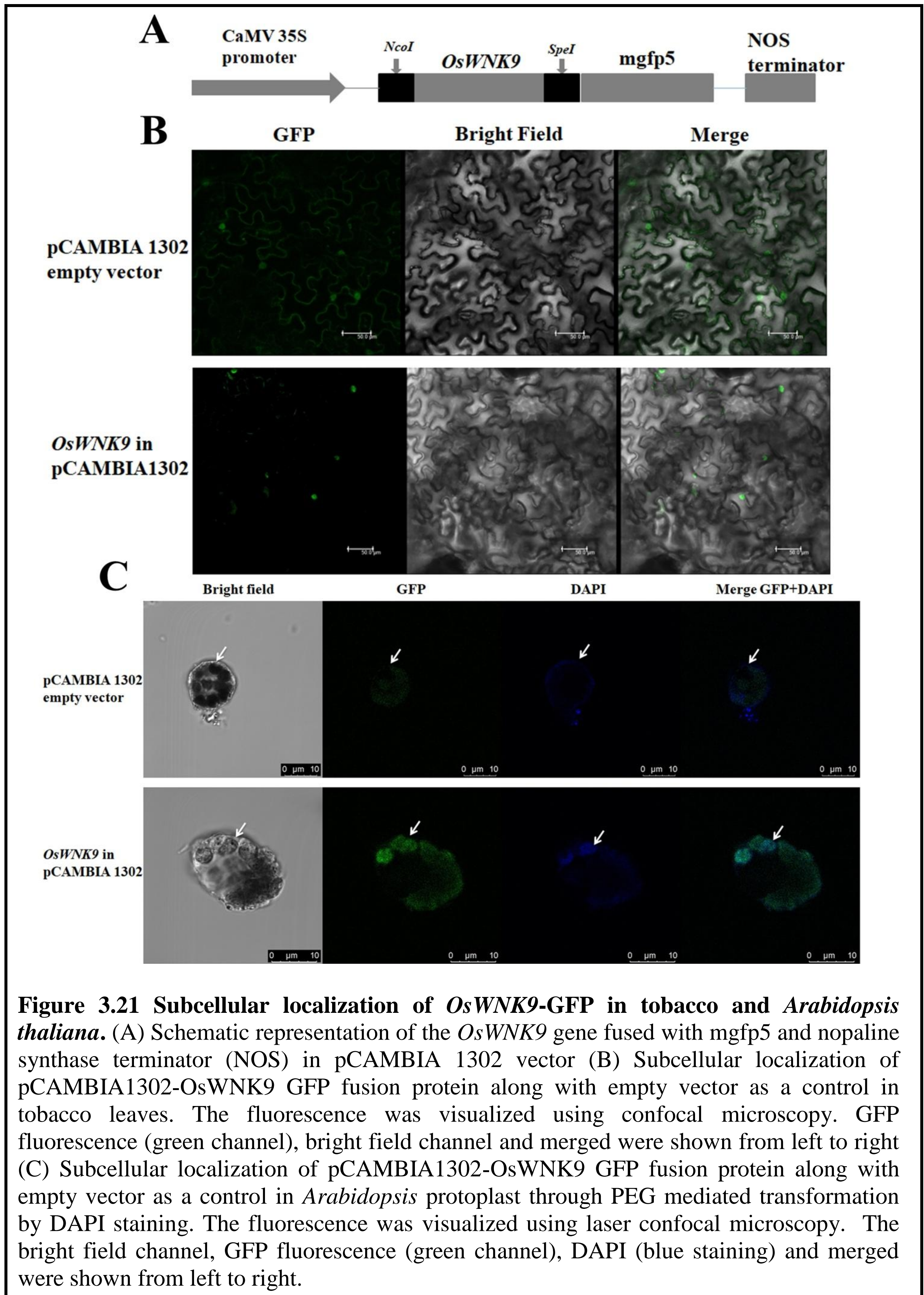


Figure 3.21 Subcellular localization of *OsWNK9*-GFP in tobacco and *Arabidopsis thaliana*. (A) Schematic representation of the *OsWNK9* gene fused with mgfp5 and nopaline synthase terminator (NOS) in pCAMBIA 1302 vector (B) Subcellular localization of pCAMBIA1302-OsWNK9 GFP fusion protein along with empty vector as a control in tobacco leaves. The fluorescence was visualized using confocal microscopy. GFP fluorescence (green channel), bright field channel and merged were shown from left to right (C) Subcellular localization of pCAMBIA1302-OsWNK9 GFP fusion protein along with empty vector as a control in *Arabidopsis* protoplast through PEG mediated transformation by DAPI staining. The fluorescence was visualized using laser confocal microscopy. The bright field channel, GFP fluorescence (green channel), DAPI (blue staining) and merged were shown from left to right.

3.4 Discussion

Based on our findings, we targeted *OsWNK9* gene which was differentially regulated under various abiotic stresses. In the present study, we successfully cloned a rice protein kinase *OsWNK9* from *Oryza sativa cv. IR64*. The comparison between sequences of cloned *OsWNK9* with rice sub species Japonica *OsWNK9* sequences showed 3 amino acid variations at the protein level. However, a recently characterized *Arabidopsis* WNK9 was distantly related to OsWNK9 and shared only 54.1% similarity (Xie et al., 2014). OsWNK9 showed the conserved features of the WNK Kinase protein family such as kinase and auto-inhibitory domains. Similarly, same domains were observed in human and plants WNK family members (Xu et al., 2002; Nakamichi et al., 2002; Wang et al., 2010). The unique feature of OsWNK9 was the replacement of catalytic lysine (K) residue of subdomain II by serine (S), while in AtWNK9 it was replaced by asparagine (N) (Xie et al., 2014). However, the catalytic lysine residue of soybean GmWNK1 and human WNK1 was replaced by cysteine (C) residues (Xu et al., 2000; Wang et al., 2010). The signature serine/threonine kinase motifs 'IIHRDLKCDNIFI' in subdomain VIb and `GTPEFMAPE' in subdomain VIII were conserved in kinase domain (Taylor et al., 1995). Similarly, the serine/threonine kinase motifs were also observed in OsWNK9 and underscored that OsWNK9 belongs to ser/thr protein kinase group. The OsWNK9 had a C-terminal autoinhibitory domain containing FXF motif. The mutant studies revealed that both phenylalanines (F) residues were equally important to the autoinhibition process (Xu et al., 2002). *OsWNK9* was significantly down regulated in shoot tissues under abiotic stresses such as salt, drought, and cold stresses (Manuka et al., 2015). Similarly, rice *WNK1* showed differentially expression pattern under several abiotic stresses like cold,

heat, salt, and drought at the transcription level (Kumar et al., 2011). Similar results were observed for *OsWNK9* under salt stress and showed significantly lower transcript abundance at 3 and 12 h in the shoot. Under drought and ABA stress, *OsWNK9* demonstrated down regulation pattern in shoots. Compilation of this data suggested that down regulation of *OsWNK9* in shoot may have the regulatory function during abiotic stress conditions. Surprisingly, when we performed transcript abundance analysis of *OsWNK9* under salt, drought and ABA stresses, it was highly up regulated in root tissues compared to shoot. Specific expression pattern was observed for soybean *WNK1* and it was demonstrated that *GmWNK1* is involved in the regulation of root system architecture. *GmWNK1* is also involved in regulation of multiple osmotic stresses (Wang et al., 2010). The mutant and overexpressed analysis of *AtWNK9* showed a reduction in root length (Xie et al., 2014). Taken together, this data underscored that the functional involvement of *OsWNK9* in the root tissue and modulation of abiotic stresses.

The physiological and cellular functions of most of the plant WNK family members are still unknown except for few members (Nakamichi et al., 2002; Wang et al., 2010; Xie et al., 2014). Earlier reports on plant WNK demonstrated that the WNK members were involved in regulation of circadian rhythm, root architecture, and modulation of various abiotic stresses (Nakamichi et al., 2002; Wang et al., 2010; Wang et al., 2011; Kumar et al., 2011; Xie et al., 2014; Manuka et al., 2015). The mutant analysis and overexpression studies of *GmWNK1*, *AtWNK8,* and *AtWNK9* showed significant regulation under ABA and various abiotic stresses (salt and drought) (Wang et al., 2010; Wang et al., 2011; Xie et al., 2014). However, animal WNK family members were involved in the regulating ion homeostasis and mutation led to a salt sensitive

hypertension (Wilson et al., 2001). They can regulate the renal potassium channel (ROMK), the sodium potassium chloride cotransporter type 2 (NKCC2), the sodium chloride cotransporter (NCC), and the epithelial sodium channel (ENaC) (Hoorn et al., 2011). In order to understand the role of *OsWNK9* under abiotic stress, the *OsWNK9* was overexpressed in *Arabidopsis*. The constitutive over expression of *OsWNK9* in *Arabidopsis* significantly improved physiological parameters such as higher fresh biomass and higher root length, higher chlorophyll content, low water loss through stomata, drought tolerance compared to WT. However, the OE lines showed most significant phenotype against salt stress compared to drought and ABA stress. It could be possible that *OsWNK9* may have primary roles in the regulation of plant ion transporter which mitigates salinity stress. It has been demonstrated that *Arabidopsis* WNK8 interacts with vacuolar H^+-ATPase (V-ATPase) through C-terminal domain and predicted that it might be involved in regulating ion transport in plants (Hong et al., 2006). Further MALDI-TOF MS analysis reported that AtWNK8 phosphorylates the vacuolar H^+-ATPase subunit C at multiple sites. Taken together, we hypothesized that *OsWNK9* may regulate salt transporters and provide salinity stress tolerance. Similar phenotypes were recorded in *AtWNK9* over expressed and mutant lines under drought stress (Xie et al., 2014). Moreover, when *GmWNK1* was overexpressed in *Arabidopsis*, it showed enhanced tolerance during seed germination and seedling development under salt and osmotic stress (Wang et al., 2011). The mutant analysis of *AtWNK8* observed that it improved tolerance against salt and osmotic stresses through modulating proline content and activities of catalase and peroxidase (Zhang et al., 2013). *AtWNK9* overexpressed lines showed better sensitivity to exogenous ABA and led to inhibition of root growth

(Xie et al., 2014). However, we observed under exogenous ABA, *OsWNK9* overexpressed lines showed significantly increased in root length. It could possible that ABA positively responds and promotes root growth. Chlorophyll is one of the major components in the plant cell photosynthesis which is sensitive to salt stress (Gill and Tuteja, 2010). In our study, transgenic lines showed the higher content of chlorophyll content compared to WT plants under salinity stress condition. This could be one of the reasons that the high content of chlorophyll related to the high photosynthetic activity which leads to enhancing the fresh weight of transgenic line under salt stress. Similarly, less water loss was observed in transgenic lines compared to wild type. Drought stress triggered the osmotic stress in the plant which needs to be control by minimizing water loss through stomata and cuticles, and increase water uptake through root (Roychoudhury et al., 2013). Compilation of phenotypic data and physiological studies, we inferred that *OsWNK9* may have regulatory roles under stress and overexpression improved transgenic plant condition to cope with unfavorable conditions.

The investigation of *cis*-acting elements in the promoters regulates the gene expression at the transcriptional level are crucial for improving our basic understanding of gene regulation (Hernandez and Finer, 2014). In the previous studies, we identified several putative regulatory elements in the *OsWNK9* promoter region (Manuka et al., 2015). The predicted *cis*-acting elements belonged to various categories such as ABA responsive element (ABRE), environmental signal response (G-box), dehydration responsive elements (MBS), heat shock element (HSEs), antioxidant responsive element (ARE) and circadian regulatory elements (Manuka et al., 2015). In the present study, we observed that OsWNK9 was expressed in almost all the tissues throughout the

developmental stages as well as under abiotic stress but most significant GUS activity were recorded in roots. As we predicted, *OsWNK9* had stress responsive *cis*-acting elements (ABRE, MBS and ARE) in their promoter region. We found that, when transgenic lines were exposed to NaCl, drought, and ABA, the GUS expression steadily increased in the crown region of rice seedlings. The results suggested that the OsWNK9 promoter gets activated by osmotic stress and played an important role in regulating downstream genes and transcription factors during stress conditions, previous reports of soybean WNK1 expression showed that it was significantly expressed in primary root and involved in root architect (Wang et al., 2010). Subcellular localization of maize protein gibberellin 3-Oxidase with GFP was dual localized to the nucleus and the cytosol and confirmed by confocal microscopy (Chen et al., 2014). However, protein kinase AtWNK8 physically interacted with EDM2 in the nucleus and when transiently expressed individually in tobacco leaves, both GFP–EDM2 and GFP–WNK8 fusions were nuclear-localized (Tsuchiya and Eulgem, 2010). Rice WNK1 protein with GFP was transiently expressed in the tobacco and localized to cell nucleus (unpublished data Kumar et al., 2009). Similarly, OsWNK9 subcellular localization was predicted using an mPLoc server to the nucleus. Further, it was confirmed the localization of OsWNK9 to the cell nucleus through transient expression in tobacco epidermal cells and *Arabidopsis* protoplast. However, an animal WNK GFP-tagged was predominantly localized to cytoplasm in the HeLa cells line (Jordan, 2011).

The previous reports demonstrated that primary effect of salt and drought stresses led to osmotic stress and was conveyed through ABA-dependent and ABA independent pathways (Kumar and Saddhe, 2018). A high salt induced osmotic stress led to increased

abscissic acid biosynthesis, thus regulating ABA dependent stress response pathway (Kumar et al., 2013). The abiotic stress-related genes such as *AtRD22, AtNCED3, AtABA3, AtDREB2C, AtWRKY28, AtNAC019,* and *AtEREBP* have been widely used as markers in studying plant response and regulate to abiotic stress. Previous studies indicated that these genes in plants are often responsive to osmotic, cold, drought and salt stresses. The expression analysis of ABA biosynthesis such as *ABA3* and *NCED3* induced under dehydration and showed higher *NCED3* in the transgenic plants but lower in the wnk9 mutant than WT plants (Xie et al., 2014). The ABA synthesis genes *ABA3* and *NCED*3 (9-cis-epoxycarotenoid dioxygenases genes) were up regulated in *OsWNK9* overexpressed plants under salt stress compared to WT. Under drought and ABA stresses, both the transcript *ABA3* and *NCED3* were highly up regulated, it was suggesting that *OsWNK9* may modulate ABA synthesis and mitigate abiotic stress through ABA dependent. The expression of *OsNCED3, OsNCED4,* and *OsNCED5* was observed at 1 h after salt stress and their expressions were correlated to the level of ABA in rice roots (Welsch et al., 2008). After stress response, ABA synthesis was triggered and accumulates in plants. The MYC and MYB transcription factors were synthesized after ABA accumulation and cooperatively activate responsive dehydration 22 (*RD22*) expressions (Abe et al., 2003). In the present study, we observed transgenic lines showed significant up regulation of ABA biosynthesis genes as well as *RD22* under salt stress. Similarly, under drought and ABA stress *RD22* transcript abundances were highly up regulated. The ABA-up regulated genes *RD22* transcription levels were increased in the *AtWNK9* overexpressing plants but decreased in the mutant compared with WT (Xie et al., 2014). In *Arabidopsis* several stress induced TFs families were up regulated and

further, categorized into four groups: dehydration-responsive element binding protein (DREB), NAC (NAM, ATAF1, 2, and CUC2) and zinc- finger homeodomain (ZF-HD), AREB/ABF(ABA-responsive element binding protein/ABA-responsive element binding factor) and MYC (myelocytomatosis oncogene) and MYB (myeloblastosis oncogene) (Nakashima and Yamaguchi, 2005). Besides these families, TFs belonging to WRKY, HSF family have been reported to play a major role under abiotic stress (Babitha et al., 2013). Plant dehydration-responsive element binding factors (DREBs) are transcriptional regulators of the APETELA2/ethylene responsive element-binding factor (AP2/ERF) family that regulate expression of abiotic stress-related genes (Je et al., 2014). The overexpression studies of *DREB2C* showed ABA hypersensitive and induced expression under salt and cold stress (Lee et al., 2010). Ethylene responsive element binding proteins (EREBPs) also known as ERE binding factor (ERF) proteins were involved in regulation of abiotic stresses such as wounding, salt, cold, and drought (Fujimoto et al., 2000). The *M. truncatula* was exposed to salt-stress and identified 84 unique transcription factors which were showing significant expression pattern. Some of these transcription factors belonged to the AP2/EREBP and MYB transcription factor family (Gruber et al., 2009). Microarray analysis of *Arabidopsis thaliana* showed a total of 33 genes up regulated by both NaCl and ABA stress including genes transcription factor *NAC019* (Liu et al., 2013). Over expression studies of *AtNAC019* revealed its role under drought, high salinity, and ABA siganling (Tran et al., 2004). The co-expression of *AtbHLH17* and *WRKY28* exhibited enhanced tolerance to salt, mannitol and oxidative stress in *Arabidopsis* transgenic lines (Babitha et al., 2013). Similar up regulation expression pattern of *DREB2C*, *NAC019*, *WRKY28*, and *EREBP*, were observed in *OsWNK*9

transgenic line compared to WT under salt stress. Same up regulation of these transcription factors was observed under drought and ABA stresses. Compilation of this data is helpful to understand the role of *OsWNK9* under salt stress and it can be hypothesized that OsWNK9 modulate salt, drought and ABA stresses through ABA dependent pathways.

3.5 Conclusion

We have cloned full-length rice *OsWNK9* sequence. Further, we concluded that the *OsWNK9* belongs to protein kinase family confirm by conserved signature motifs and domains analysis, phylogeny and homology modeling with established protein kinase family members. Transcript analysis of shoots and roots suggested significantly differential expression pattern under salt, drought and ABA stresses. Overexpression of *OsWNK9* in *Arabidopsis* improved tolerance against salt, drought, and ABA stress. Transgenic lines showed significant phenotypes such as higher fresh biomass and long root length compared to WT. The overexpressed line showed improved performance such as less water loss, higher drought tolerance and low bleaching of chlorophyll pigments under salt stress compared to WT. Promoter activity of rice under developmental stages and abiotic stresses revealed that it is expressed throughout developmental stages of vegetative and reproductive organs, while highly expressed in the root. The green fluorescent protein tagged with OsWNK9 protein was localized to cell nucleus in epidermal cells of tobacco and in protoplast of *Arabidopsis*. An ABA biosynthesis gene (*ABA3, NCED3*) and abiotic stress marker genes (*DREB2C, WRKY28, NAC019*, and

EREPB) showed significantly higher expression pattern under salt, drought and ABA stress compared to WT. Transgenic lines showed higher endogenous ABA content compared to WT. Taken together, we concluded that *OsWNK9* may be a prospective candidate gene to develop abiotic stress tolerant crop plants through genetic engineering strategy.

Chapter 4

OsWNK9 mitigates salt and drought stress effects through the modulation of multiple antioxidant systems in *Arabidopsis*

4.1 Introduction

In rice 9 genes and in *Arabidopsis*, 11 genes of WNK family have been predicted so far and few of the genes are characterized (Huang et al., 2007; Wang et al., 2008). *AtWNK1* is involved in the regulation of flowering time, which was confirmed through knockout analysis of *wnk1* showing delayed flowering during the long-day hours in *Arabidopsis* (Wang et al., 2008). However, more WNK kinase members were reported to be involved in the regulation of flowering and confirmed by T-DNA mutant analysis of *AtWNK2, AtWNK5*, and *AtWNK8* which showed early flowering under long-day hours. In all these mutants, the transcript levels of photoperiod pathway genes such as ELF4, TOC1, CO, and FT were found to be regulated, indicating that *Arabidopsis* WNK gene family regulates flowering time through the photoperiod pathway (Wang et al., 2008). The C-terminal domain of *AtWNK8* was found to interact with the vacuolar H^+-ATPase (V-ATPase) subunit C and postulated to have a role in the regulation of ion transport in plants (Hermesdorf et al., 2006). *AtWNK8* was reported to interact with the upstream member of EDM2 and regulates flowering in FLC-dependent under short-day hours (Tsuchiya and Eulgem, 2010). Rice *OsWNK1* expression pattern has been observed and suggested for its role in the regulation of biological circadian cycle and also the abiotic stresses (Kumar et al., 2011). The knockout studies of *AtWNK8* significantly improved tolerance to salt and osmotic stresses by regulating the proline content, catalase and peroxidase enzymes activity (Zhang et al., 2013).The involvement of *OsWNK9* against abiotic stress environment has been reported in our previous chapter. The constitutive expression of the *OsWNK9* gene has been shown to impart some morphological and physiological changes in *Arabidopsis* showing tolerance to salt and drought stress. These

changes include maintenance of healthy morphological features such as green leaves, ample fresh weight, longer roots and also the physiological features like proper seed germination, maintained chlorophyll content and reduced water loss via ABA-dependent stress regulatory pathway.

The present research work was pivoted on the analysis of biochemical changes that occurs in wild type (WT) and *OsWNK9* transgenic *Arabidopsis* plants under salt and drought conditions. WT and overexpressed lines were analyzed for the proline content, MDA content, H_2O_2 content, activities of peroxidase, catalase, ascorbate peroxidase as well as total antioxidant capacity, Na^+, K^+ content after treatment with the stressful conditions and compared with control experiments. Depending upon the type of biochemical alterations observed, tolerance level of wild and transgenic lines was concluded.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

The *Arabidopsis thaliana* plants, WT and transgenic lines (T_9, T_{11}, $T_{12,}$ and T_{13}) were grown onto ½ MS agar plates under the controlled conditions of 16 h light and 8 h dark with 60% humidity at 22°C for 6 days. The plantlets were transferred to MS hydroponic conditions, grown for a month followed by salt and drought stress treatments (200 mM NaCl and 300 mM Mannitol) for 0 (control), 24, 48 and 72 h. Plants were then harvested, ground into a fine powder in liquid nitrogen and stored at -80°C for further use.

4.2.2 Proline content

Plant sample (500 mg) was homogenized in 3% sulphosalicylic acid (2 ml) and centrifuged at 5000 g for 5 minutes. To 500 µl supernatant in a test tube, acetic acid (500 µl) and ninhydrin reagent (500 µl) was added, incubated at 95°C for 45 minutes and the tube was placed on ice for 30 minutes. An equal volume of toluene was added to the solution, vortexed for a minute and centrifuged at 1000 g for 5 minutes. The supernatant was transferred to a fresh tube and absorbance was measured at 520 nm using a UV-Visible spectrophotometer (SHIMADZU). Proline content was determined by extrapolating the absorbance values onto the standard graph. For each plant sample, the assay was performed in three biological replicates (Bates et al., 1973).

4.2.3 Malondialdehyde (MDA) content

To the plant sample (100 mg) 0.1% TCA (1 ml) was added and mixed well by inverting tube several times. The sample was then centrifuged at 10,000 g for 10 minutes and supernatant was transferred to a fresh tube. To the supernatant, 20% TCA (4 ml) containing 0.5% TBA was added, mixed well and boiled at 95°C for 15 minutes followed by cooling on ice. The sample was then centrifuged at 10,000 g for 5 minutes and absorbance of supernatant was measured at 532 nm using a UV-Visible spectrophotometer. MDA content was determined by extrapolating the corresponding absorbance value onto the standard graph. For each plant sample, the assay was performed in three biological replicates (Heath and Packer, 1968).

4.2.4 Guaiacol Peroxidase (POD) assay

Plant sample (100 mg) was homogenized in ice-cold phosphate buffer (pH 7) and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and placed on ice. POD activity was determined by observing the formation rate of yellow colored guaiacol dehydrogenation product (GDHP) in presence of 0.33% guaiacol solution and H_2O_2 followed by recording absorbance at 420 nm. Simultaneously, the concerned protein concentration in crude extract was determined by Bradford's method. The specific activity was expressed as mol.UA/mg protein. For each plant sample, the assay was performed in three biological replicates (Putter, 1974).

Unit Activity (Units/min/g FW) = [Change in abs./min x Total volume(ml)] / [Ext. coefficient x Volume of the sample taken].

Specific Activity (mol.UA/mg protein) = Unit Activity (Units/min/g FW) / Protein content (mg/g FW).

4.2.5 Ascorbate Peroxidase (APX) assay

Plant sample (100 mg) was homogenized in ice-cold phosphate buffer (pH 7.8) containing 1mM ascorbic acid, 1 mM EDTA and 2% PVP and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and kept on ice for assay. APX activity in crude extract was determined by observing the rate oxidation of ascorbate followed by decrease in absorbance at 290 nm in presence of phosphate buffer (pH 7) containing 0.2 mM ascorbic acid, 0.2 mM EDTA and 20 µM H_2O_2. The protein concentration in crude extract was determined by Bradford's method. The specific

activity was expressed as U/mg protein. For each plant sample, the assay was performed in three biological replicates (Nakano and Asada, 1981).

Unit Activity (Units/min/g FW) = [Change in abs./min x Total volume(ml)] / [Ext. coefficient x Volume of the sample taken].

Specific Activity (mol.UA/mg protein) = Unit Activity (Units/min/g FW) / Protein content (mg/g FW).

4.2.6 Catalase assay

Plant sample (100 mg) was homogenized in ice-cold phosphate buffer (pH 7), vortexed and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and kept on ice for assay. Catalase activity was determined by recording the change in absorbance of supernatant (50µl) at 240nm in presence of 500 µl phosphate buffer and 200 µl hydrogen peroxidase (150Mm). The protein concentration in crude extract was determined by Bradford's method. The specific activity was expressed as U/mg protein. For each plant sample, the assay was performed in three biological replicates (Aebi, 1983; Anderson et al, 1995).

Unit Activity (Units/min/g FW) = [Change in abs./min x Total volume(ml)] / [Ext. coefficient x Volume of the sample taken].

Specific Activity (mol.UA/mg protein) = Unit Activity (Units/min/g FW) / Protein content (mg/g FW).

4.2.7 Hydrogen Peroxidase content

Plant sample (100 mg) was homogenized in 0.1% TCA (1 mL) and mixture was centrifuged at 10,000 g for 15 minutes. The supernatant was transferred to fresh vial and stored on ice for further use. Supernatant (25 µl) was mixed with 50 µl potassium iodide (1M) and 125 µl phosphate buffer (pH 7) in 96 well microtiter plate and absorbance was recorded at 390 nm using multiwell plate reader. The standard graph was prepared by using same protocol with standard H_2O_2 solutions. The concentration of H_2O_2 was determined by extrapolating absorbance values onto the standard graph. For each plant sample, the assay was performed in triplicate (Velikova et al., 2000).

4.2.8 DPPH-radical scavenging activity

Plant sample (100 mg) was homogenized in 1 ml of ethanol (chilled), vortexed and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and stored on ice. To 50 µl supernatant, 200 µl of DPPH reagent was added in a microtiter plate and plate was incubated in dark for 30 minutes. After incubation, the absorbance was recorded at 517nm using a multiwell plate reader. The percentage of radical scavenging activity was calculated by using the following formula (Kang and Saltveit, 2002).

% of radical scavenging = [Absorbance of control-Absorbance of sample/ Absorbance of control] x 100.

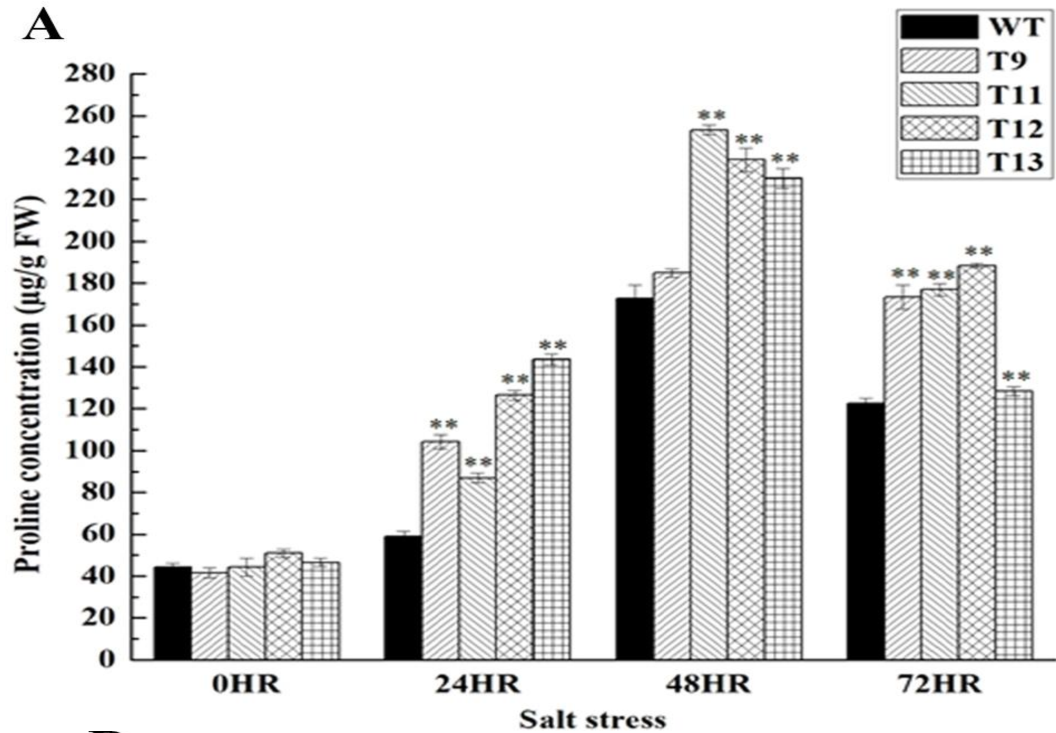
4.2.9 Measurement of total Na^+ and K^+ ion content under salt stress

Plant samples of WT and transgenic lines were harvested at 0 h and 48 h time points. The samples were dried at 95°C for 2-3days, digested with 1.5 ml concentrated HNO_3 at 90°C for 20 minutes and centrifuged at 12,000 rpm for 10 min at room temp (Mishra et al., 2014). The suspension was diluted with 8.5 ml of sterile milliQ water and analyzed for Na^+ and K^+ content with the flame photometer (SYSTRONICS flame photometer 128). Elemental analysis experiments were performed with three biological replicates.

4.3. Results

4.3.1 *OsWNK9* promotes osmotic balance by raising proline levels under salt and drought stress

Proline level in wild type and *OsWNK9* transgenic lines under normal physiological conditions as well as salt and drought stress was determined to assess their ability to regulate osmotic balance. Under normal growth environment, both wild type and transgenic lines showed similar proline concentrations. Upon application of salt stress, it was found that proline levels increased linearly up to 48 hours incubation period with stress-inducing agent; being comparatively maximum in transgenic lines and declined at 72 hours incubation period. Same was the case for mannitol as drought stress-inducing agent with proline levels almost similar under normal growth conditions, increased linearly till 48 hours stress treatment and decreased at 72 hours stress treatment period (Fig. 4.1). Thus, transgenic *Arabidopsis* expressing *OsWNK9* may stimulate the accumulation of proline to withstand the damaging effects caused by reactive species overproduced due to the abiotic stress.



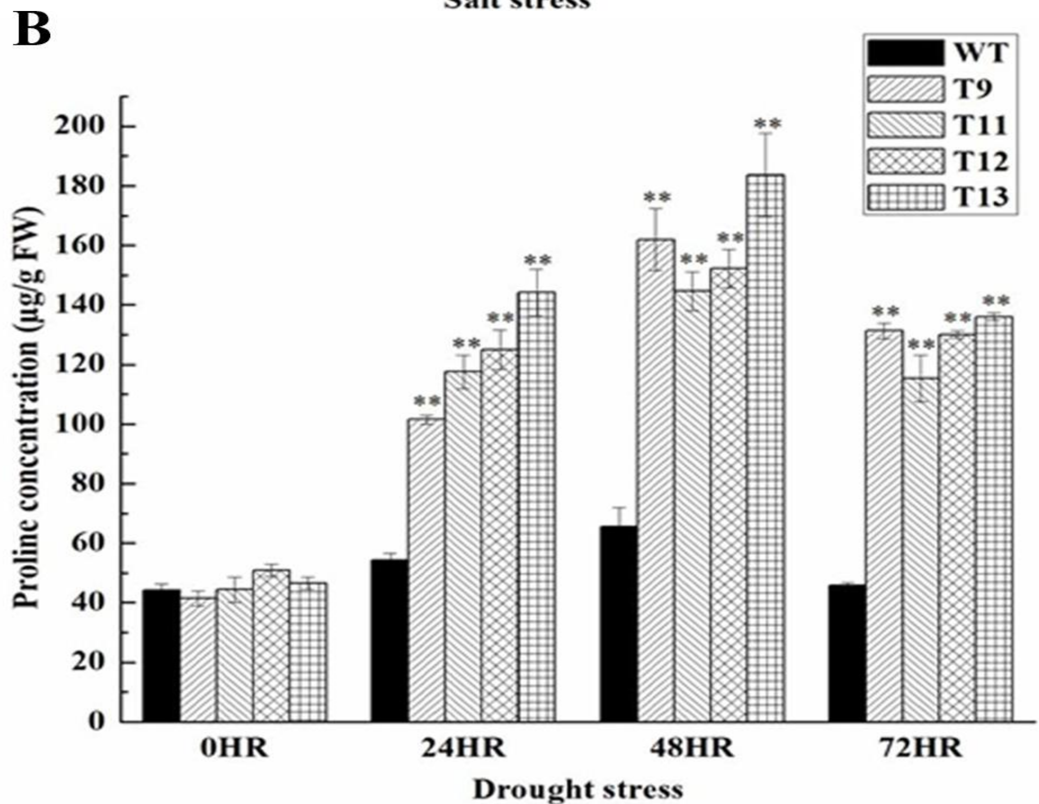


Figure 4.1 Proline content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.2 Transgenic plants show reduced membrane damage under salinity and drought stress

MDA levels in wild type and transgenic *Arabidopsis* under normal physiological conditions as well as salt and drought stress was determined to assess the extent of membrane damage caused due to stress treatments. Both wild type and transgenic plants showed similar minimum MDA levels in normal physiological conditions. The MDA concentration got raised in all plants when treated with salt as a stress agent for 48 hours. However, wild type plants showed maximum MDA levels compared to the transgenic ones thus signifying the capability of transgenic plants to withstand the salt stress by reducing lipid peroxidation induced membrane damage. The MDA concentration and thereby membrane damage at 72 hours of incubation with salt stress agent was significantly reduced in transgenic plants while it was found still maximum in case of wild type plants. Wild type and transgenic plants exposed to mannitol as drought stress-inducing agent shown similar MDA concentration profile like in case of salinity stress (Fig. 4.2).

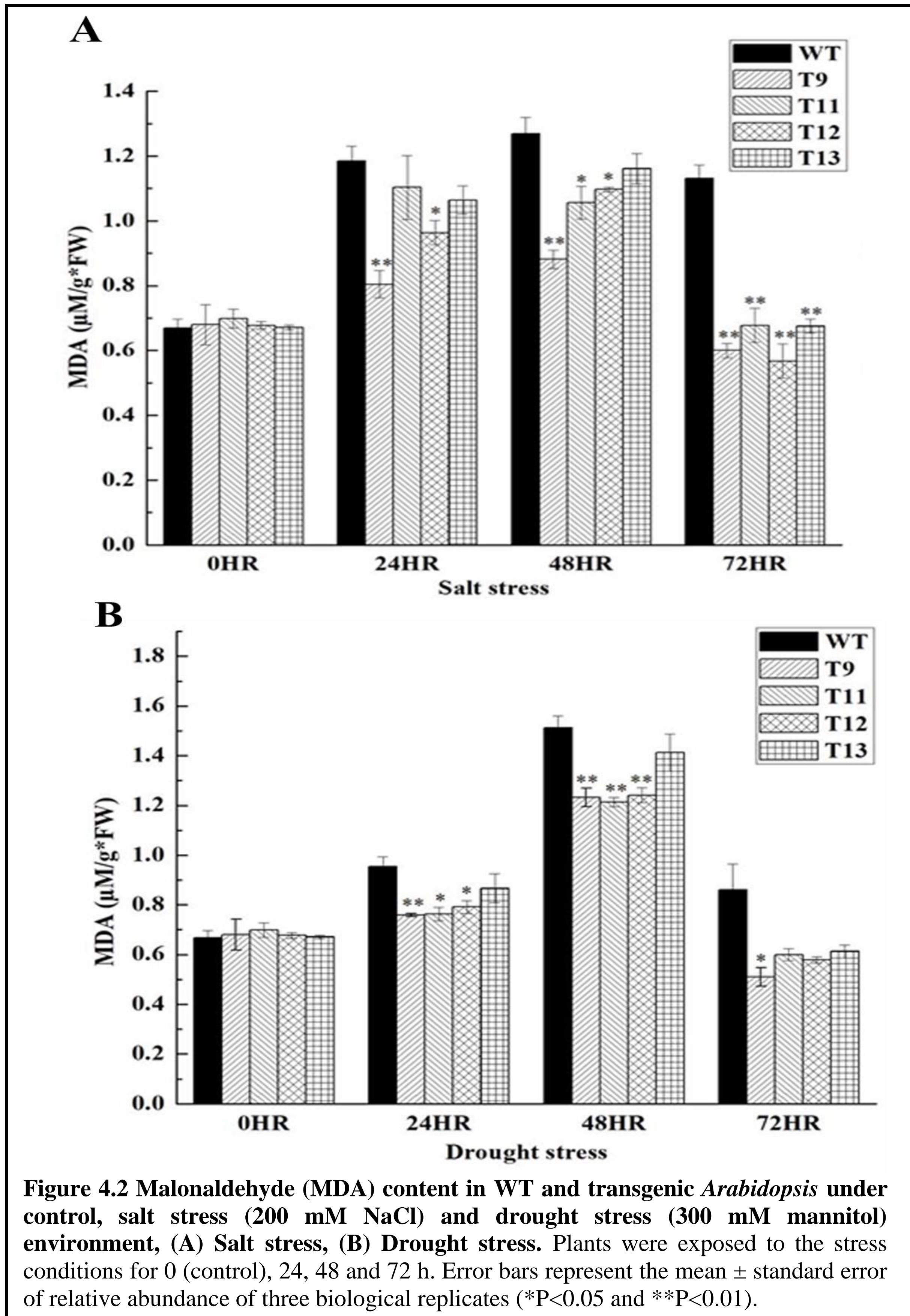
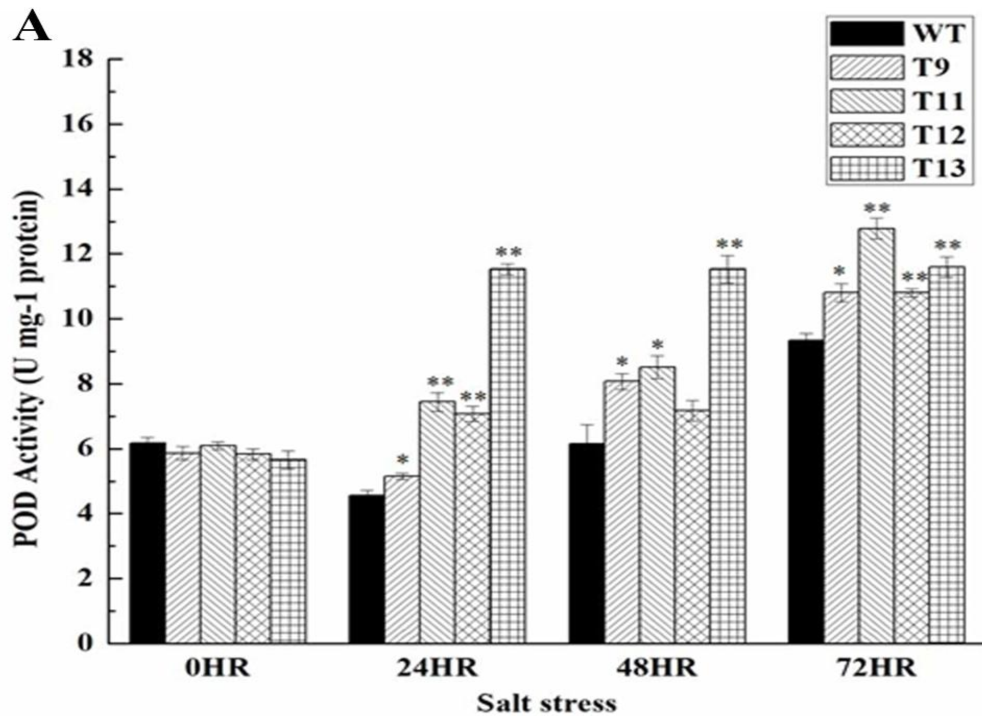


Figure 4.2 Malonaldehyde (MDA) content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.3 WT and transgenic plants exhibit a dynamic pattern of peroxidase activity

Guaiacol peroxidase (POD) assay in wild type and transgenic plants showed a much more variable pattern of its activity under normal physiological as well as stress conditions. WT and T9 showed an initial decrease in POD activity at 24 hours of salt stress exposure whereas the activities got increased subsequently at 48 and 72 hours exposure periods. T_{11} transgenic line has shown a linear increase in the peroxidase activity till 72 hours stress exposure. In the case of a T_{12} transgenic line, POD activity increased at 24 hours, remained similar at 48 hrs and again rose at 72 hours of stress treatment. Transgenic line T_{13} showed almost two-fold increase in POD activity at 24 hours and maintained the activity similar throughout 48 and 72 hours of salt stress treatments. A similar type of variable pattern of POD activity was observed in case of drought stress treatment. The T_{13} line showed a linear increase in POD activity with about two-fold increases in activity at 72 hours drought stress treatment. No significant change in the peroxidase activity in WT plants was observed under normal as well as stress conditions. T_9 and T_{12} plants exhibited increased POD activity at 24 hours, maintained the similar at 48 hours and rose at 72 hours of stress treatment period. In the case of a T_{11} transgenic line, POD activity increased at 24 hours, maintained at 48 hours while decreased at 72 hours of stress treatment (Fig. 4.3). Overall, in the case of all the plants including WT and transgenic lines, dynamic pattern of POD activity was observed.



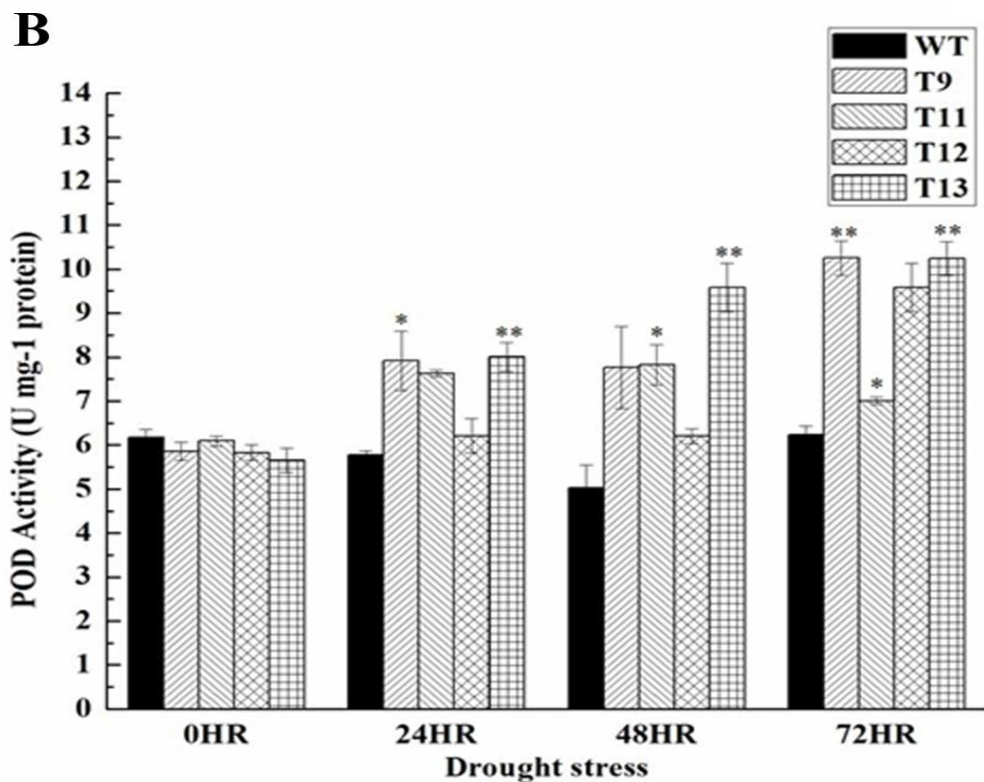


Figure 4.3 Peroxidase (POD) activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.4 Transgenic plants show improved APX activity against salt and drought stress

Ascorbate peroxidase uses ascorbate as a cofactor for the scavenging of H_2O_2 accumulated by virtue of stress conditions. No significant change in the APX activity was observed in WT plant under control and salt-stressed conditions. In case of transgenic lines, a linear increase in APX activity was observed till 48 hours and got decreased at 72 hours of stress treatment period which was also same trend for accumulated proline content. Out of four transgenic lines, T_{13} exhibited more than 8 fold increase in the APX activity being highest among all of them. In drought stress treatment, a similar pattern of APX activity was found in WT plants showing no significant change under a stressed condition. Similar to salt stress, all transgenic lines shown a linear increase in the activity of APX being highest for the T_{13} transgenic line with about 6 fold rise at 48 hours of mannitol stress treatment. In all plants, APX activity got decreased at 72 hours of the drought stress treatment period (Fig. 4.4). Overall, all transgenic plants showed an improved APX activity compared to wild type.

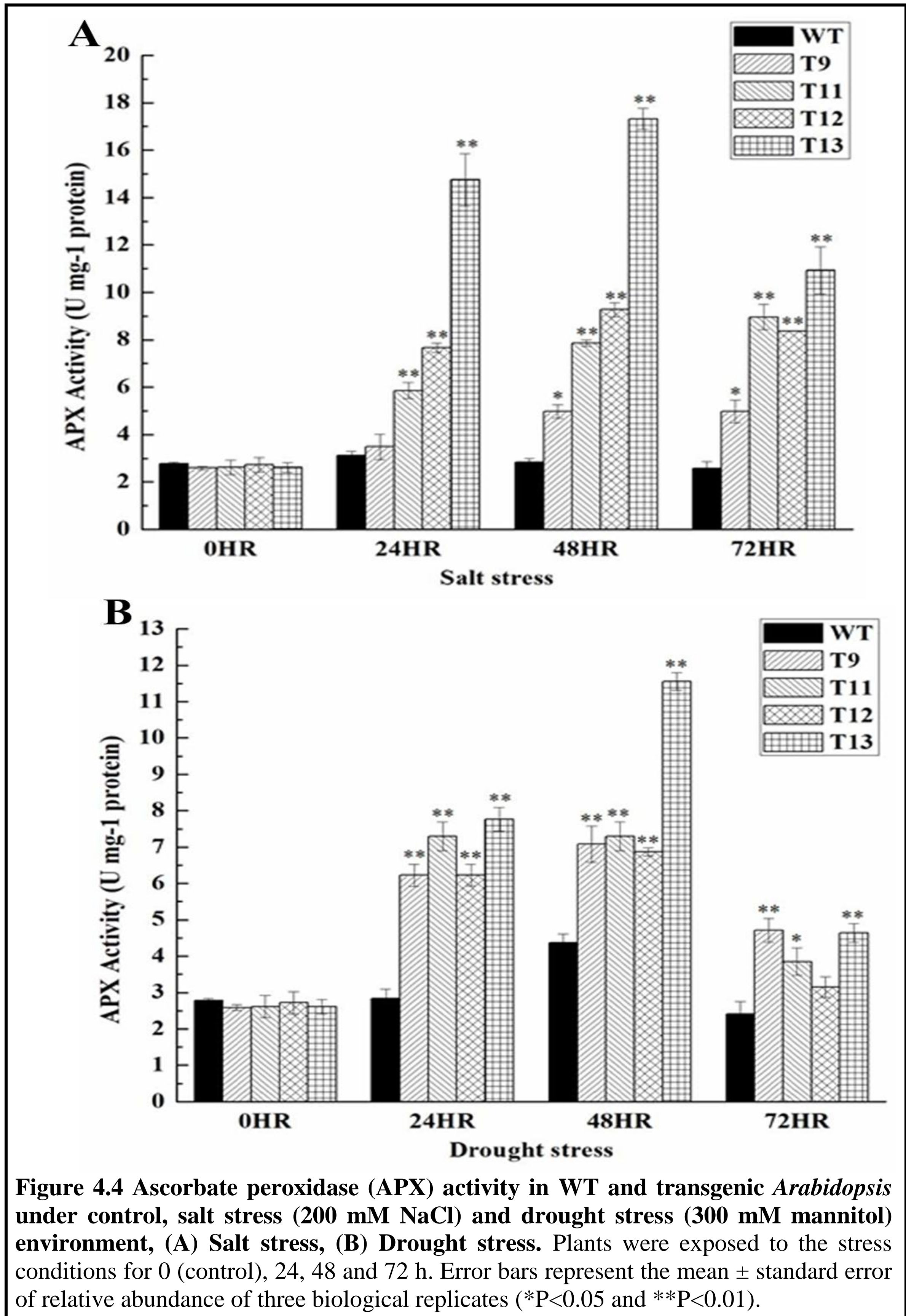
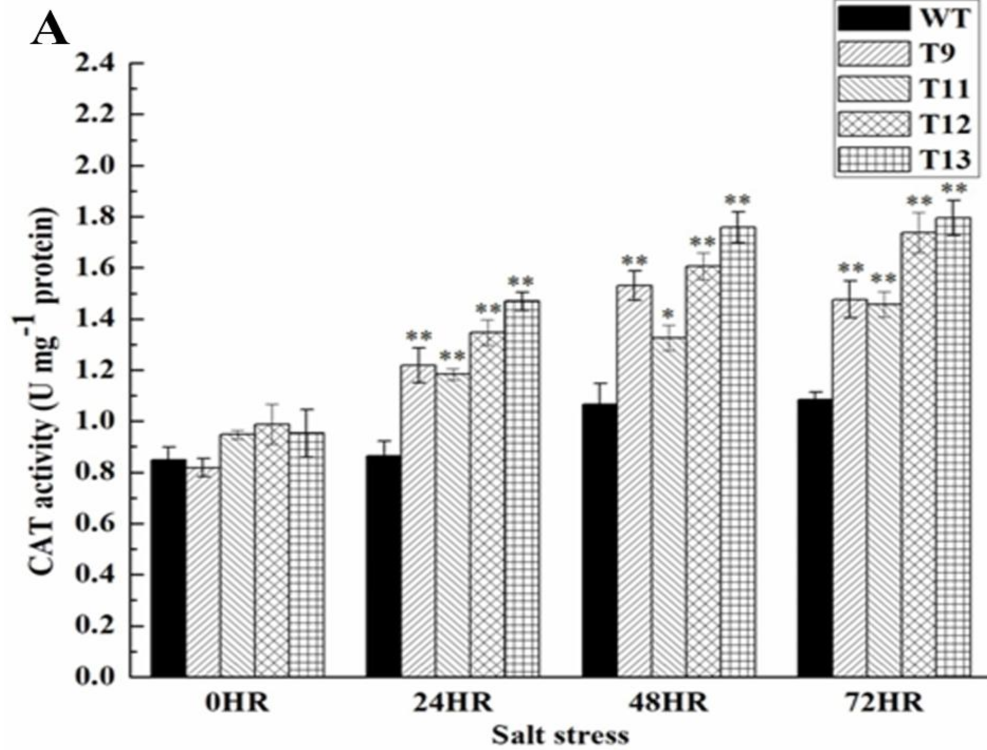


Figure 4.4 Ascorbate peroxidase (APX) activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.5 Transgenic plants maintained increased catalase activity under salt and drought stress

H_2O_2 scavenging activity of catalase in WT plant under salt and drought stress showed no significant change compared to control. Under salt stress, T_{11}, T_{12} and T_{13} transgenic lines maintained linearly increased catalase activity throughout the stress treatment, while T_9 showed linear increase till 48 hours and a slight decrease in the activity at 72 hours of stress treatment period. Under drought stress, all transgenic lines showed linear increased catalase activity compared to that in control (Fig. 4.5). Observing the trend of catalase activity in both the stress conditions, transgenic lines exhibit well maintained H_2O_2 scavenging activity signifying the role of OsWNK9 in antioxidant defense mechanism against non-radical species over-accumulated in stressful environments.



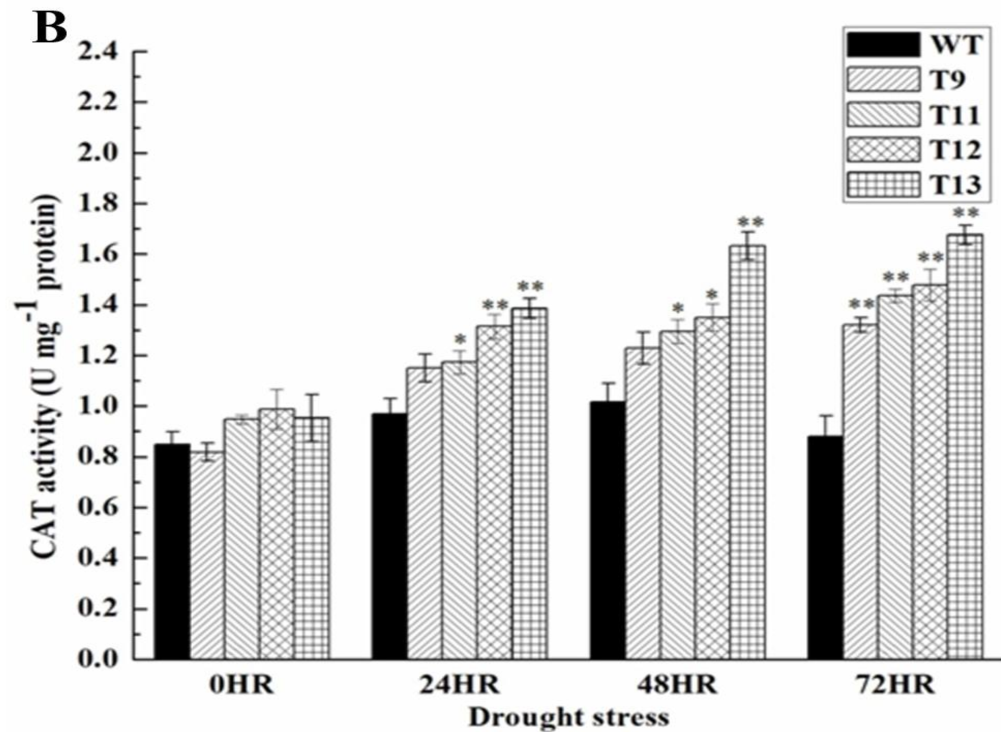


Figure 4.5 Catalase activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*$P<0.05$ and **$P<0.01$).

4.3.6 Antioxidant defenses in transgenic lines alleviate over-accumulated H_2O_2

Under salinity stress, transgenic lines showed decreased content of H_2O_2 which is an indication of active involvement of antioxidant defense system against it. Contrary to this, WT plants shown high levels of H_2O_2 indicating the oxidative damage inducing conditions in plant cells. In transgenics at 24 hours of salt stress treatment, a slight rise in H_2O_2 content was observed with no significant change while it was reduced at 48 hours with again a slight rise at 72 hours treatment period, whereas it was linearly increased to higher levels in WT plant. Compared with WT plant, all transgenic lines showed positively significant H_2O_2 scavenging activity. In drought condition, the H_2O_2 content was more or less similar in all plants but increased to high levels in WT, whereas it maintained at low concentrations in transgenic lines. In transgenic plants, the H_2O_2 concentration was decreased at 24 hours with slight increase at 48 and 72 hours of stress treatment in comparison to that in WT plant (Fig. 4.6). Overall, all transgenic lines showed less H_2O_2 contents under the stress conditions applied, indicative of actively scavenging system within transgenic lines.

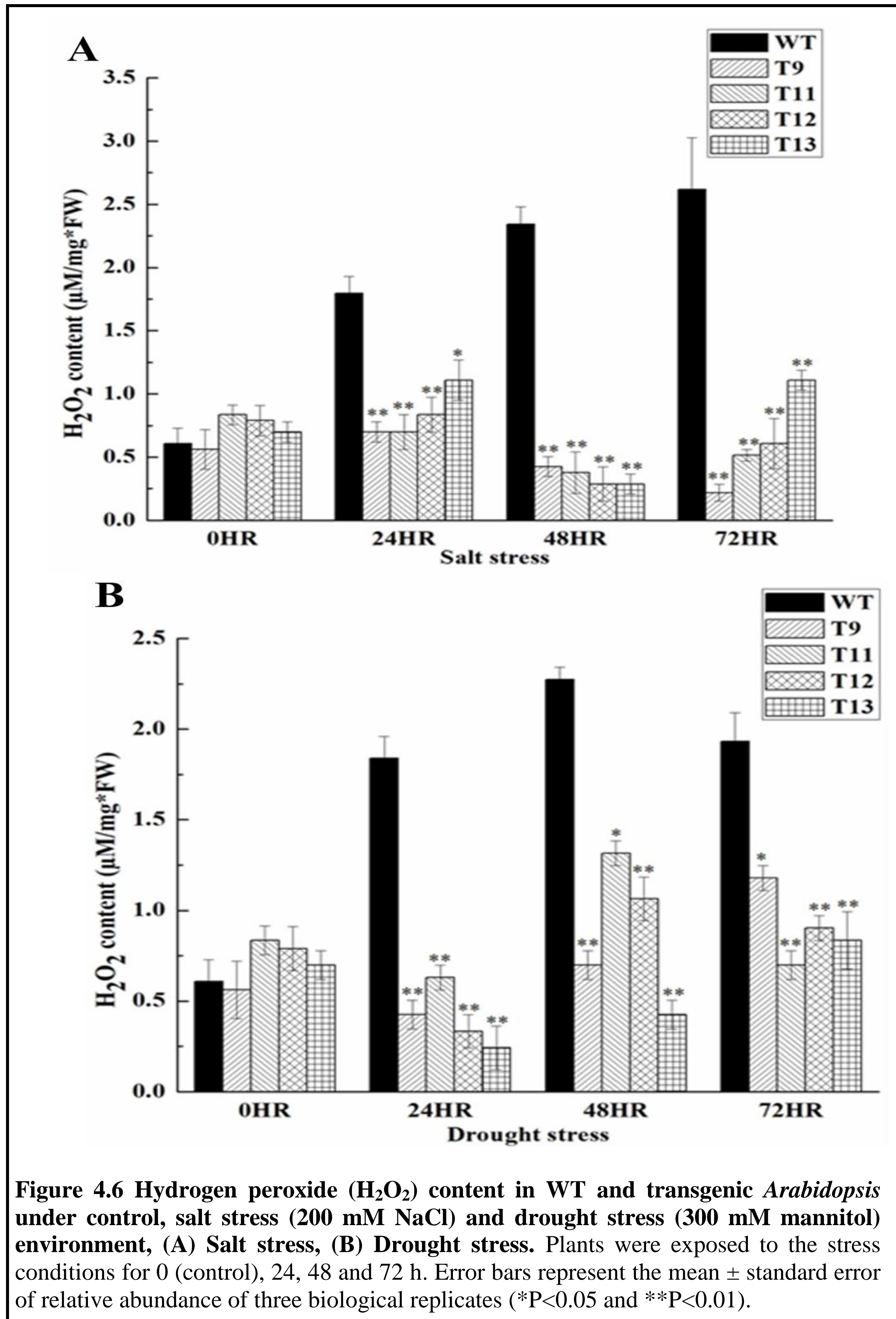


Figure 4.6 Hydrogen peroxide (H_2O_2) content in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.7 Transgenic lines maintain higher antioxidant activity

The DPPH free radical scavenging antioxidant activity of WT and transgenic lines showed a significant difference in radical scavenging percentage. Under salt and drought stress, the percent of scavenging was reduced to a greater extent in WT plants, while it was increased in all transgenic plants from 24 hours and maintained to high levels till 72 hours of stress treatment period (Fig. 4.7). Overall comparison between the total antioxidant activity of ethanolic extracts of WT and transgenic lines showed that the later possess abiotic stress tolerance characteristics. Transgenics able to withstand the damaging effects of reactive species overproduced during stress conditions, through the rise in overall antioxidant defense machinery including enzyme and other compounds.

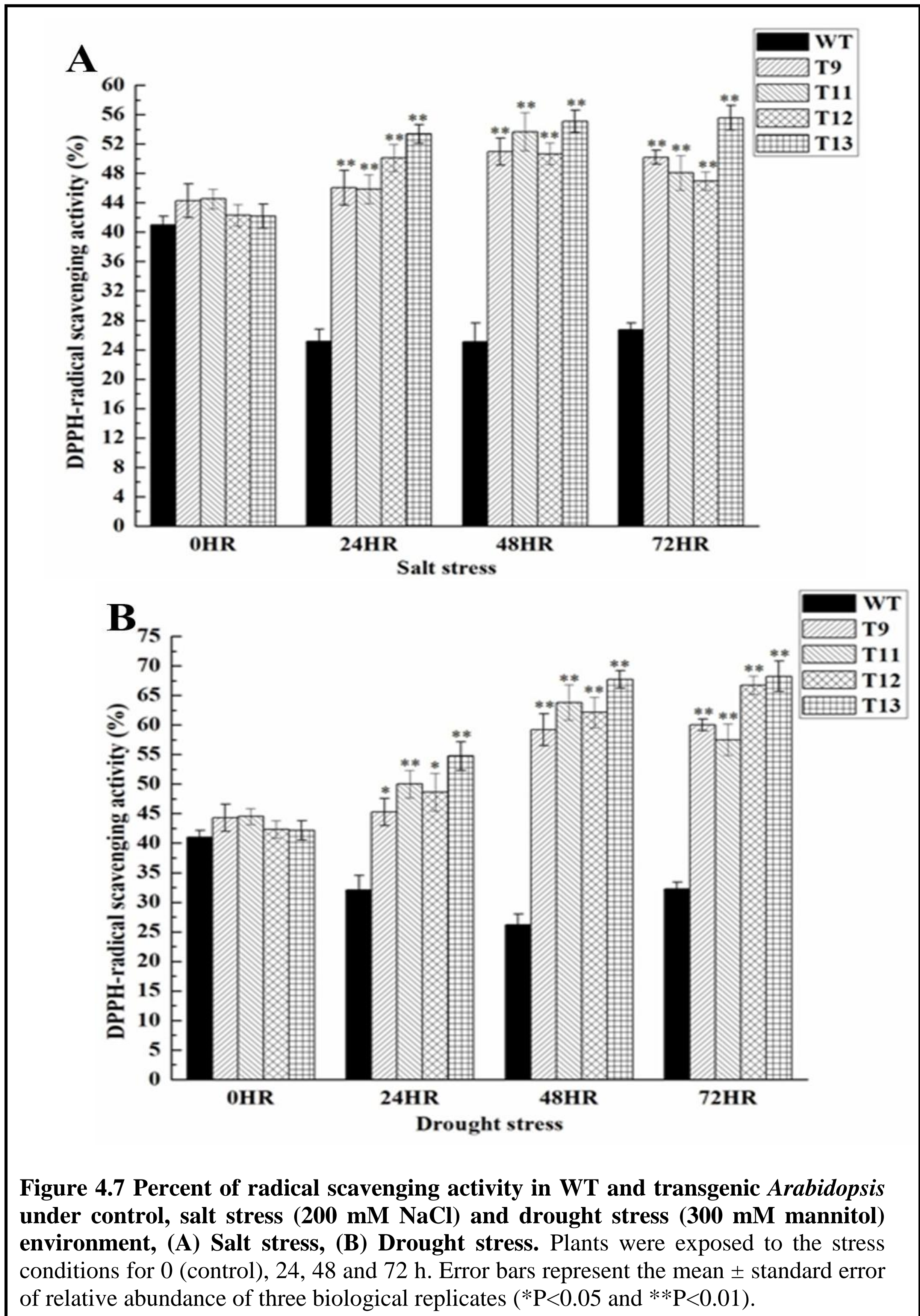


Figure 4.7 Percent of radical scavenging activity in WT and transgenic *Arabidopsis* under control, salt stress (200 mM NaCl) and drought stress (300 mM mannitol) environment, (A) Salt stress, (B) Drought stress. Plants were exposed to the stress conditions for 0 (control), 24, 48 and 72 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.3.8 Transgenic lines exhibit an active ion homeostasis under salt stress

The ion homeostasis, especially of Na^+ and K^+ is one of profound mechanisms involved during salt stress tolerance in plants. Under control and salt-stressed conditions wild type as well as transgenic lines shown a significant difference in the intracellular Na^+ content (Fig. 4.8A). In case of no stress (control), a negligible amount of Na^+ was detected in all plants whereas in case of salt stress treatment for 48 hours, the Na^+ was found to be significantly increased indicating the influx of Na^+ within plant cells. The Na^+ accumulation was comparatively less in transgenic lines being lowest in the T_{13} line, whereas wild type plants shown highest accumulated contents. The level of K^+ was found to be maintained at an optimal level in transgenics being compared with control. In wild type the K^+ content is decreased at 48 h while compared with 0 h (Fig. 4.8B). Also, Na^+/ K^+ ratio was found highest in wild type plants and less among the transgenic lines being lowest in the T_{13} lines (Fig. 4.8C). All-inclusive, compared to wild type, transgenic lines exhibited the salt tolerance ability through an active balance of intracellular Na^+/ K^+.

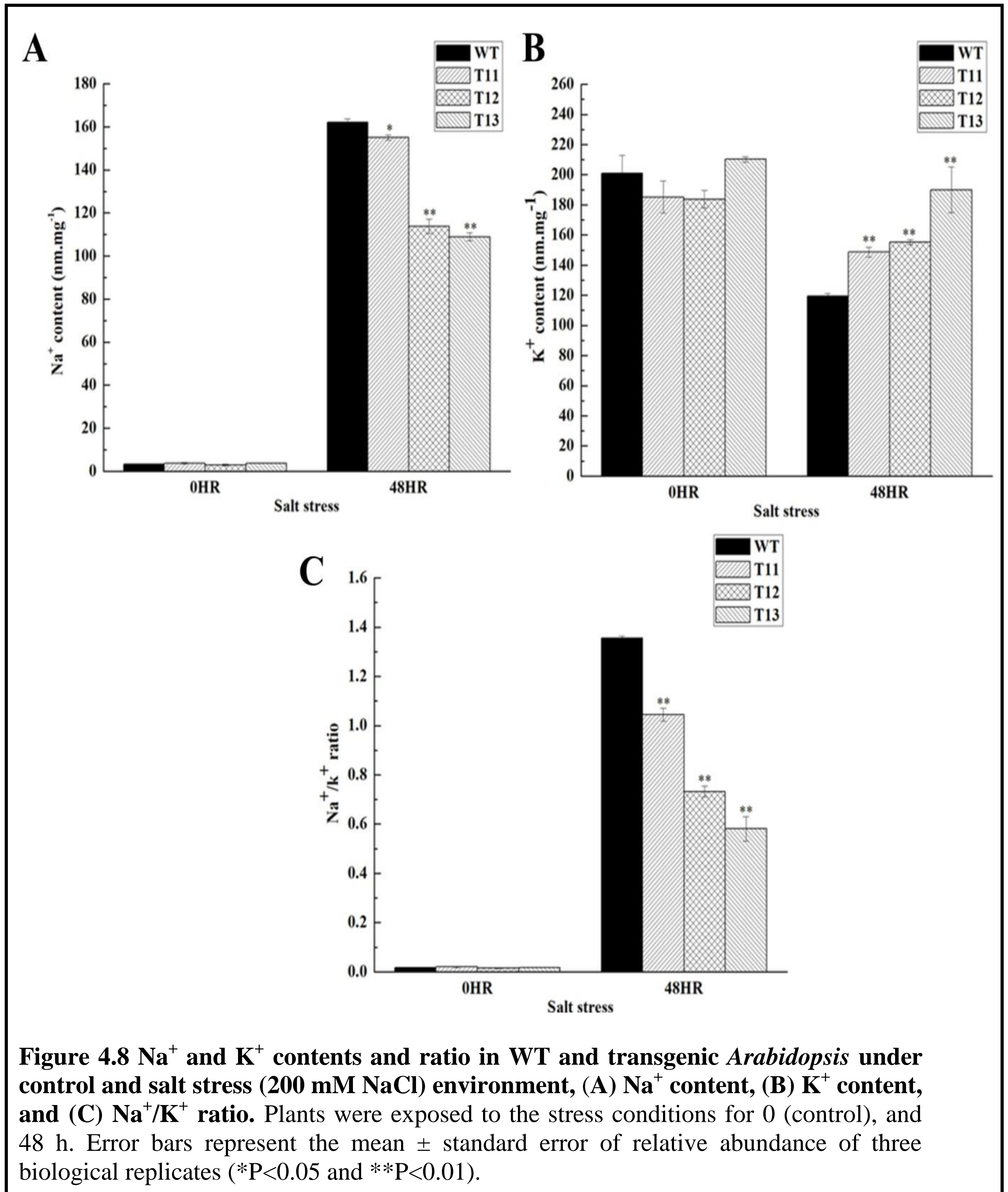


Figure 4.8 Na^+ and K^+ contents and ratio in WT and transgenic *Arabidopsis* under control and salt stress (200 mM NaCl) environment, (A) Na^+ content, (B) K^+ content, and (C) Na^+/K^+ ratio. Plants were exposed to the stress conditions for 0 (control), and 48 h. Error bars represent the mean ± standard error of relative abundance of three biological replicates (*P<0.05 and **P<0.01).

4.4 Discussion

Proline is an important amino acid which functions to maintain osmotic balance in plant cells against the reactive species formed under stressful conditions such as salt and drought stress. Similar to our results (Fig. 4.1), Mzid et al (2018) showed maximum proline accumulation in transgenic *VvWRKY2 Nicotiana tobacum* compared with wild type subjected to salinity and drought stress. This indicates the importance of proline in protecting the cellular structures against reactive species generated due to abiotic stress through regulation of turgor pressure, hence maintains osmotic balance (Sarmast and Saheli, 2015). Maximal levels of accumulated proline in transgenic plants suggests regulatory role of *OsWNK9* transgene in the same. The ROS and other reactive compounds render plant cells to oxidative stress by peroxidation of membrane lipids leading to membrane damage; as a result, the ultimate leakage of ions through damaged membranes results into ionic imbalance that eventually brings on to cell death. Lipid peroxides are less stable and their degradation give rise to certain reactive products including carbonyl compounds. These are also called as TBA-reactive substances (TBARS) and their concentration is increased under oxidative stress conditions. The MDA is one of TBARS largely produced by degradation of polyunsaturated fatty acid lipid hydroperoxides and can be used as a biomarker for oxidative stress. The spectrophotometric measurement of chromophore (MDA-TBA adduct) generated by reaction between one molecule of MDA and two molecules of TBA reflects MDA concentration and thereby extent of oxidative stress induced membrane damage (Zhao et. al., 2009). Our MDA concentration measurements for normal physiological and stressful conditions (Fig. 4.2) are in agreement with those mentioned by Zhao et al, 2009. They

showed increased MDA levels after plants exposed to 200 mM salt stress for 48 hours, the raised levels of MDA being comparatively higher in wild type *Arabidopsis* than those in transgenic one carrying yeast transcription factor *YAP1* gene. Similarly, same pattern found in transgenic rice plants co-overexpressing *OsGS1;1* and *OsGS2* genes; MDA levels in them being comparatively lower to that of wild type under osmotic and salinity stress (James et al., 2018). Liu et al (2013) reported same results for wild type and transgenic rice (expressing *OsHsf 7*) plants exposed to salt stress for 24 hours. Hence, the expression of *OsWNK9* may have a role in improvement of stability in cell membrane under salt stress and supports abiotic stress tolerance in transgenic *Arabidopsis* plants. Abiotic stress often leads to over-accumulation of non-radical reactive molecules like H_2O_2 which are reduced by antioxidant defense systems in plants. The H_2O_2 reacts with biomolecules of cell causing extensive damage eventually leading to a programmed cell death. Three enzymes peroxidase, ascorbate peroxidase and catalase are involved in the scavenging of H_2O_2 over-accumulated under stress. Each of them has different affinity to H_2O_2 and therefore a complex pattern of their activity and regulation is observed under normal physiological conditions as well as under various types of stresses (Velikova et al., 2000; Racchi, 2013). Our H_2O_2 measurement results (Fig. 4.6) are in consensus with those of three *AtOxR* transgenic lines of *Arabidopsis* which limits H_2O_2 accumulation under the salinity and drought conditions for 12, 24 and 48 hours. Peroxidase use organic compounds like phenols, aromatic amines, and quinines as substrate and bring on dehydrogenation reaction along with the use of H_2O_2. The decrease in amount of hydrogen donor or H_2O_2 or production of an oxidized compound is generally used as a measure of activity in peroxidase scavenging reactions (Putter et al., 1974). Guaiacol

peroxidase (POD) or simply peroxidase is one of the antioxidant enzymes which is involved in scavenging of H_2O_2 accumulated during various stress conditions in plants. The dynamic pattern of POD activity (Fig. 4.3) obtained in our study is consistent with results reported in case of salt-tolerant BR5033 genotype in maize, suggests involvement of this enzyme in stress tolerance (Neto et al., 2006). Similarly, such type of trend has been reported in four *Festuca arundinacea Schreb.* genotypes subjected to drought stress. Along with the POD, catalase and ascorbate peroxidase (APX) are two enzymes which perform similar function in conversion of H_2O_2 into simpler non-harmful compounds within the cell. Among, catalase and POD, APX scavenge H_2O_2 with highest affinity in almost every part of cell including cell organelles mitochondria, peroxisomes, and chloroplast as well as cytosol and apoplastic space (Sofo et al, 2015). APX using ascorbate as an electron donor substrate reduces H_2O_2 and its presence at multiple places in plant cells denotes its vital importance in protection of sub-cellular structures from the oxidative damage under abiotic stress conditions including salinity. The end product of APX scavenging action involves dehydroascorbate which is an important component of ascorbate-glutathione cycle. Our result shows concurrence with such versatility of APX activity compared to the activity pattern observed for catalase and POD (Fig. 4.3, 4.4, 4.5). Similar to our results, a linear increase in the APX activity has been reported in Jaguar variety and catalase activity in Mini-Mustang variety of maize plant subjected to drought stress for different time periods. Comparing with wild type, the highest rise in APX activity has been observed in *Arabidopsis IbZFP1* transgenic lines subjected to salt and drought stress for a period of 2 and 4 weeks respectively (Wang et al, 2016). Du et al (2017) report an increase in catalase activity of *RtWRKY* transgenic lines of *Arabidopsis*

under salinity stress. The activity trends of all three enzymes indicate complex regulatory mechanisms involved in scavenging of reactive molecules produced during stress conditions. The total antioxidant capacity of plant extracts is an outcome of an additive action considering all antioxidant molecules in plants. The antioxidant enzymes located in different organelles which represent a first line of defense and other antioxidant molecules such as ascorbic acid, glutathione, phenolic compounds, flavonoids, anthocyanins, carotenoids ,and α-tocopherol which represent a second line of defense against the oxidative damage (Racchi, 2013). DPPH is a stable free radical which upon scavenging gets turned from red to yellow. Thus, DPPH free radical scavenging activity is a measure of total antioxidant capacity of plant extracts. In our research, ethanolic extracts from leaves of transgenic plants showed a higher percentage of radical scavenging as compared with wild type plants (Fig. 4.7); these results are in accordance by Cheng et al (2013). They reported transgenic potato plants expressing IbMYB1 transcription factor showed rise in the DPPH scavenging percentage for 2, 4 and 6 days of salt stress treatment period (Cheng et al., 2013). Also, compared to wild type, increase in the total antioxidant capacity in terms of DPPH scavenging percentage has been reported to occur in transgenic tobacco lines over-expressing Snapdragon *del* gene (Naing et al, 2017). Over all correlation of biochemical assays, the transgenic lines harboring *OsWNK9* transgene could be a promising candidate gene for alleviation of excess reactive molecules produced under stressful environments. The transgenic plants showed reduced H_2O_2 and MDA content, high levels of proline accumulation and overall rise in various antioxidant defense systems as compared with the wild type plant. The intracellular balance of Na^+ and K^+ is one of mechanisms for conferring salinity stress

tolerance characteristics in plants (Mishra et al., 2014). Our results indicate that the transgenic lines exhibit salt tolerance by virtue of ion homeostasis as Na^+ to K^+ ratio was well maintained by them. Moreover, it also resembles with the report published by Yao et al., 2010 showing that transgenic tobacco BY2 lines expressing two transporters: OsHKT2;1 and OsHKT2;2 mediated salt tolerance through ionic balance. Thus, the results obtained from biochemical assays implicates *OsWNK9* has a stellar role in conferring salt tolerance by regulation of multitude mechanisms, includes antioxidant enzyme systems, various antioxidant molecules and regulation of Na^+, K^+ balance within the cells.

4.5 Conclusion

The transgenic plants adopted stress tolerance mechanisms against salinity and drought stress, they encompass free radical scavenging by diverse enzymes of antioxidant system namely peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) as well as accumulation of osmolytes like proline, reduction in membrane damage and enhancement of total antioxidant capacity of plants. Thus, *OsWNK9* clearly has a significant role in abiotic stress tolerance through a variety of mechanisms, also indicating the complexity of stress management in plants. The gene can be a good candidate for generation of the genetically modified and economically important plant species.

Summary

Summary

The main objective of present thesis is identification and characterization of *OsWNK* gene family in rice plant. To identify the OsWNK gene family first we performed some of *in-silico* analysis for identifying the OsWNK family belongs to protein kinase. We identified 9 members of WNK in rice, that showed resemblance to *Arabidopsis* and human WNK. Phylogenetic analysis of all WNK was performed and categorized into five main clades and subclades. Conserved motif and domains in the deduced amino acid sequences of rice WNK strongly supported their identity as members of WNK kinase protein. Their gene expression pattern was further investigated using qRT-PCR in tissue specific and various abiotic stresses. Significant expression level expression of most *OsWNK* was observed in root. However, *OsWNKs* showed differential transcript accumulation pattern under various abiotic stresses *viz*. salt, drought, cold and heat. The presence of various abiotic stress responsive *cis*-elements in promoter region of OsWNK will also predict their importance in providing basal tolerance to rice during critical conditions.

Further, we have selected *OsWNK9* because of its differential transcript expression and cloned full-length rice *OsWNK9* sequence. We have concluded that *OsWNK9* belongs to protein kinase family by the observation of conserved signature motifs and domains with phylogeny and homology modeling using established protein kinase family members. Transcript analysis of shoots and roots suggested significantly deferential expression pattern under salt, drought and ABA stresses. Overexpression of *OsWNK9* in *Arabidopsis* improved tolerance against salt, drought, and ABA stress. Transgenic lines showed remarkable phenotypes such as higher fresh biomass and long

root length compared to WT. The overexpressed line showed better performance such as less water loss, higher drought tolerance and low bleaching of chlorophyll pigments under salt stress compared to WT. Promoter activity of rice during the developmental stages revealed that it is expressed throughout, in vegetative and reproductive organs, while being highly expressed in roots under the abiotic stresses. The green fluorescent protein tagged with OsWNK9 protein was localized in cell nucleus of epidermal cells in tobacco and protoplast of *Arabidopsis*. ABA biosynthesis (*ABA3, NCED3*) and abiotic stress marker genes (*DREB2C, WRKY28, NAC019*, and *EREPB*) shown significantly higher expression pattern under salt, drought and ABA stress compared to WT. Transgenic lines showed higher endogenous ABA compared to WT.

Further research work was undertaken in accordance with the above findings. Various stress tolerance mechanisms adopted by these transgenic plants against salinity and drought stress were investigated. Some of these studies included free radical scavenging by diverse enzymes of the antioxidant system such as peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX). Accumulation of osmolytes like proline, reduction in the membrane damage and enhancement of the total antioxidant capacity of the plants was also elucidated. Thus, OsWNK9 clearly shows a significant role in abiotic stress tolerance through a variety of mechanisms. This further substantiates the complexity of signaling cascades with stress management in plants.

The schematic model for the regulatory function of OsWNK9 under the salt and drought stress in the plant cell is represented in the conclusion figure (Fig. 5.1). Considering the repertoire of the research data generated in this thesis, we have

concluded that *OsWNK9* may be a prospective candidate gene to develop abiotic stress tolerant crop plants through genetic engineering strategy.

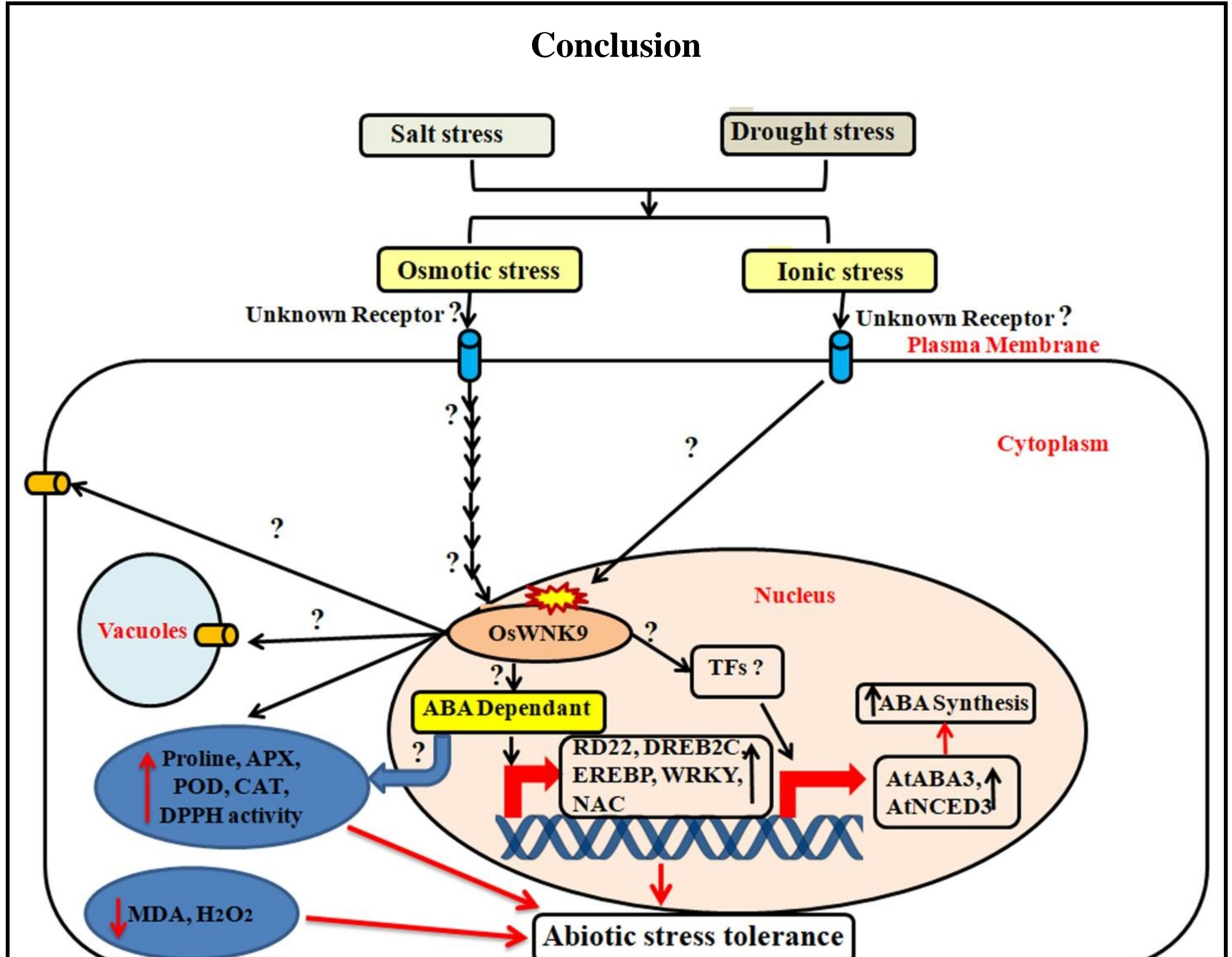


Figure 5.1 Hypothetical schematic model for the regulatory function of OsWNK9 under the salt and drought stress. Salt and drought stress triggered cumulatively osmotic and ionic stress which is sensed by unknown plasma membrane receptors and activate the downstream response. The probable unknown mechanisms (marked with '?') is represented here. OsWNK9 might be involved in the abiotic stress responsive cascade through ABA-dependant pathways. The activated OsWNK9 further phosphorylate other unknown proteins as well as transcription of abiotic stress-related genes. It may activate vacuolar and plasma membrane transporter by phosphorylating and maintaining ion homeostasis.

Future scope of study

Future scope of study

- This thesis is mainly focused on characterization of *OsWNK9* gene. It discovered the importance of *OsWNK9* gene under abiotic stress conditions, but the signaling pathway of WNK gene family in the plant system are yet to be identified.
- Activation studies of OsWNK9 kinase in *Arabidopsis* transgenic lines by raising OsWNK9 specific antibodies and performing kinase activation experiments.
- Hunting for the interacting partner proteins for the OsWNK9 by yeast two hybrid assays through protein-protein interaction.
- Subcellular localization of OsWNK9 in rice system.
- Over-expressing of *OsWNK9* gene in rice system is an important future course for this research work. This can be carried out through genetic engineering strategies and field trials of *OsWNK9* transgenic plants for developing the abiotic resistant crop fields.
- The commercial availability of genetically modified *OsWNK9* transgenic rice lines will help farmers by enabling greater crop tolerance against salt and drought conditions.
- The characterization of remaining genes (*OsWNK1-OsWNK8*) of OsWNK family in rice is also yet to be undertaken.

References

1) Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2003). *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *The Plant Cell*, *15*(1), 63-78.

2) Aebi, H. E. (1983). Catalase. *Methods of enzymatic analysis*.

3) Agarwal, P., Agarwal, P. K., & Gohil, D. (2018). Transcription Factor-Based Genetic Engineering for Salinity Tolerance in Crops. In *Salinity Responses and Tolerance in Plants, Volume 1* (pp. 185-211). Springer, Cham

4) Ali, M. A., Azeem, F., Nawaz, M. A., Acet, T., Abbas, A., Imran, Q. M., & Bohlmann, H. (2018). Transcription factors WRKY11 and WRKY17 are involved in abiotic stress responses in *Arabidopsis*. *Journal of plant physiology*, *226*, 12-21.

5) Almeida Trapp, M., De Souza, G. D., Rodrigues-Filho, E., Boland, W., & Mithöfer, A. (2014). Validated method for phytohormone quantification in plants. *Frontiers in plant science*, *5*, 417.

6) Amin, U. S. M., Biswas, S., Elias, S. M., Razzaque, S., Haque, T., Malo, R., & Seraj, Z. I. (2016). Enhanced salt tolerance conferred by the complete 2.3 kb cDNA of the rice vacuolar Na+/H+ antiporter gene compared to 1.9 kb coding region with 5′ UTR in transgenic lines of rice. *Frontiers in plant science*, *7*, 14.

7) Anderson, M. D., Prasad, T. K., & Stewart, C. R. (1995). Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant physiology*, *109*(4), 1247-1257.

8) Ashikari, M., & Ma, J. F. (2015). Exploring the power of plants to overcome environmental stresses.

9) Babitha, K. C., Ramu, S. V., Pruthvi, V., Mahesh, P., Nataraja, K. N., & Udayakumar, M. (2013). Co-expression of AtbHLH17 and AtWRKY28 confers resistance to abiotic stress in *Arabidopsis*. *Transgenic research*, *22*(2), 327-341.

10) Barnabas, B., Jager, K., & Feher, A. (2008). The effect of drought and heat stress on reproductive processes in cereals. *Plant, cell & environment*, *31*(1), 11-38.

11) Bates, B. C., Kundzewicz, Z. W., Wu, S., & Palutikof, J. P. (2008). Climate change and water. Technical paper of the intergovernmental panel on climate

change, IPCC secretariat, Geneva. *Climate Change Policy with a Renewed Environmental Ethic*, *21*, 85-101.

12) Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and soil*, *39*(1), 205-207.

13) Bruinsma, J. (2017). *World agriculture: towards 2015/2030: an FAO study*. Routledge.

14) Cai, R., Zhao, Y., Wang, Y., Lin, Y., Peng, X., Li, Q., & Cheng, B. (2014). Overexpression of a maize WRKY58 gene enhances drought and salt tolerance in transgenic rice. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *119*(3), 565-577.

15) Cao-Pham, A. H., Urano, D., Ross-Elliott, T. J., & Jones, A. M. (2018). Nudge-nudge, WNK-WNK (kinases), say no more?. *New Phytologist*, *220*(1), 35-48.

16) Carranco, R., Almoguera, C., & Jordano, J. (1999). An imperfect heat shock element and different upstream sequences are required for the seed-specific expression of a small heat shock protein gene. *Plant Physiology*, *121*(3), 723-730.

17) Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osório, M. L., & Pinheiro, C. (2002). How plants cope with water stress in the field? Photosynthesis and growth. *Annals of botany*, *89*(7), 907-916.

18) Chen, H., Liu, L., Wang, L., Wang, S., & Cheng, X. (2016). VrDREB2A, a DREB-binding transcription factor from Vigna radiata, increased drought and high-salt tolerance in transgenic *Arabidopsis* thaliana. *Journal of plant research*, *129*(2), 263-273.

19) Chen, L. J., Wuriyanghan, H., Zhang, Y. Q., Duan, K. X., Chen, H. W., Li, Q. T., & Lin, Q. (2013). An S-domain receptor-like kinase OsSIK2 confers abiotic stress tolerance and delays dark-induced leaf senescence in rice. *Plant physiology*, pp-113.

20) Chen, X., Wang, Y., Lv, B., Li, J., Luo, L., Lu, S., & Ming, F. (2014). The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway. *Plant and Cell Physiology*, *55*(3), 604-619.

21) Chen, Y., Hou, M., Liu, L., Wu, S., Shen, Y., Ishiyama, K., & Tan, B. C. (2014). The maize DWARF1 encodes a gibberellin 3-oxidase and is dual-localized to the nucleus and cytosol. *Plant physiology*, pp-114.

22) Cheng, Y. J., Kim, M. D., Deng, X. P., Kwak, S. S., & Chen, W. (2013). Enhanced salt stress tolerance in transgenic potato plants expressing IbMYB1, a sweet potato transcription factor. *J Microbiol Biotechnol*, *23*(12), 1737-1746.

23) Chinnusamy, V., & Zhu, J. K. (2009). Epigenetic regulation of stress responses in plants. *Current opinion in plant biology*, *12*(2), 133-139.

24) Chinnusamy, V., Jagendorf, A., & Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. *Crop Science*, *45*(2), 437-448.

25) Chou, K. C., & Shen, H. B. (2010). Plant-mPLoc: a top-down strategy to augment the power for predicting plant protein subcellular localization. *PloS one*, *5*(6), e11335.

26) Crooks, G. E., Hon, G., Chandonia, J. M., & Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome research*, *14*(6), 1188-1190.

27) Das, P., Nutan, K. K., Singla-Pareek, S. L., & Pareek, A. (2015). Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles. *Frontiers in Environmental Science*, *2*, 70.

28) de Azevedo Neto, A. D., Prisco, J. T., Enéas-Filho, J., de Abreu, C. E. B., & Gomes-Filho, E. (2006). Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany*, *56*(1), 87-94.

29) Dhankher, O. P., & Foyer, C. H. (2018). Climate resilient crops for improving global food security and safety. *Plant, cell & environment*, *41*(5), 877-884.

30) Diedhiou, C. J., & Golldack, D. (2006). Salt-dependent regulation of chloride channel transcripts in rice. *Plant Science*, *170*(4), 793-800.

31) Ding, Z., Li, S., An, X., Liu, X., Qin, H., & Wang, D. (2009). Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. *Journal of Genetics and Genomics*, *36*(1), 17-29.

32) Dresselhaus, T., Barcelo, P., Hagel, C., Lörz, H., & Humbeck, K. (1996). Isolation and characterization of a Tritordeum cDNA encoding S-adenosylmethionine decarboxylase that is circadian-clock-regulated. *Plant molecular biology*, *30*(5), 1021-1033.

33) Du, C., Zhao, P., Zhang, H., Li, N., Zheng, L., & Wang, Y. (2017). The Reaumuria trigyna transcription factor RtWRKY1 confers tolerance to salt stress in transgenic *Arabidopsis*. *Journal of plant physiology*, *215*, 48-58.

34) Eynard, A., Lal, R., & Wiebe, K. (2005). Crop response in salt-affected soils. *Journal of Sustainable Agriculture*, *27*(1), 5-50.

35) Filichkin, S. A., Breton, G., Priest, H. D., Dharmawardhana, P., Jaiswal, P., Fox, S. E., & Mockler, T. C. (2011). Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and cis-regulatory modules. *PloS one*, *6*(6), e16907.

36) Fujimoto, S. Y., Ohta, M., Usui, A., Shinshi, H., & Ohme-Takagi, M. (2000). *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. *The Plant Cell*, *12*(3), 393-404.

37) Gagnon, K. B., England, R., & Delpire, E. (2006). Volume sensitivity of cation-Cl− cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *American Journal of Physiology-Cell Physiology*, *290*(1), C134-C142.

38) Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The proteomics protocols handbook* (pp. 571-607). Humana press.

39) Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry*, *48*(12), 909-930.

40) Goldberg, T., Hecht, M., Hamp, T., Karl, T., Yachdav, G., Ahmed, N., & Bernhofer, M. (2014). LocTree3 prediction of localization. *Nucleic acids research*, *42*(W1), W350-W355.

41) Gomez-Porras, J. L., Riaño-Pachón, D. M., Dreyer, I., Mayer, J. E., & Mueller-Roeber, B. (2007). Genome-wide analysis of ABA-responsive elements ABRE and CE3 reveals divergent patterns in *Arabidopsis* and rice. *BMC genomics*, *8*(1), 260.

42) Grattan, S., Zeng, L., Shannon, M., & Roberts, S. (2002). Rice is more sensitive to salinity than previously thought. *California Agriculture*, *56*(6), 189-198.

43) Gruber, V., Blanchet, S., Diet, A., Zahaf, O., Boualem, A., Kakar, K., & Crespi, M. (2009). Identification of transcription factors involved in root apex responses to salt stress in Medicago truncatula. *Molecular Genetics and Genomics*, *281*(1), 55-66.

44) Guo, A. Y., Zhu, Q. H., Chen, X., & Luo, J. C. (2007). GSDS: a gene structure display server. *Yi chuan= Hereditas*, *29*(8), 1023-1026.

45) Gupta, B., & Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *International journal of genomics*, *2014*.

46) Hanks, S. K., & Quinn, A. M. (1991). [2] Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. In *Methods in enzymology* (Vol. 200, pp. 38-62). Academic Press.

47) Hao, Y. J., Wei, W., Song, Q. X., Chen, H. W., Zhang, Y. Q., Wang, F., & Ma, B. (2011). Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. *The Plant Journal*, *68*(2), 302-313.

48) Hashimoto, M., & Komatsu, S. (2007). Proteomic analysis of rice seedlings during cold stress. *Proteomics*, *7*(8), 1293-1302.

49) Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of biochemistry and biophysics*, *125*(1), 189-198.

50) Hemmer, W., McGlone, M., Tsigelny, I., & Taylor, S. S. (1997). Role of the glycine triad in the ATP-binding site of cAMP-dependent protein kinase. *Journal of Biological Chemistry*, *272*(27), 16946-16954.

51) Hernandez-Garcia, C. M., & Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. *Plant Science*, *217*, 109-119.

52) Ho, H. L. (2015). Functional roles of plant protein kinases in signal transduction pathways during abiotic and biotic stress. *J. Biodivers. Biopros. Dev*, *2*, 147-154.

53) Hong, Y., Zhang, H., Huang, L., Li, D., & Song, F. (2016). Overexpression of a stress-responsive NAC transcription factor gene ONAC022 improves drought and salt tolerance in rice. *Frontiers in plant science*, *7*, 4.

54) Hong-Hermesdorf, A., Brüx, A., Grüber, A., Grüber, G., & Schumacher, K. (2006). A WNK kinase binds and phosphorylates V-ATPase subunit C. *FEBS letters*, *580*(3), 932-939.

55) Hoorn, E. J., Nelson, J. H., McCormick, J. A., & Ellison, D. H. (2011). The WNK kinase network regulating sodium, potassium, and blood pressure. *Journal of the American Society of Nephrology*, ASN-2010080827.

56) Hou, L., Chen, L., Wang, J., Xu, D., Dai, L., Zhang, H., & Zhao, Y. (2012). Construction of stress responsive synthetic promoters and analysis of their activity in transgenic *Arabidopsis thaliana*. *Plant molecular biology reporter*, *30*(6), 1496-1506.

57) Huang, C. L., Cha, S. K., Wang, H. R., Xie, J., & Cobb, M. H. (2007). WNKs: protein kinases with a unique kinase domain. *Experimental & molecular medicine*, *39*(5), 565.

58) Hugouvieux, V., Kwak, J. M., & Schroeder, J. I. (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. *Cell*, *106*(4), 477-487.

59) Hwang, I., Sze, H., & Harper, J. F. (2000). A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca2+ pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *97*(11), 6224-6229.

60) Ishimaru, T., Horigane, A. K., Ida, M., Iwasawa, N., San-oh, Y. A., Nakazono, M., & Yoshida, M. (2009). Formation of grain chalkiness and changes in water distribution in developing rice caryopses grown under high-temperature stress. *Journal of Cereal Science*, *50*(2), 166-174.

61) Jaleel, C. A., Manivannan, P., Lakshmanan, G. M. A., Gomathinayagam, M., & Panneerselvam, R. (2008). Alterations in morphological parameters and photosynthetic pigment responses of Catharanthus roseus under soil water deficits. *Colloids and Surfaces B: Biointerfaces*, *61*(2), 298-303.

62) James, D., Borphukan, B., Fartyal, D., Ram, B., Singh, J., Manna, M., & Reddy, M. K. (2018). Concurrent overexpression of OsGS1; 1 and OsGS2 genes in transgenic rice (Oryza sativa L.): Impact on tolerance to abiotic stresses. *Frontiers in plant science*, *9*.

63) Je, J., Chen, H., Song, C., & Lim, C. O. (2014). *Arabidopsis* DREB2C modulates ABA biosynthesis during germination. *Biochemical and biophysical research communications*, *452*(1), 91-98.

64) Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant molecular biology reporter*, *5*(4), 387-405.

65) Jena, K. K., Kim, S. M., Suh, J. P., Yang, C. I., & Kim, Y. G. (2012). Identification of cold-tolerant breeding lines by quantitative trait loci associated with cold tolerance in rice. *Crop science*, *52*(2), 517-523.

66) Ji, H., Pardo, J. M., Batelli, G., Van Oosten, M. J., Bressan, R. A., & Li, X. (2013). The Salt Overly Sensitive (SOS) pathway: established and emerging roles. *Molecular plant*, *6*(2), 275-286.

67) Jonak, C., Kiegerl, S., Ligterink, W., Barker, P. J., Huskisson, N. S., & Hirt, H. (1996). Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proceedings of the National Academy of Sciences*, *93*(20), 11274-11279.

68) Jones, C. G., Hare, J. D., & Compton, S. J. (1989). Measuring plant protein with the Bradford assay. *Journal of chemical ecology*, *15*(3), 979-992.

69) Joo, J., Choi, H. J., Lee, Y. H., Kim, Y. K., & Song, S. I. (2013). A transcriptional repressor of the ERF family confers drought tolerance to rice and regulates genes preferentially located on chromosome 11. *Planta*, *238*(1), 155-170.

70) Jordan, P. (2011). WNK2 (WNK lysine deficient protein kinase 2).

71) Kahle, K. T., Rinehart, J., Ring, A., Gimenez, I., Gamba, G., Hebert, S. C., & Lifton, R. P. (2006). WNK protein kinases modulate cellular Cl− flux by altering

the phosphorylation state of the Na-K-Cl and K-Cl cotransporters. *Physiology*, *21*(5), 326-335.

72) Kahle, K. T., Wilson, F. H., & Lifton, R. P. (2005). Regulation of diverse ion transport pathways by WNK4 kinase: a novel molecular switch. *Trends in Endocrinology & Metabolism*, *16*(3), 98-103.

73) Kandpal, R. P., & Rao, N. A. (1985). Alterations in the biosynthesis of proteins and nucleic acids in finger millet (Eleucine coracana) seedlings during water stress and the effect of proline on protein biosynthesis. *Plant Science*, *40*(2), 73-79.

74) Kang, H. M., & Saltveit, M. E. (2002). Antioxidant enzymes and DPPH-radical scavenging activity in chilled and heat-shocked rice (Oryza sativa L.) seedlings radicles. *Journal of agricultural and food chemistry*, *50*(3), 513-518.

75) Kanneganti, V., & Gupta, A. K. (2008). Overexpression of OsiSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice. *Plant molecular biology*, *66*(5), 445-462.

76) Kim, S. J., Lee, S. C., Hong, S. K., An, K., An, G., & Kim, S. R. (2009). Ectopic expression of a cold-responsive OsAsr1 cDNA gives enhanced cold tolerance in transgenic rice plants. *Molecules and cells*, *27*(4), 449-458.

77) Knight, H., & Knight, M. R. (2001). Abiotic stress signalling pathways: specificity and cross-talk. *Trends in plant science*, *6*(6), 262-267.

78) Komis, G., Samajova, O., Ovecka, M., & Samaj, J. (2018). Cell and Developmental Biology of Plant Mitogen-Activated Protein Kinases. *Annual review of plant biology*, *69*, 237-265.

79) Kondamudi, R., Swamy, K. N., Chakravarthy, D. V. N., Vishnuprasanth, V., Rao, Y. V., Rao, P. R., & Voleti, S. R. (2012). Heat Stress in Rice–Physiological Mechanisms and Adaptation Strategies. In *Crop Stress and its Management: Perspectives and Strategies* (pp. 193-224). Springer, Dordrecht.

80) Kosova, K., Vítamvas, P., Prasil, I. T., & Renaut, J. (2011). Plant proteome changes under abiotic stress contribution of proteomics studies to understanding plant stress response. *Journal of proteomics*, *74*(8), 1301-1322.

81) Kumar, K., & Saddhe, A. A. (2018). Targeting Aquaporins for Conferring Salinity Tolerance in Crops. In *Salinity Responses and Tolerance in Plants, Volume 1* (pp. 65-84). Springer, Cham.

82) Kumar, K., Kumar, M., Kim, S. R., Ryu, H., & Cho, Y. G. (2013). Insights into genomics of salt stress response in rice. *Rice*, *6*(1), 27.

83) Kumar, K., Rao, K. P., Biswas, D. K., & Sinha, A. K. (2011). Rice WNK1 is regulated by abiotic stress and involved in internal circadian rhythm. *Plant signaling & behavior*, *6*(3), 316-320.

84) Kumar, K., Rao, K. P., Sharma, P., & Sinha, A. K. (2008). Differential regulation of rice mitogen activated protein kinase kinase (MKK) by abiotic stress. *Plant physiology and biochemistry*, *46*(10), 891-897.

85) Kurusu, T., Hamada, H., Koyano, T., & Kuchitsu, K. (2012). Intracellular localization and physiological function of a rice Ca2+-permeable channel OsTPC1. *Plant signaling & behavior*, *7*(11), 1428-1430.

86) Lai, J. G., Tsai, S. M., Tu, H. C., Chen, W. C., Kou, F. J., Lu, J. W., & Yuh, C. H. (2014). Zebrafish WNK lysine deficient protein kinase 1 (wnk1) affects angiogenesis associated with VEGF signaling. *PLoS One*, *9*(8), e106129.

87) Latha, G. M., Mohapatra, T., Geetanjali, A. S., & Rao, K. R. S. (2017). Engineering Rice for Abiotic Stress Tolerance: A Review. *Current Trends in Biotechnology & Pharmacy*, *11*(4).

88) Lee, S. J., Kang, J. Y., Park, H. J., Kim, M. D., Bae, M. S., Choi, H. I., & Kim, S. Y. (2010). DREB2C interacts with ABF2, a bZIP protein regulating ABA-responsive gene expression, and its overexpression affects ABA sensitivity. *Plant physiology*, pp-110.

89) Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic acids research*, *30*(1), 325-327.

90) Leung, J., & Giraudat, J. (1998). Abscisic acid signal transduction. *Annual review of plant biology*, *49*(1), 199-222.

91) Liang, W., Ma, X., Wan, P., & Liu, L. (2018). Plant salt-tolerance mechanism: a review. *Biochemical and biophysical research communications*, *495*(1), 286-291.

92) Liu, A. L., Zou, J., Liu, C. F., Zhou, X. Y., Zhang, X. W., Luo, G. Y., & Chen, X. B. (2013). Over-expression of OsHsfA7 enhanced salt and drought tolerance in transgenic rice. *BMB reports*, *46*(1), 31.

93) Liu, C., Mao, B., Ou, S., Wang, W., Liu, L., Wu, Y., & Wang, X. (2014). OsbZIP71, a bZIP transcription factor, confers salinity and drought tolerance in rice. *Plant molecular biology*, *84*(1-2), 19-36.

94) Liu, L., Zhang, Z., Dong, J., & Wang, T. (2016). Overexpression of MtWRKY76 increases both salt and drought tolerance in Medicago truncatula. *Environmental and experimental botany*, *123*, 50-58.

95) Liu, Y., Ji, X., Zheng, L., Nie, X., & Wang, Y. (2013). Microarray analysis of transcriptional responses to abscisic acid and salt stress in *Arabidopsis thaliana*. *International journal of molecular sciences*, *14*(5), 9979-9998.

96) Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.

97) Lopez-Carbonell, M., & Jáuregui, O. (2005). A rapid method for analysis of abscisic acid (ABA) in crude extracts of water stressed *Arabidopsis thaliana* plants by liquid chromatography—mass spectrometry in tandem mode. *Plant physiology and Biochemistry*, *43*(4), 407-411.

98) Lu, M., Ying, S., Zhang, D. F., Shi, Y. S., Song, Y. C., Wang, T. Y., & Li, Y. (2012). A maize stress-responsive NAC transcription factor, ZmSNAC1, confers enhanced tolerance to dehydration in transgenic *Arabidopsis*. *Plant cell reports*, *31*(9), 1701-1711.

99) Lutts, S., Kinet, J. M., & Bouharmont, J. (1995). Changes in plant response to NaCl during development of rice (Oryza sativa L.) varieties differing in salinity resistance. *Journal of Experimental Botany*, *46*(12), 1843-1852.

100) Ma, T. L., Wu, W. H., & Wang, Y. (2012). Transcriptome analysis of rice root responses to potassium deficiency. *BMC Plant Biology*, *12*(1), 161.

101) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., & Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science*, *298*(5600), 1912-1934.

102) Manuka, R., Saddhe, A. A., & Kumar, K. (2015). Genome-wide identification and expression analysis of WNK kinase gene family in rice. *Computational biology and chemistry*, *59*, 56-66.

103) Manuka, R., Saddhe, A. A., & Kumar, K. (2018). Expression of OsWNK9 in *Arabidopsis* conferred tolerance to salt and drought stress. *Plant Science*, *270*, 58-71.

104) McCormick, J. A., & Ellison, D. H. (2011). The WNKs: atypical protein kinases with pleiotropic actions. *Physiological reviews*, *91*(1), 177-219.

105) Min, X., Lee, B. H., Cobb, M. H., & Goldsmith, E. J. (2004). Crystal structure of the kinase domain of WNK1, a kinase that causes a hereditary form of hypertension. *Structure*, *12*(7), 1303-1311.

106) Mishra, S., Alavilli, H., Lee, B. H., Panda, S. K., & Sahoo, L. (2014). Cloning and functional characterization of a vacuolar Na+/H+ antiporter gene from mungbean (VrNHX1) and its ectopic expression enhanced salt tolerance in *Arabidopsis thaliana*. *PloS one*, *9*(10), e106678.

107) Mishra, S., Singh, B., Panda, K., Singh, B. P., Singh, N., Misra, P., & Singh, N. K. (2016). Association of SNP haplotypes of HKT family genes with salt tolerance in Indian wild rice germplasm. *Rice*, *9*(1), 15.

108) Mitsui, T., Shiraya, T., Kaneko, K., & Wada, K. (2013). Proteomics of rice grain under high temperature stress. *Frontiers in plant science*, *4*, 36.

109) Mittler, R., & Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. *Annual review of plant biology*, *61*, 443-462.

110) Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., & Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *93*(2), 765-769.

111) Morita, S. (2008). Prospect for developing measures to prevent high-temperature damage to rice grain ripening. *Japanese Journal of Crop Science (Japan)*.

112) Moustafa, K., AbuQamar, S., Jarrar, M., Al-Rajab, A. J., & Trémouillaux-Guiller, J. (2014). MAPK cascades and major abiotic stresses. *Plant cell reports*, *33*(8), 1217-1225.

113) Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.*, *59*, 651-681.

114) Murakami-Kojima, M., Nakamichi, N., Yamashino, T., & Mizuno, T. (2002). The APRR3 component of the clock-associated APRR1/TOC1 quintet is phosphorylated by a novel protein kinase belonging to the WNK family, the gene for which is also transcribed rhythmically in *Arabidopsis thaliana*. *Plant and cell physiology*, *43*(6), 675-683.

115) Mzid, R., Zorrig, W., Ayed, R. B., Hamed, K. B., Ayadi, M., Damak, Y., & Hanana, M. (2018). The grapevine VvWRKY2 gene enhances salt and osmotic stress tolerance in transgenic Nicotiana tabacum. *3 Biotech*, *8*(6), 277.

116) Naing, A. H., Park, K. I., Ai, T. N., Chung, M. Y., Han, J. S., Kang, Y. W., & Kim, C. K. (2017). Overexpression of snapdragon Delila (Del) gene in tobacco enhances anthocyanin accumulation and abiotic stress tolerance. *BMC plant biology*, *17*(1), 65.

117) Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and cell physiology*, *22*(5), 867-880.

118) Nakashima, K., & Yamaguchi-Shinozaki, K. (2005). Molecular studies on stress-responsive gene expression in *Arabidopsis* and improvement of stress tolerance in crop plants by regulon biotechnology. *Japan Agricultural Research Quarterly: JARQ*, *39*(4), 221-229.

119) Nakashima, K., Tran, L. S. P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., & Yamaguchi-Shinozaki, K. (2007). Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *The Plant Journal*, *51*(4), 617-630.

120) O'Reilly, M., Marshall, E., Speirs, H. J., & Brown, R. W. (2003). WNK1, a gene within a novel blood pressure control pathway, tissue-specifically generates radically different isoforms with and without a kinase domain. *Journal of the American Society of Nephrology*, *14*(10), 2447-2456.

121) Pandey, G. K., Pandey, A., Prasad, M., & Böhmer, M. (2016). Abiotic stress signaling in plants: Functional genomic intervention. *Frontiers in plant science*, *7*, 681.

122) Peng, S., Huang, J., Sheehy, J. E., Laza, R. C., Visperas, R. M., Zhong, X., & Cassman, K. G. (2004). Rice yields decline with higher night temperature from global warming. *Proceedings of the National academy of Sciences of the United States of America*, *101*(27), 9971-9975.

123) Putter, J. (1974). Peroxidases. In *Methods of Enzymatic Analysis (Second Edition), Volume 2* (pp. 685-690).

124) Qin, Y., Tian, Y., & Liu, X. (2015). A wheat salinity-induced WRKY transcription factor TaWRKY93 confers multiple abiotic stress tolerance in *Arabidopsis thaliana*. *Biochemical and biophysical research communications*, *464*(2), 428-433.

125) Qiu, Y., & Yu, D. (2009). Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. *Environmental and experimental botany*, *65*(1), 35-47.

126) Racchi, M. L. (2013). Antioxidant defenses in plants with attention to Prunus and Citrus spp. *Antioxidants*, *2*(4), 340-369.

127) Rajendran, K., Tester, M., & Roy, S. J. (2009). Quantifying the three main components of salinity tolerance in cereals. *Plant, cell & environment*, *32*(3), 237-249.

128) Reddy, I. N. B. L., Kim, B. K., Yoon, I. S., Kim, K. H., & Kwon, T. R. (2017). Salt tolerance in rice: focus on mechanisms and approaches. *Rice Science*, *24*(3), 123-144.

129) Roy, A., Kucukural, A., & Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nature protocols*, *5*(4), 725.

130) Roy, S. J., Negrao, S., & Tester, M. (2014). Salt resistant crop plants. *Current Opinion in Biotechnology*, *26*, 115-124.

131) Roychoudhury, A., Paul, S., & Basu, S. (2013). Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. *Plant cell reports*, *32*(7), 985-1006.

132) Saddhe, A. A., & Kumar, K. (2015). In silico identification and expression analysis of MscS like gene family in rice. *Plant Gene*, *1*, 8-17.

133) Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., & Izui, K. (2000). Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *The Plant Journal*, *23*(3), 319-327.

134) Sandhu, D., Cornacchione, M. V., Ferreira, J. F., & Suarez, D. L. (2017). Variable salinity responses of 12 alfalfa genotypes and comparative expression analyses of salt-response genes. *Scientific Reports*, *7*, 42958.

135) Sanghera, G. S., Wani, S. H., Hussain, W., & Singh, N. B. (2011). Engineering cold stress tolerance in crop plants. *Current genomics*, *12*(1), 30.

136) Sarmast, M. K., Salehi, H., & Niazi, A. (2015). Biochemical differences underlie varying drought tolerance in four Festuca arundinacea Schreb. genotypes subjected to short water scarcity. *Acta physiologiae plantarum*, *37*(9), 192.

137) Sato, Y., Masuta, Y., Saito, K., Murayama, S., & Ozawa, K. (2011). Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, OsAPXa. *Plant cell reports*, *30*(3), 399-406.

138) Serysheva, E., Berhane, H., Grumolato, L., Demir, K., Balmer, S., Bodak, M., & Jenny, A. (2013). Wnk kinases are positive regulators of canonical Wnt/β-catenin signalling. *EMBO reports*, *14*(8), 718-725.

139) Shou, H., Bordallo, P., & Wang, K. (2004). Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize. *Journal of experimental Botany*, *55*(399), 1013-1019.

140) Simillion, C., Vandepoele, K., Van Montagu, M. C., Zabeau, M., & Van de Peer, Y. (2002). The hidden duplication past of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *99*(21), 13627-13632.

141) Singh, A. K., Choudhury, B. U., and Bouman, B. A. M. (2002). "Water-wise rice production," in Proceedings of the international workshop on water-wise rice production, eds B. A. M. Bouman, H. Hengsdijk, B. Hardy, P. S. Bindraban, T. P. Tuong, and J. K. Ladha (Los Baños, Philippines: International Rice Research Institute),237–248.

142) Singh, C. M., Kumar, B., Mehandi, S., & Chandra, K. (2012). Effect of drought stress in rice: a review on morphological and physiological characteristics. *Trends in Biosciences*, *5*(4), 261-265.

143) Singh, R. K., Mishra, B., & Singh, K. N. (2004). Salt tolerant rice varieties and their role in reclamation programme in Uttar Pradesh. *Indian Farming*, 6-10.

144) Singh, R., & Jwa, N. S. (2013). Understanding the responses of rice to environmental stress using proteomics. *Journal of proteome research*, *12*(11), 4652-4669.

145) Sirault, X. R., James, R. A., & Furbank, R. T. (2009). A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. *Functional Plant Biology*, *36*(11), 970-977.

146) Sofo, A., Scopa, A., Nuzzaci, M., & Vitti, A. (2015). Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. *International journal of molecular sciences*, *16*(6), 13561-13578.

147) Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., ... & Miller, H. L. (2007). IPCC, 2007: Climate change 2007: The physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. *SD Solomon (Ed.)*.

148) Spensley, M., Kim, J. Y., Picot, E., Reid, J., Ott, S., Helliwell, C., & Carré, I. A. (2009). Evolutionarily conserved regulatory motifs in the promoter of the *Arabidopsis* clock gene LATE ELONGATED HYPOCOTYL. *The Plant Cell*, *21*(9), 2606-2623.

149) Sun, X., Sun, M., Luo, X., Ding, X., Ji, W., Cai, H., & Zhu, Y. (2013). A Glycine soja ABA-responsive receptor-like cytoplasmic kinase, GsRLCK, positively controls plant tolerance to salt and drought stresses. *Planta*, *237*(6), 1527-1545.

150) Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

151) Taylor, S. S., Radzio-Andzelm, E., & Hunter, T. (1995). How do protein kinases discriminate between serine/threonine and tyrosine? Structural insights from the insulin receptor protein-tyrosine kinase. *The FASEB Journal*, *9*(13), 1255-1266.

152) Teige, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., ... & Hirt, H. (2004). The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. *Molecular cell*, *15*(1), 141-152.

153) Tester, M., & Bacic, A. (2005). Abiotic stress tolerance in grasses. From model plants to crop plants.

154) Todaka, D., Nakashima, K., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. *Rice*, *5*(1), 6.

155) Todaka, D., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2015). Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Frontiers in plant science*, *6*, 84.

156) Tran, L. S. P., Nakashima, K., Sakuma, Y., Simpson, S. D., Fujita, Y., Maruyama, K., & Yamaguchi-Shinozaki, K. (2004). Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. *The Plant Cell*, *16*(9), 2481-2498.

157) Triboi, Eugene, and Anne-Marie Triboi-Blondel. "Productivity and grain or seed composition: a new approach to an old problem." *European Journal of Agronomy* 16.3 (2002): 163-186.

158) Tsuchiya, T., & Eulgem, T. (2010). The *Arabidopsis* defense component EDM2 affects the floral transition in an FLC-dependent manner. *The Plant Journal*, *62*(3), 518-528.

159) Tsukaguchi, T., & Iida, Y. (2008). Effects of assimilate supply and high temperature during grain-filling period on the occurrence of various types of

chalky kernels in rice plants (Oryza sativa L.). *Plant Production Science*, *11*(2), 203-210.

160) Uchida, S., Sohara, E., Rai, T., & Sasaki, S. (2014). Regulation of with-no-lysine kinase signaling by Kelch-like proteins. *Biology of the Cell*, *106*(2), 45-56.

161) Urano, D., Czarnecki, O., Wang, X., Jones, A. M., & Chen, J. G. (2015). *Arabidopsis* receptor of activated C kinase1 phosphorylation by WITH NO LYSINE8 KINASE. *Plant physiology*, *167*(2), 507-516.

162) Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant science*, *151*(1), 59-66.

163) Verissimo, F., & Jordan, P. (2001). WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. *Oncogene*, *20*(39), 5562.

164) Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., & Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal*, *45*(4), 523-539.

165) Wakamatsu, K., Sasaki, O., Uezono, I., & Tanaka, A. (2007). Effects of high air temperature during the ripening period on the grain quality of rice [Oryza sativa] in warm regions of Japan. *Japanese Journal of Crop Science (Japan).*

166) Wang, H., Zhang, M., Guo, R., Shi, D., Liu, B., Lin, X., & Yang, C. (2012). Effects of salt stress on ion balance and nitrogen metabolism of old and young leaves in rice (Oryza sativa L.). *BMC plant biology*, *12*(1), 194.

167) Wang, J., Ding, H., Zhang, A., Ma, F., Cao, J., & Jiang, M. (2010). A novel mitogen-activated protein kinase gene in maize (Zea mays), ZmMPK3, is involved in response to diverse environmental cues. *Journal of integrative plant biology*, *52*(5), 442-452.

168) Wang, L., Liu, Y., Cai, G., Jiang, S., Pan, J., & Li, D. (2014). Ectopic expression of ZmSIMK1 leads to improved drought tolerance and activation of systematic acquired resistance in transgenic tobacco. *Journal of biotechnology*, *172*, 18-29.

169) Wang, R. K., Cao, Z. H., & Hao, Y. J. (2014). Overexpression of a R2R3 MYB gene MdSIMYB1 increases tolerance to multiple stresses in transgenic tobacco and apples. *Physiologia plantarum*, *150*(1), 76-87.

170) Wang, Y., Liu, K., Liao, H., Zhuang, C., Ma, H., & Yan, X. (2008). The plant WNK gene family and regulation of flowering time in *Arabidopsis*. *Plant Biology*, *10*(5), 548-562.

171) Wang, Y., Sun, T., Li, T., Wang, M., Yang, G., & He, G. (2016). A CBL-interacting protein kinase TaCIPK2 confers drought tolerance in transgenic tobacco plants through regulating the stomatal movement. *PloS one*, *11*(12), e0167962.

172) Wang, Y., Suo, H., Zheng, Y., Liu, K., Zhuang, C., Kahle, K. T., & Yan, X. (2010). The soybean root-specific protein kinase GmWNK1 regulates stress-responsive ABA signaling on the root system architecture. *The Plant Journal*, *64*(2), 230-242.

173) Wang, Y., Suo, H., Zhuang, C., Ma, H., & Yan, X. (2011). Overexpression of the soybean GmWNK1 altered the sensitivity to salt and osmotic stress in *Arabidopsis*. *Journal of plant physiology*, *168*(18), 2260-2267.

174) Wani, S. H., & Sah, S. K. (2014). Biotechnology and abiotic stress tolerance in rice. *J Rice Res*, *2*, e105.

175) Welsch, R., Wüst, F., Bär, C., Al-Babili, S., & Beyer, P. (2008). A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. *Plant Physiology*, *147*(1), 367-380.

176) Wilson, F. H., Disse-Nicodeme, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., & Feely, M. P. (2001). Human hypertension caused by mutations in WNK kinases. *Science*, *293*(5532), 1107-1112.

177) Xie, G., Kato, H., & Imai, R. (2012). Biochemical identification of the OsMKK6–OsMPK3 signalling pathway for chilling stress tolerance in rice. *Biochemical Journal*, *443*(1), 95-102.

178) Xie, G., Kato, H., Sasaki, K., & Imai, R. (2009). A cold-induced thioredoxin h of rice, OsTrx23, negatively regulates kinase activities of OsMPK3 and OsMPK6 in vitro. *FEBS letters*, *583*(17), 2734-2738.

179) Xie, M., Wu, D., Duan, G., Wang, L., He, R., Li, X., & Liu, X. (2014). AtWNK9 is regulated by ABA and dehydration and is involved in drought tolerance in *Arabidopsis*. *Plant physiology and biochemistry*, *77*, 73-83.

180) Xin, Z., & Browse, J. (2000). Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell & Environment*, *23*(9), 893-902.

181) Xing, Y., Jia, W., & Zhang, J. (2007). AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering H2O2 production in *Arabidopsis*. *Journal of Experimental Botany*, *58*(11), 2969-2981.

182) Xiong, L., & Yang, Y. (2003). Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid–inducible mitogen-activated protein kinase. *The Plant Cell*, *15*(3), 745-759.

183) Xu, B. E., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J., & Cobb, M. H. (2000). WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *Journal of Biological Chemistry*, *275*(22), 16795-16801.

184) Xu, B. E., Min, X., Stippec, S., Lee, B. H., Goldsmith, E. J., & Cobb, M. H. (2002). Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. *Journal of Biological Chemistry*.

185) Xu, J., Tian, Y. S., Peng, R. H., Xiong, A. S., Zhu, B., Jin, X. F., & Yao, Q. H. (2010). AtCPK6, a functionally redundant and positive regulator involved in salt/drought stress tolerance in *Arabidopsis*. *Planta*, *231*(6), 1251-1260.

186) Yamada, K., Park, H. M., Rigel, D. F., DiPetrillo, K., Whalen, E. J., Anisowicz, A., & Caplan, S. L. (2016). Small-molecule WNK inhibition regulates cardiovascular and renal function. *Nature chemical biology*, *12*(11), 896.

187) Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *The Plant Cell*, *6*(2), 251-264.

188) Yang, A., Dai, X., & Zhang, W. H. (2012). A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. *Journal of experimental botany*, *63*(7), 2541-2556.

189) Yang, Z., & Rannala, B. (2012). Molecular phylogenetics: principles and practice. *Nature Reviews Genetics*, *13*(5), 303.

190) Ying, S., Zhang, D. F., Fu, J., Shi, Y. S., Song, Y. C., Wang, T. Y., & Li, Y. (2012). Cloning and characterization of a maize bZIP transcription factor, ZmbZIP72, confers drought and salt tolerance in transgenic *Arabidopsis*. *Planta*, *235*(2), 253-266.

191) Yokotani, N., Ichikawa, T., Kondou, Y., Matsui, M., Hirochika, H., Iwabuchi, M., & Oda, K. (2009). Tolerance to various environmental stresses conferred by the salt-responsive rice gene ONAC063 in transgenic *Arabidopsis*. *Planta*, *229*(5), 1065-1075.

192) Yoo, S. D., Cho, Y. H., & Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature protocols*, *2*(7), 1565.

193) Zhang, B., Liu, K., Zheng, Y., Wang, Y., Wang, J., & Liao, H. (2013). Disruption of AtWNK8 enhances tolerance of *Arabidopsis* to salt and osmotic stresses via modulating proline content and activities of catalase and peroxidase. *International journal of molecular sciences*, *14*(4), 7032-7047.

194) Zhang, L., Zhang, L., Xia, C., Zhao, G., Liu, J., Jia, J., & Kong, X. (2015). A novel wheat bZIP transcription factor, TabZIP60, confers multiple abiotic stress tolerances in transgenic *Arabidopsis*. *Physiologia plantarum*, *153*(4), 538-554.

195) Zhang, Q., Chen, Q., Wang, S., Hong, Y., & Wang, Z. (2014). Rice and cold stress: methods for its evaluation and summary of cold tolerance-related quantitative trait loci. *Rice*, *7*(1), 24.

196) Zhang, X., Liu, X., Wu, L., Yu, G., Wang, X., & Ma, H. (2015). The SsDREB transcription factor from the succulent halophyte Suaeda salsa enhances abiotic stress tolerance in transgenic tobacco. *International journal of genomics*, *2015*.

197) Zhao, H., Tan, Z., Wen, X., & Wang, Y. (2017). An improved syringe agroinfiltration protocol to enhance transformation efficiency by combinative use of 5-azacytidine, ascorbate acid and Tween-20. *Plants*, *6*(1), 9.

198) Zhao, J., Guo, S., Chen, S., Zhang, H., & Zhao, Y. (2009). Expression of yeast YAP1 in transgenic *Arabidopsis* results in increased salt tolerance. *Journal of Plant Biology*, *52*(1), 56.

199) Zhao, K., Shen, X., Yuan, H., Liu, Y., Liao, X., Wang, Q., & Li, T. (2013). Isolation and Characterization of Dehydration-responsive Element-binding Factor 2c (msdreb2c) from malus sieversii Roem. *Plant and cell physiology*, *54*(9), 1415-1430.

200) Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annual review of plant biology*, *53*(1), 247-273.

201) Zhu, J., Dong, C. H., & Zhu, J. K. (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Current opinion in plant biology*, *10*(3), 290-295.

202) Zulawski, M., Schulze, G., Braginets, R., Hartmann, S., & Schulze, W. X. (2014). The *Arabidopsis* Kinome: phylogeny and evolutionary insights into functional diversification. *BMC genomics*, *15*(1), 548.

Appendix

Appendix I- Reagents, buffers and media composition

A. TE Buffer (pH 8.0)

Ingredients **$gm.L^{-1}$**

1. Tris-HCl (10 mM)……1.21
2. Na_2EDTA (1 mM)……0.372

B. CTAB Buffer in MilliQ water (DNA isolation)

Ingredients **$gm.L^{-1}$**

1. CTAB………………...20
2. EDTA (25mM)……….7.3
3. NaCl (1.4 M)…………81.8
4. PVP…………………..20
5. β-mercaptoethanol……10
6. SDS…………………..100

C. Hoagland nutrient solution

Ingredients **$gm.L^{-1}$**

1. Ca $(NO_3)_2.4H_2O$…….236.1
2. KNO_3………………...101.1
3. KH_2PO_4……………..136.1
4. $MgSO_4.7H_20$………...246.5
5. Fe $(NO_3)_3.9H_2O$……..13.31
   EDTA………………...8.68
6. MnCl2.4H2O………...2.34
   $ZnSO_4.7H_2O$………...0.88
   $Na_2MoO_4.2H_2O$……..0.26

D. Murashige and Skoog Medium (Himedia PT021-4.4gm/l)

Ingredients **$gm.L^{-1}$**

MACROELEMENTS

Ammonium nitrate……………………..1650.000

Calcium chloride……………………….332.200

Magnesium sulphate…………………...180.690

Potassium nitrate……………………1900.000

Potassium phosphate monobasic………170.000

MICROELEMENTS

Boric acid…………………………...6.200

Cobalt chloride hexahydrate…………..0.025

Copper sulphate pentahydrate………...0.025

EDTA disodium salt dehydrate………37.300

Ferrous sulphate heptahydrate………..27.800

Manganese sulphate monohydrate…...16.900

Molybdic acid (sodium salt)………….0.213

Potassium Iodide…………………..0.830

Zinc sulphate heptahydrate………..8.600

VITAMINS

myo-Inositol……………………100.000

Nicotinic acid (free acid)………….0.500

Pyridoxine HCl……………………0.500

Thiamine hydrochloride…………...0.100

AMINO ACID

Glycine…………………………… 2.000

Sucrose………………………………30

Agar/ Gelatin......……………………15/4

E Murashige and Skoog Macroelements (Himedia TS1068-4.2g/l)

Ingredients **$gm.L^{-1}$**

MACROELEMENTS

Ammonium nitrate……………………..1650.000

Calcium chloride……………………….332.200

Magnesium sulphate…………………...180.690

Potassium nitrate……………………1900.000

Potassium phosphate monobasic…….170.000

F. Phosphate buffer (pH- 7.4)

Ingredients **$gm.L^{-1}$**

Monobasic sodium phosphate………….23.796

Dibasic sodium phosphate……………...28.392

G. Assay buffer for catalase (CAT)

Ingredients **$gm.L^{-1}$**

Monobasic sodium phosphate………….23.796

Dibasic sodium phosphate……………...28.392

H_2O_2……………………………………5.1

H. Extraction buffer for Ascorbate peroxidase (APX)

Ingredients **$gm.L^{-1}$**

Monobasic sodium phosphate………….23.796

Dibasic sodium phosphate……………...28.392

Ascorbic acid…………………………...0.176

EDTA…………………………………..0.292

PVP……………………………………2%

I. Assay buffer for Ascorbate peroxidase (APX)

Ingredients **$gm.L^{-1}$**

Monobasic sodium phosphate………….23.796

Dibasic sodium phosphate……………...28.392

Ascorbic acid…………………………...0.035

EDTA…………………………………..0.058

H_2O_2……………………………………6.8

J. Assay buffer for Guaicol peroxidase (POD)

Ingredients **gm.L^{-1}**

Monobasic sodium phosphate…………23.796

Dibasic sodium phosphate……………...28.392

Guaicol………………………………….0.33%

H_2O_2……………………………………0.418

Appendix II

List of Publications

1) Manuka, R., Saddhe, A. A., & Kumar, K. (2018). Expression of OsWNK9 in *Arabidopsis* conferred tolerance to salt and drought stress. *Plant Science*, *270*, 58-71.

2) Manuka, R., Saddhe, A. A., & Kumar, K. (2015). Genome-wide identification and expression analysis of WNK kinase gene family in rice. *Computational biology and chemistry*, *59*, 56-66.

Appendix III

Poster presentation in conferences

1) Rakesh Manuka, Kundan kumar. The role of OsWNK9 in conferring increased tolerance to salt and drought stress. 16th International Symposium on Rice Functional Genomics (ISRFG 2018), September 5-7, 2018, Tokyo University of Agriculture, Tokyo, Japan.

2) Rakesh Manuka, Kundan kumar. Identification and expression analysis of *OsWNK9* gene in *Arabidopsis thaliana* and rice, Inter Drought-V, February 21-25, 2017, Hyderabad International conventional center, organized by ICRISAT, Hyderabad.

3) Rakesh Manuka, Kundan kumar. Differential transcript accumulation of with-no-lysine kinase in rice under abiotic stress. National Seminar on New Frontiers in plant Sciences and Biotechnology, January 29-30, 2015, Department of Botany, Goa University.

4) Rakesh Manuka, Kundan kumar. Identification of abiotic stress responsive with no lysine kinase in rice. International Symposium on Plant Signaling and Behavior, March 7-10, 2014, Department of Botany, University of Delhi.

Appendix IV

Brief Biography of the Candidate

Name Rakesh Manuka

Date of birth 02/04/1990

Education M.Sc. Biotechnology

Loyola Degree and PG College, Secunderabad, Telangana

B.Sc. Biotechnology

Nalanda Degree College, Jagitial, Telangana

Email ID rakesh_manuka@yahoo.com, p20140002@goa.bits-pilani.ac.in

Working experience

- August 2016- December 2018: Institute Fellowship, BITS Pilani, K. K. Birla, Goa campus, India.
- August 2013- July 2016: Sponsored project JRF from DST-SERB, India.
- January 2013- July 2013: Internship at ICRISAT, patancheru, Hyderabad, India.

Research Publications

02 publications in International journal.

Awards/ Fellowship

- DST-SERB International travel grant: Attended 16th International Symposium on Rice Functional Genomics (ISRFG 2018), Tokyo University of Agriculture, Tokyo, Japan.
- Research Fellowship: 2016- 2018, Research scholar from BITS Pilani K.K. Birla Goa campus.
- Research Fellowship: 2013-2016, JRF from DST-SERB sponsored project.
- PhD representative and senate member for the time period of August 2017- December 2018 at BITS PILANI K.K. Birla goa campus.

Appendix V

Brief Biography of the Supervisor

Name Dr. Kundan Kumar

Education Ph.D. (2009): National Institute of Plant Genome Research, New Delhi

Ph.D. Thesis Title Investigation of the role of mitogen activated protein kinase kinase in *Oryza sativa* under abiotic stress conditions

Contact Details Chamber No: B112, BITS PILANI, K.K. Birla Goa Campus NH17B, Zuarinagar, Goa, India

Email ID kundan@goa.bits-pilani.ac.in

Phone 0832-2580196

Research Interest Plant molecular biology and stress physiology, Plant signaling, Phytoremediation and Plant natural products

Professional Experience

- May 2009-Oct 2011 Postdoctoral Research Associate, University of Massachusetts, Amherst, USA
- Nov 2011-April 2012 Postdoctoral Fellow, McGill University, McDonald Campus, Sainte-Anne-de-Bellevue, Canada
- May 2012-June 2018 Assistant Professor, Department of Biological Sciences, BITS Pilani K K Birla Goa Campus, Goa, India
- July 2018-present Associate Professor, Department of Biological Sciences, BITS Pilani K K Birla Goa Campus, Goa, India

Sponsored Research Project

Completed

1. Identification and characterization of abiotic stress responsive With No Lysine (WNK) kinase in rice (*Oryza sativa* L.): Science & Engineering Research Board) under DST Fast Track scheme. (2013-16)

2. DNA Barcoding of Goan Mangroves: Research Initiation Grant from BITS. (2013-15)

Ongoing

1. Screening, isolation and identification of novel antimicrobial compounds from potential mangroves of Goa. Funded by CSIR EMR-II (2016-19)
2. Study on the role of TIP family members in arsenite accumulation and their transport in rice, Funded by BRNS (2017-2020)

Publications

32 publications in International journals, 03 book chapters

Reviewer for international journals

Plant Molecular Biology, Scientific Reports, International Journal of Phytoremediation, Phytochemistry, Plant Science, PeerJ, Plant Physiology and Biochemistry, Scientific Reports and BMC Genomics.

No of Ph.D. Students

Registered 04

Member of professional body

Life member of Indian Science Congress Association and Life member of society of Plant Biochemistry and Biotechnology.

Reprints of publications